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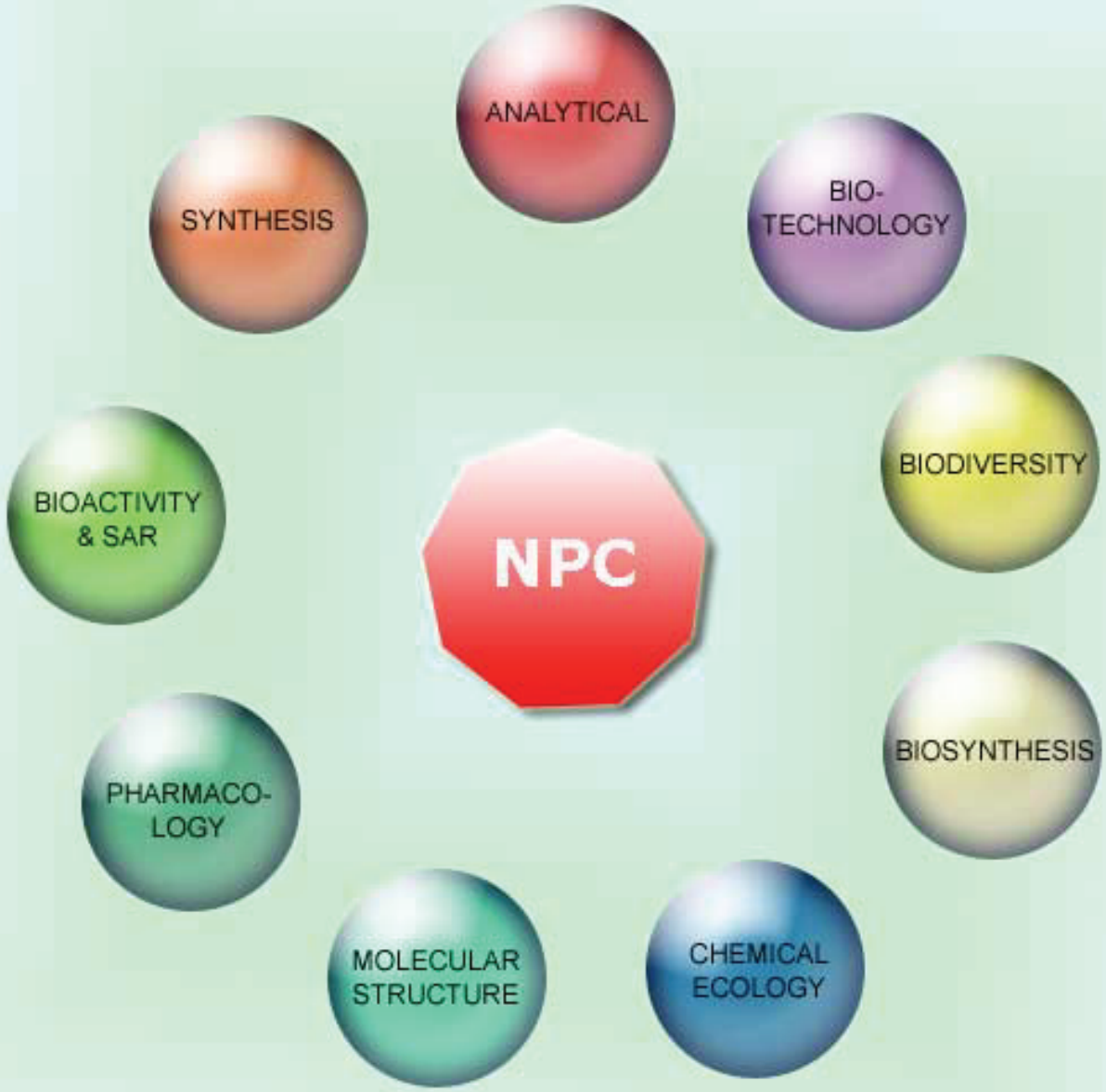

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Editorial

NPC-Bromo: Special Issue

I am very grateful to Prof. Bambang Prajogo, Chairman, Bromo Conference (Symposium on Natural products & Diversity), and Dr. Tutik Sri Wahyuni, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, and the Organizing Committee for arranging this issue, originating from the Bromo Conference-2018, which was held in Surabaya, Indonesia, from July 11–12, 2018, and attended by a large number of participants.

The first part of the December 2018 edition is devoted to selected manuscripts (8) presented at Bromo-2018. I am very grateful to Profs. Bambang Prajogo and Tutik Sri Wahyuni for extending an invitation to participate in this scientific meeting, as well as for organizing this issue. The editors join me in thanking Profs Prajogo and Wahyuni, the authors, and the reviewers for their efforts that have made this issue possible, and to the production department for putting it into print.

Pawan K. Agrawal
Editor-in-Chief

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Introduction to NPC Bromo Issue

This special issue contains selected papers previously presented at the Bromo Conference: Symposium of Natural Products and Biodiversity held in Surabaya on July 11-12, 2018. This symposium was organized by Universitas Airlangga, Surabaya, Indonesia in collaboration with the Indonesian Association of Natural Drug Researchers (PERHIPBA) and the Phytochemical Society of Asia (PSA). It was held to commemorate the 10th anniversary of the IOCD seminar in Surabaya. The Bromo Conference provides a forum for the exchange of information on Natural Products within all of the related topics, as well as with the aim to build and strengthen scientific cooperation between the research institutions.

Academic and other researchers, industrial practitioners and students participated in the symposium. The topics of interest covered in the Bromo Conference included ethnomedicine, implementation of the Nagoya Protocol, sustainable valorization of biodiversity, bioactivity of natural products, metabolomics, phytopharmaceutical technology, clinical trials and other related subjects. The manuscripts have been reviewed by the Organizing Committee members, Prof. Katsuyosi Matsunami, Prof Gunawan Indrayanto, and Prof. Angela Calderon, and edited by Dr Pawan Agrawal. The manuscripts underwent further rigorous peer review and were revised before being accepted for publication.

This special issue of *Natural Product Communications* is intended to help readers gain knowledge from the contributors, as well as to provide an overview of the various fields to improve natural products research.

We would like to present a special thanks to the authors and reviewers. Also, we are grateful to Dr Pawan K Agrawal, the Editor-in-Chief of *Natural Product Communications* and the editorial team for their assistance in the preparation of this issue and for the continued support and collaboration between Universitas Airlangga, Surabaya, Indonesia and *NPC*.

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Screening of Biological Activities of *Ligustrum lucidum* Berries: a Comparative Approach

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Ligustrum lucidum Aiton including its berries have been used in Chinese Traditional Medicine for around 2000 years. Scientific studies developed on the last decades provided evidence on some biological properties of these products, mostly from particular geographic origins. In the present study, *L. lucidium* berries harvested in two regions of Portugal were considered. Extracts obtained with 100% ethanol, with 50% ethanol/water (v/v) and in boiled water were prepared and several parameters were assessed: antioxidant activity (using two methodologies), antimicrobial activity and phenolic profile. Results suggest that the different biological activities varied according to the region where samples were collected but also with the extraction methodology. Superior antioxidant potential was observed in water extracts, according to both assays, while for the remaining activities the ethanol 50% extracts had the highest activity. In these regions, the *L. lucidum* berries showed promising biological activities and may be interesting sources of compounds for the development of new drugs for diseases where oxidant reactive species and enzymatic disruption are believed to play a role as well as adjuvants for current antibiotic therapy.

Keywords: *Ligustrum lucidum* Aiton berries, Antioxidant activity, Antimicrobial activity, Phenolic compounds, Natural compounds, Biological properties.

Ligustrum lucidum Aiton is a Chinese native plant and belongs to the *Oleaceae* family. In ancient time, this plant was believed to have protective role as a tonic [1] as well as in the prevention of osteoporosis, diabetes and coronary heart disease [2-6]. On the last years, some studies focused on its berries, reporting the presence of several biological activities among which immunomodulatory, anti-inflammatory, hepatoprotective, anti-tumor and antioxidant [7,8]. He *et al*. [9] reported an inhibition of the free radical induced hemolysis in red blood cells and highlighted the presence of the compounds oleoside dimethylester, oleuropein and neonuezhenide. Liu *et al*. [10] also studied the anti-obesity effect of (8-E)-niizhenide from *Ligustrum lucidum* in mice. Other compounds that can be found in these berries such as oleanolic and ursolic acids usually play a role in control of inflammation, hepatotoxicity, pain and hyperlipidemia [11,12]. Compounds like triterpenes, phenolics and secoiridoid glycosides are also present in the *Ligustrum* spp [13-15]. However, despite this promising therapeutic potential, broad-leaf privet berries contain a toxic principle named ligustrin (syringin glycosides) that may be poisonous by oral ingestion inducing symptoms such as abdominal pain, nausea, vomiting, diarrhea, headache, weakness, low blood pressure, cold and clammy skin, lasting from 48 to 72 hours [16].

The search for healthy products with beneficial properties has been increasing recently due to the strong scientific evidence that correlates these chemical structures to the prevention or neutralization of oxidative stress effects, which have been claimed to have a key role in a wide range of pathological processes [2], despite the discussion around the issue. In addition, antioxidants have an important role as stabilizers in the food industry increasing the shelf lifetime of food products [17].

Some previous studies have been performed with flavonoids from *Ligustrum vulgaris* leaves showing potential as antioxidants [18-20]. Nevertheless, none of them was carried out with broad-leaf or berries belong to plants collect in Portugal. The inclusion of Traditional Chinese Medicine in the legislation of diverse occidental counties [21] as Portugal, bring a special motivation on the understanding and research correlated to medicinal plants from this therapy, once they could be in a near future introduced as drugs. The knowledge of the composition and biological effects of these plants harvest in different geographical origins will be important for the pharmacological impact in further studies.

Following this background, this study aimed to characterize and evaluate some biological activities of berries harvested in plants grow in two different Portuguese regions [Lisbon (R1) and Castelo Branco (R2)]. First of all the antioxidant potential will be checked as, antioxidants compounds have broad applications and could be useful in some pathological conditions. Recently, Sayah *et al*. shown a potential correlation of the antioxidant activity of certain plant extracts and their antidiabetic effect [22]. In this previous screen the potential antimicrobial activity is also evaluated, once one of the actual highlighted situations is the resistance to current antibiotics. New therapeutic approaches should be study in this fields, and to the best of our knowledge, there are no previous

reports of any data collected as we did in this screening work with the crude material, harvested in Portugal.

Antioxidant activity: In Table 1 are presented the results of the antioxidant activity (using two different methodologies) and the total reducing capacity (TRC) of *L. lucidum* berries extracts. All assays were performed using two replicates and each analysis was performed in duplicate. Afterwards, an ANOVA test was made in order to test whether the two extracts for each solvent were significantly different. In all cases no significant differences were found between extracts replication with a Least Significant Difference (LSD) test (with a significant level of the $p<0.05$). Therefore, the results were analyzed together.

Table 1: Antioxidant activity and total reducing capacity of *L. lucidum* extracts samples from the three extraction solvents.

	Extract (E)	Region (R)	DPPH – EC_{50} (mg plant/mL)	FRAP (mg Fe(II) equivalent/mL)	TRC (mg GAE/mL)
	100% Ethanol	**R1**	5.0±0.4^{d}	20.3±0.6^{d}	2.0±0.3^{b}
		R2	8.0±0.2^{e}	8.1±0.5^{a}	1.7±0.2^{a}
	50% Ethanol	**R1**	2.7±0.2^{b}	20.2±0.7^{d}	4.0±0.4^{e}
		R2	4.8±0.2^{d}	12.2±0.9^{b}	3.5±0.3^{d}
	Boiled water	**R1**	2.1±0.0^{a}	31.5±1.9^{e}	2.5±0.1^{c}
		R2	3.4±0.0^{c}	18.6±1.9^{c}	1.6±0.0^{a}
ANOVA	**Region (R)**		34.0***	33.9***	1.1***
	Extractive method (E)		59.6***	62.2***	98.4***
	RxE		5.8***	3.4***	n.s
	Residual		0.6	0.5	0.5

Mean±SD. Different letter (a-e) on the same column mean that results are significantly different ($p<0.05$).

As far as we know, there are no studies regarding the antioxidant activity of *L. Lucidum* berries. However, the antioxidant capacities of various colored berries from other species have been well established [23-25].

The DPPH and FRAP methods are commonly used to assess radical-scavenging activities in vitro. The extracts of *L. lucidum* berries exhibited appreciative scavenging capacities against both radicals. The inhibition percentage was different amongst the two regions and for the three extraction solvents analyzed. Regarding DPPH assay, the highest antioxidant activity was always found for R1 region (EC_{50}: 2.1-5.0 mg plant/mL) and in all cases the extract performed with boiled water had higher antioxidant activity (EC_{50}: 2.12-3.35 mg plant/mL) than the other ones (EC_{50}: 2.68-8.04 mg plant/mL).

On the other hand, concerning FRAP method, the highest values were found for *L. lucidum* extracts from region 1 (R1) (20.20-31.50 mg Fe(II) equivalent/mL). The extracts obtained using boiling water showed, once more, the highest antioxidant activity (18.59-31.50 mg Fe(II) equivalent/mL).

Regarding the Total Reducing Capacity (TRC), the higher values were attributed to berries extracted with 50% ethanol (3.5-4.0 mg GAE/mL) (Table 1). Similar results were observed for the extracts with 100% ethanol and boiling water (1.7-2.0 mg GAE/mL and 1.6-2.5 mg GAE/mL, respectively). Concerning the TRC assays the results obtained for R1 were statistically higher ($p<0.05$) than those obtained for R2, for the three extraction methods.

For both methods, the major discriminant factor was the extraction method that allowed to explain 59.6%, 62.2% and 98.4% of the total variance for DPPH, FRAP and TRC assays, respectively. The results suggest that the region where the berries were harvested has an important weight in the antioxidant capacity and, therefore, that the chemical composition differs depending on the climatic conditions and soils to which plants are exposed. However, this study is a first step to understand the potentiality of these berries growing in Portugal and further studies are needed to confirm this hypothesis.

Antimicrobial activity: Antimicrobial activity was tested against both Gram-positive and Gram-negative bacteria only with the extract with 100% ethanol. Gram-positive bacteria are believed to influence cell metabolism and Gram-negative bacteria affect membrane integrity, leading to cell death [26,27]. The antimicrobial activity was also evaluated against yeast. In Table 2 it is presented the results obtained for the antimicrobial activity against the microorganisms under study.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), *L. lucidum* collected in two regions (R1 - Lisbon and R2 – Castelo Branco).

	MIC (mg/mL)		MBC (mg/mL)	
	R1	**R2**	**R1**	**R2**
Escherichia coli ATCC 29998™	0.4±0.1^{a}	0.6±0.1^{b}	3.0±0.6^{a}	4.6±0.3^{b}
E. coli ESA 37 Cephalosporins-resistant	0.6±0.1^{a}	0.8±0.1^{b}	4.1±0.3^{b}	0.8±0.1^{a}
Streptococcus mitis ATCC	0.1±0.0^{a}	0.1±0.0^{a}	1.5±0.4^{a}	2.1±0.3^{b}
S. mitis ESA65 Penicillin-resistant	0.1±0.0^{a}	0.2±0.0^{b}	2.2±0.3^{a}	3.3±0.4^{b}
Candida albicans ATCC 10231™	3.7±0.4^{a}	4.8±0.4^{b}	12.9±0.7^{a}	14.9±0.3^{b}
Anfotericine B-resistant	4.5±0.4^{a}	5.5±0.4^{b}	14.9±0.7^{a}	16.3±0.4^{b}

Mean ± SD. Similar small letter superscripts on the same row means that there are not significantly different ($p<0.05$).

Streptococcus mitis ATCC® 49456 D5 ™ was the most sensitive microorganism, with MIC values around 0.1±0.0 mg/mL and MBC values ranging from 1.5±0.4 mg/mL to 2.1±0.3 mg/mL.

The less sensitive bacterial strain was *Cephalosporins*-resistant *Escherichia coli* ESA 37 in the presence of the extract R2, with a MIC value of 0.8±0.1 mg/mL. The MBC value in the presence of extract R1 was 4.1±0.3 mg/mL.

Regarding the antifungal activity, the *Anfotericine* B-resistant *C. albicans* ESA 100 strain was the most resistant particularly to the action of R2 extract (MIC = 5.5±0.4 mg/mL and MBC = 16.3±0.4 mg/mL).

The results obtained in the present study for *L. lucidum* extracts were similar to those reported by Bajpai *et al*. [28] in *L. lucidum* oil. However, Bacha *et al*. [29] observed lower antimicrobial activity potential in selected medicinal plants from Ethiopia.

For "*Streptococcus mitis* ATCC" and "*S. mitis* ESA65 *Penicillin*-resistant" the R1 extract was also the most active against, being the correspondent ratios1.6 and 1.8.

Regarding "*Candida albicans* ATCC 10231™" and "*Anfotericine* B-resistant *C. albicans* ESA 100" the more active extract it was from R1 region, presenting a ratio of 1.3 and 1.2, respectively.

The correlation between MIC and MBC assays for all the microorganism was 1.1 and it was consistent among all of them.

The analysis of the relative concentration of the various constituents in the most active extract comparing to the other was not so easy for this bioactive property.

HPLC/DAD phenolic profile: Figure 1 represents the HPLC/DAD phenolic profile for the three solvents used to extract samples from the two regions (R1 and R2). For the same concentration, all

extracts had similar profiles with the same compounds (UV spectral data from HPLC/DAD). The ratio among the relative concentrations is depicted in Table 3.

On Figure 2 the different peak areas of the 3 extracts are plotted for the three solvents studied in the two regions, to allow better comparisons of compounds' concentration.

Comparing the results of the three extracts for each region (Figure 2), boiling water shows the best extraction for phenolic compounds **3**, **4** and 5 in sample R1 and 3 and 4 for sample R2. Nevertheless, the concentration for both was the same for compound **4** and two times higher for compounds **3** and **5** in sample R1. This may, at least partly, justify the higher antioxidant activity of sample R1. Compounds **8** and 9 were observed in higher concentration for region R2 considering the extraction with ethanol 50% and Ethanol 100%. However, concerning the ratios that could be obtained with the results plotted in Table 1, seems that no implication occurs in terms of the antioxidant bioactivity, because their ratio of concentration (Table 3) is very different from those observed for the antioxidant activity.

For the antioxidant bioactivity, the ratio R1/R2 obtained for ethanol 50% and 100 % extract was 1.7 (compound **8** and **9**) and 1.1(compound **8**) and 1.6 (compound **9**), respectively. These results suggest that none of these compounds is relevant to explain the previously described differences.

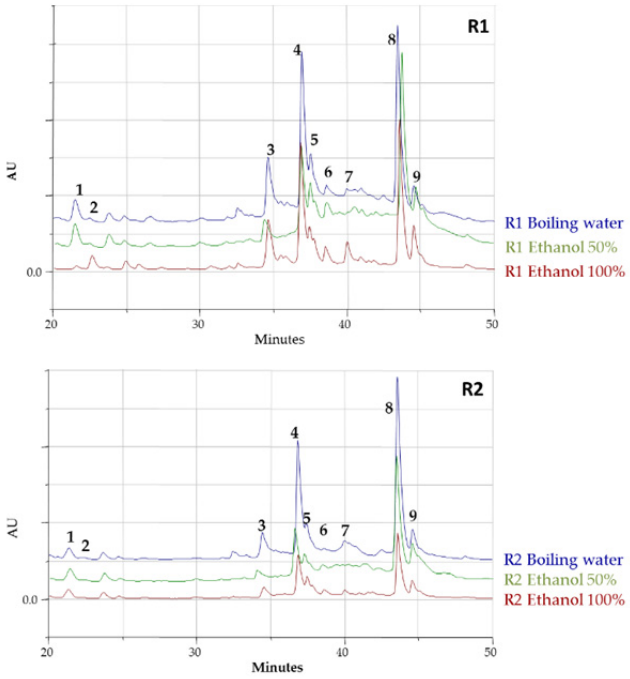

Figure 1: HPLC/DAD Profile of compounds with absorption at 260 nm, for the three solvents studied in the two Portuguese regions, R1 and R2.

Comparing the differences found in the chemical compounds and those of the bioactive properties under study it was possible to conclude that region R1 and ethanol 50% (V/V) was the best extract for a possible isolation on the compound **6**, the more concentrated constituent in this extract compared to a similar extract for R2 (Figure 1). In fact, the extract R1 was 1.6 times more active than R2 and compounds **1**, **3** and **5** are 1.7 more concentrated in extract R1.

Regarding to DPPH and FRAP results, boiling water extracts show the best activity and R1 was 1.6 and 1.7 more active than R2

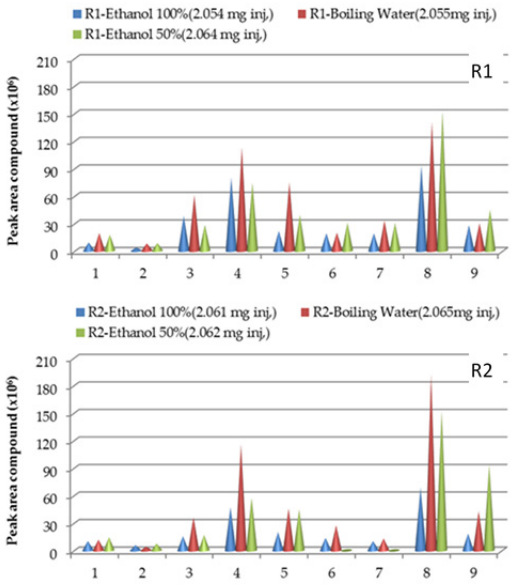

Figure 2: Concentration of the three extracts for each region, R1 and R2.

Table 3. Ratio of the concentration of each compound in different extracts.

Compounds	R1/R2 (Boiling Water extract corresponding to 2.065 mg of sample injection)	R1/R2 (Ethanol 50% extract corresponding to 2.062 mg of sample injection)	R1/R2 (Ethanol 100% extract corresponding to 2.061 mg of sample injection)
1	1.7	1.2	0.9
2	2.0	1.1	0.6
3	1.7	1.7	2.5
4	1.0	1.3	1.7
5	1.6	0.9	1.1
6	0.7	--	1.4
7	2.5	--	1.8
8	0.7	1.0	1.3
9	0.7	0.5	1.5

respectively. Correlating the relative concentration of the constituents in the more active extract, compounds **1**, **3** and **5** (see HPLC/DAD profile) were 1.7, 1.7 and 1.6 more concentrated in R1 samples than in R2 respectively. These constituents seem to be related to the increase of the bioactivity in samples from region R1. Compounds **4**, **6**, **8** and **9** showed ratios of 1.0, 0.7, 0.7, and 0.7 (lower concentrations) and compounds **2** and **7** had an increase in the concentration of 2.0, 2.5 which would be too high compared to the increase of the bioactivity of 1.6 and 1.7 (Figures 1 and 2).

Considering these ratios it was possible to speculate that compounds **1**, **3** and **5** will be responsible for this bioactivity, what is reasonable, once constituents **1** and **3** are derivatives of caffeic acid (see results of HPLC/DAD) that shown important antioxidant bioactivity in several literature reports [19,30]. Additionally, compound **5** would not be involved in this bioactivity, because for instance the ratio among the water and ethanol extract concentrations was 3.4 and the ratio among the bioactivity ranging between (0.5 and 2.3) (values for the ratio calculation in Table 1).

Compound **1** corresponds to the structure identified by Agati *et al*. [20] and Tattini *et al*. [19] named echinacoside, which is a caffeic acid derivative in the carboxylic acid function (Figure3A). Compound **3** was identified to be 7,8-di-*O*-methylherbacetin-3-*O*-R [R = Phenolic acid] (Figure 3B) [30].

The same evaluation among the other extracts was carried out and the data was beyond these values which validates the hypothesis presented above.

Compounds **6** and **7** (possible luteolin derivatives according to Tattini *et al*. [19]), in R2 extract, shown values of bioactivity similar to those in the extract from region R1 (13.5±0.2 µg/mL for R2 and 10.7±0.5 µg/mL for R1).

In the matter of the other compounds in the extracts, compound **8** has the same concentration in both extracts and compounds **5** and **9** had lower concentrations (Figures 1 and 2).

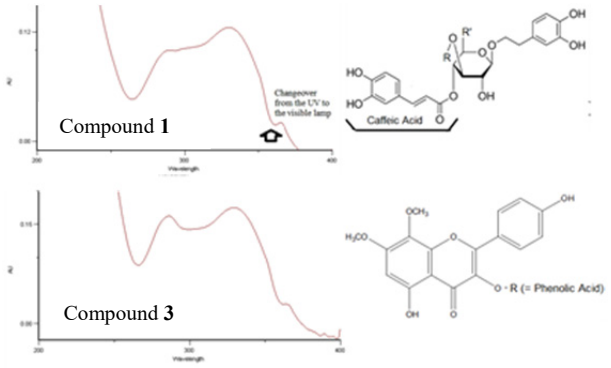

Figure 3: HPLC/DAD Profile of the extracts. A. Compound **1**: derivative of Caffeic Acid; B. Compound **3**: 7,8-di-*O*-methylherbacetin-3-*O*-R [R = Phenolic acid].

Considering these results further analyses will be carried out with compounds **1** and **4** that showed a probable involvement on the bioactivity.

Despite the data above is not being able to attribute and justify the variance on the bioactivity using only this preliminary data, this is a good pathway to be followed in further studies. Indeed, by characterizing the bioactive properties and the better extraction methodology, it will be possible to identify and quantify the most important compound or compounds related to the properties of the *L. lucidum*.

In conclusion, even though some discrepancy was observed amongst the greatest activity on DPPH and FRAP assays, the berries extracted with ethanol 50% were those that showed the best results. Highest activity was observed for berries harvested in Lisbon region and in this case probably the proximity of the sea can influence the biosynthesis of the compounds.

Results of the antimicrobial assays highlighted the potential of *L. lucidum* berries extracts for being used as adjuvants in antimicrobial therapy, mostly against antibiotic-resistant strains.

The biological activities of *L. lucidum* berries appear to be influenced by their place of origin. Further research must be carried out in order to identify the optimum climatic conditions and geographic origins for increasing the biological potential of this phytotherapic agent.

Experimental

Plant samples: The broad-leaf privet berries (*L. lucidum* A.) used in this study were acquired in Portugal in December 2016 and frozen at -20 °C until the extraction procedures were carried out. In order to study the effect of different climatic conditions on the characteristics and biological properties of the plants, samples from two regions (Lisbon, R1; and Castelo Branco, R2) were analyzed and compared.

Reagents: 2,2-diphenyl-1-picrylhydrazyl [CAS Nº 1898-66-4]; Ethanol [CAS Nº 64-17-5, purity 99.8%]; Methanol [CAS Nº 67-56-1, purity 99.8%]; Ferrous Sulphate Heptahydrate [CAS Nº 7782-63-0, purity > 98%]; Gallic acid [CAS Nº 149-91-7, purity > 98%]; Sodium Carbonate [CAS Nº 497-19-8]; Acetonitrile (HPLC grade) [CAS Nº 5-05-8, purity 99.8%]; o-Phosphoric Acid [CAS Nº 8017-16-1] was all purchased from Sigma. Folin-Ciocalteu was all purchased from Panreac

Extraction conditions: The extraction procedure was performed as previously described by Delgado *et al*. [31]. Since the antioxidant activity observed on the *L. lucidum* berries extracts was high and a small increase occurred upon increase of the extraction time, samples were extracted with 50% and 100% of ethanol following 15 hours of stirring at 120 rpm, at room temperature. After this, samples were centrifuged at 5000 rpm during 10 minutes and the supernatant was transferred to a vial and stored at -20 °C until further analysis. Concerning water extraction, the extraction method chosen was boiled water during 45 minutes. All extractions were carried out in duplicate, as well as all further measurements and analysis.

Antioxidant activity

Free-radical-scavenging (DPPH) assay: The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to the method reported by Delgado *et al*. [32]. Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6 × 10-5 mol/L). The mixture was shaken vigorously and left to stand in the dark for 1 hour. The absorption values were obtained at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation:

$$\%\ Scavenging\ effect = \left(\frac{A_{DPPH} - A_{Sample}}{A_{DPPH}}\right) \times 100 \quad (1)$$

where A_{Sample} is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The extract concentrations providing 50% inhibition (EC_{50}) were calculated from the graph of scavenging effect percentage against extract concentration.

FRAP assay: The modified FRAP assay (with incubation) was performed according to the reported by Berker *et al*. [33]. A sample extract (0.1 mL) was diluted with 0.3 mL distilled water and 3 mL FRAP reagent. The mixture was shaken and incubated at 37 °C for 20 min. Absorbance was read at 595 nm with the solutions cooled to room temperature. Aqueous solutions of known ferrous sulphate heptahydrate (FeSO4.7H2O) concentrations in the range of 1.4 × 10-4 to 1.4 × 10-3 mol/L were used to construct a standard curve.

Antimicrobial activity: The minimal inhibitory concentration (MIC) of the samples was determined against reference strains and hospital isolates: *Escherichia coli* ATCC 29998™, *Escherichia coli* ESA37, *Cephalosporins*-resistant *Streptococcus mitis* ATCC® 49456D5™, *β-Lactam* Resistant *Streptococcus mitis* ESA65, *Candida albicans* ATCC 10231™ and *Anfotericine B*-resistant *C. albicans* ESA 100.

The in vitro activity was evaluated as recommended by the Clinical and Laboratory Standards Institute Guidelines (CLSI) [34,35]. The antimicrobial activity was measured using two-fold serial dilutions ranging from 8.0000 to 0.0625 µL/mL. Time of incubation was kept the same for all inoculum used and plates were read visually. The lowest sample concentration at which reduction of initial bacterial count was 99.9%, which was considered to be the minimal bactericidal concentration (MBC). MBC was determined by plating

10 µL from the wells without microbial growth in order to classify the type of inhibition (reversible versus permanent).

Each assay was performed in triplicate in three independent occasions and the results are expressed as mean ± standard deviation.

Total phenolic quantification

Total reducing capacity: The total reducing capacity (TRC) was estimated using the microplate assay based on the Folin-Ciocalteu method described by Attard [36]. Briefly, Folin-Ciocalteu reagent was diluted tenfold and 100 µL was added to 10 µL of sample, and immediately after 80 µL of 1M sodium carbonate solution was added. Color was allowed to develop for 20 mins and the samples were measured against a sample black at 630 nm in a SPECTRAmax PLUS 384 spectrophotometer. A standard curve of gallic acid was prepared in a concentration range between 0 and 150 µg/mL in the same conditions described previously and sample concentration was determined as µg of gallic acid equivalents/mL of extract (µg GAE/mL).

Phenolic profile: The HPLC/DAD analysis was carried out in a Gilson 170 system with a Waters Spherisorb ODS2 (5 mm) (4.6 × 250 mm) column stabilized at 25 °C by using an eluent mixture of water-acetonitrile gradient with a flow rate of 0.8 ml/min. The water phase was previously acidified to pH 2.4+0.1 with o-phosphoric acid. The chromatograms were acquired at 260 and 340 nm [37,38] and spectral data was collected between 220 and 400 nm (spectral zone of the phenolic acids and flavonoids in this study). A previous screening between 220 and 600 was carried out before to check the possible presence of antocyanidins in the extract. The different compounds' structures present in the *L. lucidum* extract were determined by UV absorption spectroscopy according to Campos and Markham [38].

Statistical analysis: ANOVA was performed to assess the effects of the three extraction solvents and of the L. lucidum berries region of origin. The variance percentage was calculated and an LSD post-hoc test with 95% confidence level was applied to identify significant differences between samples ($p<0.05$). The statistical analysis was performed on Statistica 7.0 software. A factorial variance analysis was performed to assess the effects of the region and extractive method as fixed factors. The factorial design was performed with two levels. For each significant effect or interaction the variance percentage was calculated and a Least Significant Difference (LSD) test post-hoc test with 95% confidence was applied to the corresponding variables.

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Accounts/Reviews

Natural Product Communications
2018
Volume 13, Number 12

Contents

Continued inside backcover