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Chemical and Functional Characterization of Extracts from Leaves and Twigs of *Acacia dealbata*

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Abstract: The purpose of this work was to evaluate the recovery of bioactive extracts from *Acacia dealbata* leaves and twigs and to characterize their chemical composition and functional properties. Fresh and air-dried samples were extracted by maceration at room temperature and by hot extraction at 60 °C using aqueous solutions of acetone, ethanol, and methanol. The highest extraction yields (14.8 and 12.0% for dried leaves and twigs, respectively) were obtained with 70% acetone, for both extraction procedures. Extracts were characterized for total phenolics content (TPC), total flavonoid content (TFC) and total proanthocyanidin content (TPrAC). Bioactive extracts with high TPC (526.4 mg GAE/g extract), TFC (198.4 mg CatE/g extract), and TPrAC (631.3 mg PycE/g extract) were obtained using maceration, a technically simple and low-energy process. The non-polar fraction of selected extracts was characterized using gas chromatography and time of flight mass spectrometry (GC-TOFMS). The main components detected were phytol, squalene, α -tocopherol, lupenone, and lupeol. The antioxidant activity of the extracts was characterized through DPPH and FRAP assays. Antimicrobial activity of the extracts against different bacteria was also determined. The highest DPPH and FRAP activities were obtained from dried twigs from Alcobça (1068.3 mg TE/g extract and 9194.6 mmol Fe²⁺/g extract, respectively). Extracts from both leaves and twigs showed antimicrobial properties against *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus mutans*, and *Streptococcus mitis*. The results obtained demonstrate the feasibility of recovering valuable components from these biomass fractions that may be further valorized for energy production in a biorefinery concept.

Keywords: *Acacia dealbata*; leaves; twigs; antioxidant; antimicrobial; invasive species



Citation: Correia, R.; Duarte, M.P.; Maurício, E.M.; Brinco, J.; Quintela, J.C.; da Silva, M.G.; Gonçalves, M. Chemical and Functional Characterization of Extracts from Leaves and Twigs of *Acacia dealbata*. *Processes* **2022**, *10*, 2429. <https://doi.org/10.3390/pr10112429>

Academic Editor: Maria Angela A. Meireles

Received: 3 October 2022

Accepted: 12 November 2022

Published: 17 November 2022

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1. Introduction

The genus *Acacia* comprises more than 1350 species distributed throughout tropical and warm temperate areas of the world [1]. Most of those species are native to Australia but spread all over the world due to a wide variety of useful applications such as sand and dunes stabilization, extraction of tannins, essences or gums, valorization as timber or fodder crop, and production of biofuels [2–4]. The ability to easily adapt to changing environments, the large seed production and accumulation of massive seed banks for long

periods, the high capacity for reproduction after fires and cuttings, and the allelopathic properties are some of the characteristics that contribute to the success of *Acacia* spp. as invaders, leading to negative impacts on ecosystem biodiversity [5–7].

A. dealbata is one of the most widespread *Acacia* species [7,8] and is classified as invasive in Portuguese territory [9]. Periodic removal operations to minimize its proliferation generate high amounts of biomass that is usually burned for energy production or landfilled, but these solutions are not economically sustainable due to the high costs of biomass collection and transportation. However, different fractions of *Acacia* spp. biomass have been evaluated as raw materials to produce functional extracts that can be used in the nutraceutical, cosmetic, or food industries. In fact, it is widely documented that bark, wood, leaves, flowers, pods, seeds or roots of *Acacia* spp. are rich in bioactive secondary metabolites (e.g., amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, gums, non-protein amino acids, terpenes, tannins and other flavonoids, and simple phenolics) [4,10] and have been used in traditional medicine for a wide range of ailments, such as diabetes, worm infection, dysmenorrhea, eczema, malaria, gout, jaundice, abdominal pain, kidney problems, constipation, leprosy, piles, pneumonia, rheumatism, fever, and cancer [11].

Plant extracts are known for their ability to act as antioxidants and reduce oxidative stress [12], a physiologic condition considered to play a key role in the pathogenesis of several degenerative diseases, such as cardiovascular diseases, diabetes neurodegeneration, or cancer [13]. Additionally, it has been reported that plant secondary metabolites also possess antimicrobial properties, which is important in the development of alternatives to antibiotics due to the increasing resistance to conventional antimicrobial agents [14]. Therefore, the production of bioactive extracts from *A. dealbata* biomass is an additional pathway for the valorization of these biomass materials, complementing energy applications and contributing to the sustainability of the forest cleaning and management actions, reducing the risk of fire, and improving the social-economic development of rural areas.

Extraction of value-added components from *Acacia* spp. has been focused mainly on the bark, flower, wood, and leaves as reviewed by Correia et al. [15]. Concerning the leaves fraction, antioxidant or antimicrobial activities have been determined for *A. farnesiana* [16], *A. karroo* [17–20], *A. longifolia* [21], *A. pycnantha* [22,23], *A. saligna* [24–27], or *A. nilotica* [28]. The antioxidant and antimicrobial activities of *A. dealbata* leaves were evaluated by Borges et al. [29], for acetic and ethanolic extracts of fresh *A. dealbata* leaves, obtained with different extraction methods. Ethanolic extracts of dried *A. dealbata* leaves were also found to have antimicrobial activity against the food poisoning agent *Bacillus cereus* [30].

Research on extraction of functional components from twigs of *Acacia* spp. is scarce. Extracts of *A. nilotica* twigs were characterized for antimicrobial activity against oral pathogens [31–34], while extracts of *A. pennata* twigs were described as having some potential application in the prevention of Alzheimer's disease [35].

The extraction methods explored so far included solid-liquid extraction for a pre-determined period under agitation at room temperature (1 h [29], 8 h [34], or 2 days [30,33]) ultrasound-assisted extraction [29], Soxhlet [29,35], microwave-assisted extraction [29], cold percolation [31], and supercritical fluid extraction [32,36]. Most of these methods are energy-consuming or involve high investment costs and may promote thermal degradation of the extract components [37].

This study aimed to investigate the production of bioactive extracts from leaves and twigs of *A. dealbata*, using maceration at room temperature, a method with low energy requirements and easy to implement on an industrial scale. Extraction with different aqueous solvents (acetone, ethanol, and methanol) was applied to fresh and dried biomass, collected in two different locations to evaluate the influence of solvent polarity, biomass water content, and geographic origin on the characteristics of the extracts, namely their yield, composition, and properties. To the best of our knowledge, this is also the first study on the production and characterization of bioactive extracts from *A. dealbata* twigs. The

extracts were characterized for mass yield, total phenolic content, total flavonoid content, total proanthocyanidins content, in vitro antioxidant activity tests, and antimicrobial activity against several bacteria and yeasts. To identify non-polar components that might contribute to the antioxidant or antimicrobial properties of the extracts, a representative set of leaves and twigs extracts were fractionated and characterized by GC-TOFMS.

2. Materials and Methods

2.1. Plant Material

Branches of at least ten different *A. dealbata* trees of Caparica (CAP) and Alcobaça (ALC) regions were collected. Samples of around 500 g of branches were collected from each tree and combined to obtain a composite sample from that geographical origin. For each composite sample, leaves were separated from the twigs and both fractions were analyzed separately. Fresh and dried leaves and twigs were manually cut into small pieces about 1 cm in length before impregnation with the solvent.

2.2. Chemicals and Reagents

Acetone, ethanol 96°, methanol, and petroleum ether used in the extraction processes were purchased from CarloErba Reagents (Val de Reuil, France). Folin-Ciocalteu reagent (Panreac, Barcelona, Spain), sodium carbonate (Labkem, Barcelona, Spain), and gallic acid (Merck, Darmstadt, Germany) were used to determine phenolics content. The reagents sodium nitrite (NaNO₂), sodium hydroxide (NaOH), and aluminium chloride (AlCl₃) used in the determination of flavonoids were all purchased from Merck (Darmstadt, Germany). Butanol (Panreac, Barcelona, Spain), ferrous sulfate heptahydrate (FeSO₄·7H₂O) (Panreac, Barcelona, Spain), hydrochloric acid (Chem-Lab NV, Zedelgem, Belgium), and Pycnognol® (generously provided by Horphag Research Ltd, Geneva, Switzerland) were used in the acid-butanol assay. The reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) was used in the DPPH assay. The reagents 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), acetate buffer 3.6, iron (III) chloride (FeCl₃), and iron (II) sulfate (FeSO₄) used in the ferric reducing antioxidant power (FRAP) assay were also purchased from Merck (Darmstadt, Germany). Ketoconazole, ofloxacin and vancomycin were purchase from Sigma-Aldrich (St. Louis, MO, USA). Brain heart infusion agar (BHIA) was purchased from Merck (Darmstadt, Germany). Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA) and Trypto casein-soy agar (TSA) were purchase from Biokar diagnostics (Allonne, France).

2.3. Extraction Procedure

A portion of leaves and twigs was processed in a fresh state, during the first 24 h from harvest, while the remaining biomass was left to dry in air, at room temperature, until a constant weight was attained. Two extraction methods were compared with the fresh and dried raw material: maceration at room temperature for 48 h in the case of the leaves and 24 h for the twigs; extraction times were selected for leaves and twigs as the maceration time necessary to achieve constant values of extract yield and extract composition according to a previous study (Supplementary Materials; Table S2). Hot extraction was performed at 60 °C for 1 h, for both leaves and twigs, to evaluate the effect of temperature in extract yield and properties. Higher temperatures were not selected to avoid thermal degradation of the extracts. Maceration and hot extraction were performed in a single step, at a biomass:solvent ratio of 1:6 (w:v), using 70% of aqueous acetone (70% ACE), 70% aqueous ethanol (70% ET), and 70% of aqueous methanol (70% MET) as extraction solvents. After the extraction period, the liquid extract was separated from the biomass by filtration and stored at −20 °C until analysis. An adequate aliquot was evaporated to dryness at reduced pressure to determine the mass yield (expressed as percentage, on a wet basis). The extraction process was made in duplicate for each one of the four biomass samples of leaves and twigs (fresh and dried material, from Caparica and Alcobaça).

Extraction of spent raw material was also assessed after the removal of lipophilic components by petroleum ether. Dried leaves and twigs were extracted with petroleum ether by maceration at room temperature using a biomass:solvent ratio of 1:5, and the lipophilic extracts were stored for subsequent analysis and not included in this study. The spent biomass was left to dry in air at room temperature and then submitted to extraction of polar components by maceration at room temperature with 70% ACE at the same conditions that were used for the raw leaves and twigs. Mass yields were determined as described above, and the extracts were stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.4. Extracts Characterization

Total phenolic content (TPC) of the extracts was measured by the Folin-Ciocalteu method, following an adaptation of the method described by Singleton et al. [38]. Briefly, 0.5 mL of extract, 2 mL of distilled water, and 0.5 mL of Folin-Ciocalteu reagent were added to a test tube and allowed to stand for 5 min. Then, 2 mL of aqueous sodium bicarbonate (10% m/V) were added, and the mixture was incubated for 1 h in the dark. Absorbance was then measured at 760 nm and total phenolic content was determined using a calibration curve constructed with gallic acid standards. Results were expressed as gallic acid equivalents (mg GAE/g of dry extract, and mg GAE/g of fresh biomass).

Total flavonoid content was determined as described by Barros et al. [39]. Briefly, 0.5 mL of extract was mixed with 2 mL of distilled water and subsequently with 0.15 mL of NaNO_2 solution (5%). After 6 min, 0.15 mL of AlCl_3 solution (10%) was added and allowed to stand further 6 min. After this period 2 mL of NaOH solution (4%) was added to the mixture, and distilled water was then added to bring the final volume to 5 mL. Then, the mixture was completely mixed and allowed to stand for 15 min. Finally, absorbance was measured at 510 nm (Biochrom Libra S4). The results were expressed in catechin equivalents (mg CatE/g of dry extract, and mg CatE/g of fresh biomass). A calibration curve was also made using rutin as a standard for comparison of the results with the ones found in the literature expressed in rutin equivalents (mg RE/g of dry extract, and mg RE/g of fresh biomass).

Total proanthocyanidin content (TPrAC) was determined by the butanol-HCl assay, exactly as described by Skerget et al. [40]. Briefly, 5 mL of an acidic ferrous solution (77 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 500 mL HCl/BuOH (2/3)) was added to 0.5 mL of the extract. The tubes were covered and put in a water bath at $95\text{ }^{\circ}\text{C}$ for 15 min. The absorbance was read at 540 nm (Biochrom Libra S4). The same procedure was made using a proanthocyanidin commercial extract (Pycnogenol[®]) to build a calibration curve, and the results were expressed as Pycnogenol[®] equivalents (mg PycE/g of dry extract, and mg PycE/g of fresh biomass).

Antioxidant activity was evaluated by the DPPH free radical scavenging assay and by the ferric reducing antioxidant power assay. For the DPPH assay [41], 0.5 mL of extract were mixed with 4 mL of DPPH solution (2,2-diphenyl-1-picrylhydrazyl at 45 mg/L in methanol), and then incubated at room temperature for 30 min. After incubation, absorbance was measured at 517 nm and the results were expressed in Trolox equivalents by using a calibration curve obtained with Trolox as a standard (mg TE/g of dry extract, and mg TE/g of fresh biomass).

The FRAP assay was carried out using the procedure of Benzie and Strain with slight modifications [42]. The FRAP reagent was prepared from 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. This reagent was freshly prepared before analysis and warmed to $37\text{ }^{\circ}\text{C}$ in a water bath prior to use. One hundred microliters of sample were added to 3 mL of the FRAP reagent and the mixture was kept at $37\text{ }^{\circ}\text{C}$ for 30 min. After this period, absorbance was recorded at 593 nm. A standard curve was made using iron (II) sulfate solution, and the results were expressed as mmol Fe(II)/g of dry extract and as mmol Fe(II)/g of fresh biomass.

In all these characterization assays, two replicates of each sample were analyzed in triplicate.

2.5. Antimicrobial Activity

Antimicrobial activity was assayed against Gram-negative bacteria (*Escherichia coli* ATCC[®] 8739[™] and *Pseudomonas aeruginosa* ATCC[®] 9027[™]), Gram-positive bacteria (*Staphylococcus aureus* ATCC[®] 6538[™], *Staphylococcus epidermidis* ATCC[®] 12228[™], methicillin resistant *Staphylococcus aureus* (MRSA) ATCC[®] 33591[™], *Enterococcus faecalis* ATCC[®] 29212[™], *Bacillus cereus* ATCC[®] 11778[™], *Streptococcus mutans* ATCC[®] 25175[™] and *Streptococcus mitis* NCIMB[®] 13770) and a yeast (*Candida albicans* ATCC[®] 10231[™]). All microbial strains were kept at $-70\text{ }^{\circ}\text{C}$ in broth with glycerol (15% *v/v*). The antimicrobial activity was assessed by the well-diffusion assay, according to Pereira et al. [43]. Briefly, microorganisms were subcultured on TSA (*E. coli*, *P. aeruginosa*, *S. Choleraesuis*, *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis* and *B. cereus*), BHIA (*S. mutans* and *S. mitis*) or SDA (*C. albicans*) and incubated at $30 \pm 2\text{ }^{\circ}\text{C}$ (*B. cereus* and *C. albicans*) or $35 \pm 2\text{ }^{\circ}\text{C}$ (remaining bacteria). Isolated colonies were suspended in saline medium (NaCl, 0.85% *w/v*), and the turbidity was adjusted to 0.5 on the McFarland scale (approx. $1\text{--}2 \times 10^8$ CFU/mL for bacteria and $1\text{--}5 \times 10^6$ CFU/mL for yeasts) (DEN-1B McFarland Densitometer, Grant-bio). Subsequently, microbial suspensions were spread on BHIA (*S. mutans* and *S. mitis*) or MHA (remaining microorganisms) Petri dishes, wells (6 mm in diameter) were aseptically punched, and 50 μL of extracts (10 mg/mL) were poured into the wells. The plates were incubated, in the dark, for 24 h at $30 \pm 2\text{ }^{\circ}\text{C}$ (*B. cereus* and *C. albicans*) or $35 \pm 2\text{ }^{\circ}\text{C}$ (remaining bacteria). Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zone (mm) around the well. Paper disks impregnated with vancomycin (Gram-positive bacteria), ofloxacin (Gram-negative bacteria) and ketoconazole (*C. albicans*) were used as positive controls. Ethanol (70% *w/v*) was used as a negative control. Data are presented as the mean \pm standard deviation. All the determinations were performed in triplicates.

2.6. GC-TOFMS Analysis

The extracts selected for analysis of non-polar components were derivatized using a procedure adapted from Popova et al. [44]. Briefly, 2 mL of each extract was evaporated to dryness and the residue was diluted in 100 μL of pyridine and 100 μL of the derivatizing agent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA); the mixture was incubated at room temperature for 12 h, diluted in petroleum ether (1.5 mL), dried with anhydrous sodium sulphate and kept at $-5\text{ }^{\circ}\text{C}$ until analysis. Analyses were performed with an Agilent 7890B (Palo Alto, CA, USA) gas chromatograph equipped with a multi-mode inlet. An Agilent HP-5MS UI fused silica capillary column (30 m \times 0.25 mm i.d., 0.25 μm d f—film thickness) was used in all separations. An aliquot of the derivatized sample (1 μL) was injected via a LECO L-PAL3 autosampler fitted with a 10 μL syringe. The injector was operated in solvent vent mode, with the split valve opened at 100 mL/min for 25 s, and the injector at $70\text{ }^{\circ}\text{C}$. Then, the split valve was closed for 120 s, and the inlet temperature was raised to $300\text{ }^{\circ}\text{C}$ at $700\text{ }^{\circ}\text{C}/\text{min}$. Finally, the split valve was opened again at 20 mL/min until the end of the run. The oven program was as follows: $50\text{ }^{\circ}\text{C}$ for 1 min, then $7\text{ }^{\circ}\text{C}/\text{min}$ until $100\text{ }^{\circ}\text{C}$, then $3\text{ }^{\circ}\text{C}/\text{min}$ until $220\text{ }^{\circ}\text{C}$, and finally $10\text{ }^{\circ}\text{C}/\text{min}$ until $295\text{ }^{\circ}\text{C}$, held for 8 min. The transfer line to the MS was kept at $300\text{ }^{\circ}\text{C}$. Detection was performed with a LECO Pegasus BT Time-of-Flight mass spectrometer (Saint Joseph, MI, USA). The MS was operated with the ion source at $250\text{ }^{\circ}\text{C}$, electron ionization at 70 eV, acquisition from *m/z* 40 to 550, 10 spectra per second and an acquisition delay of 480 s for liquid samples. Data acquisition, system control and spectra deconvolution were performed using LECO ChromaTOF version 5.40. NIST MS Search Program Version 2.3 g was used for spectra matching (NIST, 2015). Linear retention index (LRIs) values for sample peaks were calculated by analyzing the commercial alkane standard solution C8–C40, using the aforementioned chromatographic conditions [45].

2.7. Statistical Analysis

Statistical analysis of the results was performed using the SPSS software, version 23 (IBM Analytics, Armonk, NY, USA). Analysis of variance (ANOVA) was performed to

evaluate significant differences between averages, and the Tukey post hoc test ($p < 0.05$) was used to perform multiple comparisons between averages.

3. Results and Discussion

3.1. Extract Yield and Composition

The recovery of bioactive extracts from *A. dealbata* leaves and twigs was evaluated for different extraction methods (maceration at room temperature and hot extraction), different solvents (70% ACE, 70% ET, and 70% MET), different geographical origins (Caparica and Alcoaça) and for different biomass water contents (fresh biomass and dried biomass).

Leaves and twigs were collected in Alcoaça (39°36'36.5" N; 8°59'45.0" W, at 32–40 m of altitude) and Caparica (38°37'22.6" N; 9°10'38.5" W, at 44–48 m of altitude). Both locations are classified as Csb (Warm-summer Mediterranean climate) according to Köppen-Geiger's classification system for climate but with some differences between them. Monthly average daily temperatures are in similar ranges (10.6–20.4 °C for Alcoaça and 11.6–21.9 °C for Caparica), but Alcoaça is characterized by a higher level of annual rainfall (696 mm against 591 mm) and relative humidity (73–81% against 68–80%) [46,47]. The fresh leaves and twigs that were processed in the first 24 h from the collection had moisture contents in the ranges of 47.7–58.3% and 43.4–51.9%, respectively. After air-drying at room temperature, moisture contents were in the range of 8.1–9.3% for leaves and 8.8–11.2% for twigs.

Extraction yields and compositions (TPC, TFC, and TPrAC contents) for the extracts obtained leaves and twigs, and the different extraction conditions are presented in Table 1.

Making an overall analysis of this set of data, significantly higher extraction yields were obtained for dried raw material, and significantly higher TPC, TFC, and TPrAC contents were found in the extracts produced with 70% ACE (except for hot extraction from twigs). The TPC, TFC, and TPrAC contents of twigs extracts with 70% ACE were also higher than those contents in the leaves extracts obtained with the same solvent.

The extraction yield obtained for leaves with 70% ACE (10.5–15.8%, on a wet basis) was higher than the ones reported by Borges et al. for *A. dealbata* leaves, extracted with different solvents (water, methanol, ethanol, acetone, dichloromethane, and hexane) and extraction methods (maceration under agitation, Soxhlet, and the use of ultrasound and microwaves), and solvent-to biomass ratio of 1:10, that were in the range of 2.8–12.0% of fresh weight [29]. Extraction yields were also higher than those observed by Luís et al. [48] for ethanolic, hydroethanolic (1:1 in volume basis), methanolic, and acetic extracts of aerial parts (wood, bark, and leaves) of *A. dealbata* (3.26–9.51% dry weight) using Soxhlet extraction and were in the same range of the described for *A. melanoxydon* aerial parts (11.88–15.41% dry weight) by the same authors. Concerning twigs, Lomarat et al. found an extraction yield of dried *A. pennata* twigs of 15.1%, obtained by Soxhlet extraction with methanol [35].

For the best extraction solvent (70% ACE), TPC in leaves and twigs extracts varied in the range 345.8–478.4 mg GAE/g extract and 367.1–526.5 mg GAE/g extract, respectively. These contents are higher than those found by Luís et al. [48] in extracts of aerial parts of *A. dealbata* (203.10–290.65 mg GAE/g extract) and *A. melanoxydon* (100.10–138.76 mg GAE/g extract), and higher than the reported for *A. farnesiana* leaves extracts (63.2–247.9 mg GAE/g extract) [49], similar to the TPC contents found for *A. mearnsii* (163.9–646.6 mg GAE/g extract) [50], *A. tortilis* (260.7–512.4 mg GAE/g extract) [49], *A. nilotica* (42.18–116.60 mg GAE/g extract) [51], and *A. ataxacantha* (63.26–115.57 mg GAE/g extract) [51] leaves extracts, but lower than the observed for *A. longifolia* leaves extracts (524.9–858.8 mg GAE/g extract) [49]. Leaves and twigs are important sources of phenolics, with contents of 36.2–71.2 mg GAE/g of fresh leaves and 30.1–59.9 mg GAE/g of fresh twigs, respectively (see full results in Supplementary Material, Table S1). These are in a similar range as what was observed by Ferreira-Santos et al. [52] with *Pinus pinaster* bark, a known source of phenolic compounds, which gave 65.1 and 68.2 mg GAE/g of bark by extraction with 70% and 50% aqueous ethanol, respectively.

Table 1. Extraction yields (% wet basis), TPC (mg GAE/g extract), TFC (mg CatE/g extract), and TPrAC (mg PycE/g extract) of extracts from fresh and dried leaves and twigs from Alcobaca (ALC) and Caparica (CAP), using different extraction solvents and extraction methods; TPC, TFC, and TPrAC expressed relative to the mass of biomass are presented in the Supplementary Material, Table S1.

Samples		Maceration at Room Temperature			Hot Extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
Yield	Fresh (ALC)	12.2 ± 0.1 ^h	10.8 ± 0.1 ^j	11.6 ± 0.1 ⁱ	12.2 ± 0.1 ^h	11.0 ± 0.1 ^j	9.9 ± 0.1 ^l
	Fresh (CAP)	10.5 ± 0.2 ^k	10.0 ± 0.1 ^l	9.8 ± 0.1 ^l	12.3 ± 0.1 ⁱ	9.8 ± 0.1 ^l	9.9 ± 0.1 ^l
	Dried (ALC)	14.8 ± 0.1 ^c	14.4 ± 0.1 ^{de}	14.1 ± 0.1 ^{de}	15.8 ± 0.1 ^a	13.5 ± 0.1 ^f	13.0 ± 0.1 ^g
	Dried (CAP)	14.0 ± 0.1 ^e	14.3 ± 0.1 ^{de}	13.5 ± 0.1 ^f	15.6 ± 0.1 ^a	15.8 ± 0.1 ^a	15.2 ± 0.1 ^b
TPC	Fresh (ALC)	449.1 ± 8.0 ^b	338.9 ± 6.6 ^{ghi}	329.0 ± 7.6 ^{ijk}	428.4 ± 3.7 ^c	361.0 ± 5.9 ^{de}	336.7 ± 6.9 ^{hj}
	Fresh (CAP)	345.8 ± 4.0 ^{fgh}	295.8 ± 6.4 ^m	288.3 ± 3.4 ^{mn}	352.6 ± 5.8 ^{def}	324.5 ± 9.5 ^{kl}	314.7 ± 7.6 ^l
	Dried (ALC)	478.5 ± 4.5 ^a	324.9 ± 3.3 ^{ijkl}	336.1 ± 3.2 ^{hijk}	450.6 ± 3.7 ^b	362.2 ± 3.6 ^d	351.3 ± 5.4 ^{def}
	Dried (CAP)	350.2 ± 4.4 ^{efg}	292.3 ± 4.3 ^{mn}	255.2 ± 5.3 ^p	352.6 ± 5.4 ^{def}	272.4 ± 4.4 ^o	282.4 ± 4.7 ^{no}
TFC	Fresh (ALC)	98.7 ± 2.5 ^{cde}	81.4 ± 2.5 ^{kl}	75.3 ± 2.6 ^m	90.7 ± 3.1 ^{gh}	83.1 ± 3.4 ^{ijkl}	77.7 ± 3.3 ^{lm}
	Fresh (CAP)	94.9 ± 2.4 ^{efg}	97.7 ± 3.4 ^{cde}	89.3 ± 3.7 ^h	106.5 ± 2.8 ^b	96.6 ± 3.9 ^{def}	95.7 ± 2.8 ^{defg}
	Dried (ALC)	114.8 ± 2.0 ^a	82.7 ± 1.8 ^{ijkl}	81.7 ± 1.3 ^{ijkl}	103.2 ± 1.5 ^{bc}	88.2 ± 1.8 ^{hi}	87.0 ± 1.9 ^{hij}
	Dried (CAP)	105.4 ± 1.7 ^b	92.1 ± 1.8 ^{fgh}	78.9 ± 2.3 ^{klm}	101.2 ± 2.8 ^{bcd}	83.6 ± 2.3 ^{ijk}	82.1 ± 1.9 ^{ijkl}
TPrAC	Fresh (ALC)	330.7 ± 7.9 ^b	192.4 ± 6.2 ^h	203.7 ± 9.3 ^{gh}	292.7 ± 9.4 ^c	191.0 ± 6.1 ^h	211.6 ± 8.4 ^g
	Fresh (CAP)	255.4 ± 5.5 ^{de}	140.2 ± 6.0 ^j	120.0 ± 5.1 ^k	263.6 ± 8.1 ^d	142.2 ± 8.3 ^j	146.4 ± 5.3 ^j
	Dried (ALC)	357.0 ± 8.0 ^a	201.5 ± 5.5 ^{gh}	189.4 ± 6.3 ^{hi}	331.5 ± 5.1 ^b	241.9 ± 8.8 ^{ef}	238.1 ± 7.8 ^f
	Dried (CAP)	279.3 ± 4.5 ^c	211.1 ± 4.9 ^g	133.5 ± 5.1 ^{jk}	280.0 ± 7.6 ^c	174.9 ± 8.5 ⁱ	198.2 ± 5.8 ^{gh}
Twigs							
Yield	Fresh (ALC)	7.9 ± 0.1 ^h	6.9 ± 0.1 ^{kl}	6.9 ± 0.1 ^{kl}	9.0 ± 0.1 ^e	8.3 ± 0.1 ^g	7.7 ± 0.1 ^{hi}
	Fresh (CAP)	7.3 ± 0.1 ^j	6.7 ± 0.1 ^l	7.0 ± 0.1 ^k	8.0 ± 0.2 ^g	7.6 ± 0.1 ⁱ	7.0 ± 0.1 ^k
	Dried (ALC)	12.0 ± 0.1 ^a	10.7 ± 0.1 ^b	10.6 ± 0.1 ^b	8.8 ± 0.1 ^{ef}	7.1 ± 0.1 ^k	10.1 ± 0.1 ^c
	Dried (CAP)	9.0 ± 0.1 ^e	8.6 ± 0.1 ^f	10.1 ± 0.1 ^c	8.8 ± 0.1 ^{ef}	8.3 ± 0.1 ^g	9.5 ± 0.1 ^d
TPC	Fresh (ALC)	526.5 ± 4.5 ^a	406.6 ± 4.3 ^g	348.3 ± 6.3 ^j	514.6 ± 2.2 ^b	420.9 ± 3.3 ^{ef}	419.1 ± 3.0 ^{ef}
	Fresh (CAP)	412.6 ± 6.7 ^{fg}	294.2 ± 4.9 ⁿ	273.7 ± 7.9 ^o	367.1 ± 3.8 ⁱ	344.8 ± 5.3 ^{jk}	324.0 ± 6.1 ^l
	Dried (ALC)	499.0 ± 3.9 ^c	389.5 ± 7.3 ^h	329.0 ± 8.3 ^l	485.6 ± 3.5 ^d	412.5 ± 5.5 ^{fg}	402.6 ± 6.2 ^g
	Dried (CAP)	419.1 ± 3.7 ^{ef}	311.0 ± 6.5 ^m	251.6 ± 3.4 ^p	425.1 ± 5.8 ^e	341.5 ± 6.1 ^{jk}	334.9 ± 3.3 ^{kl}
TFC	Fresh (ALC)	166.2 ± 4.3 ^{cd}	139.1 ± 4.0 ^{ijkl}	130.9 ± 2.1 ^m	166.3 ± 3.9 ^c	137.6 ± 3.3 ^{ijkl}	135.3 ± 2.6 ^{klm}
	Fresh (CAP)	178.7 ± 4.7 ^b	147.6 ± 2.3 ^{fgh}	130.0 ± 3.8 ^{mn}	153.0 ± 2.8 ^f	153.2 ± 2.4 ^{ef}	144.7 ± 4.5 ^{ghi}
	Dried (ALC)	166.3 ± 3.0 ^c	143.0 ± 3.4 ^{hij}	133.8 ± 2.0 ^{lm}	163.8 ± 1.7 ^{cd}	139.7 ± 2.8 ^{ijkl}	141.0 ± 2.9 ^{hijk}
	Dried (CAP)	198.4 ± 2.7 ^a	149.8 ± 3.3 ^{fg}	123.8 ± 2.4 ⁿ	201.0 ± 1.9 ^a	159.6 ± 1.4 ^{de}	151.5 ± 2.7 ^f
TPrAC	Fresh (ALC)	585.8 ± 10.3 ^b	307.5 ± 7.1 ⁱ	286.4 ± 11.6 ^j	553.3 ± 7.0 ^c	375.2 ± 6.8 ^g	387.7 ± 9.0 ^g
	Fresh (CAP)	440.3 ± 9.1 ^e	94.2 ± 6.9 ^m	72.3 ± 6.3 ⁿ	388.3 ± 9.2 ^g	209.1 ± 7.8 ^k	142.1 ± 7.3 ^l
	Dried (ALC)	576.4 ± 5.5 ^b	340.3 ± 9.5 ^h	305.1 ± 6.9 ⁱ	516.9 ± 8.2 ^d	351.0 ± 8.1 ^h	384.6 ± 6.5 ^g
	Dried (CAP)	631.3 ± 7.9 ^a	379.9 ± 9.8 ^g	306.0 ± 7.2 ⁱ	614.3 ± 8.4 ^a	417.6 ± 6.1 ^f	432.4 ± 6.4 ^{ef}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 × 3); different letters indicate significant differences of means within samples, for each determined parameter (one-way ANOVA, Tukey test at p < 0.05).

For 70% ACE, TFC in leaves and twigs extracts lied between 90.7 and 114.8 mg CatE/g extract and 153.0 to 198.4 mg CatE/g extract, respectively. A calibration curve was made using rutin as a standard for comparison purposes, giving 228.8–348.0 mg RE/g extract for leaves, and 375.5–601.4 mg RE/g extract (Supplementary Material, Table S3). TFC in leaves extracts were in the range observed by Tung et al. [53] with leaves of *A. nilotica* (2.3–355.3 mg RE/g extract), while TFC in twigs extracts were higher. The TFC values found in the extracts of *A. dealbata* twigs with 70% ACE were higher than those

observed in bark extracts of various *Acacia* spp. including *A. dealbata* (74.0–134.0 mg RE/g extract) [54], in leaves and bark extracts of *A. nilotica* and *A. ataxacantha* (0.34–49.90 mg RE/g extract) [51], or in *Pinus pinaster* bark extracts, a known source of flavonoids (77–161 mg CatE/g extract) [52]. Relatively to the mass of raw material, TFC contents varied in the range 9.9–17.0 mg CatE/g of leaves (30.2–51.4 mg RE/g of leaves), and 12.6–20.0 mg CatE/g of twig (38.2–60.5 mg RE/g of twig) (see full results in Supplementary Material, Table S4), which are much superior to the reported by Sowndhararajan et al. for the bark of *A. dealbata* and other *Acacia* spp. (2.4–15.5 mg RE/g of bark), and also other known sources of flavonoids such as yellow, green, and black teas (0.2–3.8 mg CatE/g of tea) [55], and red fruits (0.2–0.8 mg CatE/g of tea) [56].

Regarding TPrAC, the results were expressed relative to a standard source of proanthocyanidins, the commercial extract Pycnogenol[®], a mixture of proanthocyanidins extracted from bark of Atlantic *Pinus* trees. As observed for TPC and TFC, the extracts with higher TPrAC contents were obtained with 70% ACE, with TPrAC values in the range of 255.4–357.0 mg PycE/g extract for leaves, and in the range of 388.3–631.3 mg PycE/g extract for twigs.

The results of this study showed that the leaves and twigs of *A. dealbata* from Caparica and from Alcobaça regions can be an abundant source of extracts rich in phenolics, especially flavonoids and proanthocyanidins, with average contents similar or higher than comparable extracts described in the literature.

For a better interpretation of the impact of the extraction method (regardless of the drying status or the origin of the plant), the average of the measured variables for all extracts produced by maceration was compared with the average of all extracts produced by hot extraction for each solvent. The same approach was followed to evaluate the average values of the measured variables for all extracts obtained from fresh and dried leaves (regardless of other extraction parameters). The average values of the measured variables for the extracts obtained from a specific origin (Caparica or Alcobaça) were also determined and compared. These results were then subject to analysis of variance (ANOVA) followed by Tukey's test at $p < 0.05$ and are shown in Supplementary Material, Table S5.

For the extraction yield, statistically significant differences were only found between fresh and dried raw materials, as shown in the Figure 1. Yields in the range 13.9–15.0%, and 10.3–11.6% were found for dried and fresh leaves, respectively and yields in the range of 8.7–10.1%, and 7.2–8.1% were found for dried and fresh twigs, respectively.

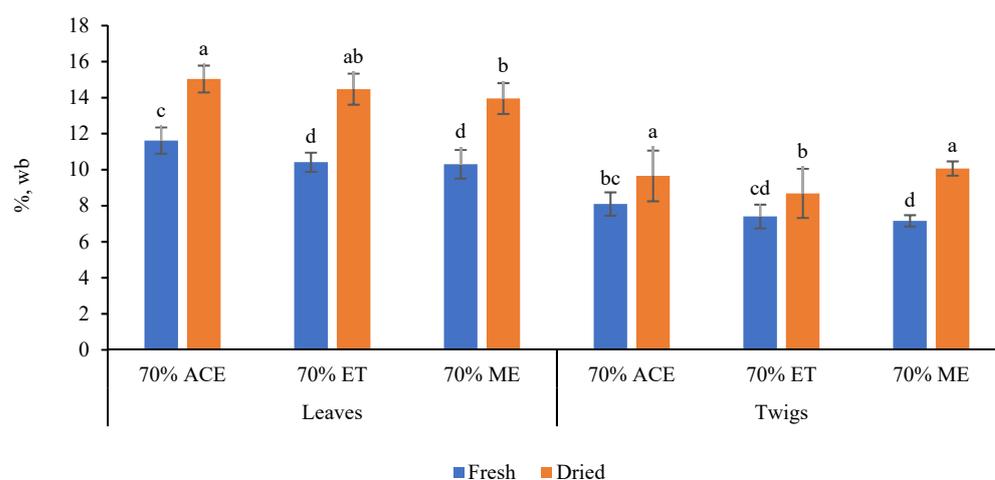


Figure 1. Comparison of the average extraction yields for fresh and dried leaves and twigs (regardless the extraction method and the provenience of the raw material); different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

In what concerns the TPC, TFC, and TPrAC of the extracts, no significant differences were found between maceration and hot extraction, except for methanolic extracts that

presented slightly higher contents for hot extraction (Supplementary Material, Table S5). Figure 2 highlights differences coming from the drying status and origin of the raw material, for all determined composition parameters.

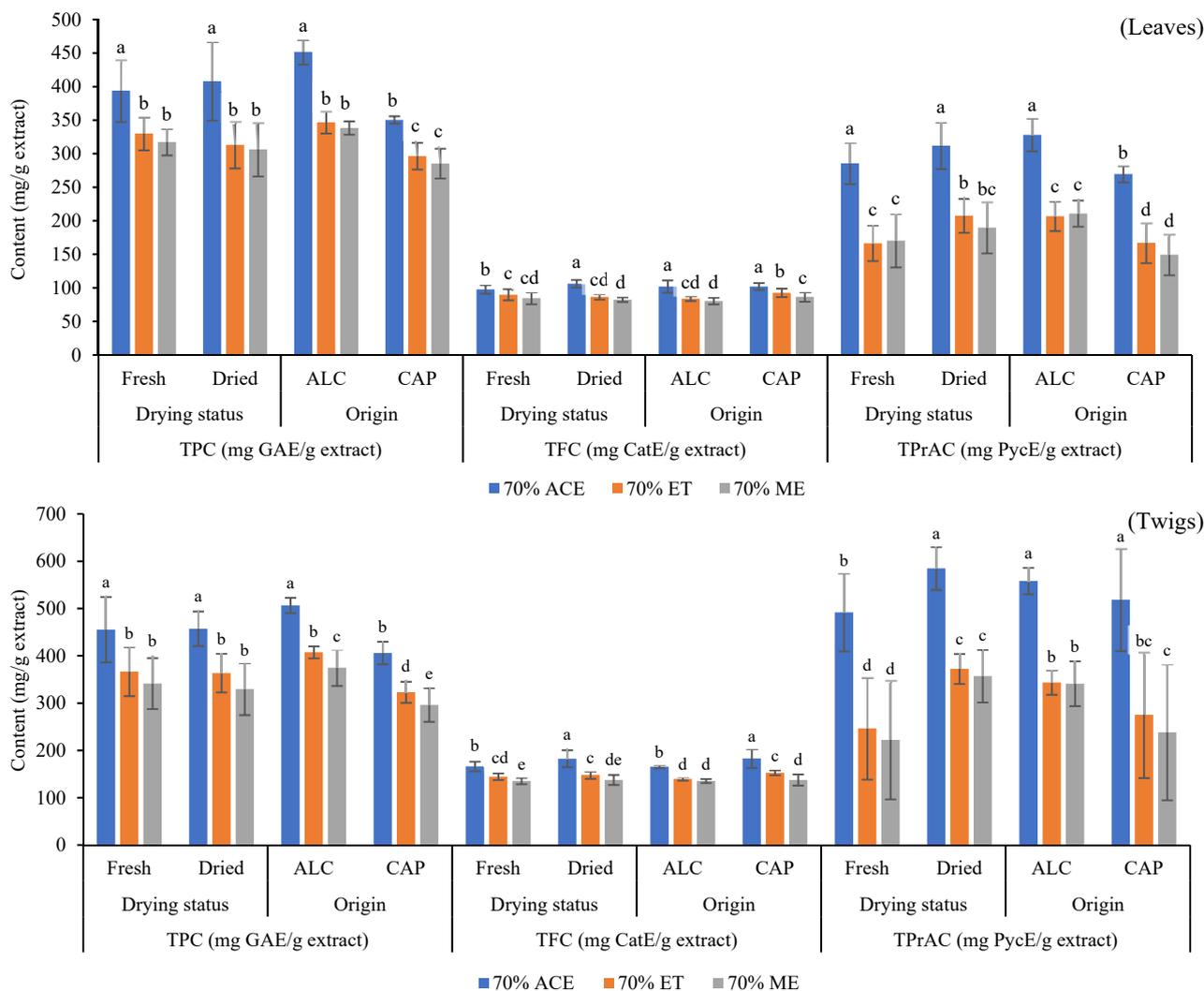


Figure 2. Comparison of the average values of TPC (mg GAE/g extract), TFC (mg CatE/g extract), and TPrAC (mg PycE/g extract) for fresh or dried material (regardless the extraction method and the provenience of the raw material), and for plants from Alcobaça (ALC) and Caparica (CAP) (regardless the extraction method and the drying status); different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

Overall, it was confirmed that the higher average values for all the parameters considered were higher for the extracts obtained with 70% ACE. Regarding leaves, the extracts from the Alcobaça region had higher average values of TPC and TPrAC and higher average values of TFC (only for 70% ET and 70% ME extracts) than those from Caparica region. In the case of twigs, extracts from dried biomass had higher average values of TFC and TPrAC than those obtained from wet biomass with the same solvents. Extracts of twigs from Alcobaça region showed higher average values of TPC than those from the Caparica region. Differences in the average values comparing fresh and dried plants were most noticed in the extraction yield, TFC, and TPrAC, with dried material originating better results. The differences of extraction yield or extract composition between fresh or dried biomass may be explained by the change of the extraction solvent characteristics, since the presence of biomass moisture corresponds to a reduction of the concentration of the organic component (acetone, ethanol, or methanol). Differences coming from the plant origin can

be explained by the fact that secondary metabolites are synthesized by plants to protect them in adverse conditions from pathogens and environmental stresses, being influenced by several biotic and abiotic factors, such as water, drought, salinity, temperature, radiation, or chemical stresses [57]. Thus, differences in edaphoclimatic conditions between Alcobaça and Caparica can explain the differences observed.

3.2. Antioxidant Activity

The antioxidant activity of the extracts obtained from *A. dealbata* leaves and twigs was assessed by DPPH and FRAP assays, and the results are presented in Table 2.

Table 2. Results from DPPH (mg TE/g extract) and FRAP (mmol Fe²⁺/g extract) of extracts from fresh and dried leaves and twigs from Alcobaça (ALC) and Caparica (CAP), using different extraction solvents and extraction methods; DPPH and FRAP results expressed relative to the mass of biomass are presented in Supplementary Material, Table S1.

Samples	Maceration at Room Temperature			Hot Extraction			
	70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET	
Leaves							
DPPH	Fresh (ALC)	740.8 ± 10.1 ^b	597.9 ± 8.3 ^e	535.8 ± 8.5 ⁱ	740.2 ± 6.1 ^b	636.7 ± 6.0 ^d	526.8 ± 5.3 ⁱ
	Fresh (CAP)	560.0 ± 7.2 ^h	348.5 ± 5.7 ⁿ	335.4 ± 5.9 ⁿ	540.0 ± 8.4 ^{gh}	428.4 ± 9.0 ^k	393.6 ± 7.2 ^m
	Dried (ALC)	893.4 ± 10.8 ^a	578.2 ± 6.5 ^{fg}	576.4 ± 8.2 ^{fg}	886.5 ± 7.5 ^a	634.3 ± 9.1 ^d	685.4 ± 5.5 ^c
	Dried (CAP)	446.3 ± 7.0 ^j	412.2 ± 5.6 ^l	337.0 ± 8.3 ⁿ	588.9 ± 5.5 ^{ef}	392.6 ± 7.3 ^m	406.6 ± 6.0 ^{lm}
FRAP	Fresh (ALC)	7664.1 ± 167.6 ^b	6285.3 ± 95.4 ^{ef}	5620.3 ± 118.5 ^{gh}	7702.8 ± 164.7 ^b	6501.3 ± 132.2 ^{de}	5423.2 ± 142.5 ^{hi}
	Fresh (CAP)	6612.1 ± 167.8 ^d	4386.9 ± 93.0 ^l	4403.9 ± 129.7 ^l	6015.2 ± 177.6 ^{def}	5072.4 ± 225.1 ^{jk}	4835.8 ± 178.6 ^k
	Dried (ALC)	8885.4 ± 93.1 ^a	6299.9 ± 71.9 ^{ef}	5334.0 ± 79.0 ^{ij}	6962.5 ± 63.4 ^c	6175.8 ± 138.3 ^f	3300.7 ± 98.7 ⁿ
	Dried (CAP)	5846.6 ± 90.7 ^g	4855.0 ± 58.6 ^k	3934.8 ± 103.5 ^m	5766.3 ± 81.2 ^g	4097.2 ± 124.8 ^m	4449.8 ± 82.2 ^l
Twigs							
DPPH	Fresh (ALC)	996.8 ± 6.7 ^b	757.2 ± 11.6 ^e	511.1 ± 11.3 ^j	994.7 ± 6.2 ^{bc}	778.8 ± 14.0 ^d	782.7 ± 8.1 ^d
	Fresh (CAP)	675.5 ± 11.0 ^h	254.2 ± 6.6 ^q	230.2 ± 9.2 ^r	596.3 ± 11.5 ⁱ	379.7 ± 11.8 ⁿ	358.4 ± 11.4 ^o
	Dried (ALC)	1068.3 ± 8.5 ^a	665.5 ± 11.2 ^h	514.2 ± 11.8 ^j	974.2 ± 6.4 ^c	791.2 ± 14.3 ^d	782.1 ± 7.1 ^d
	Dried (CAP)	731.3 ± 6.1 ^f	418.5 ± 7.9 ^m	321.3 ± 8.3 ^p	710.2 ± 5.2 ^g	474.5 ± 9.9 ^k	452.6 ± 8.5 ^l
FRAP	Fresh (ALC)	6852.4 ± 208.0 ^{de}	6546.5 ± 221.0 ^e	3989.4 ± 61.6 ^j	7829.9 ± 140.9 ^c	6851.8 ± 225.2 ^{de}	6554.3 ± 112.4 ^e
	Fresh (CAP)	6951.8 ± 146.0 ^d	3306.8 ± 109.3 ^k	3075.8 ± 75.0 ^k	6192.0 ± 53.1 ^f	4626.8 ± 255.7 ^{hi}	4461.1 ± 137.7 ⁱ
	Dried (ALC)	9194.6 ± 165.8 ^a	4770.3 ± 86.1 ^h	3783.0 ± 84.8 ^j	8804.2 ± 112.4 ^b	6987.7 ± 163.4 ^d	7098.5 ± 87.1 ^d
	Dried (CAP)	7754.4 ± 119.0 ^c	5354.9 ± 141.1 ^g	3969.0 ± 85.0 ^j	8003.8 ± 171.7 ^c	5585.2 ± 140.0 ^g	5429.1 ± 83.3 ^g

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 × 3); different letters indicate significant differences of means within samples, for each antioxidant determination method (one-way ANOVA, Tukey test at p < 0.05).

The highest antioxidant activity of extracts from leaves or twigs was observed when using 70% ACE as extraction solvent, regardless of the extraction method, drying status, or the geographical origin of the plant. When using this solvent, the antioxidant activity of twigs extracts was higher than the one of corresponding leaves extracts.

The DPPH assay measures the ability of extract components to act as free radical scavengers or hydrogen donors antioxidants [58]. For 70% ACE extracts, the values lied between 446.3–893.4 mg TE/g extract (58.6–132.1 mg TE/g of leaves) for leaves extract and between 596.3–1068.3 mg TE/g extract (49.0–128.3 mg TE/g of twigs). These values are higher than those reported by Zheleva-Dimitrova et al. for extracts from leaves (31.94–493.90 mg TE/g extract) and stem bark (8.51–349.17 mg TE/g extract) of *A. nilotica* and *A. ataxacantha* [51]. These results are also higher than the ones reported for known antioxidant sources such as yellow, green, and black teas (11.930–26.521 µmol TE/100 g, which corresponds to 0.030–0.066 mg TE/g of tea leaves) [55], red fruits (64.14–177.11 µmol TE/g, corresponding to 16.1–44.3 mg TE/g of fruit) [56], or pine bark (approximately between 50 and 350 mg TE/g of extract) [59].

The reducing power of the obtained extracts was measured through the FRAP assay, which evaluates the reduction of Fe³⁺ in the complex (Fe³⁺-TPTZ) to a ferrous form

(Fe²⁺-TPTZ), by donation of electrons from the antioxidants, resulting in stopping the free radical production chain [13,60,61]. For 70% ACE the FRAP values were in the range 5766.3–8885.4 µmol Fe²⁺/g extract and 6192.0–9194.6 µmol Fe²⁺/g extract, for leaves and twigs extracts, respectively. These values higher than those found in leaves (233.17 µmol Fe²⁺/g extract), pods (254.42 µmol Fe²⁺/g extract), and seed (178.14 µmol Fe²⁺/g extract) extracts of *A. leucophloea* [62]. Values of 152 mg Fe²⁺/g (corresponding to 2721.2 µmol Fe²⁺/g extract) and 575 mg Fe²⁺/g (corresponding to 10296.4 µmol Fe²⁺/g extract) were found for *Pinus radiata* bark extract and standardized *Pinus pinaster* bark extract Pycnogenol[®], respectively [63].

The extraction solvent significantly affected the antioxidant activity, with the best results being obtained when using 70% ACE, regardless of the origin and the drying status of the raw material. Higher antioxidant activities of extracts obtained with aqueous acetone solutions were also observed with stem bark extracts of *Butea monosperma* (Lam.) Kuntze [64], bark extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata* and *A. pennata* [54], and flower extracts of safflower (*Carthamus tinctorius* L.) [65].

A better understanding of the influence of the extraction method, drying status, and provenience of the plant on the antioxidant activity can be derived from the analysis of variance applied using the same approach as for the impact of these factors on the composition and extract yield. The results are presented in Table 3. Besides the confirmation of 70% ACE as the best extraction solvent for the removal of antioxidant compounds, it can be seen that the geographical origin of the plant had the major impact on the antioxidant activity of the produced extracts, since the extracts of leaves and twigs from Alcobaça showed significantly higher antioxidant activity than the corresponding extracts from biomass collected in the Caparica region. The extraction method only gave significant differences on methanolic and ethanolic extracts of twigs, with hot extraction giving the highest antioxidant activity by both DPPH and FRAP assays. The effect of using fresh or dried material was most reflected in the antioxidant activity of twigs given by the FRAP assay, with dried twigs extracted with 70% ACE originating a significantly higher value than the one obtained using fresh leaves.

Table 3. Average values of DPPH (mg TE/g extract) and FRAP (mmol Fe²⁺/g extract) assays for extracts obtained by maceration and hot extraction (regardless drying status and origin), from fresh and dried material (regardless extraction method and origin), and from plants from Alcobaça (ALC) and Caparica (CAP) (regardless extraction process and drying status).

Samples	Leaves			Twigs		
	70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
DPPH						
Maceration	660.1 ± 174.7 ^a	484.2 ± 109.0 ^b	446.1 ± 113.5 ^b	868.0 ± 171.5 ^a	523.8 ± 203.4 ^{bc}	394.2 ± 125.7 ^c
Hot extraction	696.2 ± 131.2 ^a	523.0 ± 115.9 ^b	503.1 ± 120.0 ^b	818.9 ± 174.4 ^a	606.0 ± 186.4 ^b	593.9 ± 195.7 ^b
Fresh	652.5 ± 90.2 ^a	502.9 ± 121.4 ^b	447.9 ± 88.0 ^b	815.8 ± 186.2 ^a	542.5 ± 235.1 ^b	470.6 ± 210.4 ^b
Dried	703.8 ± 197.2 ^a	504.3 ± 106.5 ^b	501.3 ± 140.5 ^b	871.0 ± 157.5 ^a	587.4 ± 152.6 ^b	517.5 ± 171.7 ^b
Alcobaça	815.2 ± 76.8 ^a	611.8 ± 26.2 ^b	581.1 ± 64.7 ^{bc}	1008.5 ± 37.0 ^a	748.2 ± 51.7 ^b	647.5 ± 138.1 ^c
Caparica	541.1 ± 57.3 ^c	395.4 ± 31.3 ^d	368.1 ± 33.6 ^d	678.3 ± 53.1 ^c	381.7 ± 83.2 ^d	340.6 ± 81.9 ^d
FRAP						
Maceration	7252.1 ± 1174.0 ^a	5456.8 ± 873.7 ^b	4823.3 ± 746.5 ^{bc}	7688.3 ± 969.5 ^a	4994.6 ± 1199.0 ^c	3704.3 ± 386.4 ^d
Hot extraction	6693.0 ± 746.5 ^a	5461.7 ± 981.1 ^b	4502.4 ± 801.6 ^c	7707.5 ± 976.6 ^a	6012.9 ± 1007.8 ^b	5885.8 ± 1046.0 ^b
Fresh	7079.9 ± 644.8 ^a	5561.5 ± 898.9 ^b	5070.8 ± 509.5 ^b	6956.5 ± 610.9 ^b	5333.0 ± 1491.8 ^{cd}	4520.2 ± 1306.5 ^d
Dried	6865.2 ± 1287.9 ^a	5357.0 ± 946.6 ^b	4254.8 ± 765.0 ^c	8439.3 ± 611.2 ^a	5674.5 ± 841.4 ^c	5069.9 ± 1364.5 ^{cd}
Alcobaça	7803.7 ± 715.8 ^a	6315.6 ± 159.6 ^b	4919.5 ± 966.2 ^c	8170.3 ± 940.4 ^a	6289.1 ± 926.3 ^c	5356.3 ± 1518.6 ^d
Caparica	6141.4 ± 380.2 ^b	4602.9 ± 412.3 ^{cd}	4406.1 ± 347.9 ^d	7225.5 ± 737.5 ^b	4718.4 ± 921.4 ^{de}	4233.7 ± 873.4 ^e

Different letters indicate significant differences of means within samples for each set of parameters (one-way ANOVA, Tukey test at $p < 0.05$).

3.3. Extraction of Spent Raw Material

Leaves and twigs of *Acacia* spp. contain lipophilic compounds such as terpenes, long-chain alcohols, fatty acids, sterols, aromatic compounds, among others [66]. These lipophilic components may be valorized in different application fields, and their presence might limit the access of polar solvents to the matrix, affecting extraction of the bioactive components. To test this hypothesis the dried leaves and twigs were treated with petroleum ether, to remove non-polar components and extraction with 70% ACE was performed on the spent leaves and twigs using equivalent conditions to those used with the raw biomass. The characterization of the lipophilic extracts will be addressed elsewhere.

The comparison of composition and antioxidant activity of extracts obtained from dried material macerated at room temperature with 70% ACE, and those of extracts obtained from spent material in similar conditions is shown in Table 4.

Table 4. Extract yield, composition, and antioxidant activity of extracts obtained from raw and spent leaves and twigs, using maceration at room temperature with 70% ACE as extraction solvent.

Sample		Yield	TPC	TFC	TPrAC	DPPH	FRAP
Leaves	Alcobaça-spent	15.5 ± 0.1	436.4 ± 11.1	116.1 ± 2.8	441.0 ± 6.8	796.0 ± 9.9	8807.8 ± 225.1
	Alcobaça-raw	14.8 ± 0.1	478.5 ± 4.5	114.8 ± 2.0	357.0 ± 8.0	893.4 ± 10.8	8885.4 ± 93.1
	Caparica-spent	14.5 ± 0.1	350.5 ± 2.4	97.0 ± 1.8	356.9 ± 1.6	661.7 ± 3.5	6431.1 ± 85.8
	Caparica-raw	14.0 ± 0.1	350.2 ± 4.4	105.4 ± 1.7	279.3 ± 4.5	446.3 ± 7.0	5846.6 ± 90.7
Twigs	Alcobaça-spent	12.9 ± 0.1	448.3 ± 8.0	152.8 ± 3.6	610.1 ± 5.8	838.6 ± 8.6	8171.1 ± 153.0
	Alcobaça-raw	12.0 ± 0.1	499.0 ± 3.9	166.3 ± 3.0	576.4 ± 5.5	1068.3 ± 8.5	9194.6 ± 165.8
	Caparica-spent	10.2 ± 0.1	389.0 ± 5.3	147.6 ± 2.2	544.2 ± 4.7	644.8 ± 9.6	7151.6 ± 160.4
	Caparica-raw	9.0 ± 0.1	419.1 ± 3.7	198.4 ± 2.7	631.3 ± 7.9	731.3 ± 6.1	7754.4 ± 119.0

The extracts produced from spent leaves and twigs had a slightly higher yield and slightly higher proanthocyanidin contents than those obtained from the raw dried materials. However, TPC and TFC, as well as the antioxidant activity, were slightly lower when using spent raw material. The removal of lipophilic compounds from the plant matrix may facilitate the penetration of the solvent, thus allowing obtaining higher extract yields. The slight decrease in the antioxidant activity may be explained by the previous removal of lipophilic components with antioxidant activity, such as terpenes or sterols. Despite this, it should be noted that the values are quite close and that this option allows more efficient use of this resource by obtaining a wider range of products from the same amount of raw material.

3.4. Correlations between Composition and Antioxidant Activity

The correlation analysis between TPC, TFC, TPrAC, and antioxidant activities by DPPH and FRAP assays for the extracts of fresh and spent flowers are given in Table 5.

All the correlations found in this work were statistically significant at $p < 0.01$, with the exception made for the correlation between TFC and antioxidant activity by the DPPH assay for leaves (non-significant) and twigs (significant at $p < 0.05$) and for the correlation between TFC and FRAP assay in the case of leaves (significant at $p < 0.05$). The Pearson's correlation coefficients found indicate very strong correlations between TPC and antioxidant activity given by DPPH and FRAP assays, and strong correlations between TPrAC and the same assays [67], for both leaves and twigs. Moderate correlation was found between TFC of leaves and twigs and both antioxidant assays. Ref. [68] also found strong correlations between TPC, TFC and DPPH values ($r = 0.771$ and $r = 0.815$), for extracts of *A. nilotica* leaves. However, [69] found non-significant correlation between TPC of extracts of different components (leaves, flowers, and pods) of three *Acacia* species (*A. nilotica*, *A. seyal*, and *A. laeta*) and DPPH results, having found a significant correlation between TFC and DPPH

values instead. Significant and strong correlations between TPC and the DPPH and FRAP results were found in *Pinus densiflora* bark extract [59] and in medicinal herbs and spices [70].

Table 5. Correlation analysis between total phenolics content (TPC), total flavonoid content (TFC), total proanthocyanidins content (TPrAC) and antioxidant activities given by DPPH and FRAP assays, for leaves extracts; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

		TPC	TFC	TPrAC	DPPH	FRAP
Leaves	TPC	1				
	TFC	$r = 0.563^{**}$ $p = 0.003$	1			
	TPrAC	$r = 0.801^{**}$ $p = 0.000$	$r = 0.553^{**}$ $p = 0.003$	1		
	DPPH	$r = 0.922^{**}$ $p = 0.000$	$r = 0.373$ $p = 0.061$	$r = 0.766^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.864^{**}$ $p = 0.000$	$r = 0.490^{*}$ $p = 0.011$	$r = 0.742^{**}$ $p = 0.000$	$r = 0.778^{**}$ $p = 0.000$	1
	Twigs	TPC	1			
	TFC	$r = 0.511^{**}$ $p = 0.008$	1			
	TPrAC	$r = 0.754^{**}$ $p = 0.000$	$r = 0.671^{**}$ $p = 0.000$	1		
	DPPH	$r = 0.967^{**}$ $p = 0.000$	$r = 0.396^{*}$ $p = 0.045$	$r = 0.765^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.861^{**}$ $p = 0.000$	$r = 0.603^{**}$ $p = 0.011$	$r = 0.856^{**}$ $p = 0.000$	$r = 0.884^{**}$ $p = 0.000$	1

** correlation is significant at $p < 0.01$ (two-tailed); * correlation is significant at $p < 0.05$ (two-tailed).

These findings demonstrate that phenolic compounds have an important role in the observed antioxidant activity given by DPPH and FRAP assays. Moreover, the abundance of these compounds in the produced extracts suggests that leaves and twigs have a strong potential to be explored for obtaining antioxidants for diverse applications, such as in nutraceutical or cosmetic industries.

3.5. Antimicrobial Activity

A representative group of extract samples was selected to assess antimicrobial activity against several microorganisms responsible for foodborne disease, skin infections, caries, and oral infections. Some of these microorganisms, namely *S. aureus*, *Enterococcus* spp., *P. aeruginosa*, *E. coli*, and *C. albicans*, are usually involved in hospital-acquired infections and have developed resistance to antibiotics, and virulence factors can exacerbate microbial drug resistances [71,72]. Antibiotic resistance is emerging as a serious worldwide problem and natural extracts with antimicrobial activity have been suggested as a solution to this problem [73]. Natural products from plants could also target microbial virulence factors and thus play an important role to combat microbial infections and overcoming antibiotic resistances [74]. Microbial virulence factors encompass a wide range of molecules produced by pathogens, such as toxins, enzymes, exopolysaccharides, cell surface structures such as capsules, lipopolysaccharides, glyco- and lipoproteins [75]. Moreover, natural extracts with antimicrobial activity could be an less polluting and more safe alternative to synthetic antimicrobial substances applied in food industry and in oral, cosmetics, and pharmacological formulations [43].

Both leaves and twigs extracts were able to inhibit the growth of Gram-positive bacteria but were inactive against Gram-negative bacteria and *C. albicans*. The inhibition zones against bacteria *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis* are presented in Table 6. Overall, extracts were more effective against *B. cereus* (inhibition zones in the range 11.0–12.1 mm for leaves extracts and 9.3–13.0 mm for twigs extracts),

while *E. faecalis* showed a sensitivity lower than the remaining microorganisms (inhibition zones in the range 8.3–8.8 mm for leaves extracts and 9.3–9.8 mm for twigs extracts).

Table 6. Inhibition zones (mm) of selected extracts of *A. dealbata* leaves and twigs against *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis*.

Samples (0.5 mg/well)	<i>S. aureus</i>	MRSA	<i>S. epidermidis</i>	<i>E. faecalis</i>	<i>B. cereus</i>	<i>S. mutans</i>	<i>S. mitis</i>
Leaves							
Dried-70%ACE-maceration (ALC)	10.3 ± 0.1	10.3 ± 0.0	9.8 ± 0.1	8.5 ± 0.1	12.1 ± 0.1	11.0 ± 0.0	11.3 ± 0.6
Dried-70%ET-maceration (ALC)	8.8 ± 0.0	9.5 ± 0.1	8.5 ± 0.1	8.0 ± 0.0	11.5 ± 0.0	10.0 ± 0.0	11.0 ± 0.0
Dried-70%MET-maceration (ALC)	8.5 ± 0.1	9.3 ± 0.1	8.0 ± 0.0	nd	11.0 ± 0.0	10.0 ± 0.0	nd
Dried-70%ACE-maceration (CAP)	9.3 ± 0.0	9.0 ± 0.0	8.5 ± 0.1	8.8 ± 0.0	11.0 ± 0.0	10.0 ± 0.0	9.0 ± 1.7
Dried-70%ACE-hot extraction (ALC)	9.5 ± 0.1	10.5 ± 0.1	9.5 ± 0.1	8.8 ± 0.1	12.0 ± 0.0	10.0 ± 0.0	nd
Fresh-70%ACE-maceration (ALC)	9.5 ± 0.1	9.8 ± 0.0	9.3 ± 0.1	8.3 ± 0.1	11.3 ± 0.0	10.0 ± 0.0	9.3 ± 0.6
Spent leaves-70%ACE-maceration (ALC)	10.5 ± 0.1	10.5 ± 0.1	9.3 ± 0.0	8.5 ± 0.1	11.8 ± 0.0	11.3 ± 0.0	nd
Twigs							
Dried-70%ACE-maceration (ALC)	10.8 ± 0.0	10.5 ± 0.0	10.3 ± 0.1	9.5 ± 0.1	12.5 ± 0.1	9.0 ± 0.0	12.0 ± 0.0
Dried-70%ET-maceration (ALC)	nd	7.8 ± 0.0	nd	nd	10.0 ± 0.0	9.0 ± 0.0	11.0 ± 0.0
Dried-70%MET-maceration (ALC)	nd	nd	nd	nd	9.3 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
Dried-70%ACE-maceration (CAP)	11.0 ± 0.0	11.0 ± 0.0	10.0 ± 0.0	9.5 ± 0.1	13.0 ± 0.0	12.0 ± 0.0	10.0 ± 0.0
Dried-70%ACE-hot extraction (ALC)	10.0 ± 0.0	10.8 ± 0.1	9.8 ± 0.0	9.5 ± 0.1	13.0 ± 0.0	9.0 ± 0.0	11.0 ± 0.0
Fresh-70%ACE-maceration (ALC)	11.0 ± 0.0	10.7 ± 0.0	9.5 ± 0.1	9.3 ± 0.0	12.8 ± 0.1	11.0 ± 0.0	12.0 ± 0.0
Spent twigs-70%ACE-maceration (ALC)	11.0 ± 0.0	11.0 ± 0.0	9.5 ± 0.1	9.8 ± 0.0	12.1 ± 0.0	9.0 ± 0.0	10.0 ± 0.0
Positive control	12.5 ± 0.5 ^a	13.9 ± 0.7 ^a	15.7 ± 1.0 ^a	12.5 ± 0.5 ^a	16.4 ± 1.1 ^a	19.3 ± 0.6 ^b	25.7 ± 0.6 ^b

^a Vancomycin (5.0 mg); ^b Vancomycin (50.0 mg); nd: not detected.

Comparing leaves and twigs, twigs originated slightly higher inhibition diameters than leaves extracts, whenever acetone was used as extraction solvent. 70% ACE was the best extraction solvent regarding antimicrobial activity, for both leaves and twigs extracts, having produced significantly higher inhibition diameters than 70% ET or 70% MET extracts.

The origin of the raw material had a marked influence in the case of leaves extracts, with extracts obtained with leaves from Alcobaça having better results with all microorganisms, except for *E. faecalis*. In the case of twigs extracts, the differences were not so visible but extracts from Caparica twigs gave better results, exception made against *S. epidermidis*, *E. faecalis*, and *S. mitis*.

For both leaves and twigs extracts, the extraction process (maceration versus hot extraction), as well as the drying state of the raw material (dried versus fresh raw material), and the use of dried versus spent raw material did not significantly affect the antimicrobial activity, with extracts producing very similar inhibition zones against the microorganisms. Regarding the obtention of antibacterial extracts, these are important findings, once it supports the suggestion of using a more sustainable process (maceration at room temperature), with facilitated logistics due to not having the necessity of using fresh raw material. Moreover, the results show the possibility of retrieving a broader range of products from the same amount of raw material, since the previous extraction with petroleum ether to get lipophilic compounds did not affect the antimicrobial activity of the extracts that were then obtained with 70% aqueous acetone.

Considering the concentration of 10 mg/mL used in the antimicrobial assays for comparison purposes, it can be observed that the leaves extracts obtained in this study produced similar effects on the microorganisms to the ones shown by other extracts from leaves of *Acacia* spp. reported in the literature with concentrations ranging from 5 mg/mL to 200 mg/mL. Antimicrobial effects of leaves extracts expressed by inhibition zones were already described against *S. aureus* with *A. dealbata* aqueous and ethanolic extract (10.0–10.2 mm of inhibition, at 5 mg/mL) [29], with *A. saligna* (15 mm by an ethyl acetate extract at 10 mg/mL [24], and 24.7 mm by an ethanolic extract at 200 mg/mL [26]), with extracts of *A. etbaica*, *A. laeta*, and *A. origina* (7.3–21.0 mm at 500 mg/mL) [23], with *A.*

pycnantha (10.2–21.0 mm at 500 mg/mL [23], and 9–12 mm by aqueous and ethanolic extracts, no concentration reported [22]), and with *A. rigidula* and *A. berlandieri* acetic, methanolic, and acetic acid extracts (6–11.4 mm at 500 mg/mL); against MRSA with *A. saligna* ethanolic extract (22.7 mm at 200 mg/mL) [26]; against *S. epidermis* (6–10 mm by aqueous and ethanolic extracts, no concentration reported) [22]; against *E. faecalis* with *A. rigidula* and *A. berlandieri* acetic, methanolic, and acetic acid extracts (6–12.1 mm at 500 mg/mL); and against *B. cereus* with *A. dealbata*, *A. melanoxylon*, *A. baileyana*, and *A. nicholli* aqueous, ethanolic, and methanolic extracts (6–19 mm at 10 mg/mL) [30], and with *A. saligna* ethyl acetate extract (inhibition zone of 16 mm at 10 mg/mL) [24].

Concerning twigs in particular, just a few studies on antimicrobial effects involving *Acacia* spp. were found in the literature, most of them involving *A. nilotica* twigs [31–34]. Antimicrobial activity of twigs extracts produced in this work was in a lower range than the reported by Kumari et al. for *A. nilotica* twigs against *S. aureus* (10.8–40.2 mm), *E. faecalis* (16.2–38.0 mm), and *C. albicans* (14.7–27.0 mm) [31]. However, these results were obtained by applying 2 mg of extract/well [31], while in this work only 0.5 mg of extract/well was applied, which can at least partially explain the differences observed. To the best of our knowledge, no results of antimicrobial effects against MRSA, *S. epidermidis*, or *B. cereus* for twigs extracts of other *Acacia* spp. have been reported in the literature.

It is known that Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, due to the protection of the lipopolysaccharide layer in the outer membrane [73]. *Candida albicans* was included in this study as a model to determine the antifungal activity of the extracts. Despite being a common commensal yeast fungus of the human oral, gastrointestinal, and genital mucosal surfaces and skin, under specific circumstances, such as perturbation of barrier integrity or host immune responses, *C. albicans* causes opportunistic infections that range from superficial infections of the skin to life-threatening systemic infections [76,77]. All tested samples revealed ineffective against Gram-negative *P. aeruginosa* and *E. coli*, which was equally reported by El-Toumy et al. with aqueous, methanolic, and ethyl acetate extracts of *A. saligna* leaves [24]. The tested samples were also ineffective against *C. albicans* yeast, as observed by Silva et al. with aqueous and methanolic extracts of *A. dealbata* leaves [30], by Ramli et al. with ethanolic extract of *A. farnesiana* leaves [16], or by Cock and van Vuuren with aqueous and methanolic extracts of *A. karroo* leaves [17]. Antibacterial effects against Gram-negative *E. coli* bacteria [22,25,26,29,30] and *C. albicans* yeast [24,26,30,31] were detected in leaves extracts of *Acacia* spp., but, in some cases, using extract concentrations that were more than double [31] or even twenty times higher [26] than the ones used in this work. In general, a positive correlation was observed between the antimicrobial activity of extracts from leaves and twigs of *A. dealbata*, and their total phenolic content, but this association should be confirmed by additional quantitative studies.

3.6. Characterisation of the Non-Polar Components of the Extracts

To understand if the phenolic extracts also contained non-polar components with antioxidant or antimicrobial activities that might influence the properties of the extracts, a group of selected extracts were derivatized and fractionated to isolate these less polar components and analysed by GC-TOFMS. A total of 32 compounds were tentatively identified using the NIST mass spectra library matching (Supplementary Material, Table S6). Among them, phytol, squalene, α -tocopherol, lupeol, and lupenone (lup-20(29)-en-3-one) (Figure 3) are the most predominant, showing the highest peak areas. Phytol, squalene, lupeol, lupenone, and α -tocopherol have been previously identified in *A. dealbata* leaves [36,66,78].

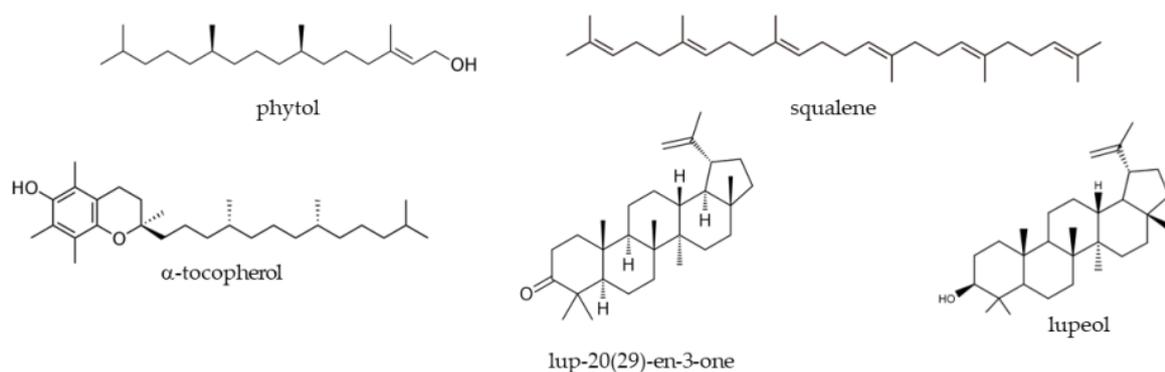


Figure 3. Chemical structures of phytol, squalene, α -tocopherol, lupenone (lup-20(29)-en-3-one), and lupeol.

Phytol is a diterpene known for its wide range of biological activities, such as antimicrobial, antioxidant, cytotoxic, anxiolytic, anticonvulsant, immunomodulatory, antinociceptive, and anti-inflammatory activities, besides its role in the induction of apoptosis and protective autophagy and in the treatment of metabolic disorders [79]. Phytol is the most abundant compound in the analyzed extracts, especially in the leaves fraction, with the extracts obtained from dried leaves showing higher content than the one obtained from fresh leaves (Figure 4).

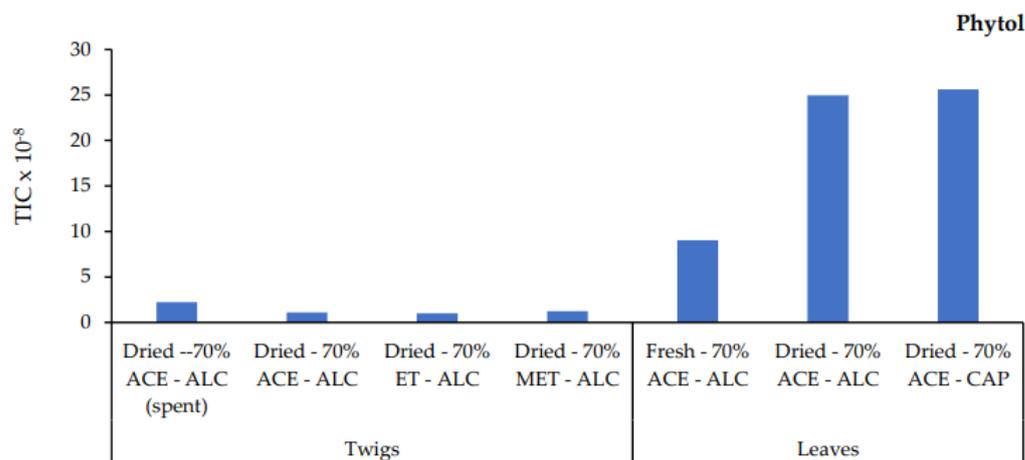


Figure 4. Absolute peak areas of phytol in the analyzed extracts; TIC: Total Ion Current.

Figure 5 shows the absolute peak areas of squalene, α -tocopherol, lupenone, and lupeol for the selected extracts.

Squalene is a triterpene with reported antioxidant, anti-inflammatory, and anti-atherosclerotic properties [80], as well as presenting the potential for cosmetic dermatology applications due to its emollient and antioxidant properties, and for hydration and its antitumor activities [81]. Squalene is more abundant in 70% ACE extracts than in 70% ET or 70% MET ones, with the highest amounts being observed in the dried leaves extracts.

α -Tocopherol is the main form of vitamin E, which has antioxidant, anti-inflammatory, and antitumor properties already described in the literature [82]. α -Tocopherol is not present in the 70% ET and 70% MET extracts, being particularly abundant in the dried leaves extracts.

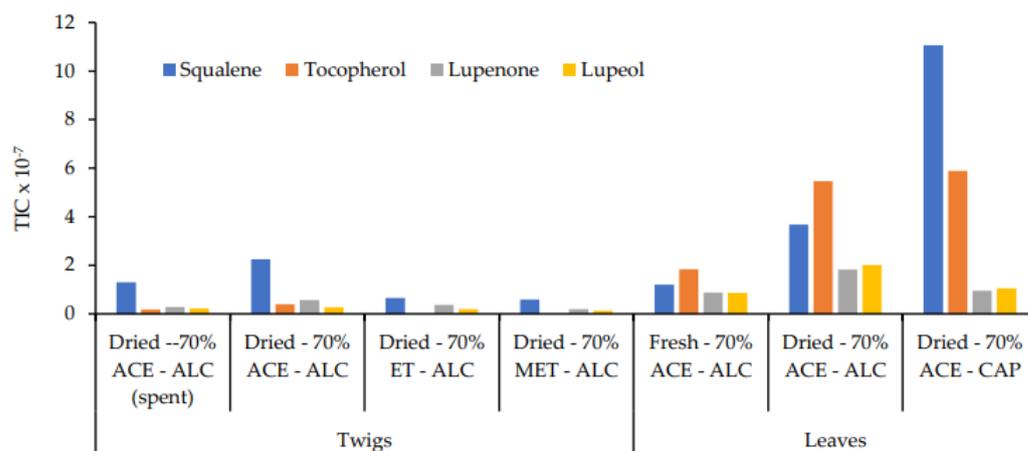


Figure 5. Absolute peak areas of squalene, α -tocopherol, lupenone (lup-20(29)-en-3-one), and lupeol in the analyzed extracts; TIC: Total Ion Current.

Lupenone and lupeol are pentacyclic triterpenes showing a broad spectrum of biological activities. For the former, its importance as a therapeutic and chemo preventive agent for the treatment of inflammation, virus infection, diabetes, cancer, and Chagas disease was already described in the literature [83]. For lupeol, antioxidant, antiprotozoal, anti-inflammatory, antitumor, and antimicrobial activities were previously reported, in addition to its cardio- and hepatoprotective effects and as a cancer chemo preventive agent [84]. Both compounds are more abundant in leaves extracts than in twigs extracts. These results show the ability of acetone to extract both polar and non-polar compounds, which occurred to a lesser extent when using aqueous ethanol or methanol, given the higher polarity of these solvents. The use of dry material favored the extraction of this non-polar fraction as well. The previous extraction of the raw material with petroleum ether naturally decreased the amount of these non-polar compounds in the extract obtained from spent material. The presence of these bioactive compounds in a higher amount in the leaves extracts does not agree with the highest antioxidant and antimicrobial effects observed in the twig's extracts, which suggests that these effects are predominantly due to the action of the more polar compounds present in the extracts. In fact, it was observed a strong positive correlation between TPC and antioxidant activity.

4. Conclusions

This investigation demonstrated the possibility of producing extracts rich in phenolic compounds with antioxidant and antimicrobial properties using leaves and twigs of Portuguese *A. dealbata* by a simple maceration process at room temperature. The highest values of extraction yield, TPC, TFC, and TPrAC as well as antioxidant activity measured by the DPPH and FRAP assays were obtained with 70% ACE using leaves and twigs from Alcobaca. The drying status of the raw material also affected some characteristics of the extracts, improving extraction yield, TFC, and TPrAC for dried leaves and twigs.

As a rule, better results were observed in extracts from the twigs than in the corresponding extracts from the leaves, except for extraction yield. This observation shows the possibility of using the entire fraction of the branches, where the leaves represent by far the highest weight fraction, without the necessity of separating the twigs from the leaves once they add quality to the produced extracts.

Moreover, this work showed the possibility of obtaining a wider range of products from the same amount of raw material via sequential extraction with petroleum ether to remove lipophilic compounds followed by the best extraction solvent to remove phenolic compounds, all without significantly decreasing the quality of the extract.

Significant and strong correlations between TPC and TPrAC and antioxidant activity by DPPH and FRAP assays shows that those phenolic components are the main responsible

for the antioxidant and antimicrobial properties of the extracts. Nevertheless, bioactive lipophilic components, such as phytol, squalene, α -tocopherol, lupenone, and lupeol were also detected, especially in the extracts of leaves. Therefore, this work demonstrates the possibility of obtaining high-value extracts with antioxidant and antimicrobial properties from fractions of *A. dealbata* biomass that can be collected regularly to reduce widespread dissemination of this species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr10112429/s1>, Table S1: TPC (mg GAE/g of biomass, wet basis), TFC (mg CatE/g of biomass, wet basis), and antioxidant activity by DPPH (mg TE/g of biomass) and FRAP (mmol Fe²⁺/g of biomass) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods; Table S2: Extraction yield, TPC and DPPH values for different times of maceration at room temperature, of plants collected in Caparica using 70% ACE as extraction solvent; Table S3: TFC (mg RE/g extract, wet basis) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods; Table S4: TFC (mg RutE/g of raw material, wet basis) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods; Table S5: Average values of extraction yield (% wet basis), TPC (mg GAE/g extract), TFC (mg CatE/g extract) and TPrAC (mg PycE/g extract) for extracts obtained by maceration and hot extraction (regardless drying status and origin), from fresh and dried material (regardless extraction method and origin), and from plants from Alcobaça and Caparica (regardless extraction process and drying status); Table S6: Chromatographic peak areas of non-polar components co-extracted with phenolic compounds for a group of selected extracts obtained by maceration at room temperature; Figure S1: Chromatographic profile of the non-polar fraction of the extract obtained with 70% acetone by maceration of dried Acacia leaves collected in Alcobaça region. Part 1—Low retention time components; Figure S2: Chromatographic profile of the non-polar fraction of the extract obtained with 70% acetone by maceration of dried Acacia leaves collected in Alcobaça region. Part 2—High retention time components; Figure S3: Chromatographic profile of the non-polar fraction of the extract obtained with 70% methanol by maceration of dried Acacia twigs collected in Alcobaça region. Part 1—Low retention time components.

Author Contributions: Conceptualization, R.C. and M.G.; methodology, R.C. and M.G.; investigation, R.C., E.M.M. and J.B.; writing—original draft preparation, R.C.; writing—review and editing, M.G., M.P.D., J.C.Q., M.G.d.S. and R.C.; supervision, M.G., M.P.D., M.G.d.S. and J.C.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by national funds through the Fundação para a Ciência e a Tecnologia, I.P. (Portuguese Foundation for Science and Technology) by the project (UIDB/04077/2020-2023 and UIDP/04077/2020-2023) of Mechanical Engineering and Resource Sustainability Center—MEtRICs, by the project UIDB/05064/2020 of VALORIZA—Research Centre for Endogenous Resource Valorization), by the project (PTDC/CTA-AMB/6587/2020) of CENSE “Center for Environmental and Sustainability Research” and by the project (UID/QUI/50006/2020) of LAQV/REQUIMTE; human resources were also supported by the PhD grants SFRH/BD/133300/2017 and UI/BD/150867/2021.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge Fundação para a Ciência e a Tecnologia, I.P. (Portuguese Foundation for Science and Technology) for financing the projects (UIDB/04077/2020-2023 and UIDP/04077/2020-2023), UIDB/05064/2020, (PTDC/CTA-AMB/6587/2020) and (UID/QUI/50006/2020) that support the activities of the research units that participated in this work. The authors also acknowledge Fundação para a Ciência e a Tecnologia, I.P. for the PhD grants SFRH/BD/133300/2017 and UI/BD/150867/2021.

Conflicts of Interest: The authors declare no conflict of interest.

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