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Full Length Research Papers

Evaluation of the antibacterial activity of the essential oil and antioxidant activity of aqueous extracts of the *Eucalyptus globulus* Labill. leaves

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The purpose of this study was valorizing *Eucalyptus globulus* Labill. (*Myrtaceae*) residues in terms of phenolic compounds extracted to aqueous extract and essential oils extract to leaves. The leaves were studied are collected in Gardunha Mountains near Alpedrinha (Portugal). Essential oil was extracted of leaves in Clevenger apparatus and the hot-water was used for tested the phenolic content. The essential oils were obtained and analyzed by GC-MS and tested for antibacterial activity using a *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. These tests were performed using two different techniques: agar diffusion with cylindrical cavities and vapor diffusion in agar plate. The aqueous extract is submitted to study antioxidant activity. This way the phenolic content was determined by the Folin-Ciocalteu method, flavonoid content was also determined by colorimetric method and antioxidant activity was measured by DPPH method. These results show that *E. globulus* leaves have a high amount of compounds derived from monoterpene allowing show good antibacterial activity. For other side the aqueous extract has a very strong antioxidant activity and been possible to identify six components by HPLC-DAD: gallic, caffeic and ellagic acids, rutine, luteolin and quercetin. To our knowledge this is the first report on flavonoid content, antioxidant activity and composition of the aqueous phase from *E. globulus* leaves.

Keywords: *Eucalyptus globulus*; leaves; aqueous extract; oil composition; antimicrobial activity; phenolic content; antioxidant activity.

INTRODUCTION

Eucalyptus is an evergreen tall tree, native to Australia, effectively introduced worldwide, now extensively cultivated in many other countries including Portugal.

Eucalyptus is a large genus of the *Myrtaceae* family that includes about 900 species and subspecies (Tyagi and Malik, 2011). This characterized for the sources of biologically active terpenoids, tannins, flavonoids and phloroglucinol derivatives (Ito et al., 2000).

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In Portugal, the planting of *Eucalyptus globulus* occupies about 20% of the forest area and is mainly used by the pulp industries, as source of cellulosic fiber, but some parts of the plant (principally leaves and bark) continue to be rejected by the paper industry (Silvestre et al. 1997).

Each time that the paper industry let leaves in the forest increases the fire risk allowing this way the destruction of forest, animal life and the flora

The essential oil of *eucalyptus* is commonly used in traditional medicine because of its expectorant and balsamic activity (Della Porta et al., 1999). The leaves could also be a promising source of phenolic compounds, which can be used for possible applications in food, pharmaceutical and cosmetic industries.

Because of their antioxidant activity leaf extracts of *E. globulus* have been proposed as food additives. Therefore, this species might be a good candidate for further development as a nutraceutical. However, detailed information has not been published about the phenolic composition of leaves of this species. In other works (Boulekbache-Makhlouf L et al., 2010), reported the characterization of 55 phenolic constituents in fruits of *E. globulus*, including gallic acid, hydrolysable tannins and flavonols using extracts with acetone/water (70:30 v/v) and 0.5% acetic acid. The study on the phenolic content of the leaves of *E. globulus* were performed by (Boulekbache-Makhlouf L et al., 2013) which reported the existence of 39 phenolic compounds, including 26 compounds that have not previously been detected in leaves of *E. globulus*, extracted with 70% acetone containing 0.5% acetic acid. It was detected gallic, ellagic and methylellagic acids, eucaglobulin, quercetin derivatives and others.

We have studied the secondary metabolites of wood and bark, where pentacyclic triterpenes were found, some esterified with *E,Z p*-methoxycinnamic acids. Of acetone extract of bark were isolated, by dissolution in benzene and chromatographed on column, the following products: vomifoliol, 2,6-dimethoxy-*p*-benzoquinone and acetates of 3,4,5-trimethoxyphenol and 2,4,6-trimethoxyphenol (Santos et al., 1997).

Other researchers (Santos et al., 2011) conducted the study of phenolic compounds in bark extract *E. globulus* using dichloromethane, and subsequently separate it into two fractions, one of methanol and other of water. In this work it was possible to detect the presence of 29 compounds belonging to the classes of flavonoid glycosides, ellagic acid and derivatives, galloylglucose derivatives and ellagittannins.

Phenolic compounds are a group of plant secondary metabolites that are synthesized during normal development and in response to stress such as infection, wounding and UV radiation (Boulekbache-Makhlouf L et al., 2013).

Phenolic compounds are usually associated with health beneficial features, primarily due to their application as antioxidant, such as its use in decrease of the risk of cardiovascular diseases, this compounds include several classes, such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and tannins. These compounds have attracted a great deal of scientific interest because of their biological activities (Santos et al., 2012).

Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plant may contribute directly to their antioxidant actions.

Within a plant species the variation of the composition and yield of essential oil in the leaves might be attributed to three major factors (Silvestre et al., 1997): genetics, leaf age and type of environmental (e. g. the soil and weather). These factors usually cause complex variation patterns in the oil composition.

In this work was studied the composition of water extraction and antioxidant activity, the oil composition and the inhibitory effect against three microorganism.

MATERIALS AND METHODS

Raw material

E. globulus leaves were collected since March a June of 2013, in Gardunha Mountains, near Alpedrinha, Portugal.

The plant material was air dried for 10 days and stored at ambient temperature (25±1°C) without exposure to direct sunlight.

Extraction method

The essential oils were prepared by hydrodistillation (100 g of leaves), using a standard apparatus recommended in the European Pharmacopeia during 2 hours.

The aqueous extract was obtained after to hydrodistillation; the water was separated from solid waste by filtration and preserved.

Chemical analysis of essential oils

GC

The oils were analyzed on Agilent Technologies 7890A GC-System apparatus equipped with a DB5-MS fused silica capillary column, 30 mx0.25 mm i.d., film thickness 0.25 µm of polydimethyl siloxane (J&W LTM Column Module). The initial column temperature is 60 °C (5 min), rising from 60 to 250 °C at 10 °C/min, and the final temperature is 250 °C (20 min); injector temperature: 250 °C; carrier gas: He (1 mL/min). The injected volume was 1 µL.

The relative concentration was calculated using the *Chemstation Software*, which allowed the assimilation of the percentages of the peak areas to the percentages of the various constituents and the NIST Mass Spectral Software.

GC-MS

Samples were analyzed by GC-MS on Agilent Technologies 5975C, Inert XL MSD with Triple-Axis detector using same experimental conditions as described in section 2.4.1. The mass spectrometer operating conditions were: ionization voltage, 70 eV; ion source 230 °C.

Antibacterial activity

Characteristics of the strains and culture medium

The microorganisms used in these tests were obtained from American Type Culture Collection (ATCC), distributed by Culti-loop® (OXOID LTD.). The selected strains were *Staphylococcus aureus* (ATCC 25923/Lot 902 840), *Escherichia coli* (ATCC 25922/Lot 931 370) and *Pseudomonas aeruginosa* (ATCC 27853/Lot 931 372), kept at 2-8 ° C. Working cultures were obtained from strains original.

The culture medium was a Muller Hinton agar (MH2-Ref. 43301; BioMérieux SA). This commercial medium has 90mm in diameter and 4mm thickness.

Inoculum preparation

The standardization of the inoculum was performed once with the same density affects the results of screening. The isolated colonies were diluted in sterile 85% NaCl saline physiology. To ensure the concentration of colonies, based on measuring the turbidity of 0.5 McFarland scale, thereby resulting in a concentration of 108 CFU / ml approximate. The wavelength of 550 nm ($T \sim 74.9\%$ A~0125) for such a densimeter DENSIMAT (BioMérieux Code 99234) was used. Subsequently, dilutions and addition of colonies ensured the optimal concentration for the screening of bacterial.

Diffusion in agar plate with cylindrical cavities

The plates were prepared using a metal cylindrical rod of 5 mm diameter and 4 mm in depth in order to create the deposition of oil wells ; 3 wells per plate , were placed evenly apart . As also performed with the control board 2 antibiotics, Penicillin G (0.05 mg / ml) and Gentamicin (10 mg / ml) , and a solvent DMSO; The reference plates , which were pure oils 3, 1,8-cineole, β -Pinene and limonene were performed for each microorganism.

To perform the tests 70 μ L of crude oil were added to each well, allowed to stand for 15 minutes and then incubated for 24 hours at 37 ± 1 ° C. (Ostrosky et al., 2008).

Vapor diffusion in agar plate

Plates of inoculum were prepared as in point 2.4.2 and then reversed, making the top of the base plate. The amount of oil was placed at the same point 2.4.2. The plates were incubated for 24 hours at 37 ± 1 ° C (Lisin et al., 1999).

Total phenols

Total phenol (TP) concentration was measured by the colorimetric Folin–Ciocalteu assay (Swain and Hillis 1959) using a UV–Vis spectrophotometer, model Evolution 160 (Thermo Fisher Scientific, Madison, USA) at a wavelength of 765 nm. TP concentration was calibrated ($R^2 = 0.992$) using standard methanolic solutions of gallic acid (50–500 μ g/mL), and expressed as milligrams of gallic acid equivalent per gram of dry biomass (mgGAE/gDB) by means of the linear relationship:

$$ABS_{765} = 0.0019TP + 0.0125 \quad (1)$$

Analyses were performed in triplicate.

Total flavonoids

The total flavonoids (TF) content of the extracts was determined using the colorimetric method described by (Latoui et al., 2012) of deionized water, we added 0.075 mL of 5% sodium nitrite solution; after 5 min of reaction 0.15 mL of 10% aluminum chloride; after additional 6 min 0.5 mL of 1.0 M NaOH, and finally deionized water into a final volume of 3 mL. The absorbance of the mixture was determined at 415 nm using the same spectrophotometer as above. Analyses were performed in triplicate. The calibration straight line ($R^2 = 0.999$) was made using standard methanolic solutions of quercetin in the range 12.5–200.0 μ g/mL:

$$ABS_{415} = 0.0056TF + 0.0072 \quad (2)$$

Antioxidant activity

The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability by means of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Brand-Williams et al., 1995) which is widely used to describe the antiradical power of different matrices (Aliakbarian et al., 2009). For each sample, seven different dilutions ranging from 50.0 to 1500.0 μ g/mL in methanol were prepared. For each extract, 0.10

mL of diluted sample was mixed with 3.90 mL of DPPH• methanolic solution (0.10 mM). The reaction mixtures were shaken and incubated for 90 min in the dark at room temperature, and then the absorbance was read at 517 nm using the same spectrophotometer as above. The inhibitory activity was calculated and the IC₅₀ (concentration of the extract able to inhibit 50%) was calculated graphically with the concentration of the extract versus the corresponding inhibitory effect (% I). The antioxidant activity was expressed as the antioxidant activity index (AAI) Analyses were performed in triplicate. ($R^2=0.996$).

$$ABS_{517} = 0.0248DPPH + 0.0132 \quad (3)$$

HPLC–DAD analysis

The samples were analyzed by HPLC (Merck-Hitachi Elite Lachrom) on a reverse phase C-18 Purospher® Star (Merck) column (particle size 5 µm, 4 × 250 mm), thermostated at 25 °C, according to an adaptation of a method described elsewhere (Santos et al., 2013). The identification of polyphenols compounds was carried out using a photodiode array detector with a variable working wavelength between 220 and 600 nm, to set λ_{max} used for quantification. The mobile phase used two eluents: A (H₂O–CH₃COOH at (999:1; v/v)) and B (CH₃OH–CH₃COOH at (999:1; v/v)). Before starting the analysis, the chromatographic column was washed with 100% B and then stabilized with an initial mobile phase up to five minutes. The gradient profile used was A:B (10:90) up to 5 min, changed to A:B (50:50) for 30 min and held for 8 min; changed to A:B (10:90) for 1 min and held for 16 min. The flow rate was 1.0 mL/min and the sample injection volume (filtered through a 0.45 µm filter) was 20 µL. All of these solutions were prepared with water purified by a Milli-Q water system (Millipore, Billerica, USA) using reactants and solvents (HPLC grade) supplied by Sigma–Aldrich.

RESULTS AND DISCUSSION

Chemical composition analysis

In the present study, *E. globulus* essential oil showed the presence of 22 components in 15 samples (collected in different months), which formed 100% of the total oil composition with 1,8-cineole, pinocarvenol, α-Pinene, alloaromadendrene, globulol, L-Pinocarveol, Aromadendrene and pinocarvone as major component (table 1). The oil contained monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes.

The 1,8-cineole content of *E. globulus* oil reported in the literature varied between 18.566 and 89.95% while the α-Pinene represent 0.70%-14.15% 0.70%-14.15% (Tyagi

and Malik, 2011; Silvestre et al., 1997; Kumar et al., 2012; Jimenez-Carmona and Luque de Castro, 1999; Cimanga et al., 2002; Baranska et al., 2005; Vilela et al., 2009; Elaissi et al., 2012; Song et al., 2009; Ait-Ouazzou et al., 2011). This wide variation in chemical composition of oil could be attributed to environmental and agronomic factors as well as on extraction procedure (Jimenez-Carmona and Luque de Castro, 1999).

Antibacterial activity

Antibacterial activity of diffusion in agar plate with cylindrical cavities

The results of antibacterial screening are obtained by the measurement of the inhibition halo observed in mm (zone of inhibition) for the each different oil obtained. In figure 1 and 2 are represented a zone of inhibition the sample and references. The comparison with weak, moderate and strong it does following the literature (Ostrosky et al., 2008).

In the diffusion in agar plate with cylindrical cavities, the extractions tested showed inhibition extractions tested showed a level of inhibition between moderate and strong against *S. aureus*, particularly extracting the month of March and April, with 38 and 37 mm, respectively, being still the oil with a moderate inhibition collected in the month of July with 21 mm.

Against *E. coli* all samples showed a moderate inhibitory effect, ranging from 10 to 25 mm, where the sample with more effect is, again March, 25mm, but the minimum value of inhibition is the month of May with 10 mm.

When tested against *P. aeruginosa* all samples showed a very weak inhibitory effect, only the oil of the month of March shows a stronger inhibition, but still weak, with 13 mm.

Finally, when analyzing the results obtained of references is possible to verify that 1,8-cineol has a weak inhibitory effect against *S. aureus* (14 mm), moderate effect against *E. coli* (18 mm) and against *P. aeruginosa* has no inhibitory effect; β-pinene has a very weak inhibitory effect against *S. aureus* (8 mm), moderate effect against *E. coli* (15 mm) and against *P. aeruginosa* has no inhibitory effect; ; limonene has a moderate inhibitory effect against *S. aureus* (18 mm), strong effect against *E. coli* (33 mm) and against *P. aeruginosa* has no inhibitory effect.

Antibacterial activity of vapor diffusion test in agar plate

The vapor diffusion in agar plate has as showed interesting results in in each month: in April only has a strong inhibition against *E. coli* (37 mm); May has inhibition against *S. aureus* and *E. coli*, respectively 25

Table 1. Chemical constituents of *E. globulus* leaves essential oil.

		March	April	May	June	July
Retention time	Compound	Percentage				
6.7	α -Pinene	2.77	2.79	5.53	7.32	
8.8	p-Cymene	0,71	0.50	0,47		
8.9	Limonene	0,43	0,19	0,95	1,43	
9.0	1,8-Cineole	82.6	84.7	71,7	80,5	89.0
11.0	Trans-pinocarveol	3,95	1.5		1,73	2,84
11,1	L-pinocarveol	2,78	3,35	4,42		
11.2	Pinocarveol					4,17
11.5	Pinocarvone	1,42	2,44	3,21	0,95	1,74
11.8	Pinocamphone		0.75	0,55	0,29	
12.0	α -Terpenol			0,69	1,08	
12.5	Trans-Carveol			0,16		
12.8	Verbenone			0,18		
14.4	α -Terpenyl acetate	0,3		1,12		
15,3	α -Gurjunene			0,13	0,31	
15,7	β -Gurjunene			0,12	0,10	
16.0	Aromadendrene	2,7	0,23	0,39	0,59	
17,5	(+) Aromadendrene		1.15	1,86	2,92	
17.6	Globulol			5,19	0,27	2,25
17.7	Viridiflorol			3,08		
18.3	β -Eudesmol			0,38	0,73	
19.1	β -Terpenyl acetate	0,77	0,53		0,49	

Table 1. Continue

22.3	Alloaromadendrene	1,63	1.30		1,30
<hr/>					
	Monoterpene hydrocarbons	3,91	3,48	6,95	8,75
<hr/>					
	Oxygenated monoterpenes	91,82	93,27	82,03	85,04 97,75
<hr/>					
	Sesquiterpene hydrocarbons	4,33	2,68	2,5	5,22 0
<hr/>					
	Oxygenated sesquiterpenes	0	0	8,65	1 2,25
<hr/>					

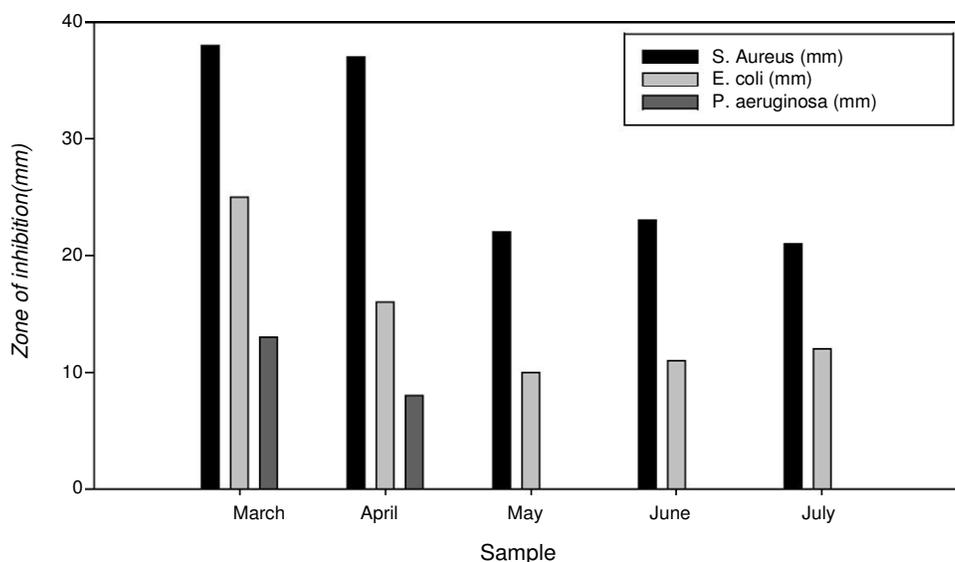


Figure 1. Inhibition halos - Diffusion in agar plate with cylindrical cavities

(moderate) and 32 mm (strong); June has inhibition against *S. aureus* and *E. coli*, respectively 25 (moderate) and 30 mm (strong); July has inhibition against *S. aureus* and *E. coli*, respectively 25 (moderate) and 30 mm (strong). All samples have no inhibition against *P. aeruginosa*, as showed in figure 3.

Total phenols

The total phenolic content in the examined water extraction using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid. Using equation (1) it was obtained a TP value of 0.389 mg of gallic acid/ mL methanolic

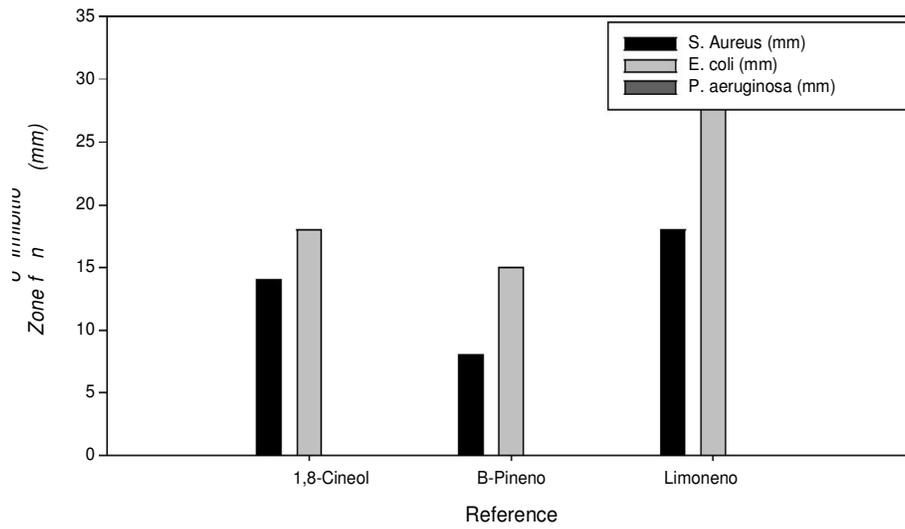


Figure 2. Zone of inhibition of the reference and the control using a diffusion in agar plate test with cylindrical cavities

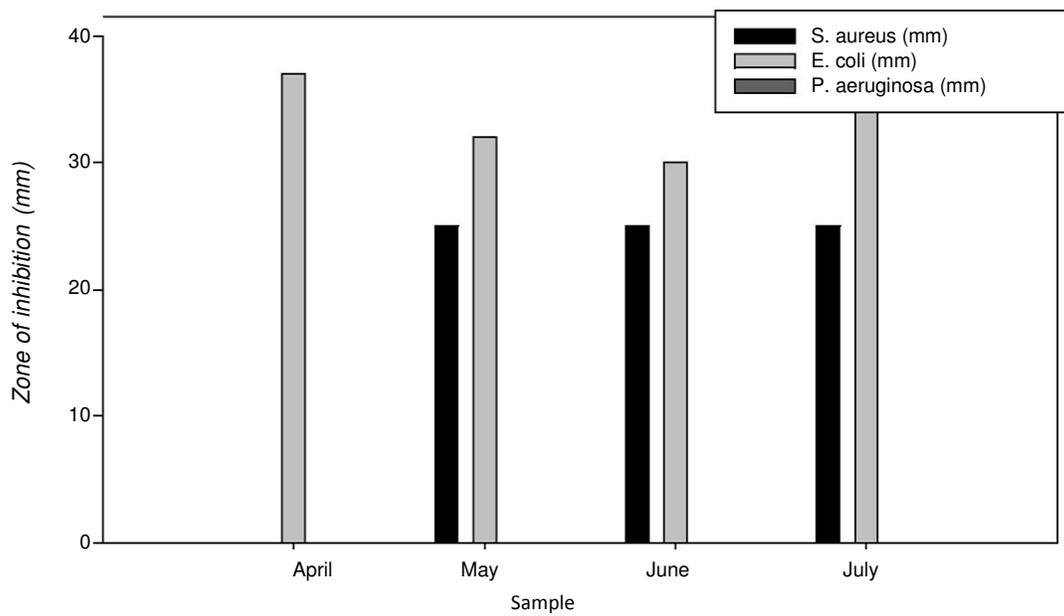


Figure 3. Zone of inhibition of samples using a vapor diffusion test in agar plate

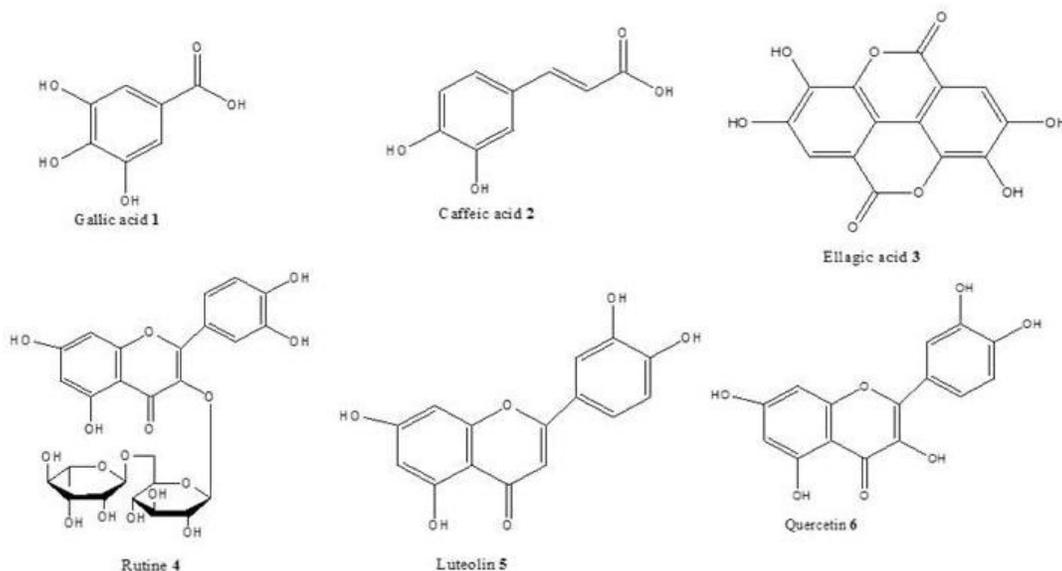


Figure 4. Structures of the phenolic compounds identified in *E. globulus* leaves aqueous extra

Table 2. Total phenols, flavonoids and antioxidant activity of aqueous extract from *E. globulus* leaves

Total phenols content (mg GAE/g DB)	Total flavonoids content (mg QE/g DB)	IC ₅₀ (µg/mL)	AAI
67.92 ± 2.39	30.98 ± 1.58	426.8	84.4

Values are averages ± standard deviation of triplicate analysis

solution, with the dry biomass obtained (0.0057g dry biomass/mL sample) the total phenols value is 67.92±2.39 mg GAE/gDB, as we can see in table 2.

Comparing the obtained results with recent published works, as it is the work of (Pereira et al., 2014), it is verified that the aqueous extract presents the highest amount of total phenols.

Total flavonoids

The concentration of flavonoids in water extraction was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent. Flavonoids were presented in small amounts compared to the

phenols.

Using equation (2) was obtained a TF value of 0.178 mg of quercetin / mL methanolic solution, with the dry biomass obtained (0.0057g dry biomass/mL sample) the total phenols value is 30.98±1.58 mg QE/gDB (see table 2).

The study of flavonoids in aqueous extract from this specie of *Eucalyptus* is not to be studied, as it is with extract using other solvents. However, we can compare the results obtained with studies in other *Eucalyptus* species, such as *Eucalyptus gilli*. In study (Hassine et al., 2012) was achieved to a value of flavonoids to 14.4 mg QE/kgDB in aqueous extract. Therefore it can be stated that the aqueous extract is the most suitable for the extraction of phenolic and flavonoids compounds.

Table 3. Calibration data used for the HPLC-DAD quantification of phenolic components from aqueous extract of *E. globulus* leaves

Compound n°.	Compound	$\lambda_{\text{máx}}$ (nm)	conc. range ($\mu\text{g/mL}$)	calibration curve	R^2
1	Gallic acid	271	1-50	$y=305460x-51463$	0.999
2	Caffeic acid	324	1-50	$y=1000000x-571716$	0.999
3	Ellagic acid	254	1-50	$y=437111x-340808$	0.998
4	Rutine	257	1-50	$y=1066434x-103535$	0.998
5	Luteolin	350	1-50	$y=317306x-101326$	0.999
6	Quercetin	255	1-50	$y=400481x-708491$	0.998

Antioxidant activity

A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules eliminate DPPH free radicals.

Analyzing seven samples it was found that the sample with concentration 1500 $\mu\text{g/mL}$ showed the highest value of %I with 95.36%, the sample with the lowest value of %I is a sample with concentration 50 $\mu\text{g/mL}$ with 3.72%. The values of %I obtained for each concentration constructed a linear relationship with equation (3); this equation allowed calculating a value of IC_{50} , which were 426.8.

The antioxidant activity of aqueous extract from leaves of *E. globulus* using DPPH test showed an IC_{50} of 426.8 $\mu\text{g/mL}$.

Because of their antioxidant activity, the leaves of *E. globulus* have been proposed as food additives (Amakura et al., 2009).

HPLC–DAD analysis

The HPLC–DAD identification of phenolic compounds was carried out by comparing peak retention times and UV spectra with reference compounds run under the same experimental conditions. The phenolic compounds identified in the water extract and the calibration data used Table 3 quantify them are reported in Table 2, the corresponding structures are shown in Figure.1. The components identified and quantified were gallic (21.72 $\mu\text{g g/mL}$), caffeic (8.19 $\mu\text{g g/mL}$) and ellagic (49.47 $\mu\text{g g/mL}$) acids, rutine (3.92 $\mu\text{g g/mL}$), luteolin (55.06 $\mu\text{g g/mL}$) and quercetin (50.24 $\mu\text{g g/mL}$) and the results correspond to the average value estimated from injection of three

aliquots analyzed in triplicate with a standard deviation are < 5%.

Plants as *E. globulus* with higher content of quercetin and luteolin should be considered as important sources of bioactivitie (Pereira et al., 2014).

CONCLUSIONS

In summary, the results presented here contribute to the knowledge of chemical composition and the effects antibacterial activity against same bacterial strains obtained from *E. globulus*.

The method proposed for the isolation of *Eucalyptus globulus* essential oil, hydrodistillation with a Clevenger apparatus is quicker, cheaper and effective and the most important is not harmful to the environment.

E. globulus having a good composition of constituents, but its composition varies from month to month, thus giving notice that the height at which the plant produces more or less oil.

The same observation can be given with respect to antibacterial testing where it can be seen that the influence of the oil composition, of yet unknown way, the inhibition effect against certain bacterial strains.

After analyzing all results is possible, say that the diffusion with cavities and the vapor diffusion are very different. The most important difference is the inhibitory effect against *E. coli* and *S. aureus*. Since the diffusion test cavities is greater inhibition against *S. aureus* and against a weak/ moderate inhibition against *E. coli*.

To our knowledge the flavonoid contents, antioxidant activity and phenolics profiles of the aqueous extract from leaves of *E. globulus* were determined by HPLC–DAD and their quantification were reported here for the first time.

It was possible to observe that the hot-water extract has constituted a great amount of phenolic compounds, thus resulting its high content in total phenols (67.92 ± 2.37 mg GAE/g DB) and flavonoids (30.98 ± 1.58 mg QE/g DB). Because of its high composition of phenolic compounds, their antioxidant activity it becomes of considerable interest since it is quite high compared to other plants. It was thus detected high antioxidant activity by the DPPH method ($IC_{50} = 426.8$ mg/L).

In the HPLC analysis we can observe six compounds, of which only two (ellagic and gallic acid) had already been previously identified by other studies conducted on leaves. To the knowledge, the remaining four (caffeic acid, rutine, quercetin and luteolin) never are found in the *Eucalyptus globulus* Labill. leaves.

The pulp and paper industries, in Portugal, produce considerable amounts of residues such as leaves from *E. globulus* that, if they are left in the forest, could be a problem because of the fires. All this biomass could be a source of interesting compounds, which can be used for possible applications in food, pharmaceutical and cosmetic industries. The extraction with water is a safety and economical way to extract these compounds.

This way we can take advantage of all the biomass in the forest for use in several areas and protected the forest.

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