ANTIMICROBIAL ACTIVITY OF *Diaporthe terebinthifolii* AGAINST *Staphylococcus aureus* AND METHICILLIN-RESISTANT *S. aureus* (MRSA)

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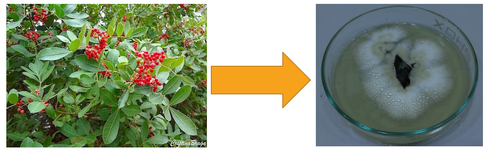
Abstract

Medicinal plants represent a great source of endophytic fungi, potential producers of novel bioactive compounds that may be useful to pharmaceutical industries. *Diaporthe terebinthifolii* is a fungus described in 2013 and isolated from *Schinus terebinthifolius*, which is used in popular medicine to combat many health problems. In this study, the strain LGMF907 was tested in two different fermentation conditions and its biological activity was investigated. The crude extracts were obtained from malt extract broth, pH 5.8, 28°C, 120 rpm and 10 days of fermentation, demonstrating high antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). The compounds diaporthin and orthosporin were identified, and showed low activity against both *S. aureus* and MRSA. *Diaporthe terebinthifolii* can be a great source of bioactive compounds and further studies should be carried out in order to isolate and examine their bioactivity.

Introduction

Fungal endophytes are hyper-diverse and abundant groups that can be found colonizing the tissues of all plants, regardless of their taxonomical affiliation or environmental preferences (Ismaiel et al. 2010) . Throughout centuries medicinal plants have been used as a source of molecules with therapeutic potential, and until today they represent an important pool for the identification of novel drug leads (Atanasov et al. 2002). For clinical use, the ultimate source of the majority of all known antibacterial drugs are natural products (NPs), and the current push to find new therapeutics to combat antibacterial drug resistance stimulates renewed interest in NP sources for new leads (Silver 2015). *Schinus terebinthifolius* Raddi, also known as Brazilian peppertree, rose pepper or "aroeira-da-praia", is used in folk medicine to combat diseases of the urogenital system and has antipyretic, analgesic, depurative, antimicrobial, anti-inflammatory, and anti-ulcerogenic activities (Carvalho et al. 2013).

The genus *Diaporthe* comprises a widely-studied group of fungi for its ability to generate metabolites for several applications (Flores et al. 2013). *Diaporthe terebinthifolii* is a species isolated from *Schinus terebinthifolius* Raddi and described by Gomes et al. (2013). In order to access and expand the knowledge about the biological activity of strain LGMF907, extracts originated from the large-scale production and major compounds were evaluated against important clinical pathogens associated with hospital infections: *S. aureus* and methicillin-resistant *S. aureus*.



Reference: pinterest.com

Reference: the authors

Fig. 1 - The endophytic fungus *Diaporthe terebinthifolii* LGMF907 isolated from *Schinus terebinthifolius* Raddi

Materials and Methodology

Large-scale fermentation

The strain *Diaporthe terebinthifolii* LGMF907 was obtained from leaves of *Schinus terebinthifolius* Raddi collected in Curitiba, Paraná, Brazil (25° 28' 31.847" S, 49° 17' 5.629" W). The microorganism is stored in the biological bank of the Laboratory of Genetics of Microorganisms (LabGeM) from Federal University of Paraná (http://www.labgem.ufpr.br/).

Two different fermentation conditions were used in order to scale up secondary metabolite fungal production: **a**) inoculation of three 10 mm diameter agar plugs into a baffled 250 mL Erlenmeyer flask containing 100mL of Malt Extract Broth (MEB; 20 g L^{-1} malt extract, 20 g L^{-1} glucose, and 1 g L^{-1} peptone); **b**) inoculation of 24 agar plugs into a baffled 2 L Erlenmeyer flask containing 800 mL of MEB. Follow, they were incubated at 28 °C under shacking at 120 rpm for 10 days.

Extraction, isolation and identification of compounds

The liquid was separated from mycelia by filtering the culture media over a Whatman qualitative filter paper Grade 4 and then subjected to exhaustive extraction with EtOAc on a separating funnel. All EtOAc extracts were combined and the solvent removed under reduced pressure on rotary evaporator at 40 °C, yielding crude extract.

The crude extracts, from both production methods, were initially subjected to silica gel column chromatography, eluted with a gradient system consisting of increasing amounts of methanol in chloroform (from 0 to 100, *v/v*) yielding 13 and 17 fractions, respectively. Resulting fractions were further submitted to HPLC separation on a Waters 600 HPLC system coupled with a photodiode diode array detector. The system was equipped with a semi-preparative reversed phase C_{18} column (10.0 I.D. x 250 mm long, 5 µm particle diameter). Chromatography separations were performed by a binary gradient system consisting of acetonitrile and water at a flow rate of 3.0 mL min^{-1}. The solvents were filtered through a 0.45µm HV filter (Millipore) then degassed for 30 min in an ultrasonic bath. Finally, major compounds from HPLC were further purified on a Sephadex LH-20 eluted with MeOH, yielding orthosporin (2.3 mg) and diaporthin (0.25 mg) in pure forms as pale-yellow solids from fermentation condition (*a*) and diaporthin (2.0 mg) from fermentation condition (*b*). The remaining fractions and sub-fractions were excluded due to the limited quantities.

HR-MS analyses were recorded on an Amershan Biosystem ABSciex 4000 Q-Trap MS system. NMR data were acquired on the Agilent DD2 500 and DD2 600 NMR spectrometers, operating at 11.7 and 14.1 Tesla, observing ^{1}H and ^{13}C at 500 and 600 and 125 and 150 MHz, respectively. The spectrometer was equipped with a 5-mm multinuclear direct detection probe with z-gradient. For help to confirm the substructure of the compounds, Antibase was used (Laatsch, 2012).

Antibacterial tests

The bioactivity of the isolated compounds and the crude extracts were tested against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591. An adapted disk diffusion essay protocol was used, standardized by the National Committee for Clinical Laboratory Standards (NCCLS) and followed by the Brazilian Health Surveillance Agency (ANVISA) (NCCLS 2003). Both microorganisms were maintained in LB agar. Thus, one colony of each organism was inoculated into a 10 mL culture of LB broth and incubated in a 37 °C orbital shaker at 200 RPM for 15 h. Using a cotton swab, each test bacterium (10^8 CFU/mL) was spread homogenously on the surface of a sterile 90 mm polystyrene plate containing Mueller-Hinton agar (CRITERION™, Hardy Diagnostics). Sterile paper disks (6 mm diameter) were placed on the plates and loaded with 100 µg amount of each crude extract or compound dissolved in methanol. The plates were incubated at 37 °C for 24 h. Antibiotics ampicillin (100 µg/mL) and oxacillin (100 µg/mL and 600 µg/mL) were used as positive controls; pure methanol (v/v) and saline solution (0,85%) were used as negative controls. The experiment was carried out in triplicate with the inhibition zones measured in millimeters, statistical parameters (Normality test, ANOVA and Tukey test) were calculated using R software (2011).

Results

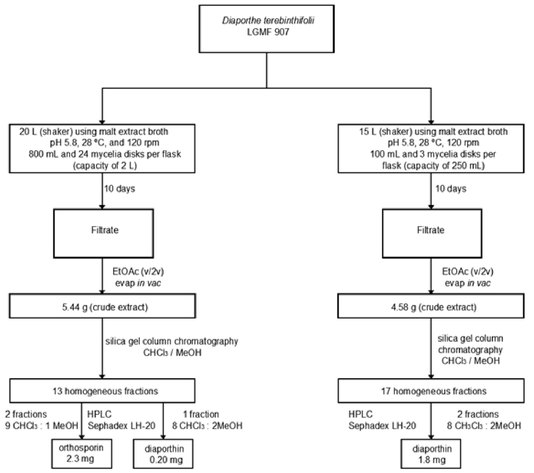
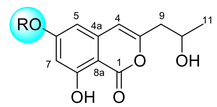


Figure 2- Work-up scheme of *Diaporthe terebinthifolii* LGMF907 using malt extract broth (MEB) with two different methods.



1: R=CH_3; Diaporthin
2: R=H; Orthosporin

1: (+)-HRESI-MS: m/z 273.0732 $[M + Na]^+$ (Calcd. 273.0739 for $C_{13}H_{14}O_5Na$ $[M + Na]^+$)
2: (+)-HRESI-MS: m/z 237.0763 $[M + H]^+$ (Calcd. 237.0763 for $C_{12}H_{13}O_5$ $[M + H]^+$)

Fig. 3 - Chemical structures and HR-MS data of diaporthin (1) and orthosporin (2) produced by the fungus *Diaporthe terebinthifolii* LGMF907.

Table 1 - Evaluation of the inhibition zone (mm) of LGMF907 crude extracts and compounds tested against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) at 100 µg/disk

Microorganism	methanol[#]	ampicillin 100µg/mL	oxacillin 100µg/mL	oxacillin 600µg/ml	diaporthin	orthosporin	crude extract 1*	crude extract 2*
S. aureus	0Ag	15.67Ae	26.67Ab	31.00Aa	9.0Af	9.33Af	16.00Ad	19.67Ac
MRSA	0Ad	0Bd	15.67Bb	20.00Ba	9.5Ac	9.00Ac	16.33Ab	18.00Aab

Tukey results significative with p-value < 0.001. The capital letters compare the microorganisms between the lines whereas the lowercase letters compare the treatments among the columns. [#] Solvent.
*Crude extract 1 was obtained from fermentation method (*a*) and crude extract 2 from fermentation method (*b*)

Acknowledgements

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Discussion and Conclusion

The ^{1}H and ^{13}C NMR spectral data of the isolated compounds corroborate with those described previously (Lee et al. 2006). They are known as isocoumarins and phytotoxins (Hallock et al. 1988; Sparapano et al. 1989). Diaporthin was firstly isolated from *Endothia parasitica* (Boller et al. 1957) and the first report of orthosporin was from the plant pathogenic fungus *Drechslera siccans*. In addition, both diaporthin and orthosporin, were co-isolated from *Daldinia concentrica*, *Aspergillus ochraceus* (Miller et al. 2012; Huang et al. 2015) and in a hypovirulent strain of *Cryphonectria parasitica* (Arnone et al. 2002).

The two culture conditions (methods *a* and *b*) that were carried out using different amount of cells, oxigenation, flask size and liquid volume, resulted in different amounts of diaporthin, as well as the non-detection of orthosporin, when method (*b*) was used. Fungi have specific response to different growing environments that affects the biosynthesis of active secondary metabolites and changes in the environment can alter chemical and biological profiles leading to metabolite diversification and, consequently, to novel pharmacological applications (Bracarense et al. 2014; Ismaiel et al. 2010).

In our study, compounds diaporthin and orthosporin showed activity against the clinical bacteria *S. aureus* and MRSA, however the crude extract 1 and 2 showed high activity, even superior to the antibiotic ampicillin. These data suggest that the activity observed in the extracts is possibly due to the compounds diaporthin and orthosporin in synergism with other compounds that are present in small amounts in the extracts. The synergism is also supported by the higher activity against MRSA than *S. aureus*, produced by crude extract 1, which may suggests the presence of two different mechanisms of action (Redwan et al. 2016).

Diaporthe terebinthifolii can be an important source of bioactive compounds, but it is necessary more investigations to elucidate which compounds are responsible for its great antimicrobial activity.

References

Arnone A, Assante G, Nasini G, Strada S, Vercesi A (2002) Cryphonectric acid and other minor metabolites from a hypovirulent strain of *Cryphonectria parasitica*. J Nat Prod 65:48-50.

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM et al (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotech Adv 33(8):1582–1614.

Boller A, Gauman E, Hardegger E, Kugler K, Naef-Roth, Rosner M (1957) Wellkstotte und antibiotike: diaporthin ein wellketoxin aus kultaren von *Endothia parasitica* (Murr.). Helv Chim Acta 40:875-880.

Bracarense AAP, Takahashi JA (2014) Modulation of antimicrobial metabolites production by the fungus *Aspergillus parasiticus*. Braz J Microbiol 45(1):313–321.

Carvalho, C.R., Ferreira, M.C., Amorim, S.S., Florindo, R.H.S., Assis, J.C.S., Zani, C.L., Rosa, L.H. Bioactive compounds of endophytic fungi associated with medicinal plants, Recent Advancement in White Biotechnology Through Fungi, (2019) 303-361.

Flores AC, Pamphile JA, Sarragiotto MH, Clemente E (2013) Production of 3-nitropropionic acid by endophytic fungus *Phomopsis longicolla* isolated from *Trichilia elegans* A. JUSS ssp. *elegans* and evaluation of biological activity. World J Microbiol Biotechnol 29:923–932.

Gomes RR, Glienke C, Videira SIR, Lombard L, Groenewald JZ, Crous, PW (2013) *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Fung Div 60:161-170.

Ismaiel AA, El-Sayed AES, Mahmoud AA (2010) Some optimal culture conditions for production of cyclosporin a by *Fusarium roseum*. Braz J Microbiol 41(4):1517-8382

Hallock YF, Clardy J, Kenfield DS, Strobel G (1988) De-*O*-methyldiaporthin, a phytotoxin from *Drechslera siccans*. Phytochemistry 27:3123–3125.

Huang Q, An H, Song H, Mao, H, Shen, W, Dong J (2015) Diversity and biotransformative potential of endophytic fungi associated with the medicinal plant *Kadsura angustifolia*. Res Microbiol 166(1):45-55.

Laatsch H (2012) AntiBase. Wiley-VCH, Weinheim.

Lee IK, Seok SJ, Kim WG, Yun BS (2006) Diaporthin and orthosporin from the fruiting body of *Daldinia concentrica*. Mycobiology 34(1):38-40.

Miller KI, Qing C, Sze DM-Y, Roufogalis BD, Neilan BA (2012) Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microb Ecol 64:431-449.

NCCLS (2003) Performance Standards for Antimicrobial Disk Susceptibility Tests; approved standard—8th edn. Clinical and Laboratory Standards Institute document M2-A8. Clinical and Laboratory Standards Institute, Wayne.

R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org/.

Redwan EM, El-Baky NA, Al-Hejin AM, Baeshen MN, Almehdar HA, Elzawy A, Gomaa ABM, Al-Masaudi SB, Uversky VN (2016) Significant antibacterial activity and synergistic effects of camel lactoferrin with antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA). Res Microbiol 167(6):480-491.

Silver LL (2015) Natural products as a source of drug leads to overcome drug resistance. Future Microbiol 10(11):1711-1718.

Sparapano L, Mairota P, Lerario P (1989) Phytotoxic Substances from virulent and hypovirulent strains of *Cryphonectria Parasitica*. Phyto Plant Pathol 27:399-401.

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Screening of the antioxidant and enzyme-inhibiting potential of essential oils of some commercial Lamiaceae species

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Introduction

- Lamiaceae species, especially those belonging to the subfamily Nepetoideae, are extensively used for centuries in traditional medicine, cookery, perfumery and cosmetics due to their prominent flavoring and curative properties.
- There is a hot topic on oxidative stress as one of the main triggers of diabetes mellitus and neurodegenerative diseases, hence **this research was aimed to examine the antioxidant effects as well as α-amylase, acetylcholinesterase and tyrosinase inhibiting potential of essential oils of 11 selected Lamiaceae species.**





Material and Methods

- Plant material of *Hyssopus officinalis (Ho), Lavandula angustifolia (La), Mentha x piperita (Mp), Ocimum basilicum (Ob), Origanum heracleoticum (Oh), Origanum vulgare (Ov), Rosmarinus officinalis (Ro), Salvia officinalis (So), Satureja montana (Sm), Thymus serpyllum (Ts)* and *Thymus vulgaris (Tv)* was collected on the fields of the Institute for Medicinal Plant Research „Dr Josif Pančić" in Pančevo, Serbia. The essential oils (EOs) were isolated from aerial parts by hydrodistillation and tested at concentrations of 0.5, 1, and 2 mg/mL.
- Antioxidant effects were assessed using DPPH and β-carotene bleaching assays, and compared to positive controls BHT and BHA, while enzymes inhibition potential was tested *in vitro* using α-amylase, acetylcholinesterase and tyrosinase along with acarbose, galanthamine (GAL) and kojic acid (KA) as positive controls, respectively.
- The results were expressed as percent of inhibition and presented as mean averaged from three measurements ± standard error.

















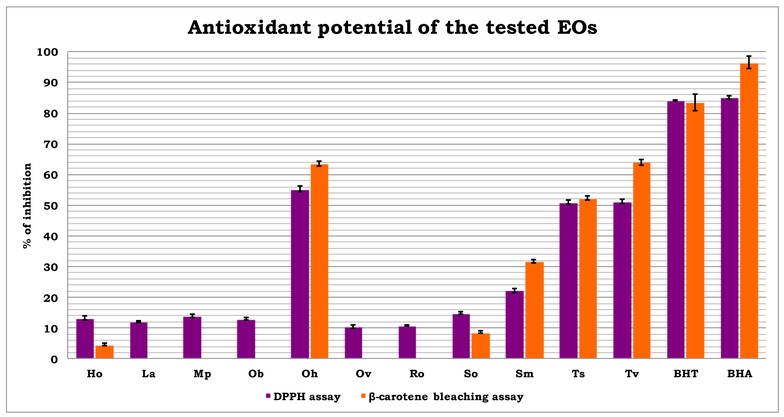
Results and Discussion

- The presented results were obtained for the EOs and positive controls tested at concentrations of 2 mg/mL and 1 mg/mL., respectively.

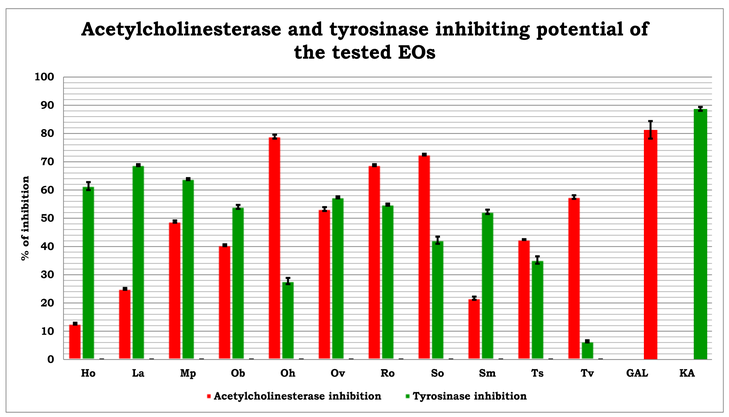
The results obtained by **DPPH and β-carotene bleaching assays** indicated that *Oh*, *Tv,* and *Ts* oils inhibit above 50% free radicals in both tests, which is in accordance with our previous findings obtained by total reducing power assay [1].

The tested EOs did not inhibit efficiently **α-amylase** (values were ranged from 0.11-5.62%), while the results of our previous study showed a promising α-glucosidase inhibitory activity, especially for *Ts*, *Tv,* and *Oh* oils [1].

The strongest **inhibition of acetylcholinesterase** was performed by *Oh* (even 78.90%), followed by *So* and *Ro* essential oils. The essential oil of *La* showed the strongest **inhibition of tyrosinase** (68.77%), followed by *Mp* and *Ho*.



Species	α-amylase inhibition (%)
Ho	3.97±0.36
La	0.11±0.04
Mp	1.27±0.22
Ob	2.48±0.13
Oh	not active
Ov	1.63±0.30
Ro	not active
So	5.62±0.22
Sm	not active
Ts	not active
Tv	not active
Acarbose	82.99±1.18



Conclusions

Comparing the obtained results with positive controls and literature data, it could be concluded that essential oils of the examined Lamiaceae species, particularly those of *Ts*, *Tv,* and *Oh*, have significant potential for further research as antioxidant and antineurodegenerative agents.

Acknowledgments
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References
[1] S. Janković, D. Pljevljakušić, M. Oalđe, A. Alimpić Aradski, S. Duletić-Laušević, 6th International Electronic Conference on Medicinal Chemistry, November 1-30, (2020), doi:10.3390/ECMC2020-07271.

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EVALUATION OF AZOLE-RESISTANT MYCOBIOTA AND MYCOTOXINS PROFILE IN TEA

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TECNOLOGIA DA SAÚDE DE LISBOA
INSTITUTO POLITÉCNICO DE LISBOA

H&TRC
Health and Technology Research Center

Background

- Tea (infusion of the *Camellia sinensis* leaves) is one of the most common beverages worldwide, namely, black tea and green tea (1).
- Fungal contamination of tea by mycotoxins (produced by filamentous fungi such as *Aspergillus)* confer potential risks to human health and can cause serious diseases (2).
- Continued exposure of *Camellia sinensis* to fungicides in conventional agricultural can promote the development of azole resistance among fungal species, such as *A. fumigatus* (3).
- Azole resistance limits therapeutic options against fungal diseases (e.g. aspergillosis), representing a threat to public health.

Study Goals

- Identify and quantify the mycobiota through microbiological and molecular analysis.
- Characterize the susceptibility profile of fungi to antifungal drugs of the azole group.
- Assess the contamination of tea by mycotoxins.
- Relate the results obtained with the origin (organic or conventional agriculture) and the type of tea packaging (bulk or tea bags).

Materials & Methods

Gathering Samples

Supermarkets	Herbalist Shops

Sample Treatment (4)

Extraction	Infusion
4,4g tea per 40ml of 0.9% NaCl with 0.05% Tween80 ™	2g per 100ml boiling water for 6 minutes

Fungal Burden
- MEA
- DG18

Azole Resistance
- Saboraud (control)
- Saboraud + Itraconazole 4 mg.L^{-1}
- Saboraud + Voriconazole 2 mg.L^{-1}
- Saboraud + Posaconazole 0,5 mg.L^{-1}

Mycotoxins

HPLC with MS-MS
- Aflatoxins
- Ochratoxin A

Colony Forming Units → Microscopy → Real-Time PCR

Aspergilus sections: Circumdati, Flavi, Fumigati, Versicolores

Independent variables	Measured variables
Tea bags vs bulk Conventional vs biological origin	Fungal burden Toxigenic species Azole resistance Mycotoxins distribution

Expected Outcomes

- Detailed characterization of the contamination present in tea samples commercially accessible in Portugal, focusing on the distribution of fungal species azole and toxigenic resistant, as well as in the characterization of the mycotoxin profile.
- Risk assessment of tea consumption for human health.
- Propose measures to monitor and control fungal contamination (including resistant and toxigenic fungi) in commercially available tea.

References

1. Pakshir K, Mirshekari Z, Nouraei H, Zareshahrabadi Z, Zomorodian K, Khodadadi H, et al. Mycotoxins Detection and Fungal Contamination in Black and Green Tea by HPLC-Based Method. J Toxicol. 2020;2020.
2. Chuaysrinule C, Maneeboon T, Roopkham C, Mahakarnchanakul W. Occurrence of aflatoxin- and ochratoxin A-producing Aspergillus species in Thai dried chilli. J Agric Food Res [Internet]. 2020;2(June):100054. Available from: https://doi.org/10.1016/j.jafr.2020.100054
3. Barber AE, Riedel J, Sae-Ong T, Kang K, Brabetz W, Panagiotou G, et al. Effects of Agricultural Fungicide Use on Aspergillus fumigatus Abundance, Antifungal Susceptibility, and Population Structure. MBio. 2020;11(6):1–18.
4. Viegas C, Sá F, Mateus M, Santos P, Almeida B, Aranha Caetano L, et al. Commercial green tea from Portugal: Comprehensive microbiologic analyses. Int J Food Microbiol [Internet]. 2020;333(July):108795. Available from: https://doi.org/10.1016/j.ijfoodmicro.2020.108795

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IN VITRO ASSAY OF BIOACTIVE POTENTIAL OF *ZIZIPHUS JUJUBA* MILL. EXTRACTS

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Abstract

The fruit of *Zizifus jujuba* has high energy and nutritional value, contains a lot of sugar, dietary fiber, vitamin C, minerals, phenolic compounds. Therefore, this plant is a valuable source of phytochemicals, bioactives, vitamins etc. which makes it to be effective in curing or prevention from various diseases. The present work investigates quantification of total phenols and flavonoids, antioxidant and antimicrobial potential of four different extracts *Z. jujube* (methanol, water, dichloromethane and *n*-butanol). The results showed that these extracts are rich in phenolic compounds, but do not contain a high percentage of flavonoids. The best antioxidant potential in the DPPH test was shown for *n*-butanol extracts, while in the ABTS test another three extracts were shown to be significantly better antioxidants.

All extracts possessed antibacterial/antifungal activity against tested bacterial species, and dermatomycetes. The extracts showed very high antimicrobial potential, with the aqueous extract being slightly weaker compared to the other three ones. The extracts from all three localities show approximately the same antifungal effect. The obtained results show that fruit of *Z. jujuba* and its extracts represent a potential source of new compounds with antioxidant and antimicrobial activity. It is an appropriate candidate for treatment of microbial infections and for therapeutic application.

Introduction

Human infectious diseases have been markedly on the rise, especially in immunocompromised patients. Despite of a number of the available treatments, bacterial and fungal infections still remain a major therapeutic problem (1). Likewise, the increasing resistance of pathogens to commercial drugs is a serious medical problem, which has resulted in the need for novel antimicrobial agents. Plants, vegetables, fruits and their products have proven to be great source of novel compounds with biological potential (2).

Jujube (*Zizifus jujuba* Mill., Rhamnaceae) is an important medicinal plant used in traditional medicine. Phytochemical studies have confirmed their biological effects, such as antioxidant, anticancer, anti-inflammatory, immunostimulatory and hepatoprotective. Also, Jujube is used for the treatment of diabetes, diarrhea, skin infections, urinary disorders, obesity, fever, bronchitis, anemia, cancer (3-6).

Therefore, the aim of this study was to investigate antioxidant and antimicrobial potentials of different extracts *Z. jujuba* on several strains of gram-negative and gram-positive bacteria and dermatomycetes.

Materials

Ziziphus jujuba Mill (fruit) was collected from three localities of Montenegro: Podgorica (PG), Ulcinj (UL) and Šušanj (ŠŠ) during the autumn of 2018. The fleshy part of fruit was dried for 5 days at 25ºC.



Ziziphus jujuba Mill

Antibacterial/antifungal activities of *Z. jujuba* extracts were evaluated against:

- bacterial species (human infectious agents and food contaminants): *Staphylococcus aureus* (ATCC 6538), oral isolate *S. aureus* (SAK2), Methicillin-resistant isolate *S. aureus* (MRSA 12), *Escherichia coli* (ATCC 25922), resistant isolate *E. coli* (H2b), *Pseudomonas aeruginosa* (ATCC 27853), resistant isolate *P. aeruginosa* (01-05392);
- dermatomycetes: *Microsporum gypseum* (MG003), *M. canis* (MC10), *Trichophyton mentagrophytes* (TM005), *T. tonsurans* (TT001).

Methodology

Dried fruits were prepared for extraction with methanol (MeOH). The extracts were obtained using ultrasonic assisted extraction. Obtained extracts were fractionized to obtain the dichlormetane (CH_2CL_2), *n*-buthanol (n-BuOH) and water (dH_2O) extracts.

The total phenolic and flavonoids content along with determination of antioxidant activity of extracts was measured by spectrophotometric methods using Jenway 7315 UV-Visible spectrophotometer.

The content of phenolic compounds was determined using Folin-Ciocalteau reagent and expressed as gallic acid equivalents (GAE) in mg per g of dried extract weight (DW).

Total flavonoid content was measured using aluminium nitrate nonahydrate, and expressed as quercetine hydrate equivalents in mg per g of dried extract weight (DW) (7).

Antioxidant activity of extracts was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assays (7).

A modified microdilution method (96 system) (8) was used to examine potential antimicrobial activity of extracts.

Results

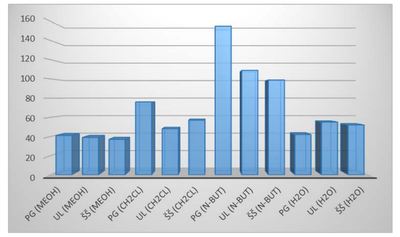


Figure 1. Total phenolic content (mg GAE/g SE) in *Ziziphus jujuba* extracts.

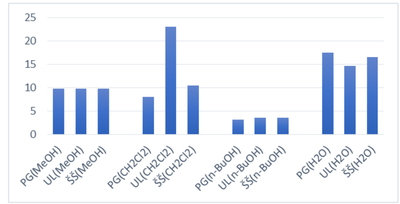


Figure 3. DPPH radical scavenging activity (IC_{50} mg/mL) of *Ziziphus jujuba* extracts.

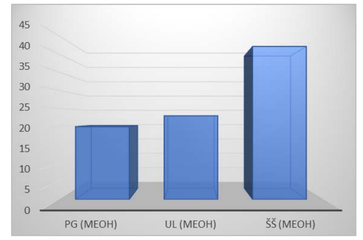


Figure 2. Total flavonoids content (mg QE/g SE) in *Ziziphus jujuba* methanol extracts.

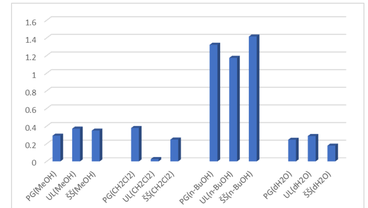


Figure 4. ABTS radical scavenging activity (IC_{50} mg/mL) of *Ziziphus jujuba* extracts.

Table 1. Antibacterial activity of *Ziziphus jujuba* extracts and conventional antibiotic (mg/mL).

		S.aureus (ATCC)	*S. aureus* - oral	*Rez. S. aureus*	*E. coli* (ATCC)	*Rez. E. coli*	*P. aeruginosa* (ATCC)	*Rez. P. aeruginosa*
PG MEOH	MIC	0.40	0.40	0.80	0.40	0.80	0.20	0.10
	MBC	0.80	0.80	1.60	0.80	1.60	0.40	0.20
UL MEOH	MIC	0.20	0.40	0.40	0.025	0.40	0.10	0.20
	MBC	0.40	0.80	0.80	0.05	0.80	0.20	0.40
ŠŠ MEOH	MIC	0.40	0.20	0.40	0.20	0.80	0.20	0.20
	MBC	0.80	0.40	0.80	0.40	1.60	0.40	0.40
PG CH_2CL_2	MIC	0.10	0.05	0.10	0.20	0.20	0.20	0.80
	MBC	0.20	0.10	0.20	0.40	0.80	0.40	1.60
UL CH_2CL_2	MIC	0.10	0.20	0.20	0.40	0.40	0.20	0.40
	MBC	0.20	0.40	0.40	0.80	0.80	0.40	0.80
ŠŠ CH_2CL_2	MIC	0.20	0.20	0.20	0.40	0.20	0.20	0.40
	MBC	0.40	0.40	0.40	0.80	0.40	0.40	0.80
PG n-BuOH	MIC	0.40	0.20	0.40	0.10	0.80	0.20	0.20
	MBC	0.80	0.40	0.80	0.20	1.60	0.40	0.40
UL n-BuOH	MIC	0.20	0.10	0.20	0.20	0.20	0.10	0.20
	MBC	0.40	0.20	0.40	0.40	0.40	0.20	0.40
ŠŠ n-BuOH	MIC	0.20	0.20	0.40	0.40	0.40	0.20	0.05
	MBC	0.40	0.40	0.80	0.80	0.80	0.40	0.10
PG dH_2O	MIC	-	1.60	-	0.20	0.80	0.80	0.10
	MBC	-	-	-	0.40	1.60	1.60	0.20
UL dH_2O	MIC	0.80	0.80	0.80	0.20	0.80	0.20	0.20
	MBC	1.60	1.60	1.60	0.40	1.60	0.40	0.40
ŠŠ dH_2O	MIC	0.40	0.40	0.40	0.40	0.40	0.20	0.10
	MBC	1.60	1.60	1.60	0.80	0.80	0.40	0.20
Streptomycin	MIC	0.15	0.10	0.10	0.10	0.10	0.10	0.05
	MBC	0.30	0.20	-	0.20	0.20	0.20	0.10

Table 2. Antifungal activity of *Ziziphus jujuba* extracts and conventional fungicide (mg/mL).

		M. gypseum	*M. canis*	*T. mentagrophytes*	*T. tonsurans*
PG MEOH	MIC	0.80	0.10	0.05	0.10
	MFC	1.60	0.20	0.10	0.40
UL MEOH	MIC	0.20	1.60	0.10	0.40
	MFC	0.40	-	0.20	0.80
ŠŠ MEOH	MIC	0.80	0.05	0.20	0.80
	MFC	1.60	0.10	0.40	1.60
PG CH_2CL_2	MIC	0.05	0.10	0.05	1.60
	MFC	0.10	0.20	0.10	-
UL CH_2CL_2	MIC	0.05	0.05	0.10	0.20
	MFC	0.10	0.10	0.20	0.40
ŠŠ CH_2CL_2	MIC	0.10	0.10	0.20	0.20
	MFC	0.20	0.20	0.40	0.40
PG n-BuOH	MIC	0.20	0.10	0.20	0.10
	MFC	0.40	0.20	0.40	0.20
UL n-BuOH	MIC	0.10	0.05	0.10	0.20
	MFC	0.20	0.10	0.20	0.40
ŠŠ n-BuOH	MIC	0.10	0.10	0.20	0.40
	MFC	0.40	0.20	0.40	0.80
PG dH_2O	MIC	0.10	0.10	0.80	0.10
	MFC	0.40	0.20	1.50	0.40
UL dH_2O	MIC	0.40	0.05	0.40	0.80
	MFC	1.60	0.10	0.80	1.60
ŠŠ dH_2O	MIC	0.40	0.20	0.40	0.80
	MFC	1.60	0.40	0.80	1.60
Ketoconazole	MIC	0.80	0.40	0.20	1.60
	MFC	1.60	0.80	0.40	-

Conclusion

The obtained results indicate that extracts of *Z. jujuba* are rich in phenolic compounds and possessed valuable biological potential. Generally, Jujube is a promising natural source of compounds with meaningful bioactive properties, which could be of significant interest for further research from various applicative aspects.

Acknowledgements

This research was financially supported by the bilateral project Serbia-Montenegro "Biocontrol of phytopathogenic fungus by natural products from subtropical plants order Rosales" and Science and Technological Development of Republic of Serbia (Contract No. 451-03-68/2020-14/200007).

References

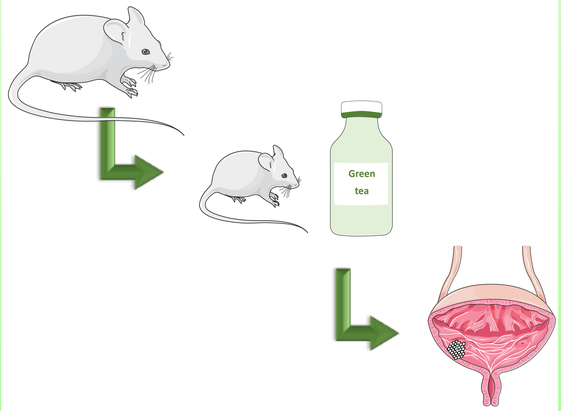
1. Demain, LA., Sanchez, S. 2009. Microbial drug discovery: 80 years of progress. J. Antibiot. 62, 5-16.
2. Ferreira, I.C.F.R., Morales, P., Barros, L. 2016. Wild Plants, Mushrooms and Nuts, Wild Plants, Mushrooms and Nuts: Functional Food Properties and Applications. John Wiley & Sons, Ltd, Chichester, UK.
3. Hung C-F, Hsu B-Y, et al. 2012. Antiproliferation of melanoma cells by polysaccharide isolated from *Zizyphus jujuba*. Nutrition 28, 98-105.
4. Al-Reza SM, Yoon JI, et al. Anti-inflammatory activity of seed essential oil from Zizyphus jujuba. Food Chem. Toxic. 48, 639-43.
5. Al-Reza SM, Rahman A, et al. 2010. Potential roles of essential oil and organic extracts of *Zizyphus jujuba* in inhibiting food-borne pathogens. Food Chem. 19, 981-6.
6. Tahergorabi, Z., Abedini, M. et al. 2015. "*Ziziphus jujuba*": A red fruit with promising anticancer activities. Pharm. Reviews, 9, 99–106.
7. Matejić, J.S., Stojanović-Radić, Z.Z., et al. 2018. Chemical characterization, *in vitro* biological activity of essential oils and extracts of three *Eryngium* L. species and molecular docking of selected major compounds. J. Food Sci. Technol., 55, 2910-2925
8. Stojković, D., Dias, M.I., et al. 2020. Methanolic extract of the herb ononis spinosa L. Is an antifungal agent with no cytotoxicity to primary human cells. Pharmaceut. 13, 1-13.

EFFECTS OF GREEN TEA IN URINARY BLADDER CANCER: DATA FROM A MOUSE MODEL

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Abstract



Introduction

Urinary bladder cancer is one of the most common diseases around the world, associated with several risk factors. It is more frequent in men than in women, representing the fourth and eighth causes of cancer, respectively. Urinary bladder cancer development in developed countries is associated with risk factors, namely tobacco smoking, chemical carcinogens, and ionizing radiation [1-2].

N-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) is a carcinogen able to induce the development of preneoplastic and neoplastic urothelial lesions in rodents [3].

Green tea (GT) is one of the most popular beverages whose beneficial effects on health have been demonstrated. Major polyphenols present in GT are flavonoids, especially catechins, that have garnered considerable attention due to beneficial effects on health, including antioxidant, anti-inflammatory, and chemopreventive effects [4].

This study aimed to evaluate the effects of whole GT on urinary bladder cancer in male and female mice.

Materials

The design and experimental procedures were performed in accordance with the EU regulations (Directive 2010/63/EU) on protection of animals used for experimental and other scientific purposes.

Forty-one, five-week-old ICR mice (21 males and 20 females), were obtained from Harlan-Interfauna (Spain).

BBN was purchased from Tokyo Kasei Kogyo (Japan). The GT leaves (Thea Sinensis L.) were purchased from Augusto Coutinho Ervanários (Portugal).

Methodology

Animals from each gender were randomly divided into three experimental groups, as follows: Males - group I (BBN+GT) (n=8); group II (BBN) (n=7); group III (GT) (n=6); Females - group IV (BBN+GT) (n=7); group V (BBN) (n=7); group VI (GT) (n=6) (Fig. 1).

BBN was administered to animals from groups I, II, IV and V by gavage, at a dose of 7.25 mg/mouse, 2 times/week, during 10 consecutive weeks. The whole GT (0.5%) was daily prepared and given *ad libitum* to groups I, III, IV and VI for 20 consecutive weeks. Animals were sacrificed by pentobarbital sodium overdose and a complete necropsy was performed. A histological analysis of the urinary bladder was performed.

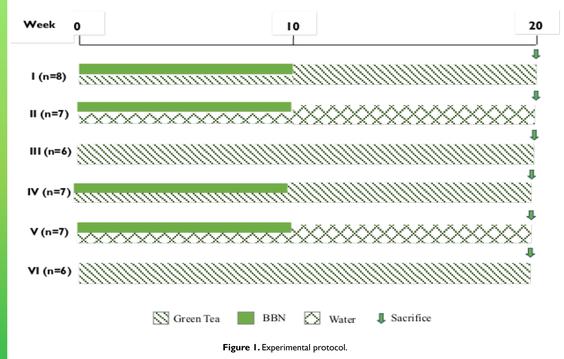


Figure 1. Experimental protocol.

Data was analyzed with ANOVA. Results were considered statistically significant for $p<0.05$.

Results

No changes in animals' behavior were observed. Clinical signs of distress or discomfort were not observed throughout the study.

The groups showed a similar food and water intake. Conversely, initial and final weights and weight gain were similar among groups.

Animals from groups not exposed to BBN (III and VI) did not develop any urothelial lesion.

Animals from groups BBN+GT (I and IV) and BBN (II and V) developed only preneoplastic lesions. The number of inflammatory aggregates was lower in animals exposed to BBN that drank GT (I and IV), when compared with those only exposed to BBN (II and V).

A statistically significant difference was observed between groups BBN (II and V) and groups GT (III and VI) ($p<0.05$) (Tables 1 and 2, Fig. 2).

Table 1: Urothelial histopathological analysis (n; %) and inflammatory aggregates in male rats (mean±S.D.).

Analysis	Groups	Male		
		I (BBN+GT) n=8	II (BBN) n=7	III (GT) n=6
Histological analysis	Normal urothelium	0 (0%)	0 (0%)	6 (100%)
	Simple hyperplasia	6 (75%)	3 (50.0%)	0 (0%)
	Dysplasia	7 (87.5%)	5 (83.3%)	0 (0%)
	Papilloma	2 (25%)	0 (0%)	0 (0%)
	Squamous metaplasia	0 (0%)	0 (0%)	0 (0%)
	Inflammatory aggregates	3.50 ± 4.46	4.67 ± 2.99[a]	0.42 ± 0.90

[a] $p<0.05$ *vs* Group III

Table 2: Urothelial histopathological analysis (n; %) and inflammatory aggregates in female rats (mean±S.D.).

Analysis	Groups	Female		
		IV (BBN+GT) n=7	V (BBN) n=7	VI (GT) n=6
Histological analysis	Normal urothelium	0 (0%)	0 (0%)	6 (100%)
	Simple hyperplasia	3 (42.8%)	4 (66.7%)	0 (0%)
	Dysplasia	4 (57.1%)	4 (66.7%)	0 (0%)
	Papilloma	0 (0%)	0 (0%)	0 (0%)
	Squamous metaplasia	0 (0%)	2 (16.7%)	0 (0%)
	Inflammatory aggregates	3.50 ± 2.56	3.83 ± 3.13[b]	0.25 ± 0.45

[b] $p<0.05$ *vs* Group VI

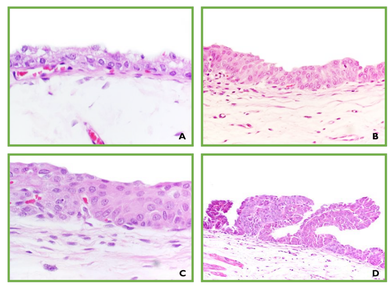


Figure 2. Histological analysis of urothelium. **A.** Normal urothelium (H&E, 400 x). **B.** Simple hyperplasia (H&E, 400 x). **C.** Dysplasia (H&E, 600 x). **D.** Papilloma (H&E, 200 x).

Conclusion

Animals from BBN (II and V) and BBN+GT (I and IV) groups only developed preneoplastic lesions. The number of inflammatory aggregates was lower in animals that drank GT.

We can conclude that the whole GT infusion had no effect on urinary bladder cancer development, but reduced urothelial inflammation.

For future investigations, the use of different GT infusion concentrations is recommended.

References

[1] A. Jemal, F. Bray, MM. Center, J. Ferlay, E. Ward, D. Forman, CA Cancer J Clin, 61 (2011) 69.

[2] RC. Fernandez, TR. Driscoll, DC. Glass, D. Vallance, A. Reid, G. Benke, L. Fritschi, Aust N Z J Public Health, 36 (2012) 111.

[3] C. Palmeira, PA. Oliveira, R. Arantes-Rodrigues, A. Colaço, PLF. De la Cruz, C. Lopes, L. Santos, Oncol Rep, 21(2009) 247.

[4] C. Cabrera, R. Artacho, R. Gimenez, J Am Coll Nutr, 25 (2006) 79.

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Characterization and biological activity of extracts from artichoke leaves

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Introduction

Cynara scolymus L. is an herbaceous and perennial crop. Originally native to the Mediterranean basin, the arid, high temperatures and elevated salinity conditions are ideal for growth and reproduction. Its cultivation dates the ancient times when Greek and Roman civilizations used the artichoke plant for its varied medicinal and nutritional properties (Ceccarelli et al., 2010; Velez et al., 2012). Artichoke is also known for its antioxidant activity and for the ability to lower cholesterol levels in blood (Biel et al., 2020). The increasing interest for these biological properties in the diet and for healthier foods triggered the worldwide production and economic valorization of artichoke. Artichoke presents a wide spectrum of potential applications with major importance for scientific research and the pharmaceutical industry due, particularly, to its great potential to produce commercial extracts rich in polyphenols (De Menna et al., 2016).

Artichoke leaves, non-edible parts often discarded, are rich in bioactive secondary metabolites, mainly polyphenolic compounds like flavonoids (0.1-1.0%) and phenolic acids (2%), and sesquiterpenes, such as cynaropicrin (0.44-1.6%) (Alonso et al., 2006; Pieri and Stuppner, 2011). Cynaropicrin, a common caffeoylquinic acid, has anti-inflammatory, antioxidant, choleretic, antibacterial, and antitumoral properties and prevents skin photoaging induced by UV radiation (Cho et al., 2004). While chlorogenic acid, a phenolic acid, has antioxidant, hepatoprotective and antitumoral properties, and reduces blood cholesterol (Falco et al., 2015).

The polarity of the solvent used in the extraction influences the composition of artichoke extracts, so phenolic compounds are mainly extracted with polar solvents, while sesquiterpenes are mainly extracted with non-polar solvents (Gálvez et al., 2005). Moreover, the chemical composition of artichoke plant is highly variable, depending on the harvest time, type of extraction, drying methods (Alonso et al., 2006), storage, plant age (Lutz et al., 2011), among others, which, consequently, affect its biological properties.

The non-edible parts of the plant that are waste from the food industry could be used for extracting pharmacologically active compounds or in the production of extracts for nutraceutical and cosmeceutical applications. Since artichoke industrial bio-products (stems, leaves, other bracts) represent about 80% of the biomass (Ceccarelli et al., 2010). Thus, this work aims to characterize the structure and the biological potential of the extracts obtained from *C. scolymus* by infusion of leaves in hot water. The components of the aqueous extract were partitioned by liquid-liquid extraction, and aqueous (Aq) and organic (O) fractions were examined separately to assess the *in vitro* and *in vivo* antioxidant activities of their components.

Materials

Plant material Artichoke, *C. scolymus* L., leaves were selected as the biological material for the experiments. Plant specimens were provided by the Earth Essences company, in Póvoa do Lanhoso, Portugal.

Methodology

1. Extract preparation Infusion of five grams of new fresh leaves in 50 mL H_2O for 10 min, followed by vacuum filtration, and liquid-liquid extraction with ethyl acetate. The obtained aqueous (Aq) fraction was dried by lyophilization and the organic (O) fraction was dried in the rotary evaporator. The dried residue of each fraction was diluted to reach a stock solution of 50 mg/mL with either water for the aqueous fraction and ethanol 70% for the organic fraction.

2. Nuclear Magnetic Resonance (NMR) The chemical analysis of the extracts, or fractions, was carried out by NMR at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. Solutions were obtained by the dissolution of dried extracts, or fractions, in 600 µL deuterated dimethylsulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$).

3. Antioxidant activity

3.1. DPHH radical assay The scavenging capacity of antioxidants was determined using the DPPH method developed by Blois (1958). The *gallic* acid (10-100 µg/mL) was used as the standard to determine the IC_{50} for each fraction.

3.2. Reducing power assay The reducing power was determined according to the method of Oyaizu (1986). The method was adapted to use 100 µL of fraction sample, *gallic* acid (10-100 µg/mL) was used as the standard, and results expressed in GAE µg/mg of fraction.

3.3 flow cytometry

Saccharomyces cerevisiae cells, used as eukaryotic cell model, loaded with the redox-sensitive fluorochrome dichlorofluorescein diacetate (50 µM) were treated with organic (O) fraction (25, 50 or 100 µg/mL) or aqueous (Aq) fraction (1, 10 or 25 µg/mL) and 10 mM H_2O_2 for 20 min in the dark at 30°C. Subsequently, intracellular fluorescence was measured by flow cytometry in at least 20,000 cells.

4. Genotoxic potential and antigenotoxic activity

4.1. *In vitro* DNA topology assay The genotoxic and antigenotoxic potential of the Aq and O fractions were evaluated with this method using the plasmid pBR322 (0.05 µg/mL), and in the presence or absence of 0.2 mM $FeSO_4$, respectively. Samples were prepared with 0.5x TBE buffer (0.9 M Tris-HCl, 0.9 M H_3BO_3, 0.2M EDTA, pH 8) and 2 µL of loading dye solution (60% glycerol, 10 mM Tris-HCl, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF) and applied on 1% agarose gel in 0.5 TBE, containing 10 µL of 10000x (dH_2O) GelRed.

5. Colorimetric analysis of total phenolic content (TPC) Total phenols were estimated using the Folin-Ciocalteu (F-C) method adapted to a 96-well microplate (Blainski et al., 2013), using *gallic* acid (25-1000 µg/mL) as the standard.

6. Statistical analysis Statistical analysis was conducted using the GraphPad Prism 8 software. Data are shown as the mean values ± SD from at least three independent experiments (n ≥ 3). The comparison between Aq and O fractions results for the TPC content and DPPH scavenging activity was analyzed by an Unpaired t-test. The differences between each fraction and the standard *gallic* acid for the reducing power assay, and the results of Flow Cytometry were assessed by 1-way ANOVA analyses followed by Dunnett's multiple comparison test.

Results

1. Artichoke extracts are rich in chlorogenic acid and cynaropicrin according to NMR

After liquid-liquid extraction, the obtained fractions were analyzed by ^{1}H and 2D NMR spectroscopy. The NMR analysis demonstrated that chlorogenic acid (1) was the prominent constituent in the aqueous fraction (**Fig. 1a**), while cynaropicrin (2) was completely separated into the organic fraction (**Fig. 1b**).

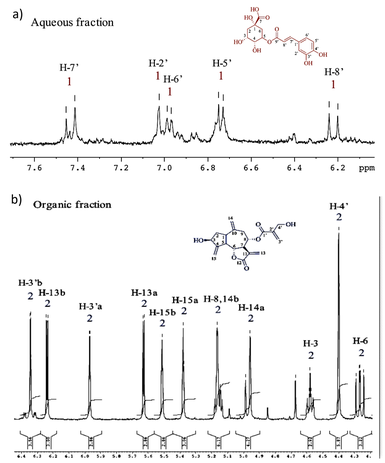


Figure 1 Excerpts of ^{1}H NMR spectra from *Cynara scolymus* L. aqueous (Aq) fraction obtained after liquid-liquid extraction with ethyl acetate, 400 MHz, DMSO-d_6 (a); and organic (O) fraction obtained after liquid-liquid extraction with ethyl acetate, 400 MHz, $CDCl_3$ (b).

2. Antioxidant activity

2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Both fractions present a similar (p=0.8064) *in vitro* antioxidant capacity by free radical scavenging activity (**Table I**).

Table I DPPH scavenging activity of aqueous (Aq) and organic (O) fractions of *C. scolymus* leaves extract expressed in IC_{50}. *Gallic* acid (GA) was used as standard antioxidant compound.

Fraction	IC_{50} (µg/mL)
GA	0.51±0.03
Aq	15.82±0.74
O	16.45±4.32

2.2. Reducing power capacity

Only the Aq fraction exhibited reducing power capacity, which is significantly different (p<0.0001) from the standard *gallic* acid (**Table II**).

Table II Reducing power capacity of aqueous (Aq) fraction of *C. scolymus* leaves extract expressed in µg GAE/mg extract. Gallic acid (GA) was used as standard antioxidant compound.

Fraction	(µg GAE/mg fraction)
GA	947±9.24
Aq	26.74±3.08 (****)

2.3. *In vivo* flow cytometry

The O fraction was able to protect living cells from oxidative stress, while for the Aq fraction this was not observed.

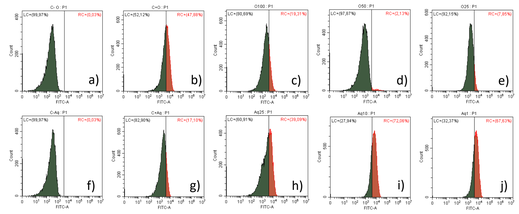


Figure 2 Antioxidant activity of *Cynara scolymus* leaves Aq and O fractions against H_2O_2 in *Saccharomyces cerevisiae* cells. Yeast cells were treated with organic (O) fraction, 100 µg/mL (c); 50 µg/mL (d); 25 µg/mL (e); or with aqueous (Aq) fraction 25 µg/mL (h); 10 µg/mL (i); 1 µg/mL (j) µg/mL; and 10 mM H_2O_2 for 20 min in the dark at 30 ºC. The positive (C+; a,f) and negative (C-; b,g) controls represent *S. cerevisiae* cells treated with 10 mM H_2O_2 and *S. cerevisiae* cells in the presence of water and 70%, respectively. The population with lower fluorescence than the C+ is depicted in green while the population with higher fluorescence is depicted in red.

3. Genotoxic potential and antigenotoxic activity

3.1. *In vitro* DNA topology

Both fractions are not genotoxic. The O fraction exhibited antigenotoxic activity for all the tested concentrations unlike the Aq extract.

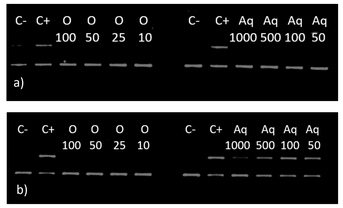


Figure 3 *In vitro* DNA topology images for genotoxicity (a). pDNA was treated with Aq fraction (50, 100, 500 and 1000 µg/mL) and O fraction (10, 25, 50 and 100 µg/mL). For antigenotoxicity (b), the pDNA was treated with Aq and O fractions, same conditions, and $FeSO_4$ (2 mM). The C$^-$, pDNA only, was used as the control for the genotoxicity, while the C$^+$, with pDNA and $FeSO_4$, was used as the control for the antigenotoxicity.

4. Total phenolic content (TPC)

Polyphenols are more abundant in the Aq fraction, which is significantly different (p=0.0099) from the O fraction (**Table III**).

Table III Total polyphenolic content (TPC) results for *C. scolymus* aqueous (Aq) and organic (O) fractions, expressed in µg GAE/mg fraction. *Gallic* acid (GA) was used as standard antioxidant compound.

Fraction	TPC (µg GAE/mg fraction)
Aq	33.11±6.64 (**)
O	14±2.65 (**)

Conclusion

- The Aq fraction has radical scavenging activity, which correlates with the high content in polyphenols.
- The O fraction exhibited *in vivo* and *in vitro* antioxidant activity and antigenotoxic activity.
- The O fraction is manly composed by cynaropicrin which suggests that cynaropicrin is the main antioxidant and antigenotoxic secondary metabolite in *C. scolymus*.
- The results recognize and value the consumption of artichoke leaves and potentiate their application in different areas such as dietary, medicinal, and cosmetic.

References

Alonso, M.R., García, M.D.C., Bonelli, C.G., Ferraro, G., Rubio, M., 2006. Validated HPLC method for cynarin determination in biological samples. Acta Farm. Bonaer. 25, 267–270.

Biel, W., Witkowicz, R., Piątkowska, E., Podsiadło, C., 2020. Proximate Composition, Minerals and Antioxidant Activity of Artichoke Leaf Extracts. Biol. Trace Elem. Res. 194, 589–595. https://doi.org/10.1007/s12011-019-01806-3

Ceccarelli, N., Curadi, M., Picciarelli, P., Martelloni, L., Sbrana, C., Giovannetti, M., 2010. Globe artichoke as a functional food. Med. J. Nutrition Metab. 3, 197–201. https://doi.org/10.1007/s12349-010-0021-z

Cho, J.Y., Kim, A.R., Jung, J.H., Chun, T., Rhee, M.H., Yoo, E.S., 2004. Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines. Eur. J. Pharmacol. 492, 85–94. https://doi.org/10.1016/j.ejphar.2004.03.027

De Falco, B., Incerti, G., Amato, M., Lanzotti, V., 2015. Artichoke: botanical, agronomical, phytochemical, and pharmacological overview. Phytochem. Rev. 14, 993–1018. https://doi.org/10.1007/s11101-015-9428-y

De Menna, F., Malagnino, R.A., Vittuari, M., Molari, G., Seddaiu, G., Deligios, P.A., Solinas, S., Ledda, L., 2016. Potential biogas production from artichoke byproducts in Sardinia, Italy. Energies 9, 1–11. https://doi.org/10.3390/en9020092

Gálvez, M., Martín-Cordero, C., Houghton, P.J., Ayuso, M.J., 2005. Antioxidant activity of methanol extracts obtained from Plantago species. J. Agric. Food Chem. 53, 1927–1933. https://doi.org/10.1021/jf048076s

Lutz, M., Henríquez, C., Escobar, M., 2011. Chemical composition and antioxidant properties of mature and baby artichokes (*Cynara scolymus* L.), raw and cooked. J. Food Compos. Anal. 24, 49–54. https://doi.org/10.1016/j.jfca.2010.06.001

Pieri, V., Stuppner, H., 2011. Quantification of cynaropicrin in artichoke leaf extracts by ^{1}H NMR spectroscopy. Planta Med. 77, 1756–1758. https://doi.org/10.1055/s-0030-1271083

Velez, Z., Campinho, M., Guerra, Â., García, L., Ramos, P., Guerreiro, O., Felício, L., Schmitt, F., Duarte, M., 2012. Biological Characterization of *Cynara cardunculus* L. Methanolic Extracts: Antioxidant, Anti-proliferative, Anti-migratory and Anti-angiogenic Activities. Agriculture 2, 472–492. https://doi.org/10.3390/agriculture2040472.

Acknowledgements

We would like to thank the company Earth Essences for their kind supply of artichoke leaves. This work was supported by the "Contrato-Programa" UIDB/04050/2020 founded by national funds through the FCT I.P. National Funds by FCT - Portuguese Foundation for Science and Technology, under the projects UIDB/04033/2020 and UIDP/04033/2020.

Antioxidant activity of Portuguese commercial essential oils obtained from forest maintenance

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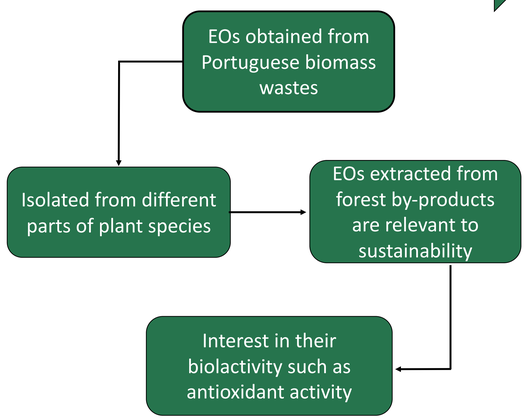
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NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Introduction

Landscaping of *Eucalyptus globulus*, *Pinus pinaster, Pinus pinea* and *Cryptomeria japonica* forest provide biomass wastes that can be used to extract EOs (Figure 1).



Aim

Investigation of the chemical composition and the antioxidant activity of eleven EO samples from:

E. globulus,

P. pinaster,

P. pinea, and

C. japonica

obtained from local producers from the mainland Portugal and Azores archipelago.

Materials and Methods

National producers kindly supplied eleven EOs, Table 1, that were stored at -20°C.

- Chemical Analysis of Essential Oils**:** Gas chromatography associated with mass spectrometry [1].
- Antioxidant activity: DDPH method [2].

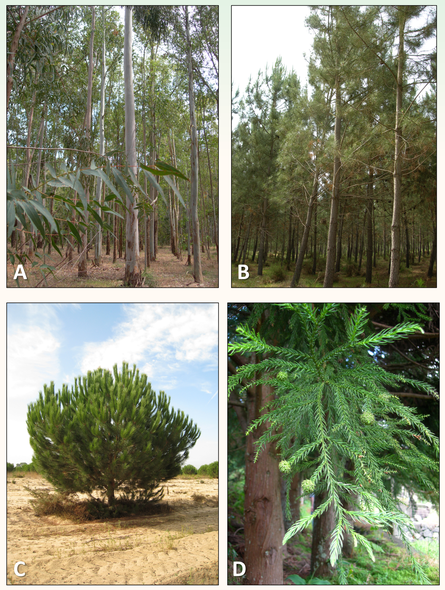


Figure 1. A. *Eucalyptus globulus*, **B.** *Pinus pinaster*, **C.** *Pinus pinea* and **D.** *Cryptomeria japonica*.

Table 1. List of the studied essential oils (EOs), corresponding codes and examples of the EOs (A to D).

Plant species EOs	Code	
Eucalyptus globulus (A)	Eg_OE_1_G	
	Eg_OE_2_B	
	Eg_OE_3_O	
	Eg_OE_4_E	
	Eg_OE_5_P	
	Eg_OE_6_S	
Pinus pinaster (B)	Pp_OE_1_G	
	Pp_OE_2_P	
	Pp_OE_3_S	
Pinus pinea (C)	Ppi_OE_1_B	
Cryptomeria japonica (D)	Cj_OE_1_M	

Results and Discussion

1,8-Cineole (eucalyptol) was the main component of the *E. globulus* EO samples (49-65%) (Table 2). From the three samples of *P. pinaster* EOs, two were dominated by α-pinene (37-45%), and the third showed similar amounts of α-pinene and β-pinene (28% and 29%, respectively) (Table 3). Limonene (73%) was the main component in *P. pinea* EO (Table 4) and α-pinene (26%) in *C. japonica* EO (Table 5).

C. japonica EO showed the highest antioxidant activity (Table 5), whereas the *E. globulus* EO samples showed the lowest antioxidant capacities (Table 2). Among *P. pinaster*, *P. pinea* and *E. globulus* EOs, the results obtained were broad, differing from the producers, probably due to each sample's composition. The ascorbic acid was used as control and the IC_{50} (µg/mL) obtained was 36.0 ± 1.1.

Table 2. *Eucalyptus globulus* EOs main components and antioxidant activity (all the values are mean ± SD).

Eucalyptus globulus							
EOs main components (≥10%)	**IR**	**Eg_OE_ 1_G**	**Eg_OE_ 2_B**	**Eg_O_ 3_O**	**Eg_OE_ 4_E**	**Eg_OE_ 5_P**	**Eg_OE_ 6_S**
α-Pinene	930	13.2	13.3	11.0	21.8	14.7	13.8
1,8-Cineole	1005	65.2	63.2	59.5	53.9	58.2	49.4
Limonene	1009	8.2	17.2	13.7	16.6	12.5	18.0
Antioxidant activity IC_{50} (mg/mL)		197.6 ± 20.4	647.3 ± 5.7	151.8 ± 0.0	652.1 ± 44.6	-	246.7 ± 54.5

Table 3. *Pinus pinaster* EOs main components and antioxidant activity (all the values are mean ± SD).

Pinus pinaster				
EOs main components (≥10%)	**IR**	**Pp_OE_1_G**	**Pp_OE_2_P**	**Pp_OE_3_S**
α-Pinene	930	28.0	44.6	36.5
B-Pinene	963	28.5	23.0	18.8
B-Myrcene	975	11.0	5.0	5.9
Antioxidant activity IC_{50} (mg/mL)		92.0 ± 1.1	-	-

Table 4. *Pinus pinea* EO main components and antioxidant activity (all the values are mean ± SD).

Pinus pinea		
EOs main components (≥10%)	**IR**	**Ppi_OE_1_B**
Limonene	1009	72.8
Antioxidant activity IC_{50} (mg/mL)		109.2 ± 0.9

Table 5. *Cryptomeria japonica* EO main components and antioxidant activity (all the values are mean ± SD).

Cryptomeria japonica		
EOs main components (≥10%)	**IR**	**Cj_OE_1_M**
α-Pinene	930	26.1
Sabinene	958	18.1
Phyllocladene	2006	13.8
Antioxidant activity IC_{50} (mg/mL)		23.1 ± 0.2

Conclusion

It is possible to conclude from these preliminary results that some EO's studied have antioxidant activity, which can be a benefit justified by their promising health-promoting properties

References

[1] A. Neves, J. Marto, A. Duarte, L.M. Gonçalves, P. Pinto, A.C. Figueiredo, H.M. Ribeiro. Flavour and Fragance Journal, 32 (2017) 392-402.

[2] A.K. Mishra, N. Sahu, A. Mishra, A. K. Ghosh, S. Jha, P. Chattopadhyay. Pharmacognosy Journal, 2 (2010) 25-28.

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Health from the sea: *Bifurcaria bifurcata* as a source of bioactive compounds

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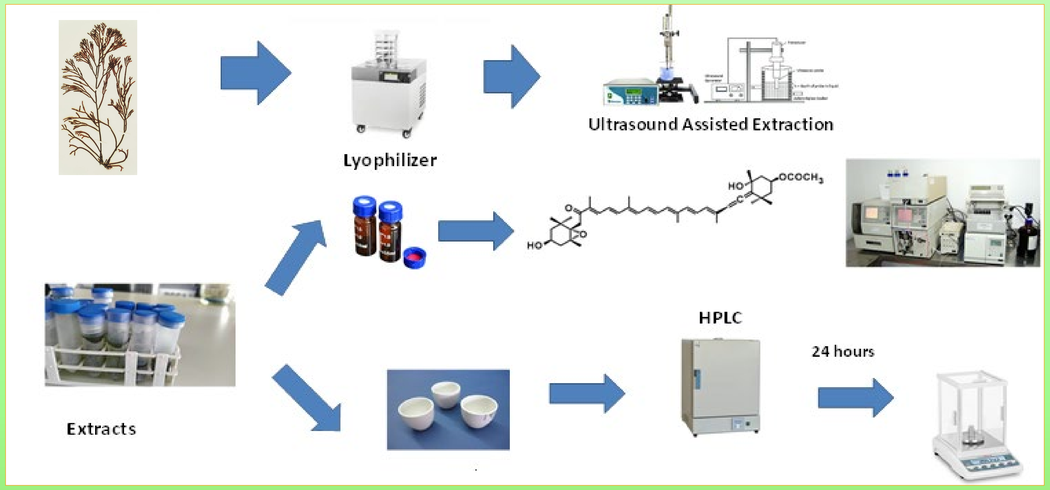
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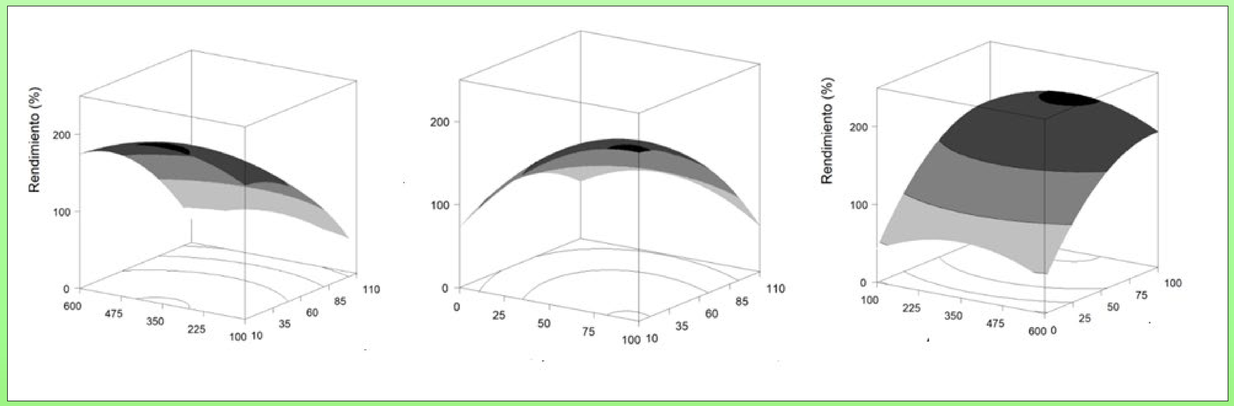
Introduction

The marine environment appears to be a source of compounds used in the manufacture of food, cosmetic and medicinal products. Within all the richness of this medium, algae are potential candidates for the discovery and extraction of bioactive compounds [1]. Brown algae are especially rich in a carotenoid compound called fucoxanthin. Fucoxanthin is considered to be the most abundant natural carotenoid and its consumption has been related to the improvement of various biochemical parameters and with a possible beneficial effect on the loss of fat weight [2].

Methodology

This work performed an optimization of the main variables of ultrasound assisted extraction (UAE) for fucoxanthin obtaining from *Bifurcaria bifurcata*, an alga typically found in the Galician coast. The optimization procedure was built with a Response Surface Methodology (RSM) with 5 levels of three variables: the extraction time (t, 5 to 55 min), the solvent as a percentage of ethanol (S, 35 to 100%) and the power applied (P, 100 to 500 W).





Results

The optimal conditions are:
High Power
Medium Time
High proportion of ethanol .

~260 mg of extract per g of dried weight sample of alga
~2984µg of fucoxanthin per g of dried weight sample of alga

Conclusions

The obtained results suggest that the application of UAE can be considered as an ecological, fast and simple method, suitable to obtain fucoxanthin in short times from *Bifurcaria bufurcata*, allowing the valorization of the algae.

References

[1] Kiuru, P et al., J. Planta Med. 2014, 80, 1234-1246.
[2] Zhang, H et al, Z. 2015, 2015.

Acknowledgements

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IMPROVING THE ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL-ISOLATED β-CARYOPHYLLENE USING A SELF-EMULSIFYING SYSTEM: AN IN VIVO STUDY

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Abstract

(-)-*β*-Caryophyllene (*β*-Cary) is reported to present anti-inflammatory, but its lipophilic character makes it difficult to interact with the gastrointestinal aqueous environment. Nano-emulsifying carriers have been shown to be effective as systems for oral administration of active compounds with low water solubility and they should improve the anti-inflammatory activity *β*-Cary. To test this hypothesis, a lipid-based self-emulsifying drug delivery system (SEDDS) containing *β*-Cary extracted from clove bud oil was prepared and its acute anti-inflammatory effect when orally administered was evaluated by using the carrageenan-induced paw edema in rats. The SEDDS system, consisting of *β*-Cary, surfactant and oily phase, was characterized in relation to morphology and rheology. Wistar rats were distributed into three groups: Controls, which received saline; CAR-50, which received unformulated 50 mg/kg *β*-Cary; and F-CAR, which received formulated 50 mg/kg *β*-Cary. All animals were treated 1 h before the carrageenan injection in the left hind paw. The edema was monitored by plethysmography at 0, 1, 2 and 4 h after the induction. SEDDS containing β-Cary were spherical droplets, whose viscosity tended to decrease following a Newtonian behavior as the temperature increased. The paw edema was similar for the 3 groups at 0, 1 and 2 h, but at 4 h it was lower in F-CAR (-40%) and CAR-50 (-20%) when compared to the control group. The results show that SEDDS containing *β*-Cary was more effective in decreasing the carrageen-paw edema than unformulated *β*-Cary.

Introduction

(-)-*β*-Caryophyllene (*β*-Cary) is a sesquiterpene reported to present anti-inflammatory activity [1] that can be obtained from asymmetric synthesis or, alternatively, from essential oils [2]. Clove buds are rich in eugenol (70-90%), which is widely used in perfumes and flavorings, but also has a considerable amount of *β*-Cary [3]. Eugenol is extracted from clove oil by fractionated distillation, which also permits to separate a fraction exceptionally rich in *β*-Cary [3].

The lipophilic character of *β*-Cary makes it difficult to interact with the gastrointestinal aqueous environment [1], but nano-emulsifying carriers have been shown to be very effective as systems for oral administration of active compounds with low water solubility. Therefore, they should improve the anti-inflammatory activity of the orally administered *β*-Cary. To test this hypothesis, a lipid-based self-emulsifying drug delivery system (SEDDS) containing *β*-Cary was prepared and its acute anti-inflammatory effect when orally administered was evaluated by using the carrageenan-induced paw edema in rats.

Materials and methods

β-Cary from clove bud oil was purchased from Quinarí Fragrâncias e Cosméticos Ltda (Ponta Grossa, Brazil) and chemically characterized by gas chromatography with mass spectrometry (GC-MS).

The SEDDS system, consisting of *β*-Cary, surfactant and oily phase, was characterized in relation to morphology and rheology. Data from rheology were used to obtain the consistency index (k) and flow behavior index (n). For *in vivo* study, Wistar rats were distributed into three groups (n=5): Controls, which received saline; CAR-50, which received unformulated 50 mg/kg *β*-Cary; and F-CAR, which received formulated 50 mg/kg *β*-Cary. All animals were treated 1 h before the carrageenan injection in the left hind paw. The edema was monitored by plethysmography at 0, 1, 2 and 4 h after the carrageenan injection. The animal protocol was approved by the Ethics Committee for Animal Experimentation of UEM.

Results

The chemical characterization of *β*-Cary from clove essential oil reveled a purity of 91% **(Table 1)**. SEDDS containing β-Cary were spherical droplets, as defined using optical microscopy **(Fig. 1)**.

Table 1. Chemical characterization of β-caryophyllene from clove essential oil determined by GC-MS. RT: retention time; LRI: linear retention index.

Compound	RT (minute)	Calculated LRI	Theoretical LRI [4]	Mean ± SD (%)
Copaene	35.89	1374	1374	0.7 ± 0.1
β-Caryophyllene	37.843	1419	1419	91.0 ± 0.2
Humulene	39.229	1453	1452	8.2 ± 0.1

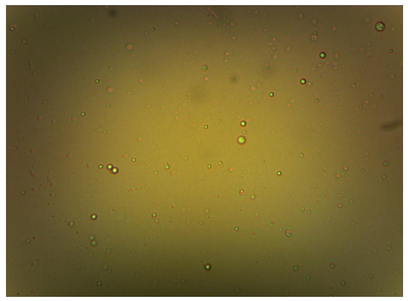


Fig 1. General view in light microscopy of SEEDS containing β-caryophyllene from clove essential oil. SEEDS were diluted with ultrapure water (1:10).

Table 2. Values obtained for the consistency index (k) and flow behavior index (n). The values are the mean ± SD of three replicates. *p < 0.05.

Temperature	37°C	25°C
K ((Pa.s)^n)	0,094000 ± 0.00544*	0.014775 ± 0,007664
n (dimensionless)	0,983875 ± 0.0143	0.995800 ± 0.002979

Rheology was carried out using a gradient and controlled-shear stress rheometer (MARS II; Haake®) and the effect of temperature increase was estimated from 25°C to 37°C. Data from rheology and the Ostwald–de Waele equation (power law) were used to obtain the consistency index (k) and flow behavior index (n) [5]. The values are shown in **Table 2**.

The consistency index (k) represents the average viscosity of all points obtained by a flow curve. The flow behavior index (n) evaluated whether the preparation was Newtonian (n = 1), shear-thinning (n <1) or dilating (n> 1) [5]. The results show that the viscosity tended to decrease following a Newtonian behavior as the temperature increased.

In relation to in vivo study, the paw edema was observed on the first hour after carrageenan injection and it was equal in all groups. At second hour the paw edema was even higher and also equal for all groups. At 4 hour, however, the paw edema was approximately 40% lower in F-CAR and 20% lower CAR-50 when compared to the control group.

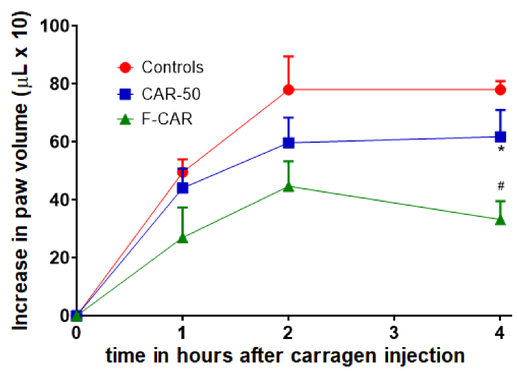


Fig. 2. Time courses of the effects of SEEDS containing (-)-*β*-caryophyllene (*β*-Cary) orally administered on carrageenan-induced paw edema in Wistar rats. Controls received saline; CAR-50 received unformulated 50 mg/kg *β*-Cary; and F-CAR received formulated 50 mg/kg *β*-Cary. * p < 0.05: for difference from Controls; # p < 0.05 for difference from CAR-50.

Conclusion

Overall, this study suggests that the lipid-based self-emulsifying drug delivery system (SEDDS) containing (-)-*β*-Caryophyllene (*β*-Cary) extracted from clove bud oil was more effective in decreasing the carrageen-paw edema in rats than unformulated β-Cary. The dose of 50 mg/Kg of β-Cary in rats would correspond to a human dose of approximately 8 mg/kg as given by the body surface area normalization method [6]. Future approaches will consider additional characterization the SEEDS in relation to continuous phase viscosity, oscillatory rheology and DLS (Dinamic light scattering).

Recommendations

[1] Ames-Sibin AP, Barizão CL, Castro-Ghizoni CV, Silva FMS, Sá-Nakanishi AB, Bracht L, Bersani-Amado CA, Marçal-Natali MR, Bracht A, & Comar JF. J. Cell. Biochem. 119 (2018) 10262–10277. https://doi.org/10.1002/jcb.27369

[2] Maffei, ME. Plant natural sources of the endocannabinoid (E)-β-caryophyllene: a systematic quantitative analysis of published literature. Int. J. Mol. Sci. 21 (2020) 6540. https://doi.org/10.3390/ijms21186540

[3] Nurdjannah N, Bermawie N. Cloves. In: Peter KV, Editor. Handbook of Herbs and Spices, second edition, vol.1. Woodhead Publishing limited, p. 197-215, 2012. https://doi.org/10.1533/9780857095671.197

[4] Adams RP. Identification of Essential Oil Compounds by Gas Chromatography and Mass Spectrometry (fourth ed.), Allured Publishing Corporation, Carol Stream, IL, USA, 2009.

[5] Bruschi ML. Strategies to Modify the Drug Release from Pharmaceutical Systems. 1st ed. Elsevier, 2015. 208 p.

[6] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2007,22:659-661. https://doi.org/10.1096/fj.07-9574LSF

Acknowledgements

We thank the funding agency CAPES and CNPq, for the support and incentive to research. The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020).

PHYTOCHEMICAL CHARACTERIZATION AND EVALUATION OF THE BIOACTIVE PROPERTIES OF TISANES PREPARED FROM AROMATIC AND MEDICINAL PLANTS

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Introduction

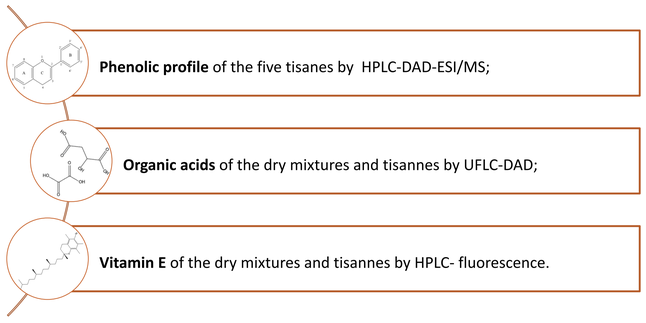
The antioxidant, antidiabetic and anti-inflammatory properties of different species of Medicinal and Aromatic plants (MAP) have been extensively described, being its most common form of consumption the tisane, or popularly known as herbal teas or infusions [1-3]. The present study aimed to characterize the phenolic compounds by HPLC-DAD/ESI-MS of five tisanes prepared from mixtures of MAP (**Figure 1**), as also study the bioactive properties, namely antioxidant, antimicrobial, anti-inflammatory, cytotoxic, anti-tyrosinase, and anti-diabetic activities. It was also proposed to characterize its composition in organic acids and tocopherols by HPLC coupled to a DAD and fluorescence detector, respectively.

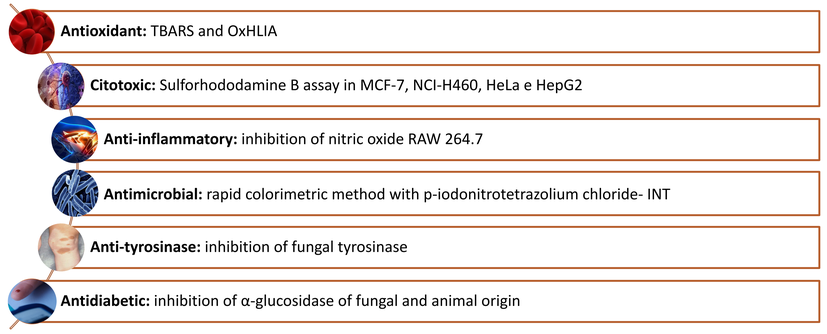
Materials



Figure 1. Description of the MAPs that constituted the five samples of studied herbal tea.

Methodology





Results

Thus, the tisane composed by lemon thyme, Shrubby St. John's Wort, cloves and cinnamon, was the most promising mixture, presenting the lowest values for the lipid peroxidation inhibition, anti-inflammatory, cytotoxicity against breast adenocarcinoma tumor cell line and anti-diabetic activity. In addition, it showed the highest concentration of tocopherols (γ-isoform), phenolic acids (caffeoylquinic acids derivatives) and flavan-3-ols (catechin derivatives). For the anti-hemolytic, antimicrobial (Gram-negative bacteria), and cytotoxic (cell lines HepG2, NCI H460 and HeLa) activities, the tisanes containing also lemon thyme as the major component, were highlighted as the best mixtures. Rosmarinic acid and its derivatives were the most abundant phenolic compounds in all tisanes, followed by glycosylated flavanols/flavones.

Table 2. Quantification (mg/g of extract) of the phenolic and non-phenolic compounds present in tisanes prepared from five MAP mixtures (mean ± SD).

	T1	T2	T3	T4	T5
Total Phenolic acids	28.51±0.28^{e}	40.59±0.13^{b}	31.73±0.23^{d}	34.34±0.10^{c}	68.30±0.78^{a}
Total isoflavones	nd	nd	17.81±0.14	nd	nd
Total flavanones	nd	0.086±0.004^{d}	6.495±0.23^{b}	7.88±0.12^{a}	3.17±0.07^{c}
Total flavonoids	1.42±0.05^{e}	19.7±0.3^{d}	53.98±0.08^{a}	20.58±0.19^{c}	27.66±0.08^{b}
Total flavan-3-ol	nd	nd	nd	nd	3.64±0.04
Total other compounds	nd	0.215±0.004	3.16±0.02	nd	nd
Total phenolic compounds	29.93±0.34^{e}	60.58±0.45^{d}	113.19±0.68^{a}	62.79±0.41^{c}	102.78±0.84^{b}

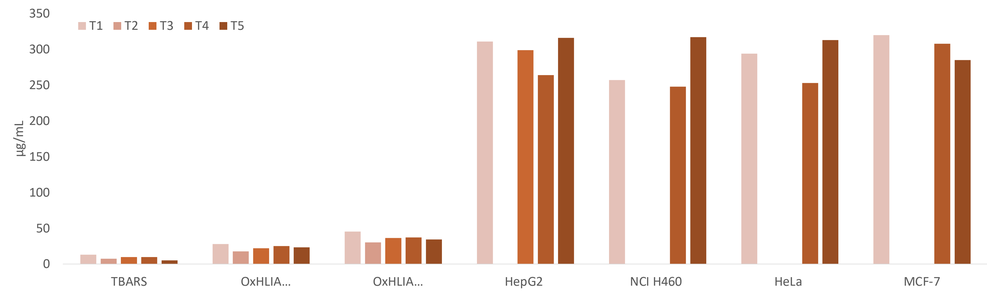


Figure 2. Antioxidant and cytotoxic EC_{50} and GI_{50} values of the five tisanes, respectively.

The competitiveness of phenolic compounds from the mixtures of aromatic and medicinal plants is noticeable, therefore, a synergism study between the various mixtures is suggested to evaluate and optimize the different proportions of the plants;

Conclusion

These results suggest that tisanes are promising sources of bioactive compounds with high added value that can be applied in several industrial fields, such as the food industry.

References

[1] P. Konieczynski, M. Wesolowski, I. Radecka, e P. Rafalski, "Bioavailable inorganic forms of essential elements in medicinal plants from Northern Poland", Chem. Speciat. Bioavailab., vol. 23, no 2, p. 61–70, 2011.
[2] R. F. Milani, M. A. Morgano, E. S. Saron, F. F. Silva, e S. Cadore, "Evaluation of direct analysis for trace elements in tea and herbal beverages by ICP-MS", J. Braz. Chem. Soc., vol. 26, no 6, p. 1211–1217, 2015.
[3] S. Nookabkaew, N. Rangkadilok, e J. Satayavivad, "Determination of trace elements in herbal tea products and their infusions consumed in Thailand", J. Agric. Food Chem., vol. 54, no 18, p. 6939–6944, 2006.

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0377_Iberphenol_6_E and and TRANSCoLAB 0612_TRANS_CO_LAB_2_P,
and also to the Ministry of Education, Science and Technological Development of Republic of Serbia (451-03-68/2020-14/200007).

ANTIMICROBIAL POTENTIAL OF *CYMBOPOGON MARTINII* ESSENTIAL OIL AND DENTAL IRRIGANT BASED ON IT AGAINST ROOT CANAL BIOFILMS

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Abstract

The interest in natural products applications as antimicrobial agents is rapidly growing, mainly due to increasing microbial resistance to conventional therapeutics. Essential oils (EOs) seem to be especially attractive, since many of them possess high antimicrobial potential and none revealed resistance issue. EOs have already found applications in endodontic practice, mainly as sealers, but their antimicrobial potential has not been explored in this field yet. Taking this into account, the objective of this study was to investigate the antibacterial and antibiofilm efficacy of *Cymbopogon martinii* EO against bacterial strains relevant for endodontic therapy. Chemical composition of EO was determined by GC-MS/FID analysis. Antibacterial potential was screened in microdilution assay, detecting minimal inhibitory and bactericidal concentrations (MICs and MBCs) and performed on *Enterococcus faecalis, Streptococcus mitis* and *Streptococcus sanguinis*. Eradicating potential against biofilms preformed of each bacterial species as well as of all three bacteria, was determined *in vitro* in crystal violet (CV) assay. For screening of *in situ* effect against biofilm preformed in root canals of extracted teeth, the EO-based irrigant was formulated. Its antibiofilm potential was evaluated by plate counting (PC) assay, performed with single *E. faecalis* biofilm and multispecies biofilm composed of all three strains. Single *E. faecalis* biofilm was selected since it is the most relevant intracanal pathogen, while multispecies biofilm was screened to better mimic clinical conditions.
Chemical analysis revealed that geraniol was the most abundant constituent (83.5%), followed by geranyl acetate (8.4%). Determined MIC and MBC values were the lowest for *E. faecalis* (0.12 and 0.25 mg mL^{-1}, respectively), and the highest for *S. sanguinis* (2.95 and 5.90 mg mL^{-1}, respectively). CV assay pointed out that EO successfully eradicated *in vitro* preformed biofilms of *E. faecalis* and *S. mitis* (approximately 30% reduction), but had no effect on *S. sanguinis* and multispecies biofilms. EO-based irrigant used for determination of *in situ* antibiofilm potential was pre-screened for its physicochemical characteristics: a mean particle diameter and polydispersity index was 101 ± 3 nm and 0.36 ± 0.06, respectively. PC assay showed that eradicating potential of applied irrigant was weak against both *E. faecalis* and multispecies intracanal biofilm (reduction of log CFU was 0.76 and 0.52, respectively). However, successive irrigation with conventional dental irrigants (sodium hypochlorite and sterile saline), followed by *C. martini* EO-based irrigant, notably increased the biofilm reduction (1.89 and 1.69, respectively).
In conclusion, *C. martini* EO possesses notable antibacterial potential that could be further investigated for different dental applications.

Introduction

The growing antimicrobial resistance constantly stimulates searching for new therapeutic agents among bioactive natural substances. The essential oils (EOs) seem to be of great importance, due to the fact that they do not support the resistance. As far as it is known nowadays, there is no documented case of bacterial resistance to EOs.
New antimicrobial agents are investigated for the use in different fields, including dentistry. Endodontics, the branch of dental medicine that concerns with the therapy of dental pulp diseases, is especially interested in new efficient antimicrobials, since some infections of pulp tissue are difficult to cure and could be recurrent. For that reason, there is an interest to study possible application of EOs as antimicrobials in endodontics, especially due to the fact that EOs have already been used in endodontic practice, mainly as sealers. To postulate adequate methodology for the study of antimicrobial potential of test substances that should be used in endodontics, it is important to bear in mind that the most prevalent mode of bacterial life within infected root canals is biofilm. Furthermore, the biofilm is mostly consisted of several bacterial species (multispecies biofilm), but the *Enterococcus faecalis* is considered to be with the highest incidence in unsuccessfully treated teeth root canals. Strains of *Streptococcus mitis* and *Streptococcus sanguinis* are also frequent as a co-participants in infected root canals.
On the other hand, when we selected the EO that should be tested, we were guided with the available data concerning their antibacterial potential, especially against the species being established in the infected root canals. The EO of *Cymbopogon martinii*, being reach in potent antimicrobial geraniol, was selected. Furthermore, the adequate delivery system of EO (EO-based irrigant), to be used within root canals, has to be developed.

The aim of the study

The aim was to chemicaly characterize EO of *C. martinii* and to investigate its antibacterial and antibiofilm activity against bacteria relevant for endodontic therapy, i.e. *E. faecalis, S. mitis* and *S. sanguinis*. Antibiofilm effect was screened on biofilms preformed *in vitro*, and *in situ* in root canals of extracted teeth.

Materials

1. Essential oil of *Cymbopogon martinii*



constituent	% (w/w)	constituent	% (w/w)
myrcene	0.2	geranyl	0.2
limonene	0.1	**geranyl-acetate**	**8.4**
cis-β-ocimene	0.3	cis-caryophyllene	1.1
trans-δ-ocimene	1.3	geranyl butanoate	0.1
linalool	1.5	caryophyllene oxide	0.1
neral (citral B)	0.4	2Z,6E-farnesol	0.7
geraniol	**83.5**	geranyl tiglate	0.5
geranial (citral A)	1.5	TOTAL	100%



2. Human premolars extracted for orthodontic indications

3. Identified bacterial isolates originated from infected root canals

Enterococcus faecalis
Streptococcus mitis
Streptococcus sanguinis



Methodology

- Chemical characterization of the *C. martinii* EO was provided by GC-MS/FID analysis.
- EO-based irrigant was prepared by adding of EO and ethanol (1:1 v/v) mixture (50 µL) in distilled water (up to 6 mL) and ultrasound treatment at 125 rpm.
- Characterization of EO-based irrigant involved determination of particle size and polydispersity index by use of Dynamic Light Scattering.
- Minimal inhibitory and bactericidal concentrations (MICs and MBCs, respectively) was determined in resazurin-incorporated microdilution assay.
- *In vitro* eradication of single species and multispecies biofilms was determined by measuring of total biofilm biomass reduction in crystal violet (CV) assay.
- *In situ* determination of single species and multispecies biofilms was monitored in root canals of extracted teeth by performing plate counting (PC) assay.
- Scanning electron microscopy (SEM) was used to visualize efficacy of EO-based irrigant in biofilm eradication.

Results

Table 1. Antibacterial effect of *C. martinii* EO

Clinical isolates		*C. martini* EO (mg mL^{-1})	TAP (µg mL^{-1})
S. mitis	MIC	1.30	0.125
	MBC	2.60	0.250
S. sanguinis	MIC	2.95	0.125
	MBC	5.90	0.250
E. faecalis	MIC	0.12	0.125
	MBC	0.25	0.500

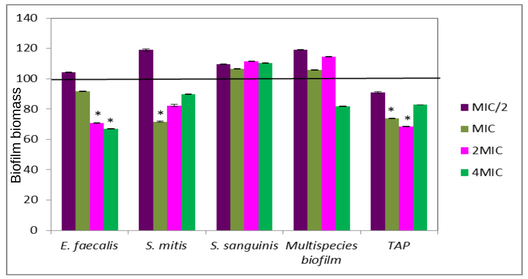


Figure 1. Antibiofilm effect of *C. martinii* EO determined against pre-formed biofilms in CV assay
* Statistically significant difference compared to untreated control (100% biomass of biofilm)

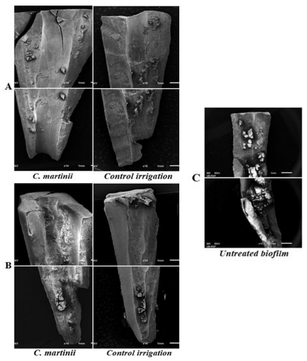


Figure 2. SEM images of biofilms irrigated singularly by EO-based irrigant or control – 1.5% NaOCl (A), successively by both EO-based irrigant and 1.5% NaOCl (B), or un-irrigated (C)

EO was screened for *in vitro* antibacterial and antibiofilm effect, while EO-based irrigant was monitored for *in situ* antibiofilm potential. Prior to activity testing, both agents were adequately characterized.
Chemical analysis revealed that the *C. martinii* EO was abundant in oxygenated monoterpenes (OM), with dominant participation of geraniol and geranyl acetate (91.9% of the total EO). Antibacterial potential, determined in microdilution assay, pointed out the MIC and MBC values (Table 1). Further on, screening of eradication potential on pre-formed biofilms, performed by CV assay, showed moderate activity (up to 30%) against *E. faecalis* and *S. mitis* (Fig. 1).
Considering EO-based irrigant, its characterization included determination of a mean particle diameter and polydispersity index (101 ± 3 nm and 0.36 ± 0.06, respectively). It was screened for the activity in *in situ* conditions that mimic clinical situations (in root canals of extracted teeth). Antibiofilm properties were determined against single *E. faecalis* and multispecies biofilms, since the first one is consisted as one of the of the most relevant intracanal pathogens, while the second one better mimic clinical conditions. Antibiofilm properties were determined for sole EO-based irrigant, as well as in procedures involving successive irrigations by conventional medicament sodium hypochlorite (NaOCl), sterile saline (intermediate irrigant) and EO-based irrigant. Obtained results (Table 2) indicated that EO-based irrigant could be used only as an adjuvant to enhance overall antibiofilm effect. Visualization of antibiofilm activity by SEM confirmed this result (Fig. 2).
Finally, observed antibacterial and antibiofilm effect of *C. martinii* EO and irrigant based on this EO used as an active principle, could be ascribed to high abundance of OM, and especially the high percentage of geraniol.

Table 2. Antibiofilm effect of *C. martinii* EO-based irrigant detrmined in PC assay performed for biofilms pre-formed in root canals of extracted teeth

	***E. faecalis* biofilm**				**multispecies biofilm***			
	EO-based irrigation		**control irrigation**		**EO-based irrigation**		**control irrigation**	
	before	after	before	after	before	after	before	after
	singular irrigation[1]				singular irrigation[1]			
log CFU	5.3±0.3	4.6±0.3	6.2±0.13	5.4±0.3	5.7±0.3	5.2±0.3	6.0±0.1	5.5±0.3
Δ(log CFU)[3]	0.8		0.9		0.5		0.5	
	successive irrigation[2]				successive irrigation[2]			
log CFU	6.1±0.1	4.2±0.9	5.5±0.2	4.2±0.3	6.1±0.1	4.4±0.9	6.2±0.2	4.7±0.3
Δ(log CFU)[3]	1.9		1.3		1.7		1.4	

*Multispecies biofilm formed from the *E. faecalis*, *S. mitis* and *S. sanguinis* inoculums.
[1]Singular irrigation by EO-based irrigant or by control (1.5% NaOCl).
[2]Successive irrigation by 1.5% NaOCl, 0.85% NaCl (both in treatment and in control), and additionally by EO-based irrigant (in treatment).
[3]The value of biofilm reduction, determined by subtraction of log CFU values of un-irrigated and irrigated biofilms.

Conclusion

This work reports the antibacterial and antibiofilm activity of *Cymbopogon martini* EO and irrigant based on it, against bacterial strains of *Enterococcus faecalis*, *Streptococcus mitis* and *Streptococcus sanguinis* isolated from infected dental root canals. The fact that EO-based irrigant enhanced disruption of multispecies biofilms, composed of these three species and pre-formed in root canals of extracted teeth, encourages further research for possible use in endodontics and other branches of dental medicine.

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ANETHOLE IMPROVES THE OXIDATIVE STATE OF RATS WITH ADJUVANT-INDUCED ARTHRITIS

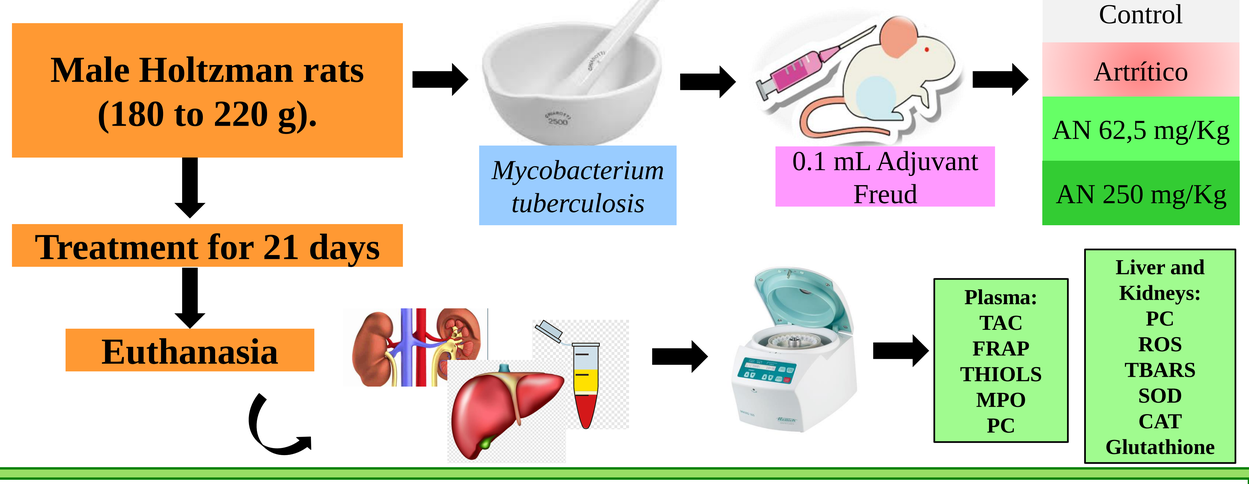
Carla Indianara Bonetti,[1] Jackeline Joyce da Silva Moreira,[1] Edvalkia Magna Teobaldo da Rocha,[2] Mariana Macedo Carmona,[1] Anacharis Babeto de Sá Nakanishi,[1] Jurandir Fernando Comar,[1] Adelar Bracht,[1] Ciomar Aparecida Bersani Amado,[2] Lívia Bracht.[1]

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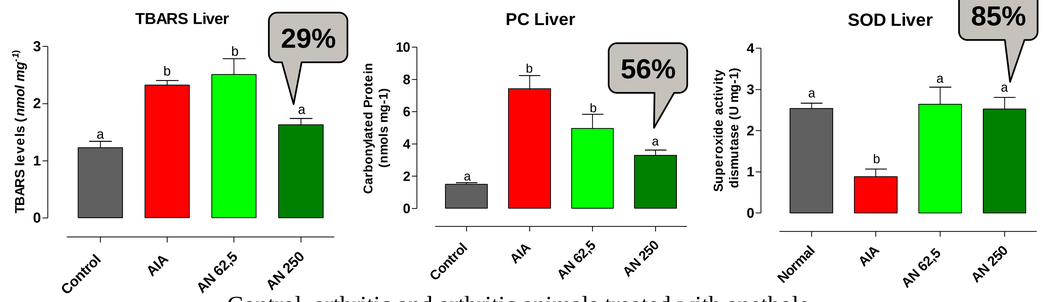
Introduction

Rheumatoid arthritis (RA) is a chronic multisystem disease, which the main feature is a persistent inflammatory synovitis [1]. Anethole, a substance present in the essential oil of star anise (*Illicium verum*), can reduce inflammation and joint damage in adjuvant-induced rheumatoid arthritis model (AIA) [2]. However, studies investigating the effects of anethole on oxidative stress induced by arthritis are lacking. Thus, the aim of the present study was to evaluate the effects of anethole treatment on oxidative stress in arthritic bearing rats.

Materials and methods

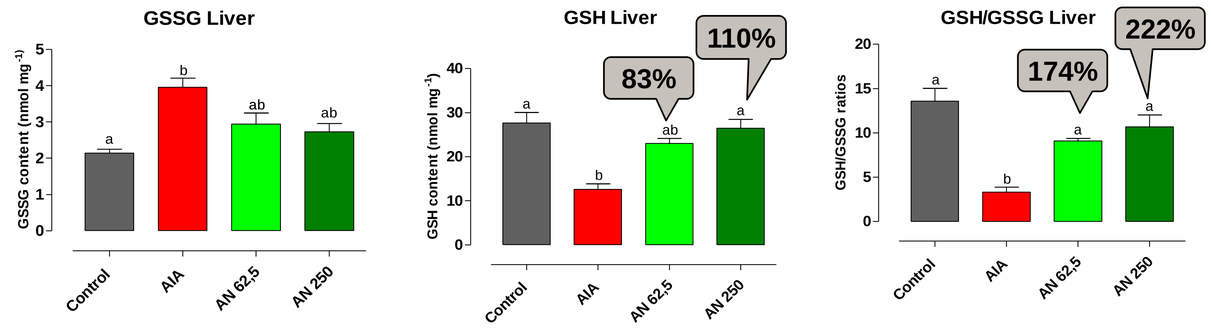


Results

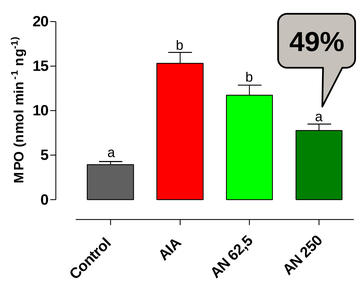


Control, arthritic and arthritic animals treated with anethole.

Results



Control, arthritic and arthritic animals treated with anethole.



Anethole treatment was not able to prevent the decreased levels of thiols or the reduced total antioxidant capacity caused by arthritis. Anethole treatment (62.5 and 250 mg/kg) was not able to prevent the decreased catalase activity in the liver and kidneys.

Conclusion

Therefore, our results demonstrate that anethole, specially at the dose of 250 mg/Kg, is able to improve the antioxidant defense and to reduce oxidative damage in arthritic animals.

References

[1] G. S. Firestein. Evolving concepts of rheumatoid arthritis. Nature. 423 (2003) 356.
[2] A. M. V. Ritter. Inflam Res. 66 (2017) 725.
[3] V. G. Correa, A. B Sá-Nakanishi, G. A. Gonçalves, L. Barros, I. C. F. R. Ferreira , A. Bracht, R. M. Peralta. Food Funct. 10 (2019) 5682.

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IN VIVO EFFECT OF AQUEOUS 20% PROPOLIS EXTRACT ON THE CONCENTRATION OF *Malassezia* SPP. IN DOGS WITH BILATERAL CHRONIC EXTERNAL OTITIS

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Abstract

Chronic external otitis (CEO) is a common syndrome in adult dogs, causing financial and psychological damage to the owner, in addition to affecting animal health [1]. In these conditions, it is common to see otopathic conditions with a marked presence of yeasts of the genus *Malassezia*, worsening the clinical presentation, making therapy difficult and preventing the restoration of the patient's health [1]. It is necessary to control this fungal population, either through traditional antimycotic drugs or other substances with similar properties, such as herbal medicines and animal derived products. Propolis is a natural resin collected by bees (*Apis mellifera*), having proven antifungal activity *in vitro*, but its effectiveness *in vivo* is still the subject of discussion in the scientific community [1]. The objective of this research was to evaluate the concentration of *Malassezia* spp. present in the ear canal of dogs with CEO and subjected to topical exposure to aqueous propolis extract (APE) 20%.

Introduction

CEO can be conceptualized as a lasting inflammation of the external auditory canal with the involvement of different etiological agents. Its prevalence in dogs in Brazil exceeds the rate of 70% of animals with otopathies already installed. The yeast *Malassezia* spp. it is considered an autochthonous inhabitant and an opportunistic pathogen in the healthy external auditory meatus of dogs, as well as in the inflamed, increasing its concentration when this microenvironment undergoes alterations that allow its selective development. Despite the lack of knowledge of the true role of this fungus in optical environments of healthy and otitic dogs, it is known that its overpopulation prevents the resolution of an already installed picture of otitis (regardless of the cause). Even with the broad antifungal arsenal available on the market, many of these products have drawbacks, such as high cost, low biological safety and / or safety due to the resistance of the microorganism. Propolis is a resin collected by bees of the species *Apis mellifera* from different parts of plants such as flower buds, leaves and bark of trees. During their collection, the bees mix the collected wax and propolis, together with enzymes present in their saliva, enriching it with their own components. This combination has antibacterial properties and produces fungicidal effects that serve to protect the hive against diseases and climatic agents. At least 180 different compounds have been identified so far in propolis, however, the therapeutic effects are attributed to the different phenolic compounds that make up this substance. Of these, flavonoids can be considered the main compounds, with some phenolic acids and their esters, phenolic aldehydes, alcohols and ketones. The application of propolis in different segments of medicine is wide, but there are no conclusive studies on its antifungal action *in vivo* on *Malassezia* spp. present in the ear canal of dogs. Recent work reveals that there is antifungal activity *in vitro* even in very small concentrations in the optical environment, with a strong inhibitory effect on the growth of *Malassezia* spp. isolated from dog ear ducts, however, nothing has been recorded so far with *in vivo* experiments using dogs with CEO.

Materials and Methodology

Fifteen adult dogs (varied weight, size and breed) were used, who had a history, clinical signs and ear exams compatible with CEO **(Figure 1)**. Three moments were selected to collect auditory exudate (D0 = pre-treatment; D5 = five days of treatment; and D10 = ten days of treatment) for yeast counting in culture medium (CFU/mLx10^2) and conventional optical microscopy (YB/F) [2]. The experiment started with the instillation of six drops / animal of the APE 20% **(Figure 2)** in each ear every eight hours, for ten consecutive days. No other medication or therapeutic maneuver was used in these patients.



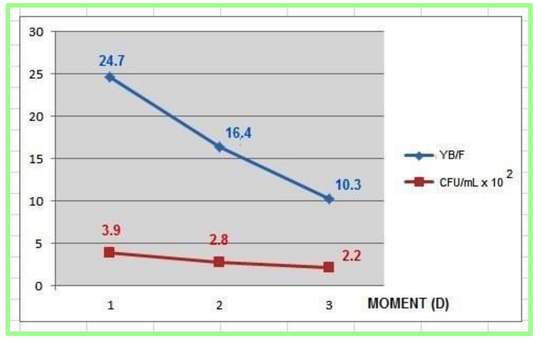
Figure 1: Labrador Retriever dog, eight years old, male, with CEO. Strong erythema and alterations of hyperkeratosis and melanin pigmentation are visible.



Figure 2: Crude propolis extract powder, ready for dilution in deionized aqueous solution.

Results

The analysis of the data shows that there was a significant regression ($p<0.05\%$) in the yeast population, both in the counting in culture medium and in conventional optical microscopy (**Graphic 1** and **Figures 3/4**). The yeast cell count per field (YB/ F) on the slides presented, proportionally, a greater drop than that verified for the culture alone **(Graphic 1)**. It is likely that the methodology used for both procedures may have interfered with this result, but even so, the falls in both tests are statistically significant ($p < 0.05$).



Graphic 1: Concentration of *Malassezia* spp. in the ear canal of dogs with chronic otitis externa before and after exposure to therapy with 20% aqueous propolis extract.



Figure 3: *Malassezia* spp. on modified Sabouraud agar.

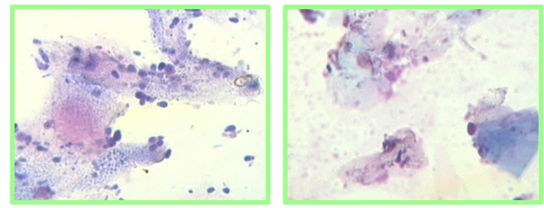


Figure 4: Conventional optical microscopy of a dog before *(left)* and after 10 days *(right)* of treatment with propolis 20%. Note the reduction in the yeast concentration at the end of therapy.

Conclusion

It is concluded that the isolated use of 20% aqueous propolis extract in dogs with chronic external otitis leads to a reduction in the yeast population of *Malassezia* spp. *in vivo*, suggesting that this practice may be associated with conventional canine otitis externa therapy.

References

[1] Betancourt, N.T. García-Contreras, L. Sánchez, T.A.C. Journal of Veterinary Medicine, 5 (2015) 11.
[2] Compendium of Methods for the Microbiological Examination of Foods. Downes, F.P., Ito, K. (Eds.), Washington, American Public Health Association, 2001.

Acknowledgements

[1] Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG.
[2] Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq.

Relevance of Horse Chestnut (*Aesculus hippocastanum* L.) Saponins in Health and Cosmetics

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NPA Natural products application: Health, Cosmetic and Food
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Abstract

The *Aesculus hippocastanum* L. saponins have a trisaccharide chain at C3 of the aglycone and a β-glucopyranosyl unit attached at C4 of the glucuronic acid. Aescin is the main active component and responsible for most of its medicinal properties, which were revised in this study.

Introduction and Aims

Saponins are steroidal glycosides or polycyclic terpenes resulting from the secondary plant metabolism and have many beneficial characteristics and applications. They can be used to fight and cure many diseases and health issues, as well as in cosmetics and as natural alternatives for food preservation and packaging [1,2]. Due to the finite resources our planet can provide, and the increasing potential for saponins in various sectors, it is necessary to find alternative sources of these pharmacologically active high-added value compounds. This study aims to provide an overview of the bioactive properties of saponins that can be found in *A. hippocastanum* L. (Figure 1).



Figure 1. *Aesculus hippocastanum* fruits.
Source: https://flora-on.pt/

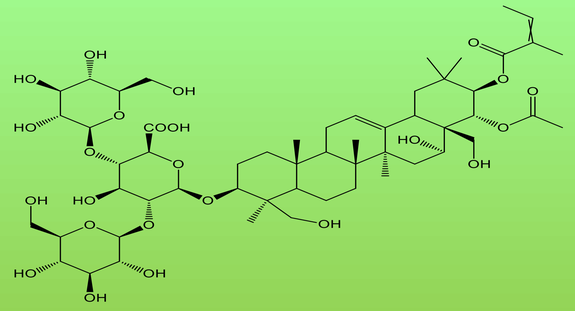


Figure 2. Chemical structure of the main component of aescin.

Discussion

The saponins found in *A. hipposcastanum* L. have a trisaccharide chain at C3 of the aglycone and a β-glucopyranosyl unit attached at C4 of the glucuronic acid. Aescin (Figure 2) is the main active component and responsible for most of its medicinal properties [2,3]. These saponins have already been described as having more antioxidants than vitamin E and can protect cells from free radicals and reactive species [2,4]. These compounds are shown to have positive effects on venous insufficiency and associated conditions, they reduce capillary fragility and help prevent the leakage of fluids into surrounding tissues, thus presenting beneficial effects on human skin [2]. Due to these properties, *A. hippocastanum* saponins can be used in cosmetic formulations with an anti-aging capacity [2]. The bark of this plant has also been used to treat dermatitis, while the leaves and seeds are used for their anti-inflammatory effects [3]. β-Aescin (the main active compound of aescin) is a natural inhibitor of leukemic cell proliferation and may be useful for new anti-leukemic drugs [5]. β-Aescin has also been reported to be effective for the treatment of colon cancer [4].

Conclusion and Future Studies

Given the high added-value of aescin, it would be interesting to develop an extraction method that maximizes the recovery of these compounds from *A. hippocastanum* in a sustainable way to promote resource-use efficiency.

References

References
[1] C.Y. Cheok, H.A. Karim Salman, R. Sulaiman, Food Research International, 59 (2014) 16.
[2] J.A. Wilkinson, A.M.G. Brown. International Journal of Cosmetic Science, 21 (1999) 437.
[3] G. Foca, et al. in Nuts and Seeds in Health and Disease Prevention. V. Preedy, R. Watson (Eds.), Academic Press, 2011, 653.
[4] Y.P. Niu, W.L. Journal of Pharmacy and Pharmacology, 60 (2008) 1213.
[5] N. Yang-Ping, W. Li-Mao, Leukemia & Lymphoma, 49 (2008) 1381.

Acknowledgements

To FCT for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020); for the M. Añibarro-Ortega PhD grant (2020.06297.BD) and the contracts of J. Pinela (CEECIND/01011/2018) and L. Barros through the individual and institutional scientific employment program-contract, respectively; to C. Albiston grant through CIMO funds; and to the FEDER-Interreg España-Portugal programme for financial support through the Project TRANSCoLAB 0612_TRANS_CO_LAB_2_P and to ERDF through the Regional Operational Program North 2020, within the scope of Project GreenHealth - Norte-01-0145-FEDER-000042.

Lee Index Variation and Glucose Clearance Rate in an Animal Model Of Obesity with Animals Supplemented with *Ganoderma lucidum*

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Abstract

The consumption of diets high in sugars and saturated fat are associated with obesity and other comorbidities, as well as glucose intolerance. Obesity can be influenced by the regular consumption of natural bioactive compounds like those found in *Ganoderma lucidum* mushroom (GL). The main objective of this work is to determine body mass variations (Lee Index) and serum glucose levels in animals fed with diets supplemented with three concentrations of an hydroethanolic extract of GL. The fruiting bodies of GL were provided by Bioreishi- Agricultura Biológica, Lda. Forty-eight male mice (C57BL/6) were acquired and divided into 5 groups: G1-Western Diet 0.2% Cholesterol (WD); G2-Western Control (WC); G3-WD+0.7%g/kg of *G. lucidum*; G4-WD+1.4%g/kg of *G. lucidum*; G5- WD+2.8%g/kg of *G. lucidum*. At 7th and 13th weeks of study, the animals were measured (nasoanal distance in cm) in order to calculate the Lee Index (cubicle root of the weight (g)/nasoanal length (cm) x 1000). Obesity was defined by a Lee index>310. Glucose intolerance test was performed on 4 animals in each group. The animals were fasted overnight and in the early morning, a 50% glucose solution (2g/kg) was injected intraperitoneally. Blood samples were collected one hour before glucose administration (time zero) and 30, 60 and 120 minutes later. Serum glucose was determined with the OGCare equipment. Glucose concentration values at time zero were considered control values. All ethical issues were followed (approval nº 8776). The chemical composition of the extract was profiled by HPLC-DAD-ESI/MS. Ganoderic acid H and p-hydroxybenzoic acid were the main triterpenic and phenolic acids found in the extract, respectively. In respect to the Lee index, the values show that all animals in the experimental groups became obese. In both weeks, G5 showed the highest values (353.45 ± 12.43 and 351.77 ± 12.24 respectively). At week 7, G1 and G2 differed statistically ($p<0,05$) from G5. Although G1 and G2 have the lowest Lee values in both weeks, they are the only ones that increase from week 7 to week 13, in contrast to the groups supplemented with GL. Regarding the glucose intolerance test, recorded glucose values at 30 minutes after the injection were increased in all groups, returning to normal values two hours later. At 13th week, the glucose values were increased in all groups in the 30 and 60 minutes after the intraperitoneal injection. At the end of two hours, the values decreased, but did not return to normal values in all the groups. Although animals in all experimental groups remained obese, animals supplemented with GL decreased their Lee Index. At 7th week the animals showed a normal glucose clearance, but in the last week of the study glucose values did not return to the values initially recorded after two hours, showing that the animals were not able to maintain the glucose clearance rate.

Introduction

Obesity is defined as the excessive accumulation of fat in adipose tissue, resulting from the imbalance in consumption patterns, energy expenditure and physical activity [1,2]. Although there are treatments for this disease (drugs or surgery), side effects or complications may arise, making it necessary to discover new products, in which natural products with anti-obesity properties stand out [3,4]. Mushrooms like *Ganoderma lucidum* have been added to human diets and have several medicinal properties, being a great value in the prevention and treatment of various diseases [5]. The main objective of this work was to determine the variations in the animals' body mass, associating with the Lee index as well as serum glucose levels. These animals were fed diets supplemented with three concentrations of a hydroethanolic extract of *Ganoderma lucidum*.

Materials & Methodology

Fruiting bodies of *G. lucidum* were supplied by Bioreishi-Agricultura Biológica, Lda. Forty-eight male mice C57BL/6 were acquired and divided into 5 experimental groups: Group1-Western Diet 0.2% Cholesterol (WD); Group 2-Western Control (WC); Group 3-WD+0.7%g/kg of *G. lucidum*; Group 4-WD+1.4%g/kg of *G. lucidum*; Group 5- WD+2.8%g/kg of *G. lucidum*. To calculate the Lee index (root of the weight cubicle (g)/nasoanal length (cm) x 1000) at the 7th and 13th weeks of study, the animals were measured (nasoanal distance in cm) and weighed. For values higher than 310, the animals are considered obese. To perform the intraperitoneal glucose tolerance test, 4 animals from each group under study were used. The animals were subjected to an overnight fast and at the beginning of the following morning a 50% glucose solution (2g/kg) was injected intraperitoneally (Fig.1A). Blood samples were collected through a minimal cut of the animals' tail tip one hour before glucose administration (time 0) and 30, 60 and 120 minutes later, using a glucometer (OGCare) (Fig.1B). The glucose concentration values at time zero were considered to be control values. All ethical questions were followed (approval nº. 8776).

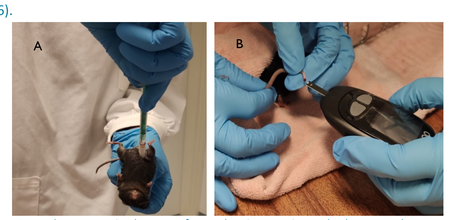


Fig 1: Procedures required to perform the intraperitoneal glucose tolerance test. A- Intraperitoneal injection of the glucose solution; B- Measurement of blood glucose concentration values with the glucometer.

Results

The chemical composition of the extract was evaluated by HPLC-DAD-ESI/MS, where ganoderic acid H and *p*-hydroxybenzoic acid were the main triterpenic and phenolic acids found in the extract, respectively. The results show that all animals in the experimental groups became obese (Lee index greater than 310) (Fig.2).

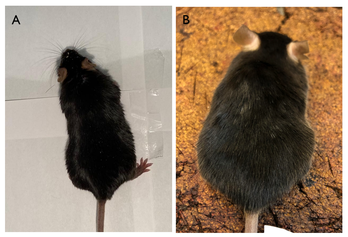


Fig 2: Development of obesity in animals under study. A- Animal at the beginning of the study G4; B- Animal at the end of the study G4.

Results

In the two weeks, the highest Lee index values were found in G5. At week 7, G1 and G2 differed statistically from G5 ($p<0.05$). Although G1 and G2 have the lowest Lee valuesin the two weeks, they are the only ones that increase from week 7 to 13, unlike the groups supplemented with GL (Table 1).

Table 1: Lee index values calculated for each experimental group, at week 7 and 13 (M ± SD). *Different statistically from G5 ($p<0,05$).

	Lee Index	
Group	**Week 7**	**Week 13**
G1 (WD)	331±9,97*	334,62±9,39
G2 (WC)	327,76±25,16*	332,03±35,16
G3 (WD+0,7%GL)	345,22±13,80	344,74±10,08
G4 (WD+1,4%GL)	348,94±7,75	344,85±7,04
G5 (WD+2,8%GL)	353,45±12,43	351,77±12,24

The intraperitoneal glucose tolerance test showed that glucose values recorded 30 minutes after injection were increased in all groups, returning to normal valuestwo hours later. At week 13, glucose values were increased in all groups 30 and 60 minutes after intraperitoneal injection. After two hours, the values decreased, but did not return to normal values in all groups (Fig.3).

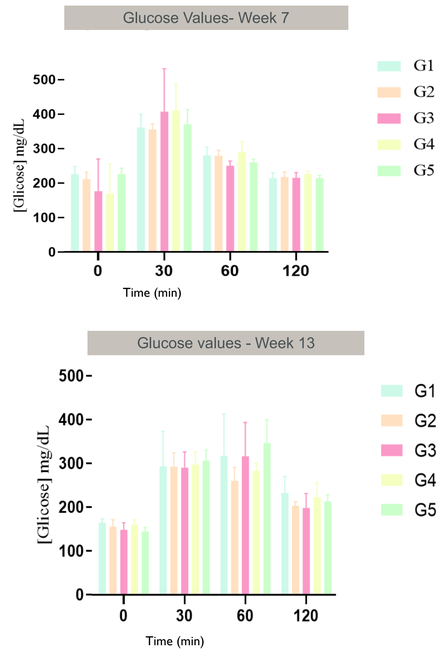


Fig.3: Glucose values recorded at week 7 and week 13 in the different groups of the experimental study.

Conclusion

According to our results, all animals in the experimental groups developed obesity. However, animals that were supplemented with GL decreased their Lee index, proving the anti-obesity properties of this natural product. Contrary to the observed at week 7, in which the animals were able to maintain normal glucose clearance, at the last week of the study the intraperitoneal glucose tolerance test showed that the animals were not able to maintain normal glucose clearance rate, suggesting a side effect of the diet used in this study.

References

[1] Kleinert, M., Clemmensen, C., Hofmann, S. M., Moore, M. C., Renner, S., Woods, S. C., Huypens, P., Beckers, J., De Angelis, M. H., Schürmann, A., Bakhti, M., Klingenspor, M., Heiman, M., Cherrington, A. D., Ristow, M., Lickert, H., Wolf, E., Havel, P. J., Müller, T. D., and Tschöp, M. H. (2018). Animal models of obesity and diabetes mellitus. Nature Reviews Endocrinology, 14(3), 140–162.

[2] Rodríguez-Pérez, C., Segura-Carretero, A., and del Mar Contreras, M. (2019). Phenolic compounds as natural and multifunctional anti-obesity agents: A review. Critical Reviews in Food Science and Nutrition, 59(8), 1212–1229.

[3] Hu, X., Tao, N., Wang, X., Xiao, J., and Wang, M. (2016). Marine-derived bioactive compounds with anti-obesity effect: A review. Journal of Functional Foods, 21, 372–387.

[4] Wan-Loy, C., and Siew-Moi, P. (2016). Marine algae as a potential source for anti-obesity agents. Marine Drugs, 14(12), 1–19.

[5] Isabel C.F.R. Ferreira, Â. F., and Heleno, S. A. (2017). Chemical, Nutritional, and Bioactive Potential of Mushrooms. Edible and Medicinal Mushrooms, 455–501.

Acknowledgements

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Determination of antiarthritic activity of *Nigella sativa* L. seeds extracts *in vitro*.

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Abstract

The present investigation was carried out the evaluation of the antiarthritic activity *in vitro* as well as the phytochemical screening of *Nigella sativa* L. seeds extracts. The dosage of flavonoids have shown that the content found in the hydroalcoholic extract (134.7 ± 0.289 mg QE / g) was significantly higher compared to the aqueous extract (48.495 ± 0.035 mg QE / g). The phytochemicals analysis of the extracts revealed the presence of tannins, saponins and reducing compounds and the absence of alkaloids. The results of antiarthritic activity showed an important effect of the aqueous and hydroalcoholic extracts on the inhibition of inflammation. The results of this study show that the seed extracts exhibited an important anti-inflammatory effect.

Key words: *Nigella Sativa* L, Inflammation, Aqueous extract, Hydroalcoholic extract, Antiarthritic activity.

Introduction

Rheumatoid arthritis is the most common chronic inflammatory rheumatism (between 0.4 and 0.8% of the general population). It is also the most serious of rheumatism in particular by the risk of developing irreversible joint destruction, joint deformities and a sometimes significant handicap.

Current treatment for inflammation involves steroidal anti-inflammatory drugs (Glucocorticoids) and non-steroidal (Diclofenac sodium). These molecules, although effective from 15 to 20%.

Nigella sativa L. is an annual herb of the *Ranunculaceae* family, with the seeds called black cumin seed or black seed. It's one of the most widely used plants in medicine traditional because of the various properties of its different parts including its seed. It is used as treatment for a variety of health conditions pertaining to the digestive tract, kidney and liver functions, respiratory system, cardiovascular system, and immune system support, as well as for general well-being. These seeds have also been used to fight intestinal worms.

For this, the aim of the present study is to test the efficacy of *Nigella sativa* L. extracts seeds on rheumatoid arthritis by the determination of anti-arthritic activity *in vitro* and the phytochemical screening of the prepared extracts.

Materials

Plant material

The seeds of *Nigella sativa* L. are bought from an herbalist located in Ain defla (Algeria). The seeds are imported from the Medea region (Algeria).

The seeds were crushed with an electric grinder to obtain a powder. The powder was then stored away from light and moisture.

Methodology

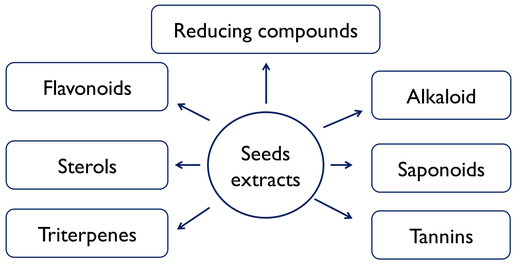
1. Preparation of the aqueous extract

The aqueous extract was prepared by macerating 10 g of *Nigella Sativa* powder in 100 ml of distilled water. After stirring, the mixture was left for 72 hours. Then the mixture was filtered and dried in an oven at 40°C.

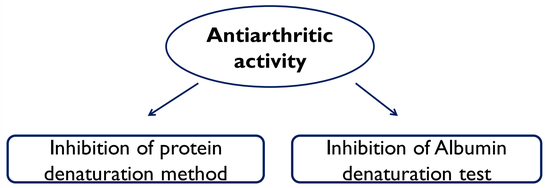
2. Preparation of the hydroalcoholic extract

The hydroalcoholic extract was removed by maceration of 10 g of *Nigella sativa* powder in 100 ml of hydroalcoholic solution. After contact for 72 hours, the mixture was filtered and dried in an oven at 40°C.

3. Phytochemical analyzes



4. Antiarthritic activity *in vitro*



Results

Table I: phytochemical tests results.

Extracts	Aqueous extract	Hydroalcoholic extract
Tannins	+	+
Flavonoids	+	+
Sterols	+	+
Triterpenes	+	+
Reducing compounds	+	-
Saponoids	-	+
Alkaloid	-	-

Table 2: Results of flavonoid content and the inhibition of protein and albumin denaturation

Extracts	Flavonoids content (mg QE/g of extract)	Inhibition of protein denaturation (IC50 mg/ml)	Inhibition of Albumin denaturation (IC50 mg/ml)
Hydroalcoholic extract	134.7 ± 0.28a	52.74 ± 1.86b	46.75 ± 1.74b
Aqueous extract	48.495 ± 0.03b	37.79 ± 0.67a	34.09 ± 1.26a
Diclofinac sodium	/	48.55 ± 1.09b	48.55 ± 1.09b

Conclusion

From the results of the study, it can be concluded extract of *Nigella sativa* seeds possessed antiarthritic property.

This property perhaps due to the presence of bioactive compounds such as polyphenoles which inhibit rheumatoid arthritis progress.

Recommendations

The activity of *Nigella sativa* seeds extracts could be attributed to the presence of phenolic acids and further work is in progress in isolating and characterizing the constituents by bioactive guided assays and to understand its effect by *in vivo* studies.

CHEMICAL CHARACTERIZATION OF ESSENTIAL OILS FROM CULTIVATED POPULATIONS IN W. GREECE OF *THYMUS VULGARIS* L., *SATUREJA MONTANA* L. AND *ORIGANUM VULGARE* L. A SURVEY OF CARVACROL AND THYMOL AS ANTIMICROBIAL AGENTS

HELLENIC REPUBLIC
National and Kapodistrian University of Athens
EST. 1837

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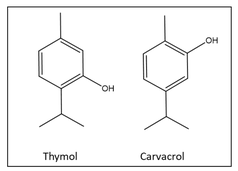
NPA
Natural products application: Health, Cosmetic and Food
Online Edition
4-5 Feb 2021

Abstract

Throughout European flora, Greece is characterized by high plant diversity, remarkable endemism, and polymorphism. A great example of this unique identity is attributed to the aromatic and medicinal plants, which are of high economic interest. The essential oils (EOs) produced by such plants are valuable products with many applications in pharmaceutical, cosmetic, food and flavouring industries. Lamiaceae is one of the most important families in the production of EOs rich in monoterpene phenols, especially thymol and carvacrol. This class of secondary metabolites are one of the most potent components of the EOs possessing significant biological properties including antioxidant, antispasmodic, antitussive, expectorant, anti-inflammatory, analgesic, antibacterial, and antimicrobial. Due to the alarming increase in antibiotic resistance, the interest in natural products containing plant antimicrobial compounds as alternative or adjunct antimicrobial agents has been revived. Thus, we investigated the EOs of *Thymus vulgaris* L., *Satureja montana* L. and *Origanum vulgare* L. from cultivated populations in W. Greece. The results herein are supported by a literature survey on the major constituents i.e. phenolic monoterpenes. The antimicrobial compounds thymol and carvacrol represented by these EOs might be useful as potential therapeutic agents alternatively or synergistically with synthetic chemical agents to enhance their antimicrobial activity.

Introduction

An emergence issue during the last decades is the antibiotic resistance and the decline in the development rate of new antimicrobial agents. Thus, more efficient herbal compounds with less damaging effects than synthetic molecules could serve as interesting alternatives. Since the antimicrobial properties of various plant EOs, associated mainly with the presence of their phenolic components such as carvacrol (CV) and thymol (TH), have been reported, EOs serve as promising substitutes to currently used antimicrobials [1,2]. The composition and concentration of multiple antimicrobial components in EOs can result in various combined effects. CV and TH have a wide antimicrobial spectrum against both Gram-positive and Gram-negative bacteria [3,4]. These hydrophobic monoterpenoids interact with the lipid bilayer of bacterial cells cytoplasmic membrane, disrupt its integrity and cause leakage of cellular material such as ions, ATP, and nucleic acids, resulting in the cell death. The presence of the free -OH group, hydrophobicity, and the phenol moiety are essential for their antimicrobial activity [1, 5].



Hence, the high amounts of the antimicrobial compounds thymol and carvacrol in *T. vulgaris* L., *S. montana* L. and *O. vulgare* L. EOs is associated with remarkable antibacterial effects and these EOs might be useful as potential alternative or synergistic therapeutic agents with synthetic chemical agents to enhance their antimicrobial activity. Thus, the present preliminary study was undertaken not only to determine the chemical composition of the essential oils of these cultivated species from W. Greece, but it also aims to provide information on the antimicrobial activities of their encapsulated end-products in the next step of our project.

Materials and Method

A comprehensive search on relevant literature of the last decade was conducted on the major scientific databases including, Pubmed, ScienceDirect, Reaxys and Google scholar. The search terms "thymol", "carvacol", "essential oil", "antimicrobial", "T. vulgaris", "O. vulgare" and "S. montana" were used for data collection. Selected data are presented in Table 2.

Plant material

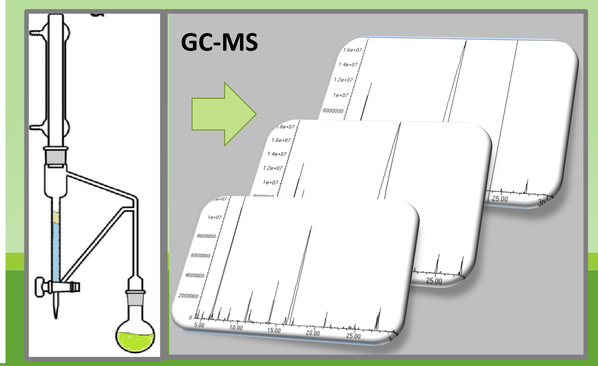
The plant materials originated from cultivated populations of *T. vulgaris, O. vulgare and S. montana* under organic farming conditions in W. Greece. The plant materials were dried for 10 days at room temperature.

Hydro-Distillation of volatile constituents and Gas Chromatography-Mass Spectrometry Analysis

To obtain the volatile constituents, 28 g of air-dried plant material from each sample were cut in small pieces, and the volatile extracts were obtained by steam distillation in 500 mL H_2O for 3 hours (Hellenic Pharmacopoeia, 2002), in a modified Clevenger apparatus with a water-cooled oil receiver to reduce hydro-distillation over-heating artifacts. The extracts were collected in 5ml pentane and were dried over anhydrous sodium sulphate and kept in closed pyrex containers, air tight at -200C. GC–MS analyses were carried out using a Hewlett- Packard 7820A-5977B MSD system operating in EI mode (70 eV), equipped with a HP- 5MS fused silica capillary column (30 m × 0.25 mm; film thickness 0.25 μm), and a split-splitless injector. The temperature program was from 60°C at the time of the injection, raised to 300 °C at a rate of 3 °C/min and subsequently held at 300°C for 10 min. Helium was used as a carrier gas at a flow rate of 2.0 mL/min. The injected volume of the samples was 2 μL. The analyses were carried out twice, resulting in reproducible results.

Identification of Compounds.

Retention indices for all compounds were determined according to the Van der Dool approach (Van Den Dool and Kratz, 1963), with reference to a homologous series of n-alkanes from C_9 to C_{26}. The identification of the chemical components was based on comparison of both relative retention times and mass spectra with those reported in the Adams, NIST/NBS and Wiley libraries and other literature data. In many cases, the volatile constituents were subjected to co-chromatography with authentic compounds (Fluka, Sigma). Component relative percentages were calculated based on GC peak areas without using correction factors.



Results

Table 1		*O.vulgare* L.	*T.vulgaris* L.	*S. montana* L.
n.	Compounds	%	%	%
1	α-pinene	-	0.6	0.9
2	camphene	0.2	0.4	1.3
3	1-octen-3-ol	0.4	0.3	1.0
4	*β*-myrcene	0.6	0.8	-
5	α-phenathrene	0.10	0.2	-
6	α-terpinene	0.90	0.9	0.7
7	*p*-cymene	5.3	6.8	24.0
8	1,8-cineole	-	-	0.5
9	*β*-ocimene	0.1	-	-
10	*β*-phellandrene	0.3	0.4	-
11	γ-terpinene	3.6	2.0	1.5
12	α-terpinolene	0.1	0.2	-
13	cis sabinene hydrate	0.2	-	-
14	linalool	0.40	1.0	1.2
15	borneol	0.2	1.7	4.1
16	terpinen-4-ol	0.6	0.8	1.1
17	thymol	2.80	80.2	2.3
18	carvacrol	79.9	0.1	52.5
19	eugenol	-	0.1	-
20	*β*-fenchyl alcohol	0.1	-	-
21	carvacrol methyl ether	0.60	-	3.7
22	*β*-caryophyllene	1.0	1.9	0.9
23	*β*-bisabolene	0.9	0.2	-
24	α-bisabolene	-	0.1	-
25	aromadendrene	0.2	-	-
26	caryophyllene oxide	0.9	0.9	1.9
27	*δ*-cadinene	0.1	-	-
28	spathulenol	0.1	-	1.3
	Total	**99.6%**	**99.6%**	**98.9%**

Table 2.	Antimicrobial activities	Literature
O. vulgare EOs	Antibacterial tests showed that the pathogenic bacteria Staphylococcus aureus and *Salmonella enterica* and the phytopathogenic bacteria *Erwinia rhapontici* and *Xanthomonas campestris* were the most susceptible to EO, with the lowest concentrations of oil necessary to inhibit their bacterial growth. Moreover, the EO showed antibacterial activity against bacteria associated with food poisoning	https://doi.org/10.3390/metabo10100414
T. vulgaris EOs	Wide antimicrobial spectrum, including Gram-positive and Gram-negative bacteria e.g., foodborne bacteria such as Salmonella, Escherichia, Pseudomonas, Listeria, and Bacillus species and some pathogens implicated in respiratory tract infections.	https://doi.org/10.1002/ptr.6109
S. montana EOs	Noticeable activity against clinical and reference Gram-positive and Gram-negative strains. S. aureus, both clinical and reference strains, with MIC values in the range 0.39–0.78 mg mL^{-1} and a MBC of 0.78 mg mL^{-1} . Against L. monocytogenes MIC was in the range 0.78–1.56 mg mL^{-1}; MBC was a concentration over the MIC value. Regarding uropathogenic E. coli strains, MIC and MBC values for oil coincided in the range 1.56–3.12 mg mL^{-1}.	https://doi.org/10.1016/j.micpath.2018.11.025
Carvacrol	Carvacrol has a high inhibitory effect against both Gram-positive and Gram-negative bacteria, except Pseudomonas aeruginosa	https://doi.org/10.1002/ptr.6103
Thymol	Thymol was more effective against Gram positive strains. 200 mg/ml could inhibit the growth of E. coli. by inducing the permeabilization and epolarization of the cytoplasmic membrane. While, at 2.5 mM inhibits the growth of S. aureus, E. coli and S. typhimurium. The ester derivatives of thymol were found to be more effective against streptococcus species. Thymol was found to possess antibacterial activity against selected verocytotoxigenic E. coli.	https://doi.org/10.3389/fphar.2017.00380

Table 1. Results on the chemical composition of the essential oils of *O. vulgare*, T. *vulgaris* and *S. montana* are presented in Table 1. The essential oil of *O. vulgare* was rich in carvacrol (79.9%), while p-cymene (5.3%) and γ-terpinene (3.6%) were also dominant constituents with slightly lower percentage. *T. vulgaris* essential oil was abundant with thymol (80.2%), followed by p-cymene (6.8%), and *S. montana* was rich in carvacrol (52.5%) followed by p-cymene (24.0%). In less concentration, borneol and carvacrol methyl ether were also presented (4.1% and 3.7%, respectively). Other constituents were present in amounts less than 15.0%.

Table 2. The essential oils are considered as low risk antimicrobial agents for resistance development. It is noteworthy that the EOs in combination with antibiotics could represent a significant tool to control bacteria growth. For example a synergistic interaction was found for thymol with all antibiotics tested against *E. coli*, *S. typhimurium, S. aureus* and *S. pyogenes*.

Conclusion

The GC-MS analysis of the essential oils shows the presence of 28 compounds in total, with the 99.6% of *O. vulg*are, 99.6% of *T. vulgaris* and 98.9 % of *S. montana* being identified. Many reports could be found representing the chemical compositions of such EOs, highlighting the influence of the different plant distributions on EOs chemical profile. In fact, our results compared with other previous similar works around the word indicate the high value of cultivated aromatic plants in Greece based on carvacrol and thymol percentages.

The plethora of literatures revealed, beyond any doubt, the antimicrobial potential of such EOs. Our future goal is to set under investigation a screening test against a broad spectrum of microorganisms not only in pure compounds but also in EOs mixtures in combination or without antibiotics duly encapsulated in end-products.

Interestingly, during our literature survey, several gaps and remarks emerge on quality and stability analysis guidelines, as well as general rules for packaging, conditioning and storage, thus our future goal is thoroughly studying these criteria.

Indicative bibliography

[1] Sakkas H, Papadopoulou C. Antimicrobial Activity of Basil, Oregano, and Thyme Essential Oils. J Microbiol Biotechnol. 2017 Mar 28;27(3):429-438. doi: 10.4014/jmb.1608.08024. PMID: 27994215.

[2] Salehi B, Mishra AP, Shukla I, Sharifi-Rad M, Contreras MDM, Segura-Carretero A, Fathi H, Nasrabadi NN, Kobarfard F, Sharifi-Rad J. Thymol, thyme, and other plant sources: Health and potential uses. Phytother Res. 2018 Sep;32(9):1688-1706. doi: 10.1002/ptr.6109. Epub 2018 May 22. PMID: 29785774.

[3] Cho TJ, Park SM, Yu H, Seo GH, Kim HW, Kim SA, Rhee MS. Recent Advances in the Application of Antibacterial Complexes Using Essential Oils. Molecules. 2020 Apr 10;25(7):1752. doi: 10.3390/molecules25071752. PMID: 32290228; PMCID: PMC7181228.

[4] Sharifi-Rad M, Varoni EM, Iriti M, Martorell M, Setzer WN, Del Mar Contreras M, Salehi B, Soltani-Nejad A, Rajabi S, Tajbakhsh M, Sharifi-Rad J. Carvacrol and human health: A comprehensive review. Phytother Res. 2018 Sep;32(9):1675-1687. doi: 10.1002/ptr.6103. Epub 2018 May 9. PMID: 29744941.

[5] Konuk HB, Ergüden B. Phenolic -OH group is crucial for the antifungal activity of terpenoids via disruption of cell membrane integrity. Folia Microbiol (Praha). 2020 Aug;65(4):775-783. doi: 10.1007/s12223-020-00787-4. Epub 2020 Mar 19. PMID: 32193708.phs and images to this section. Add your information, graphs and images to this section.

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Με τη συγχρηματοδότηση της Ελλάδας και της Ευρωπαϊκής Ένωσης
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DEVELOPMENT OF A QSAR MODEL TO EVALUATE THE ANTIOXIDANT ACTIVITY OF DI(HETERO)ARYL COMPOUNDS

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1 Abstract

QSAR (Quantitative Structure - Activity Relationship) models are useful to understand and explain the mechanism of action of compounds at a molecular level and can help in the development of new compounds with desirable biological properties. A QSAR quantitative model is represented by a mathematical equation that relates calculated properties of the studied compounds (descriptors), with their biological activities. QSAR models can have multiple applications, including in natural compounds. Per example, they may help in the prediction of different natural products bioactivities, if the QSAR model is developed using similar compounds. **[1,2]**

The QSAR study presented was carried out to model the antioxidant activity, specifically the ability to eliminate free radicals, from 70 di(hetero)aryl compounds. This model is an enhancement of a model previously developed in our group. **[3]**

The OCHEM molecular descriptor calculation tool was used to calculate a total of 8.235 molecular descriptors from which PyQSAR software selected 4 descriptors: B06 [C-O], Eig04_AEA (dm), JGI2 and J_Dz (p), in order to build the QSAR model. With this model, an equation was obtained capable of making a prediction of the antioxidant activity (pIC50) of new compounds. This model presents very good statistical parameters including the correlation coefficient (R^2=0.8905), the square root value of the average error (RMSE=0.2328) and the mean score value of the multiple linear regression method (Q^2CV=0,8676).

Finally, this model proved that it can be a useful tool for the prediction of free radical scavenging capacity of compounds with similar di(hetero)aryl structures and may guide the synthesis of new compounds with better antioxidant capabilities or the analysis of natural compounds with similar structures.

2 Introduction

More than 50 years have passed since the field of Quantitative Structure-Activity Relationship (QSAR) modeling was founded by Corwin Hansch. Initially conceptualized as the logical extension of organic chemistry, QSAR modeling has grown, diversified and evolved from its application to small series of similar compounds using relatively simple regression methods, to the analysis of much larger datasets spanning thousands of molecular structures, using a wide variety of statistical and machine learning techniques. **[4]**

QSAR modeling is widely practiced in the chemical, medical and pharmaceutical industry, as well as in government institutions around the world. These models are widely used to assess potential impacts of chemicals, materials and nanomaterials on human health and ecological systems. One of the areas of QSAR modeling that has been expanding more is the construction and use of predictive models to obtain a predicted value for a given biological activity, prior to synthesis.

A biological activity can be expressed quantitatively as the concentration of a substance necessary to cause a particular biological response. Each compound has several molecular and physicochemical characteristics which we call molecular descriptors. When these descriptors can be expressed by numbers, one can try to find a mathematical relationship or quantitative structure-activity relationship (QSAR), between biological activity and the descriptors. This mathematical expression, if carefully validated, can then be used to predict the value of the biological activity of a given compound with similar structure.

In this study, the intention was to model the antioxidant activity of various di(hetero)aryl compounds in order to obtain the mathematical equation that allows us to predict this biological activity in similar compounds. As is known a free radical can be an atom or a molecule that contains one or more unpaired electrons, that is, it has orbitals constituted only by an electron which makes, in this form, the compound to show a strong reactivity with most species. Oxygen-derived free radicals are generically known as reactive oxygen species (ROS). Thus, we can note that, although molecular oxygen (O_2) is essential for aerobic life, it can be toxic under certain conditions. We call this phenomenon the "oxygen paradox". **[5]**

The cells are equipped with several defense systems against damage caused by free radicals, including oxidizing enzymes or chemical compounds. Considering that there are many existing diseases that can be avoided if we control free radicals, it is easy to understand the great importance of knowledge about their production and control. This control can be achieved through antioxidant compounds. Synthetic products capable of having an antioxidant activity can help the endogenous defense system, therefore, it is important to obtain compounds with good antioxidant capacity for the treatment and prevention of various disorders. **[6,7]**

3 Methodology

The di(hetero)aryl compound library used as Input for the creation of the QSAR model, developed in this report, were chosen through literature search. Compounds were chosen considering the following characteristics:

- ✓ Present a structure with 2 rings connected by a linker that has an amine group in its composition (Figure 1).
- ✓ The linker cannot contain more than 3 covalent bonds.
- ✓ They have an experimental antioxidant activity (IC50) value published.
- ✓ The IC50 value was obtained using the DPPH method (2,2-diphenyl-1-picrylhydrazil).

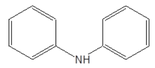


Figure 1 – Example of a compound with 2 rings with an amino group linker.

All compounds found that met these criteria were divided into groups according to the constitution of their linker. The defined groups were:

- ✓ Group A - linker composed of an amino group (NH). (Figure 2A)
- ✓ Group B - linker composed of a methanamino group (NHCH2). (Figure 2B)
- ✓ Group C - linker composed of an amide group (CONH). (Figure 2C)

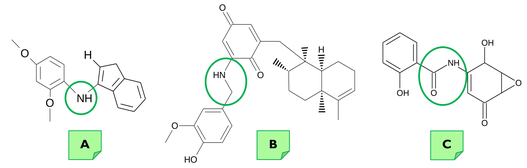


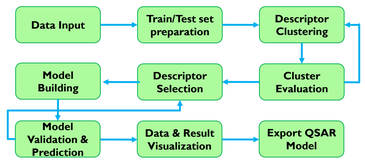
Figure 2 – Example of compounds of **A)** group A, **B)** group B and **C)** group C.

Groups A, B and C consist of 26, 24 and 20 compounds, respectively, and the final library being composed of 70 compounds. As previously mentioned, the values for antioxidant activity, more specifically for the ability to eliminate free radicals from each compound (IC50), were obtained by the DPPH method (2,2-diphenyl-1-picrylhydrazil). The experimental IC50 values were converted to experimental IC50 values using the formula:

$$pIC_{50} = - \log (IC_{50})$$

OCHEM (Online Chemical Modeling Environment) is an online platform that aims to automate and simplify the typical steps required to build QSAR models. Given the structure of our compounds, OCHEM calculated a total of 8235 molecular descriptors.

To develop the model, the PyQSAR module was used, which is based on the PYTHON programming language. PyQSAR seeks to unify, in a single workspace, all the different steps involved in creating a QSAR model, from the preparation of the data to its validation. Layout 1 shows the workflow for PyQSAR and the name of its modules.



Layout 1 – PyQSAR workflow. **[8]**

First, the descriptors were grouped using a hierarchical clustering to reduce the research space. Although OCHEM has calculated a total of 8.235 descriptors for each molecule, many of them have very little discriminating power, so descriptors with low variance are eliminated. During the descriptor selection process, a genetic algorithm (AG) was used to maintain the best descriptors for the different clusters. This method helps to prevent the selection of descriptors with similar properties (belonging to the same cluster) at the same time. After the QSAR model was built, it was also validated using the cross-validation method, implemented by PyQSAR. This validation verifies whether the model obtained is reliable for predicting the biological activity of new compounds and provides important statistical data to prove it.

4 Results

To build the QSAR model for the free radical scavenging activity, a library of 70 di(hetero)aryl compounds, belonging to 3 different groups (A, B and C), which differ in terms of their linker constitution, was used. During the construction of the model, great care was taken to avoid the inclusion of highly collinear molecular descriptors and in the end only 4 descriptors were selected by PyQSAR. The selected molecular descriptors were as follows:

- ✓ **B06[C-O]:(alvaDesc)** – "presence/absence of C-O at topological distance 6".
- ✓ **Eig04_AEA(dm):(alvaDesc)** – "eigenvalue n. 4 from augmented edge adjacency mat. weighted by edge degree".
- ✓ **JGI2:(alvaDesc)** – "mean topological charge index of order 2"
- ✓ **J_Dz(p):(alvaDesc)** – "Balaban-like index from Barysz matrix weighted by polarizability"

The QSAR model developed by PyQSAR, using the selected descriptors, presents the following equation:

$$pIC_{50(predicted)} = 4{,}3384 + 1{,}1236*B06[C\text{-}O] + (-1{,}7150)*Eig04(dm) + 1{,}5673*JGI2 + (-2{,}0874)*J_Dz(p)$$

Where the $pIC_{50(predicted)}$ value corresponds to the antioxidant activity predicted by the model after applying the equation presented above. This equation is the final objective in the realization of a QSAR model, since it is using it that the pIC_{50} value for new compounds with analogous structures can be predicted later. The statistical parameters obtained by PyQSAR for this model were as follows:

- ✓ **N**=70; R^2=0,8905; **RMSE**=0,2328;
- ✓ R^2CV=0,8909; **RMSE CV**=0,2467; Q^2CV=0,8676

Where **N** is the number of compounds used; R^2 is the correlation coefficient found when using the Linear Regression method between the various points; **RMSE** is the square root of the mean error; R^2CV and **RMSE CV** are the values of R^2 and **RMSE** after performing the Cross-Validation method. Finally, Q^2CV is the average score value of the Multiple Linear Regression (MLR) method of the five times that Cross-Validation was performed.

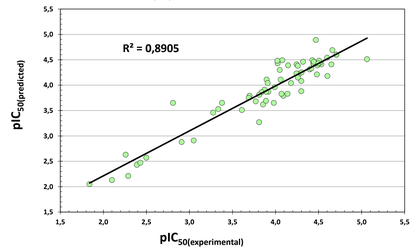
These statistical parameters define whether a model has a good predictive capacity, thus allowing to know if it can be used to accurately predict the studied biological activity and thus help in the synthesis of new compounds.

The correlation matrix between the antioxidant activity values, $pIC_{50(experimental)}$, and the chosen molecular descriptors, presented in Table 1, confirms that the descriptors do not have a strong correlation with each other, but present a strong correlation with the $pIC_{50(experimental)}$. This demonstrates that the selection of descriptors was well done by PyQSAR, since what we want are descriptors that describe different characteristics of the molecules and not descriptors that qualify the same characteristic.

Table 1 – Correlation coefficient values between descriptors and $pIC_{50(experimental)}$.

	B06 [C-O]	Eig04_AEA (dm)	JGI2	J_Dz (p)	$pIC_{50(experimental)}$
B06 [C-O]	1,0000	0,5456	0,0734	0,5617	0,8007
Eig04_AEA (dm)	–	1,0000	0,2927	0,7417	0,5565
JGI2	–	–	1,0000	0,1421	0,1706
J_Dz (p)	–	–	–	1,0000	0,7511
$pIC_{50(experimental)}$	–	–	–	–	1,0000

Graph 1 shows the representation of the relationship between the experimental and predicted pIC_{50} values, as well as the linear regression line that defines the value for the model's Correlation Coefficient (R^2).



Graph 1 – Graphical representation of the relationship between $pIC_{50(experimental)}$ and $pIC_{50(predicted)}$ by PyQSAR.

By observing the dispersion of the points, and the values of R^2, **RMSE** and Q^2CV obtained, it is possible to verify that the model developed has very good ability to predict antioxidant activity and can be used as a tool to predict the pIC_{50} value for new compounds with structures similar to the compounds used to build this model.

5 Conclusion

With this work, it is possible to conclude that the free radical scavenging activity of the 70 di(hetero)aryl compounds studied was successfully modeled using the PYTHON computer language, its PyQSAR package and the OCHEM molecular descriptors calculation system.

A QSAR model was obtained by formulating a relationship between the structures of the library compounds and their molecular descriptors. After analyzing the 8.235 descriptors initially found, PyQSAR selected 4 descriptors as the ones that best related to the structures of the 70 compounds used: **B06 [CO]**, **Eig04_AEA (dm)**, **JGI2** and **J_Dz (p)** . As can be seen from Table 1, the molecular descriptors have a weak correlation with each other, which shows that each descriptor determines a different characteristic, thus reducing the probability of having correlated values. These descriptors also proved to have a strong correlation with the activity of free radical scavenging, as shown in Table 1, thus indicating that all of them contribute significantly to the variation in pIC_{50} values predicted by the model.

The obtained QSAR model presented statistical sound parameters including correlation coefficients (R^2 **= 0.8905** and R^2CV **= 0.8909**), mean error values (**RMSE = 0.2328** and **RMSECV = 0.2467**) and average score value of the multiple linear regression method (Q^2CV **= 0.8676**), confirming it's good predictive power.

That said, it is safe to say that the built QSAR model can be used to predict the antioxidant activity of compounds similar to the di(hetero)aryl used in this work, in order to guide in the synthesis of new compounds with greater antioxidant activity.

References

[1] Roy, K., Kar, S., & Das, R. N. (2015). "Chapter 1.2: What is QSAR? Definitions and Formulism". A primer on QSAR/QSPR modeling: Fundamental Concepts. New York: Springer-Verlag Inc., 2–6.

[2] Ghasemi, F., Mehridehnavi, A., Perez-Garrido, A., & Perez-Sanchez, H. (2018). Neural network and deep-learning algorithms used in QSAR studies: merits and drawbacks. Drug Discov. Today, 23(10), 1784-1790.

[3] Abreu, R. M., Ferreira, I. C., & Queiroz, M. J. R. (2009). QSAR model for predicting radical scavenging activity of di (hetero) arylamines derivatives of benzo [b] thiophenes. European journal of medicinal chemistry, 44(5), 1952-1958.

[4] Hansch C., Maloney P. P., Fujita T., & Muir R. M. (1962). Correlation of biological activity of phenoxyacetic acids with Hammett substituent constants and partition coefficients. Nature, 194(4824), 178-180.

[5] Gilbert D. L. (2000). Fifty years of radical ideas. Annals of the New York Academy of Sciences, 899(1), 1-14.

[6] Geronikaki A.A., & Gavalas A.M. (2006). Comb. Chem. High Throughput Screen. 9, 425–442.

[7] Kontogiorgis A.C., Pontiki A.E., & Hadjipavlou-Litina D. (2005). Mini-Rev. Med. Chem. 5, 563–574.

[8] Kim S., & Cho K.-H. (Nov 2018). BULLETIN OF THE KOREAN CHEMICAL SOCIETY.

BIOHERBICIDE CYNARA: WEED CONTROL TROUGH NATURAL RESOURCES FOR SUSTAINABLE AGRICULTURE

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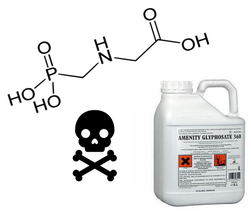
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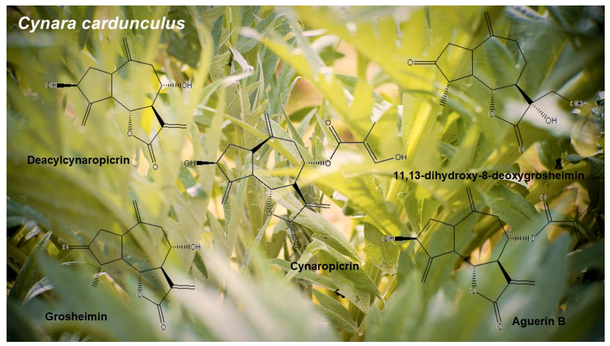
Introduction

- Weeds represent a negative impact to crop plants productivity, lowering crop productivity by 34%. The intensive use of synthetic herbicides in the last decades not only has led to herbicide-resistant weeds development, but also the bioaccumulation of synthetic compounds in the environment resulting in health and ecological concerns.[1]





- *Cynara cardunculus* belongs to the Asteraceae family, widely found in the Mediterranean basin. Cardoon is a rich source of bioactive compounds; sesquiterpene lactones (SL) have its majority content (95g/kg DW) in leaves, namely cynaropicrin [2], grosheimin, deacylcynaropicrin, aguerin B and 11,13-dihydroxy-8-deoxygrosheimin.
- SL have recently been described for its allelopathic effect on common target species showing strong evidences of their phytotoxicity and potential use as bioherbicides.[3-5]



AIM: To develop a green bioherbicide based on *Cynara cardunculus* leaves extract (CCLE), rich in SL to be used in olive orchards, grapevines and cereals production

Task 1. CCLE fractioning and phytotoxic evaluation

Cc leaves

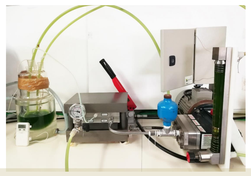


SL extraction [6]



Ethanolic Pulsed Ultrasound Assisted Extraction Method

Extract fractionation [7]



Membrane Nanofiltration Technology

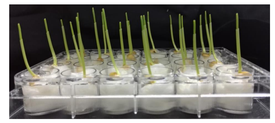
SL rich fractions



- **Increased SL bioavailability**
- **Expected enhancement of phytotoxic activity**

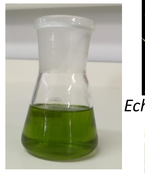
Chemical characterization HPLC-MS/MS/MS

Phytotoxic activity determination



Wheat coleoptile bioassay

Task 2. CCLE phytotoxicity against common Mediterranean weed species





Echinochloa crus-galli L.



Portulaca oleracea



Lolium multiflorum



Fumaria officialis L.



Sinapis arvensis



Calendula arvensis

Evaluation of:
Germination rate
Root length
Shoot length

Among others

CCLE fractions with higher phytotoxic activity will be used for formulation design

Task 3. CCLE based-formulation design and mode of action assessment

Formulation design parameters:

- Formulation type
- CCLE fractions stability in solution
- Ecological solvents





Echinochloa crus-galli L.



Portulaca oleracea



Lolium multiflorum



Fumaria officialis L.



Sinapis arvensis



Calendula arvensis

Evaluation of:
Germination rate
Root length
Shoot length

Among others

Elucidation of SL mode of action, within formulation context

Task 4. Application of designed formulation in agricultural crops





Grapevines



Olive orchards



Barley crop

Weeds pre-emergence (during Autumn) and on weeds post-emergence (late Winter)

Crop pre-emergence and post-emergence

The efficiency of the formulation treatment will be validated by determination of germination rate through time.

References

[1] Jabran, Khawar, et al. Crop Protection 72 (2015): 57-65; **[2]** Ramos, Patrícia AB, et al. Journal of agricultural and food chemistry 61.35 (2013): 8420-8429; **[3]** Macías, Francisco A., Francisco JR Mejías, and José MG Molinillo. Pest management science 75.9 (2019): 2413-2436; **[4]** Scavo, Aurelio, et al. Journal of agricultural and food chemistry 67.23 (2019): 6487-6496; **[5]** Rial, Carlos, et al. Journal of agricultural and food chemistry 64.33 (2016): 6416-6424; **[6]** Brás, Teresa, et al. Industrial Crops and Products 150 (2020): 112395; **[7]** Brás, Teresa, et al. Separation and Purification Technology 256 (2020): 117856.

Acknowledgements

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BIOLOGICAL ACTIVITY OF CHICORY EXTRACTS AND COMPOUNDS

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Abstract

The aim of this study was to screen the different types of chicory extracts and terpene compounds for their bioactivities and to understand better how the valuable currently unused part of chicory could be taken to use.

Antimicrobial activity of terpene samples was tested via analysing the type of growth inhibition (biostatic or biocidic) using modified microdilution method. For the determination of the antimicrobial activity of terpene samples, a modified bioautographic method was used as well. Impact of extracts and compounds on biofilm formation were determined on *Candida albicans* 475/15 and *Pseudomonas aeruginosa* (AMPR). Cristal violet assay was used for the determination of antiproliferative effect, on human gingival fibroblasts cells HGF-1 (ATCC® CRL-2014™).

It was observed that EtOAc extract of chicory root showed good antimicrobial potential against all tested bacteria (MIC 0.75-3 mg/mL) except for *P. aeruginosa* (AMPR) strain, while water root extract did not display antimicrobial effect with tested concentrations. Costunolide, lactucopicrin, 11β,13-dihydrolactucin, compounds found in chicory roots, showed the most prominent effect against resistant strain of *P. aeruginosa*. All tested standard compounds demonstrated very good antifungal activity against *Candida* species (MIC 0.03-1.00 mg/mL).

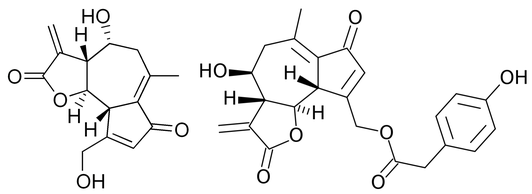
Introduction



Cichorium intybus L. (Asteraceae) or chicory is a leafy biennial or perennial plant, with a strong fleshy taproot, that can grow up to 75 cm in length.

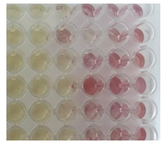
Chicory has a long history being used as medicinal plant.

The major sesquiterpene lactones (STLs) of chicory belong to the class of guaianolide sesquiterpene lactones and are commonly derived from a single sesquiterpene, germacrene A. The main STLs in chicory are lactucin, lactucopicrin, 8-deoxylactucin and their oxalate and glucoside derivatives.

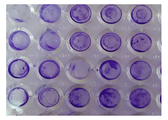


Herbal extracts usually contain a mixture of several potential antimicrobial compounds, which can act in different ways to inhibit several key points in the metabolic pathways of pathogenic microbes. It is believed that this strategy will enable a way out of the impasse caused by multiple resistances to commercial antimicrobial agents.

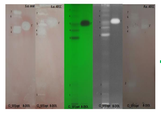
Methodology



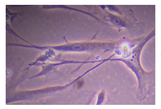
Antimicrobial activity was determined by microdilution assay in 96-well microtiter plates



For the determination of the antimicrobial activity of terpene samples, a modified bioautographic method was used as well. Impact of extracts and compounds on biofilm formation were determined on *Candida albicans* 475/15 and *Pseudomonas aeruginosa* (AMPR).



For the determination of the antimicrobial activity of terpene samples, a modified bioautographic method was used as well



Cristal violet assay was used for the determination of antiproliferative effect, on human gingival fibroblasts cells HGF-1 (ATCC® CRL-2014™).

Results

Same extracts and standard compounds were subjected to microdilution method. Accordingly with earlier, it was observed that Ci_EtOAc showed good antimicrobial potential against all tested bacteria except for *P. aeruginosa* (AMPR) strain, while Ci_H2O did not display antimicrobial effect with tested concentrations

Table 1. Antibacterial activity of extracts and compounds (MIC and MBC in mg/mL). MIC-minimal inhibitory concentration, MBC-minimal bactericidal concentration.

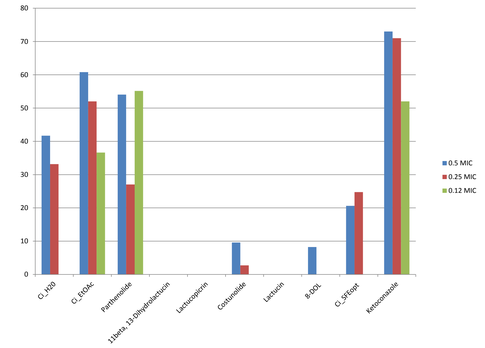
Extracts	S. aureus oral		S. aureus 11632		P. aeruginosa 27853		P. aeruginosa AMP_R		E. coli 25922	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ci_EtOAc	0.75	1.5	0.75	1.5	1.5	3	/	/	6	6
Ci_H2O	/	/	/	/	/	/	/	/	/	/
Standards										
Costunolide	/	/	/	/	/	/	0.50	1.00	/	/
Parthenolide	0.08	0.16	0.16	0.31	0.31	0.63	/	/	/	/
Lactucin	/	/	/	/	/	/	/	/	/	/
Lactucopicrin	/	/	0.16	0.31	0.31	0.63	0.50	1.00	/	/
11beta, 13-Dihydrolactucin	/	/	/	/	/	/	0.50	1.00	/	/
Controls										
Ampicillin	0.002	0.003	0.002	0.003	0.0002	0.0004	/	/	0.003	0.003
Streptomycin	0.006	0.012	0.05	0.10	0.0004	0.0008	0.05	0.1	0.03	0.03

Results

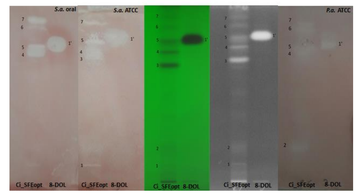
- Some chicory extracts and compoounds exhibitted strong antimicrobial potential

Table 2. Antifungal activity of terpene extracts and pure compounds (MIC (minimal inhibitory concentration) and MFC (minimal fungicidal concentration) in mg/mL).

	C. albicans475/15		*C. albicansATCC 10231*		*C. krusei*		*A. fumigatus*		*A. versicolor*		*A. ochraceus*		*A. niger*		*T. viride*		*P. ochrochloron*		*P. funiculosum*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Extracts																				
Ci_EtOAc	1.00	2.00	/	/	/	/	/	/	3.00	4.00	4.00	8.00	6.00	8.00	1.00	2.00	0.5	1.00		
Ci_H2O	1.00	2.00	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/		
8-DOL	0.06	0.12	0.06	0.12	0.06	0.12	0.13	0.26	0.03	0.06	0.25	0.50	0.50	1.00	NA	NA	NA	NA	0.25	0.5
Ci_SFE_{opt}	0.50	1.10	0.5	1.1	0.50	1.10	1.1	2.2	0.50	1.10	1.10	2.20	2.20	4.40	NA	NA	NA	NA	1.1	2.2
Standards																				
Costunolide	0.50	1.00	/	/	0.13	0.25	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Parthenolide	0.25	0.50	/	/	0.03	0.06	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Lactucin	1.00	>1.00	/	/	0.50	1.00	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Lactucopicrin	1.00	>1.00	/	/	0.50	1.00	/	/	/	/	/	/	/	/	/	/	/	/	/	/
11beta, 13-Dihydrolactucin	0.50	1.00	/	/	0.50	1.00	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Control																				
Ketoconazole	0.03	0.06	0.0016	0.032	0.006	0.01	0.18	0.25	0.045	0.062	0.25	0.5	0.375	0.5	0.375	0.5	0.156	0.031	0.15	0.2



Percentage of inhibition of *C. albicans* 475/15 biofilm formation after treatment with terpene pure standard compounds and chicory terpene extracts.



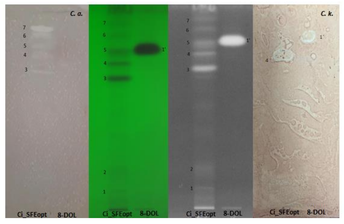


Figure 1. Bioautographic assay test on TLC plates with Ci_SFEopt and 8-DOL, developing in solvent: dichloromethane:methanol = 85:15 v/v; (in middle); **A**) antibacterial activity against *Staphylococcus aureus* (oral isolate), *Staphylococcus aureus* ATCC (left side); and *Pseudomonas aeruginosa* (right side). **B**) antifungal activity against *Candida albicans* (left side) and *Candida krusei* (right side). Spots on different Rf values: 1. Rf 0.18 (only against *S. aureus* ATCC);2. Rf 0.20 (only against *P. aeruginosa*); 3. Rf 0.65 (possessed activity against *S. aureus* ATCC and *C. albicans*); 4. Rf 0.71 (possessed antimicrobial activity against all tested bacteria and fungi); 5. Rf 0.76 (possessed activity against all tested bacteria and fungi, except *C. krusei*); 6. Rf 0.82 (possessed activity against all tested microorganisms, except *P. aeruginosa* and *C. krusei*); 7. Rf 0.88 (possessed activity against all tested bacteria and fungi, except *C. krusei*).

Results

Ci_H2O did not exhibit any cytotoxicity up to 1 mg/mL. On the other hand, Ci_EtOAc was cytotoxic to HGF-1 cell at $GI_{50(\%)}$ concentration of 0.750 mg/mL. As for the tested compounds, 11β-13-dihydrolactucin was the less toxic to HGF-1 cells. All other compounds were toxic at the lowest concentration used, indicating relatively high cytotoxicity to normal human gingival fibroblast cells.

Table 3. Antiproliferative activity of extracts and compounds expressed as GI50(%) values in mg/mL.

Extracts	$GI_{50(\%)}$
Ci_EtOAc	0.750
Ci_H2O	> 1
Compounds	GI_{50}(%)
Costunolide	< 0.030
Parthenolide	< 0.030
Lactucin	< 0.030
Lactucopicrin	< 0.030
11β-13-dihydrolactucin	0.210

Conclusion

- EtOAc extracts show higher antibacterial and antifungal activity than water extracts
- SFE seems to be most potent extraction method for high antibacterial activity
- especially interesting are the Ci_SFE and 8-DOL activities against resistant strains S. aureus MRSA and P. aeruginosa AMPR
- Ci_EtOAc extract showed wider species-specific antifungal activity than standard STL compounds
- Ci-EtOAc and Ci_H2O show higher antibiofilm activity against C. albicans than standard STL compounds
- Ci_EtOAc and standard STLs showed relatively high cytotoxicity to normal human gingival fibroblasts

Acknowledgements

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Anti-inflammatory and immunomodulatory activity of *Lentinula edodes* extracts

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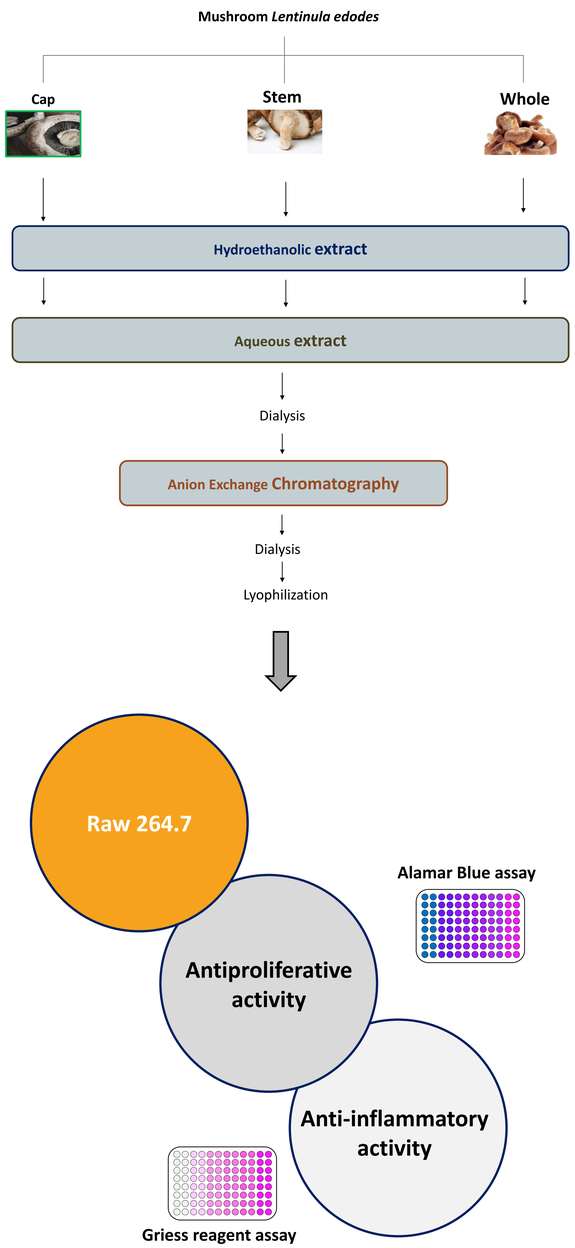
Introduction

The use of mushrooms for their medicinal and nutritional properties has been reported throughout history. Nowadays, the market for edible mushrooms is dominated by *Agaricus bisporus*, followed by *Lentinula edodes* and *Pleurotus spp.*. *Lentinula edodes*, commonly known as shiitake, is the second most cultivated mushroom in the world and classified as a medicinal species. Mushrooms have also been studied for their functional and medicinal properties and their application for pharmaceutical use. They are considered healthy food products, as they contain sugars, fatty acids, polysaccharides and several other nutritional components. These compounds, especially polysaccharides, have high medicinal value and are responsible for several biological activities, such as anti-tumor, immunomodulatory, antioxidant, anti-hypercholesterolemic, antiviral, antibacterial, hepatoprotective and anti-diabetic effects.

In this study, lyophilized *Lentinula edodes* mushroom was extracted using an exhaustive hydroethanolic extraction as well as an aqueous extraction method, followed by dialysis and subsequent purification of some fractions using ion exchange chromatography.

The extracts and fraction obtained were then evaluated for their anti-inflammatory activity as well as its anti-proliferative action.

Materials and methodology



Results

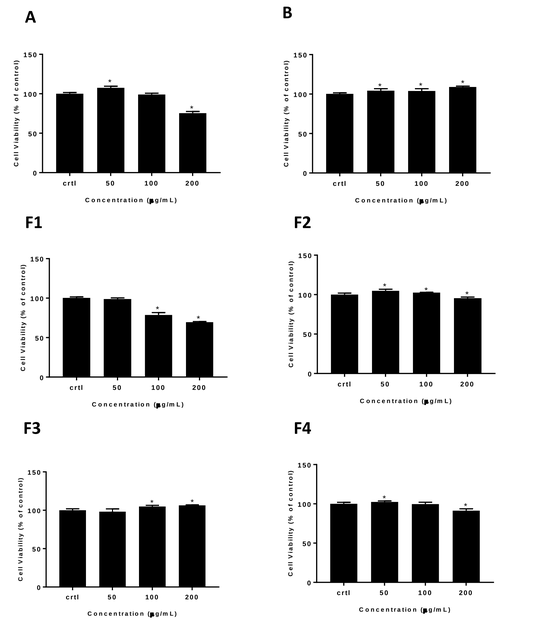


Figure 1: Viability of RAW 264.7 cells after 24 h of exposure to extracts: (A) Cap AQ, (B) Cap-HE, (F1) Whole-NaOH, (F2) Stem-NaOH, (F3) Whole-acetate buffer, (F4) Cap (purified RNA peak).

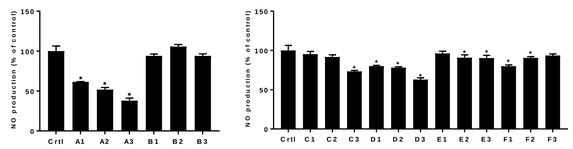


Figure 2: NO production (% control), by RAW 246.7 cells exposed to LPS, in the presence of extracts of: (A) Cap-AQ, (B) Cap-HE, (C) Whole-NaOH, (D) Stem-NaOH, (E) Whole-acetate buffer, (F) Cap (peak 10-15) at 50 (1), 100 (2) and 200 (3) µg / mL. All values were normalized by the control, 100% NO production.

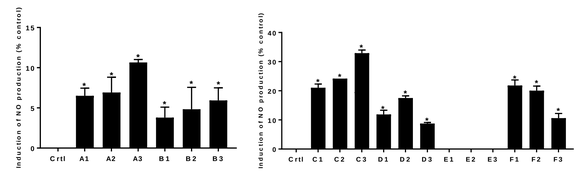


Figure 3: Induction of NO production (% control) in RAW 246.7 cells in the absence of exposure to LPS. (A) Cap-AQ, (B) Cap-HE, (C) Whole-NaOH, (D) Stem NaOH, (E) Whole-acetate buffer, (F) Cap (purified RNA fraction) at 50 (1), 100 (2) and 200 (3) µg / ml.

Discussion

By observing Figure 1, we can see that the aqueous extract reduced cell viability only at 200 µg / mL, by about 25%, compared to the control. Cap HE extract did not alter the cell viability, at all tested concentrations, more than 10%. With the exception of F1 extract (Figure 1), all the others extracts and fractions only induced slight variation o RAW 264.7 cells viability. Exposure of cells to 100 µM and 200 µM of F1 extract, reduced viability by ~22% and ~30%, respectively.

In Figure 2, it can be seen that the AQ extract from hats is the one with the greatest anti-inflammatory potential, with a dose-dependent effect.

In Figure 3, we see that extracts A, B and C induce the production, in a dose-dependent manner, of NO.

Conclusion

The results obtained allowed us to conclude that extracts A and B are more cytotoxic than their fractions F1, F2, F3 and F4;

As for the anti-inflammatory activity, it was found that the extracts are considered immunomodulatory, since they alone induce NO production.

Acknowledgements

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To FungiTech - Technology and innovation in the shiitake and other nutraceutical and medicinal mushrooms production chain, N.º 33788, financed by the European Regional Development Fund (ERDF) through NORTE 2020 (North Regional Operational Program 2014/2020).

Phytochemical screening and cytotoxic potential of siderol isolated from cultivated *Sideritis euboea* Heldr.

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Introduction

Over the last decades, natural products have been in the forefront as major drug sources in cancer and the explosion of knowledge in this area have brought forward new drug discoveries and therapeutic developments. Diterpenes represent about 20% of the terpenes, exerting a broad range of significant biological activities [1–3]. In recent years, *Sideritis* genus is one of the most economically explored in the Lamiaceae family. Diterpenes are characteristic constituents of genus *Sideritis.* Among them, siderol is a well-known *ent*-kaurene diterpenoid of the *Sideritis* spp [4]. Even though siderol consists a common major constituent of *Sideritis* genus, few biological studies have been conducted, revealing only its antimicrobial, antiviral, antioxidant and anticholinesterase activity. So far, its cytotoxic potential has not been evaluated.

In continuation of our phytochemical investigations into cultivated *Sideritis* species, herein, we report on the isolation and identification of specialized products from the non-polar extract from the aerial parts of the cultivated *S. euboea* Heldr. The current study was designed to guide the isolation of siderol and evaluate its cytotoxic activity, since this remained elusive. Whole the isolation process was monitored by NMR spectroscopy. Then, we investigated the effect of the isolated siderol against three human cancer cell lines: DLD1, HeLa and A549. As far as we know, this is the first study to report on the anticancer potential of siderol [5].

Results

The present study was focused on the isolation and identification of the bioactive compound, siderol from the dichloromethane extract of the cultivated *S. euboea.* 1D ^{1}H-NMR spectroscopy was applied in the crude extract, revealing the characteristic proton signals of siderol, as well as signals arising from phytosterols/triterpenoids, 18-hydroxy-*ent*-kaurene derivatives, and methoxylated flavones. Afterwards, the crude extract was fractionated by using CC techniques and overall, **11** compounds were isolated, including one fatty acid ester, 2-(p-hydroxyphenyl)ethylstearate (**1**); three phytosterols, namely β-sitosterol (**2**), stigmasterol (**3**), and campesterol (**4**); one triterpenoid, ursolic acid (**5**); four diterpenoids, siderol (**6**), eubol (**7**), eubotriol (**8**), 7-epicandicandiol (9) and two flavonoids, xanthomicrol (**10**) and penduletin (**11**) (Figure 1).

The cytotoxic effects of siderol *in vitro* were evaluated by MTT assay in three cancer cell lines (DLD1, HeLa, A549) in the presence of various concentrations (0–100 μM) for 72 h. The IC_{50} values of siderol in DLD1, HeLa, and A549 cells were 26.4 ± 3.7 μM, 44.7 ± 7.2, and 46.0 ± 4.9 μM, respectively (Figure 2). Interestingly, concentrations at 100 and 50 μM inhibited the growth in all three cell lines significantly. Siderol exemplified an enhanced cytotoxic effect on the DLD1 cell line compared to the others.

In order to evaluate the potential similarity of the isolated chemotype of siderol with other compounds with known bioactivity in the CHEMBL database, we conducted a 3D-based virtual screening [6]. Of interest is that overlapping anticancer bioactivity and specifically in colon cancer was also reported for another compound bearing high structural similarity with siderol.

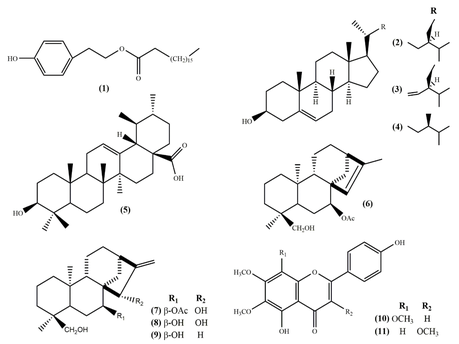


Figure 1. Structures of isolated compounds 1-11.

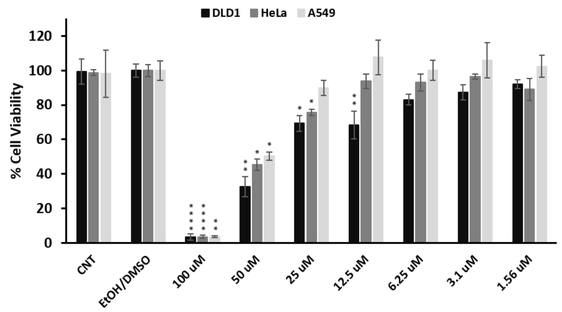
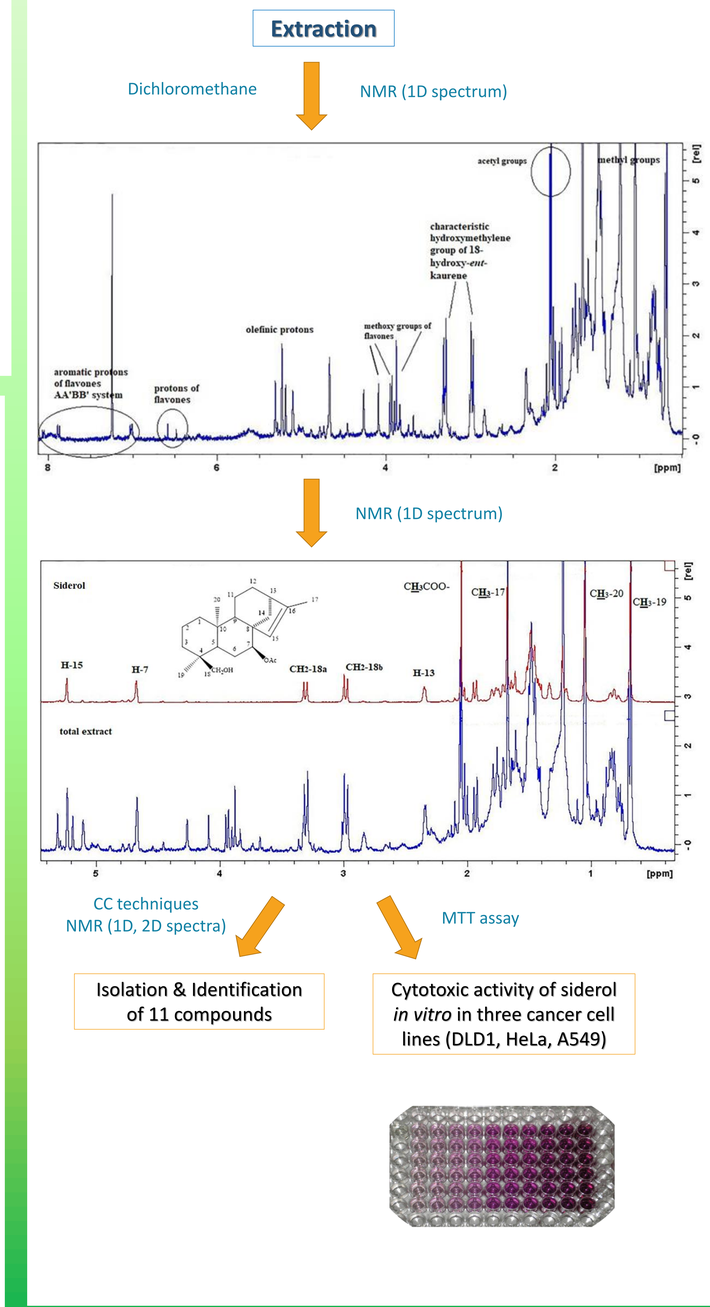


Figure 2. Cell viability of DLD1, HeLa, and A549 cells line under treatment with siderol. Cells were treated with the indicated concentrations of siderol for 72 h, and the viability was estimated by the MTT assay. CNT (control) corresponds to the untreated cells and EtOH/DMSO to the highest volume (0.1% v/v) of the ethanol: dimethyl sulfoxide (50:50 v/v) solution, which was added to the cells. Statistical analysis was conducted with the Dunnett's test. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.1$.

Methodology



Conclusion

- *S. euboea* is a Greek endemic and threatened perennial herb. Therefore, this study deals with the first chemical investigation of the dichloromethane extract of cultivated *S. euboea*. Our results showed that the cultivation of *S. euboea* was feasible without affecting its chemical composition, compared to the wild species
- 11 specialized products were isolated
- Siderol demonstrated remarkable cytotoxic effects and its derivatization or formulation should be considered in order to enhance even more its cytotoxic efficacy
- NMR application in the total extract: rapidly, non-destructive, reproducibly, and costless detection of the major chemical categories of the specialized products, as well as the identification of the known or unknown or rare compounds.
- Further studies are in progress in our labs.

References

[1]. Aneva, I.; Zhelev, P.; Kozuharova, E.; Danova, K.; Nabavi, S.F.; Behzad, S. Genus *Sideritis*, section Empedoclia in southeastern Europe and Turkey–studies in ethnopharmacology and recent progress of biological activities. DARU J. Pharm. Sci. 2019, 27, 407–421.

[2]. Bruno, M.; Rosselli, S.; Pibiri, I.; Kilgore, N.; Lee, K.-H. Anti-HIV Agents Derived from the ent –Kaurane Diterpenoid Linearol. J. Nat. Prod. 2002, 65, 1594–1597.

[3]. Erta¸s, A.; Öztürk, M.; Boğa, M.; Topçu, G. Antioxidant and Anticholinesterase Activity Evaluation of ent-kaurane Diterpenoids from *Sideritis arguta*. J. Nat. Prod. 2009, 72, 500–502.

[4]. Piozzi, F.; Bruno, M.; Rosselli, S.; Maggio, A. The Diterpenoids from the Genus *Sideritis*. In Studies in Natural Products Chemistry; Elsevier: Amsterdam, The Netherlands, 2006; Volume 33, pp. 493–540.

[5]. Tomou, E-M.; Chatziathanasiadou, M.V.; Chatzopoulou, P.; Tzakos, A.G.; Skaltsa, H. NMR-Based Chemical Profiling, Isolation and Evaluation of the Cytotoxic Potential of the Diterpenoid Siderol from Cultivated *Sideritis euboea* Heldr. Molecules 2020, 25, 2382.

[6]. Banegas-Luna, A.J.; Cerón-Carrasco, J.P.; Puertas-Martín, S.; Pérez-Sánchez, H. BRUSELAS: HPC generic and customizable software architecture for 3D ligand-based virtual screening of large molecular databases. J. Chem. Inf. Model. 2019, 59, 2805–2817

Acknowledgements

The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the HFRI PhD Fellowship grants (Fellowship Numbers: 16228 and 1090). The project/research is co-financed by the European Regional Development Fund (ERDF) under the Operational Program "Epirus 2014-2020", NSRF 2014-2020 [Grant Number: 5033143]. The research work was supported by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the "First Call for H.F.R.I. Research Projects to support Faculty members and Researchers and the procurement of high-cost research equipment grant" (Project Number: 991).

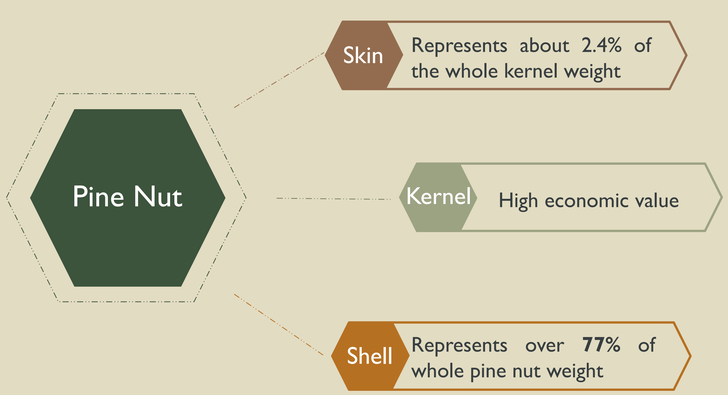
PINE NUT SHELLS AS A SOURCE OF BIOACTIVE COMPOUNDS: HEALTH-PROMOTING POTENTIAL

Élia Fogeiro[1], Susana M. Cardoso[2], Dulcineia F. Wessel[1,2,3*]

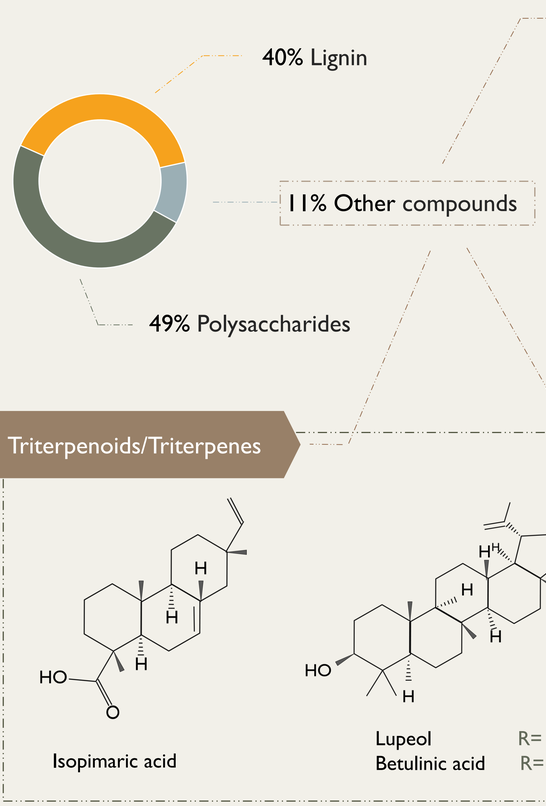
[1]School of Agriculture, Polytechnic Institute of Viseu, Quinta da Alagoa – Estrada de Nelas, Ranhados, 3500-606 Viseu, Portugal
[2]LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal; susanacardoso@ua.pt
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The agro-industrial sector generates a high quantity of by-products and waste with low economic value. Environmental concerns caused by population growth and depletion of natural resources made it urgent to seek viable solutions for waste valorisation and better use of these resources.

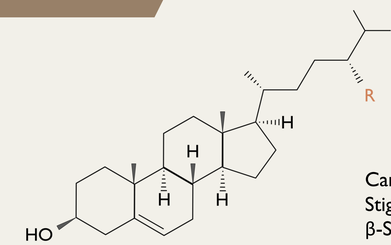
Pinus pinea L. is a forest specie widely present in the Iberian Peninsula and is well known for the economic importance of its edible pine nuts.



PINE NUT SHELL COMPOSITION



Sterols



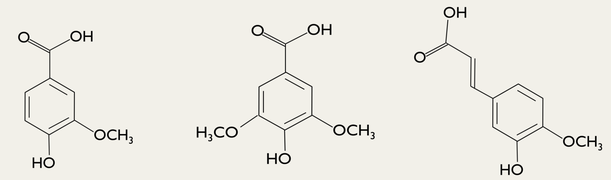
Campesterol R= $-CH_3$
Stigmasterol R= $-C_2H_5$, Δ^{22}
β-Sitosterol R= $-C_2H_5$

Triterpenoids/Triterpenes

Isopimaric acid

Lupeol R= $-CH_3$
Betulinic acid R= $-COOH$

Phenolic compounds



Vanillic acid | Syringic acid | Isoferulic acid

› Pine nut shell is the main byproduct of pine nut processing, currently used in the production of biochar and bio-oils.

› Prospective integrated valorisation as added-value compounds with health-promoting potential.

The preceding compounds have been investigated for pharmacological prospects, regarding their potential as

- Antihypercholestrolemic
- Antioxidant
- Antimutagenic
- Anti-inflammatory
- Hypoglycaemic
- Antimicrobial

Final considerations

Although the studies concerning the characterization of *Pinus pinea* pine nut shell are very limited, literature data suggests promising results concerning the potential use of this by-product as a cheap source of bioactive compounds.

References

[1] I. Evaristo, R. Tenreiro, and R. Costa, Silva Lusitana, 16 (2008) 1-19.
[2] P. Queirós, S. Cardoso, A. Lourenço et al, Biomass Conversion and Biorefinery, 10 (2020) 175-188.

Acknowledgements

Thanks are due to the Polytechnic Institute of Viseu and to FCT/MEC for the financial support to LAQV-REQUIMTE (UIDB/50006/2020) and CITAB (FCT UID/AGR/04033/2019) research units, through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. SMC acknowledges the research contract through the project AgroForWealth (CENTRO-01-0145-FEDER-000001).

EFFECT OF HIDROETHANOLIC EXTRACT OF *LAVANDULA PEDUNCULATA* (MILL.) CAV. ON MORPHOMETRIC PARAMETERS IN HPV-16 TRANGENIC

Nascimento-Gonçalves E., Ferreira T., Moutinho M. S. S., Pires M. J., Bastos M. M. S. M., Medeiros R., Nogueira A., Barros L., Ferreira I. C. F. R., Gil da Costa R., Oliveira P.A.*

*pamo@utad.pt

Abstract

Lavandula penduculata (Mill.) Cav., common English name French lavender, belongs to the Lamiaceae family and has been used as a medicinal plant in infusions for respiratory and digestive systems and as a therapeutic agent with antiseptic action for cleaning wounds [1,2]. The K14HPV16 mice is a skin squamous carcinoma model that can be used to test the antitumoral properties of several chemical and natural products [3]. The aim of this work was to evaluate the effect of the hydroethanolic French lavender extract (FLE) on body weight, relative organs weights, food and water consumption in an HPV-16-transgenic mice model. The extract was obtained from a maceration with ethanol/water (80:20, *v/v*), and the phenolic composition was determined through HPLC-DAD-ESI/MS. Twenty-eight male mice were randomly divided into four groups (n=7/group) according to their genotype: group I (HPV16- control); II (HPV16- FLE); III (HPV16+ control) and IV (HPV16+ FLE). The FLE was administrated orally in drinking water at 6.8 mg/10mL/animal to animals from groups II and IV and changed every 4 days. The animals were kept under controlled conditions such as temperature, light and humidity. Food and water were kept *ad libitum* regime. Animals' body weight, food and water consumption were measured weekly as well as animal welfare. After twenty-nine days, all animals were sacrificed by anaesthetic overdose and blood was obtained from cardiac punction. The organs were collected and immediately weighted. Data was analysed using SPSS 25. The differences were considered statistically significant at $p<0.05$. A total of thirteen compounds were identified in the hydroethanolic extract, being salvianolic acid B and rosmarinic acid the main molecules present. Moreover, the compounds revealed to be stable in the drinking water during the 5 tested days. HPV animals exposed to FLE (group IV) showed lower values of body weight variation than HPV animals not exposed to lavender (group III) in week 1, 2 and 3 ($p<0.05$), suggesting that the FLE was highly palatable. However, the values of food consumption were identical between groups and water intake was higher in transgenic animals as expected. The relative organ weight of heart, lung, kidneys, adrenals and liver did not demonstrate differences between groups ($p<0.05$). According to our results the consumption of French lavender demonstrated a favourable and safe toxicological profile using these experimental conditions.

Introduction

The K14HPV16 mice model expresses all the early HPV16 genes under of the cytokeratin 14 (*Ck14, Krt14*) gene promoter and develop cervical and cutaneous lesions. Thus, the K14HPV16 mice is a skin squamous carcinoma model that can be used to test the antitumoral properties of several chemical and natural products [3].

Lavandula penduculata (Mill.) Cav., common English name French lavender, belongs to the Lamiaceae family and has been used as a medicinal plant in infusions for respiratory and digestive systems and as a therapeutic agent with antiseptic action for cleaning wounds [1,2].

The aim of this work was to evaluate the effect of the hydroethanolic French lavender extract (FLE) on body weight, relative organs weights, food and water consumption in an HPV-16-transgenic mice model.

Methodology

Study design and animal procedures were approved by the University of Trás-os-Montes and Alto Douro Ethics Committee (10/2013) and the *Direção Geral de Alimentação e Veterinária* (0421/000/000/2014). The K14HPV16 model on FVB/n background was generously were generously donated by doctors Jeffrey Arbeit and Douglas Hanahan, from the University of California, through the National Cancer Institute's Mouse Repository. The animals were kept under controlled conditions of temperature (23 ± 2°C), light-dark cycle (12 h light/12 h dark), and relative humidity (50 ± 10%). Food and water were kept *ad libitum* regime.

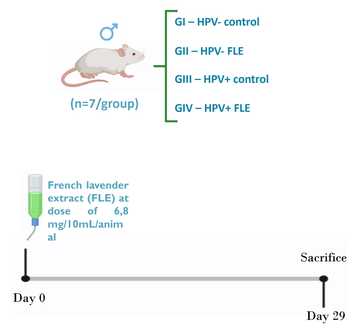


Figure. I Experimental design

Animals' body weight, food and water consumption were measured weekly as well as animal welfare. After twenty-nine days, all animals were sacrificed by anaesthetic overdose and blood was obtained from cardiac punction. The organs were collected and immediately weighted.

SPSS 25.0 software (Statistical Package for the Social Sciences Chicago, Illinois, USA) was used for data processing. Measurement data were presented as mean ± standard error (SE) and processed in one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-hoc analysis. The statistical significance was defined when $p < 0.05$.

Results

A total of thirteen compounds were identified in the hydroethanolic extract, being salvianolic acid B and rosmarinic acid the main molecules present. Moreover, the compounds revealed to be stable in the drinking water during the 5 tested days. HPV animals exposed to FLE (GIV) showed lower values of body weight variation than HPV animals not exposed to lavender (GIII) in week 1, 2 and 3 ($p<0.05$), suggesting that the FLE was not highly palatable (Figure 1). Additionally, Wild-type animals exposed to FLE (GII) showed higher values of body weight than HPV animals not exposed to lavender (GIII). However, the values of food consumption were identical between groups and water intake was higher in transgenic animals as expected (data not show).

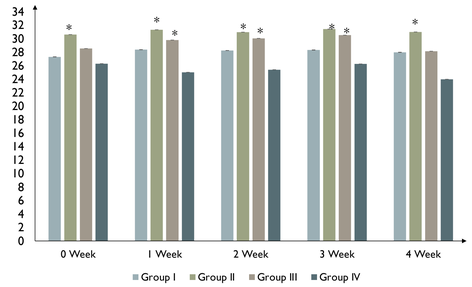


Figure 2. Weekly variation of body weight (g). *Statistically significant difference from group IV ($p < 0.05$).

The relative organ weight of heart, lung, kidneys, adrenals and liver did not demonstrate differences between groups ($p<0.05$) (table 1). Spleen relative weight of wild-type animals (GI and II) shown higher values compared to HPV control (GIII). Moreover, the thymus relative weight of GI shown higher values compared to HPV animals (GIII and IV). These results suggest that only oncogenes HPV had effect in these organs.

Table 1. Relative organ weights.

Groups	Heart	Lung	Right kidney	Left kidney	Right adrenal	Left adrenal	Spleen	Liver	Thymus
GI	0.0049 ±0.0003	0.0061 ±0.0002	0.0081 ±0.0004	0.0078 ±0.0003	0.0002 ±0.0.00007	0.00022 ±0.00006	0.0038 ±0.0002[a]	0.0520 ±0.0010	0.0011 ±0.0001[a,b]
GII	0.0044 ±0.0002	0.0059 ±0.0003	0.0079 ±0.0002	0.0074 ±0.0003	0.0002 ±0.0.00003	0.0004 ±0.00006	0.0034 ±0.0002[a]	0.0534 ±0.0011	0.0007 ±0.0001
GIII	0.0051 ±0.0002	0.0062 ±0.0003	0.0080 ±0.0004	0.0078 ±0.0005	0.0001 ±0.0.00005	0.0002 ±0.00010	0.0074 ±0.0017	0.0539 ±0.0028	0.0005 ±0.0011
GIV	0.0049 ±0.0001	0.0065 ±0.0004	0.0083 ±0.0003	0.0076 ±0.0002	0.0001 ±0.0.00004	0.0002 ±0.00003	0.0059 ±0.0007	0.0542 ±0.0026	0.0004 ±0.0001

[a] Statistically significant difference from group III ($p<0.05$); [b] Statistically significant difference from group IV ($p<0.05$).

Conclusion

According to our results the consumption of French lavender demonstrated a favourable and safe toxicological profile using these experimental conditions. Further studies will be needed as a histological analysis to assess the toxicity of the compound, especially in the kidney and liver. In addition, skin histology will be performed in order to evaluate the chemopreventive effect of lavender on HPV-induced lesions 16.

References

[1]. Neves, J.M.; Matos, C.; Moutinho, C.; Queiroz, G.; Gomes, L.R. *J. Ethnopharmacol.* 124, (2009), 270–283;

[2]. Lopes, C.; Pereira, E.; Soković, M.; Carvalho, A.; Barata, A.; Lopes, V.; Rocha, F.; Calhelha, R.; Barros, L.; Ferreira, I. *Molecules*, *23*, (2018) 1037;

[3]. Arbeit, J.; Münger, K.; Howley, P.; Hanahan, D., *J. Virol. 68*, (1994), 4358–4368.

Acknowledgements

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CASSAVA STARCH SPOUTED BED FOR MANUFACTURING INCLUSION COMPLEXES OF *Equisetum hyemale* EXTRACT

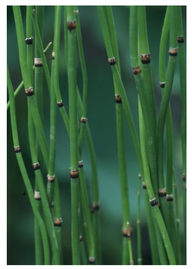
Campos E.I.A;* Borges L.L; Freitas L.V.D; Montes A.C.R; Conceição E.C; Freitas L.A.P.

*College of Pharmacy, Goiás Federal University (UFG)

INTRODUCTION

Equisetum hyemale L. (Equisetaceae) is a species rich in phenolic compounds, flavonoids, and alkaloids, being used popularly to treat hypertension, stroke and inflammatory diseases. The spouted bed technique was developed for drying wheat grains, however, is used in the pharmaceutical and food industry, due to the excellent fluid-solid contact power and efficient drying.

Image 1: General aspect of *Equisetum hyemale* species.



Source: Tropicos, 2021.

OBJECTIVE

This study aims find the best condition for the coating of granules of cassava starch, with polymeric solution containing *E. hyemale* liquid extract, with the purpose of protecting and slowly releasing the chemical markers of the plant.

MATERIALS

The materials used to perform the work were:

- *Equisetum hyemale* L liquid extract;

- Granules of cassava starch;

- Fluidized bed.

Image 2: Spouted bed equipment.



Source: Labmaq, 2021.

METHODOLOGY

A Box-Behnken factorial design (3^3) was applied varying the factors to investigate the effects over the content of total phenols and flavonoids, varying:

- The granule load (80g; 120g; 160g);

- Flow of the coating solution (1mL/min; 2mL/min; 3mL/min);

- Percentage polymeric solution (1%; 2%; 3%).

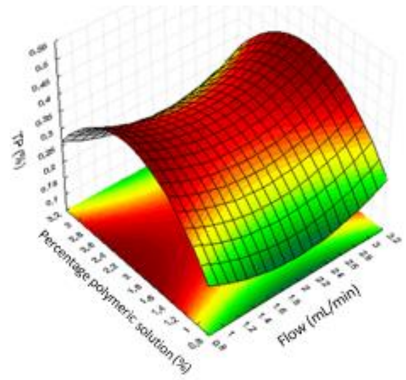
The correlation between the variables analyzed was established through the determination of total phenols and flavonoids.

RESULTS

The determination test of the levels of phenols and flavonoids for each experiment allowed to observe a correlation between the variables of load, flow of the coating solution and HPMC content applied.

The ANOVA showed that linear and quadratic effects exerted effect on the total phenols (TP).

Image 3: Response surface plot for total phenols (TP) as a function of the percentage polymeric solution and flow of the coating solution.



Source: Labmaq, 2021.

The fitted equation in terms of coded factors presented $Radj^2 = 0.85$, is given by:

$$TP=0.35-0.013A+0.000034B+0.024C+0.049A^2+0.053B^2-0.13C^2$$

Where as: Granule load (A); Flow of the coating solution (B) and Percentage polymeric solution (C).

CONCLUSION

The results presented here was suitable to predict the best condition to apply *E. hyemale* liquid extract in cassava starch.

REFERENCES

BOX, G.E.R.; HUNTER, W.G.; HUNTER, J.S. Statistics for experimenters, John Wiley & Sons, New York, 1978.

LI, H. et al. Journal of Ethnopharmacology, 144 (2012) 322-327.

MOLE, S.; WATERMAN, P. Oecologia, 72 (1987) 148-156.

PARK, B.J.; TOMOHIKO, M. Chemistry of Natural Compunds, 47(3) (2011) 363-365.

ROLIM, A. et al. Journal of AOAC International, 88(4) (2005) 1015-1019.

Tropicos.org. Missouri Botanical Garden (2021). Retrieved from https://www.tropicos.org/name/26600161. Accessed January 29, 2021.

Labmaq. Leito Fluidizado (2021). Retrieved from http://www.labmaqdobrasil.com.br/index.php/leitos-fluidizados/. Accessed January 29, 2021.

ACKNOWLEDGEMENTS

CAPES

FAPEG Fundação de Amparo à Pesquisa do Estado de Goiás

PDTI Bioprodutos

UFG

NEUROPROTECTIVE EFFECTS OF MULTICOMPONENT CHINESE HERBAL TEAS

Filipe Fernandes[1], João Moreira[1], M. Fátima Barroso[1], Mónica Dias-Teixeira[1,2], José Paulo Pereira[2], Lin Jiashen[3], Cristina Delerue-Matos[1], Clara Grosso[1*]

[1]REQUIMTE/LAQV, Instituto Superior de Engenharia do Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4249-015 Porto, Portugal;

[2]NICiTeS – Núcleo de Investigação em Ciências e Tecnologias da Saúde, Escola Superior de Saúde Ribeiro Sanches, Lisboa, Portugal;

[3]Zhuhai Lin Shangxi Health Tea Co., Ltd, China

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NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Abstract

The Traditional Chinese medicine is one of the oldest medical systems and was developed in China about 3000 years ago. One of its branches, the Chinese herbal medicine, has a long history of use for prevention and treatment of several diseases, including mental health, with several preparations being prescribed against major depressive disorder (MDD) and dementia [1,2].
In this study, four multicomponent Chinese herbal teas were evaluated, for the first time, against two enzymes related with Alzheimer's disease (acetylcholinesterase – AChE - and butyrylcholinesterase - BuChE) and MDD (monoamine oxidase A – MAO-A).

Materials



Wei Er Le tea



Honeysuckle tea



Shangxi Yiyang tea



Shangxi Fuyi tea

Methodology











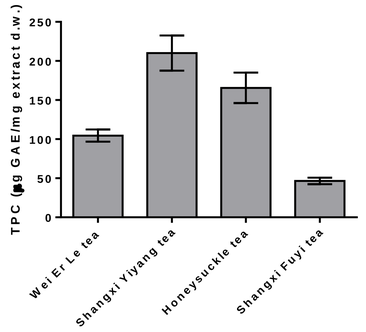
85°C, 10 min

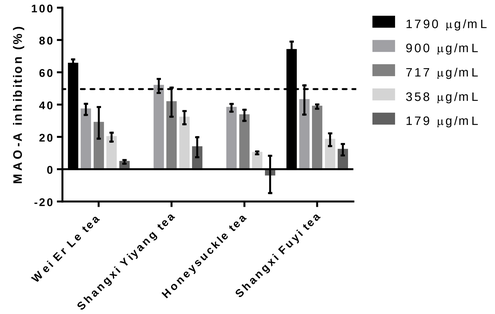
- **Total phenolic content, TPC**
- **Acetylcholinesterase (AChE) inhibition**
- **Butyrylcholinesterase (BuChE) inhibition**
- **Monoamine oxidase A (MAO-A) inhibition**

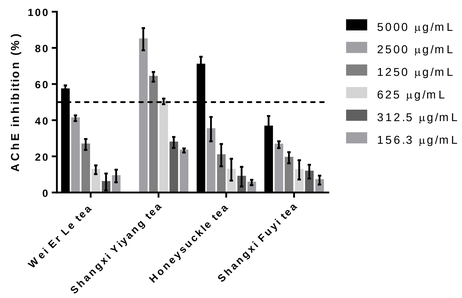
Results

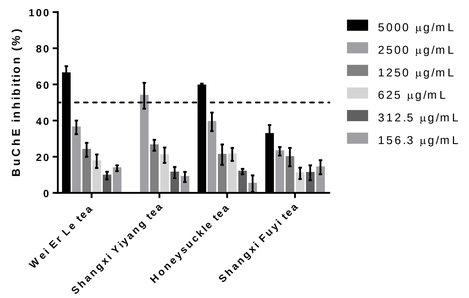
Total Phenolic Content (TPC), inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and monoamine oxidase A (MAO-A)

Sample	TPC (µg GAE/mg extract d.w.)	AChE inhibition (IC_{50}, µg/mL)	BuChE inhibition (IC_{50}, µg/mL)	MAO-A inhibition (IC_{50}, µg/mL)
Wei Er Le tea	104.5 ± 7.8	3889.0	3645.3	1306.2
Shangxi Yiyang tea	210.1 ± 22.4	619.3	2327.2	870.9
Honeysuckle tea	165.6 ± 19.5	3544.5	3825.9	> 900
Shangxi Fuyi tea	46.5 ± 4.2	> 5000	> 5000	1104.0









Total Phenolic Content (TPC), inhibition of monoamine oxidase A (MAO-A), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

Conclusion

The results obtained showed the potentialities of these phenolic-rich multicomponent herbal formulations to affect three different targets implicated in the etiology of brain disorders.

For the inhibition of cholinesterases, the most promising teas were the Shangxi Yiyang tea, the Wei Er Le tea and the Honeysuckle tea. For the inhibition of MAO-A, the order of potency was Shangxi Yiyang tea > Shangxi Fuyi tea > Wei Er Le Tea > Honeysuckle tea.

The bioactivities of the teas can be correlated with the Total Phenolic Content of the samples, of which the Sharigxi Yiyang tea displayed the highest value.

The results obtained showed the potentialities of these phenolic-rich multicomponent herbal formulations to affect three different targets implicated in the etiology of brain disorders.

References

[1] W. F. Yeung, K. F. Chung, K. Y. Ng, Y. M. Yu, S. P. Zhang, B. F. L. Ng, E. T. C. Ziea. Evidence-Based Complementary and Alternative Medicine, 2015, Article ID 160189.
[2] Z. Lin, J. Gu, J. Xiu, T. Mi, J. Dong, J. K. Tiwari. Evidence-Based Complementary and Alternative Medicine, 2012, Article ID 692621.

Acknowledgements

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Effects of *Myrciaria jaboticaba* Peel Extract on the Oxidative State of the Brain, Liver and Plasma of Rats with Adjuvant-Induced Arthritis

Heloisa Vialle Pereira Maróstica, Pâmela Alves Castilho, Gustavo Henrique de Souza, Ciomar Aparecida Bersani Amado, Rosane Marina Peralta, Adelar Bracht, Jurandir Fernando Comar, Lívia Bracht, Anacharis Babeto de Sá-Nakanishi.

State University of Maringá, Paraná, Brazil.

Abstract

Adjuvant-induced arthritis (AIA) is an experimental immunopathology in rats that shares many characteristics of rheumatoid arthritis in humans and is often used as a model for this disease [1]. Jabuticaba, *Myrciaria jaboticaba*, is a fruit native to Brazil and has been widely studied for being a good source of phenolic compounds. The results of this work showed that *M. jaboticaba* peel extract was able to reduce the levels of oxidative damage mainly in the plasma and improve the enzymatic antioxidant defense system, protecting rats tissues from oxidative stress induced by arthritis.

Introduction

Jabuticaba, *Myrciaria jaboticaba* has been widely consumed either *in natura* or used in the production of jams, wines and juices. The *jabuticaba* peel, on the other hand, is rigid and has an astringent taste. It is usually discarded as residue by the food industry, but it is also a potential source of bioactive molecules and a functional food [2]. The objective of the study was to investigate the actions of the *M. jaboticaba* peel extract (250 and 500 mg/Kg) on the oxidative state of the brain, liver and plasma of arthritic rats.





Material and methods

Controls and arthritics animals were treated once a day by oral administration (gavage) of the extract during 23 days. Healthy control and control arthritics rats received water for the same period.





Results

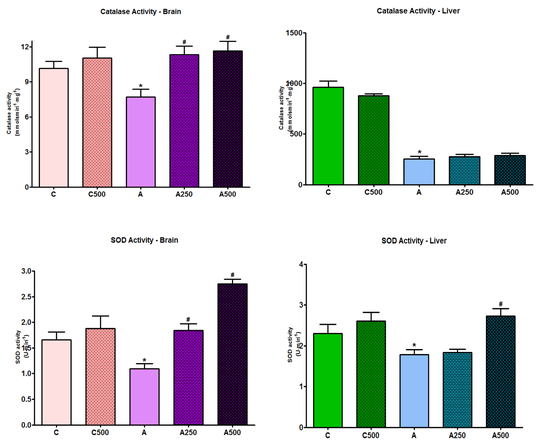


Figure 1. Effects of treatment with *Myrciaria jabuticaba* peel extract on the activity of antioxidant enzymes in the brain (on left) and in the liver (on right) of healthy and arthritic rats. C, controls; C500, controls treated with 500 mg · Kg^{-1} of the extract; A, arthritic rats; A250 and A500, arthritic rats treated with 250 and 500 mg · Kg^{-1} of the extract respectively. The data are the means ± EPM of five animals for each experimental condition. Statistical analysis: ANOVA ONE-WAY with Newman-Keuls post-hoc test. *p<0.05: different from controls (C); #p<0.05: different from untreated arthritic rats (A).

Results

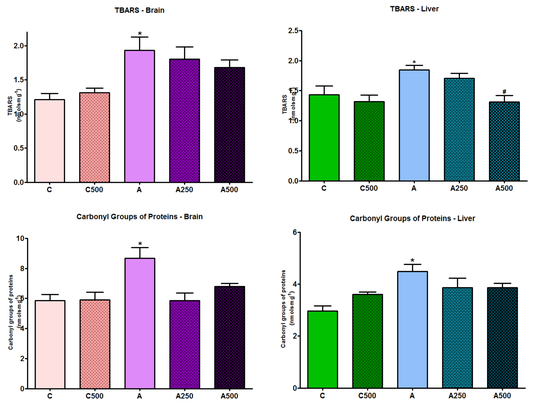


Figure 2. Effects of treatment with *Myrciaria jaboticaba* peel extract on TBARS levels and carbonyl groups of´protein in the brain (on left) and in the liver (on right) of healthy and arthritic rats. Values are expressed per mg of protein. C, controls; C500, controls treated with 500 mg · Kg^{-1} of the extract; A, arthritic rats; A250 and A500, arthritic rats treated with 250 and 500 mg · Kg^{-1} of the extract respectively. The data are the means ± standard error of the average of five animals for each experimental condition. *p<0.05: different from controls (C); #p<0.05: different from untreated arthritic rats (A).

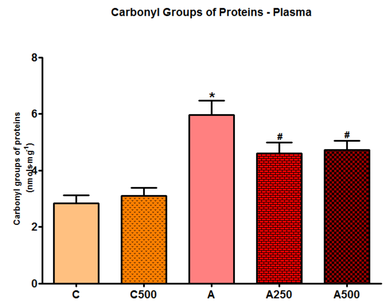


Figure 3. Effects of treatment with *Myrciaria jaboticaba* bark extract on protein carbonyl groups in the liver of healthy and arthritic rats. Values are expressed per mg of protein. C, controls; C500, controls treated with 500 mg · Kg^{-1} of the extract

A, arthritic rats; A250 and A500, arthritic rats treated with 250 and 500 mg · Kg^{-1} of the extract respectively. The data are the means ± standard error of the average of five animals for each experimental condition. Statistical analysis: ANOVA ONE-WAY with Newman-Keuls post-hoc test. *p<0.05: different from controls (C); #p<0.05: different from untreated arthritic rats (A).

Conclusion

The results showed that *M. jaboticaba* peel extract was able to reduce the levels of oxidative damage in lipids and proteins mainly in the plasma and improve the enzymatic antioxidant defense system, protecting tissues from oxidative stress induced by arthritis.

References

[1] Comar, J. F., de Sá-Nakanishi, A. B., de Oliveira, A. L., Wendt, M. M. N., Amado, C. A. B., Iwamoto, E. L. I., ... & Bracht, A. (2013). *Free Radical Biology and Medicine*, 58, 144-153.

[2] Alezandro, M. R., Dubé, P., Desjardins, Y., Lajolo, F. M., & Genovese, M. I. (2013). *Food Research International*, 54 (1), 468-477.

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Natural products application: Health, Cosmetic and Food
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Pterospartum tridentatum: phytochemical profile and antioxidant activity of methanolic extracts

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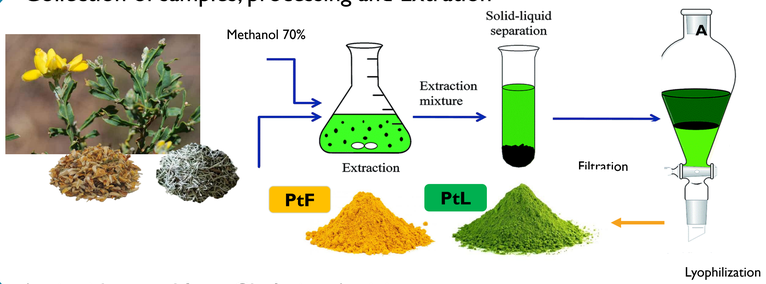
Introduction

Pterospartum tridendatum is a fabaceae plant native of the North-West Iberian Peninsula and Morocco. This plant is used in folk medicine and by populations for the management of several pathologies like, diabetes, hypertension, respiratory and digestive disorders. Nevertheless, scientific literature on *P. tridendatum* is scarce and fragmented.

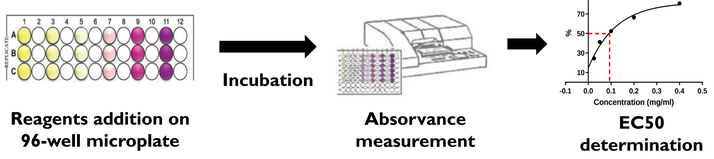
The aim of this work was i- to produce methanolic extracts (70:30 v/v) of leaves and flowers of *Pterospartum tridendatum* (PtL and PtF, respectively) collected in the littoral of Portugal (Esposende), ii- to determine the antioxidant activity of them, and iii- to correlate it with total phenolic content by Folin-Ciocalteu spectrophotometric method.

Materials and Methods

Collection of samples; processing and Extration



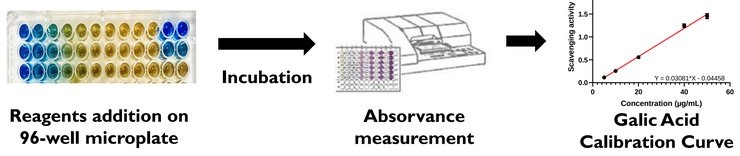
Antioxidant and Iron Chelation Activity



Reagents addition on 96-well microplate | **Absorvance measurement** | **EC50 determination**

Assay	Reagents	Incubation	Absorvance
DPPH	Replicate: 10µl extract + 140µg DPPH Blank: 10µl extract + 140µg DMSO Control: 150µl DPPH	60 min	515
SO	Replicate: 50µl extract + 50µl NADH + 150µl NBT + 50µl PMS Blank : 50µl extract + 50µl NADH + 150µl NBT + 50µl KH2PO4 Control: 50µl NADH + 150µl NBT + 50µl PMS + 50µl KH2PO4	15 min	560
NO	Replicate: 50µl extract + 50µl SNP + 50µl Griess reagent (after incubation) Blank: 50µl extract + 50µl SNP + 50µl KH2PO4 (after incubation) Control: 50µl extract + 50µl SNP + 50µl Griess reagent (after incubation) Blank control: 50µl extract + 50µl SNP + 50µl KH2PO4 (after incubation)	60 min	562
ICA	Replicate: 50µl extract + 50µl FeSO4 + 50µl Fz Blank: 50µl extract + 50µl FeSO4 + 50µl H2O Control: 50µl H2O + 50µl FeSO4 + 50µl Fz	10 min	515

Folin-Ciocalteu (F-C) Method

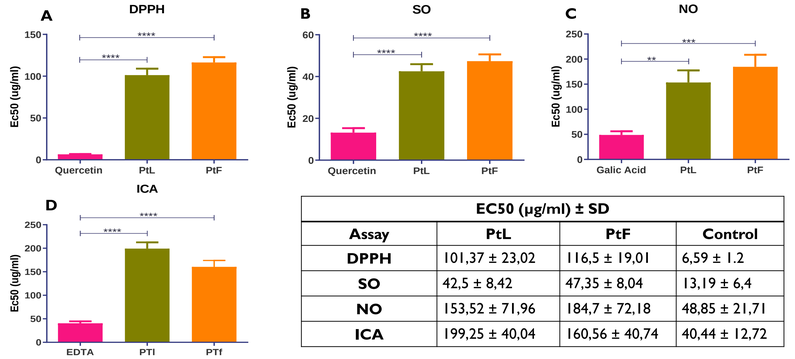


Reagents addition on 96-well microplate | **Absorvance measurement** | **Galic Acid Calibration Curve**

Assay	Reagents	Incubation	Absorvance
FC	Replicate: 10µl Extract + 50µl Folin reagent + 40µl Na2CO3 Blank: 10µl extract + 90µl H2O Control: 10µl H2O + 50µl Folin reagent + 40µl Na2CO3	60 min	762

Results

Antioxidant and Iron Chelation activity



EC50 (µg/ml) ± SD			
Assay	**PtL**	**PtF**	**Control**
DPPH	101,37 ± 23,02	116,5 ± 19,01	6,59 ± 1.2
SO	42,5 ± 8,42	47,35 ± 8,04	13,19 ± 6,4
NO	153,52 ± 71,96	184,7 ± 72,18	48,85 ± 21,71
ICA	199,25 ± 40,04	160,56 ± 40,74	40,44 ± 12,72

Figure 1. EC50 values of PtL and PtF extracts in (A) DPPH ($ANOVA_{one\text{-}way}$: $F_{(2,24)}$ =107.0, P<0.0001) , (B) SO ($ANOVA_{one\text{-}way}$: $F_{(2,24)}$ =46.98, P<0.0001), (C) NO ($ANOVA_{one\text{-}way}$: $F_{(2,24)}$ =12.59, P=0.0002), scavenging assays and in (D) Iron Chelating activity ($ANOVA_{one\text{-}way}$: $F_{(2,14)}$ =54.06, P<0.0001). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005 and ***P<0.001, ****P<0.0001

Both extracts exhibited strong antioxidant activities, as evidenced by the low EC50 values in scavenging methods and Iron Chelating Activity. Both extracts displayed low EC50 in the Super Oxide scavenging assay

Total Phenolic Content (F-C Method)

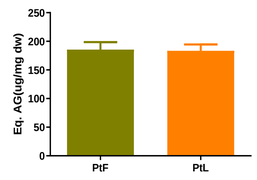
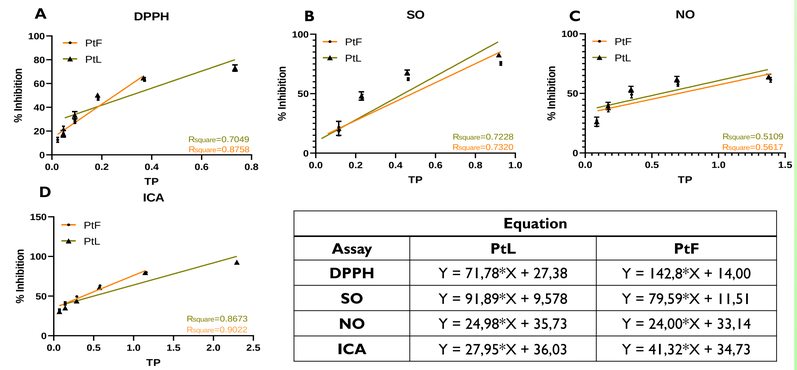


Figure 2. Content of Total phenolic of PtL and PtF extracts ($t_{(16)}$=0.3524, P=0.7291). Results are expressed as mean ± SEM.

Both extracts exhibited high levels of Total Phenolic Content (PtL: 185.447 ± 13.212; PtF: 183.464 ± 10.915)

Antioxidant activity and Total Phenolic content correlation



Equation		
Assay	**PtL**	**PtF**
DPPH	Y = 71,78*X + 27,38	Y = 142,8*X + 14,00
SO	Y = 91,89*X + 9,578	Y = 79,59*X + 11,51
NO	Y = 24,98*X + 35,73	Y = 24,00*X + 33,14
ICA	Y = 27,95*X + 36,03	Y = 41,32*X + 34,73

Figure 3. Correlation between (A) DPPH, (B) SO, (C) NO and (D) Iron Chelating activities and Total phenolic content of the extracts.

In a linear regression, a positive strong level of agreement (R>0.5) between the antioxidant activities and total phenolic content was observed in both extracts.

Conclusion

Both extracts exhibited strong antioxidant activities, as evidenced by the low EC50 values in both DPPH, nitric oxide and superoxide scavenging methods and Iron Chelating Activity (ICA). Additionally, antioxidant activity and the total phenolic content demonstrated a strong positive correlation.
Our work highlight the potential of *Pterospartum tridentatum* methanolic extracts as a source of compounds for the development of new therapies targeting oxidative stress-related diseases.

Acknowledgements

CITAB Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas
cbma centre of molecular and environmental biology
ICVS Life and Health Sciences Research Institute Instituto de Investigação em Ciências da Vida e Saúde
Universidade do Minho
PD+F FCT PhD PROGRAMMES PD/BD/ 150263/2019
POCH
PORTUGAL 2020
UNIÃO EUROPEIA Fundo Social Europeu

BIOACTIVE COMPOUNDS OF OLIVE SEEDS FROM THREE CULTIVARS WITH VALUABLE BIOLOGICAL PROPERTIES

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Abstract

The present work aimed to evaluate the phytochemical composition of seed extracts from different olive cultivars (Cobrançosa, Galega and Picual), as well as their antioxidant capacity. The content of total phenols ranged between 11.90 ± 1.56 and 14.71 ± 1.37 mg GA g^{-1}. Regarding *ortho*-diphenols and flavonoids content, Galega seed extracts revealed to present significantly higher concentrations than Cobrançosa and Picual samples with 7.05 ± 0.44 mg GA g^{-1} and 2.40 ± 0.18 mg CAT g^{-1}, respectively. The antiradicalar scavenging capacity of phenolic olive seed extracts have been performed by ABTS and DPPH methods. As expected, Galega cultivar samples presented the highest activity compared to the other cultivars studied with 0.065 ± 0.004 and 0.016 ± 0.002 mmol Trolox g^{-1} for ABTS and DPPH, respectively.

The HPLC–DAD–ESI/MSn screening of the three cultivars of olive seeds studied revealed similar chromatograms. Six phenolic compounds were identified and recorded at 280 nm: one phenyl alcohol, two flavonoids, three secoiridoids. The most abundant compounds are nüzhenide (32.45 mg g^{-1}, on average), ligstroside (4.52 mg g^{-1}, on average), and rutin (3.08 mg g^{-1}, on average).

Some studies allowed to identify some phenolic compounds in olive seeds, however, very few investigations have quantified these compounds in this matrix. Overall, our results indicate that olive seeds extracts may provide a new and alternative source of agents for medical and industrial applications.

Introduction

Olive oil production is one of the most important agro food industries in South Europe and Mediterranean area, with great economic and social importance for these countries. Nowadays, the awareness and efforts to reach "zero waste" along the chain production, has made growing attention and research about bioactive compounds coming from olive oil by-products, with the objective of developing innovative applications in cosmetic, pharmaceutical, nutritional, and technological food industry. Thus, it is possible to add value to bioactive compounds present in the waste by increasing the efficiency of the use of resources, and reducing costs and environmental impact (1,2).

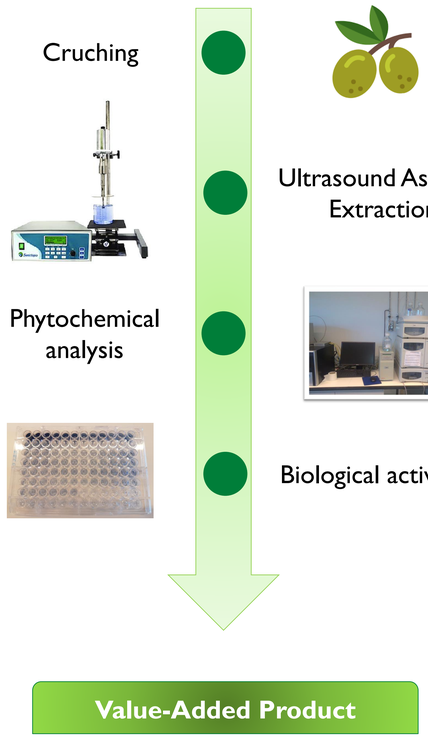
Antioxidants are chemical compounds that counteract the oxidation of proteins, DNA and lipids being one group of compounds that has captured the interest of both scientific community and general public (3). In fact, oxidative damage is related to the physiopathology of several diseases, such as metabolic syndrome, atherosclerosis, cancer, cardiovascular, and brain ageing (4).

Therefore, this work aimed to apply the ultrasound-assisted extraction for the first time in olive seeds from Cobrançosa, Galega, and Picual cultivars in order to recover phenolic compounds and study their antiradicalar scavenging potential.

Materials



Methodology



Results

Phenolic composition

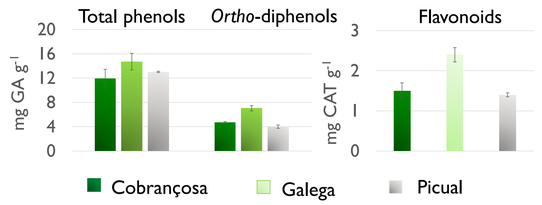


Table 1. Identification of phenolic compounds in olive seeds from different cultivars by HPLC–DAD–MS^n in negative mode.

Peak no.	Compound id.	RT (min)	λ (UV) (nm)	$[M–H]^-$, m/z	Fragments
(1)	Tyrosol	14.77	234; 275	137	119, 106
(2)	Rutin	24.00	252; 355	609	301, 179
(3)	Nüzhenide	25.16	248; 275	685	1371, 731, 523
(4)	Luteolin-7-glucoside	25.45	256; 350	447	285
(5)	Oleuropein	26.43	245; 280	539	377, 307, 275, 223
(6)	Ligstroside	26.89	249	523	361, 291, 259

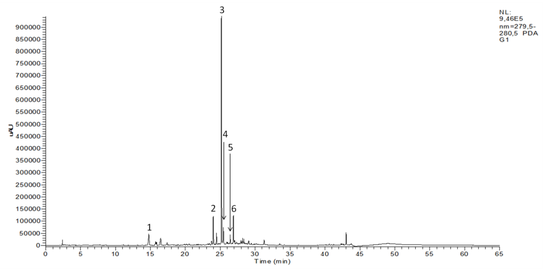
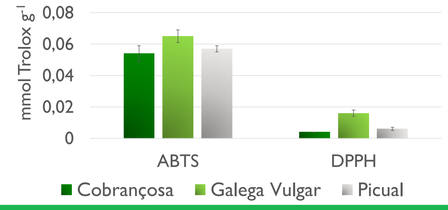


Figure 1. HPLC–DAD chromatogram (280 nm) of olive seeds from Galega cultivar: (1) Tyrosol; (2) Rutin; (3) Nüzhenide; (4) Luteolin-7-glucoside; (5) Oleuropein; (6) Ligstroside.

Antioxidant capacity



Results and Conclusion

Phenolic content of olive seeds

The phenolic content of olive seeds from Cobrançosa, Galega, and Picual cultivars was assessed, being the results presented in Table 1. As it can be observed, no significant differences ($P > 0.05$) were found between the cultivars concerning the content of total phenols, values ranging between 11.90 ± 1.56 and 14.71 ± 1.37 mg GA g^{-1}. However, regarding *ortho*-diphenols and flavonoids content, Galega seed extracts revealed to present significantly higher concentrations than Cobrançosa and Picual samples with 7.05 ± 0.44 mg GA g^{-1} and 2.40 ± 0.18 mg CAT g^{-1}, respectively. The total phenolic content in these olive seeds revealed to be present in higher concentrations than those found in other studies, such as by Alu'datt et al. (2011) that determined concentrations between 1.03 and 2.23mg GA g^{-1} depending on the extraction temperatures (5). Just few studies investigated the total phenolic content of olive seeds, with no studies found concerning the total *ortho*-diphenols and flavonoids content, as far as we know. In this sense, Falcinelli et al. (2018) determined the total phenolic content in olive seeds from Moraiolo, Leccino and Arbequina cultivars, obtaining about 4 mg GAE g^{-1}, significantly lower than those of the present study (6). Flores et al. (2018) also determined low contents of total phenolics in Manzanilla cultivar olive seeds untreated with absicic acid of about 0.09 mg GA g^{-1} (7). This can be explained by the high efficiency of the extraction method employed in the present study (UAE) which present some advantages including short times extraction, low temperatures and low amount of solvent. However, Silva et al. (2006) determined concentrations between 5.9 and 14.9 mg g^{-1} of total phenolic content in olive seeds from ten cultivars growing in Portugal, being in the range of the present study (8).

Antioxidant capacity of olive seeds

The antiradicalar scavenging capacity of phenolic olive seed extracts have been performed by ABTS and DPPH methods, with results presented in Table 1. As expected, Galega cultivar samples presented the highest activity compared to the other cultivars studied with 0.065 ± 0.004 and 0.016 ± 0.002 mmol Trolox g^{-1} for ABTS and DPPH, respectively. As far as we know, Falcinelli et al. (2018) were the only authors which determined the antioxidant capacity of olive seeds by these methods. These authors obtained activities between 0.008 and 0.012 mmol Trolox g^{-1} and between 0.0001 and 0.012 mmol Trolox g^{-1} for ABTS and DPPH methods, respectively, revealing considerably lower values than those obtained in this study (6). However, Alu'datt et al. (2011) also investigated some variable extraction parameters such as time, temperature, solvent type, sequential extraction of solvents and the effects of multi-step extraction in order to evaluate the antioxidant potential of olive seeds (5).

References

(1) Roselló-Soto, E., Koubaa, M., Moubarik, A., Lopes, R. P., Saraiva, J. A., Boussetta, N., Grimi, N., & Barba, F. J. (2015). *Trends in Food Science and Technology*, *45*(2), 296–310.
(2) Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014). *International Journal of Molecular Sciences*, *15*(9), 15638–15678.
(3) Pandey, K. B., & Rizvi, S. I. (2009). *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.
(4) Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D., & Abete, P. (2018). *Clinical Interventions in Aging*, *13*, 757–772.
(5) Alu'datt, M. H., Alli, I., Ereifej, K., Alhamad, M. N., Alsaad, A., & Rababeh, T. (2011). *Natural Product Research*, *25*(9), 876–889.
(6) Falcinelli, B., Maranghi, S., Paoletti, A., Marconi, O., Rosati, A., Famiani, F., & Benincasa, P. (2018). *Scientia Horticulturae*, *240*(June), 558–560. https://doi.org/10.1016/j.scienta.2018.06.066
(7) Flores, G., Blanch, G. P., & Ruiz del Castillo, M. L. (2018). *Lwt*, *90*(July 2017), 556–561.
(8) Silva, S., Gomes, L., Leitão, F., Coelho, A. V., & Boas, L. V. (2006). *Food Science and Technology International*, *12*(5), 385–395.

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THERAPEUTIC EFFECTS OF GINGER (*Zingiber Officinale*): LITERATURE REVIEW

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INTRODUCTION

Over the years and due to the advancement of medicine, there is an increasing interest in herbal medicine, which consists of a set of techniques for using products of plant origin for the purpose of therapeutic, that is, to prevent or cure diseases. Among the various plants studied in phytotherapy we can highlight gengribe for its pharmacological properties, being endowed with a rich nutritional composition.



MATERIAIS AND METHODOLOGY

This work was carried out through searches conducted on various online sites. The keywords were used: Ginger, Zingiber officinale, therapeutic and pharmacological properties. Articles in Portuguese were used, being selected those that presented relevant information for the elaboration of the work and were articles of low credibility were excluded. The objective of this work was to present and discuss the effects methods of ginger, including the general use of this plant for medicinal purposes.



RESULTS

The nutritional composition of Ginger is described in Table 1, a functional food that acts as a source of fiber and potassium, which makes it quite commercialized and used in medicine.

CONCLUSION

The jobs found in the literature point to ginger as a plant originating in South Asia, where spread to other regions of the world, having recognition in Europe at the time of the discoveries having been brought from India along with other spices. In Portugal there is a record of its presence from the 16th century onwards, but before that it had been used for over 2000 years in the East. In the last Herbal medicine has become more popular over the years and while use in the past was empirically, we can now identify the active components of each plant and with the new understanding of human physiology, explain its mechanism of action. As for ginger, we have described in the Scientific Literature the main pharmacological properties and effect being endowed with action antimicrobial, anti-inflammatory, diuretic, antipyretic, hepatoprotective and antioxidant.

Valores nutricionais por 100g de Gengibre		
Valores por:		% Dose de referência*
Energia (Kcal)	81	4
Lípidos Totais (g)	1	1
Ácidos Gordos Saturados (g)	0	0
Hidratos de Carbono (g)	16	12
dos quais açúcares (g)	2	2
Fibra (g)	2	7
Proteínas (g)	2	4
Sal (g)	0,33	6
Vitamina E (mg)	1,1	9
Vitamina B6 (mg)	0,2	14
Potássio (mg)	415	21
Magnésio (mg)	43	11

* Doses de referência de acordo com Regulamento da União Europeia nº1169/2011 de 25 de outubro de 2011

Tabela 1: Valores Nutricionais do Gengibre (*Zingiber officinale*).

REFERENCES

[1] Zhang, F., Thakur, K., Hu, F., Zhang, J.-G., & Wei, Z.-J. Cross-talk between 10- gingerol and its anti-cancerous potential: a recent update. Food Funct., 8(8), 2017.

Catharanthus roseus FLOWERS AQUEOUS EXTRACT INEFFECTIVENESS LARVICIDAL ACTIVITY AGAINST *Aedes aegypti* L. IN VITRO.

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Abstract

The mosquito species *Aedes aegypti* L. are important vectors of several diseases, and control of these insects is imperative for human health. The use of insecticides for prolonged periods to control vectors without adequate monitoring of the population in relation to its consequences may promote the mosquito vectors resistant populations selection [1]. With the growing concern for the mosquito borne diseases, urge for different botanicals as mosquitocidal or larvicidal agents has increased. *Catharanthus roseus* L. belongs to the *Apocynaceae* family, is native to Madagascar, and has compounds that can stimulate the microtubules depolarization, to β-tubulins binding for polymerization preventing vinblastine and vincristine [2]. Vincristine has anti-cancer activity and toxicity different from vinblastine, even though it has similar chemical characteristics [3]. Because of all this fact, the *Catharanthus roseus* L. flowers aqueous extract was tested, in of 10, 50 and 100 µg/ml concentrations, on the Aedes aegypti L. L4 larvae, for a period of 48 hours. Its flowers aqueous extract proved to be harmless to the larvae of the mosquito Aedes aegypti L., under the conditions tested here.

Introduction

Catharanthus roseus L. belongs to the *Apocynaceae* family, is native to Madagascar, is commonly names: vinca, cat vinca, madagascar vinca, good night and shameless maria, and has compounds that are capable of stimulating the depolarization of microtubules, binding to β-tubulins preventing polymerization. The compounds that represent this class are mainly vinblastine and vincristine (DAS; SHARANGI, 2017), vincristine has anti-cancer activity and toxicity different from vinblastine, even though it has similar chemical characteristics (MARQUES; LOPES, 2015).



Figure 1. *Catharanthus roseus* L. (Google Image, 2021).

Materials

Figure 2. Larvae collect



Font: Research data. Photo: Nelcineide Almeida Simões (2019).

Methodology

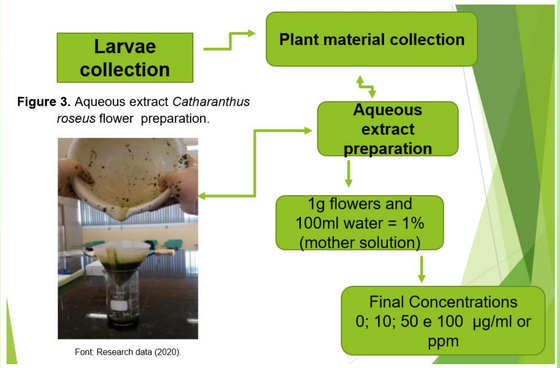


Figure 3. Aqueous extract *Catharanthus roseus* flower preparation.

Font: Research data (2020).

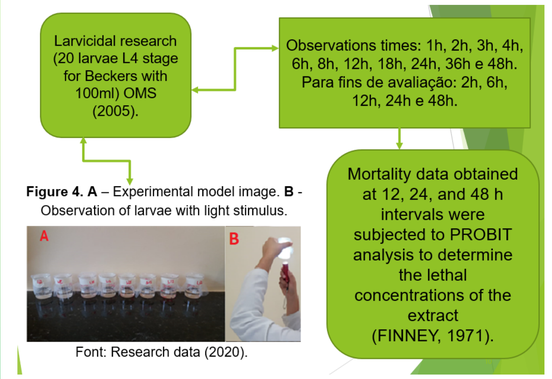
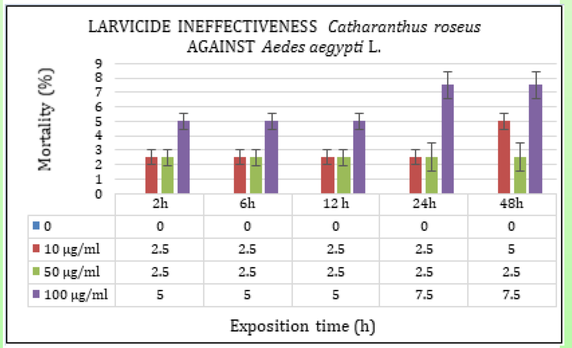
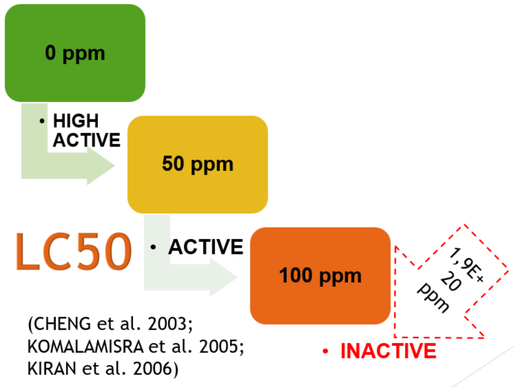


Figure 4. A – Experimental model image. **B** - Observation of larvae with light stimulus.

Font: Research data (2020).

Results





(CHENG et al. 2003; KOMALAMISRA et al. 2005; KIRAN et al. 2006)

Despite their benefits, all alkaloids have neurotoxic activity, especially vincristine, affecting neurotransmission. Vincristine and vinblastine are highly toxic antimitotics, blocking mitosis in metaphase after binding to microtubules. In addition, many side effects have been reported for these drugs including myelosuppression, alopecia, abdominal cramps, constipation, nausea / vomiting, paralytic ileus, mouth ulcerations, hepatocellular damage, kidney failure, pulmonary fibrosis, urinary retention, amenorrhea, azoospermia, orthostatic hypotension and hypertension. Dosage and administration must be carefully controlled to reduce side effects (NEJAT et al., 2015).

Conclusion

The results obtained here demonstrate that the aqueous extract of *Catharanthus roseus* L. flowerswas **not effective** as a larvicidal potential in the concentrations tested here. There is also no intervention in the dynamics of the reproductive cycle of *Aedes aegypti* L., since some of these larvae have reached the pulp stage.

Recommendations

Although the biochemical properties of the tested plant are well known, from which drugs for leukemia chemotherapy are extracted, its flowers proved to be harmless to the larvae of the mosquito *Aedes aegypti* L., under the conditions tested here

References

CHENG, S.; CHANG, H.; CHANG, S. Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. **Bioresour. Technol. 89, 99–102. 2003.**

Finney, D.J. **Probit Analysis.** third ed. Cambridge university press, Cambridge, 1971. p. 333.

KIRAN, S.R.; BHAVANI, K.; DEVI, P.S.; RAO, B.R.R.; REDDY, K.J. Composition and larvicidal activity of leaves and stem essential oils of *Chloroxylon swietenia* DC against *Aedes aegypti* and *Anopheles stephensi*. **Bioresour. Technol. 97, 2481–2484. 2006.**

KOMALAMISRA, N.; TRONGTOKIT, Y.; RONGSRIYAM, Y.; APIWATHNASORN, C. Screening for lavicidal activity in some Thai plants against four mosquito vector species. **Southeast Asian J. Trop. Med. Public Health 36, 1412–1422. 2005.**

Organização Mundial de Saúde. **GUIDELINES FOR LABORATORY AND FIELD TESTING OF MOSQUITO LARVICIDES.** WORLD HEALTH ORGANIZATION COMMUNICABLE DISEASE CONTROL, PREVENTION AND ERADICATION WHO PESTICIDE EVALUATION SCHEME. 2005. 41p.

Acknowledgements

This study was financially supported by PROPPG/IFPA and CNPq.

TOPICAL ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF COENZYME Q10

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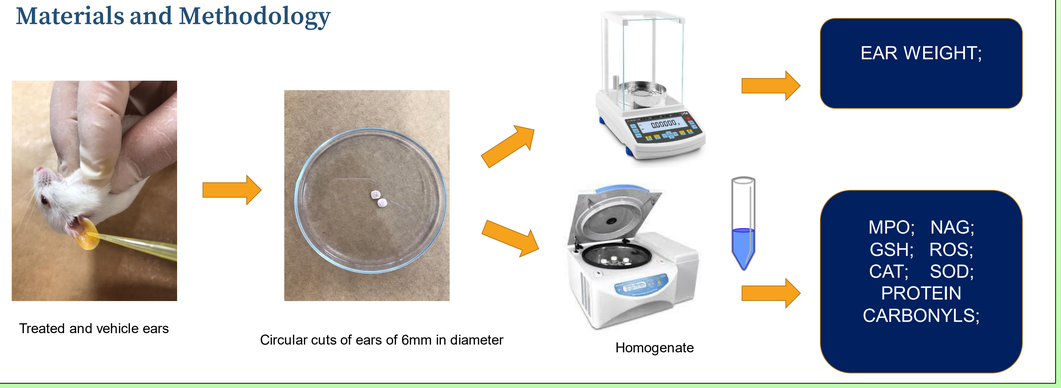
Abstract

Coenzyme Q10 (CoQ10), or ubiquinone, is a lipid-soluble benzoquinone, with a long isoprenoid side chain, which has antioxidant and photoprotective properties. Although some studies have already shown that CoQ10 has in vitro [2] and in vivo [1] anti-inflammatory activity, no studies have demonstrated the effects of topical application of CoQ10 on a model of skin inflammation. Thus, the aim of this work was to investigate the effects of CoQ10 administered topically on ear edema induced by croton oil (OC) in mice. Swiss mice weighing between 30-40 g were used. Ear edema was induced by the application of croton oil to the inner ear of mice. Animals received CoQ10 (0.03 to 0.5 mg/ear) or dexamethasone (positive control, 0.1 mg/ear) topically, dissolved in acetone. Myeloperoxidase [3] (MPO) and N-acetylglucosaminidase (NAG) [4] activities were evaluated in the homogenate supernatant of the ear sections (controls and treated). Parameters indicating oxidative stress (reduced glutathione – GSH - content, protein carbonyls, reactive oxygen – ROS - species and antioxidant enzymes) were measured in ear tissue homogenate [3]. Antioxidant capacity of CoQ10 was also evaluated in vitro in chemical assays and on the real time ROS generation using mice liver isolated mitochondria [5]. The results obtained in the present study demonstrated that the topical application of CoQ10 was able to significantly reduce the ear edema induced by croton oil and the migration of polymorphonuclear and mononuclear leukocytes. Additionally, topically applied CoQ10 reduced ROS content in the tissue and normalized the reduced GSH content. The in vitro experiments demonstrated a low direct antioxidant activity of CoQ10 in chemical tests (FRAP and ABTS). However, CoQ10 was able to significantly reduce real time generation of ROS in mice liver isolated mitochondria. Therefore, our results clearly demonstrate that topically applied CoQ10 is able to reduce the skin inflammatory process and improve some oxidative stress parameters in the croton oil ear edema model in mice.

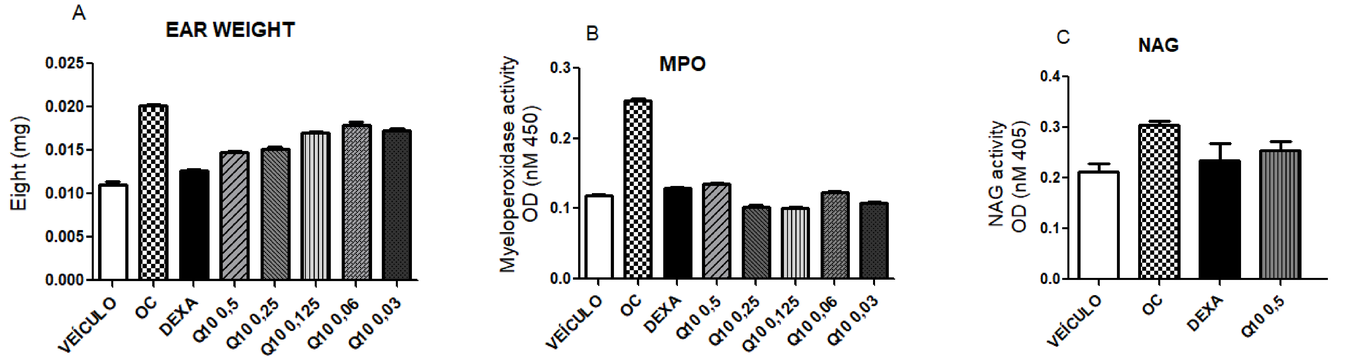
Introduction

Coenzyme Q10 (CoQ10), or ubiquinone, is a lipid-soluble benzoquinone, with a long isoprenoid side chain, which has antioxidant and photoprotective properties [1]. It is commercialized as a food supplement and used in cosmetic formulations. Although some studies have already shown that CoQ10 has in vitro [2] and in vivo [1] anti-inflammatory activity, no studies have demonstrated the effects of topical application of CoQ10 on a model of skin inflammation.

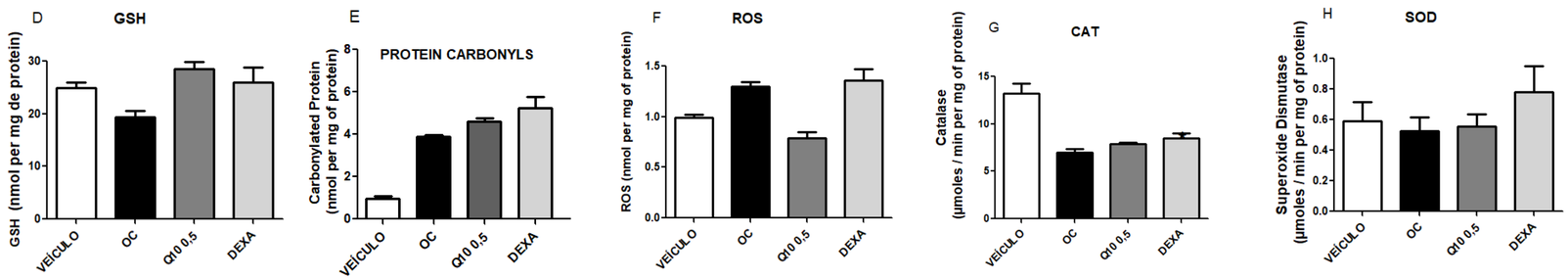
Materials and Methodology



Results



A: Ear swelling; Panel B: Myeloperoxidase (MPO) enzyme activity; Panel C: Activity of the enzyme N-acetylglycosaminidase (NAG).



Panel D: Content of reduced glutathione (GSH); Panel E: Content of total total reactive species (ROS); Panel F: Activity of the antioxidant enzyme catalase (CAT); Panel G: Activity of the antioxidant enzyme superoxide dismutase (SOD); Panel E: Content of carbonylated protein.

Conclusion

Our results clearly demonstrate that topically applied CoQ10 is able to reduce the skin inflammatory process and improve some oxidative stress parameters in the croton oil ear edema model in mice. Additionally, *in vivo* antioxidant properties could be partially related to the ability of CoQ10 to reduce mitochondrial ROS generation. At least to our knowledge, this is the first report of a direct topical anti-inflammatory effect of CoQ10 in a *in vivo* model of skin inflammation.

Acknowledgements

CAPES for the financial support.

NEW INSIGHTS INTO ANTI-STREPTOCOCCAL POTENTIAL OF SELECTED ESSENTIAL OILS RELEVANT TO DENTISTRY

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[2]Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia,
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Abstract

Essential oils (EOs) are potent antibacterial agents that have numerous applications in different fields, such as traditional medicine, pharmacy and cosmetics, food and beverage industry. However, their use in dentistry is limited and not adequately explored. In order to investigate the potential to expand their applications in dentistry, we screened for antibacterial and antibiofilm activity of selected EOs toward *Streptococcus salivarius* dental isolates. Selected EOs were originated from *Pelargonium roseum, Pelargonium graveolens*, *Leptospermum petersonii* and *Eucalyptus citriodora*. In order to take insight into the variation in sensitivity among bacterial strains of different origin, both isolates from symptomatic and asymptomatic root canal infections, as well as the reference *S. salivarius* ATCC 9222 strain, were involved in this study. EOs were chemically characterized by GC/MS-FID analysis. Microdilution assay, was used to determine minimal inhibitory concentrations (MICs). Crystal violet (CV) assay was provided to explore the potential to disturb biofilm formation. Statistical analysis performed by One Way ANOVA was used to compare sensitivity between tested strains.

Chemical characterization revealed that all EOs were abundant in oxygenated monoterpenes (76.8 – 92.4% composition). Furthermore, the content of sesquiterpene hydrocarbons was relatively high in P. roseum and P. graveolens EOs (12.3% and 16.7%, respectively). The major constituents of *Pelargonium* oils were citronellol, geraniol, citronellyl formate and geranyl formate, contributing together with 71.3% and 57.1% of total *P. roseum* and *P. graveolens* EOs, respectively. EO of *L. petersonii* was abundant with following dominant constituents: geranial, neral, citronellal, citronellol and isopulegol (accounting for 81.2% of the total EO). Some of these constituents, namely citronellal, citronellol and isopulegol, were also dominant in *E. citriodora* oil, constituting 84.8% of its composition. MIC assay demonstrated that referent strain was the most sensitive, while the strains isolated from symptomatic infections were highly resistant. Considering all tested strains, the comparison of EOs antibacterial activity pointed out the following order, calculated in mg mL-1: *E. citriodora* (MIC values 0.08±0.03 – 5.42±1.71) > *L. petersonii* (MIC values 0.53±0.00 – 4.74±0.64) > *P. graveolens* (MIC values 0.36±0.15 – 5.54±0.00) > *P. roseum* (MIC values 1.50±0.68 – 13.77±0.69). The inhibition of biofilm formation by EOs was also strain specific; clinical isolates were less sensitive than the reference strain (the ranges of biofilm inhibition were 24%-75% and 84%-89%, respectively). Similarly as in the microdilution assay, the *E. citriodora* EO was the most potent biofilm inhibitor.

Taking into account all obtained results, *Eucalyptus citriodora* EO proved promising antibacterial and antibiofilm activity and could be recommended for further investigation as alternative antimicrobial in dental practice.

Introduction

Members of the genus *Streptococcus*, including *S. salivarius*, are capable to overcome standard infected root canal treatment. Unsuccessful therapy of infected root canals leads to tooth extraction. This is why alternative antimicrobials, that can be used for infected root canal disinfection, are of the utmost interest. Bearing in mind that essential oils (EOs) possess well documented antibacterial potential and no resistance issues, they seem to be good candidates for decent alternative antimicrobial agents in dentistry.

Materials

Four EOs were tested in this study:
1) *Leptospermum petersonii* Bailey oil, from the leaves of Australian lemon scented tea tree,
2) *Eucalyptus citriodora* oil, from the leaves of lemon scented eucalyptus from Madagascar,
3) *Pelargonium graveolens* L'Hér oil, from the leaves of geranium Bourbon from Madagascar,
4) *Pelargonium roseum* Wild. oil, from the leaves of Moroccan rose geranium.

As a positive control, triple antibiotic paste (TAP), composed of metronidazole (Orvagyl®, Galenika, Belgrade, Serbia, 400 mg), minocycline (Minocin®, Pfizer, New York, USA,100 mg) and ciprofloxacin (Ciprofloxacin®, Remedica LTD, Limassol, Cyprus, 200 mg) was used.

Four clinical isolates of *S. salivarius* and reference *S. salivarius* ATCC 9222 strain were included in the study.

Methodology

The GC–MS/FID was used for chemical characterization. Assessment of activities were provided by microdilution assay and crystal violet (CV) assay, for antibacterial and antibiofilm effect, respectively. Statistical analysis included group comparisons performed using One Way Analysis of Variance test, and Post Hoc comparisons performed using Low Significant Difference test.

Conclusion

***Eucalyptus citriodora* and *Leptospermum petersonii* essential oils induced notable antibacterial potential, while *E. citriodora* essential oil additionally achieved remarkable antibiofilm activity.**

Recommendations

In order to formulate effective antimicrobials that could be used as appropriate alternatives for treatment of root canals infections, following should be provided:

(1) Analysis of antibacterial activity of sole EOs active principals, i.e. oxygenated monoterpene - aldehydes and alcohols, as well as their the most active combinations;

(2) Analysis of antibacterial potential conducted on huge number of clinical isolates, involving the ones from asymptomatic, and especially symptomatic root canal infections.

Acknowledgements

The authors appreciate financial support of the Ministry of Education, Science and Technological Development of Republic of Serbia.

Results

Table 1. Origin of *S. salivarius* isolates

Type of isolate	Isolated from
S. salivarius	
STSL1	Symptomatic infected root canal
STSL2	Symptomatic infected root canal
STSL3	Symptomatic infected root canal
STSL4	Asymptomatic infected root canal

Table 2. Antibacterial activities of essential oils against *S. salivarius* clinical isolates and corresponding ATCC strains (MICs are presented in mg/ml; means ± SD).

	S. salivarius strains				
	symptomatic infections			**asymptomatic infections**	
	STSL1	**STSL2**	**STSL3**	**STSL4**	**ATCC 9222**
L. petersonii	4.37±0.96^{a}	4.74±0.64^{a}	3.30±0.00^{a,b,c}	1.16±0.81^{b}	0.53±0.00^{b}
E. citriodora	5.42±1.71^{a}	1.35±1.60^{b}	1.08±0.00^{b}	0.16±0.00^{c}	0.08±0.03^{c}
P. graveolens	5.54±0.00^{a}	5.54±0.00^{a}	1.11±0.00^{b}	0.54±0.00^{c}	0.36±0.15^{c}
P. roseum	13.77±0.69^{a}	9.28±3.22^{a}	11.14±3.57^{a}	2.23±0.00^{b}	1.50±0.68^{b}
TAP*	0.003±0.000^{a}	0.003±0.000^{a}	0.004±0.002^{a}	0.010±0.004^{b}	0.005±0.002^{a}

*Triple antibiotic paste (TAP) was used as a positive control.
Items denoted with different small letters are significantly different within subgroup (susceptibility of different isolates against each EO), p<0.05

Table 3. Dominant constituents (>1%) of *Leptospermum petersonii, Eucalyptus citriodora , Pelargonium roseum* and *Pelargonium graveolens* essential oils.

constituents (%)	***L. petersonii***	***E. citriodora***
α-Pinene	1.5	0.8
β-Pinene	1.6	0.1
Linalool	4.3	
Isopulegol	5.2	6.1
Citronellal	15.3	74.1
iso-Isopulegol	0.3	4.1
Terpinene-4-ol	2.2	
Citronellol	5.3	4.6
Neral	23.5	
Geraniol	2.7	
Geranial	31.9	
Citronellyl formate		
trans-Caryophyllene	0.8	1.4
γ-Gurjunene	1.3	
Caryophyllene oxide		3.2
Total identified		
Monoterpene hydrocarbons (HM)	*4.33%*	*2.45%*
Oxigenated monoterpenes (OM)	*92.37%*	*92.39%*
Sesquiterpene hydrocarbons (SH)	*3.00%*	*1.57%*
Oxigenated sesquiterpenes (OS)	*0.16%*	*3.22%*
	99.85%	99.63%

constituents (%)	***P. roseum***	***P. graveolens***
Linalool	4.4	5.6
cis-Rose oxide	0.8	1.1
Menthone	1.7	1.4
iso-Menthone	1.8	6.4
Citronellol	30.7	26.4
Geraniol	16.3	14.6
Citronellyl formate	**16.2**	10.7
Geranyl formate	**8.1**	5.4
α-Copaene	1	0.4
β-Bourbonene	0.7	1.6
cis-Caryophyllene	1.2	1.5
6,9-Guaiadiene		7.4
γ-Cadinene	0.2	1
δ-Cadinene	1.1	1.3
Furopelargone A	0.6	0.4
Geranyl butanoate	0.8	1.1
2-Phenyl ethyl tiglate		1.4
Geranyl tiglate	0.8	1.5
Total identified		
Monoterpene hydrocarbons (HM)	*1.25%*	*1.54%*
Oxigenated monoterpenes (OM)	*84.44%*	*76.83%*
Sesquiterpene hydrocarbons (SH)	*12.28%*	*16.70%*
Oxigenated sesquiterpenes (OS)	*1.98%*	*4.81%*
	99.96%	99.88%

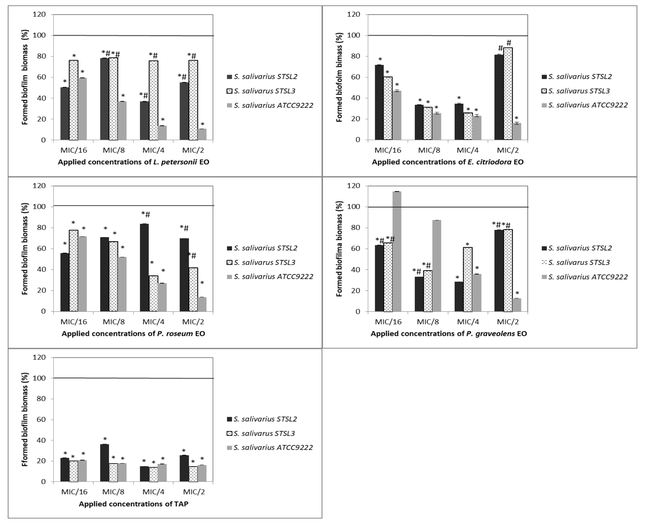


Figure 1. EOs inhibition of biofilm biomass of *S. salivarius* clinical and ATCC strains, observed in the CV assay.
Triple antibiotic paste (TAP) was used as a positive control.
*statistical difference in the biofilm biomass compared to the untreated control (100% biofilm biomass), p<0.05
#statistical difference compared to inhibition of biofilm biomass formed by referent ATCC 9222, p<0.05

The sensitivity of the *S. salivarius* strains, recovered from symptomatic and asymptomatic infected root canal infections (Table 1), has been investigated. The sensitivity of the clinical isolates differed among themselves, but also from the sensitivity of reference strain (Table 2). *E. citriodora* and *L. petersonii* EOs, abundant in oxygenated monoterpenes i.e. aldehydes and alcohols (Table 3), held the highest antibacterial potential (Table 2). In addition *E. citriodora* EO proved high antibiofilm activity (Fig. 1).

CHEMICAL CHARACTERISATION OF CHOKEBERRY SEED OIL

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Abstract

In the last decade, there is an increasing research interest in black chokeberries, still only small number of studies has been dedicated to investigation of chemical and pharmacological potential of oils obtained from its seeds. The aim of the present study was to explore the composition (fatty acids, carotenoids and tocols) of fatty oil obtained from chokeberry seeds.

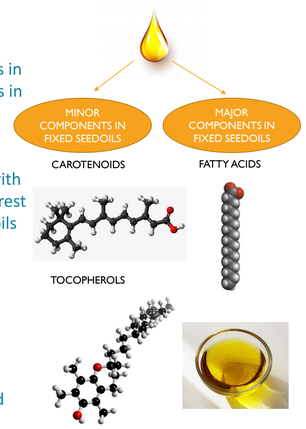
Oil was extracted from ground seeds using Soxhlet extraction with *n*-hexane. Before GC/MS analysis, fatty acids were converted to fatty acid methyl esters. Tocopherols and tocotrienols were analysed using a normal-phase HPLC equipped with a fluorescence detector. Carotenoids were analysed using reversed-phase HPLC with diode array detector.

According to our results, chokeberry seed oil is rich in linoleic acid, alpha-tocopherol and (E)-*β*-caroten.

Having this in mind from a nutritional viewpoint seeds of *Aronia melanocarpa,* that mainly represent a by-product from the berry fruits processing industry, may have potential as non-traditional sources of oils due to high content of antioxidant compounds.

Introduction

Numerous reports state that introduction of vegetable oils in the diet has beneficial effects in prevention of cardiovascular diseases, preserving healthy cholesterol levels, improving brain function and fighting with free radical species. The interest in finding newer sources of oils for edible and non-edible applications such as unconventional plant seeds increased recently. Although there is a broad range of vegetable oil sources, world consumption is prevailed by palm, soybean, rapeseed and sunflower oils.





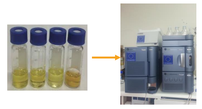
Black chokeberry (*Aronia melanocarpa*) fruits are particularly rich in polyphenol compounds and are among the fruits with the highest antioxidant activity. In the last decade, there is an increasing research interest in black chokeberries, still only small number of studies has been dedicated to investigation of chemical and pharmacological potential of oils obtained from its seeds.

Methodology

Oil was extracted from ground seeds using Soxhlet extraction with *n*-hexane.



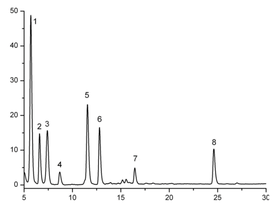
Before analysis, fatty acids were converted to fatty acid methyl esters (FAME) using standard sulfuric acid procedure (AOAC International).





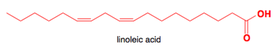
Chemical analysis of oil was carried using reversed-phase HPLC with diode array detector at 450 nm. Quantification of carotenoids was conducted based on comparison of peak areas and their specific absorption maxima with those obtained for external standards with defined concentrations considering the recovery of the internal standard (*β*-apo-8′-carotinal).

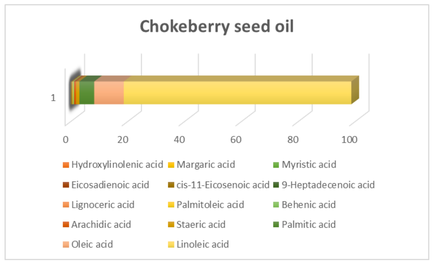
Tocopherols and tocotrienols were analysed using a normal-phase HPLC equipped with a fluorescence detector (excitation: 292 nm, emission: 330 nm). Quantification of tocols was conducted based on comparison of peak areas with those obtained for external standards with defined concentrations considering the recovery of the internal standard (α-tocopherol acetate).



Results

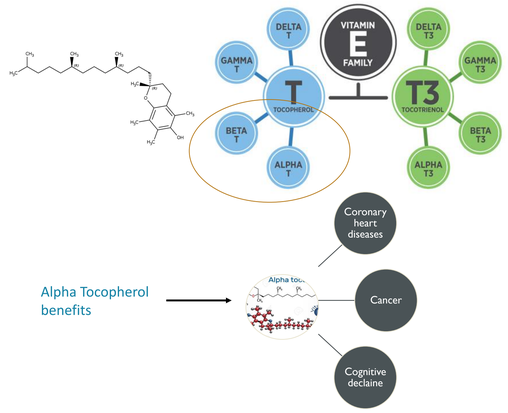
Accurate composition of fatty acids is important in order to determine possible adulterations and overall stability of the product. According to our results, unsaturated fatty acids comprised around 90% of all of the fatty acids. In GC profile of investigated sample, 16 peaks were identified and the predominant fatty acids in chokeberry seed oil were linoleic acid (80%) and oleic acid.



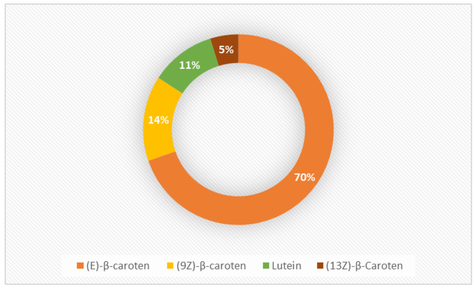


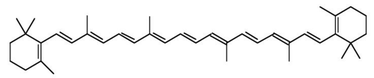
Results

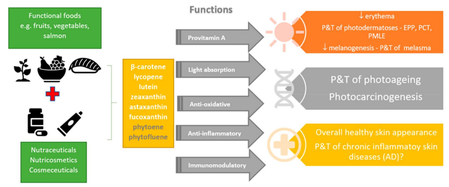
Although advantageous from a nutritional point of view, high contents of polyunsaturated fatty acids result in poorer oxidative stability and shorter shelf life of the oils. Tocols and carotenoids have strong antioxidant activity and their main role is to protect unsaturated fatty acids against oxidation. In the seed oil chokeberry two tocopherols were detected. The dominant one was *α*-tocopherol (133 mg/100 g oil) followed by *β*-tocopherol (14 mg/100 g oil). No tocotrienol compounds were detected in investigated oil.



Concerning carotenoids, (*E*)-*β*-caroten followed by (*9Z*)-*β*-caroten were predominant in the oil. (*13Z*)-*β*-Caroten and lutein were also present. The total amount of carotenoids was 31.88 µmol/100 g of oil.







Recommendations



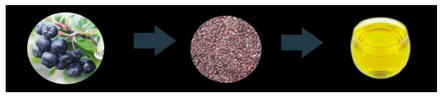
LINOLEIC ACID

E BETA CAROTEN

ALPHA TOCOPHEROL

POTENTIAL BENEFITS:

- Prevention of cardiovascular diseases
- Beneficial effects on glucose metabolism
- Skin benefits (anti-aging effect, anti-acne, hydratation)



Byproducts → Valuable seed oil

Conclusion

This study reporting data on the phytochemicals composition of chokeberry seeds as agricultural-food waste could be used as guideline for more extensive industrial valorisation of the fruit and vegetable by-products resulting from different processing procedures. The berry fruits processing industry supplies large amounts of seed residues which could be used as potential sources of lipids with important applications in the cosmetics and food industries.

The results obtained in our study demonstrated that from a nutritional viewpoint seeds of *A. melanocarpa* may have potential as non-traditional sources of oils due to high content of antioxidant compounds. It represents a good source of vitamin E, and carotenoids, as well as linoleic acid.

Acknowledgements

This work has been supported by Ministry of Education, Science and Technological Development of Republic of Serbia (451-03-68/2020-14/ 200003).

NPA
Natural products application: Health, Cosmetic and Food
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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *Luehea divaricata* MARTIUS EXTRACT

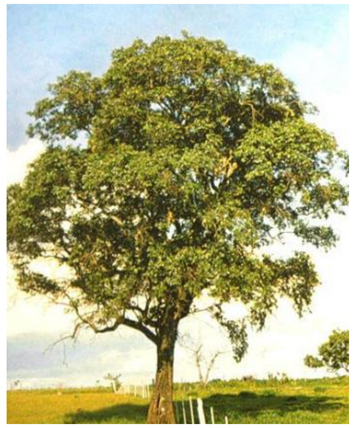
Jéssica A.A. Garcia [1], Marija Ivanov[2], Marina Soković[2], Lillian Barros[3], Isabel C.F.R. Ferreira[3], Adelar Bracht[1], Rosane M. Peralta[1]

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INTRODUCTION

The search for herbal medicines has grown in recent years, what confers a strong importance to studies about active plant of compounds. *Luehea divaricata* Martius is a tree native to Brazil that is among those widely used in folk medicine as a herbal medicine against various diseases.



OBJECTIVE

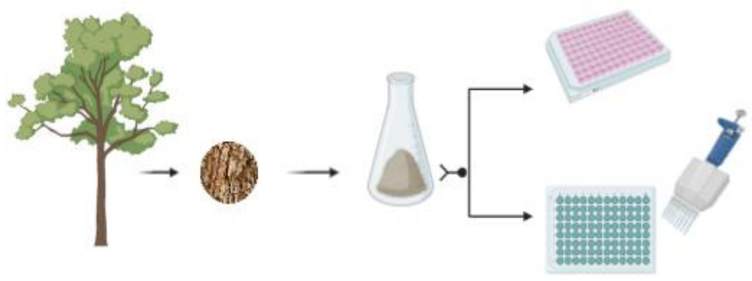


This work aims to evaluate the antioxidant and antimicrobial activities of the hydroethanolic extract of the bark of the *L. divaricata* stem.

METHODOLOGY

The extract was prepared by adding 20 mL of 70% ethanol to 1 g of the *L. divaricata* bark. The antioxidant activity of the extract was evaluated by two methods: reduction of the 2,2-diphenyl-1-picrilhydrazyl radical (DPPH) and reduction of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^+$). The results were expressed as IC_{50} values (sample concentration that causes 50% inhibition of radical reduction). The antimicrobial and antifungal activity of the extract was evaluated by the microdilution method, in which Gram-negative and Gram-positive bactéria. The antifungal activity was evaluated against *Aspergillus fumigatus Aspergillus ochraceus, Aspergillus niger, Penicillium funiculosum, Penicillium ochrochloron and Penicillium verrucosum*. The results were expressed as the minimum extract concentrations that completely inhibited bacterial growth (MICs), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC).

The antioxidant activity was evaluated by two methods: DPPH and $ABTS^+$



The antimicrobial and antifungal activity

RESULTS

The bark extract of *L. divaricata* showed high antioxidant capacity. An IC50 of 13.31 µg/mL was found for DPPH bleaching and 7.30 µg/mL in the ABTS assay. Regarding the antimicrobial activity, the values referring to the minimum inhibitory, bactericidal and fungicidal concentrations of the extract are presented in Table 1. When analyzing the results, it appears that the *L. divarica* bark extract is more active against fungi than against bactéria.

Table 1: Antibacterial and antifungal activities of *L. divaricata* stem bark extract (mg/mL)

		S. aureus	*B. cereus*	*L. monocytogenes*	*E. coli*	*S. typhimurium*	*E. cloacae*
Antibacterial activity	MIC	3	1.5	1.5	1.5	3	3
	MIB	6	3	3	3	6	6
		A. fumigatus	*A. niger*	*A.ochraceus*	*P. funiculosum*	*P. verrucosum*	*P. ochrochloron*
Antifungal activity	MIC	0.75	0.75	0.75	0.75	0.75	1.5
	MFC	1.5	1.5	1.5	1.5	1.5	3

CONCLUSION

Based on the results it can be concluded that the hydroethanolic extract of the stem bark of *L. divaricata*, in addition to having a high antioxidant capacity, also presents antimicrobial activity.

ACKNOWLEDGEMENTS

CAPES and CNPq

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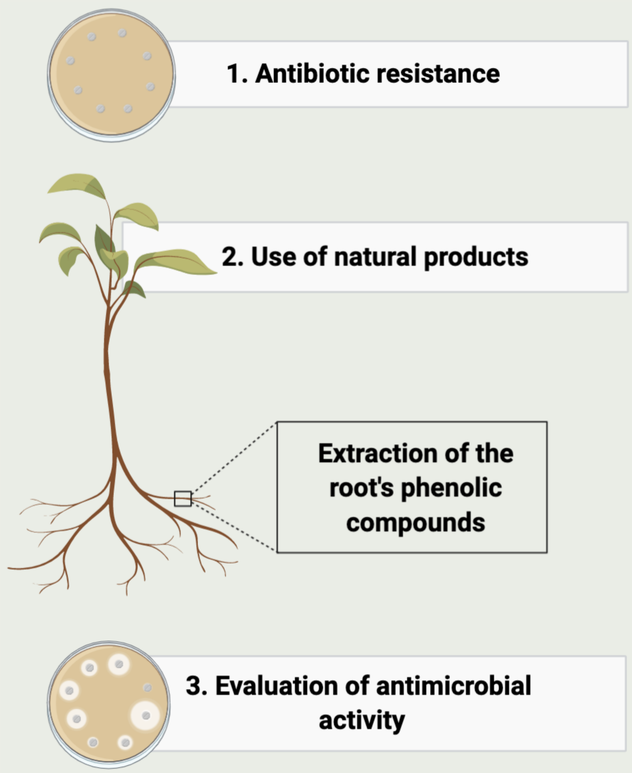
Root of *Rumex acetosa* as a possible antibiotic adjuvant

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Graphical Abstract



Introduction

The discovery of antibiotics was one of the most relevant breakthroughs of modern medicine. Antibiotics allow humans to live longer and with more quality of life because they can control bacterial infections by killing or inhibiting bacterial growth. However, due to acquisitions of resistance genes, genetic changes and nonheritable phenomena, bacteria can survive and grow in the presence of antibiotics **[1]**. Thus, the use of natural products has increased and, consequently, several microbiological studies have been conducted with plants. Common plants of our community could be an excellent way to fight this issue **[2]**. This study was designed to explore the antibacterial properties of the roots of *Rumex acetosa*.

Methodology

The extraction of the root's phenolic compounds was performed with two different solvents: a water/ethanol (20:80) mixture and an absolute solution of methanol. The roots were mill-powdered and then two grams of powder were extracted with 100 mL of solvent by stirring for 2h. The extracts were redissolved in dimethyl-sulfoxide (DMSO) to five different concentrations (100, 75, 50, 25, 10 mg/mL). The antimicrobial potential was screened against nine pathogenic bacteria (*L. monocytogenes*, *E. faecium*, *E. faecalis*, *B. cereus*, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *S. enteritidis* and *E. coli*) using the Kirby-Bauer disk diffusion method.

Results

Both extractions showed results against *L. monocytogenes*, *E. faecium*, *E. faecalis*, *S. aureus* and *B. cereus*. The methanol extraction also showed results against *S. epidermidis* with the highest inhibition zone (15 mm). The water/ethanol extraction had a minimum inhibitory concentration of 25 mg/mL while the methanol extraction had a minimum inhibitory concentration of 10 mg/mL. In general, the methanol extraction had the best antimicrobial efficiency **(Table 1)**.

Table 1. Minimum inhibitory concentration (MIC, mg/mL) and inhibition zones (mm) of the phenolic extracts of the roots of *Rumex acetosa*.

	MIC (inhibition zone)	
	Water/Ethanol Extraction	Methanol Extraction
L. monocytogenes	25 (9)	10 (9)
E. faecium	50 (9)	50 (8)
E. faecalis	25 (9)	25 (9)
B. cereus	50 (9)	50 (10)
S. aureus	25 (9)	25 (8)
S. epidermidis	0 (0)	10 (9)
P. aeruginosa	0 (0)	0 (0)
S. enteritidis	0 (0)	0 (0)
E. coli	0 (0)	0 (0)

Conclusion

Our work confirms the efficiency of the roots of *R. acetosa* as a natural antibacterial agent and a possible antibiotic adjuvant for the treatment of infectious diseases caused by drug-resistant bacteria.

References

[1] M.H. Pontes, E.A. Groisman. A physiological basis for nonheritable antibiotic resistance. *MBio* (2020), 11, 1–13, doi:10.1128/mBio.00817-20.
[2] Ç. Küçük, C. Cevheri. Preliminary study on the antifungal and antimicrobial activities of some medicinal plants of Turkey. *J. Biotechnol. Res.* (2017), 3, 110–113.

Acknowledgements

This work was supported by the Associate Laboratory for Green Chemistry - LAQV which is financed by national funds from FCT/MCTES (UIDB/50006/2020 and UIDP/50006/2020). Vanessa Silva is grateful to FCT (Fundação para a Ciência e a Tecnologia) for financial support through the PhD grant SFRH/BD/137947/2018.

Trametes versicolor (L.) Lloyd extracts: Assessment of their antioxidant and neuroprotective effects

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[1]REQUIMTE/LAQV, Instituto Superior de Engenharia do Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4249-015 Porto, Portugal; [2]CITAB – Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes e Alto Douro, 5001- 801 Vila Real, Portugal; [3]CQ – Centro de Química, University of Trás-os-Montes e Alto Douro, 5001- 801 Vila Real, Portugal; *claragrosso@graq.isep.ipp.pt

1. Introduction

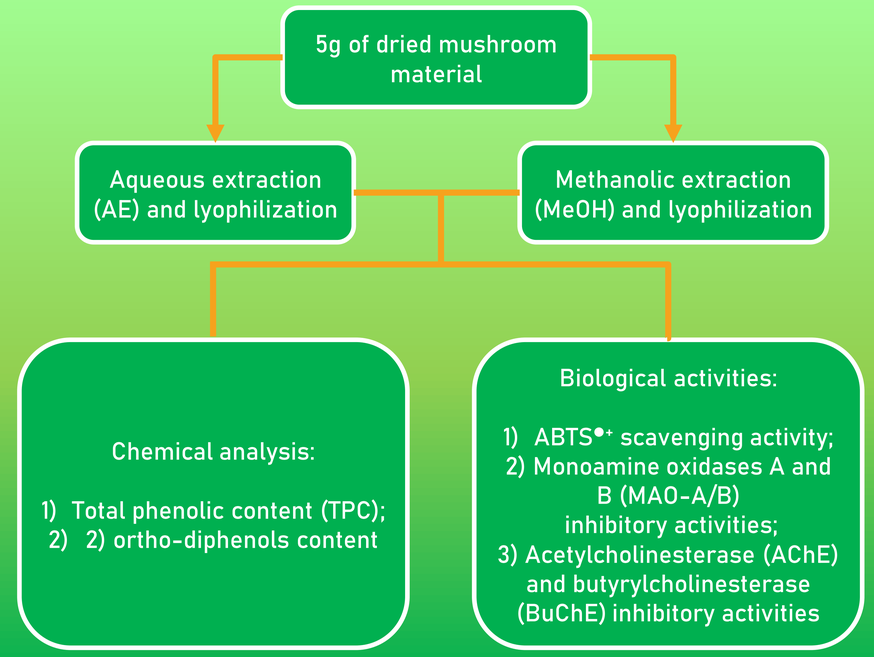
Mushrooms have been used for medicinal and food purposes for centuries due to their nutritional value and bioactive compounds; however, their pharmacological potential is still underestimated [1]. The reported medicinal properties are vast, including immunity-stimulating, antitumor, antimicrobial, antioxidant, anti-diabetic, anti-hypercholesterolemic, hepatoprotective, and anti-inflammatory effects, among others, and they are usually correlated to different classes of metabolites found in mushrooms (e.g. polysaccharides, phenolic compounds, terpenoids, ergosterols, and volatile organic compounds) [2].

This study aimed to explore the chemical composition, antioxidant and neuroprotective activities of two different extracts (aqueous and methanolic) of *Trametes versicolor* (Fig. 1).



Fig. 1. *Trametes versicolor* (L.) Lloyd sample.

2. Materials and methodology



3. Results

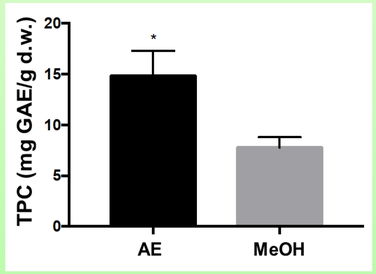


Fig. 2. TPC of AE and MeOH extracts of *T. versicolor*. (* - p<0.05)

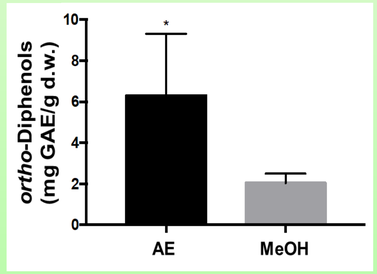


Fig. 3. ortho-Diphenol content of AE and MeOH extracts of *T. versicolor*. (* - p<0.05)

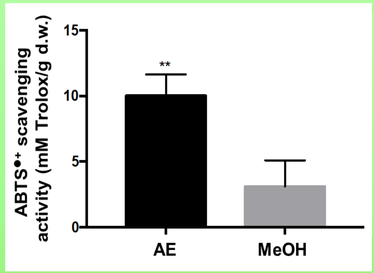


Fig. 4. ABTS•+ scavenging activity of AE and MeOH extracts of *T. versicolor*. (** - p<0.01)

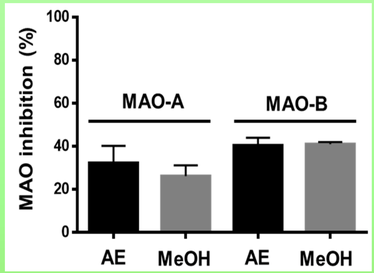


Fig. 5. MAO-A and MAO-B inhibition of AE and MeOH extracts of *T. versicolor* at 1.79 mg/mL.

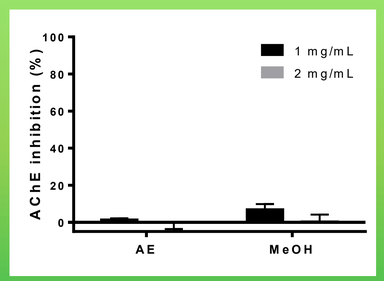


Fig. 6. AChE inhibition of AE and MeOH extracts of *T. versicolor*.

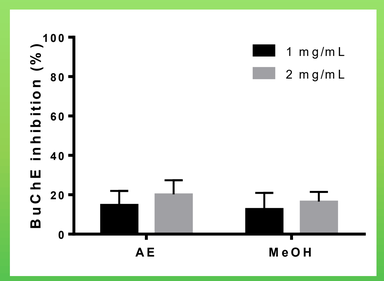


Fig. 7. BuChE inhibition of AE and MeOH extracts of *T. versicolor*.

4. Discussion and conclusions

- AE showed higher TPC and ortho-diphenol content (Figs. 2 and 3) than the MEOH one, which was reflected in stronger scavenging of ABTS$^{\bullet+}$ radical (Fig. 4).
- AE and MeOH extracts were evaluated against MAO-A (related with neuropsychiatric disorders) and MAO-B (involved in Parkinson's disease). The same pattern was observed, AE being also slightly more active against both isoforms of MAO (Fig. 5).
- The extracts showed little to no AChE inhibitory effect (Fig 6); c.a. 20% of BuChE inhibition was achieved at 2 mg/ml (Fig. 7);
- These results highlight the potential of *T. versicolor* extracts, as well as other mushrooms rich in phenolic compounds, as possible therapy against neuropsychiatric and neurodegenerative disorders.

References

[1] A. Knežević, M. Stajić, I. Sofrenić, T. Stanojković, I. Milovanović, V. Tešević, J. Vukojević, PloS one, 13(2018), Article ID e0203064.

[2] V. K. Chaturvedi, S. Agarwal, K. K. Gupta, P. W. Ramteke, M. P. Singh, 3 Biotech, 8(2018) 334.

Acknowledgments

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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY *OF LENTINULA EDODES* DONKO AND KOSHIN VARIETIES EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

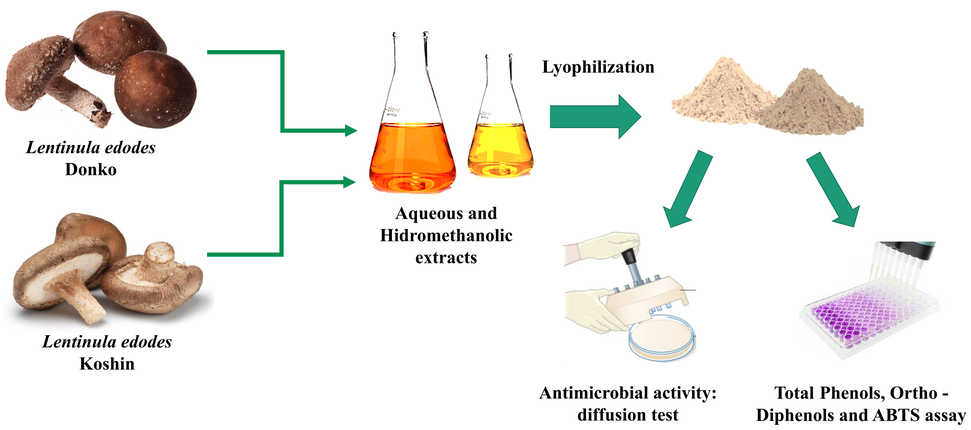
Juliana Garcia[1], Ana Afonso[2], Conceição Fernandes[2], Fernando M. Nunes[3], Guilhermina Marques[1], Maria José Saavedra[1]

[1]CITAB - Centre for the Research and Technology of Agro-Environment and Biological Sciences, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal, [2]CIMO - Centro de Investigação de Montanha, Polytechnic Institute of Bragança 5300-253 Bragança, Portugal, [3]CQ - Centro de Química, University of Trás-os-Montes e Alto Douro, 5001- 801 Vila Real, Portugal *saavedra@utad.pt

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA), a Gram-positive bacterium, has become the most recurrent global cause of skin and soft tissue infections, belonging to the WHO high priority pathogens list. Effective therapy remains challenging and entails the assessment of novel and successful antibiotics. In this context, mushrooms are considered a valuable source of single and natural compounds to be applied for fighting multidrug resistant bacteria. Consequently, the present study aimed to evaluate, by diffusion disk method, *Lentinula edodes* Donko and Koshin methanolic and aqueous extracts against to MRSA isolates from clinical wound infections. Moreover, the phytochemical constituents, as well as the antioxidant activity of these extracts were also assessed [1].

METHODS



RESULTS

Table 1. Antimicrobial activity of *L. edodes* extracts against clinical bacterial isolates

	Clinical isolates	Donko (mm)	Koshin (mm)	DMSO (mm)
Methicillin-susceptible *Staphylococcus aureus*	MJMC 018	7 ± 0,0	10 ± 0,0	6 ± 0,0
	MJMC 026	8 ± 0,0	7 ± 0,0	
	MJMC109	9 ± 0,0	11 ± 0,0	
	MJMC110	9 ± 0,0	11 ± 0,0	
Methicillin-resistant *Staphylococcus aureus*	MJMC511	10 ± 0,0	10 ± 0,0	
	MJMC 025	8 ± 0,0	8 ± 0,0	
	MJMC 027	8 ± 0,0	7 ± 0,0	
	MJMC102	8 ± 0,0	12 ± 0,0	
	MJMC111	9 ± 0,0	10 ± 0,0	
	MJMC507	10 ± 0,0	10 ± 0,0	
	MJMC534 B	11 ± 0,0	16 ± 0,0	
	MJMC 539	9 ± 0,0	9 ± 0,0	
	MJMC 545	7 ± 0,0	8 ± 0,0	
	MJMC 552	9 ± 0,0	9 ± 0,0	

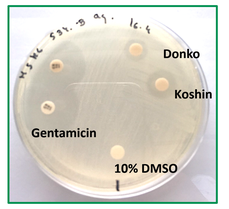


Fig. 1 – Antimicrobial activity by disk diffusion test of aqueous extracts of *L. edodes* Donko and Koshin varieties.

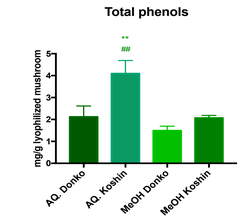


Fig. 2 – Total phenolic content of aqueous (AQ.) and hidromethanolic (MEOH) extracts of *L. edodes* Donko and Koshin varieties (**p< 0.01 vs. AQ. var. Donko extract; ##p < 0.01 vs. MeOH var. Koshin extract).

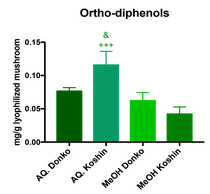


Fig. 3 – Ortho-diphenols content of aqueous (AQ.) and hidromethanolic (MEOH) extracts of *L. edodes* Donko and Koshin varieties (&p< 0.05 vs. AQ. var. Donko extract; ***p < 0.001 vs. MeOH var. Koshin extract).

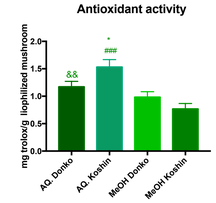


Fig. 4 – ABTS scavenging activity of aqueous (AQ.) and hidromethanolic (MEOH) extracts of *L. edodes* Donko and Koshin varieties. (*p< 0.05 vs. AQ. var. Donko extract; ###p < 0.001 vs. MeOH var. Koshin extract; &&p< 0.01 vs. MeOH var. Koshin extract).

CONCLUSIONS

- There is a strong relation between the total content of phenolic compounds, antioxidant activity, and antimicrobial activity suggesting that the presence of phenolic compounds may explain the biological effects of the extracts.
- Highest total phenolic compounds, as well as antioxidant activity was shown by Koshin variety aqueous extract, which presented the more effective antimicrobial activity against MRSA.
- Our results suggest that Koshin variety could be a significant source of compounds for the treatment of global multidrug-resistant pathogens.

REFERENCES

[1] Juliana Garcia, Ana Afonso, Conceição Fernandes, Fernando M. Nunes, Guilhermina Marques, Maria José Saavedra. (2020) Comparative antioxidant and antimicrobial properties of *Lentinula edodes* Donko and Koshin varieties against priority multidrug-resistant pathogens, SAJCE https://doi.org/10.1016/j.sajce.2020.09.008

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β-CARYOPHYLLENE ISOLATED FROM CLOVE ESSENTIAL OIL IMPROVES LIPOPOLYSACCHARIDE (LPS)-INDUCED ACUTE LUNG INJURY IN MICE

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[1] Department of Biochemistry and [2] Department of Pharmacology and Therapeutics, University of Maringá (UEM), PR, Brazil, [3]Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, Portugal. *jfcomar@uem.br

Abstract

This work evaluated the effects of (-)-β-caryophyllene (β-Cary) isolated from clove bud oil on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice, an experimental model of lung inflammation that shares many features of human ALI. β-Cary was chemically characterized by GC-MS, revealing a purity of 91%. BALB-c mice were orally treated with β-Cary at doses of 50-400 mg/kg. After 1 h, animals received 5 mg/kg LPS intranasally to induce ALI. After 24 h, the bronchial alveolar lavage (BAL) and lung tissue were collected. Total and polymorphonuclear cells (PMN) were counted in the BAL and myeloperoxidase (MPO) activity, carbonylated proteins and reduced glutathione (GSH) were measured in the lung tissue. Mice of the LPS group showed a significant increase of the leukocytes number in the BAL, which was related to the migration of PMN. Treatment with 200 and 400 mg/kg β-Cary reduced the leucocytes migration by 30% as well as the number of PMN. The MPO activity was increased after LPS administration and 200 and 400 mg/kg β-Cary reduced this parameter by 50%. Carbonylated protein and GSH were, respectively, 60% higher and 35% lower in the lung of LPS group. Treatment with 200 and 400 mg/kg β-Cary reestablished the control values for these parameters. Overall, this study suggests that β-Cary improves ALI in mice by means of attenuating the neutrophils migration to the lungs, a phenomenon that also decreases the oxidative stress.

Introduction

Acute lung injury (ALI) is a respiratory failure syndrome characterized by diffuse pulmonary infiltration, edema and severe hypoxemia. Risk factors for ALI include pneumonia, trauma, sepsis, shock and, more recently, COVID-19 [1]. Approximately 30% of patients with COVID-19 develop ALI. The pathophysiology of ALI involves an intense pulmonary inflammatory response associated with edema, macrophages activation and neutrophils recruitment. Neutrophils and activated macrophages secrete reactive species and proteases, which damage the alveolar epithelium.

The lipopolysaccharide (LPS)-induced acute lung injury (ALI) is a model of lung inflammation in mice that shares many features of human ALI. (-)-β-caryophyllene (β-Cary) is a sesquiterpene found in essential oils and has been reported to present anti-inflammatory activity [2]. This compound is the second most abundant (6-17%) of the essential oil extracted from clove buds, after eugenol that comprises 70-90% [3]. To date, no study has been carried out to verify whether β-Cary improves the inflammation in ALI. Thus, this work aimed at evaluating the effects of β-Cary isolated from clove oil on LPS-induced ALI in mice, when orally administered.

Materials and methods

β-Cary from clove bud oil was purchased from Quinarí Fragrâncias e Cosméticos Ltda (Ponta Grossa, Brazil) and chemically characterized by gas chromatography with mass spectrometry (GC-MS).

BALB-c mice were orally treated with vehicle, β-Cary at doses of 50-400 mg/kg, dexamethasone (Dexa; 1 mg/kg) or zafirlukast (Zaf; 5 mg/kg). Dexa is a standard anti-inflammatory drug and Zaf is an inhibitor of leukotrienes. After 1 h, anesthetized animals received 5 mg/kg LPS or saline (control) intranasally to induce ALI. After 24 h, the animals were anesthetized and bronchial alveolar lavage (BAL) and lung tissue was collected. Total and polymorphonuclear cells (PMN) were counted in the BAL. The activity of myeloperoxidase (MPO) and the levels of carbonylated proteins and reduced glutathione (GSH) were measured in the lung tissue homogenate as previous described [4]. The animal protocol was approved by the Ethics Committee for Animal Experimentation of UEM.

Results

The chemical characterization of β-Cary from clove essential oil reveled a purity of 91%. Humulene (8.2%) and copaene (0.7%) were also identified in this fraction. **Fig. 1** shows the numbers of total leukocytes and PMN cells in the bronchial alveolar lavage.

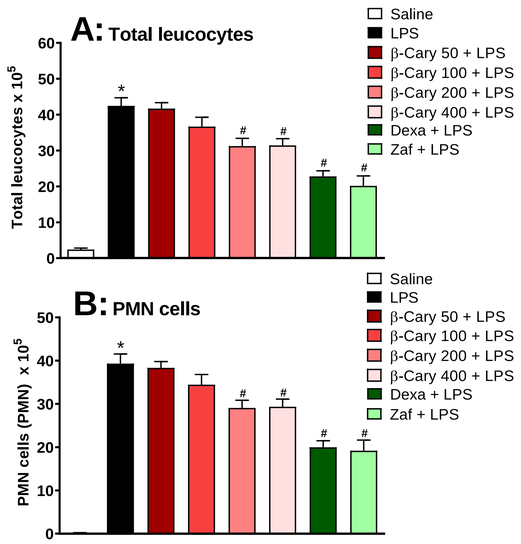


Fig. 1. Effects of (-)-β-caryophyllene (β-Cary) on the number of leukocytes in the bronchial alveolar lavage of mice with LPS-induced acute lung injury. PMN: polymorphonuclear cells, Dexa: dexamethasone, Zaf: zafirlukast. *p<0.05: for difference of LPS from saline; #p<0.05 for difference from LPS.

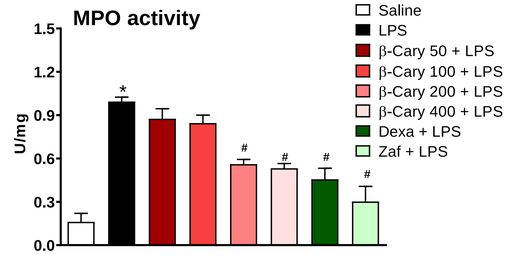


Fig. 2. Effects of (-)-β-caryophyllene (β-Cary) on myeloperoxidase (MPO) activity in the lung tissue of mice with LPS-induced ALI. Dexa: dexamethasone, Zaf: zafirlukast. *p<0.05: for differences of LPS from saline; #p<0.05 for difference from LPS.

Mice of the LPS group showed a significant increase of the number of leukocytes in the BAL, which was related to the migration of PMN. Treatment with 200 and 400 mg/kg β-Cary reduced the leucocytes migration by 30% as well as the number of PMN **(Fig. 1)**. The animals treated with zafirlukast and dexamethasone had this count reduced by approximately 40%. The accumulation of neutrophils in the lung was assessed by the MPO activity, which increased significantly after LPS administration **(Fig. 2)**. Treatment with 200 and 400 mg/kg β-Cary reduced MPO activity by 50%. The same value was obtained for animals treated with zafirlukast and dexamethasone.

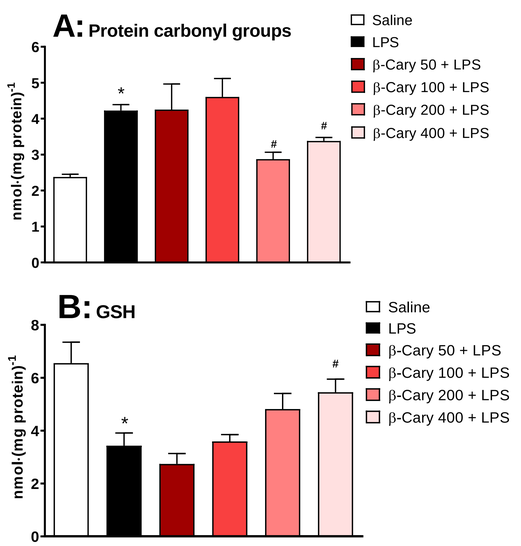


Fig. 2. Effects of (-)-β-caryophyllene (β-Cary) on oxidative stress in the lung tissue of mice with LPS-induced acute lung injury. GSH: reduced glutathione; β-Cary: β-Caryophyllene; LPS: lipopolysaccharide. * p<0.05: for differences of LPS from saline; # p<0.05 for differences from LPS.

Fig. 3 shows the results of the lung oxidative stress. The levels of protein carbonyl groups, a pro-oxidative parameter, was 60% higher in the lung of mice of the LPS group and the treatment with 200 and 400 mg/kg β-Cary reduced considerably these levels. The levels of GSH in the lung, an antioxidant parameter, was reduced after LPS administration **(Fig. 3B)**. The treatment of mice with 400 mg/kg β-Cary practically reestablished the control values and GSH.

Conclusion

Overall, this study suggests that β-Cary improves acute lung injury in mice by means of attenuating the neutrophils migration to the lungs. The lung oxidative stress is also reduced with the treatment and the phenomenon seems to be the consequence of the anti-inflammatory action of β-Cary. The dose of 200 and 400 mg/Kg of β-Cary in mice would correspond to a human dose of approximately 16 and 32 mg/kg as given by the body surface area normalization method [5] and, consequently, the compound could be a promising compound to the current approaches aiming the acute lung injury therapy.

Recommendations

[1] Li L, Huang Q, Wang Dc, Ingbar Dh, Wang X. Acute lung injury in patients with COVID-19 infection. Clin Transl Med. 2020, 10:20-27. https://doi.org/10.1002/ctm2.16

[2] Ames-Sibin AP, Barizão CL, Castro-Ghizoni CV, et al. B-Caryophyllene, the major constituent of copaiba oil, reduces systemic inflammation and oxidative stress in arthritic rats. J Cell Biochem. 2018, 119 10262–10277. https://doi.org/10.1002/jcb.27369

[3] Nurdjannah N, Bermawie N. Cloves. In: Peter KV, Editor. Handbook of Herbs and Spices, second edition, vol.1. Woodhead Publishing limited, p. 197-215, 2012. https://doi.org/10.1533/9780857095671.197

[4] Sá-Nakanishi AB, Soni-Neto J, Moreira LS, Gonçalves GA, et al. Anti-Inflammatory and Antioxidant Actions of Methyl Jasmonate Are Associated with Metabolic Modifications in the Liver of Arthritic Rats. Oxid Med Cell Longev. 2018, 1–16. https://doi.org/10.1155/2018/2056250

[5] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2007,22:659-661. https://doi.org/10.1096/fj.07-9574LSF

Acknowledgements

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LARVICIDAL ACTIVITY OF *Parahancornia amapa* (HUBER) DUCKE LATEX AGAINST *Aedes aegypti* L. LARVAE.

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Abstract

The mosquito species *Aedes aegypti* is one of the main vectors of arboviruses, including yellow fever, dengue, Zika, chikungunya, oropouche and mayaro. A vector control remains an important strategy because the absence of vaccines to prevent these diseases and insecticides resistance acquired by the mosquito, contribute to a serious public health problem. The use of insecticides based on natural plant products represents an alternative to chemical insecticides, as they are less harmful to the environment and health, not to mention less toxic to other insects. Tests were carried out with the *Parahancornia amapa* (Huber) Ducke latex, at 0, 10, 50 and 100 µg/ml concentrations, on the *Aedes aegypti* L. L4 larvae, for a period of 48 hours. The results obtained here demonstrate that *P. amapa* latex showed effective larvicidal activity in the 100 µg/ml concentration, for an exposure period of 48 hours, LC50 <50 µg/ml, with a result of 36.84 µg/ml, being considered as a potent larvicide or highly active, under the experimental conditions tested here.

Introduction

The amapazeiro, *Parahancornia amapa* (Huber) Ducke, *Apocynaceae*, is a typical plant from Brazilian Amazon, whose barks and latex are used for medicinal purposes. Phytochemicals studies of this species report the presence of various compounds in the roots, latex, bark, having its latex confirmed the presence of mixtures of 3-O-acillupeol and triterpenes.

In the search for new agents capable to control the dengue vector, *Aedes aegypti*, bioassays were performed to assess the larvicidal activity of *Parahancornia amapa* latex under laboratory conditions.



Figure 1. *Parahancornia amapa* (Huber) Ducke, *Apocynaceae* (Google Image, 2021).

Materials

Figure 2. Larvae collect



Font: Research data. Photo: Nelcineide Almeida Simões (2019).

Methodology

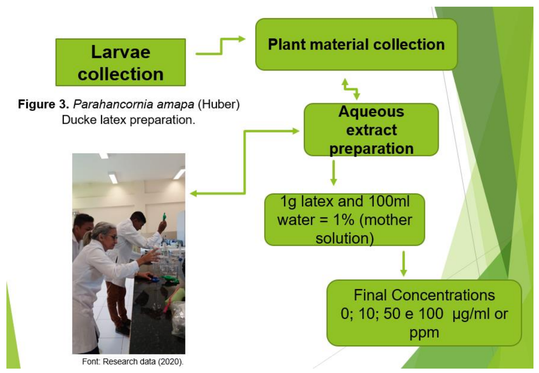


Figure 3. *Parahancornia amapa* (Huber) Ducke latex preparation.

Font: Research data (2020).

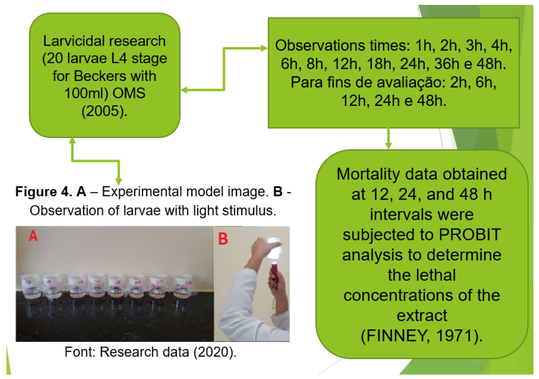
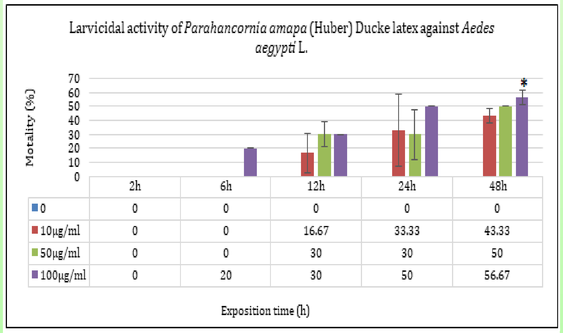
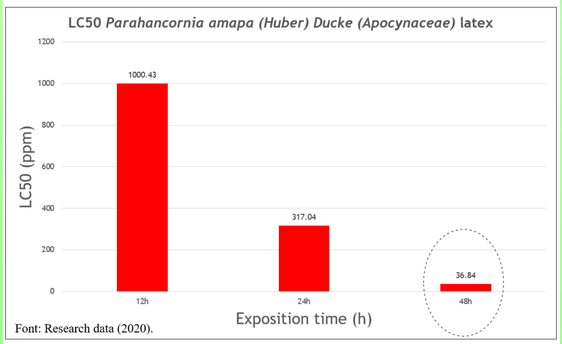


Figure 4. A – Experimental model image. **B** - Observation of larvae with light stimulus.

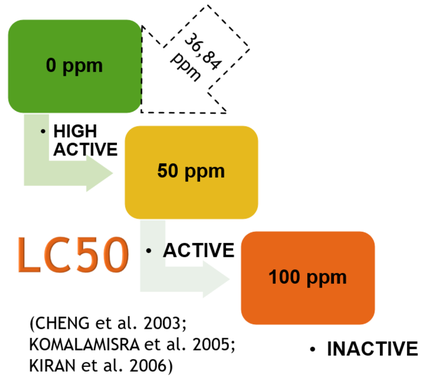
Font: Research data (2020).

Results





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The LC50 value for the 48 h reading differed from the others by presenting a high chisquare value; was used as reference. These results suggested that *Parahancornia amapa* (Huber) Ducke latex presents bioactive substances capable of killing mosquito larvae.

Conclusion

Add your information, graphs and images to this section.

The present work demonstrates that *Parahancornia amapa* (Huber) Ducke (*Apocynaceae*) latex could be considered as a novel and effective source for use in vector control programs because of its larvicidal effect against the larval stages of *Aedes aegypti* L., at experimental conditions presented here.

Recommendations

Thus, these results can be useful in the search for new, more selective and biodegradable natural larvicidal compounds. Further investigations are currently underway to isolate these compounds.

References

CHENG, S.; CHANG, H.; CHANG, S. Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. **Bioresour. Technol. 89, 99–102. 2003.**

Finney, D.J. **Probit Analysis.** third ed. Cambridge university press, Cambridge, 1971. p. 333.

KIRAN, S.R.; BHAVANI, K.; DEVI, P.S.; RAO, B.R.R.; REDDY, K.J. Composition and larvicidal activity of leaves and stem essential oils of *Chloroxylon swietenia* DC against *Aedes aegypti* and *Anopheles stephensi*. **Bioresour. Technol. 97, 2481–2484. 2006.**

KOMALAMISRA, N.; TRONGTOKIT, Y.; RONGSRIYAM, Y.; APIWATHNASORN, C. Screening for lavicidal activity in some Thai plants against four mosquito vector species. **Southeast Asian J. Trop. Med. Public Health 36, 1412–1422. 2005.**

Organização Mundial de Saúde. **GUIDELINES FOR LABORATORY AND FIELD TESTING OF MOSQUITO LARVICIDES.** WORLD HEALTH ORGANIZATION COMMUNICABLE DISEASE CONTROL, PREVENTION AND ERADICATION WHO PESTICIDE EVALUATION SCHEME. 2005. 41p.

Acknowledgements

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THE ROLE OF COSMETOLOGIST IN THE REDUCTION OF ACNE VULGARIS

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Abstract

Acne vulgaris is a disease that occurs in up to more than 80 % of people. Acne may be present as comedones (whiteheads, blackheads), pustules, nodules, cysts and papules .The study was examining the effectiveness of hydrogen purification- cosmetological new treatment (containing alkaline water) on improving the skin condition of the women with A. vulgaris.

Introduction

Hydrogen purification treatment uses alkaline water (electrochemically reduced water, ERP), rare in nature. However, with the chemical process of electrolysis, ERP can be produced immediately with a pH range between 8–10. It has both high alkaline and negative oxidation-reduction potential (ORP). The electrolysis process consists of passing a direct current between two electrodes (anode and cathode) separated by a semi-permeable membrane. During this process, the elements contained in the water break down into hydrogen ions H+, focusing on the cathode, and hydroxy ions OH-, which focus on the anode. Ionized alkaline water consists of H+ ions; it also contains alkaline elements, such as calcium, magnesium, potassium, hydrogen, and alkaline hydroxyl ions. Negative OH- ions form acid water, containing chlorine, sulfur, phosphorus, positive acid ions, and oxygen.

Materials

Group included 30 young adult women with a higher sebum level and acne vulgaris . Participants were diagnosed with mild facial acne vulgaris using the Hellgren–Vincent Scale (HVS) to determine acne lesions' severity . The HVS accurately determines the number of skin lesions in the form of lumps, pustules, and blackheads. The AV group's inclusion criteria were a young age, ranging between 18–25 years old, and severe acne, with a second degree in the HVS (n = 23, 76.67%) and a third-degree in the HVS (n = 7, 23.33%).

Methodology

A cosmetic makeup removal (micellar fluid) was performed at the first step of the procedure. Next, hydrogen cleansing was conducted using the H2 peel head, sucked the skin fold under a vacuum (supplying clean water with one cable), and flushed it with hydrogenated water. Used water was sucked in the second wire connected to the head, which drained it to the wastewater tank. The device's power was set for each of the four treatments at about a 10% vacuum; a higher dose could cause the appearance of ecchymosis on the skin. This stage of the procedure was performed for 10 minutes. The next stage of the procedure was applying an H2 jet head, using the potential of active hydrogen (hydrogenated water injection), which had a nozzle "ejecting" the hydrogenated water under pressure. Power of 2 bars (intended for the face area) was used here for each series of treatments. This head was guided at a distance of 2–3 cm from the client's skin for 5 minutes. Participants were treated at a 1-week interval for four sessions.

Results

At baseline, most participants in the AV group were diagnosed with a second degree (76.67% of the sample) and several people with a third-degree (23.33%) of AV. Two weeks after finishing hydrogen purification treatment, around half of the participants were diagnosed with the first (53.33%) and second (46%) AV degree. This shows that the level of AV decreases under the influence of hydrogen purification treatment in most individuals, which was confirmed by the *Wilcoxon matched-pairs test*, $n = 21$, $T = 0.00$, $Z = 4.02$, $p < 0.0001$. The treatment was significant in the following parameters: pH around the bottom lip, moisture between the eyebrows and around the nose, and oily skin in all three face sites. According to expectations, the sebum level decreased, and moisture levelsincreased under the influence of hydrogen purification treatment. Fisher's LSD posthoc test showed significant differences between the baseline test and retest after the first session (p <0.0001). The differences between the first measurement at baseline and two weeks after the end of all four treatment sessions persisted (p <0.0001). However, the second and third measurements (7 and 14 days after the last sessions) did not differ significantly from each other (p >0.05).

Conclusion

Treatment with hydrogen purification is safe and effective in reducing acne skin eruptions and canimprove the skin's condition and an appearance by decreasing sebum and increasing the moisture level in young women. However, it is essential to note that hydrogen purification cannot replace drug treatment but may be used as a supportive topical cure for acne vulgaris, combined with other forms of therapy, according to clinicians' recommendations.

Recommendations

This treatment can be a recommendation to popole sufferig from Acne vulgaris, who have skin eruptions. Also this treatment can be recommended to healthy people with too high sebum level.

Acknowledgements

This work was supported by Department of Health Sciences, University of Opole, Poland

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Antimicrobial activity of chosen essential oils from *Lamiaceae* family

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Introduction

Essential oils (EOs) are complex, natural volatile compounds of plants origin, produced as their secondary metabolites. These natural substances are mixtures of many chemical compounds, in various concentration ranges, therefore they exhibit broad biological activity as antioxidant or antimicrobial effect. One of the plant group well-known for EOs content is *Lamiaceae* family. *Lamiaceae* family is a group of flowering plants known from ancient times which are cultivated worldwide and are very common in Mediterranean Europe. The family includes over 7 thousand species. Most species are trees or shrubs, sometimes vines. A great number of *Lamiaceae* members contain EOs, among which the most popular are rosemary, thyme, oregano, and sage, which are used e.g. as a flavors, fragrances and also pharmaceuticals thanks to their properties.

Materials

Studies were carried out on five commercial EOs from *Lamiaceae* family herbs: thyme (*Thymus vulgaris*), sage *(Salvia sclarea*), spearmint (*Mentha piperita*), oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*). EOs were stored in darkness at 6°C. Indicator microorganisms chosen for the research included *Pseudomonas putida* ATCC 11774, *Candida albicans* ATCC 10231 and *Micrococcus luteus* ATCC 4698 bought from American Type Culture Collection (ATCC) and stored in sterile conditions in Microbank vials at -22°C. Before the experiment, microorganisms were propagated on the appropriate medium: Nutrient broth (BioMaxima, Poland) for *P. putida,* TSB (Trypticase Soy Broth, BioMaxima, Poland) for *M. luteus* and Sabouraud Dextrose broth (Oxoid, Canada) for *C. albicans*.

Methodology

Serial dilution method, with 96-well microtiter plates, was used to determine the minimal inhibitory concentration (MIC) of thyme, sage, spearmint, oregano and rosemary EOs. Chosen essential oils were tested in concentration range 0.04 - 5.00 mg/ml. The microbial suspensions, in Mueller-Hinton broth (Oxoid, Canada) and in Sabouraud broth (Oxoid, Canada) for yeasts, were prepared from 24 h cultures. The plates were incubated at 30°C or 37°C for 24 h, depending on the indicator organism. Spectrophotometric measurements were made using the BioTek Instruments EPOCH 2 microplate reader. The MIC value was defined as the concentration of the EOs, inhibiting the growth of the microorganism by at least 90%.

Results

Conducted laboratory trials indicate on differentiated antimicrobial activity of tested herbs EOs. The degree of growth inhibition by the essential oils depended on the strain of the microorganism used in the research (Table 1).

Among tested volatile compounds the lowest MIC values were obtained for thyme EOs: 0.31 mg/ml for tested indicator bacteria and 0.62 mg/ml for yeasts. The other 4 tested EOs minimal inhibitory concentration, for tested microorganisms, ranged beetwen 0.31 and 1.25 mg/ml.



Results

The greatest susceptibility to tested EOs showed *P. putida*. The MIC value for spearmint and thyme EOs was 0.31 mg/ml, and for others MIC was 0.62 mg/ml. Gram-positive bacteria *M. luteus* was also very sensitive to the action of tested *Lamiaceae* family EOs. Three, out of five tested, EOs inhibited the growth of this bacteria at a low concentration (MIC < 1 mg/ml). *C. albicans* was also susceptible towards tested volatile compounds as MIC for four out of five tested EOs was below 1 mg/ml. The lowest MIC values for all tested microorganisms were obtained for thyme and sage essential oils (0.62 mg/ml).

Essential oil	**Minimal inhibitory concentration of EOs (mg/ml)**			
	Gram-negative bacteria	Gram-positive bacteria	Yeasts	**Total MIC***
	P. putida	***M. luteus***	***C. albicans***	
Rosemary	0.62	0.62	1.25	**1.25**
Spearmint	0.31	1.25	0.62	**1.25**
Sage	0.62	0.62	0.62	**0.62**
Oregano	0.62	1.25	0.62	**1.25**
Thyme	0.31	0.31	0.62	**0.62**

Table 1. Minimal inhibitory concentration (MIC) value of choosen EOs for *P. putida, M. luteus and C. albicans*

* MIC for 3 tested microorganisms

Conclusions

Serial dilution assay is a valuable method for determination of minimal inhibitory concentration of EOs. This study shows that all tested EOs presented inhibitory effect on the growth of indicator microorganisms between 0.62 and 1.25 mg/ml concentration (for three microorganisms in total). MIC analysis shows that the best inhibitory properties have sage and thyme EOs, as they were effective in the lowest concentration for all of tested microorganisms.

Recommendations

1. To design a product with antimicrobial prosperities, most suitable *Lamiaceae* family EOs, are sage oil and thyme oil – they were effective for all analyzed microorganisms in low concentration.
2. In order to preserve the product with tested EOs, the concentration inhibiting the growth of microorganisms in the range from 0.62 to 1.25 mg/ml should be used.

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DIET, MICROBIOTA AND EPIGENETICS: A DYNAMIC AND ADJUSTABLE TRIADE TO EXPLORE TREATMENT APPROACHES

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Abstract

Recently, diet-driven **gut microbiota changes** emerged as a pathway in influencing the host's physiology, establishing **microbiotic metabolites** as a key mechanistic link underlying microbial actions.

In connection with these notions, **epigenetics** has brought new insights to our understanding of how **environmental factors** influence cellular genetic expression, pinpointing gut microbiota metabolites as one of the relevant factors.

Herein, we discuss the effect of **diet** in shaping **microbiotic intestinal populations** and how microbiotic metabolites interact with the host, key notions to address the modulatory role of microbiotic metabolites on **epigenetic changes**. Finally, we propose a dynamic **diet-microbiota-epigenetics** triade as concept of relevance in terms of health and disease, suggesting that a **dietary planning** according to specific epigenetic targets constitutes a **potential innovating strategy** for treatment and disease prevention [1].

Introduction

The gut microbiota produce metabolites through fermentative reactions. These can have several effects on the host through epigenetic modifications. The enzymes responsible for these epigenetic modifications use and interact with several metabolites, including gut metabolites [2,3].

Because metabolic effects underly both physiologic and pathologic conditions, the interaction between microbial metabolites and epigenetic enzymes has great potential regarding novel therapeutic approaches. Indeed several studies have shown interesting results based on the implementation of specific metabolite-based treatment [4,5].

Considering that gut microbiota's metabolites are, in great part, influenced by food consumption, several studies have explored the modulatory effects of diet on gut microbiota and ensued health impact on the host [4,5]. Intense efforts have been dedicated to mechanistically establish the links between aberrant epigenetic modifications and disease, and how variations in specific metabolites influence epigenetic agent's activity.

Methodology

Table 1

Nutritional behaviour	Microbial populations
Urbanized diet	Overall Bacteroides dominance; ↓ in microbiota diversity.
Rural diet	↑ Bacteroidetes (including the genera Xylanibacter and Prevotella); ↑ in microbiota diversity.
Vegan/vegetarian diet	↑ Coliforms (vegan). ↑ Prevotella (vegetarian). ↓ Bacteroides, Bifidobacteria, Escherichia coli and Enterobacteriaceae.
Omnivorous	↑ Bacteroides and Bifidobacterium species, Escherichia coli, and Enterobacteriaceae.
Fibre-rich diet	↑ bacterial abundance; ↑ Bacteroides, Bifidobacterium, Lactobacilli, Roseburia, Eubacteria and Ruminococcus; ↓ Enterococcus and Clostridium species; Actinobacteria depleted.

Verifying alterations in the microbial population as a response to some dietary patterns. Populational oscillations closely reflect ingested nutrients and bacterial degrading functions. ↑ - increase; ↓ - decrease.

Table 2

Food component	Metabolizing bacteria	Metabolites
Dietary fibre (undigested complex carbohydrates)	Faecalibacterium prausnitzii, Bifidobacterium spp., Lactobacillus spp., Roseburia spp., Eubacterium hallii.	**Short-chain fatty acids (SCFAs).**
L-carnitine, choline and phosphatidylcholine (particularly from red meat	Proteus mirabillis. Bacteria present in higher abundances in omnivores populations.	**Trimethylamine N-oxide (TMAO).**
Tryptophan	Escherichia coli, Clostridium spp. and Bacteroides spp. Enterococcus faecalis.	**Indole and indole derivates.**
Ellagic acid	C. coccoides, Bifidobacterium spp., and Lactobacillus spp.	**Urolithins.**

Some food components are metabolized by known bacteria and the resulting metabolites are of great interest.

Results

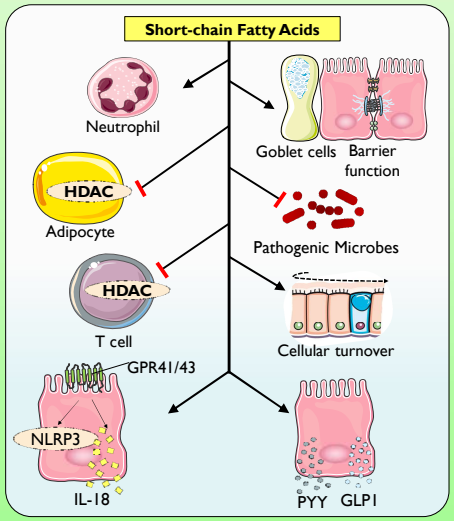


Figure 1. Bacterial metabolites have several effects on the host. Here we focus on SCFAs. These are considered anticancer agents for inhibiting histone deacetylases (HDACs).

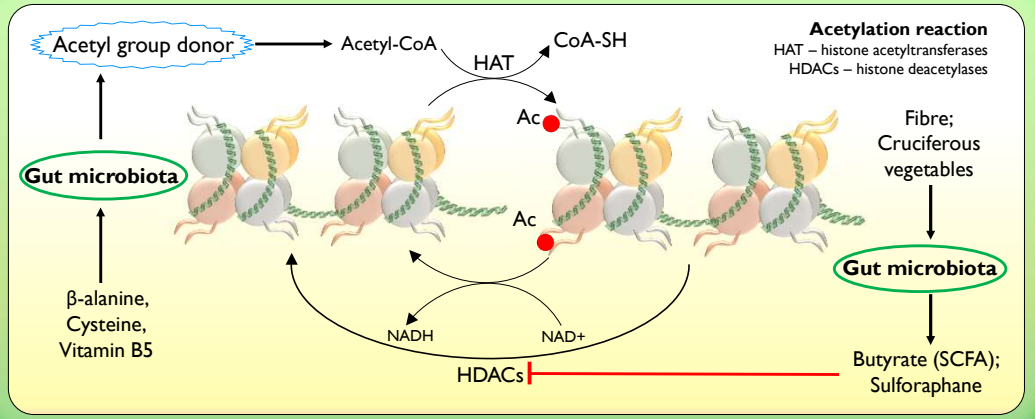


Figure 2. Diet-microbiota-epigenetics triade, with examples of HATs and HDACs as epigenetic targets.

Conclusion

Although there is plenty of evidence connecting diet, microbiota and epigenetic modifications, the **underlying mechanisms** remain largely unclear in humans.

Research faces some **challenges** within these interdisciplinary studies. Hitherto **murine models** are the systematic organism of choice and there are some **physiological differences** that cannot be ignored, including gut microbiota composition. Besides this, nutrients are rarely consumed in isolation and that **manipulating specific nutrients** can invariably alter the intake of other ones.

This way, more studies are required in **humans**, both to sharpen the knowledge of **dietary-microbial shaping** and the food components that **trigger** microbiotic and epigenetic activity. Together with the knowledge of crucial **epigenetic targets** for certain pathological states we establish the conceptual background opening the possibility to manage epigenetic activity via a properly designed diet, as a **new approach** for health and disease issues.

References

[1] K. Reva, (2020), este trabalho está publicado na UC no formato de monografia de conclusão de mestrado.
[2] C. Tiffon, Int J Mol Sci, 19 (2018) 3425.
[3] M. Hullar, B. C. Fu, Cancer J, 20 (2014) 170-175.
[4] N. Zmora, J. Suez, E. Elinav, Nat Rev Gastroenterol Hepatol, 16 (2019) 35-56.
[5] A.A. Kolodziejczyk, D. Zheng, E. Elinav, Nat Rev Microbiol, 17 (2019) 742-753.

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Enzyme inhibitory activity of *Laserpitium siler* L. (Apiacee) extracts

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Introduction

Laserwort (*Laserpitium siler* L., **Figure 1**) is one of 14 European *Laserpitium* species wide distributed at mountains of Southern and Central Europe and one of five laserworts that can be found on rocky meadows and limy talus in Serbia [1]. The root of this species popularly known as "*raskovnik*" has been intensively collected for its "miraculous powers" [2].
Numerous traditional uses of *Laserpitium* plants are known in Europe, especially Alps and in Russian folk medicine. *L. siler* roots and rhizomes are utilized in the treatment of gastrointestinal diseases as bitter herbal preparations with a sharp taste. Also, it is applied in the treatment of toothache, heart and liver dysfunction, pulmonary tuberculosis, rheumatism, as diuretics, and locally in pruritic dermatoses [3, 4].
The study on *L. siler* from Serbia presents the enzyme inhibitory activity of methanol, etanol, methylene chloride and ethyl acetate extracts from fruits and aerial parts of the plant. Performed bioassays included determination of *in vitro* inhibition of acetylcholinesterase, tyrosinase, α-amylase and α-glucosidase enzymes by tested extracts.

Methodology

L. siler extracts were tested in concentrations of C=0.25-5 mg/mL, and results were expressed as IC_{50} values in mg/mL. The extract concentrations required for inhibition of 50% of tested enzyme activity were calculated by the following equation:
Inhibitory concentration (IC_{50}) = (Absorbance of (control-blank) – Absorbance of (sample-blank))/Absorbance of (control-blank) x 100.

Cholinesterase test
The assessment of cholinesterase inhibitory activity of tested extracts was conducted using acetylcholinesterase (AchE) VI-S from *Electrophorus electricus* (Sigma), following the Ellman's method [5, 6]. Galanthamine was applied as reference compound. The absorbances were recorded at 405 nm on Multiskan Sky Thermo Scientific reader.

Tyrosinase test
The inhibitory effect of the extracts on tyrosinase (Tyr) from mushroom (Sigma) was evaluated by using L-3,4-dihydroxyphenylalanine, (L-DOPA) as supstrat [7, 8]. Kojic acid was used as a standard (**Figure 2**). Turbidity of the reaction mixtures was measured spectrophotometrically at 492 nm on a microplate reader.

α-Amylase test
The procedure for determination of *L. siler* extrats inhibitory effects on α-amylase (Amy) from porcine pancreas (Sigma) was perfomed with iodine/potassium iodide [6, 9]. Drug acarbose was used as positive control. The samples measurements were recorded at 620 nm.

α-Glucosidase test
In vitro α-glucosidase (Glu) inhibition assay was done by slightly modified protocols of Zengin et al. (2014) [6] and Hyun et al. (2016) [10] using α-glucosidase (Type I, from *Saccharomyces cerevisiae*,

Materials

Plant material of Laserpitium siler L. subsp. siler was collected in June, 2018, near the Kosovska Mitrovica in Serbia (N 42º 59.26 ' ; E 20 º 49.42 ' ; 489 m alt.) and determined by prof. dr Zoran Krivošej. The separated and dried fruits and aerial parts were powdered and extracted by ultrasonic extraction technique: 10 g of dry plant material was treated 24h with 100 mL of methanol, etanol, methylene chloride and ethylacetate, The obtained yields of *L. siler* samples are presented in **Table 1**.

Results

Obtained results are expressed as IC_{50} values (mg/mL) in **Table 2** and **Figure 3**. Enzyme inhibitory effects of tested extracts showed that aerial parts of the plant possessed higher potential than the fruits. Still, laserwort extracts showed lower activity in comparison to used standards in all applied assyas. The highest inhibitory effects showed methanol and particularly ethyl acetate extract obtained from aerial parts, which exhibited slightly lower activity on diabetes-associated enzymes, α-amylase and α-glucosidase than standard acarbose (**Table 2**, **Figure 3**). Previously, it was proven that methanol extracts of some Apiaceae species also inhibited selected enzymes in different ranges [11].

Conclusion Obtained results provide initial information on anti-hyperglycemic and neuroprotective effects of *L. siler* extracts.



Fig. 1. *Laserpitium siler L.*

Table 1. Obtained yields of ***L. siler*** crude extracts

L. siler extracts			Yields (g)	Yields (%) (w/w)
Extracts (Es)	Fruits (Fr)	Methanol (MeOH)	1.056[b]	9.72[b]
		Ethanol (EtOH)	0.919[b]	9.31[b]
		Methylene chloride ($MeCl_2$)	0.459[a]	4.59[a]
		Ethyl acetate extract (EtOAc)	0.626[a]	6.23[a]
	Aerial parts (Ap)	Methanol	2.374[c]	23.75[c]
		Ethanol	2.180[c]	21.80[c]
		Methylene chloride	0.248[a]	2.48[a]
		Ethyl acetate extract	0.233[a]	2.33[a]

Indicated letters mean significant difference in the same column ($p < 0.05$).

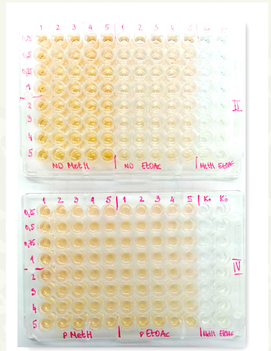


Fig. 2. Inhibitory activity of methanol and ethyl acetate extracts of aerial parts and fruits on tyrosinase

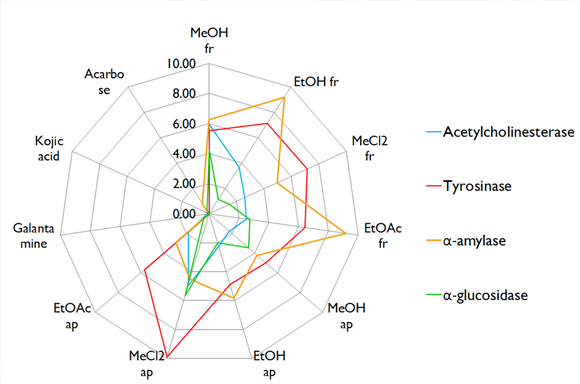


Fig. 3. Inhibitory activity of ***L. siler*** extracts on selected enzymes.

Table 2. Inhibitory activity of ***L. siler*** extracts on selected enzymes expressed as IC_{50} in mg/mL (means ± SD).

L. siler extracts (C=0.25-5 mg/mL)/ Standards (C= 0.00625-1 mg/mL)		Acetylcholine esterase	Tyrosinase	α-amylase	α-glucosidase
Fruits	MeOH	5.954 ± 0.024[c]	5.522 ± 0.516[b]	6.252 ± 0.050[b]	4.250 ± 0.181[d]
	EtOH	3.671 ± 0.014[b]	7.154 ± 0.365[c]	9.239 ± 0.285[c]	1.145 ± 0.091[b]
	$MeCl_2$	2.605 ± 0.108[b]	7.168 ± 0.145[c]	4.956 ± 0.080[b]	1.491 ± 0.069[b]
	EtOAc	2.510 ± 0.002[b]	6.424 ± 0.036[b]	9.174 ± 0.168[c]	2.737 ± 0.000[c]
Aerial parts	MeOH	1.760 ± 0.344[a]	4.973 ± 0.100[b]	4.189 ± 0.011[b]	3.454 ± 0.063[c]
	EtOH	2.223 ± 0.036[b]	4.891 ± 0.089[b]	5.831 ± 0.076[b]	1.985 ± 0.154[b]
	$MeCl_2$	4.939 ± 0.001[c]	9.909 ± 0.201[c]	4.514 ± 0.045[b]	5.655 ± 0.159[d]
	EtOAc	1.837 ± 0.038[a]	5.687 ± 0.147	2.919 ± 0.006[a]	0.479 ± 0.016[a]
Standards	Galantamine/ Kojic acid	0.130 ± 0.005[a]	0.180 ± 0.001[a]		
	Acarbose			0.832 ± 0.057[a]	0.296 ± 0.013[a]

Indicated letters mean significant difference in the same column ($p < 0.05$).

References

[1] Nikolić, V. (1973). Flora of Republic of Serbia, 5th ed., in *Laserpitium* L., M. Josifivić (Ed.), Belgrade, Serbian Academy of Sciences and Arts, 308- 313.
[2] Ranđelović, V., Zlatković, B., Jušković, M., Živojinović, Lj. (2000). Endangered of flora of Mt. Suva planina. Proceeding of 6th Symposium on Flora of the Southeastern Serbia, Sokobanja, 303-322
[3] Chen, Y. L., Lee, C. Y., Huang, K. H., Kuan, Y. H., Chen, M. (2015). Prescription patterns of chinese herbal products for patients with sleep disorder and major depressive disorder in Taiwan. Journal of Ethnopharmacology, 171, 307–316.
[4]Lechner, D., Gibbons, S., Bucar, F. (2006). Antimycobacterial activity of *Laserpitium siler* L. roots. Scientia Pharmaceutica 2 Supplement 1, 74, 94.
[5] Aktumsek, A., Zengin, G., Guler, O. G., Cakmak, S.Y., Duran, A. (2013). Antioxidant potentials and anticholinesterase activities of methanolic and aqueous extracts of three endemic *Centaurea* L. species. Food and Chemical Toxicology, 55, 290–296.
[6] Zengin, G., Sarikurkcu, C., Aktumsek, A., Ceylan, R. (2014). *Sideritis galatica* Bornm.: A source of multifunctional agents for the management of oxidative damage, Alzheimer's's and diabetes mellitus. Journal of Functional Foods, 11, 538–547.
[7] Zengin, G., Sarikurkcu, C., Aktumsek, A., Ceylan, R., Ceylan, O. (2014). A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Industrial Crops and Products, 53, 244–251
[8] Masuda, T., Yamashita, D., Takeda, Y., Yonemori, S. (2005). Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*, Bioscience, Biotechnology, and Biochemistry, 69 (1), 197–201.
[9] Yang, X.-W., Huang, M.-Z., Jin, Y.-S., Sun, L.-N., Song, Y., Chen, H.-S. (2012). Phenolics from *Bidens bipinnata* and their amylase inhibitory properties. Fitoterapia, 83, 1169–1175.
[10] Hyun, K. T., Kim, H.-C., Ko, Y.-J., Kim, J.-S. (2016). Antioxidant, a-glucosidase inhibitory and anti-inflammatory effects of aerial parts extract from Korean crowberry (*Empetrum nigrum* var. *japonicum*). Saudi Journal of Biological Sciences, 23, 181–188.
[11] Zengin. G, Sinan. K. I, Gunes. A, Mahomoodally. M. F, Paksoy. M. Y, Picot-Allain. C, Glamocilja. J, Sokovic. M, Jekő. J, Cziáky. Z, Rodrigues. M. J, Pereira. G. C, Custodio. L, (2020). Industrial Crops and Products, 153, 112572.

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COMMELINA ERECTA AS A SOURCE OF NATURAL BIOACTIVE COMPOUNDS

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Introduction

Plants are an important source for the discovery of new products of medicinal value and unique sources for food additives, flavors, and other compounds with industrial value *Commelina erecta* L. (Commelinaceae) is an herbaceous flowering plant, popularly known as "trapoeraba" and "erva-de-santa-luzia". In folk medicine, *C. erecta* has been used as antiviral, for the treatment of haemorrhages, skin rashes and sores, and for the treatment of infections [1]. Moreover, it is also used as a wild edible plant in salads, preserves or decorating the dishes [1]. The present study aims at evaluating the bioactive potential, namely, antioxidant, cytotoxicity to tumor and non-tumor cell lines, and anti-inflammatory activities of the stem and flower of *C. erecta*.

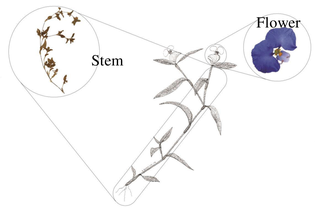
Materials

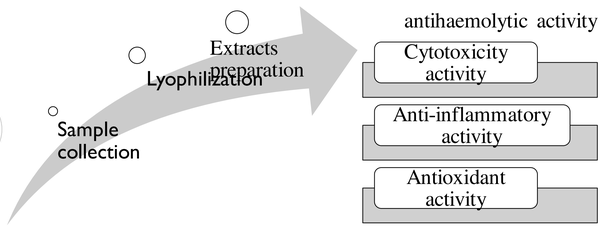
Samples of *C. erecta* were collected and lyophilized in Brazil in partnership with the University Technological Federal of Paraná and later sent to the Institute Polytechnic of Bragança to carry out bioactivity analysis of hydroenthanolic extracts.



Methodology

- The cytotoxicity was tested using four human tumor cell lines: AGS (gastric adenocarcinoma), CaCo (colorectal adenocarcinoma), NCI-H460 (non-small cell lung cancer), MCF-7 (breast carcinoma), as well as a non-tumor culture from African green monkey (Vero).
- Anti-inflammatory activity was determined based on the nitric oxide (NO) production by a murine macrophage (RAW 264.7) cell line.
- Antioxidant activity was evaluated in the hydroethanolic extracts by two *in vitro* assays measuring inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) assay and antihaemolytic activity (OxHLIA).





Results

- **Cytotoxicity to tumor cell lines**

Stem sample presented effective results in the inhibition of the tested tumor cell lines, namely, AGS, CaCo and MCF-7, while flower is more effective against NCI-H460. Both extracts exhibited toxicity against non-tumoral cell lines, denoting that these extracts have cytotoxicity.

Cytotoxicity to tumor cell lines (GI_{50} µg/mL)[a]	Flower	Stem	Ellipticine	*t*-student test *p*-value
AGS	288±59	222±8	0.22±0.02	<0.001
CaCo	294±2	195±1	0.20±0.02	<0.001
NCI-H460	84±1	135±4	0.249±0.002	<0.001
MCF-7	193±12	155±11	0.251±0.001	<0.001
Cytotoxicity to non-tumor cell lines (GI_{50} µg/mL)[b]				
Vero	104.9±0.3	112±3	0.15±0.02	<0.001

Anti-inflammatory activity (EC_{50} µg/mL)[c]	Flower	Stem	Dexamethasone	*t*-student test *p*-value
RAW 264.7	41±1	157±7	6.3±0.4	<0.001

- **Antioxidant activity**

The hydroethanolic extract showed the best activity in the TBARS assay for the stem sample, with EC_{50} value of 0.63±0.01 mg/mL. On the other hand, in the OxHLIA assay, flower hydroethanolic extract present an IC_{50} of 5.1±0.2 µg/mL at Δt = 60 min.

- **Anti-inflammatory activity**

The *in vitro* anti-inflammatory potential of extracts was evaluated by measuring NO inhibition and the most effective extract was the flower with an EC_{50} values of 41±1 µg/mL.

	Flower	Stem	Positive controls	*t*-student test *p*-value
Antioxidant activity[d]			**Trolox**	
TBARS formation inhibition (EC_{50}; mg/mL)	0.71±0.01	0.63±0.01	0.0054±0.0003	<0.001
OxHLIA (EC_{50}; µg/mL)				
Δt = 60 min	5.1±0.2	19±1	21.8±0.3	<0.001

[a, b] GI_{50}: extract concentration responsible for 50% inhibition of growth of human tumour (AGS, CaCo, NCI-H400 and MCF-7) or non-tumor cell line (Vero); [c] EC_{50}: extract concentration responsible for achieving 50% of the inhibition of NO-production; [d] EC_{50}: extract concentration corresponding to 50% of antioxidant activity. Statistically significant differences ($p < 0.05$) were assessed by a Student's *t*-test.

Conclusion

These results indicate that stems and flowers of *C. erecta* might be a potential source of natural biomolecules for pharmaceutical and food applications.

References

[1] Bezerra, J. A., Corrêa, R. F., Salomé, K., Santos, A. D. C., Lima, E. S., Campos, F. R., Barison, A., & Pinheiro, M. L. B. Revista Virtual de Química, 11(2019), 255-263.

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COPAIBA OIL PREVENTS OBESITY AND IMPROVES ANTIOXIDANT DEFENSES IN ADIPOSE TISSUE

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Introduction

Obesity, a worldwide public health problem, is a chronic disease characterized by an excessive or abnormal body fat accumulation that may lead to adipose tissue dysfunction [1, 2]. Active compounds were described in copaiba oil (CO), a product of a typical tree at the north of Brazil, which present anti-inflammatory, antimicrobial and antioxidant properties [3, 4]. However, there is no data regard the effects of CO in adipose tissue.

Objective

This study evaluated the effect of CO on the obesity development and adipose tissue dysfunction in high-sucrose feed rats.

Material and Methods



Control group (C, n=8)

30% Sucrose solution group (S, n=8)

S + Copaiba oil group (S+CO, n=8)

CO was given by gavage – 200 mg/kg/d

8 weeks

- Blood sample and epididymal adipose tissue was collected for biochemists and oxidative stress determinations.
- Visceral fat was calculated by the sum of the adipose tissue depots (epididymal, retroperitoneal and mesenteric).
- Adiposity index was determined by visceral fat normalized by final body weight

Statistical Analysis

Data were expressed as mean and standard deviation. The comparison between groups was performed using one-way ANOVA complemented *post-hoc* Tuke's test, with significance level adopted at $P<0.05$

Results

Nutritional and morphological parameters

Variables	Groups C	S	S+CO
Inicial body weight (g)	323 ± 23^{a}	323 ± 24^{a}	323 ± 26^{a}
Final body weight (g)	448 ± 25^{a}	498 ± 24^{b}	444 ± 28^{a}
Weight gain (g)	125 ± 21^{a}	175 ± 24^{b}	121 ± 37^{a}
Food inatke (g)	26.5 ± 0.5^{a}	12.1 ± 1.7^{b}	12.3 ± 0.4^{b}
Water intake (mL)	43.3 ± 1.4^{a}	51.6 ± 1.5^{b}	46.0 ± 2.2^{b}
Calorie intake(Kcal)*	101 ± 1.9^{a}	108 ± 7.1^{b}	105 ± 0.9^{ab}
Epididymal fat (g)	9.84 ± 0.9^{a}	12.8 ± 3.5^{a}	10.1 ± 2.4^{a}
Mesenteric fat (g)	6.18 ± 0.8^{a}	12.9 ± 3.2^{b}	6.84 ± 2.4^{a}
Retroperitoneal fat (g)	14.1 ± 1.8^{a}	19.5 ± 3.5^{b}	13.6 ± 3.8^{a}
Visceral fat (g)	30.1 ± 3.0^{a}	45.3 ± 9.3^{b}	30.7 ± 7.2^{a}
Adiposity index (%)	6.82 ± 0.6^{a}	9.26 ± 1.6^{b}	6.95 ± 1.3^{a}

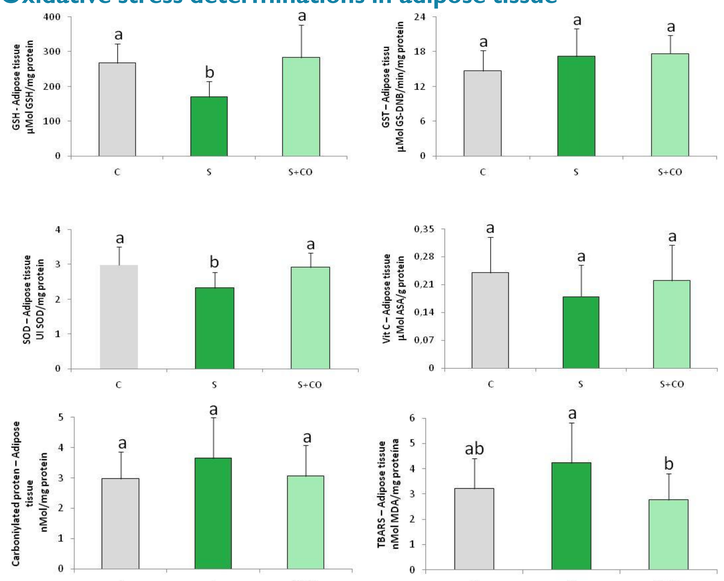
C: control group; S: 30% sucrose solution group; S+CO: S + copaiba oil (200 mg/kg/day), for 8 weeks. Data are presented as mean ± SD, n = 8. One-way ANOVA followed by Tukey's post hoc test was used to compare groups. Mean values with unlike superscript letters were significantly different at $P < 0.05$.
*Caloric intake includes energy from diet and sucrose in the drinking water

Serum biochemical analyzes

Variables	Groups C	S	S+CO
Fasting glucose (mg/dL)	97.4 ± 12^{a}	112 ± 9^{b}	105 ± 6^{ab}
Total cholesterol (mg/dL)	184 ± 66^{a}	306 ± 117^{b}	227 ± 61^{ab}
HDL (mg/dL)	31.6 ± 11^{a}	32.9 ± 10^{a}	31.1 ± 8^{a}
LDL (mg/dL)	91 ± 41^{a}	172 ± 713^{b}	126 ± 41^{ab}
VLDL (mg/dL)	60.9 ± 22^{a}	102 ± 39^{b}	69.8 ± 23^{ab}
Triglycerides (mg/dL)	304 ± 109^{a}	508 ± 194^{b}	349 ± 115^{ab}
TG/HDL ratio	10.3 ± 3.2^{a}	17.1 ± 4.3^{b}	12.6 ± 2.1^{a}

C: control group; S: 30% sucrose solution group; S+CO: S + copaiba oil (200 mg/kg/day), for 8 weeks. Data are presented as mean ± SD, n = 8. One-way ANOVA followed by Tukey's post hoc test was used to compare groups. Mean values with unlike superscript letters were significantly different at $P < 0.05$.

Oxidative stress determinations in adipose tissue



C: control group; S: 30% sucrose solution group; S+CO: S + copaiba oil (200 mg/kg/day), for 8 weeks. Data are presented as mean ± SD, n = 8. One-way ANOVA followed by Tukey's post hoc test was used to compare groups. Mean values with unlike superscript letters were significantly different at $P < 0.05$.

Conclusion

CO supplementation prevented weight gain, adiposity and insulin resistance, in addition, partially inhibited the increase of fasting glucose levels and dyslipidemia, and improved the antioxidant defense system in adipose tissue. Therefore, it is possible to conclude that CO displays benefic effects, suggesting its use as a good strategy for preventing of obesity and its complications.

References

[1] World Health Organization - WHO. Obsesity. Available at: <https://www.who.int/health-topics/obesity> Accessed: January 2021.

[2] U.J . Jung, M.S. Choi. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. International Journal of Molecular Science. 15 (2014) 6184-223.

[3] J.A. Boaroto, A.B. Fernandes, L.M. Moreira, et al. Identification and quantification of β-caryophyllene in copaiba oil using Raman spectroscopy. Instrumentation Science & Technology. 46 (2018) 265-276.

[4] A.P. Ames-Sibin, C.L. Barizão, C.V. Castro-Ghizoni, et al. β-Caryophyllene, the major constituent of copaiba oil, reduces systemic inflammation and oxidative stress in arthritic rats. Journal of Cellular Biochemistry. 119 (2018) 10262-10277.

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Sambucus nigra L. extract-loaded polymeric nanoparticles as a potential anti-inflammatory topical formulation

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Abstract

Sambucus nigra L. is widely used in traditional medicine with different applications. However, confirmative studies are strongly required. This study aimed to assess the biological activities of the *S. nigra* flower's extract encapsulated into two different types of nanoparticles. Different nanoparticles (poly(lactide-co-glycolide, PLGA) and poly-Ɛ-caprolactone (PCL) were prepared and characterized. The biological activities were studied in terms of *in vivo* anti-inflammatory and *in vitro* cell viability. Moreover, biochemical analyses confirmed that the extract-loaded PLGA-based nanoparticles displayed the highest anti-inflammatory activity. In addition to supporting the previously reported evidence of potential therapeutic uses of *S. nigra*, these results could draw the pharmaceutical industry's interest to the novelty of the nanoproducts.

Introduction

Natural products and their derivatives have been recognized for many years as a source of therapeutic agents that can be less toxic and more effective. It has been estimated that nearly 75,000 species of higher plants exist on the earth, and only 10% have been used in traditional medicine. Only 1 to 5% have been studied scientifically and are known to have therapeutic value.

Sambucus nigra L. has been used since ancient times [1,2]. In our previous study, some important biological activities have been found in twenty-two extracts of *S. nigra* flowers (elderflowers) and berries (elderberries), suggesting potential anti-inflammatory activity [3,4]. Thus, the present study reports for the first time the combination of a methanolic extract of *S. nigra* flowers with polymeric nanoparticles (NPs) for potential topical use. In the current study, two polymers were used to prepare NPs: poly(lactide-co-glycolide) (PLGA) and poly-Ɛ-caprolactone (PCL). To the best of our knowledge, and specifically for *S. nigra*, a small number of studies combining *S. nigra* and NPs are described in the literature. Thus, taking advantage of the biological properties of the methanolic extract of elderflowers and of the combination with the carriers' nanoparticulate nature, a new nanoformulation is herein proposed as an alternative to the classical anti-inflammatory products in the market. Extract-loaded PLGA NPs and extract-loaded PCL NPs were prepared and characterized in terms of mean size, size distribution, surface charge and morphology. Safety, and *in vitro* and *in vivo* anti-inflammatory activity (AIA), were also investigated.

Materials

Plant Material: *S. nigra L.* flowers (elderflowers) were supplied by Régiefrutas—Cooperativa Agrícola Távora-Varosa, CIPRL, collected from commercial crops at Tarouca, Beira Alta, Portugal (lat. 40°59'06" N; long. 7°37'03" W; 695 m alt.) in May 2019.

Chemicals: Poly(lactide-co-glycolide) (Purasorb® PLDG 5002-PLGA Ratio L/G % 50:50; molecular weight 45.000–75.000 Da) was supplied by Purac Biomaterials (Gorinchen, The Netherlands). Poly-Ɛ-caprolactone's molecular weight is 14.000 g/mol (PCL) and Pluronic® F127 (PF127) were purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

Cell Lines: HaCaT cell line was supplied by Cell-Line-Service cat: 300493, Eppelheim, Germany. Human fibroblast (HFF) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

Animals: Adult female albino Wistar rats weighing around 200 g were supplied from the *Instituto de Higiene e Medicina Tropical* (Lisboa, Portugal). They were housed in polypropylene cages at room temperature (RT, 20–24 °C), with relative humidity (55 ± 5%), a 12 h light/dark cycle, and received a standard diet and water ad libitum. All animal experiments were conducted in line with the recommendations of the animal welfare board (ORBEA) of the Faculty of Pharmacy, Universidade de Lisboa, as well as approved by the competent national authority *Direção-Geral de Alimentação e Veterinária* (DGAV) (Protocol EXPL/DTP-FTO/0308), and in accordance with the EU Directive (2010/63/EU), the Portuguese laws (DL 113/2013, 2880/2015, 260/2016 and 1/2019), and all relevant legislation.

Methodology

Extract Preparation
- Methanolic extraction by ultrasonication method

Preparation of Extract-Loaded NPs
- PLGA NPs - emulsification/solvent diffusion
- PCL NPs - solvent-displacement method

Physical Characterization
- Size, PdI and Zeta Potential - Dynamic Light Scattering
- pH - pH meter
- Measurements were performed over time and at different temperatures

Morphological Characterization
- Transmission Electron Microscopy (TEM)
- Atomic Force Microscopy (AFM)

In vitro Safety Assessment
- Human Keratinocytes - HaCat (MTT)
- Human Fibroblasts - HFF (PrestoBlue®)

In vivo Anti-inflammatory Activity
- Wistar Rats
- Carrageenan paw oedema model
- Biochemical Analysis

Results

Small size, stable over the time and with different temperatures.

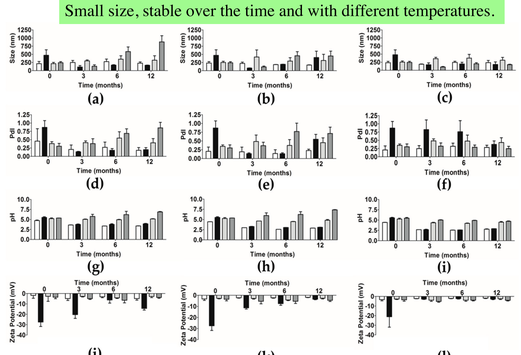
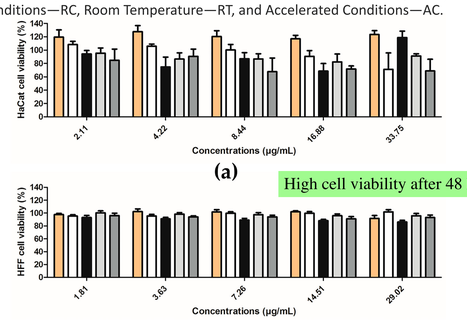


Figure 1. Mean size, PdI, pH, and zeta potential of empty PLGA NPs (white columns), empty PCL NPs (light grey columns), extract-loaded PLGA NPs (black columns) and extract-loaded PCL NPs (dark grey columns) suspension over time: mean size (a–c), PdI (d–f), pH (g–i), and zeta potential (j–l). (a) size at RC, (b) size at RT, (c) size at AC, (d) PdI at RC, (e) PdI at RT, (f) PdI at AC, (g) pH at RC, (h) pH at RT, (i) pH at AC, (j) zeta potential at RC, (k) zeta potential at RT, and (l) zeta potential at AC (mean ± S.D., n = 9). Refrigerated Conditions—RC, Room Temperature—RT, and Accelerated Conditions—AC.



High cell viability after 48 h of incubation

Figure 5. Cell viability (%) of (a) HaCaT and (b) HFF cells after 48 h incubation (%, mean ± S.D.; n > 4): free extract (orange columns), empty PLGA NPs (white columns), extract-loaded PLGA NPs (black columns), empty PCL NPs (light grey columns) and extract-loaded PCL NPs (dark grey columns).

Well-defined morphology.

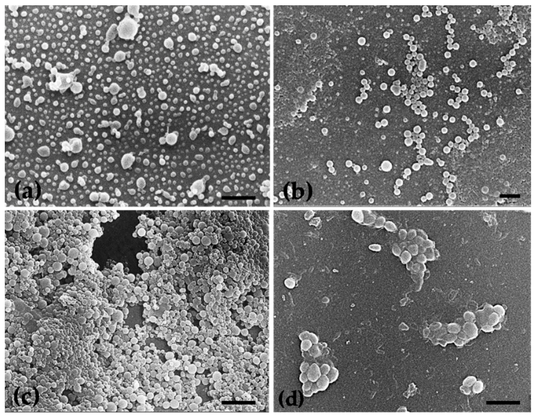


Figure 3. SEM micrographs showing the morphology of NPs. (a) Empty PLGA NPs, (b) Extract-loaded PLGA NPs, (c) Empty PCL NPs and (d) Extract-loaded PCL NPs. Scale bars = 2 µm (a, b), 10 µm (c, d).

High AIA

Table 1. AIA of topically applied extract formulations on the carrageenan-induced rats' hind paw oedema model (mean ± S.E.M, n = 5, unless otherwise indicated; a—n = 6; b—n = 8, * p < 0.05 and *** p < 0.001, when compared to negative control).

Group	Formulations	Dose (mg/kg)	Inhibition (%)
Negative Control	Carbopol 940® gel	--	0.0 ± 0.0
Positive Control [a]	Diclofenac in gel	1.0	133.5 ± 12.6 ***
Free Extract [b]	Extract in gel	1.0	41.2 ± 15.3
Extract-loaded PLGA NPs	Extract-loaded PLGA NPs in gel	1.0	60.7 ± 9.0 *
Extract-loaded PCL NPs	Extract-loaded PCL NPs in gel	1.0	21.7 ± 12.7

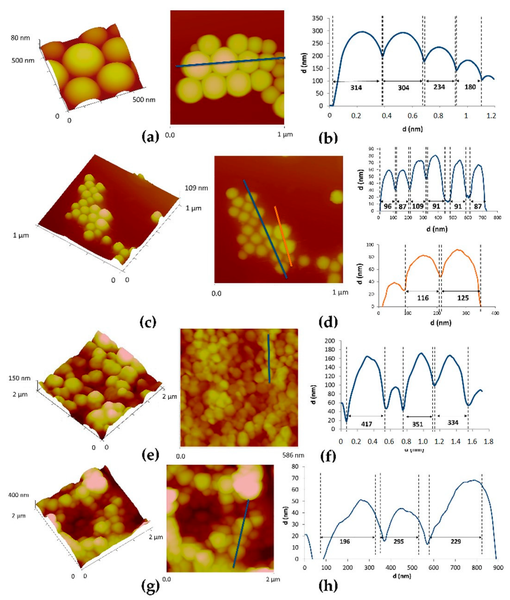


Figure 4. AFM topographic images of NPs under study. (a, b) Empty PLGA NPs, (c, d) Extract-loaded PLGA NPs, (e, f) Empty PCL NPs, (g, h) Extract-loaded PCL NPs. 3D images (a, c, e, g) and cross section analysis (b, d, f, h).

Similar values to positive control

Table 2. Status of the different parameters evaluated in the plasma of the rats for each group (mean ± S.D.).

Group	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
Negative Control	168.5 ± 42.2	109.9 ± 1.2	11.4 ± 1.6
Positive Control	95.0 ± 9.6	95.3 ± 9.0	28.8 ± 6.7
Free Extract	133.3 ± 26.5	106.9 ± 7.7	10.7 ± 1.2
Extract-loaded PLGA NPs	117.6 ± 15.1	96.3 ± 7.6	27.3 ± 10.9
Extract-loaded PCL NPs	123.4 ± 17.3	114.6 ± 16.1	18.8 ± 2.9

Conclusion

For the first time, both *in vitro* and *in vivo* methodologies supported the potential topical use of methanolic extract of *S. nigra* obtained from flowers. Comparing the two polymers used as carriers, PLGA NPs displayed a higher value of *in vivo* AIA by topical route, reaching a mean value of 61% of inhibition of oedema. Similar values of IL-6 and IL-10 were observed between extract-loaded PLGA NPs and the positive control, a commercial formulation of diclofenac. On the other hand, PLGA NPs did not cause cytotoxicity in HaCat and HFF cell lines. More detailed studies should be performed to identify the exact mechanism associated to this AIA activity, as well as to verify if the developed system in this work can be applied in a chronic model of inflammation. Overall, the obtained results could certainly attract interest in extract-loaded PLGA NPs as a potential nanoproduct in a very near future.

Acknowledgements

The authors are grateful to the Régiefrutas, Fundação para a Ciência e a Tecnologia (FCT) (UIDB/04138/2020, UIDB/00100/2020, UIDP/50017/2020+UIDB/50017/2020) and the Portuguese National and Regional Budget, through CCMAR/Multi/04326/2019 project.

References

[1] European Medicines Agency. European Union Herbal Monograph on *Sambucus Nigra* L., Flos (EMA/HMPC/611512/2016); Committee on Herbal Medicinal Products: London, UK, 2007; Volume 44 (accessed on 18 November 2020). **[2]** European Medicines Agency. Assessment Report on *Sambucus nigra* L., Flos (EMA/HMPC/611504/2016); Committee on Herbal Medicinal Products: London, UK, 2016; (accessed on 9 October 2020). **[3]** Mota, A.H.; Andrade, J.M.; Ntungwe, E.N.; Pereira, P.; Cebola, M.J.; Bernardo-Gil, M.G.; Molpeceres, J.; Rijo, P.; Viana, A.S.; Ascensão, L.; et al. Green extraction of *Sambucus nigra* L. for potential application in skin nanocarriers. Green Mater. 2020, 1–13. **[4]** Mota, A.H.; Andrade, J.M.; Rodrigues, M.J.; Custódio, L.; Bronze, M.R.; Duarte, N.; Baby, A.; Rocha, J.; Gaspar, M.M.; Simões, S.; et al. Synchronous insight of in vitro and in vivo biological activities of *Sambucus nigra* L. extracts for industrial uses. Ind. Crops Prod. 2020, 154, 1–11

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Development and validation of an HPTLC densitometry method for psoralen and bergapten quantification in *Brosimum gaudichaudii* Trécul.

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[1]Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, Brasil.

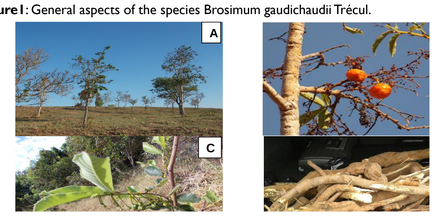
[2]Farmanguinhos/Fiocruz, Rio de Janeiro, Brasil.

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INTRODUCTION

The species *Brosimum gaudichaudii* Trécul (Figure 1) is popularly known as "mama cadela", "arbóreo de cadela" or"algodãozinho do campo ". Traditionally, its roots are used as a therapeutic resource for the treatment of vitiligo (Figure 2), which is a specific form of depigmenting leukoderma.

Figure 1: General aspects of the species Brosimum gaudichaudii Trécul.



Note: A: Adult specimens of B .gaudichaudii; B: Fruits of B. gaudichaudii; C: B. gaudichaudii leaves; D: Roots of *B.gaudichaudii*.

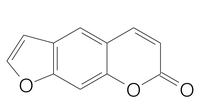
Figure 2: Example of vitiligo stains on the hands (A) and example of generalized vitiligo stains (B).



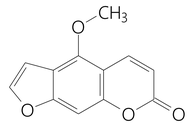


The action of the mama-cadela in the treatment of vitiligo is mainly due to the presence of secondary metabolites called linear furanocoumarins(Figure 3), and these belong to a subgroup of metabolites secondary to coumarins.

Figure 3:Representation of the chemical structures of psoralen and bergaptene



Psoraleno



Bergapteno

OBJECTIVES

The objective of the present work was to develop an analytical method by CCDAE for the simultaneous quantification of psoralen and bergaptene in lyophilized extract of *B. gaudichaudii* in order to become an alternative method for the quality control of the extracts produced from this species.

MATERIALS

- Sample of lyophilized extract of *B. gaudichaudii* was prepared at a concentration of 10 mg / mL;
- Psoralene and bergaptene standards (Sigma Aldrich®) were prepared separately at a concentration of 1 mg / mL;
- HPLC grade methanol was used as solvent;
- Automatic applicator (ATS4), densitometer ;
- TLC Visualiser coupled to a DXA 525 camera and heating plate;
- chromatoplates of Silica Gel60 F254 and Silica RP 18 F254 (reverse phase), both from Merck®.

METHODOLOGY

The CCD / densitometry analyzes were carried out in collaboration with the Farmanguinhos, Fiocruz, Rio de Janeiro.

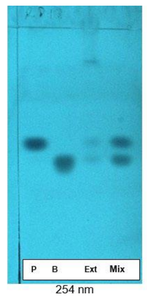
For analytical development, a screening was carried out in the literature in order to establish the best mobile phase and the best stationary phase.

The method was validated for the following parameters: Linearity, selectivity, precision, accuracy.

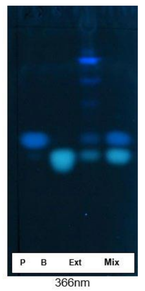
RESULTS

The stationary phase was then varied for a reverse phase silica chromatoplate (RP 18), that is, with an apolar characteristic. With this stationary phase it was possible to obtain good resolution and separation between psoralen and bergaptene, using acetonitrile: water 60:40 (Figure 4 and Figure 5)

Figure 4:Photos of the RP18 reverse phase silica chromatoplates (10 cm x 10 cm), mobile phase acetonitrile: water 60:40 (v / v), a condition that allowed the best separation of psoralen and bergaptene in the extract of *B. gaudichaudii*.



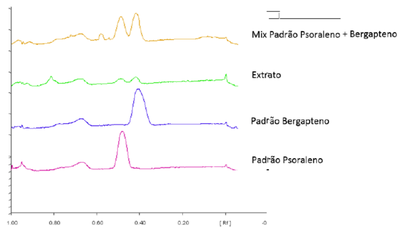
254 nm



366nm

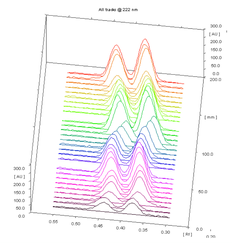
Note: P- Psoralen; B- Bergapteno; Ext- Lyophilized extract of *B. gaudichaudii*; Mix- Standard of psoralen + bergaptene (1: 1).

Figure 5: Densitograms of the patterns and lyophilized extract of B. gaudichaudii, confirming the presence of the markers and with good resolution.



The average recovery for psoralen was 95.95% and the average recovery for bergaptene was 91.45%. Below (FIGURE 6),

Figure 6: Densitograms for the three-level accuracy test



CONCLUSION

✓Densitometry by CCD or CCDAE is a fast, reproducible, accurate and low-cost technique;

✓In addition, lyophilized extracts of *B. gaudichaudii* have high concentrations of these constituents and can be used to prepare formulations for the treatment of vitiligo;

✓The advantage of CCD / densitometry is the use of small amounts of samples and solvents, minimizing the cost, which is a determining factor for the quality control of laboratories and industries that work with inputs and products of plant origin.

REFERENCES

- Pozetti GL. Brosimum gaudichaudii Trécul (Moraceae): da planta ao medicamento. Rev Ciên Farm Bás Apl. 2005;
- Khovacs SR. Vitiligo. J Am Acad Dermatol. 1998
- Steiner D, Villas RT, Bedin V, Steiner T, Moraes MB. Review Article: Vitiligo. **Anais Bras Dermato**. 2004; 79 (03): 335-351. ISSN 1806-4841.
- Ali J, Pramod K, Tahir MA. Current remedies for vitiligo. Autoimm Rev. 2010; 9: 516-520. ISSN 1568-9972. [CrossRef] [PubMed].
- Fatani MI, Alshrif SH, Alfif KA, Khan AS, Hussain WA, Banjar AA. The clinical patterns of vitiligo "hospital-based study" in Makah region, Saudi Arabia. J Dermato Dermatol Surgery. 2014; 18(1-2): 17-21. ISSN 2210-836X. [CrossRef]
- Ribeiro CVC, Kaplan MAC. Tendências evolutivas de famílias produtoras de cumarinas em Angiospermae. Quím Nova. 2002; 25(4): 533-538. ISSN 0100-4042.11. [CrossRef].
- Morais MCM, Almeida PHG, Ferreira NLO, Arruda RL, Borges LL, Freitas O, Conceição EC. Validation of a photostability indicating method for quantification of furanocoumarins from Brosimum gaudichaudii soft extract. Rev Bras Farmacog. 2018; 28(1): 118-123. ISSN 0102-695X. [CrossRef].
- Domínguez A, Jarne C et al. A Hyphenated Technique based on High-Performance Thin Layer Chromatography for Determining Neutral Sphingolipids: A Proof of Concept. Chromat. 2015, 2: 167-187. ISSN 2227-9075. [CrossRef].
- Martins FS, Pascoa H, Paula JR, Conceição EC. Technical aspects on production of fluid extract from Brosimum gaudichaudii Trécul roots. Pharmacogn Mag. 2015; 11 (41): 226-231. ISSN 0973-1296. ISSN 0976-4062. [CrossRef] [PubMed].

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PDTI Bioprodutos
Ministério da Saúde FIOCRUZ Fundação Oswaldo Cruz
farmanguinhos

NPA
Natural products application: Health, Cosmetic and Food
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OREGANO AND THYME HYDROLATS AS POTENTIAL NUTRACEUTICALS

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1. BACKGROUND

Medicinal plants contain pharmacologically active secondary metabolites that exhibit beneficial health effects and their extracts, essential oils and hydrolats are often used as **therapeutic agents**.

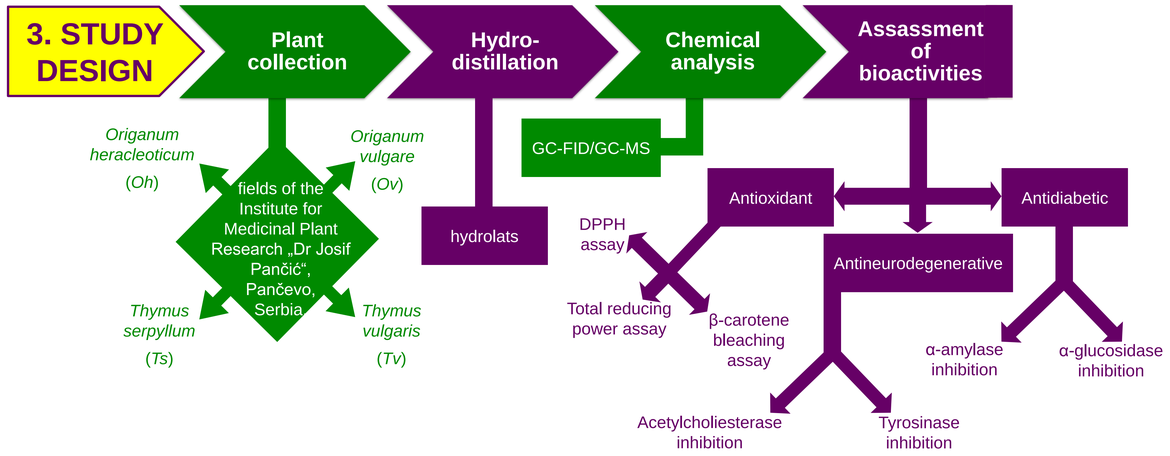
Species from genera ***Origanum* L. (oregano) and *Thymus* L. (thyme)** belong to the Lamiaceae family and are commonly recognized as spicy, fragrant, and ornamental plants, while a number of them are often used in **traditional medicine** for treating a plethora of conditions, such as sore throat, bronchitis, cold and gastrointestinal disorders [1].



2. AIM

The aim of this research was to examine the chemical composition, as well as antioxidant, antidiabetic and antineurodegenerative activities of hydrolats of **two *Origanum* and two *Thymus* species**, commercially grown on the fields of the Institute for Medicinal Plant Research „Dr. Josif Pančić" in Pančevo, Serbia.

3. STUDY DESIGN



5. CONCLUSIONS

Medicinal plants, such as oregano and thyme, establish their **health-boosting actions** through various mechanisms.

Altogether, *Tv*, *Ts*, and *Oh* exerted **excellent antioxidant, antidiabetic and antineurodegenerative potential**, while carvacrol and thymol seem to be responsible for them being considered auspicious nutraceuticals.

4. RESULTS

GC-FID/GC-MS ANALYSIS

Compound (%)	*Oh*	*Ov*	*Ts*	*Tv*
1-octen-3-ol		4.35		
p-cymene	0.11			0.15
1,8-cineole		8.70		
benzene-acetaldehyde		7.59		
γ-terpinene	0.06			
cis-sabinene hydrate			0.80	0.24
cis-linalool oxide		0.37		
trans-linalool oxide		0.93		
linalool		47.92		0.63
camphor	0.04			
δ-terpineol		0.28		
borneol			1.00	0.27
4-terpinenol		6.01	0.08	0.10
p-8-cymenol	0.07		0.08	0.07
α-terpineol		23.87		
thymol	0.72		98.04	94.70
carvacrol	99.00			3.84

BIOACTIVITIES

ANTIOXIDANT

Tv* and *Ts hydrolats showed the highest DPPH scavenging and total reducing power, while ***Oh*** most effectively protected β-carotene from bleaching, which was caused by free radicals.

ANTIDIABETIC

The examined hydrolats **did not inhibit α-amylase** activity at all, while they exhibited a higher inhibition effect on **α-glucosidase**, especially ***Ts* and *Tv***, which exhibited higher activity than acarbose at the concentration of 0.5 mg/mL.

ANTINEURODEGENERATIVE

The hydrolats were rather inactive against the activity of **tyrosinase**, however all of them had a **high acetylcholinesterase inhibition** potential, especially ***Oh***, which exerted higher inhibition than galanthamine at 0.5 mg/mL.

Biological activity	Assay	Plant species				
		Oh[a,b]	*Ov*	*Ts*	*Tv*	Positive controls[c]
Antioxidant	DPPH	54.12±0.55	14.45±1.94	56.78±1.46	**59.00±0.34**	71.70±0.29 (**BHT**)
	Total reducing power	57.12±1.45	7.24±0.72	**67.10±0.62**	**67.17±0.87**	-
	β-carotene bleaching	**44.04±0.12**	nd	nd	40.83±3.75	70.88±1.38 (**ascorbic acid**)
Antidiabetic	α-amylase	**0.96±0.12**	0.07±0.26	**0.97±0.32**	nd	80.15±4.02 (**acarbose**)
	α-glucosidase	60.49±1.03	nd	**87.39±0.21**	83.44±0.74	79.23±0.25 (**acarbose**)
Antineurodegenerative	Acetylcholinesterase	**78.51±0.55**	43.74±2.20	64.96±1.16	67.42±0.37	76.00±0.46 (**galanthamine**)
	Tyrosinase	nd	7.09±1.20	**11.81±0.91**	nd	49.25±1.12 (**kojic acid**)

[a] The results are expressed as percentages of inhibition for all the assays, except for the total reducing power (μg AAE/g), nd – not detected.
[b] The results are presented as a mean of three measurements ± standard error.
[c] The results for the positive controls are presented at 0.5 mg/mL.

Reference:
[1] Oalđe, M.M., Kolarević, S.M., Živković, J.C., Vuković-Gačić, B.S., Jovanović Marić, J.M., Kračun Kolarević, M.J., Đorđević, J.Z., Alimpić Aradski, A.Z., Marin, P.D., Šavikin, K.P., Duletić-Laušević, S.N. Saudi Pharmaceutical Journal, 28 (2020): 1592-1604.

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ROSMARINIC ACID –PLANT POLYPHENOL WITH ANTIMICROBIAL AND ANTIBIOFILM POTENTIAL

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Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia

NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Abstract

Increasing antimicrobial resistance accompanied by range of side effects linked to antibiotics overuse are urging the demand for the novel antimicrobials with the ones obtained from natural sources considered as an appealing alternative. Rosmarinic acid is naturally occurring plant polyphenol. This study aimed to enlighten its potential role as inhibitor of planktonic and biofilm microorganism growth, along with its antifungal mechanisms.

In this study, rosmarinic acid has shown promising anticandidal (MIC 0.1-0.2 mg/mL) and antibacterial (MIC 0.002->0.8 mg/mL) activity. To some extent rosmarinic acid was able to reduce attachment of fungal cells to abiotic surface, first stage in biofilm formation process, and eradicate preformed biofilms. Rosmarinic acid antifungal mode of action involves interference with membrane integrity, but not binding to membrane lipid, ergosterol, or reduction in protease production; while its antibiofilm activity is moderately attributed to the decrease in exopolysaccharide production.

Considering its wide antimicrobial and antibiofilm capacity rosmarinic acid could be further examined as antimicrobial agent along with range of medicinal plants with rosmarinic acid as the dominant compound.

Introduction

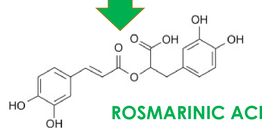
Increased incidence of fungal infections including candidiasis

Mortality rates as consequences of invasive candidiasis are up to 55%

Candida albicans is the dominant cause of invasive candidiasis

Candida spp. have developed a range of adaptation traits in order to successfully avoid host immune system reactions and establish infection often in association with bacterial pathogens

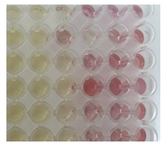
URGING THE NEED FOR THE DEVELOPMENT OF NOVEL ANTIMICROBIAL MOLECULES



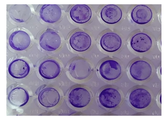
ROSMARINIC ACID

Naturally occurring polyphenol abundantly present in herbs, spices and medicinal plants – could it be novel antimicrobial lead and how it works?

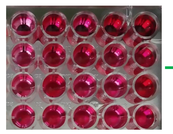
Methodology



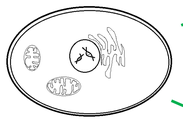
Antimicrobial activity was determined by microdilution assay in 96-well microtiter plates



Antibiofilm activity (inhibition of cell attachement and biofilm eradication) was determined by crystal violet assay in 96-well microtiter plates with adhesive bottom



Reduction in exopolysaccharide production in *Candida albicans* biofilm was determined by Congo red binding assay



Impact of rosmarinic acid on fungal membrane integrity was determined by cristal violet uptake and ergosterol binding assay

Impact of rosmarinic acid on fungal protease production was determined with azocasein as a substrat

Results

- Rosmarinic acid efficently reduces growth of different *Candida* species

Table 1. Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations of rosmarinic acid, mg/mL.

Yeasts	Rosmarinic acid		Ketoconazole	
	MIC	MFC	MIC	MFC
***C. albicans* 475/15**	0.1	0.2	0.003	0.006
***C. albicans* 13/15**	0.1	0.2	0.0016	0.05
***C. albicans* 17/15**	0.1	0.2	0.0016	0.05
***C. albicans* 527/14**	0.15	0.3	0.0031	0.0062
***C. albicans* 10/15**	0.15	0.3	0.0031	0.05
***C. albicans* 532/15**	0.1	0.2	0.0031	0.0062
***C. albicans* ATCC 10231**	0.2	0.4	0.0016	0.006
***C. krusei* H1/16**	0.2	0.4	0.0016	0.003
***C. glabrata* 4/6/15**	0.1	0.2	0.0016	0.006
***C. tropicalis* ATCC 750**	0.2	0.4	0.0016	0.006
***C. parapsilosis* ATCC 22019**	0.1	0.2	0.003	0.006

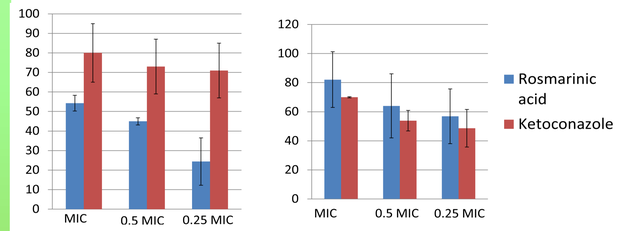
Results

- Rosmarinic acid exhibits antibacterial properties

Table 2. Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of rosmarinic acid, mg/mL.

Bacteria	Rosmarinic acid		Amoxicillin + Clavulanic Acid		Cefixime	
	MIC	MBC	MIC	MBC	MIC	MBC
Micrococcus luteus	0.1	0.2	0.0002	0.0004	0.002	0.003
Rothia mucilaginosa	0.1	0.2	0.007	0.014	0.002	0.003
Streptococcus agalactiae	0.05	0.1	0.007	0.014	0.002	0.004
Streptococcus angiosus	0.05	0.1	0.028	0.056	0.0002	0.0004
Streptococcus dysgalactiae	0.05	0.1	0.007	0.014	0.0002	0.0004
Streptococcus oralis	0.05	0.1	0.0004	0.001	0.002	0.003
Streptococcus parasanquinis	0.05	0.1	0.004	0.01	0.003	0.006
Streptococcus pyogenes	0.1	0.2	0.0004	0.001	0.0008	0.004
Streptococcus salivarius	0.002	0.004	0.01	0.014	0.013	0.027
Staphylococcus aureus	>0.8	>0.8	0.001	0.002	0.003	0.006
Staphylococcus hominis	0.4	0.8	0.004	0.007	0.002	0.003
Enterobacter cloacae	0.1	0.2	0.028	0.056	0.003	0.007
Stenotrophomonas maltophilia	0.4	0.8	0.003	0.007	0.003	0.006

- Rosmarinic acid reduces ability of *Candida* cells to attach to abiotic surface – limiting their ability to start the process of biofilm formation



Candida albicans 475/15 *Candida glabrata* 4/16/15

Figure 1. Percentage of inhibition of *Candida* cell attachment (100% means no biofilm was established) after treatment with rosmarinic acid in range of concentration 0.25 MIC- MIC.

- Rosmarinic acid eliminates 24 h pre-formed fungal biofilms

Table 3. Minimal biofilm eradication concentrations of rosmarinic acid towards different *Candida* strains.

	Rosmarinic acid	Ketoconazole
***C. albicans* 475/15**	0.4	0.012
***C. albicans* 13/15**	0.4	0.05
***C. albicans* 17/15**	0.8	0.05
***C. albicans* ATCC 10231**	0.4	0.012
***C. krusei* H1/16**	>1.6	0.1
***C. glabrata* 4/6/15**	0.8	0.012
***C. tropicalis* ATCC 750**	1.6	0.025
***C. parapsilosis* ATCC 22019**	0.8	0.006

Results

- Rosmarinic acid mechanism of antibiofilm activity is moderatly linked to reduction in exopolysaccharide content

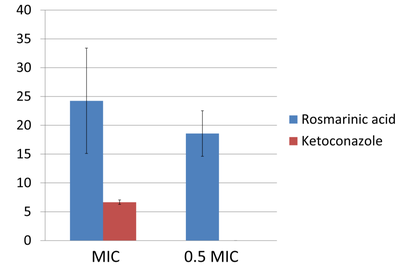


Figure 2. Inhibition of congo red binding (%) to *C. albicans* 475/15 biofilm.

- Rosmarinic acid antivirulence mechanism is linked to slight reduction in proteases production

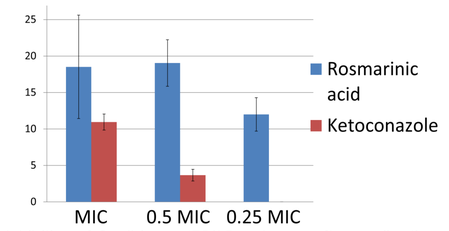


Figure 3. Inhibition of *C. albicans* 475/15 proteases after application of rosmarinic acid (%).

- Rosmarinic acid antifungal effect is due to the interference with the integrity of cell membrane

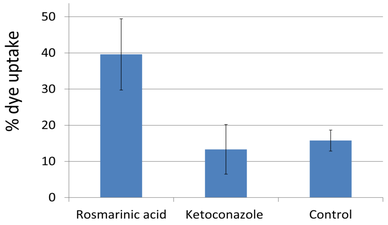


Figure 4. Crystal violet uptake of rosmarinic acid (MIC, 0.1 mg/mL) treated *C. albicans* 475/15 (%).

Conclusion

In this work we have elucidated rosmarinic acid as a prominent antimicrobial agent with promising inhibitory activity towards range of fungal and bacterial pathogens. We have evidenced its ability to reduce biofilm formation/formed biofilms in range of *Candida* species. Also, disruption in membrane integrity has been observed as potential antifungal mechanism. In the era urging for novel antibiotics rosmarinic acid should be further explored due to its broad antimicrobial spectrum and wide presence in natural bioactive products.

Acknowledgements

This research is funded by the Serbian Ministry of Education, Science and Technological Development [Contract No. 451-03-68/2020-14/200007].

Seasonal changes in the biochemical profile and antioxidant properties of *Gelidium corneum* from São Martinho do Porto, Portugal

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Cofinanciado por:

Abstract

The marine environment holds a few of the richest ecosystems on the planet and astounding genetic diversity in the species that inhabit it [1]. The existing species of marine algae remain a mystery to scientist even today, but their biotechnological potential has become appealing to various industries [2,3]. Besides the natural production of bioactive compounds, abiotic factors such as nutrient starvation, UV radiation or temperature may trigger metabolic reorientations [1]. Nowadays the algae resources from São Martinho do Porto, Portugal, namely the red algae *G. corneum* is mostly used for agar extraction and soil fertilization [4]. In this study, the seasonal variations of antioxidant capacity, carbohydrate content and lipidic content were evaluated to further deepen the biotechnological potential of these macroalgae.

The antioxidant potential was evaluated through the DPPH, ABTS and FRAP assays and total phenolic content (TPC) was determined [5,6,7,8]. Antioxidant activities for DPPH, ABTS, FRAP and TPC assays show average values (5-15% inhibition, 10-14% inhibition, 0.009-0.02 AAE/$g_{extract}$ and 4-7 GAE/$g_{extract}$, respectively), in line with the expected. The lipidic content, evaluated through the Folch method [9], showed the lowest and highest values in springtime and summertime, respectively. The carbohydrate content was evaluated through the Dubois method [10]. Samples from springtime showed the highest values (61.1 ± 2.0% DW).

Introduction

The marine environment holds one of the richest and most complex ecosystems. Algae are known for possessing a high variety of nutritional and bioactive compounds such as antioxidants (polyphenols, tocopherols and micosporine-like amino acids), antimicrobials, immunostimulants, and others. Besides the natural production of bioactive compounds, abiotic factors such as UV radiation, nutrient starvation or even temperature may trigger metabolic reorientations and, as such, it is important to know these biochemical variations to ascertain the true biotechnological potential of algae biomass.

Variation
Carbohydrate content
Lípid content
Antioxidants



Our goal was to evaluate the variations of carbohydrate and lipid content in *G. corneum* biomass harvested in São Martinho do Porto and determine the antioxidant activity of its aqueous extracts to assess the biotechnological potential.

Methodology



Antioxidant Activity

Aqueous Extraction: Percolation method described in Fatima and coworkers (2016)

Total Phenolic Content (TPC) | **DPPH assay** | **FRAP assay** | **ABTS assay**

Carbohydrate content:

Carbohydrate content was quantified using the Dubois method (1956)

Lipid content:

Lipidic content was evaluated through the Folch method (1957)

Statistical analysis:

All data was subjected to means analysis (*ANOVA*), after Levene´s test to ensure homogeneity of variations, to determine statistical differences between seasonal values, using IBM SPSS Statistics v.27. Differences at $p < 0.05$ were considered to be significantly different.

Results

***Table** 1 - Variation of carbohydrate content, lipidic content, protein content and antioxidant activity of aqueous extracts from G. corneum throughout the year, * # ǂ indicate statistical differences between the Summer, Autumn, and Winter harvests, respectively.*

Harvest	Carbohydrate content	Lipidic content	DPPH	ABTS	TPC	FRAP
Summer (H1)	[1)]34.05 ± 2.35	[1)]2.75 ± 0.28	[2)]7.60 ± 2.47	[2)]10.93 ± 1.26	[3)]6.02 ± 0.78	[4)]0.91 ± 0.22
Autumn (H2)	36.23 ± 1.76*	2.16 ± 0.10*	10.89 ± 1.46	13.90 ± 1.54*	6.82 ± 0.26	0.77 ± 0.0165
Winter (H3)	24.84 ± 5.06#	2.57 ± 0.04	5.57 ± 0.62#	10.85 ± 1.25#	6.70 ± 0.19	0.78 ± 0.05
Spring (H4)	61.05 ± 2.03*#ǂ	0.93 ± 0.04*#ǂ	9.92 ± 0.72	13.55 ± 1.50*ǂ	4.59 ± 0.37#ǂ	2.73 ± 0.13*#ǂ

[1)]Mean ± SE (%DW), [2)]Free radical scavenging activity (%) ± SE [3)]GAE/g ± SE [4)]AAE/g ± SE (n=3).

Conclusion

- In the springtime *G. corneum* biomass showed the highest production of carbohydrates. This value is higher than the values reported in many red and green algae and comparable to several brown algae.
- Antioxidant activity was evaluated through complementary assays and although there were noticeable variations along the seasons the aqueous extracts show average antioxidant activity.
- Lipid content showed variations along the seasons with values comparable to other red macroalgae.
- Although the aqueous extracts of *G. corneum* presented average antioxidant activity there is, in fact, significant variation in lipids and carbohydrates levels along the seasons. Considering this, one can assume the harvest season is essential in determination of the possible application of the collected biomass.

References

[1] Mimouni V, Ulmann L, Pasquet V, Mathieu M, Picot L, Bougaran G, Cadoret JP, Morant-Manceau A, Schoefs B. Current pharmaceutical biotechnology, 13(2012) 2733
[2] Guiry MD, Journal of Phycology, 48 (2012) 1057
[3] Rocha J, Peixe L, Gomes N, Calado R. Marine drugs, 9 (2011) 1860
[4] Díaz, RTA, Arrojo, VC, Agudo, MA, Cárdenas, C, Dobretsov, S, Figueroa, FL. Marine Biotechnology, 21 (2019) 577
[5] Berker, KI, Güçlü, K, Tor, İ, Apak, R. Talanta, 72 (2007) 1157
[6] Brand-Williams, W, Cuvelier, M E, Berset, CLWT. LWT-Food science and Technology, 28 (1995) 25
[7] Nenadis, N, Wang, L F, Tsimidou, M, Zhang, H Y. Journal of agricultural and food chemistry, 52 (2004) 4669
[8] Singleton, VL, Rossi, JA. American Journal of Enology and Viticulture, 16 (1965) 144
[9] Folch, J, Lees, M, Stanley, GS. Journal of biological chemistry, 226 (1957) 497
[10] Dubois, M, Gilles, KA, Hamilton, JK, Rebers, PT, Smith, F. Analytical chemistry, 28 (1956) 350

Acknowledgements

This study had the support of Fundação para a Ciência e Tecnologia (FCT), through the strategic project UIDB/04292/2020 granted to MARE, and the project Limo no Cais – Exploração sustentada dos recursos endógenos de S. Martinho do Porto (MAR-04.03.01-FEAMP-0101).

Medical and pharmacological potential of low-molecular compounds derived from the latex of a medicinal plant *Chelidonium majus* L.

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Abstract

Chelidonium majus L., also known as Greater Celandine, is a perennial, herbaceous plant widely used in traditional medicine. The plant produces yellow milky sap which is rich in biologically active substances, like alkaloids, flavonoids, phenolic acids and defence-related proteins with antipathogenic activities. The milky sap is used in folk medicine to treat warts, papillae and condylomas, which are caused by human papillomavirus (HPV) infections. The plant's extracts, containing mostly hydrophilic molecules such as alkaloids, have various medicinal properties as antimicrobial, anti-inflammatory, antiviral, antifungal and antitumor activities. *C. majus* alkaloids, such as chelidonine, sanguinarine or protoberberine compounds, are intensively researched for potentially medical properties. For instance, the antibacterial and antiviral activity of *C. majus* alkaloids was confirmed. Moreover, the alkaloids' properties such as cytotoxicity and ability to overcome the multidrug resistance mechanisms in tumor cells extend the potential use of the plant extracts in tumor therapies. In the presentation, the current state of knowledge about medical and pharmacological potential of *C. majus* low-molecular compounds, with special emphasis on its alkaloids, will be discussed. In the future study, the antitumor and antiviral properties of two compounds of *C. majus* milky sap, defense proteins and alkaloids, will be examined. This approach will allow investigating the potential synergistic action of milky sap compounds.

Chelidonium majus L. and its milky sap

Chelidonium majus L., also known as Greater Celandine, is a perennial, herbaceous plant belonging to the Papaveraceae family. Yellow, little flowers are characteristic of *C. majus*, as well as the presence of yellow, milky sap, which exudes after wounding of the stem, leaf, or root (Fig. 1). The milku sap serves as a defense against herbivore (mostly insects) and pathogens activity.

***C. majus* milky sap is a rich source of biologically active substances.** Alkaloids belonging to the low-molecular components, and proteins are the most abundant groups of chemicals. Other chemicals as phenolic acids (caffeic acid, chelidonic acid), flavonoids (rutin, luteolin), unsaturated fatty acids (linolenic acid, oleic acid) are less abundant in milky sap.

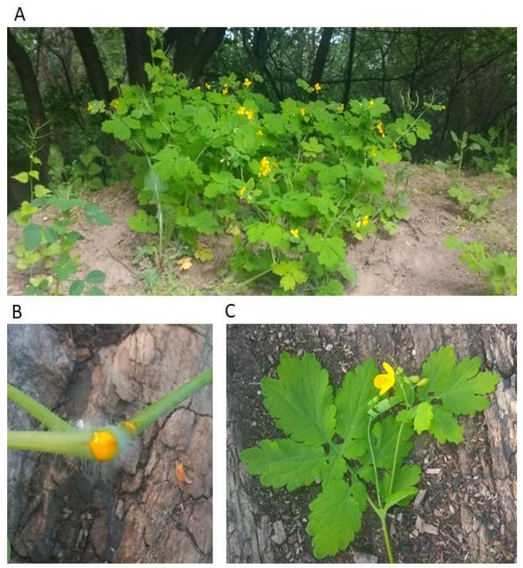


Figure 1. [A] *Chelidonium majus* L. in the natural habitat, Morasko, Poznań, Poland; [B] *C. majus* milky sap; [C] *C. majus* leaves and flowers.

Medicinal properties of the plant

C. majus in folk and traditional medicine

The milky sap from *C. majus* was used in folk medicine to treat digestive system and eyes conditions. Fresh milky sap was used also to treat warts and condylomas.

Preparations containing extracts or dried parts of *C. majus* are approved for production and use both internally and externally. Drugs regulating the work of the digestive system, e.g. Artecholin, contain *C. majus* extracts.

Antifungal properties of alkaloids

The antifungal activity of *C. majus* **chelerythrine** and **sanguinarine** was confirmed in *Candida albicans* studies.

Antitumor properties of alkaloids

A variety of *in vitro* studies were provided to investigate the antitumor properties of certain alkaloids present in the *C. majus* milky sap. The Table 1 presents results of the selected experiments.

Table 1. The antitumor properties of alkaloids against tumor and normal cell lines. After: Zielińska et al., 2018. Modified.

Cell line	Tested alkaloid(s)	General effect
HaCaT – human keratynocites	chelidonine, chelerythrine, sanguinarine, hydrastinine	antiproliferative activity
Murine Nk/Ly lymphoma	chelidonine, chelerythrine, sanguinarine, coptisine	strong cytotoxicity, strong correlation to DNA intercalation and genotoxicity
Human T cell lymphoblastic leukemia	chelidonine, sanguinarine	cytotoxicity, apoptosis induction; chelidonine induces G2/M arrest
HeLa (human cervical cancer)	chelidonine	selectively cytotoxic, apoptosis induction, cel cycle arrest
MCF-7 (human breast cancer)	chelidonine	strong cytotoxicity, telomerase inhibition

Antibacterial properties of alkaloids

The components of milky sap exhibit antibacterial properties against many microorganisms:

- **chelidonine** and **sanguinarine** derivates - antimicrobial effect against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella gallinarum*
- **protopine**, **berberine**, **chelidonine**, **chelerythrine** and **sanguinarine** - inhibit the growth of both Gram-negative and Gram-positive bacteria;

Antiviral properties of alkaloids

One of the most crucial manners of *C. majus* usage is the application of the fresh milky sap on the skin disorders, such as warts and condylomas caused by the Human Papillomavirus (HPV) infection. The diverse studies showed the antiviral activity of certain latex alkaloids against different animal viruses. The activity is shown on Fig. 2.

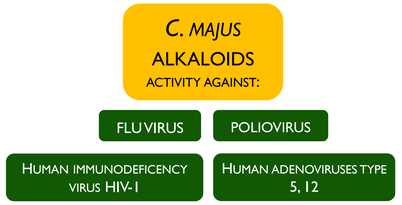


Figure 2. The antiviral activity of *C. majus* alkaloid extracts against animal viruses.

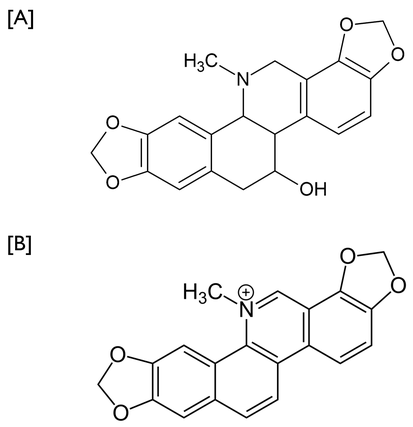


Figure 3. Chemical structure of *C. majus* alkaloids. [A] chelidonine [B] sanguinarine

Future prospects

Low-molecular coumpounds of *C. majus* milky sap, especially alkaloids, present a variety of properties potentially useful in medicine and pharmacology. However, the milky sap contains not only alkaloids, but also other, high-molecular compounds, e.g. proteins, which also present antimicrobial properties. Thus, additional studies should be provided in order to verify the potential synergistic action of alkaloids and proteins derived from *C. majus* milky sap.

Recommendations

M.L. Colombo and E. Bosisio, Pharmacological Research 33 (1996) 2.

R. Nawrot, J. Barylski, R. Lippmann et al., Planta 244 (2016) 1055.

A. Warowicka, L. Popenda, G. Bartkowiak et al., Phytomedicine, 64 (2019) 152919.

S. Zielinska, A. Jezierska-Domaradzka, M. Wojciak-Kosior et al., Frontiers in Pharmacology, 9 (2018) 299.

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Health promoting properties of Bee Royal Jelly: Food of the Queens

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NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Abstract

Royal Jelly (RJ) demand is growing every year and so is the market for functional foods in general. RJ is formed by different substances, mainly carbohydrates, proteins and lipids, but also vitamins, minerals and phenolic or volatile compounds in lower proportion. Major Royal Jelly Proteins (MRJP) are together with 10-hydroxy-2-decenoic acid (10-HDA), key substances of RJ for their different biological properties. In particular, 10-HDA is a unique substance of this product. RJ has been historically employed as health enhancer and is still very relevant in China due to the traditional medicine and the apitherapy. Nowadays, it is mainly consumed as functional food or found in supplements and other formulations for its health beneficial properties. Within these, anti-lipidemic, antioxidant, antiproliferative, antimicrobial, neuroprotective, anti-inflammatory, immunomodulatory, antiaging or estrogenic activities have been reported on RJ or its specific components. This manuscript is aimed at reviewing the actual knowledge on RJ components, their assessment in terms of authenticity, their biological activities and related health applications.

Introduction

Royal jelly is a substance secreted by the hypopharyngeal glands of the head of young worker bees of honey bees, which serves as food for the queen bee larva during its development. The composition of the RJ induces changes in gene expression, allowing the queen's development to continue.



Figure 1. RJ production in royal cells inside a hive. **A.** RJ production in queen cell base with worker bee larvae inside. **B.** Hive for breeding Honeybees and queen bees for RJ production. **C.** Removal of worker larvae from royal cells. **D.** Collection of RJ from the cells.

RJ Composition

RJ is an acidic colloid, whose pH usually ranges from 3.6 to 4.2. Water is the main component, followed by proteins, carbohydrates, lipids, trace elements, vitamins, phenols and amino acids. The percentage of each compound fluctuates within a certain range, due to the heterogeneous nature of RJ and the different origins of it.

Proteins account for >50% of dry weight and so-called Royal Jelly Main Proteins (MRJP) make up about 80-90% of the soluble RJ proteins. MRJP are recognized as the main factor involved in RJ-specific physiological actions. MRJP1 is a weak acid glycoprotein comprising about 48% of all RJ proteins. Other proteins besides MRJP but in much smaller quantity are jelleins and royalisin, which provide extensive antimicrobial protection. Most lipids (80-90%) are free fatty acids. 10-HDA constitutes about 70% of total RJ lipids and more than 50% of free fatty acids, without HDA and its precursor HDAA specific RJ components. 10-HDA is known to have many pharmacological and health effects.

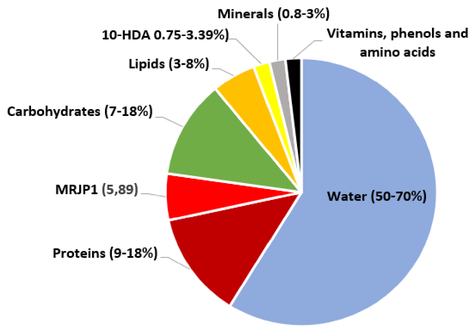


Figure 2. Pie diagram indicating the main components of the RJ.

RJ authenticity and adulteration

RJ is considered an attractive functional food, but also an expensive product that can be adulterated as a means to reduce production costs. In addition, the geographical origin, testing time or presence of polluting residues are parameters that affect the final quality of the RJ. Naturally, RJ is produced by transforming nectar and pollen collected by bees. The most common sources of sugar for bee feeding are sugar syrups or honey and yeast powder or pollen as protein sources. The use of cheap power supplies is an alternative to reduce production costs. In addition, the adulteration has been done by adding milk, yogurt, egg white and corn starch.

The most common criteria for assessing the quality and authenticity of RJ are sugar, moisture, proteins and 10-HDA. In addition, other studies have revealed amino acids, amines, carbohydrates and vitamins as potential markers. The 10-HDA is the main indicator of quality and authenticity of RJ. However, neither the content of 10-HDA nor protein can be considered a good indicator of freshness. New methods such as the 13C/12C and 15N/14N stable isotope ratio mass spectrometer are also being used to assess authenticity.

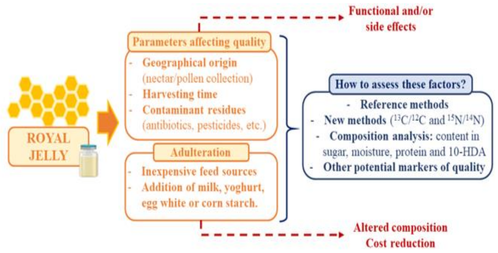


Figure 3. Summary of the main parameters affecting RJ quality, adulteration techniques and measures to assess these factors and guarantee the final quality of the product.

Biological and health promoting properties

Anti-lipidemic activity. Studies showed that treatments with RJ could reduce total cholesterol in the blood and increase HDL-C level. For instance, as it´s shown in Figure 4, these changes could be caused by the inhibition of the absorption of cholesterol in the jejunum by the MRJP. Moreover, MRJP1 can also block the reabsorption of bile acids. Furthermore, MRJP1 upregulates the expression of the enzyme cholesterol 7-α-hydroxylase (CYP7A1), which converts Cholesterol into bile acids. Bile acids are then excreted or reabsorbed at the jejunum.

Antioxidant activity. In several studies, the multiple benefits of antioxidant activity of specific RJ compounds have been demonstrated. This activity was found to come from their proteins (MRJP 1-9) and peptides. Hepatoprotective activity of RJ against non-alcoholic fatty liver disease (NAFLD), the most prevalent liver disease in the world, has also been proven.

Antiproliferative activity. RJ has been shown to have antitumor activity against Lewis lung carcinoma and colorectal adenocarcinoma cells. This antiproliferative activity may be linked to 10-ADH. 10-HDA has also shown antiproliferative effect against fibroblastic-like synoviocytes in patients with rheumatoid arthritis (RA) via PI3K-AKT. 10-HDA acts as HADACIs (histone deacetylase inhibitors), which have been considered as drugs with strong anti-inflammatory activity, related to the downward regulation of PI3K and the phosphorylation of AKT

Antimicrobial activity. RJ compounds such as gelatin, royalisin, MRJP, royalactin and apisimin can be classified as antimicrobial peptides (AMPs). In addition, there are other RJ compounds with antimicrobial activity such as 10-HAD.

Neuroprotective effect. For example, cadmium exposure causes neurodevelopmental impairment associated with Alzheimer's disease (AD). Recently, a study has been conducted on the ability of RJ to reduce cadmium-induced neuronal damage in vivo. In addition, beneficial neurological effects against postmenopausal neurological disorders have also been reported.

Anti-inflammatory activity. RJ has shown anti-inflammatory activity in different diseases related to abnormal inflammation. Cells treated with RJ have a better response to inflammation. This protective effect could be caused by inhibition of transcription of TNF-α, IL-1β, and IL-6 cytokines that are pro-inflammatory. In addition, RJ may inhibit the expression of the pro-inflammatory protein COX-2.

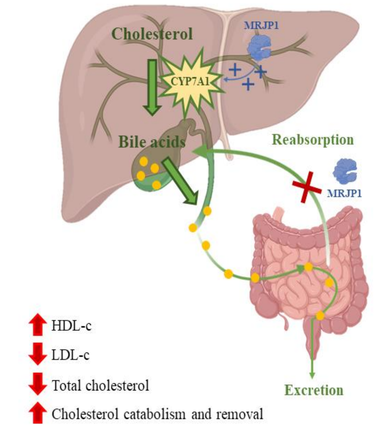


Figure 4. How treatment with RJ could reduce total cholesterol in the blood and increase HDL-C levels.

RJ health applications

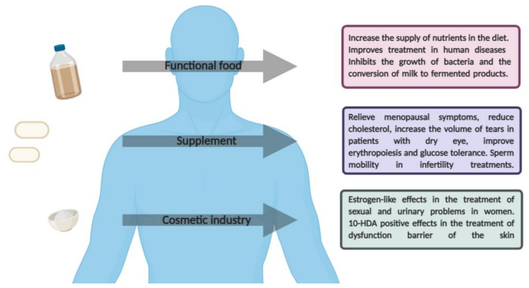


Figure 5. Royal Jelly industry applications and their benefits to promote human health.

Conclusion

Royal jelly is a complex mixture of substances which is commonly utilized by the nutraceutical and cosmetic industry. Its composition is mainly formed by water, proteins, carbohydrates, lipids and in minor proportion, trace minerals, vitamins and phenols. Among proteins, MRJP and FAA are essential components of RJ whereas regarding lipids, 10-HDA is the most important substance since it is a unique active compound. Phenolic and volatile compounds can be also important for their biological properties and their potential use as markers to differentiate RJ of different origins or harvesting times. RJ composition is highly variable and so, new analytical techniques are essential to study and address authenticity and quality of the product. In respect of its biological properties, research focuses on anti-lipidemic, antioxidant, antimicrobial, anti-inflammatory and other effects such as antiaging or estrogenic properties. However, further study of its mechanism of action is essential. RJ is consumed worldwide in different ways, being its main use as a functional food although supplements have also been commercialized. Cosmetic industry has produced creams with estrogenic-like effects, but future lines are aimed at investigating RJ causing agents of antiaging effects. Due to this rapid expansion and increase in demand for RJ, the need to regulate the RJ market, currently dominated by China, becomes evident. These measures should not only be aimed at establishing check measures on the authenticity and origin of the RJ, but should also be aimed at preserving the environment and bees. Future research should focus on the full understanding of the routes of RJ substances to develop new applications and products for the nutraceutical, cosmetic but also the pharmaceutical industry. Besides, it is essential to develop LC-MS methodology among other techniques to study the entire composition of RJ.

Acknowledgements

The research leading to these results was supported by MICINN supporting the Ramón y Cajal grant for M.A. Prieto (RYC-2017-22891); by Xunta de Galicia for supporting the post-doctoral grant of M. Fraga-Corral (ED481B-2019/096) and the pre-doctoral grant of P. García-Oliveira (ED481A-2019/295) and the program EXCELENCIA-ED431F 2020/12 that supports the work of F. Chamorro; by EcoChestnut Project (Erasmus+ KA202) that supports the work of M. Carpena, by UP4HEALTH Project that supports the work of P. Otero.

Effects of a *Myrciaria jaboticaba* Peel Extract and role of cyanidin-3-O-Glucoside on α-amylase e lipase in mice.

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Abstract

The *M. jaboticaba* peel extract inhibited both α-amylase and lipase but acted 13.6 times more strongly on the latter. Cyanidin-3-*O*-glucoside contributed minimally to these inhibitions. The *Myrciaria jaboticaba* extract, but not cyanidin-3-*O*-glucoside, inhibited starch absorption. Both the extract and cyanidin-3-*O*-glucoside inhibited triglyceride absorption, but at doses that were considerably smaller than those predicted by their strength in inhibiting the pancreatic lipase *in vitro*. It was concluded that inhibition of triglyceride absorption by extract can result from another action mechanism that is concomitant or even independent of the inhibition of lipases.

Introduction

*Myrciaria jaboticaba*is a fructiferous plant from Brazil. The fruits, known as jaboticaba, are globular with a thick skin and juicy pulp. The jaboticaba skin, is rigid and It is usually discarded as a residue by the food industry, but it is also a potential source of bioactive molecules and a functional food.

In a recent study, several phenolic compounds have been identified in extract of the skins among anthocyanins (e.g., cyanidin-3-O-glucoside) and non-anthocyanins. Then this study consists of a parallel and comparative investigation of the effects of a *Myrciaria jaboticaba* peel extract and one of its most prominent constituents, cyanidin-3-*O*-glucoside, on the pancreatic α-amylase and lipase activities.



Methodology

All procedures in animals were previously approved by the Ethics Committee for Animal Experimentation.

Animals

Male Swiss mice weighing between 35 and 40g were used.

Starch tolerance assay in mice



Starch 1g/kg
Water
Starch 1g/kg + acarbose 50mg/kg
Starch 1g/kg + *M. Jaboticaba* 250mg/kg
Starch 1g/kg + M. Jaboticaba 500mg/kg
Starch 1g/kg + Cyanidin-3-O-glucoside 10mg/kg
Starch 1g/kg + Cyanidin-3-O-glucoside 20mg/kg

Plasma glucose was determined at times 0, 30, 60, 90, and 120 minutes after starch administration.

Triglyceride tolerance assay in mice



Olive oil 5ml/kg
Water
Olive oil 5ml/kg + orlistat 50mg/kg
Olive oil 5ml/kg + *M. jaboticaba* 1mg/kg - 5mg/kg - 25mg/kg - 250mg/kg
Olive oil 5ml/kg + Cyanidin-3-O-glucoside 0,2mg/kg
Olive oil 5ml/kg + Cyanidin-3-O-glucoside 20mg/kg

The plasma triglyceride levels were determined at 0, 1.5, 3.0, 4.5, and 6.0 hours in blood samples collected from the tail vein.

Results

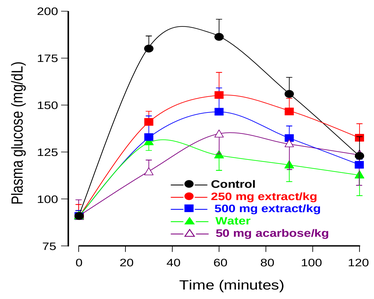


Figure 1. Blood glucose concentration profiles after intragastric starch loads in mice: the effect of the *M. jaboticaba* residues extract. The oral administration of commercial starch (1 g per kg body weight) was done immediately after the oral administration of the extracts or acarbose. Each value represents the mean ± mean standard error of 4 mice.

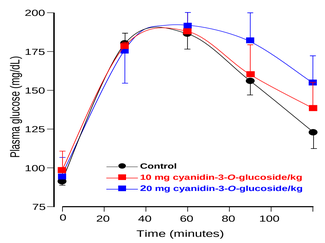


Figure 2. Blood glucose concentration profiles after intragastric starch loads in mice: the effect of cyanidin-3-O-glucoside administration. The oral administration of commercial starch (1 g per kg body weight) was done immediately after the oral administration of cyanidin-3-O-glucoside. Each value represents the mean ± mean standard error of 3 mice.

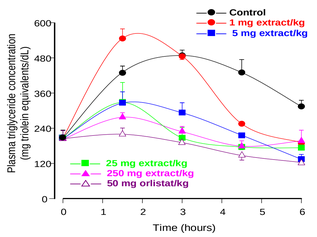


Figure 3. Blood triglyceride concentration profiles after intragastric olive oil loads in mice: the effect of the *M. jaboticaba* residues extract. The oral administration of olive oil (5 mL/kg) was done immediately after the oral administration of the extracts or orlistat. The doses and their estimated contents in cyanidin-3-O-glucoside are given on the graphs. Each value represents the mean ± mean standard error of 4 mice.

Results

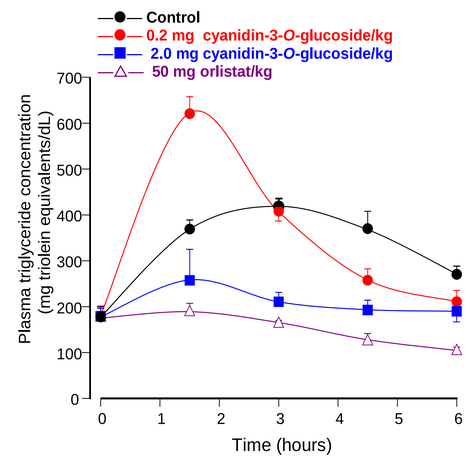


Figure 4. Plasma triglyceride concentration profiles after intragastric olive oil loads in mice: the effect of cyanidin-3-O-glucoside. The oral administration of olive oil (5 mL/kg) was done immediately after the oral administration of cyanidin-3-O-glucoside or orlistat. Each value represents the mean ± mean standard error of 3 mice.

Conclusion

It can be concluded that the jaboticaba peel extract presents many favorable perspectives as an inhibitor of fat absorption and that cyanidin-3-O-glucoside, one of its main constituents, seems to play a decisive role. In such a context further mechanistic and perhaps clinical studies are certainly highly desirable.

Acknowledgements

Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES)

Developmental effects of natural fungicides (*Equisetum arvense*, *Mimosa tenuiflora*, and Thymol) on zebrafish (*Danio rerio*)

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Introduction

The use of agrochemicals to control pests in agriculture has become crucial in modern, with a great variety of commercial products over the last years. The improper application and extensive use of these compounds have raised public concerns related to environmental pollution and animal health.

Synthetic pesticides residues spread in aquatic systems, compromising not only aquatic food resources but also fisheries and aquaculture. However, the effects of fungicides in non-target species have received less attention when compared to herbicides and insecticides. The use of natural products with fungicidal properties has been claimed as one of the most viable alternatives. Yet, there is a lack of ecotoxicological information on these products. Therefore, the objective of this study was to evaluate the effects of three natural extracts (*Equisetum arvense*, *Mimosa tenuiflora*, Thymol) with antifungal properties, by evaluating the teratogenic, oxidative stress and behavioural effects on the early development of zebrafish.

Methodology

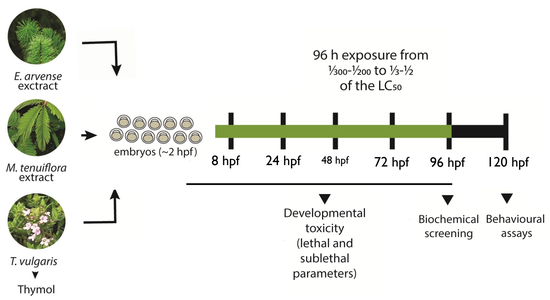


Figure 1. Schematic diagram of the zebrafish exposure to the plant-based fungicides. Collected embryos at around 2 h post-fertilization (hpf) were exposed to different concentrations of *Equisetum arvense* and *Mimosa tenuiflora* extracts and to Thymol for a period of 96 h. The selected concentrations varied from 1/300–1/200 to 1/3–1/2 of the determined LC50. During the exposure period, daily lethal and sublethal parameters were assessed. After 96 h exposure, eleutheroembryo were collected for biochemical screening of different biomarkers associated with oxidative stress, energetic metabolism, and neurotransmission. At 120 hpf, the locomotor activity of the eleutheroembryo was assessed using different behavioural paradigms.

Results

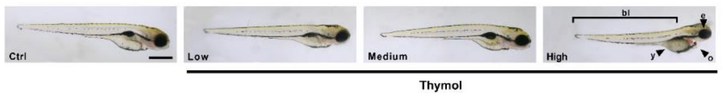


Figure 2. Representative views of the malformations observed in zebrafish larvae exposed to Thymol. Malformations were observed after exposure to the highest concentration of Thymol (T3) namely as abnormal eye (e), yolk (y), and pericardiac oedema (o) and by the decreased body length (bl). The scale bar represents 500 µm.

Results

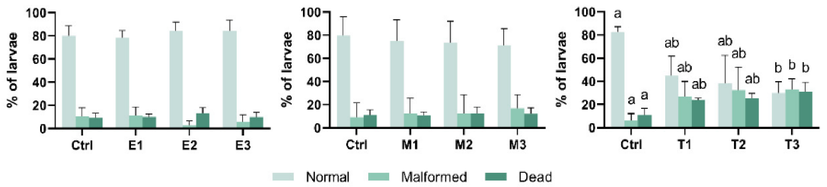


Figure 3. Percentages of normal, malformed and dead eleutheroembryo at the end of the exposure period (at 98 hpf). Values are presented as mean ± SD of five replicates per treatment (n = 10 random embryos per replicate). Different lowercase letters represent statistical differences among treatment groups (one-way ANOVA, $p < 0.05$).

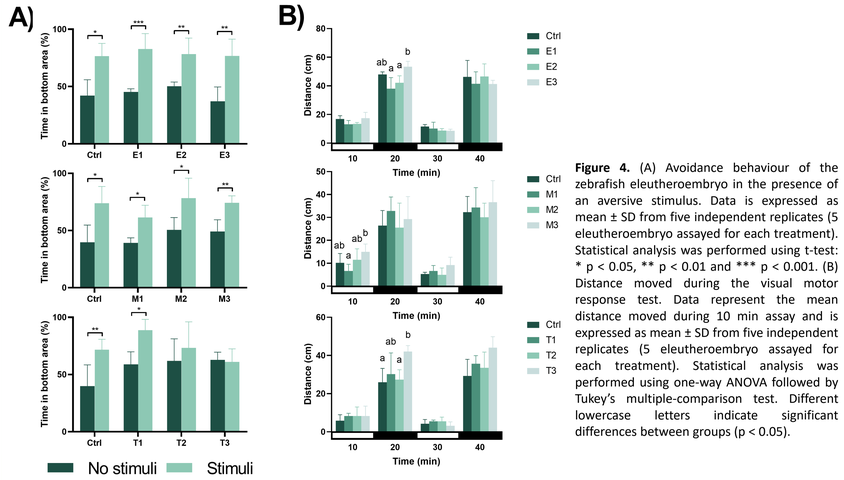


Figure 4. (A) Avoidance behaviour of the zebrafish eleutheroembryo in the presence of an aversive stimulus. Data is expressed as mean ± SD from five independent replicates (5 eleutheroembryo assayed for each treatment). Statistical analysis was performed using t-test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (B) Distance moved during the visual motor response test. Data represent the mean distance moved during 10 min assay and is expressed as mean ± SD from five independent replicates (5 eleutheroembryo assayed for each treatment). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

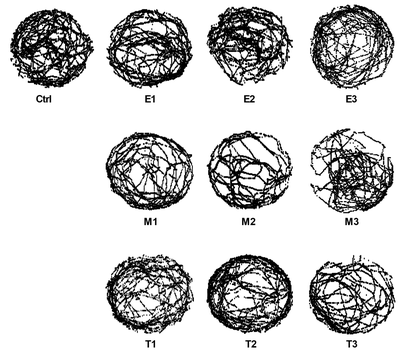


Figure 5. Effects of the different concentrations of the phyto-fungicide on zebrafish eleutheroembryo motor behaviour. Representative swimming tracks of untreated and treated zebrafish eleutheroembryo at 120 hpf. No significant changes were observed between the different treatments and the control group during the 10 min recording period.

Results

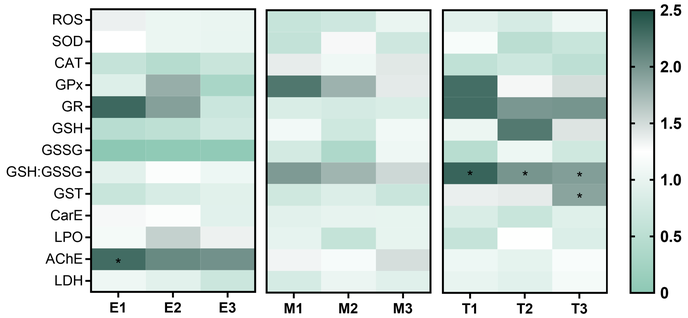


Figure 6. Heatmap of biochemical parameters measured in zebrafish eleutheroembryo at the end of the exposure to the different phyto-fungicide. Data from at least five independent samples (n = 100 individuals per replicate). The data used for the evaluation of the biochemical parameters were normalised to the control group value. Parametric data is expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. The * indicate significant differences relative to the control group ($p < 0.05$).

Conclusion

- ✓ Thymol increased lethality, pericardial oedema, yolk and eye deformations, and decreased body length;
- ✓ The higher concentration of Thymol induced an increase in the reduced and oxidized glutathione (GSH:GSSG) ratio and increased the glutathione-s-transferase (GST) activity;
- ✓ The disruption of behavioural states (fear- and anxiety-like disorders) were noted in Thymol-exposed animals;
- ✓ The results evidenced the teratogenic effects of Thymol, which might have consequences for non-target species, while showed the safety profile of *E. arvense* and *M. tenuiflora* at sublethal concentrations.

Acknowledgements

This research was funded by European Investment Funds by FEDER/COMPETE/POCI– Operational Competitiveness and Internationalization Programme, under the project POCI-01–0145FEDER-006958 and National Funds by FCT—"Fundação para a Ciência e a Tecnologia", under the project UIBD/04033/2020 and under the PhD grant number SFRH/BD/144904/2019.

Results published in Toxics journal of MDPI

NPA
Natural products application: Health, Cosmetic and Food
Online Edition
4-5 Feb 2021

THE EFFECT OF A TANNIN ENRICHED TOPICAL FORMULATION ON MICE EARS FUNGAL BIODIVERSITY

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NPA
Natural products application: Health, Cosmetic and Food
Online Edition
4-5 Feb 2021

Introduction

Cytinus hypocistis (L.) L. is a parasitic plant of the vast *Cistaceae* family occurring in the Mediterranean region.

This genus is characterized by rootless, stemless, and leafless holoparasitic plants that have a vegetative body limited to an endophytic system which exclusively grows within the roots of its host, which serves as the source of nutrients and water for these plants.

This species has long been used in European popular medicine to treat dysentery and inflammation of the eyes and throat, due to their astringent and haemostatic properties.

A study reported that extracts of *C. hypocistis* had antibacterial action on both Gram-positive and Gram-negative bacteria from a concentration of 62, 5 to 250 $\mu g.ml^{-1}$. Current studies attributed these antioxidant, anti-inflammatory, and antimicrobial properties to its hydrolysable tannin content.

Therefore, the topical application of its extract might be a natural alternative treatment for many skin conditions, resulting in the improvement of the overall heath of this organ not only by having an effect in the organism, but also in the skin flora.

Having this in mind, the main objective of the present work was to study the outcome of a *C.hypocistis* enriched topical formulation (EF) in mice ears fungal microbiota.



Figure 1: *Cytinus hypocistis* (L.) L.

Materials and Methodology

- Three EF were prepared with different concentrations of *C. hypocistis* extracts: a) 3.1 mg extract (E)/g of cream; b) 6.2 mg E/g of cream; and c) 12.4 mg E/g of cream. The formulations were carefully mixed to guarantee sample homogeneity. A negative control (base cream) and the three EF were stored at 4°C during the experiments.
- Thirty samples were collected in the ears of 30 female mice. The mice were divided in six groups of 5 mice each.
- Two of the groups were of wild type, one was treated with the base cream (G6), to assist as a negative control, and the other with the cream with highest concentration of extract (G4), to access toxicity.
- The remaining four groups were of Papillomavirus models, a positive control on which was applied the base cream (G5) and rest were treated with three creams with rising concentrations of EF (G1, G2 and G3, respectively), in order to determine the most efficient concentration for the treatment. The experiment procedure was approved by an Animal Ethics Committee-DGAV number 0421/000/000/2014, 24/09/2014 (020172).
- The samples were collected before the application and on da 17 of the essay, the surface of the ear was rubbed for 1 minute, using a swab that was previously emerged in saline solution.
- The samples were transferred to Petri dishes with PDA .
- The identification of fungal genera followed routine mycological techniques.



Figure 2: Papilomavirus models at day 17 of the essay.

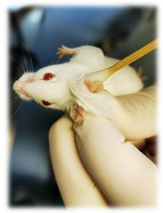


Figure 3: Collection of the samples.

Results

In the entirety of samples collected on the first collection, 4 filamentous fungal genera were isolated:

- ✓ *Penicillium* (16.7%) in G1, G4 and G5;
- ✓ *Mucor* (16.7%) G1, G2 , G4 and in G5;
- ✓ *Cladosporium* (3.33%) in G1;
- ✓ *Aspergillus* (3.33%) in G6.

In total, fungi were isolated in 14 (46.7%) animals of the 30.
Fungal genera were not identified in three mice, from G1 and G5.
There was no growth detected in three mise of G6.
The remaining samples exhibiting growth of other types of microorganisms.

After the application of *C. hypocistis* cream for 16 days in the ears, only one fungal colony was isolated in a mouse from G4. The genus was not identified.



Figure 4: Samples 2, fungal growth of the genus *Mucor* (left) and *Penicillium* (right).

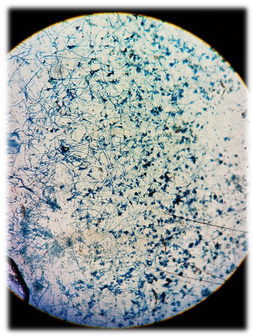


Figure 5: Microscopic image of Sample 2's *Penicillium* at 400x magnification.

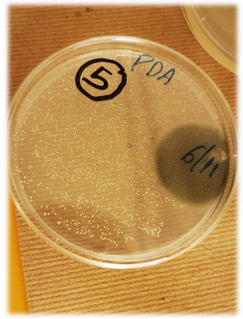


Figure 8: Samples 5, fungal growth of the genus *Cladosporium* .

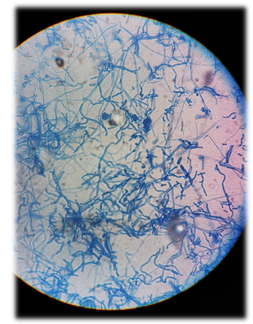


Figure 9: Microscopic image of Sample 5's *Cladosporium* at 400x magnification.



Figure 6 Samples 8, fungal growth of the genus *Mucor* (bottom).

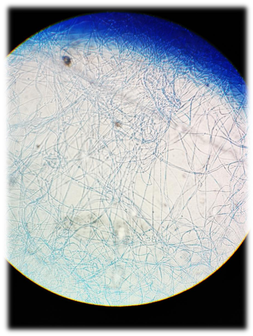


Figure 7: Microscopic image of Sample 8's *Mucor* at 400x magnification.

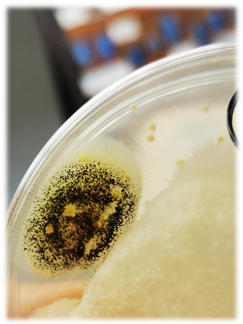


Figure 10: Samples 20, fungal growth of the genus *Aspergillus*.

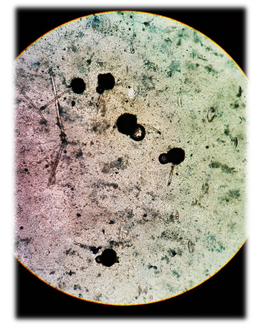


Figure 11: Microscopic image of Sample 20's *Aspergillus* at 400x magnification.

Conclusion

- ✓ The results obtained suggest that *C. hypocistis* reduces the presence of fungal in the ears of mice.
- ✓ However, more essays are needed to confirm this conclusion.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020). A. R. Silva is grateful to FCT for her Doctoral Grant (SFRH/BD/145834/2019) and L. Barros for her contract through the institutional scientific employment program. This work is funded by the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of Project GreenHealth - Norte-01-0145-FEDER-0.

ANTIOXIDANT ACTIVITY OF AGRO-INDUSTRIAL WASTE FROM GUAVA (*PSIDIUM GUAJAVA*)

Rejanne Lima Arruda; Edemilson Cardoso da Conceição

Universidade Federal de Goiás, Faculdade de Farmácia, Goiânia-GO

NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Abstract

Guava (Psidium guajava) belongs to the family Myrtaceae. Guava fruit is gaining more and more visibility in agribusiness, due to good properties, such as good appearance, pleasant taste, presence of nutrients (proteins, lipids, carbohydrates and minerals) and bioactive compounds with functional food properties, such as compounds phenolics, flavonoids, carotenes and ascorbic acid. The production of Brazilian agribusinesses requires, more and more, more developed techniques for the treatment of the eliminated residues. The environmental liability generated by the food industries is a waste with great potential for reuse and must have an appropriate destination, since in addition to generating negative environmental impacts such as environmental problems such as the proliferation of insects and pests, consequently possible disorders in the quality of the products offered.

The characterization of the guava residue was carried out according to pharmacopoeial parameters of: loss on desiccation, determination of the sweelling index, total ash, total tannins and flavonoids. The antioxidant activity was carried out by the methods of capturing the free radical 2,2-diphenyl-1-picryl-hydrazil (DPPH) following that described by Sanchez-Moreno et al (1998), as well as by capturing ferrous sulfate (FRAP) described by Pulido et al (2000). The drying loss content found in the powder of P.guajava residues was 8.39% ± 0.06%, the sweeling index was found to be 0.00 ±1.8mL; the total ash content was 1.91%± 0.01%, the content of total tannins found in residues from the processing of guava was 4.80% ±0.01% and flavonoids 1.85%±0.03. Regarding the antioxidant potential, with an EC 50 of 31.24 mg/mL for the capture of DPPH and a content of 3.67 10-6 µM of ferrous sulfate capture per gram of residue.

Introduction

Psidium guajava L. is a plant originated in Mexico, belonging to Mytarceae family. The guava fruit (Figure 1) is growth in economic value for the production of derivatives in the Brazilian agribusiness. However the use of guava pulp generates waste discarded in the environment, without the correct use, ie an environmental liability. The guava residue consists of pulp, peel, but mainly seeds.

With the increase in food production, there is an increase in the generation of waste and several studies around the world are being carried out in order to create solutions to reduce or eliminate the environmental liabilities generated by agro-industries (ALBURQUERQUE et al., 2012; VIRMONDE et al., 2012; CASTRO; SATO, 2013). However, for waste to be used and to have greater added value, it is necessary to know the chemical composition from scientific and technological investigations (HOFFMANN et al., 2009).

Figure 1 – Guava fruit (Psidium guajava L), guava variety "Paluma"



Materials

An oven with forced air circulation, knife mill, semi-analytical scale, muffle, distilled water, test tubes, serum bovine albumin solution, 0.2 M acetate buffer solution with pH = 4.9, were used ferric chloride solution, spectrophotometer, acetic acid, acetone, 0.06 mM DPPH solution, methanol, ferrous sulfate solution and FRAP reagent.

Methodology

The guava residues used in the work were supplied by the company Predilecta Alimentos®, located in the city of Aparecida de Goiânia. The material was dehydrated in an oven with forced circulation and air renewal at 40 ° C for seven days. Afther dehydration, the dry material was ground un a knife mill and stored under light and moisture, under refrigeration (-20°C). The characterization of the guava residue was carried out according to pharmacopoeial parameters of: loss on desiccation, determination of the sweelling index, total ash, total tannins and flavonoids. The 96% ethanolic extract was then produced using the maceration method, followed by percolation. The antioxidant activity was carried out by the methods of capturing the free radical 2,2-diphenyl-1-picryl-hydrazil (DPPH) following that described by Sanchez-Moreno et al (1998), as well as by capturing ferrous sulfate (FRAP) described by Pulido et al (2000).

Results

The drying loss content found in the powder of *P.guajava* residues was 8.39% ± 0.06%, remaining within all limits and favorable to the conservation of the product. The sweeling index was found to be 0.00 ±1.8mL. The total ash content was 1.91%± 0.01%. The content of total tannins found in residues from the processing of guava was 4.80% ±0.01% and flavonoids 1.85%±0.03. Regarding the antioxidant potential, with an EC 50 of 31.24 mg/mL for the capture of DPPH and a content of 3.67 10^{-6} µM of ferrous sulfate capture per gram of residue. A study with several guava species, carried out in Colombia, demonstrated a correlation between the content of phenolic compounds and antioxidante activity by the FRAP and DPPH methodologies (Rojas-Barquera; Narvaez-Cuenca, 2009).

Conclusion

The literature considers it important to study the physical and chemical composition of plant materials, since methods used to determine antioxidant activity, when applied alone, may not provide safe results. The activity against the DPPH radical and against ferrous sulfate demonstrated the potencial of guava residue in preventing oxidative, which can be useful for the development of new pharmaceutical formulations.

Recommendations

Further studies with Psidium guajava residues are necessary in order to improve and test its antioxidant activity.

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Faculdade de Farmácia/UFG

PDI Bioprodutos

UFG

Optimization And Recovery Of Vitamin D_2 From Surplus Production Of *Agaricus bisporus* Portobello

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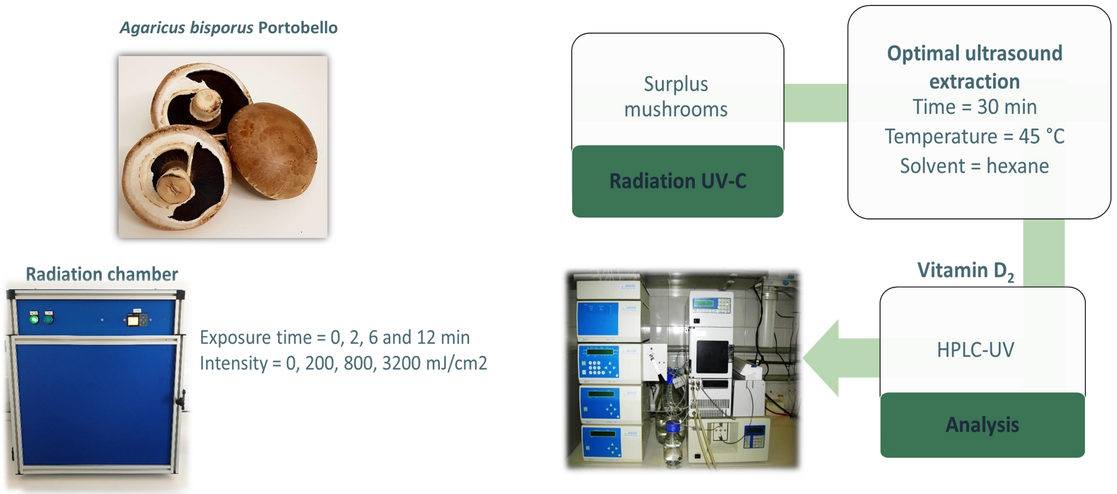
Introduction

During mushroom production, a percentage as high as 20% of surplus can be generated. These unused mushrooms have high nutritional value and valuable chemical compounds. In this sense, finding innovative alternatives to valorizing this surplus mushroom production needs to be explored [1,2]. Irradiation of mushrooms surplus to obtain vitamin D_2 is a sustainable strategy to increment vitamin D availability.

Under this perspective, the objective of this study was setting the UV-C irradiation and extraction conditions that maximize vitamin D_2 contents in surplus mushrooms production (*Agaricus bisporus* Portobello). The bioactive effects and potential toxicity of vitamin D_2-enriched extracts were also evaluated.

Material and Methods

The surplus production from *A. bisporus* Portobello were supplied by the Ponto Agrícola, Baião, North of Portugal. The irradiation was performed using an ultraviolet (UV-C at 200 mJ/cm², 800 mJ/cm² and 3200 mJ/cm²) radiation chamber with different exposure times: 0, 2, 6 and 10 min. Sensitivity and linearity of the high performance liquid chromatography (HPLC) coupled to ultraviolet detector (UV) were determined and the method was validated by the instrumental precision, repeatability and accuracy, and the extracts rich in vitamin D_2 were also quantified by HPLC-UV. The cytotoxicity of the vitamin D_2 extract were evaluated using three tumoral cell lines (MCF-7 - breast adenocarcinoma, NCI-H460 - non-small cell lung cancer and AGS - gastric cancer) and one non-tumoral cell line of bone origin (h-FOB 1.19 - human osteoblasts).



Results

The chromatographic method revealed a great reproducibility and accuracy, thus being the method validated. Independently of the UV-C irradiation dose, the effect in the conversion of vitamin D_2 concentration was very high, allowing it to increase from ~3 µg/g dw to more than 100 µg/g dw in *A. bisporus* portobello. The extract enriched in vitamin D_2 presented an effective activity in AGS (82 µg/mL) tumoral cell line and a moderate activity in NCI-H460 (293 µg/mL) and CaCo (377 µg/mL) tumoral cell lines. Furthermore, the extract did not show cytotoxicity against the non-tumor bone cell h-FOB 1.19 (GI50> 400 µg/mL).

Table 1: Antiproliferative and cytotoxicity activities of extracts using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm²) and pure vitamin D_2 (mean ± SD, n=9).

	MCF-7	NCI-H460	AGS	CaCo	h-FOB 1.19
Extract enriched in vitamin D_2 (GI_{50} µg/mL)	>400	293 ± 17^{b}	82 ± 9^{c}	377 ± 24^{a}	>400
Vitamin D_2 pure (GI_{50} µg/mL)	>400	>400	>400	>400	>400

The citotoxicity results were expressed as GI50 values: corresponding to the sample concentration that inhibited 50% of the net cell growth. In row different letters mean significant differences ($p < 0.05$).

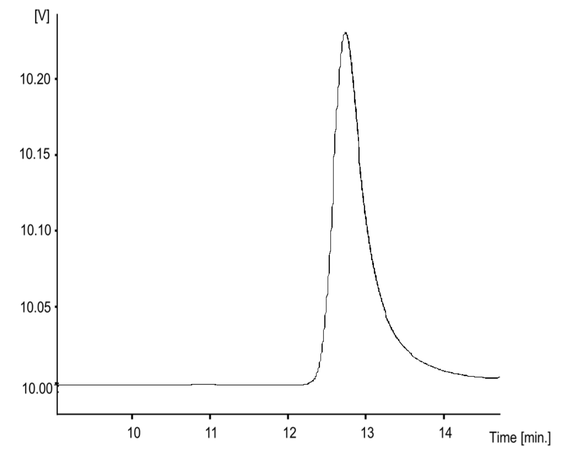


Figure 1: Vitamin D_2 profile of irradiated *Agaricus bisporus* Portobello, during 6 min at 3200 mJ/cm².

Conclusion

Accordingly, development food applications of mushroom extracts enriched in vitamin D_2, from surplus mushroom production, can be considered and valorized, supporting and adding value to the agriculture sector or pharmaceutical industries.

References

[1] Papoutsis, K., Grasso, S., Menon, A., Brunton, N. P., Lyng, J. G., Jacquier, J. C., & Bhuyan, D. J., Trends in Food Science and Technology, 99 (2020) 351–366.

[2] Pedrali, D., Gallotti, F., Proserpio, C., Pagliarini, E., & Lavelli, V., LWT - Food Science and Technology, 125 (2020) 109248.

Acknowledgements

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REPRODUCTIVE TOXICITY OF PLANT EXTRACTS PRODUCED IN PORTUGAL

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Abstract

Plant extracts are increasingly being used in healthcare and cosmetics, as well as pharmaceutical formulations. The reasons behind this interest are related with the bioactivity of plant extracts, specifically their intrinsic antimicrobial activity, antioxidant, anti-proliferative and anti-inflammatory, among many other beneficial effects. Nonetheless, not much is known about the overall safety of these plant extracts for the use in humans. In this study, we aim to determine the reproductive toxicity of extracts of plants produced/endogenous in Portugal: *Cistus ladanifer*, *Thymbra capitata*, *Helichrysum italicum* and *Cupressus lusitanica*. Hydrolates for each plant were obtained by hydrodestillation of aerial parts. The chemical composition of the hydrolates were determined by GC-MS (gas chromatography mass spectrometry). Reproductive toxicity was assessed by exposing freshly harvested bovine oocytes to five different concentrations of each hydrolate (0.2%-0.002%). After incubation, the oocytes were visually inspected to assess maturation by optical microscopy. In parallel, oocyte viability was determined for each concentration by staining with trypan blue. We found that for all concentrations tested, the viabilities of the bovine oocytes for each plant were superior to 70% (*C. ladanifer:* 79.02±7.25; *T. capitata*: 76.56±4.26; *H. italicum*: 87.28±4.87; *C. lusitanica*: 86.72±7.04). The EC50 was determined by estimating the concentration of the hydrolate able to inhibit maturation of 50% of bovine oocytes *in vitro* (*T. capitata*: 1.26%; *H. italicum*: 1.27%; *C. ladanifer:* 2.07%; *C. lusitanica*: 2.46%). The results show that tested hydrolates do not compromise oocyte viability. However, we observed some inhibition of oocyte maturation, at concentrations ranging 1-2.5%. In our study, *C. ladanifer* and *C. lusitanica* were less toxic to oocytes than *T. capitata* and *H. italicum*. Taking our results into account plant extracts shall be tested for reproductive toxicity prior to their inclusion in formulations to be used in humans, to assess the safe concentration to be used in the final product. This information should be complemented with dermal permeation experiments to properly assess the reproductive toxicity of the ingredient by considering its body absorption and distribution.

Plants under study



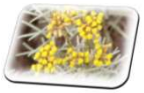
Cistus ladanifer (Esteva)

Plant that grows spontaneously in the western part of the Mediterranean region. The leaves release an aromatic resin-labdanum- used in perfumes. Amato Lusitano (doctor, botanist, writer, anatomist- 16th century) used the oil as an ointment.



Thymbra capitata (Tomilho-cabeçudo)

Occurs in xerophilic bush in the mainland Portugal. Used as a condiment in several dishes of Mediterranean cuisine, mainly as a seasoning for meat and salads. Theofrasto and Dioscórides mentioned medicinal properties, namely in the fight against respiratory diseases.



Helichrysum italicum (Perpétua-das-areias)

In Portugal coastline we can find spontaneously subsp. *picardii*. Traditionally used in the form of an infusion prepared from its inflorescences, yet is excellent healer, anti-inflammatory, analgesic and fungicide.



Cupressus lusitanica (Cedro-do-bussaco)

It is native to Central America, the plant was introduced in Portugal in the 17th century in the woods of the old Convento do Bussaco. Traditionally used to treat circulatory problems, such as varicose veins, heavy legs, strokes in the legs, varicose ulcers and hemorrhoids.

Results

Table 1. Major component of plant extracts as determined by GC-MS analysis. The proportion of the predominant compound in each extract is also showed, as well as their chemical class.

Hydrolate	Major component	Distribution (%)	Class
C. ladanifer	**4-Hydroxy-3-methylacetophenone**	**21.58**	**Alkyl-phenylketones**
T. capitata	**Carvacrol**	**98.11**	**Monoterpenes**
H. italicum	**L-α-Terpineol**	**30.55**	**Monoterpenes**
	Carvacrol	**29.58**	
C. lusitanica	**Terpinen-4-ol**	**38.93**	**Monoterpenes**

Table 2. Concentration of hydrolate [EC50 (efficient concentration; 50% oocyte maturation rate), %, V/V] able to reduce 50% of matured oocytes, after exposure.

Hydrolate	EC50 (%, V/V)
C. ladanifer	2.46
T. capitata	1.26
H. italicum	1.27
C. lusitanica	2.07

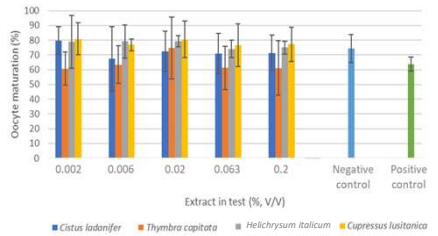


Fig. 1. Oocyte maturation (%) in the presence of maturation medium supplemented with each hydrolate (%, V/V). Negative control: maturation medium only. Positive control: 0.39 μM cycloheximide. The results represent three independent experiments.

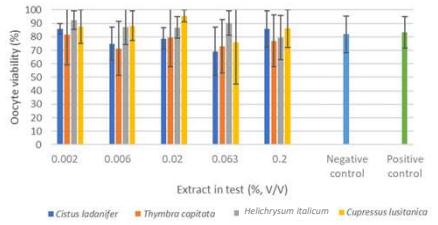


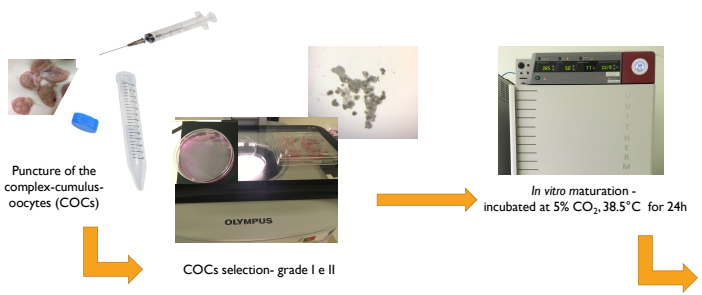
Fig. 2. Oocyte viability (%) in the presence of maturation medium supplemented with each hydrolate (%, V/V). Negative control: maturation medium only. Positive control: 0.39 μM cycloheximide. The results represent three independent experiments.

Materials

Hydrolates were obtained by hydrodestillation of aerial parts. The chemical composition was assessed by GC-MS spectrometry analysis. The bovine ovaries enrolled in this study were collected at the slaughterhouse "OVIGER", located at Alcains, Portugal. The biological material was obtained from female bovines (crossbred beef cattle) aged between 11 and 17 months, selected for slaughter; and corresponded to material that was going to be discarded. The slaughtering lasted 4 hours. At the slaughter line, the ovaries were macroscopically assessed for the presence of follicles, which was the single criteria to be considered for this study. The ovaries collected from cows at the slaughterhouse in order to be transported to the laboratory at room temperature (25-30°C) were immediately submerged in a Phosphate Buffer Saline 1x supplemented with antibiotics and kept in a thermos bottle at 34-35°C. At their arrival at the laboratory, the ovaries were washed twice with PBS and kept in a water bath at 34-35°C. The plant hydrolates were dissolved in reconstituted maturation medium and prepared in different serial dilutions [(half log) 0.2/ 0.063/ 0.02/ 0.006/ 0.002 %, V/V] (ECVAM DB-ALM Protocol n° 129).

Methodology:

The experimental procedure used was the one described in ECVAM DB-ALM Protocol n° 129 -Toxicity test on *in vitro* maturation of bovine oocytes.



Conclusion

- We found that the studied extracts affected oocyte maturation rates (Fig. 1), but did not affect oocyte viability, since ≥70% of cellular viability was obtained for all concentrations in test (Fig. 2).
- *T. capitata* and *H. italicum* hydrolates presented a lower EC50 (Table 2), which might be related with the presence of carvacrol in their composition.
- *C. ladanifer* and *C. lusitanica* hydrolates are better candidates to be introduced in natural products due to their higher EC50 regarding reproductive toxicity.
- All extracts except *T. capitata* were less toxic than the positive control in the tested concentrations (Fig. 1).

Acknowledgements

This work was supported by "INOVEP project – Innovation with Plant Extracts", I&DT projects for companies in collaboration with scientific entities, project number 33815, Centro2020.

Potential of sweet cherry by-products: phenolic profile and antimicrobial activity of stems and kernels

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Abstract

During sweet cherry processing, large amounts of by-products are generated. There is no substantial use of this waste, which increase environmental and managements costs each year to deal with the excess of such residues. These by-products only recently received attention and this new interest is focused in finding ways to achieve their valorization.

Thus, we conducted a study in which saponins, phenolic profile composition of stems and kernels of four sweet cherry cultivars (*Early Bigi* (grown under net cover (C) and without net cover (NC)), *Burlat*, *Lapins,* and *Van*) and antibacterial activities against important Gram negative and Gram positive bacterial human isolates were examined. Extracts from stems of cv. *Lapins* and kernels of *Early Bigi* NC presented high levels of saponins. Apart from cv. *Early Bigi* NC, major phenolic compounds identified in stems and kernels were sakuranetin and catechin, respectively. In cv. *Early Bigi* NC the most abundant compounds were ellagic acid for stems and protocatechuic acid for kernels. Antimicrobial activity assays showed that only stem's extracts were capable of inhibiting the growth of Gram positive isolates, although extracts from kernels resulted in only a small synergistic effect in three situations.

This new data is intended to provide new possibilities of valorization of these by-products and their valuable properties.

Introduction

Although sweet cherry is mainly commercialized as fresh fruit, a considerable quantity is used after processing as jam, jelly or juice, that generates large amounts of by-products, namely stems and pits (Figure 1). There is no substantial use of this waste, which increase environmental and managements costs each year to deal with the excess of such residues. Therefore, a solution to achieve valorization of the excess of this material is urgent.

While cherry stems are known by traditional medicine and widely used in infusions and decoctions, due to their claimed sedative, diuretic and anti-inflammatory properties, their study, as well as the characterization of pits, commonly used for therapeutic pillows, only recently have received attention. Various hydroxycinnamic acids, such as *p*-cumaric, ferulic, caffeic, chlorogenic and neochlorogenic acids, have been reported in stems of *P. avium*, and important anthocyanins were found in kernels, whose oil is rich in lipophilic compounds, such as tocopherols and carotenoid.

In this context, the objective of the current work is to evaluate the biological potential of such less used residues, providing a detailed study on their phenolic profile and antimicrobial activities.

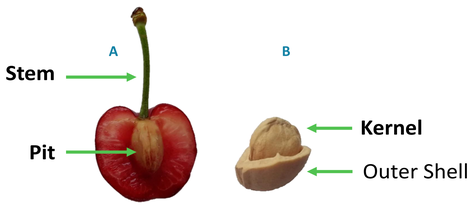
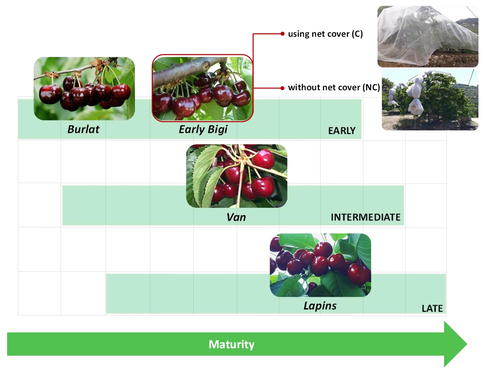


Figure 1- Unvalued sweet cherry by-products: view of sweet cherry stem and pit (A) and view of sweet cherry pit (B).

Materials

Selected 4 sweet cherry cultivars grown in an orchard located in Resende



Methodology

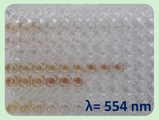


Removal of stems and kernels

Saponins quantification

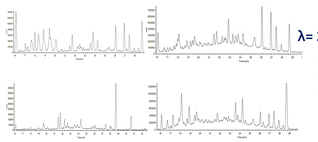


Extraction with ethanol 95%



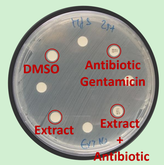
Phenolic profile





Antimicrobial activity

Faecal sample: Gram +	Urine sample: Gram -
Enterococcus faecalis	*Klebsiella pneumoniae*
Staphylococcus aureus	*Enterobacter aerogenes*
Staphylococcus aureus ATCC	*Escherichia coli* ATCC



$$\%\ RIZD = \frac{IZD\ sample - IZD\ negative\ control}{IZD\ antibiotic\ standard} \times 100$$

where IZD is the inhibition zone diameter (mm)

Results

Saponins were present in **higher amount** in **stems of cv. *Lapins*** (181.12 mg/g), with similar amount recorded for cv. *Early Bigi* NC (101.79 mg/g). For cvs. *Early Bigi* C and *Burlat*, the content of saponins were lower, with 42.45 and 38.05 mg/g, respectively. No previously data is available regarding saponin content in sweet cherry stems, being these the first data about the presence of those compounds in this samples.

For **kernel´s extracts**, saponin content ranged from 8.34 mg/g in cv. *Lapins* to 49.62 mg/g in **cv. *Early Bigi* C**, results opposed to those verified for stems, with regard to their content in both cultivars. As for stems, there is no previously data available regarding kernels saponin content, being this work the first one concerning saponins content in kernels of sweet cherry (Figure 2).

Table 1- Phenolic composition (average ± SD) of sweet cherry stems (mg/100 g DW). Different letters indicate significant differences ($p < 0.05$).

	Early Bigi C	*Early Bigi* NC	*Lapins*	*Burlat*	*Van*	*p* value
Catechin	15.02±2.12b	22.26±0.51a	17.45±0.53b	15.64±0.71b	17.86±0.31b	0.000
Epicatechin	59.05±4.19b	74.88±5.05a	50.23±0.99c	61.82±0.72b	49.39±1.63c	0.000
Naringenin-7-*O*-glucoside	22.78±2.42bc	27.52±1.11a	26.61±0.40ab	26.37±0.44ab	20.77±1.75c	0.000
Hydroxycinnamic acid	18.16±3.89a	19.14±0.20a	8.51±0.08b	18.09±0.75a	9.52±0.40b	0.000
Sakuranetin	139.73±11.06ab	128.31±7.79b	140.88±0.58ab	158.30±10.08a	131.63±0.85b	0.006
Ellagic acid	106.87±14.56b	145.25±4.69a	96.37±5.59b	106.42±3.93b	95.59±5.26b	0.000
Neochlorogenic acid + isomer	19.11±2.09d	25.63±0.28c	40.62±0.20a	26.38±0.79c	33.11±1.25b	0.000
Chlorogenic acid + isomer	43.29±4.90b	51.13±0.57a	23.77±0.41c	19.18±0.45c	25.23±1.69c	0.000
p-Coumaric acid + isomer	18.81±2.58bc	22.47±0.71ab	18.38±0.69c	22.56±0.85a	13.46±1.02c	0.000
Ferulic acid	14.95±1.82a	14.28±0.07a	9.56±0.25bc	10.99±0.57b	7.59±0.65c	0.000
Syringic acid	12.63±1.38	13.89±0.22	9.48±0.29	7.37±0.33	9.15±0.17	0.470
Taxifolin	7.07±1.00a	8.34±0.32a	4.94±0.29b	4.09±0.30b	4.85±0.18b	0.000
Kaempferol-3-*O*-rutinoside	5.94±0.60c	11.69±0.20a	7.60±0.17b	12.92±0.69a	6.28±0.67bc	0.000
Kaempferol-3-*O*-glucoside	3.20±0.41c	5.95±0.07a	Nd	4.29±0.22b	2.70±0.28c	0.000
Genistein	4.93±0.74b	6.63±0.13a	4.97±0.08b	1.53±0.07d	3.25±0.09c	0.000
Quercetin-3-*O*-glucoside	16.17±1.50a	8.61±0.31b	Nd	3.78±0.18c	0.65±0.12d	0.000
Total	508.57±46.57ab	589.99±21.20a	493.02±51.30b	501.71±19.35ab	434.02±9.74b	0.003

Table 2- Phenolic composition (average ± SD) of sweet cherry kernels (mg/100 g DW). Different letters indicate significant differences ($p < 0.05$).

	Early Bigi C	*Early Bigi* NC	*Lapins*	*Burlat*	*Van*	*p* value
Gallic acid	4.05±0.27c	13.19±0.78a	3.12±0.10cd	8.27±0.55b	2.74±0.04d	0.000
Protocatechuic acid	6.17±0.41c	30.34±0.86a	5.92±0.14c	21.05±0.42b	4.92±0.06c	0.000
Catechin	10.32±0.41c	20.06±0.55b	6.66±0.15d	22.87±1.39a	7.06±0.15d	0.000
p-coumaric acid + isomer	4.24±0.15c	11.99±0.32a	1.26±0.05d	10.27±0.48b	1.87±0.31d	0.000
Total	24.78±1.18c	75.58±2.49a	16.97±0.35d	62.46±2.73b	16.58±0.40d	0.000

Table 3- Antimicrobial activity (% RIZD) of cherry stems and kernels relatively to antibiotic gentamicin.

		Early Bigi C		*Early Bigi* NC		*Lapins*		*Burlat*		*Van*	
Standard bacterial strains		**S**	**K**	**S**	**K**	**S**	**K**	**S**	**K**	**S**	**K**
Gram +	*S. aureus* ATCC	66.6	0	58.8	0	56.3	0	68.8	0	62.5	0
	S. aureus MJS241	*	0	*	0	*	0	*	0	*	0
	E. faecalis MJS257	*	0	*	0	*	0	*	0	*	0
Gram -	*E. coli* ATCC	0	0	0	0	0	0	0	0	0	0#
	K. pneumoniae MJH812	0	0	0#	0#	0#	0#	0#	0	0	0
	E. aerogenes MJH813	0	0	0	0	0	0	0	0	0	0

Means ± SD (n = 3). 0-extract without effect; 0–100– extract less effective than an antibiotic;*-extract effective and antibiotic without effect; #-slight synergistic effect when antibiotic used in combination with extract, with increased inhibition zone comparing to the former. Abbreviations: S- stem´s extracts; K- kernel´s extracts.

Extracts from **kernels** were **not able to inhibit the growth** of any of the bacterial stains tested, and their use resulted in only a small synergistic effect in three situations.

By other hand, extracts from cherry **stems** proved to be more **effective**, namely for **Gram positive** bacteria. Indeed, for *S. aureus* ATCC, all samples were able to inhibit its growth, with values of % RIZD above 55 %. More interestingly, for the clinical isolates of ***S. aureus* MJS241** and ***E. faecalis* MJS257**, all the extracts of **stems** were able to **inhibit the growth** of those bacteria that proved to be **resistant** to the **tested antibiotic**. Extracts of cv. *Lapins* exhibited the strongest growth suppression for the Gram positive *S. aureus* MJS241 and *E. faecalis* MJS257.

For the tested **Gram negative** bacteria, **no inhibitory effect** was found, although extracts of cvs. *Early Bigi* C and NC and *Lapins* potentiated the antimicrobial effect of when used in combination (Table 3).

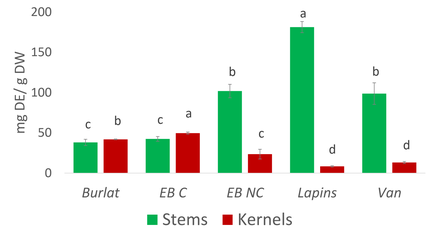


Figure 2- Saponin content (average±SD) of stem and kernel extracts. Different letters indicate significant differences ($p < 0,05$).

Sixteen individual compounds were identified in extracts of **stems**. The content of each phenolic compound varied significantly when comparing cultivars, with only syringic acid present in similar amounts in all cultivars. In all samples, the **most abundant** compound was **sakuranetin**, with the exception of stems of cv. *Early Bigi* NC, where the most abundant compound was ellagic acid (Table 1).

Four individual compounds were identified in extracts of **kernels**. The total amount of phenolic compounds in kernel´s extracts ranged from 16.58 mg/100g to 75.58 mg/100g in cvs. *Van* and *Ealy Bigi* NC, respectively. The **most abundant** phenolic compound was **catechin**, in four of the five samples, with protocatechuic acid being the major one in kernels of cv. *Early Bigi* NC, again showing the effect of tree cover in chemical parameters of sweet cherries (Table 2).

Conclusion

This work shows that **sweet cherry by-products** have very **interesting bioactive properties**, regarding to the content in **phenolics**, **saponins** and **antimicrobial activities**.

However, these by-products should be **further explored** and **more studies are required**, in order to explore **nutraceutical and pharmacological formulations** or **antioxidant preservatives for the food industry** and their effects on human health.

Sweet cherry **stem's extracts** may provide **valuable solutions** to the global problem of **antibiotic resistance** due to their **antimicrobial activity** linked to this unvalued by-product, although more research should be performed to test its applicability.

Acknowledgements

Sílvia Afonso is grateful to FCT, MCTES and FSE for the PhD Fellowship SFRH/BD/139922/2018. Work supported by National Funds by FCT - Portuguese Foundation for Science and Technology, under the project UIDB/04033/2020. Work funded by "Fundo Europeu Agrícola de Desenvolvimento Rural (FEADER)" and by "Estado Português" in the context of "Ação 1.1 «Grupos Operacionais»", integrated in "Medida 1. «Inovação» do PDR 2020 – Programa de Desenvolvimento Rural do Continente"- Grupo Operacional para a valorização da produção da Cereja de Resende e posicionamento da subfileira nos mercados (iniciativa n. º 362).

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EDIBLE FLOWERS AS FUNCTIONAL FOODS WITH THERAPEUTIC POTENTIAL

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Abstract

Edible flowers can be considered as functional foods with therapeutic potential due to their polyphenolic composition.

Flowers from *Borago officinalis* and *Viola x wittrockiana* have shown, through in vitro experiments, antioxidant and antidiabetic properties.

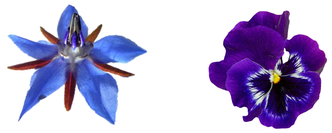
Introduction

Plants have been used in traditional medicine throughout centuries, being flowers some of the most important organs not only for ornamental purposes but also due to bioactive compounds and applications.

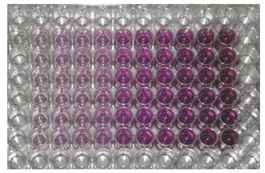
Edible flowers are currently widely used in gastronomy as healthy ingredients, but little it is known about their bioactive properties as functional foods

Materials and Methods

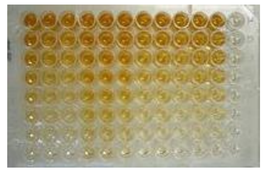
Polyphenolic extracts obtained from Borago officinalis and Viola x wittrockiana flowes have been used as starting material



The antioxidant properties were quantified by in vitro elimination of superoxide radicals (O^{2-}) generated through the xanthine/xanthine oxidase reaction [1].



The antidiabetic potential of the extracts was quantified by inhibition of the enzyme α-glucosidase [1] and the capacity of these extracts to inhibit advanced glycation end products (AGEs) formation [2].



Results

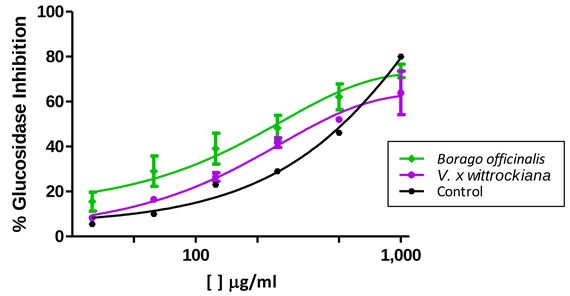


Fig. 1. Dose-response inhibition of α-glucosidase by the *Borago* and *Viola* extracts. (n = 3)
Acarbose used as control; IC_{50} = 380 µg/ml
B. officinalis IC_{50} = 249,406 µg/ml
V. x wittrockiana IC_{50} = 387,661 µg/ml

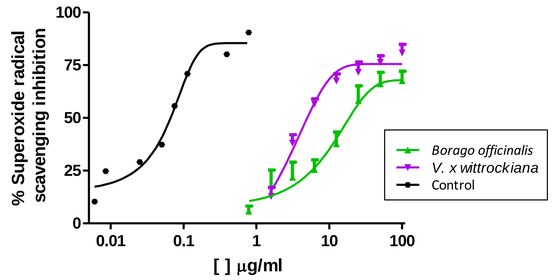


Fig. 2. Dose-response antioxidant activity of the extracts by elimination of superoxide radicals (O^{2-}). (n = 3)
Gallic acid used as control; IC_{50} = 0.044 µg/ml
B. officinalis IC_{50} = 18,799 µg/ml
V. x wittrockiana IC_{50} = 4,981 µg/ml

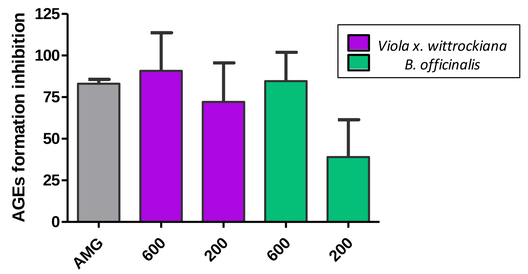


Fig. 3. Advanced glycation end products (AGEs) formation inhibition by the extracts at concentrations 600 and 200 µg/ml. (n = 3)
Aminoguanidine (AMG), anti-glycan substance, used as control at concentration 600 µg/ml.

Conclusions

Viola extract showed a lower IC_{50} value than acarbose meanwhile both *Viola* and *Borago* extracts showed great antioxidant ability against the superoxide radicals, they both also displayed important activity inhibiting protein glycation.

Flowers of *Borago officinalis* and *Viola x wittrockiana* can be considered as source of **bioactive compounds** with interesting properties in the field of nutrition as functional foods or food supplements in the prevention and improvement of metabolic of chronic diseases such as diabetes.

References

[1] Cásedas, G., Les, F., Gómez-Serranillos, M. P., Smith, C., & López, V. (2017). Anthocyanin profile, antioxidant activity and enzyme inhibiting properties of blueberry and cranberry juices: A comparative study. Food and Function, 8(11), 4187–4193. https://doi.org/10.1039/c7fo01205e.

[2] Séro, L., Sanguinet, L., Blanchard, P., Dang, B.T., Morel, S., Richomme, P., Séraphin, D., Derbré, S., 2013. Tuning a 96-well microtiter plate fluorescence-based assay to identify AGE inhibitors in crude plant extracts. Molecules 18, 14320e14339.

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Antibiofilm activity of emodin on *Staphylococcus aureus* and its effect on aerobic respiration

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Abstract

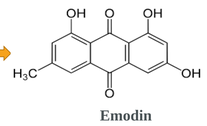
Bacterial resistance to conventional antibiotics is a global problem nowadays and it is necessary to find an appropriate solution in the form of new therapeutics, and strategies in the fight against resistance. Bearing in mind that plants are rich in secondary metabolites and often used in traditionally medicine, they represent an interesting source of new and alternative chemotherapeutics. Emodin, (derivative of anthraquinones) is an abundant constituent of many plants and possesses a wide range of biological activities. Guided by the fact that *Staphylococcus aureus* is main causative of numerous infections and resistant to many antibiotics, the aim of the study was to investigate antibiofilm potential of emodin and its influence on bacterial respiration of *S. aureus* ATCC 25923 (MSSA), ATCC 43300 (MRSA) and clinical isolates from nasal carriage – Gp19 (MSSA) and Gp7 (MRSA). For the effect of emodin on biofilm disruption, crystal violet staining of biomass was performed. O2 consumption and CO2 production in treated and untreated biofilms was monitored using Micro-Oxymax respirometer. RT-PCR was used to investigate the effect of emodin on the expression of *ica*A and *ica*D, (responsible for biofilm formation), and *srr*A and *srr*B (staphylococcal respiratory regulatory complex). Results obtained in this study revealed that emodin disrupted biofilm of both MRSA strains at all tested concentrations (up to 33%) while biofilm of MSSA strains was not affected by emodin. Aerobic respiration of ATCC 43300 strain was decreased in treated biofilm while in other strains that effect was less pronounced. In addition, emodin induced expression of *ica*A and *ica*D genes in all tested strains except ATCC 43300 where emodin downregulated this genes. On the other hand, expression of *srr*A and *srr*B was slightly increased in all MSSA strains and decreased in both MRSA strains. The obtained results demonstrated that emodin possesses significant antibiofilm activity which is related to specific strain. Furthermore, the results indicate multiple pathways through which emodin acts and affects on the biofilm and metabolism of *S. aureus*. Overall, emodin could be recommended for further investigation in order to be used in medicine treatment of infections.

Introduction

Last years, bacterial resistance is spreading world-wide leading to less successful treatment of infections. Moreover, bacteria able to form biofilms represent a special obstacle in the fight against resistance since bacteria in this form are more resistant, up to 1000 times than planktonic form. *Staphylococcus aureus* is one of the main causative of wide spectrum of human infections, both acute and chronic as well as nosocomial infections. Therefore, these facts arises that new therapeutics and strategies are urgently needed to solve the problem of bacterial resistance. The search for novel antimicrobial agents among natural products becomes very intensive since they could expressed less side effects. Plants represent an inexhaustible source of novel agents because they product various groups of secondary metabolites which possesses numerous biological activities. Antraquinones are interesting group known for their purgative properties and emodin is its common derivative with various activities, antibacterial among others.







Emodin

Scientific Institution
Institute of Chemistry, Technology and Metallurgy
National Institute

UNIVERSITY OF BELGRADE
FACULTY OF MEDICINE

1969

Results

Based on the microdillution assay, emodin showed strong antibacterial activity, especially on MRSA strains (Table1). Next, crystal violet stainning demonstrated that emodin disrupted preformed biofilm of ATCC 43300 and Gp7 (Figure1). Furthermore, aerobic respiration was decreased only in ATCC 43300 biofilm (Figure2). Emodin slightly increased expression of *ica* genes in all tested strains with exception of ATCC 43300 while expression of *srr* genes in MSSA strains was decreased and increased in MRSA strains (Figure3).

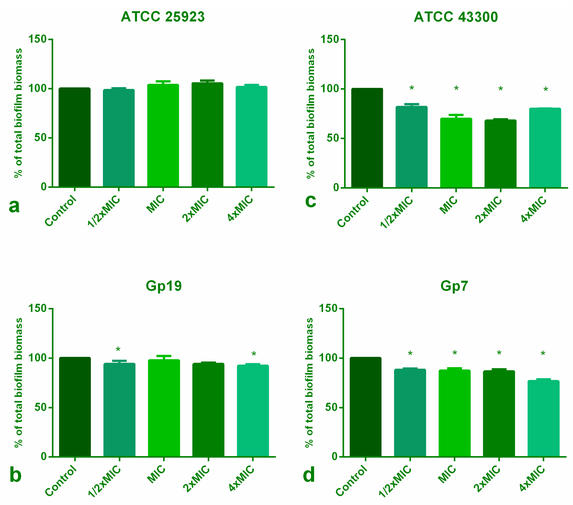


Figure 1. The effect of emodin on biofilm disruption of *S. aureus* ATCC 25923 (a), Gp19 (b), ATCC 43300 (c) and Gp7 (d). *statistical significance ($p < 0.05$)

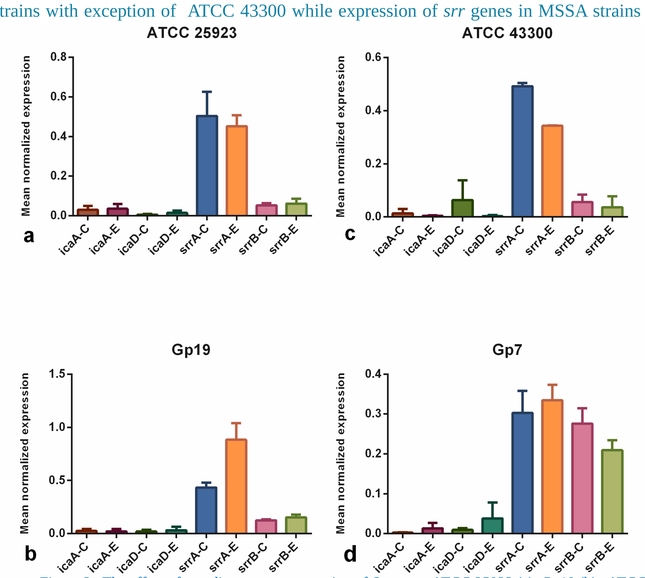


Figure 3. The effect of emodin on gene expression of *S. aureus* ATCC 25923 (a), Gp19 (b), ATCC 43300 (c) and Gp7 (d). C-gene expression in untreated biofilms; E-gene expression in emodin treated biofilm

Table 1. Minimal inhibitory concentrations of emodin

Strains		Emodin μg/mL
ATCC 25923	MSSA	25
Gp19		6.25
ATCC 43300	MRSA	6.25
Gp7		3.125



Design for monitoring of aerobic respiration in biofilms on Micro-Oxymax respirometer

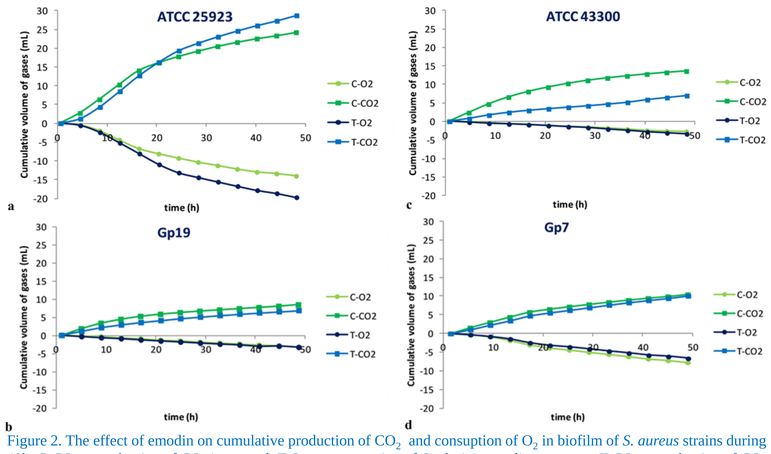


Figure 2. The effect of emodin on cumulative production of CO_2 and consuption of O_2 in biofilm of *S. aureus* strains during 48h. C-CO_2 – production of CO_2 in control; T-O_2 – consumption of O_2 during emodin treatment; T-CO_2 – production of CO_2 during emodin treatment

Aim of the study

The aim of the study was to investigate antibacterial and antibiofilm activity of emodin against *S. aureus* strains as well as its activity on aerobic respiration and expression of *icaA, icaD, srrA* and *srrB* genes.

Methodology

In this study four *S. aureus* strains was tested: ATCC 25923 (MSSA), ATCC 43300 (MRSA), Gp19 (MSSA-nasal cariage) and Gp7 (MRSA-nasal carriage) isolates. In order to determine minimal inhibitory concentrations (MICs) of emodin, microdillution assay with resazurin as cell viability indicator was applied. The effect on preformed biofilms was investigated by crystal violet (CV) staining of biofilm biomass. Next, O_2 consumption and CO_2 production in treated and untreated bacterial biofilms was monitored using Micro-Oxymax respirometer during 48h. In addition, expression of the genes from *ica* operon and *srrAB* complex under emodin treatment was quantified by RT-PCR. Gene expression was normalized to housekeeping *rpO* gene.

Decreased aerobic respiration

srrAB complex
Regulates aerobic-anaerobic switch, energy metabolism, biofilm formation

ica operon
Biofilm formation and production of main component of *S. aureus* extracellular matrix

Conclusion

Based on the obtained results it could be noticed that emodin possess strong antibacterial activity, as well as antibiofilm activity. Further, respiration monitoring revealed that respiration was desturbed only in ATCC 43300 strain. Gene expression was mostly strain-specific but in general emodin affected gene expression of all tested strains. Furthermore, based on the all results obtained for ATCC 43300 strain it could be concluded that emodin eradicate biofilm by affecting aerobic respiration and blocking expression of genes necessary for biofilm formation. Finaly, results pointed out that emodin has stronger activity on MRSA strains and could be recomended for further investigation.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia (451-03-68/2020-14/ 200178).

Assessment of *Frangula alnus* ethyl-acetate extract on biofilm disruption and bacterial respiration of *Staphylococcus aureus* strains

Tea Ganić,[1*] Stefana Đukanović,[1] Branka Lončarević,[2] Stefana Cvetković,[1] Biljana Nikolić,[1] Dina Tenji,[3] Dušan Kekić,[4] Dragana Mitić-Ćulafić[1]

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NPA Natural products application: Health, Cosmetic and Food Online Edition 4-5 Feb 2021

Abstract

Staphylococcus aureus is one of the most serious pathogens, known to the world health community. It is able to form biofilm, which can cause numerous acute and chronic infections [1]. These infections can be difficult to treat because of its wide range of resistance on different antibiotics in biofilm form. Therefore, scientific community has been looking for new approach in order to overcome this problem. It is proven that many secondary metabolites produced by plants have antibacterial activities [2]. *Frangula alnus* has been used in traditional medicine for years and previous studies have shown that plants from genus Frangula express various biological activities [3]. During lack of knowledge about antibiofilm activity of *Frangula alnus* (FA) extract, the aim of this study was to examine influence of previously chemically characterized ethyl-acetate extract on *Staphylococcus aureus* ATCC strains (MSSA-25923 and MRSA-43300) and clinical isolates (Gp19 and Gp7 both from nasal carriage). Antibiofilm activity was studied using crystal violet staining of biomass, while Scanning electron microscopy (SEM) attested effect of FA extract on biofilm structure and morphology. Respiration, O_2 consumption and CO_2 production was monitored by Micro-Oxymax respirometer. Furthermore, the effect of FA extract on expression of *ica*A, *ica*D, *srr*A, *srr*B and RNA III was investigated by RT-PCR. Gathered results, showed that disruption of biofilm biomass was significant decreased in ATCC 43300 biofilm (up to 28%), but increased in ATCC 25923. Scanning electron micrographics showed changes in matrix structure for Gp19 isolate, slight disruption of biofilm biomass for ATCC 43300 and also slight changes in structure and morphology of biofilms for Gp7. Respiration in treated biofilm of both clinical isolates, has been decreased, while respiration of tested ATCC strains in treated biofilms have not been changed. Gene expressions of MSSA strain are slightly decreased, except *ica*D gene, but in MRSA *ica*A, *srr*B and RNA III are increased and *ica*D and *srr*A decreased. In clinical isolates, Gp19 all gene expressions are slightly increased, except for *ica*A gene. In Gp7 *ica*A and *ica*D genes were upregulated, but all other were decreased. Taking into account the obtained results, FA extract has demonstrated great potential for further studies, which could provide better knowledge about its mechanism of action and provide its potential use as therapeutic agent.

Introduction

Frequency of antibiotic resistant bacteria have grown and one of well known pathogen is *Staphylococcus aureus*. It causes infections which can be difficult to treat. This hospital-associated bacteria can provide biofilm what makes it even harder to treat with antibiotics. In order to find a solution for this problem pharmaceuticals and scientific communities are searching for new treatmen options against planktonic and biofilm form of pathogenic bacteria. Their attention remained focused on plants. Plants produces secondary metabolites, which may show antioxidant, antimicrobial, antifungal, and anticancer effects. *Frangula alnus* Miller (*Rhamnus frangula* L.) belongs to Rhamnaceae family and has been used in traditional medicine for decades (Figure 1). Its most common use is as laxative, but its antimicrobial activity is not researched enough. Its bark is consistent from anthraquinone glycoside derivatives, glucofrangulins and frangulins. The most dominant anthraquinone in this plant is emodin.

Aim of study

The aim of this study was to examine antibiofilm activity of *Frangula alnus* (FA) extract on *Staphylococcus aureus* strains and clinical isolates in biofilm form.



Fig.1: Experimental setup for monitoring respiration in biofilm form on *Staphylococcus aureus* on Micro-Oxymax respirometer

Materials and methods

Two reference strains of *Staphylococcus aureus* from ATCC colection were used in this study: methiciline sensitive (MSSA) ATCC 25923 and methiciline resistant (MRSA) ATCC 43300. Also two clinical isolates were used: methiciline sensitive Gp19 and methiciline resistant Gp7 both from nasal carriage. Bacteria were grown in Lauria Bertoni (LB) broth, at 37˚C for 24 h. *Frangula alnus* (FA) ethyl acetate extract was made from its bark. Extract was previously chemically characterized with qualitative GCxGC-MS and quantitative LC-MS/MS of the selected phenolics compounds. Microdilution assay was used to determine minimal inhibitory concentration (MIC) of ethyl acetate extract. Minimal inhibitory concentration was determinated as the lowest concentration of FA extract which inhibited bacterial growth. Biomass of pre-formed biofilm was quantified using crystal violet (CV). Concentrations of FA extract used in antibiofilm testing was in range from 1/2 MIC to 4 MIC. Scanning Electron Microscopy (SEM) was used to monitor the effect of FA on the bacterial biofilm structures. Respiration, consumption of O_2 and production of CO_2 was monitored by using Micro-Oxymax respirometer (Figure 1). Concentration of gasses were measured for 48 h at the intervales of every 2 h. Real time PCR(RT-PCR) was preformed in order to monitor gene expression. In this experiment selected genes were: *ica*A and *ica*D (intercellular adhesion genes), *srr*A and *srr*B (staphylococcal respiratory regulation genes) and RNA III.

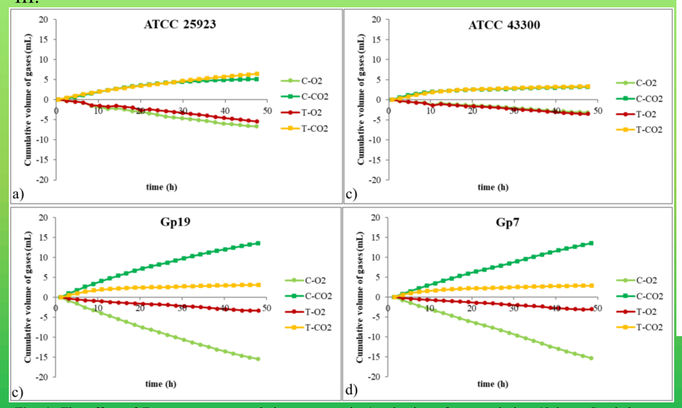


Fig. 4: The effect of FA extract on cumulative consumption/production of gasses during 48 h on *Staphylococcus aureus* biofilm: ATCC 25923 (a), ATCC 43300 (b), Gp19 (c), Gp7 (d); C- O_2 – production of O_2 in control; C – CO_2 – production of CO_2 in control; T – O_2 – consumption of O_2 during FA treatment; T – CO_2 – production of CO_2 during FA treatment

Results

Minimal inhibitory concentrations are presented in Tabele 1. Concentration range was from 62,5 µg/mL to 500 µg/mL. Disruption of pre-formed biofilm, using FA extract showed that there is no significant changes between control and treatment, concerning clinical isolates. On the other hand, in case of ATCC 25923 there was significant increase of biomass biofilm up to 143%, but in ATCC 43300, distruption was detected up to 28% (Fig. 2).

Tabele 1 : Minimal inhibitory concentrations of FA extract on *Staphylococcus aureus*

Strains		FA (µg/mL)
ATCC 25923	MSSA	500
ATCC 43300	MRSA	62,5
Gp19	MSSA	62,5
Gp7	MRSA	125

The highest concentrations of FA extract were applied on preformed biofilms. Its effect was followed by SEM (Fig. 3). Only in case of ATCC 43300 strain came to slight distruption of biofim. Also, light changes in structure and density of biofilms in Gp19 and Gp7 isolates were detected. The cumulative consumption of O_2 and production of CO_2 is presented in the Fig. 4. There was no big differences between control and treatment conserning ATCC strains. Meanwhile, in clinical isolates came to decreasing production of CO_2 and inhibition of consumptoin of O_2. Gene expression was monitored with RT-PCR (Fig. 5). Significant changes in gene expression was not observed in almost all tested strains and isolates, with exception of ATCC 25923 strain, where RNA III gene expresion was notably decreased.

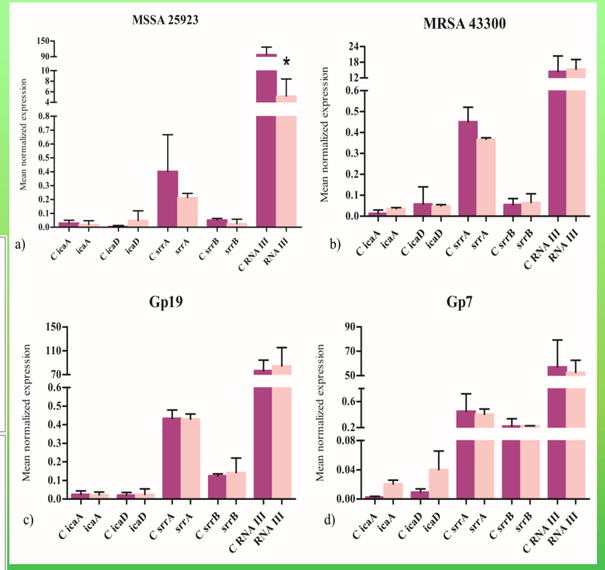


Fig. 5: Mean normalized expression of genes *ica*A , *ica*D, *srr*A, *srr*B and RNA III on *Staphylococcus aureus* strains ATCC 25923, ATCC 43300, Gp19 and Gp7 under the effect of FA extract; C-*ica*A–control of *ica*A gene expression, *ica*A-gene expression during FA treatment, C–*ica*D–control of *ica*D gene expression, *ica*D–gene expression durin FA treatment, C–*srr*A–control of srrA gene expression, *srr*A–gene expression during the FA treatment, C-*srr*B–control of *srr*B gene expression , *srr*B–gene expression during FA treatment, C-RNA III - control of RNA III gene expression, RNA III- gene expression during the treatment. *statistical significance ($p < 0,05$)

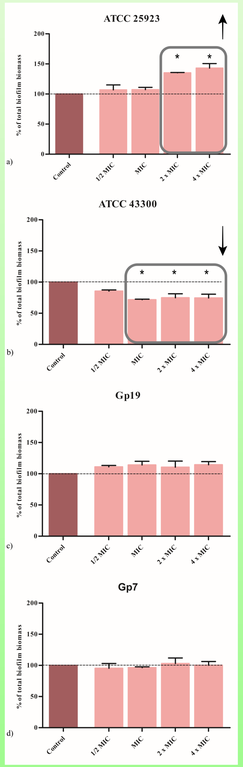


Fig. 2: Effect of FA extract on pre-formed biofilm on *Staphylococcus aureus* ATCC 25923, ATCC 43300, Gp19 and Gp7. *statistical significance ($p < 0,05$)

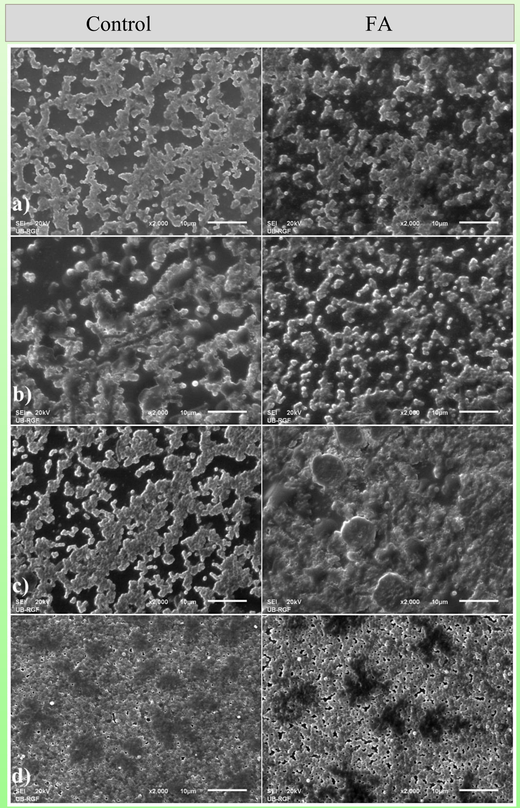


Fig. 3: Scanning electron microscopy micrographs without (left) and with (right) the treatment of FA extract on *Staphylococcus aureus* strains ATCC 25923, ATCC 43300, Gp19 and Gp7.

Conclusion

Thanks to obtained results in this study, *Frangula alnus* extract showed antibacterial activity. It has demonstrated potential to distrupt already formed biofilm of *Staphylococcus aures* ATCC 43300 strain. Aerobic respiration of clinical isolates were hinder. Further researched colud provide better knowledge and understanding on direct connection between respiration and this extract. RT-PCR showed that there are some slight changes in gene expression, but potentially there are more mechanisms to discovered in order to better understand this pathogen. With all of the results, *Frangula alnus* extract managed to express potential role as new therapeutic agent, but further research are imoprtant for its development as one.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia (451-03-68/2020-14/200178; 451-03-68/2020-14/ 200026 and 451-03-68/2020-14/200168). The authors also thank Kristina Šarić and Jovana Malbašić, from SEM Laboratory, University of Belgrade – Faculty of Mining and Geology for SEM micrographs.

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FROM OXIDATIVE STRESS PROTECTION TO CELL CYCLE ARREST: *CUCURBITA FICIFOLIA* BOUCHÉ EFFECTS

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Introduction

Cucurbita ficifolia Bouché (chila pumpkin) is a type of squash native from México, being used for human nutrition especially in crystallized pastry and deserts and also as medicinal purposes, such as for the treatment of diabetes type 2, in wound-healing and for fever. There are a few ethnopharmacological studies reporting its usage as well as some studies performed in which a hypoglycaemic effect was demonstrated in animal models fed with this pumpkin, but there is a lack of information concerning its phytochemical profile as well as of other bioactivities.

Material and Methodology

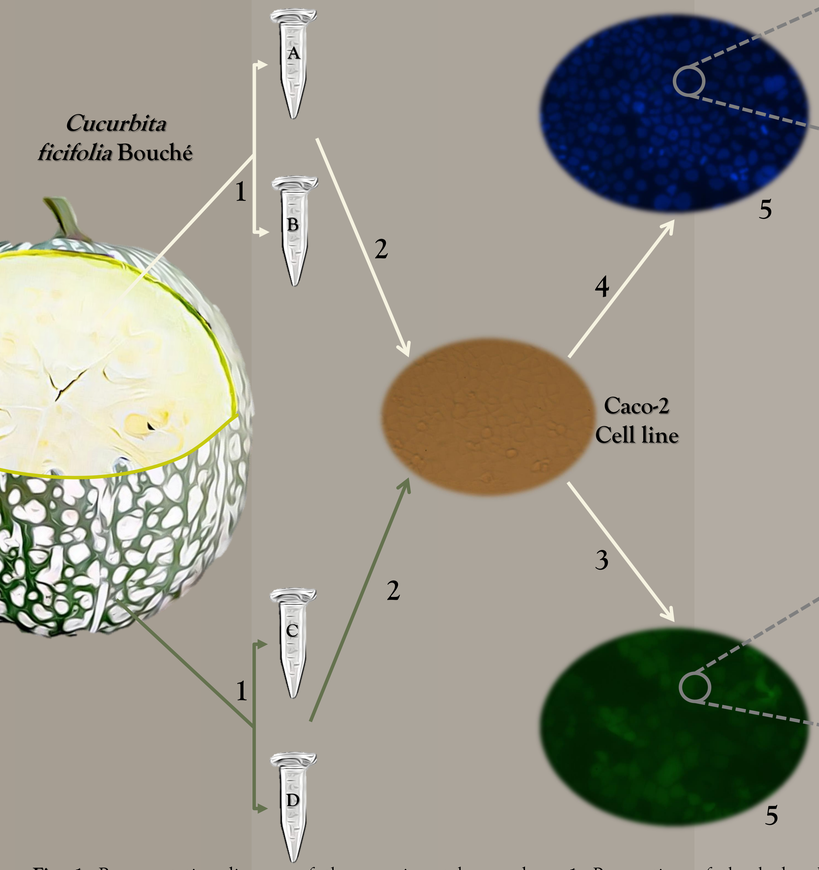


Fig. 1- Representative diagram of the experimental procedure: **1-** Preparation of the hydroethanolic ((80:20)%v/v) extract, from pulp and peel, that were further fractionated into methanolic and aqueous, obtains **A (Pulp Ex. H.E Fr.Aq)**, **B (Pulp Ex. H.E Fr. MeOH)**, **C (Peel Ex. H.E Fr. Aq)** and **D (Peel Ex. H.E Fr. MeOH)**; **2-** The Caco-2 cells were exposed for 24 hours to the different extracts at different concentration; **3-** Evaluation of ROS content. Cells were incubated with the probe DCFDA and were evaluated by flow cytometry **(5)**; **4-** Cell cycle analysis. Cells were fixed with cold EtOH/PBS ((70:30) %v/v) for 2 hours, washed with PBS and incubated with the probe PI, then were analyzed by flow cytometry **(5)**.

Results

Cell Cycle Arrest

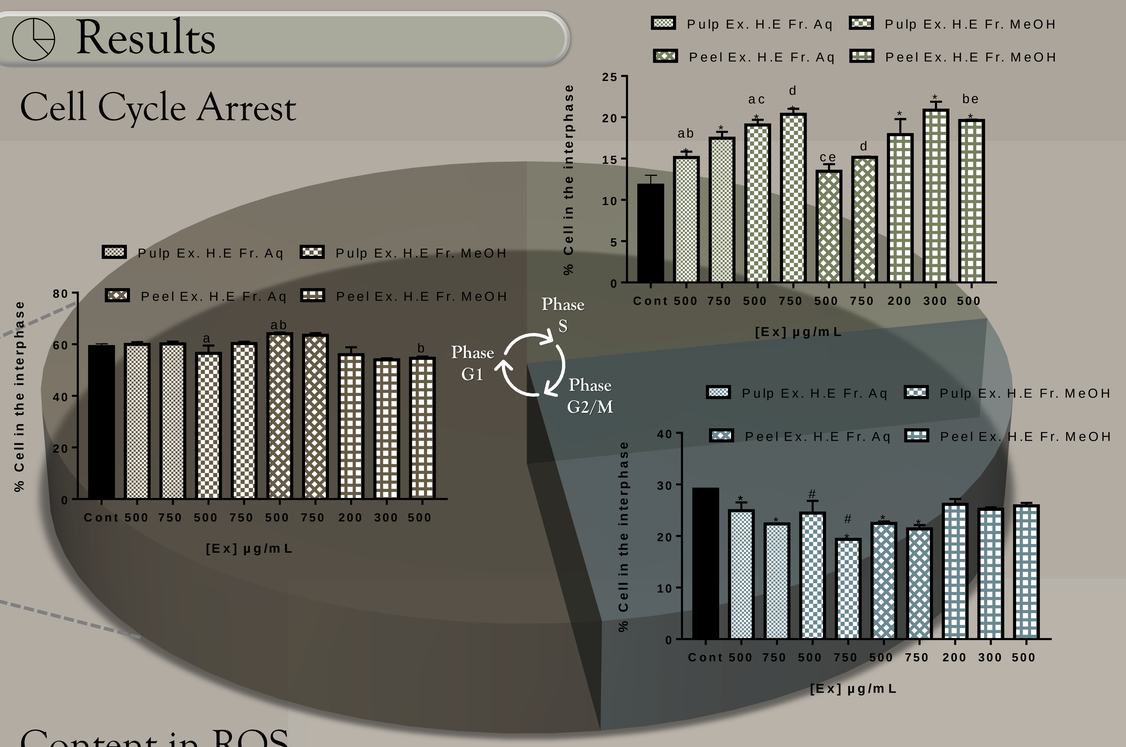


Fig. 2- Cell cycle analysis of Caco-2 cells exposed to the different extracts at different concentrations. Results are presented as % Cell in the interphase vs concentration of the extract in µg/mL; Values represent the mean ± SD of three experiments (n=3); Statistical analysis was performed using one-way ANOVA followed by Turkey's multiple comparison test and the (*) represents statistical difference with control, the (a,b,c,d and e) represents statistical difference with the same concentration in different extract and the (#) represents statistical difference with the same extract at different concentration, at $p<0.05$.

Content in ROS

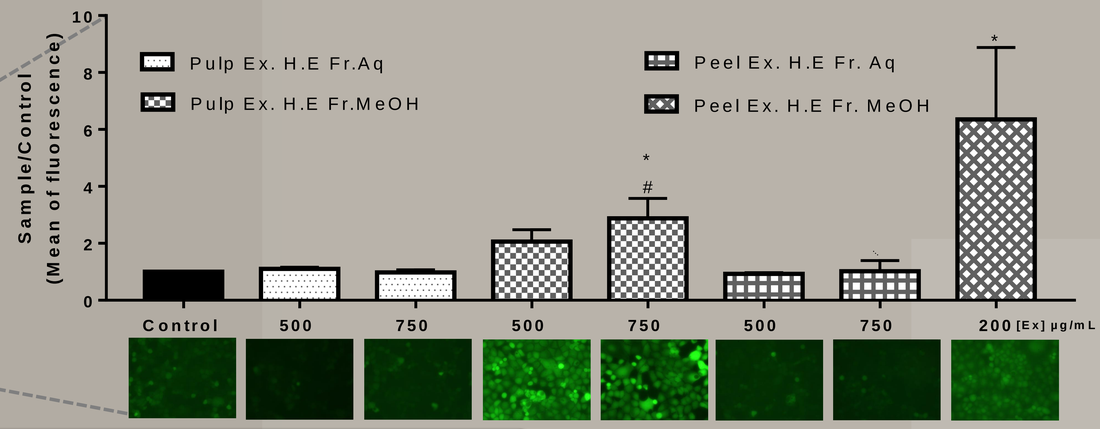


Fig. 3- Content in ROS of Caco-2 cells exposed to the different extracts at different concentrations, the results are presented as (Sample Fluorescence/ Control Fluorescence) vs (Concentration of the extract in µg/mL); Values represent the mean ± SD of three experiments (n=3); Statistical analysis was performed using one-way ANOVA followed by Turkey's multiple comparison test and the (*) represent statistical difference with control, the (#) represent statistical difference with the same concentration in different extract, at $p<0.05$.

Conclusion

We observed that the pre-exposure of Caco-2 cells to aqueous fraction of pulp extract protected the cells against, but when cells were pre-exposed to the methanolic fractions this protection was not observed. We also have observed that methanolic extracts dose-dependently increased intracellular ROS and modulated the cell cycle in respect to control cells. In conclusion, consumption of *Cucurbita ficifolia* pulp has a positive effect concerning to ROS protection and has a moderate role as anti-cancer agent against colorectal carcinoma cells (Caco-2 cells).

Acknowledgments
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The anti-tumor effect of spearmint (*Mentha spicata*) in a HPV-16-transgenic mouse model

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Abstract

Infection by Human Papillomavirus (HPV) is the main cause of cervical cancer, highlighting the importance of studying compounds that may reduce viral activity and its lesions/symptoms. The aromatic herb spearmint (Mentha spicata) (MS) has proven anti-tumor properties [1]. Thus, this study aimed to evaluate the effects of an hydroethanolic extract obtained from spearmint in HPV16-transgenic (HPV+/-) mice. The extract was obtained through maceration with ethanol/water (80:20, v/v), and the phenolic composition was determined through HPLC-DAD-ESI/MS. Thirty-three female mice (16 HPV-/- and 17 HPV+/-) were randomly divided into six groups: Group (G) I – HPV-/- w/o Mentha (n=5); G II – HPV-/- w/ Mentha (0.5 mg/ml; n=6); G III – HPV-/- w/ Mentha (0.55 mg/ml; n=5); G IV – HPV+/- w/o Mentha (n=6); G V – HPV+/- w/ Mentha (0.5 mg/ml; n=6); G VI – HPV+/- w/ Mentha (0.55 mg/ml; n=5). The spearmint extract was administered in the animal's drinking water for 28 days. During the study, water and food intake as well the animals' weights were recorded weekly. Afterwards, the animals were sacrificed, and their organs were collected for oxidative stress and genetic damage analysis. A total of thirteen compounds were identified in the hydroethanolic extract, being salvianolic acid B, rosmarinic acid and luteolin-7-*O*-glucuronide, the main compounds found. Moreover, the compounds revealed to be stable in the drinking water during the 5 tested days. Results show that HPV+/- (GIV, V and VI) have lower weight but higher water and food intake, as expected. Analysis of HPV+/- mice GIV showed a significant increase of superoxide dismutase activity when compared with GV (p=0.0029) and VI (p=0.0011). This suggests that group IV was subjected to a higher oxidative stress, as expected. These results could also mean that MS is responsible for a decrease in the oxidative stress known to be induced by HPV, which in turn decreases SOD, further the role of MS as an antioxidant. Regarding genetic damage, no statistically significant changes between groups were found in the comet and micronucleus assays, implying that spearmint has no influence on genotoxicity at the concentrations employed. There were no significant differences concerning basal DNA damage between WT and transgenic individuals as seen in other studies [2]. Further studies are required to clarify the antioxidant and antigenotoxic effects of spearmint.

Introduction

HPV is responsible for inducing a series of epithelial changes, being the cause of 90% of cancers of the anus, 40% of cancers of the penis and vagina and 35% of cancers of the head and neck [3].

Mentha spicata, is an aromatic plant whose foliage is widely used in the food industry, particularly in infusions and as a condiment. Essential oils, on the other hand, are used as aromatic agents in several food products, personal hygiene, cosmetics, cleaning and pesticides [4].

K14HPV16 mice were first conceived in the 1990s, by expressing the early region of HPV-16 in basal cells of squamous epithelium of FVB / n mice, using the human cytokeratin 14 (K14) promoter, thus allowing expression to be directed for this cell type [5].

Thus, this work aims to evaluate the effect of the aromatic herb Mentha spicata as a food supplement through its incorporation in the drinking water of mice transgenic for HPV16.

Materials and Methods

The extract was obtained through maceration with ethanol/water (80:20, v/v), and the phenolic composition was determined through HPLC-DAD-ESI/MS.

Animal protocol is shown in **Figure 1**.

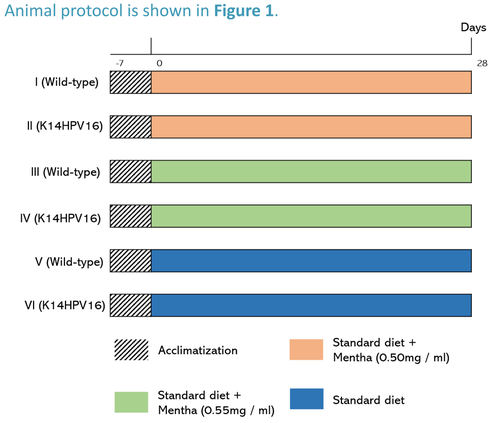


Figure 1. Experimental protocol.

Weekly, the animals were individually weighed, and water and food consumption were recorded. After 28 days, the animals were euthanized by anesthetic overdose. During euthanasia, liver samples were taken from the animals for analysis of oxidative stress and Mentha toxicity by the comet assay and micronucleus assay. All ethical issues followed the guidelines of the Portuguese *Direção Geral de Alimentação e Veterinária*.

Results

The evolution of the animals' body weight throughout the test is shown in **Figure 2**. The groups whose body weights decreased were group IV (HPV$^{(+)}$ + Mentha (0.55)) and group VI (HPV$^{(+)}$ without Mentha).

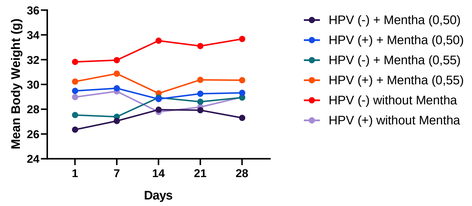


Figure 2. Mean body weight over the study days.

As we can see in **figure 3A**, the HPV$^{(+)}$ groups had a higher water consumption when compared with the wild-type groups. The group that consumed more water was the one whose Mentha was incorporated in the food at a concentration of 0.55 mg/ml. Regarding the food consumption by group, there is a higher consumption of food by the HPV$^{(+)}$ groups (**Figure 3B**).

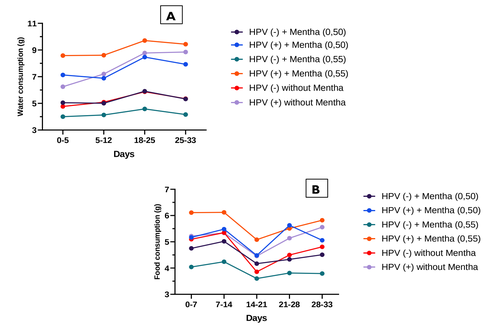


Figure 3. Mean daily water consumption **(A)** and mean daily food consumption *per* group **(B)**.

In the several tests carried out to analyze oxidative stress, only statistically significant changes were observed in the analysis of superoxide dismutase (SOD). In this, there was a significant increase in group VI (HPV$^{(+)}$ without Mentha) compared to groups II (HPV$^{(+)}$ with Mentha) (p=0.0029) and IV (HPV +/- with Mentha 0.55 mg/ml) (p=0.0011). Regarding the frequency of micronucleus, the only statistically significant change occurs between groups IV (HPV$^{(+)}$ with Mentha 0.55) and I (HPV$^{(-)}$ with Mentha 0.50) (**Figure 4A**). There were no statistically significant differences between groups regarding the comet assay (**Figure 4B**).

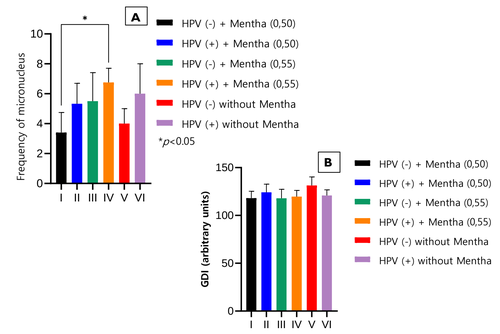


Figure 4. Mean frequency of micronuclei per 2000 erythrocytes for each group **(A)** and mean genetic damage index (GDI) by group **(B)**.

Conclusion

Based on the results obtained, it is possible to state that the animal model K14HPV16 is an excellent candidate for the study of lesions caused by HPV-16 as well as the evaluation of potential treatments. The animals did not show any side effect when exposed to the extract of Mentha spicata, nor were there any changes in biological parameters, oxidative stress or comet assay. The concentration used proved to be safe and tolerable by the animals. However, no results have been obtained that prove the antigenotoxic effect in reducing cell damage caused by HPV-16, which indicates that further studies with different concentrations and other exposure times are necessary, in order to better understand its effect and the mechanisms of action.

Refferences

[1] Yeruva, L. et al. (2007). Cancer Letters, 257(2), 216–226.

[2] Saad, A et al. (2018). Applied Physiology, Nutrition, and Metabolism, 43(1), 77–83.

[3] Parkin, D. M., and Bray, F. (2006). Vaccine, 24, S11–S25.

[4] Zheljazkov, V. D., Cantrell, C. L., Astatkie, T., and Hristov, A. (2010). Journal of Agricultural and Food Chemistry, 58(21), 11400–11407.

[5] Arbeit, J. M., Münger, K., Howley, P. M., and Hanahan, D. (1994). Journal of Virology, 68(7), 4358–4368.

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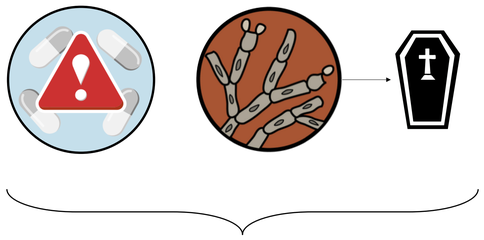
Epigallocathechin-3-gallate Antimycotic and Azole Resistant Modulator Potencial Against Triazole-Resistant *Aspergillus fumigatus*

Tomás Saraiva Ponte,[1] Edna Ribeiro,[1] Liliana Aranha Caetano,[1,2]*

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Introduction

Systemic–opportunistic fungal infections caused by azole-resistant fungi, including *Aspergillus fumigatus*, are emerging as an important cause of human disease with high associated morbimortality rates worldwide. The resistance to the limited arsenal of antifungal drugs is a serious concern, making it imperative to assess new compounds with antimicrobial properties and no cytotoxic effects, to be utilized as adjuvants in fungal therapy.





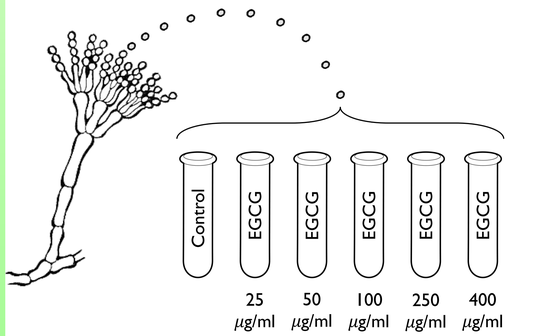
Green tea (*Camellia sinensis*)

Epigallocatechin-3-gallate (EGCG), the largest constituent of green tea, has demonstrated anti-infective properties in several *in vitro* studies however, information regarding EGCG antimycotic activity and synergistic interactions between EGCG and triazoles against *A. fumigatus* is scarce.

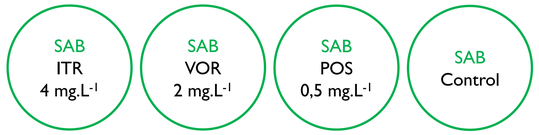
This work aimed to assess the antimycotic potential of EGCG against azole-resistant isolates of A. fumigatus, and the potential synergistic effects between EGCG and commonly used triazole antifungal drugs, namely itraconazole (ITR), voriconazole (VOR) and posaconazole (POS).

Methodology

Azole-resistant A. fumigatus isolates (N=4) collected during exposure assessment campaigns performed in the last ten years in Portugal and abroad, and previously characterized through conventional culture-based (agar-plate screening) and molecular (sequencing the calmodulin or β-tubulin genes for Aspergillus spp.) methods, were grown in MEA medium.



The obtained pure colonies were suspended in 1mL solutions with EGCG at final concentrations of 400 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and with no EGCG at all, for control purposes.



Each suspension was then spread onto Sabouraud dextrose agar media supplemented with 4 mg.L^{-1} ITR, 2 mg.L^{-1} VOR or 0.5 mg.L^{-1} POS and with no supplementation, for screening of antifungal resistance (adapted from the EUCAST 2020 guidelines).



The inoculated plates were incubated at 25°C for 7 days.

Results

Table 1. Growth pattern of azole-resistant *A. fumigatus* isolates suspended in EGCG solutions and spread onto Sabouraud dextrose agar media supplemented with triaoles

Sample	EGCG (µg/ml)	W/o Triazole	2 mg.L^{-1} VOR	0.5 mg.L^{-1} POS	4 mg.L^{-1} ITR
A	0	+	+	-	-
	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
	250	-	-	-	-
	400	-	-	-	-
B	0	+	-	-	-
	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
	250	-	-	-	-
	400	-	-	-	-
C	0	+	-	-	-
	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
	250	-	-	-	-
	400	-	-	-	-
D	0	+	-	-	-
	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
	250	-	-	-	-
	400	-	-	-	-

After the incubation period, all resistant isolates grew on Sabouraud dextrose agar media with no azole supplementation.

The addition of 25 µg/ml of EGCG to the suspensions canceled the growth observed in Sabouraud dextrose agar media of the 4 isolates.

One isolate grew on Sabouraud dextrose agar media supplemented with 2 mg.L^{-1} VOR but showed no growth when 25 µg/ml of EGCG was added to the suspension.

Conclusion

These results build evidence towards antimycotic potential of EGCG against azole-resistant isolates of *A. fumigatus*.

Sample A growth pattern indicates sensitization of the azole-resistant isolate when EGCG and VOR were used together.

At last, this study suggests that EGCG is a potential therapeutic adjuvant for therapies against azole-resistant fungi.

Recommendations

We recommend increasing the sample size in further studies and evaluating EGCG potential against other fungal pathogens of clinical interest, such as dermatophytes.

Acknowledgements

This work was supported by Instituto Politécnico de Lisboa, Lisbon, Portugal for funding the Project "Resistant mycobiota and mycotoxigenic profile of tea and medicinal plants" (IPL/2020/TEAResMyc_ESTeSL). H&TRC authors gratefully acknowledge the FCT/MCTES national support through the UIDB/05608/2020 and UIDP/05608/2020

Role of mTOR Hyperactivation in Disease

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Abstract

Chronic diseases of civilization are largely associated with hyperactivation of mTOR (a key regulator of cell growth and metabolism): acne, cancer, type 2 diabetes [1]. After 25 years, a person's growth ends, and excessive mTOR activity makes not only muscles grow, but also increases the growth of malignant tumors, moles, and acne.

Introduction

Excessive activation of mTOR work leads to uncontrolled cell division - that is, to their transformation into cancerous ones.

In particular, mTOR is hyperactive in almost all cases of prostate cancer (which is the most common cancer in men) and in several other cancers.

Materials

References

[1] Albert, V. & Hall, M. N. mTOR signaling in cellular and organismal energetics, Curr Opin Cell Biol, 33 (2015) 65.

[2] Andrew C. Hsieh, Yi Liu, Merritt P. Edlind et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis, Nature. V. 485 (2012) 57.

[3] Saxton, R. A. and Sabatini, D. M. mTOR Signaling in growth, metabolism, and disease, Cell, 169 (2017) 371.

Methodology

Normally, the work of mTOR primarily regulates cell growth and reproduction. These processes are very subtle: the slightest bias in one direction can lead to aging or death of the cell, in the other - to its uncontrolled multiplication and the development of a cancerous tumor. Therefore, too weak, and, especially, too "zealous" work of mTOR is very dangerous.

A group of American researchers led by Davide Ruggero and Christian Rommel investigated the relationship between mTOR activity and cancer development [2].

Results

What activates it the most? Dairy and meat products stimulate mTOR as strongly as possible. Cow's milk is an extremely potent evolutionary rapid growth program that overstimulates mTOR in people who consume this product. Increase in calf weight during the first year of feeding on 0.8 kg per day. This is nearly 40 times faster than that of a human infant [3]. Also, the essential amino acid leucine, which is found in large quantities in meat, has the ability to directly stimulate mTOR. mTOR reduction factors (**Figure 1**):

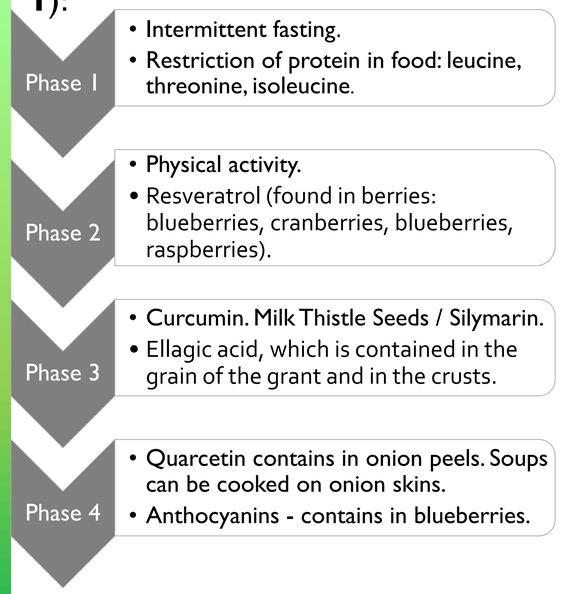


Figure 1: mTOR reduction factors

Conclusion

Take as an example the traditional diet of Okinawa island inhabitants, where there are only 10% of protein and no dairy products. They eat mostly plant foods. Hence the secret of centenarians who do not have acne, diabetes, and women have menopause after 60. Less than 1% of their diet consists of fish and meat (one serving of meat a month). Their life expectancy is the highest among any population groups [3].

Recommendations

In order to avoid mTOR hyperactivation and chronic diseases caused by its excessive work a person should follow intermittent fasting, restrict of protein in food: leucine, threonine, isoleucine, have physical activity every day, follow a diet and eat more fruits, vegetables and berries, which are rich in fiber and vitamins. So, eat mostly plant foods and exclude diary products. What is even more crucial – limit consumption of red meat and fish, which in today's conditions are full of parasites, microplastics and heavy metals.

Acknowledgements

Solid Dispersions of leaves extract of *Eugenia florida*

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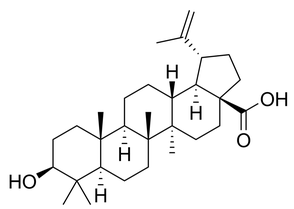
Introduction

Eugenia florida DC. (Figure 1) belongs to the Myrtaceae family and is commonly found throughout the Brazilian territory[1]. Betulinic acid (Figure 2) is a triterpene found in the leaves of *E. florida* and presents several biological activities such as anti-inflamatory, antimalarial, antimicrobial and antiviral [2,3,4,5]. However, the most studied activity is the anticancer action [6,7,8].

Figure 1. *Eugenia florida* . a) *Habitat* ; b) Flowers; c) Fruits.



Figure 2. *Structure Betulinic Acid*



Objectives

BA low aqueous solubility limits its bioavailability. The aim of the present study was the increase in the solubility *using solid dispersion technique*.

Materials

- Standarded lyophilized extract of *Eugenia florida;*
- Betulinic acid standard Sigma Aldrich® 98%;
- Gelucire ® 50/13 ;
- Monohydrate Lactose;
- Heating plate IKA (350°C± 10%);
- HPLC LaChrom (Merck-Hitachi)

Methodology

The leaves of *E. florida* were collected at Fundação Oswaldo Cruz, Rio de Janeiro, Brazil and a voucher was deposited in the Botanical Garden Herbarium of Rio de Janeiro, Brazil (RB 328.061). The leaves were dried in oven with air circulation, The extraction was carried out with ethanol by static maceration. The extract was dryed by lyophilization.

The solid dispersions were prepared by the fusion method where the lyophilized extract of *Eugenia florida* leaves was added to the molten carrier (Gelucire® 50/13), with continuous homogenization until the formation of a homogeneous dispersion, which was then solidified by cooling in an ice bath and powdered. The samples were kept in amber glass bottles until analysis.

The solid dispersions were analyzed using a validated HPLC method.

Results

Figure 3. Betulinic acid solubility present in crude extract at different pHs**.**

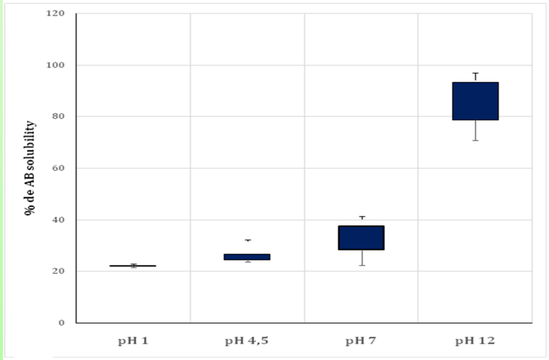
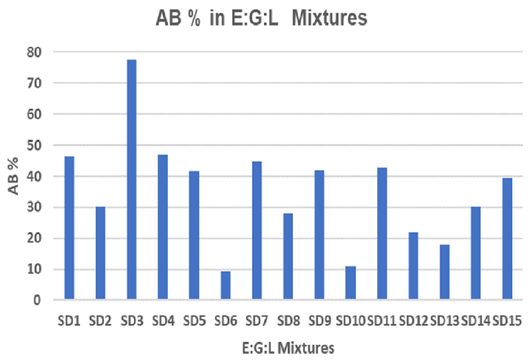


Figure 4. Solubility response in pH 7 buffer solution of the betulinic acid present in the *Eugenia florida* leaves extract in solid dispersions**..**



Solid dispersions of extract *Eugenia florida* leaves, Gelucire® 50/13 and lactose in the following proportions: SD1 (50:500:100); SD2 (500:500:100); SD3 (50:950:100); SD4 (500:950:100); SD5 (50:500:300); SD6 (500:500:300); SD7 (50:950:300); SD8 (50: 950:300); SD9 (50:725:200); SD10 (500:725:200); SD11 (275:50:200); SD12 (275:95:200); SD13 (275:725:100); SD14 (275:725: 300); SD15 (275:725:200).

The experimental design was evaluated the best proportions between extract and carriers. The best combination was SD3 with extract, Gelucire and lactose in respective proportions (50:950:100), increasing the solubillity of BA.

Conclusion

The solid dispersion technique increased in eleven times the solubility of betulinic acid in *Eugenia florida* leaves extract, suggesting a higher bioavailability of the chemical marker. These results justify the continuity of studies to develop a phyto pharmaceutical drug.

References

[1] Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas do Brasil. Lorenzi H., Nova Odessa, São Paulo,
2000.
[2] Frighetto N et al. Phytochemical Analysis. 16(6) (2005) 411-414.
[3] Junges MJ. Caderno de Farmácia, Fundação Universidade de Rio Grande, 13(2) (1997)103-104.
[4] Farmacognosia: da Planta ao Medicamento. Simões CMO et al. Ed. Universidade / UFRSC/ED.UFSC, Porto Alegre, 2007.
[5] Mukherjee PK et al. Planta Med. 63 (1997) 367-369.
[6] Cichewicz RH and Kouzi SA. Med. Research Reviews. 24(1) 92004) 90-114.
[7] Pisha E et al. Nature Medicine. I (1995) 1046-1051.
[8] Csuk R. Expert Opinion on Therapeutic Patents, 24(8) (2014) 913–23.

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MICROCRYSTAL SCOPE DETERMINATION OF CALCIUM SALTS IN TOOTHPASTES

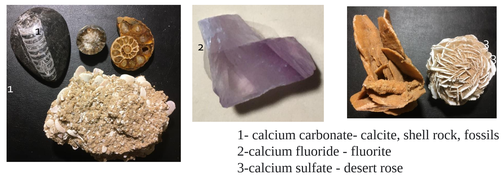
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Abstract.
The method of toothpaste sample preparation for analysis has been developed. Qualitative analysis showing the presence of calcium salts in the examined objects was fulfilled by the method of $CaSO_4$*$2H_2O$ microcrystal optical microscope determination. The work was carried out in the frame of scientific young scientist's research supervision.

Introduction.
In the course of inorganic chemistry students of St. Petersburg State Technical University (TU) perform laboratory experiments for the qualitative determination of certain elements in different compounds. Calcium salts are the objects of study, their analysis allow to get acquainted with the chemical properties of this element.
In nature, calcium salts are found mainly in the form of poorly soluble aluminosilicates (feldspar), carbonates (limestone, chalk, marble, dolomite), fluorides (fluorite), sulfates (gypsum, alabaster) and phosphates (apatite) [1-4].



1- calcium carbonate- calcite, shell rock, fossils
2-calcium fluoride - fluorite
3-calcium sulfate - desert rose

Dental and bone tissues are examples of poorly soluble compounds in the living systems, that demonstrate the unique structure, mechanical and physiological properties, enormous strength and high metabolic activity. Violation of the optimal content of both macro- and microelements in such tissues causes many diseases.

The tooth has a multi-layered complex structure. The most durable layes outside and is called tooth enamel, its main component is hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$.

Bone tissue is capable for isomorphic replacement of its components in its crystal lattice structure. Calcium, phosphate and hydroxide ions are included in the composition of bone tissue as the main components, but fluoride, chloride, sodium, potassium, magnesium, ions are found as well. During formation of the tooth, along with calcium hydroxide phosphate, calcium fluoride phosphate $Ca_5(PO_4)_3F$ is also formed in the tooth enamel, being less soluble and mechanically stronger compound [2]. In its composition and mechanical characteristics, tooth enamel is relative to the mineral apatite $Ca_{10}(PO_4)_6(OH, F, Cl)_2$.



Natural mineral apatite and tooth samples

Based on the chemical composition of bone tissue, it can be concluded that for the successful existence of tooth enamel, the active form of elements forming it - phosphorus, calcium and fluorine, have to be presented in food and in the composition of toothpaste.

Most of the modern toothpastes contain fluoride. The use of these pastes is aimed at the strengthening the enamel due to the formation of fluorapatite. But the frequent use of fluorides has a negative toxic effect, causing, for example, fluorosis. The use of hydroxyapatite is a promising direction in the development of toothpastes, because due to the similarity of the chemical and physical properties of the mineral and tooth enamel, it can contribute to the remineralization of damaged teeth [5].

Materials.

The Table shows Ca, P, F compositions of the pastes that we analyzed to determine the presence of calcium salts. The eggshell was analyzed, because SPLAT BIO CALCIUM paste includes it in processed form, that determines the presence of calcium salts in the composition.

Table

Name	Calcium	Fluoride	Phosphate	Manufact.
New Perl Calcium	Calcium carbonate Calcium lactate	-	-	RF
Splat Professional Bio-Calcium	Hydroxyapatite Calcium carbonate from eggshell Calcium lactate	-	Hydroxyapatite	RF
R.O.C.S. Active Calcium	Calcium glycerophosphate	-	Sodium Glycerophpsphate	RF
Karex	Hydroxyapatite	-	Hydroxyapatite Tetrapotassium pyrophosphate Phosphoric acid	Germany
SPLAT Professional Whitening Plus	Dicalcium phosphate dihydrate Calcium pyrophosphate	Sodium monofluorophosphate	Pentasodium triphosphate Dicalcium phosphatedihydrate Calcium pyrophosphate	RF
Colgate Sensitive	Calcium carbonate	Sodium monofluorphosphate	-	Poland
Blend-a-med 3D White	-	Sodium fluoride	Disodium pyrophosphate	Germany
Colgate Total	-	Sodium fluoride	-	China
Sensodyne	-	Stannous fluoride Sodium fluoride	Pentasodium triphosphate	GB
Eggshell	Calcium carbonate	-	-	-
Elmex Kids	-	Olaflur $C_{27}H_{60}F_2N_2O_3$	-	Swiss

The inclusion of calcium salts in the toothpaste serves to achieve two main effects - enamel strengthening with restoration and abrasive action. Calcium carbonate $CaCO_3$ and calcium hydrogen phosphate dihydrate $CaHPO_4$ * $2H_2O$ are the least abrasive. Other used abrasive compounds - sodium bicarbonate $NaHCO_3$, sodium chloride NaCl, aluminum oxide Al_2O_3 and titanium dioxide TiO_2 are coarser. Hydrated silica SiO_2 * H_2O is highly abrasive, but does not damage the enamel. Some enzymes (for example, papain) are introduced into the composition of modern pastes to prevent the formation of plaque.

The majority of toothpastes contains fluoride. These are sodium fluoride NaF, tin fluoride SnF_2, sodium monofluorophosphate Na_2PO_3F or most effective aminofluoride $C_{27}H_{60}F_2N_2O_3$.The simultaneous presence of calcium and fluorine ions in the paste is not recommended for it leads to the formation of poorly soluble calcium fluoride $Ca^{2+} + 2F^- \rightarrow CaF_2$. Nevertheless, a number of manufacturers offer pastes with both calcium and fluorine - for example, Colgate, Blend-a-med, Splat.

Most of the studied toothpastes contained calcium salts in combination with phosphates and fluorides.

Methodology.
The calcium salts found in toothpastes or eggshell are weak acid salts. These are carbonates, glycerophosphates, lactates (lactic acid salts), or hydroxyapatites, so they dissolve in strong acids. Sample preparation for analysis started from dissolving a small amount of paste in hydrochloric acid.
In this case, calcium cations pass into the solution. For example:

$$CaCO_3 + 2H^+ = Ca^{2+} + CO_2 + H_2O.$$

The resulting suspension is subjected to centrifugation, the centrifugate is analyzed for the presence of calcium salts. For this, a few drops of the studied solution are placed on a glass slide and slightly evaporated on heating. When the sulfuric acid is added, the gypsum microcrystals are formed [6].

The method is based on the reaction of obtaining crystalline calcium sulfate:

$$Ca^{2+}+SO_4^{2-}=CaSO_4$$

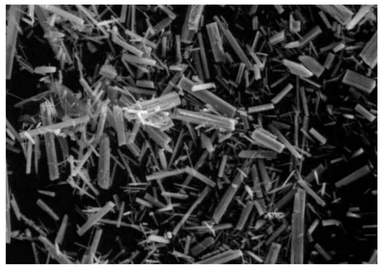
$$2CaSO_4 * H_2O + 3H_2O = 2 (CaSO_4 * 2H_2O).$$

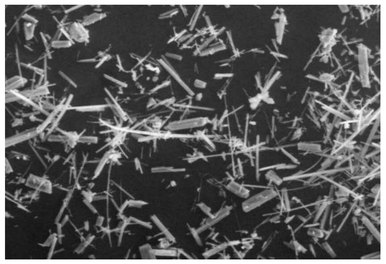
Regardless the initial concentration of calcium ions in the analyzed solution, evaporation allows to obtain crystals visible under a microscope. Depending on the concentration of the initial analyzed solution, the shape of the resulting crystals may differ - from accrete whiskers to large needle-like crystals and rods [7,8].

Experiment.
The analyzed sample (200 mg) was dissolved in hydrochloric acid (4-5 ml of 2 M), centrifuged, a drop of filtrate was evaporated on a glass slide. Sulfuric acid (3 M) was added, and the crystals formed were observed using an optical microscope. The formation of characteristic gypsum needle-shaped crystals indicated the presence of calcium salts. The Table shows the composition of the initial calcium salts in the analyzed substances.



In a separate experiment, we observed the calcium fluoride precipitation from solutions of various concentrations. In a test tube with a 0.1 M solution of calcium chloride, 0.1 M ammonium fluoride solution was added dropwise. The formation of a white crystalline calcium fluoride precipitate was observed.





The formation of characteristic needle-shape gypsum microcrystals [7] can be observed with an optical microscope



Results.

All ingredients the toothpaste consists of are divided into active and passive. Active substances have a therapeutic and pharmacological effect. Calcium ions, fluorides and phosphates are active components, aimed at protecting teeth from caries, reducing sensitivity, whitening and restoring enamel. The leading position in the protection against caries is occupied by fluoride-containing toothpastes. But such pastes should not contain calcium salts in their composition to prevent the formation of poorly soluble calcium fluoride. As it was proved by our experiment, calcium fluoride forms a sediment even from diluted solutions. Sensodyne and Blend-a Med contain together fluorides and phosphates, for example, but exclude calcium salts.
Calcium salts are not only abrasive, that is necessary for mechanical removal of plaque and whitening. They are also able to integrate into the crystal lattice of the tooth enamel, leading to the restoration of the enamel. In our study we tried to determine the reasonable composition of toothpaste. Often we met recommendations for the correct use of toothpastes - in the morning after meals to brush teeth with a fluoride-containing paste, and in the evening with a paste containing calcium salts. This will contribute to the layer-by-layer introduction of fluorides and calcium salts into the tooth enamel. Many modern pastes contain microparticles of hydroxyapatite, which is the closest in chemical composition to the tooth enamel. Apatite microparticles are able to penetrate microcracks and fill them. SPLAT and KAREX are of that kind.

We have carried out a qualitative analysis of various pastes for the presence of calcium salts and developed a method for preparing a sample for the analysis. When strong acid is added, the paste sample does not dissolve completely, a suspension is formed. Attempts to filter the precipitate in order to obtain a clear solution were unsuccessful. Therefore, centrifugation was applied. After centrifugation, the clear solution above the precipitate was placed on the slide, evaporated, and sulfuric acid was added.
The formation of gypsum microcrystals confirmed the presence of calcium salts in the studied pastes. In those pastes where, according to manufacturers, calcium salts were absent, gypsum was not formed.
For every person, the choice of toothpaste is an urgent problem, so toothpastes can be an interesting object for students to study. The use of the microcrystal analysis allows students to get acquainted with the conditions and features of sediments and crystal formation, to work out practical skills in performing heterogeneous reactions in the laboratory.

Conclusions. The microcrystal analytical method allows to fulfill a qualitative analysis of the calcium salts present in natural objects and toothpastes. The microscopic analysis of the investigated substances confirmed the expected presence or absence of calcium salts. Natural substances, such as limestone, eggshells, as well as calcium toothpastes can be offered as interesting objects for research in the course of inorganic chemistry.

References

[1] J. E. Safra, S. Y. Constantine, J. E. Goulka, The New Encyclopedia Britannica, 15th edn (Britannica, London) 1998.
[2] V. I. Slesarev, Chemistry: Fundamentals of the Chemistry in the Living Systems: a textbook for universities.- 4th edn., Revised- SPb: Khimizdat, 2007.-784 p., in Russian.
[3] L. Pauling, General Chemistry, Dover Publications, 2014.- 992 p.
[4] D. F. Shriver, P. W. Atkins, Inorganic Chemistry, Oxford University Press, 2009.-864 p.
[5]H. Sudradjat, F. Meyer, K. Loza, M. Epple, J. Enax, Eur. J. Dent. 14(2)(2020) 206–211.
[6] Z. E. Goldbricks, Workshop on Inorganic Chemistry: Textbook - 4th ed., rev. and added - M .: Alliance, 2008.-350 p., in Russian.
[7]http://www.bullchemicals.com/index.php/news/What_is_the_Function_of_Calcium_Sulfate_Whiskers_i.html.
[8]G. Sirokman, Journal of Chemical Education, 91(4) (2001) 557.
https://doi.org/10.1021/ed400004b