Health promoting potential of herbal teas and tinctures from *Artemisia campestris* subsp. *maritima*: from traditional remedies to prospective products

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This work explored the biotechnological potential of the medicinal halophyte *Artemisia campestris* subsp. *maritima* (dune wormwood) as a source of health promoting commodities. For that purpose, infusions, decoctions and tinctures were prepared from roots and aerial-organs and evaluated for *in vitro* antioxidant, anti-diabetic and tyrosinase-inhibitory potential, and also for polyphenolic and mineral contents and toxicity. The dune wormwood extracts had high polyphenolic content and several phenolics were identified by ultra-high performance liquid chromatography–photodiode array–mass-spectrometry (UHPLC-PDA-MS). The main compounds were quinic, chlorogenic and caffeic acids, coumarin sulfates and dicaffeyloyquinic acids; several of the identified phytoconstituents are here firstly reported in this *A. campestris* subspecies. Results obtained with this plant’s extracts point to nutritional applications as mineral supplementary source, safe for human consumption, as suggested by the moderate to low toxicity of the extracts towards mammalian cell lines. The dune wormwood extracts had in general high antioxidant activity and also the capacity to inhibit α-glucosidase and tyrosinase. In summary, dune wormwood extracts are a significant source of polyphenolic and mineral constituents, antioxidants and α-glucosidase and tyrosinase inhibitors, and thus, relevant for different commercial segments like the pharmaceutical, cosmetic and/or food industries.

Medicinal plants are increasingly explored by the food industry for their health-promoting benefits either as readily available for herbal teas (e.g. *Matricaria chamomilla* [chamomile], *Cymbopogon citratus* [lemongrass]) or as sources of additives for functional foods and drinks (e.g. *Aloe vera* [aloe], *Aspalathus linearis* [rooibos])¹⁻¹². Yet, medicinal halophytes remain largely unexplored and underutilized despite their outstanding potential as a reservoir of bioactive compounds and innovative health promoting products¹. Recently, different scientific efforts have unveiled some of these halophytes’ prospective commercial uses namely as food (e.g. *Arthrocennum macrostachyrum⁴*), herbal functional beverages (e.g. *Helichrysum italicum* subsp. *picardii⁵*, *Crithmum maritimum⁶*, *Limonium algarvense⁷*), or as raw material for pharmaceutical and other related industries (e.g. *Lithrum salicaria⁸*, *Polygonum maritimum⁹*).

Halophytes live and thrive in saline biotopes characterized by highly fluctuating abiotic constraints. To deal with such unfavourable conditions these salt-tolerant plants developed adaptive responses including the synthesis of highly bioactive molecules with potent antioxidant capacity, such as phenolic compounds, terpenoids and vitamins, to counteract reactive oxygen species (ROS) production and accumulation, inhibit oxidative chain-reactions and protect cellular structures⁵. These natural antioxidants usually display strong biological activities, like radical-scavenging, metal-chelating and enzyme-inhibiting abilities, leading to beneficial therapeutic

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properties, which can help explain the use of some halophytes in traditional medicine and as dietary plants. For example, the aromatic Crithmum maritimum is used in folk medicine as diuretic, antiscorbutic, digestive or anti-inflammatory, and is traditionally consumed as condiment, pickle, and in salads. Another aromatic halophyte, Helichrysum italicum subsp. picardii, is often used as a spice and has folk therapeutic uses such as anti-inflammatory, analgesic or anti-microbial. Besides their traditional use as food and folk remedies, halophytes can be produced in otherwise uncultivable saline soils and marine-influenced environments and serve as alternative cash crops in saline agriculture. In fact, these plants could be explored for diverse commercial segments, from human and animal nutrition to pharmaceutical and cosmetic industries.

Artemisia campestris L. subsp. maritima Arcangeli (Asteraceae), commonly named dune wormwood ("madonaira" or "erva-lombriqueira" in Portugal), is an aromatic and medicinal halophytic shrub common in coastal sand dunes throughout the temperate European Atlantic coast. Usually consumed as herbal tea made from stems and leaves, it is described as a remedy to treat gastric disorders, hypertension and rheumatics, being also used for its anthelmintic and abortifacient properties. The species, A. campestris, has additional ethnomedical uses described such as anti-diabetic, anti-inflammatory and antipyretic. Although several studies have already profiled the phytochemical content and bioactivities of A. campestris (revised in Dib et al., 2020), only few reports focused on the sub-species A. campestris subsp. maritima. Research on this particular plant reports compounds like phenolic acids, flavonoids, coumarins, sesquiterpenes and acetophenone derivatives, determined on organic extracts, and describes the antioxidant and anti-microbial activities of methanolic extracts.

In folk medicine, water (infusions and decoctions) and hydro-alcoholic (tinctures) extracts are commonly used to convey the plants’ healing properties. Considering the potential health benefits of such botanical extracts, medicinal plants can offer a wide range of bioactive components (e.g. polyphenols) and can be explored as raw material for herbal beverages, foods products or constituents in health promoting commodities. In fact, natural products are currently in high demand and substances with anti-ageing or beauty-enhancement properties (e.g. skin whitening) are on top of consumers list of interest. Other sought beneficial outcomes include management of diabetes mellitus and improvement of cognitive functions, associated with the intake of antioxidants. Biochemical studies on medicinal plants can therefore be extremely useful to identify new sources of relevant products for pharmaceutical, cosmetic and/or food industries, and many Artemisia species already find extensive uses as food additives and in perfumery. In this sense, Artemisia campestris subsp. maritima could be a potential reservoir of bioactive compounds, representing a commercial underexplored opportunity. Therefore, this work’s goal was to explore the dune wormwood’s biotechnological potential as source of bioactive phytochemicals. For that purpose, infusions, decoctions and tinctures were prepared from above and below-ground organs of A. campestris subsp. maritima and assessed for polyphenolic and mineral contents, and in vitro antioxidant, anti-diabetic and tyrosinase-inhibition potential. A preliminary study with this plant.

**Results and Discussion**

**Phytochemical profile.** The polyphenolic content of the extracts was firstly assessed in terms of their total contents of phenolics (TPC), flavonoids (TFC), condensed tannins (CTC), hydroxycinnamic acid derivatives (HAD), flavonols and anthocyanins (Table 1). Phenolic compounds are some of plants most widely occurring secondary metabolites. Although there is no instituted classification in terms of high/low values of total phenolics, some authors state that natural extracts can be considered rich in phenolic compounds when their TPC is higher than 20 mg GAE/g DW. In this sense, all of A. campestris subsp. maritima extracts have high phenolics content considering that TPC was between 114 and 134 mg GAE/g DW, with the highest value determined in aerial-organs’ tincture. This extract also had the highest flavonoid content (40.8 mg CAE/g DW), higher HAD together with aerial-organs’ infusion and decoction (89.4–88.4 mg CAE/g DW), and higher anthocyanins along with roots’ tincture (3.46 and 3.36 mg CCE/g DW). Flavonols, on the other hand, were highest in roots’ tincture (66.2 mg QE/g DW). As for tannins content, it was not found in the dune wormwood samples (below the limit of quantification, which was 0.78 mg/g DW). Working with the same sub-species, Megdiche-Ksouri et al., 2020 reported...
other authors determined different levels of TPC and TFC, either higher, similar or lower than those presently found. These discrepant phytochemical contents may be explained by species-specific factors, harvesting time and/or environmental characteristics, since these variables affect the biosynthesis of secondary metabolites in plants. Nevertheless, authors generally consider A. campestris rich in phenolic compounds.

To further explore the phytochemical profile of infusions, decoctions and tinctures from A. campestris, a generic LC-PDA-MS (liquid chromatography – photodiode array – mass spectrometry) method for moderately polar phytochemicals was employed. The analytical methodology was adapted from De Paepe et al. and is fully detailed in Pereira et al. including performance characteristics, quantification procedures and compound tentative identification specifics. The aim was to (tentatively) identify phytochemical constituents in the dune wormwood extracts, getting an estimate of their concentrations and/or relative abundances when no reference standards were available. The phenolics and respective concentrations are presented in Table 2. As some standards can be expensive or not available, tentative identification of other compounds was accomplished based on available chromatographic and spectral information (Table 3). To get clean product ion spectra of the detected analytes, data dependent fragmentation was used. Product ions are substrates of precursor ions (ions of a particular mass over charge range [m/z-range]), formed during fragmentation: structures were assigned to unknown peaks when both the m/z-values and molecular formulae/structures of the precursor and product ions were in agreement. Further information for de-replication was obtained from PDA spectra, in-house and commercial compound databases (PubChem, Dictionary of Natural Products, ChemSpider) and peer reviewed publications (a more detailed explanation is given in Pereira et al.). MS and diagnostic chromatographic data used for compound identification plus literature used for confirmation of compound identity can be found in Table S2 (supplementary material). It is important to mention that during LC-MS analysis different compounds can have different ionization efficiencies and so no absolute quantitative comparison can be made, although relative abundances per compound in-between samples can be calculated (based on the area of their most abundant ion). In this sense, the “maximum area detected” provides semi-quantitative information of compound abundance. Table 3 shows the relative abundances of these tentatively identified constituents. To visualize the extracts’ main detected compounds, the UV-chromatograms at combined wavelengths (280–330 nm, the absorption maxima of phenolics) are represented in Fig. 1, despite not showing all the constituents identified (compounds with no assigned peaks had low abundances or possibly their peaks overlapped).

Table 2. Concentrations of compounds in infusions, decoctions and tinctures from Artemisia campestris subsp. maritima organs (mg/g DW), calculated with reference standards using LC-amMS. Quantitation limits are presented as ≤ LOQs (µg/mg DW). *Corresponding peak number in the chromatograms on Fig. 1. **RT – retention times.
According to Table 2, the dune wormwood aerial-organs' extracts had greater diversity and higher levels of practically all phenolics found. Aerial-organs' tincture in particular had higher concentrations of most of the determined compounds adding up to a total of 45 µg/mg DW. From this total, quinic acid amounts to half (24 µg/mg DW),

**Table 3.** Average relative abundances (peak area/mg DW, %) of the tentatively identified compounds in extracts from *Artemisia campestris* subsp. *maritima* organs, analysed by LC-PDA-amMS. NF – not found.

| Peak n° | Tentative ID | Roots | Aerial-organs | Maximum area detected |
|---------|--------------|-------|---------------|----------------------|
|         |              | Infusion | Decoction | Tincture | Infusion | Decoction | Tincture |           |
| 1       | Chlorogenic acid isomer (isochlorogenic acid A, B or C) | 7.77 | 91 | 100 | 62 | 91 | 100 | 88 | 410 976 815 |
| 2       | Hydroxybenzoic acid isomer | 8 | 99 | 100 | 98 | 66 | 77 | 79 | 169 361 809 |
| 3       | Hexoside of scopoletin (scopolin) | 8.48 | 30 | 28 | 43 | 73 | 66 | 100 | 185 080 059 |
| 4       | Hexoside of coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin) | 9.02 | 43 | 46 | 65 | 69 | 64 | 100 | 403 174 862 |
| 5       | Chlorogenic acid isomer (isochlorogenic acid A, B or C) | 9.33 | 48 | 50 | 49 | 55 | 56 | 100 | 1 097 618 207 |
|         | Aesculetin | 9.61 | 19 | 19 | 44 | 43 | 47 | 100 | 68 226 925 |
| 6       | Chlorogenic acid isomer (isochlorogenic acid A, B or C) | 9.88 | 65 | 61 | 68 | 68 | 65 | 100 | 108 537 786 |
| 7       | Fraxetin | 10.19 | 100 | 93 | 82 | 60 | 62 | 87 | 82 132 702 |
| 8       | Coumaric acid hexoside isomer | 10.32 | 4 | 3 | 5 | 63 | 64 | 100 | 180 996 161 |
| 9       | Coumaric acid hexoside isomer | 10.76 | 6 | 4 | 7 | 69 | 70 | 100 | 102 579 661 |
| 10      | Coumarin sulfate with 2 methoxy moieties (iso-fraxidin or fraxidin) | 11.54 | 71 | 68 | 100 | 55 | 55 | 69 | 9 544 319 167 |
| 11      | Coumarin sulfate (fraxetin-O-sulfate isomer) | 11.65 | 50 | 53 | 31 | 82 | 86 | 100 | 4 395 956 598 |
| 12      | Not identified (C_{12}H_{18}O_{7}S) | 11.74 | 6 | 4 | 9 | 62 | 63 | 100 | 5 434 495 637 |
| 13      | Coumarin sulfate (fraxetin-O-sulfate isomer) | 11.78 | 75 | 73 | 100 | 34 | 35 | 41 | 23 258 593 384 |
| 14      | Scopoletin | 12.03 | 68 | 62 | 100 | 46 | 49 | 72 | 231 549 513 |
| 15      | Coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin) | 12.11 | 93 | 90 | 95 | 64 | 64 | 100 | 256 857 167 |
| 16      | Coumarin sulfate (fraxetin-O-sulfate isomer) | 12.19 | 71 | 67 | 100 | 31 | 32 | 41 | 17 799 164 012 |
| 17      | Fraxin-caffeoyl-hexoside | 13.03 | 32 | 30 | 45 | 55 | 55 | 100 | 91 320 194 |
| 18      | Methoxy-cinnamic acid | 13.53 | 46 | 52 | 100 | 30 | 34 | 55 | 22 190 655 |
| 19      | Dicaffeoylquinic acid | 13.65 | 92 | 100 | 91 | 59 | 62 | 69 | 1 324 940 207 |
| 20      | Dicaffeoylquinic acid methyl ester | 14.35 | 71 | 62 | 100 | 53 | 47 | 95 | 11 018 820 |
| 21      | Dicaffeoylquinic acid | 14.45 | 63 | 66 | 64 | 79 | 75 | 100 | 2 500 658 869 |
| 22      | Dicaffeoylquinic acid | 14.6 | 34 | 34 | 46 | 67 | 76 | 100 | 24 968 283 |
| 23      | Dicaffeoylquinic acid methyl ester | 14.79 | 44 | 44 | 55 | 52 | 48 | 100 | 190 893 063 |
| 24      | Dicaffeoylquinic acid methyl ester | 15.03 | 20 | 19 | 23 | 69 | 70 | 100 | 35 407 749 |
| 25      | Dicaffeoylquinic acid methyl ester | 15.37 | 9 | 8 | 11 | 52 | 47 | 100 | 136 311 796 |
| 26      | Caffeic acid coupled to C_{11}H_{12}O_{6} | 15.89 | 6 | 3 | 3 | 97 | 100 | 100 | 206 701 996 |
| 27      | Flavonoid | 15.99 | 3 | 2 | 3 | 59 | 60 | 100 | 207 351 776 |
| 28      | Ethoxy or dimethoxycinnamic acid | 16.1 | 0 | 0 | 43 | 0 | 0 | 100 | 3 704 845 930 |
| 29      | Tricaffeoylquinic acid | 16.13 | 14 | 13 | 23 | 11 | 11 | 100 | 793 875 076 |
| 30      | Dimethoxyflavonoid (axillarin) | 16.41 | 2 | 1 | 3 | 48 | 51 | 100 | 635 237 416 |
| 31      | Methoxyflavonoid (tamarixetin, rhamnetin, eupafolin, queretin-3-methyl ether) | 16.72 | 2 | 2 | 3 | 54 | 55 | 100 | 806 869 736 |
| 32      | Methoxyflavonoid (lacticitrin or mearsectin) | 16.81 | 0 | 1 | 1 | 61 | 100 | 86 | 89 733 523 |
| 33      | Trimethoxyflavonoid | 17.49 | 2 | 1 | 2 | 48 | 49 | 100 | 40 757 681 |
| 34      | Dimethoxyflavonoid (cirsiliol) | 17.97 | 2 | 1 | 3 | 53 | 56 | 100 | 714 900 053 |
| 35      | Dimethoxyflavonoid | 18.13 | 2 | 1 | 3 | 53 | 56 | 100 | 171 818 097 |
| 36      | Methoxyflavonoid (hispidulin) | 18.26 | 2 | 1 | 3 | 53 | 56 | 100 | 738 797 835 |
| 37      | Methoxyflavonoid (cirsimaritin) | 18.89 | 1 | 1 | 2 | 44 | 45 | 100 | 53 729 586 |
| 38      | Tetramethoxyflavonoid | 19.25 | 2 | 1 | 3 | 53 | 56 | 100 | 40 802 811 |
| 39      | Dimethoxyflavonoid (cirsimaritin) | 20.03 | 2 | 0 | 2 | 41 | 42 | 100 | 17 338 075 |
| 40      | Linderoflavone B | 21.2 | NF | NF | NF | 37 | 47 | 100 | 591 906 |

According to Table 2, the dune wormwood aerial-organs’ extracts had greater diversity and higher levels of practically all phenolics found. Aerial-organs’ tincture in particular had higher concentrations of most of the determined compounds adding up to a total of 45 µg/mg DW. From this total, quinic acid amounts to half (24 µg/mg DW),
followed by chlorogenic (16 µg/mg DW) and caffeic (1.6 µg/mg DW) acids. In fact, these phenolic acids were the main constituents determined in all extracts particularly quinic (roots: 13–15 µg/mg DW, aerial-organs: 24 µg/mg DW) and chlorogenic (roots: 8.4–10 µg/mg DW, aerial-organs: 10–16 µg/mg DW) acids, both higher in tinctures. Rutin was also preferentially detected in aerial-organs aqueous and hydro-alcoholic samples (0.7–1.3 µg/mg DW), followed by protocatechuic acid (0.27–0.43 µg/mg DW), luteolin (0.19–0.47 µg/mg DW) and coumaric acid (0.17–0.33 µg/mg DW), along with isoquercitrin, isorhamnetin and salicylic acid (~0.1–0.2 µg/mg DW).

In roots’ extracts, protocatechuic acid in all extracts (0.09–0.11 µg/mg DW), and coumaric and salicylic acids in.
tincture (0.1 and 0.09 µg/g dw, respectively) were also found in higher levels, although in comparatively lower concentrations than in the aerial-organ's extracts. In Table 3 and Fig. 1 it is also possible to observe the higher compound diversity in extracts from aerial-organs, especially tinctures. However, relative abundance of some major constituents such as coumarin sulfates (peaks 12, 14 and 17) and dicafeoylquinic acids (peaks 25 and 26) was higher in roots' extracts, particularly tincture. Aerial-organs' extracts had higher amounts of another coumarin sulfate (peak 13) and dicafeoylquinic acid (peak 28), along with a chlorogenic acid isomer (peak 5) and an ethoxy/dimetoxycinnamic acid (peak 36). Again, it should be stated that Table 3 provides relative quantitative measures of abundance, not to be interpreted as absolute quantitative comparison. Overall, tinctures of both organs showed higher abundance and diversity of constituents comparatively to aqueous extracts and, between organs, extracts from aerial-organs had greater variety of phenolics, generally in higher levels. To the best of our knowledge, this is the first report comparing anatomical organs in this Artemisia campestris species. Medgie-Ksouri et al. also report a wide assortment of phytochemicals in dune wormwood's shoots, several of them also presently determined, but studies detailing compound abundance in A. campestris extracts other than essential oils are extremely scarce. In fact, only Jahid et al. reports levels of phenolics in leaves' hydro-alcoholic extracts with the main components catechin and vanillic acid (>20 mg/g DW), not being found in the current study, syringic (6 mg/g DW) and coumaric (0.9 mg/g DW) acids, presently determined at lower concentrations (0.05–0.08 mg/g DW and 0.06–0.33 mg/g DW, respectively), and caffeic acid (0.2 mg/g DW), being one of the current main constituents particularly in aerial-organs' tincture (1.6 mg/g dw). These authors also consider that compound nature and abundance are related to environmental conditions, a well-established notion when comparing intra-species extracts other than essential oils are extremely scarce. In fact, only Jahid et al. reports levels of phenolics in leaves' hydro-alcoholic extracts with the main components catechin and vanillic acid (>20 mg/g DW), not being found in the current study, syringic (6 mg/g DW) and coumaric (0.9 mg/g DW) acids, presently determined at lower concentrations (0.05–0.08 mg/g DW and 0.06–0.33 mg/g DW, respectively), and caffeic acid (0.2 mg/g DW), being one of the current main constituents particularly in aerial-organs' tincture (1.6 mg/g dw). These authors also consider that compound nature and abundance are related to environmental conditions, a well-established notion when comparing intra-species.
Antioxidants can be considered a group of medicinally preventive molecules also used as food additives to inhibit food oxidation. Hence, natural antioxidant sources are increasingly sought after as an alternative to synthetic antioxidants in the food, cosmetic and therapeutic industries. Antioxidants are scavengers of free radicals or ROS and deactivators of metal catalysts by chelation, among other activities, reducing oxidative stress and consequent cell damage. It is increasingly documented that dietary antioxidant phytochemicals effectively prevent oxidative damage, reducing the risk of oxidative-stress-related conditions like neurodegenerative and vascular diseases, carcinogenesis or inflammation. Their intake is also associated with the management of diabetes mellitus and amelioration of skin ageing conditions.

In this work, the antioxidant potential of the dune wormwood’s extracts was assessed by eight different methods targeting radical scavenging activity (RSA) and metal-related potential (Table 5). The extracts were overall effective as scavengers of DPPH, ABTS, NO and O$_2$– radicals and at reducing iron, but their chelating properties were moderate for copper and low for iron. In the DPPH assay the aerial-organs’ tincture had the lowest IC$_{50}$ value (240 µg/mL), lower than that obtained for the positive control (BHT; IC$_{50}$ = 320 µg/mL), followed by the roots’ aqueous extracts (340 µg/mL), decoction (370 µg/mL) and roots decoction (570 µg/mL), all similar to BHT. Figure 2. Toxicity of infusions, decoctions and tinctures (100 µg/mL extract dw) from Artemisia campestris subsp. maritima organs on mammalian cell lines: (A) N9, (B) S17 and (C) HepG2. Cells treated only with cell culture medium were used as controls; H$_2$O$_2$ was used as positive control for cell toxicity. Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each graph, different letters mean significant differences (p < 0.05).
Table 5. Antioxidant activity (IC_{50} values, mg/mL) of infusions, decoctions and tinctures from Artemisia campestris subsp. maritima organs: radical scavenging on DPPH, ABTS, NO and O_{2}•• radicals, ferric reducing antioxidant power (FRAP) and metal-chelating activities on copper (CCA) and iron (ICA). Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each column different letters mean significant differences (p < 0.05). *Positive controls.

| Samples                        | Organ   | Extract  | DPPH (µg/mL) | ABTS (µg/mL) | NO (µg/mL) | O_{2}•• (µg/mL) | FRAP (µg/mL) | CCA (µg/mL) | ICA (µg/mL) |
|--------------------------------|---------|----------|--------------|--------------|------------|----------------|--------------|-------------|-------------|
| A. campestris subsp. maritima  | Roots   | Infusion | 0.39 ± 0.02a | 0.45 ± 0.02bc | 0.74 ± 0.03c | 0.21 ± 0.01a   | 0.29 ± 0.01c | 1.64 ± 0.10a | 7.82 ± 0.37ac |
|                                |         | Decoction| 0.37 ± 0.02bc | 0.37 ± 0.01b  | 0.55 ± 0.01bc | 0.18 ± 0.01a   | 0.25 ± 0.00b | 1.64 ± 0.04  | 7.57 ± 0.34bd |
|                                |         | Tincture | 0.46 ± 0.02a  | 0.46 ± 0.01a  | 1.40 ± 0.07ad | 0.33 ± 0.01a   | 0.24 ± 0.00b | 3.60 ± 0.11bc | >10         |
| Aerial-organs                  | Infusion| 0.33 ± 0.03b | 0.41 ± 0.01c  | 0.70 ± 0.03bc | 0.23 ± 0.01bc | 0.17 ± 0.00bc  | 1.31 ± 0.05bc | 6.47 ± 0.35bd |
|                                | Decoction| 0.34 ± 0.03bc | 0.44 ± 0.01bc | 0.49 ± 0.01ab | 0.24 ± 0.00bc | 0.27 ± 0.01bc  | 1.30 ± 0.09bc | 6.33 ± 0.43bc |
|                                | Tincture | 0.24 ± 0.01a  | 0.40 ± 0.01bc | 0.29 ± 0.02a  | 0.35 ± 0.01a  | 0.23 ± 0.01bc  | 2.51 ± 0.09bc | >10         |
| BHT*                           |         |          | 0.32 ± 0.02a  | 0.11 ± 0.00a  |            |                |              |             |
| Ascorbic acid*                 |         |          | 2.31 ± 0.22a  |              |              |                |              |             |
| Catechin*                      |         |          | 0.62 ± 0.01f  |              |              |                |              |             |
| EDTA*                          |         |          | 0.13 ± 0.00e  | 0.07 ± 0.00e  |              |                |              |             |

(p < 0.05). High RSA against DPPH was also reported by Megdiche-Ksouri et al. in methanolic extracts from shoots of the same A. campestris subspecies. Aerial-organs’ tincture also had the strongest NO scavenging activity allowing an IC_{50} of 290 µg/mL, comparable to that of this organs’ decoction (490 µg/mL, p < 0.05); most interestingly all extracts were better NO scavengers than the positive control (ascorbic acid, IC_{50} = 2.31 mg/mL). This was also the case with O_{2}•• scavenging as catechin had the highest IC_{50} (620 µg/mL). For this radical’s assay, however, the lowest IC_{50} value was obtained after the application of roots’ decoction (180 µg/mL), followed by infusions from both organs (roots: 210 µg/mL, aerial-organs: 230 µg/mL). Roots decoction was also the best ABTS scavenger (IC_{50} = 370 µg/mL), statistically similar to the result obtained with the aerial-organs’ tincture (IC_{50} = 400 µg/mL, p < 0.05). As for the iron reducing capacity, the best result was obtained with the aerial-organs’ infusion with an IC_{50} of 170 µg/mL, followed by aerial-organs’ tincture (230 µg/mL), roots tincture (240 µg/mL) and decoction (250 µg/mL). This is in accordance with Megdiche-Ksouri et al. findings of a high FRAP in this subspecies. Conversely, the extracts iron-chelating activity was comparatively low, with IC_{50} values higher that 5 µg/mL, while the capacity to chelate copper was moderate (best IC_{50} = 1.3 mg/mL in aerial-organs’ water extracts). Tannins were not detected in any of the extracts, which may partially explain its low chelating potential since tannins are known metal chelating agents. The aerial-organ’s water extracts had the highest capacity to chelate both metals (CCA, IC_{50} = 1.30–1.31 mg/mL; ICA, IC_{50} = 6.33–6.47 mg/mL). Several studies previously highlighted the high antioxidant capacity of similar aqueous and hydro-alcoholic extracts from A. campestris, which confirms our results of strong in vitro antioxidant potential for this subspecies. Most of these authors also credited the pronounced antioxidant activity of the extracts to the polyphenolic content which is, in fact, an association widely reported by several studies that confirm the phenolics’ role as antioxidants, especially in halophyte plants. Accordingly, aerial-organs’ tincture had the highest levels of almost all phenolics groups (Table 1) and was also of the best-scoring extracts in terms of antioxidant activity. Actually, that extract also had overall higher abundance and variety of individual phenolic constituents (Tables 2 and 3), altogether corroborating the hypothesis that phenolics play a major role in the sample’s strong antioxidant potential. For example, the main components quinic, chlorogenic and caffeic acids, determined in higher amounts in aerial-organs’ tincture (Table 2), are known antioxidant compounds. Nevertheless, roots’ extracts showed greater relative abundances of some major constituents (Table 3), such as the dicafeoylquinic acid (peak 25, Fig. 1) in roots’ decoction, and quinic, chlorogenic and caffeic acids, although in lower levels than in aerial-organs’ samples, were the predominant constituents. Synergistic and/or additive effects between these phytoconstituents may also account for the equally high antioxidant activity of roots’ decoction.

Besides antioxidant activity, other bioactivities have been ascribed to extracts from A. campestris as for example hypoglycaemic effects. Type 2 diabetes mellitus (T2DM) is a common health disorder characterized by high blood glucose levels that can lead to major metabolic complications if left untreated. One effective strategy to manage T2DM is to inhibit carbohydrate-hydrolysing enzymes, such as α-glucosidase, delaying carbohydrate digestion and uptake and resulting in reduced postprandial blood glucose levels, therefore lowering hyperglycaemia linked to T2DM. In this sense, the dune wormwood’s extracts were tested for their capacity to inhibit microbial and mammalian α-glucosidases as an assessment of their anti-diabetic potential. All extracts had the ability to inhibit the microbial α-glucosidase but the most active samples were roots’ aqueous extracts and aerial-organs’ decoction (IC_{50} = 0.89–1.13 mg/mL). Interestingly, all of the extracts were more efficient at inhibiting the microbial α-glucosidase than the positive control used acarbose (IC_{50} = 3.14 mg/mL), a clinically used inhibitor of this enzyme. However, only the roots’ extracts were able to inhibit mammalian α-glucosidase, particularly roots’ tincture (IC_{50} = 2.90 mg/mL), still more active than acarbose (IC_{50} = 4.64 mg/mL). Roots’ extracts were less active towards the mammalian enzyme than for the microbial counterpart, an outcome already described for some compounds showing that enzyme origin can influence the extracts’ inhibition of α-glucosidase. Nevertheless, and despite the notion that the mammalian enzyme is a more reliable proxy for in vivo activity, the in vivo anti-diabetic potential of A. campestris aqueous extracts from leaves was demonstrated...
by Sefi et al., having significantly reduced blood glucose levels in diabetic rats. Those authors considered that the in vivo hypoglycaemic activity of A. campestris extracts could be related to its strong antioxidant properties, and stated the role that this plant’s water extracts can have on the treatment of diabetic patients. It is recognized that polyphenolic compounds, besides potent antioxidants, can also have glucosidase-modulating activities therefore contributing to the management of T2DM. The dune wormwood’s extracts had a high phenolic content and contained some compounds with described hypoglycaemic activity, namely chlorogenic, caffeeic and ferulic acids, and with reported α-glucosidase inhibitory activity, like isoquercitrin, luteolin, quercetin and apigenin. Overall, our results suggest that all dune wormwood’s extracts could be beneficial in managing T2DM by its capacity to inhibit dietary carbohydrate digestive enzymes, which was higher than acarbose, and consequently controlling glucose levels. Furthermore, as oxidative stress has been considered a mediator in diabetic complications, the extracts’ strong antioxidant potential can also be an adjuvant in preventing or attenuating the disease’s symptoms when used in combined anti-diabetic strategies.

Skin hyperpigmentation (e.g. melasma, freckles, age spots) is a result of melanin over-production but, as tyrosinase is essential in melanin biosynthesis, inhibition of this enzyme can help prevent and/or manage undesired skin darkening. Tyrosinase is also responsible for unwanted browning of fruits and vegetables, which decreases their market value. Hence, tyrosinase inhibitors from natural sources are increasingly sought for cosmetic and medicinal purposes but also for their potential in improving food quality. In this context, the tyrosinase inhibitory potential of the dune wormwood’s extracts was evaluated and results are depicted in Table 6. All extracts were active, particularly aerial-organs’ infusion (IC_{50} = 4.13 mg/mL), although less effective than the used positive control (arbutin, IC_{50} = 0.48 mg/mL). Tyrosinase is a copper-containing enzyme and thus the extracts’ moderate copper chelating activity could be related to their tyrosinase inhibitory capacity. In fact, metal chelating and ROS-scavenging properties are mechanisms often thought to be related with the reducing activity of flavonoids. Some flavonoids were already identified as tyrosinase inhibitors, as for example quercetin, kaempferol and taxifolin, the last being as effective as arbutin. All these compounds were detected in the dune wormwood’s extracts, possibly contributing to their tyrosinase inhibitory activity. To the best of our knowledge, this is the first report on the tyrosinase inhibitory potential of A. campestris subsp. maritima.

This study reports for the first time a comprehensive assessment of the biotechnological potential of A. campestris subsp. maritima as a source of innovative products with health promoting properties. Overall, our results point to the potential role of infusions, decoctions and tinctures of the dune wormwood in the prevention of oxidative-stress related diseases and in the management of diabetes and skin-hyperpigmentation conditions. More specifically, those formulations can be considered an unexplored source of polyphenolic and mineral constituents, antioxidants and α-glucosidase and tyrosinase inhibitors that could deliver raw material to different commercial segments including the pharmaceutical, cosmetic and/or food industries. Further studies are being pursued aiming to fully explore the health-promoting benefits of this plant’s extracts, namely their in vivo effects.

### Methods

#### Plant collection.

_Artemisia campestris_ L. subsp. maritima Arcang. (Compositae) plants were collected in South Portugal, within the area of the Ria Formosa coastal lagoon, near Faro (Lido, 37°26’5.526”N 7°58’58.465”W), in June of 2013. The taxonomical classification was carried out by Dr. Manuel J. Pinto, botanist in the National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal, and a voucher specimen (voucher code MBH34) is kept in the herbarium of Marbiotech’s laboratory. Plants were divided in roots and aerial-organs (stems and leaves), oven dried at 50°C until complete dryness (3 days), milled and stored at −20°C until use.

#### Extracts preparation: infusions, decoctions and tinctures.

Water extracts were prepared similarly to a regular cup-of-tea: 1 g of dried plant material was homogenized in 200 mL of ultrapure water. For infusions, the biomass was immersed in boiling water for 5 min; for decoctions, the biomass was boiled in water for 5 min. Hydro-ethanolic extracts were prepared similarly to a home-made tincture: 20 g of dried plant material was left homogenising in 200 mL of 80% aqueous ethanol for a week. Independent extractions (n ≥ 3) for each combination of method + plant-part were made. All extracts were filtered (Whatman n° 4), vacuum and/or freeze-dried.

| Samples      | Organ          | Extract   | Microbial α-glucosidase | Mammalian α-glucosidase | Tyrosinase |
|--------------|----------------|-----------|-------------------------|-------------------------|------------|
| _A. campestris_ subsp. maritima | Roots          | Infusion  | 0.92 ± 0.04*            | 6.09 ± 0.41*            | 7.58 ± 0.14* |
|              |                | Decoction | 0.89 ± 0.03*            | 6.62 ± 0.48*            | 5.56 ± 0.45* |
|              | Aerial-organs  | Tincture  | 2.54 ± 0.05*            | 2.90 ± 0.22*            | 5.23 ± 0.12* |
|              |                | Infusion  | 1.64 ± 0.03*            | >10                     | 4.13 ± 0.27* |
|              |                | Decoction | 1.13 ± 0.03*            | >10                     | 5.14 ± 0.35* |
|              |                | Tincture  | 1.62 ± 0.06*            | >10                     | 5.35 ± 0.25* |
| Acrbose*     |                |           | 3.14 ± 0.23*            | 4.64 ± 0.76*            | 0.48 ± 0.01* |
| Arbutin*     |                |           |                         |                         | 0.48 ± 0.01* |

*Positive controls.
and stored in a dark, cool and moist-free environment. Extracts were re-suspended in water or aqueous ethanol to a concentration of 10 mg/mL to determine (spectrophotometric) phenolic content and test for bioactivities. For these assays, no significant differences were found among corresponding extracts from the different extractions and therefore freeze-dried extracts were pooled accordingly for the remaining analyses.

**Phytochemical composition of the extracts.** Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content. The TPC, TFC and CTC were estimated by spectrophotometric methods, respectively: Folin-Ciocalteu, aluminium chloride colorimetric and 4-dimethylaminocinnamaldehyde (DMACA), as described in Rodrigues et al.\(^6\). Gallic acid, quercetin and catechin were used as standards and results are presented as milligrams of standard equivalents per gram of extract dry weight (GAE, QE and CE, respectively; mg/g dw). Further information pertained to these methods is presented in Table S1 (supplementary material).

**Hydroxycinnamic acid derivatives (HAD), flavonols and anthocyanins content.** Total contents in HAD, flavonols and anthocyanins were assessed spectrophotometrically as described previously\(^6\) using caffeic acid, quercetin and cyanidin chloride as standards, respectively. Results are presented as milligrams of standard equivalents per gram of extract dry weight (CAE, QE and CCE, respectively; mg/g dw). Further information pertained to these methods is presented in Table S1 (supplementary material).

**Profile of moderately polar compounds by UHPLC.** Standard stock solutions were prepared at 1 mg/mL in UHPLC-grade methanol and stored at 4 °C in the dark. Standard dilutions were prepared in 60:40 (v/v) methanol:40 mM ammonium formate buffer (reference standards: apigenin, apigenin-7-O-glucoside (apigetrin), catechin, cyanidin-3-O-arabinoside, cyanidin-3-O-galactoside chloride (kuromanin chloride), cyanidin-3-O-rutinoside chloride (keracyanin chloride), (+)-dihydrokaempferol ((+)-aromadendrin), epicatechin, epigallocatechin, epigallocatechin gallate, flavone, galangin, hesperidin, hesperidin methyl chalcone, 4-hydroxybenzaldehyde, kaempferol, kaempferol-3-O-glucoside (astragalin), limonin, luteolin, naringenin, naringin, neohesperidin dihydrochalcone, phloretin, phloretin-3-O-glucoside (phlorizidin), procyanidin B2, proanthocyanidin C3, propyl gallate, quercetin, quercetin-3-O-arabinose (avicularin), quercetin-3-O-galactoside (hyperin), quercetin-3-O-glucoside (isoquercitrin), quercetin-3-O-rhamnoside (quercitrin), rutin, uvaol, and caffeo, chlorogenic, coumaric, dihydrocaffeic, ellagic, ferulic, gallic, gentisic, m-hydroxybenzoic, hydroferulic, p-hydroxybenzoic, oleanolic, quinic, rosmarinic, salicylic, sinapinic and syringic acids). Freeze-dried pooled extracts (approx. 15 mg) were dissolved in 20 mL of 60:40 methanol:water +40 mM ammonium formate, followed by 1 min vortex mixing, 30 min sonication (40 kHz, 100 W, room temperature) and 10 min centrifugation (3000 rpm). Supernatants were diluted 100-fold and stored along with undiluted extracts at 4 °C, until analysis. Both undiluted and diluted extracts were analysed with a generic ultra-high performance liquid chromatography – photodiode array – accurate mass mass spectrometry (UHPLC-PDA-amMS) method for moderately polar phytochemicals adapted from De Paep et al.\(^3\) and fully detailed in Pereira et al.\(^6\). Briefly, for analysis 5 µL of extract was injected on an UPLC BEH SHIELD RP18 column (3.0 mm × 150 mm, 1.7 µm; Waters, MA) and thermostatically eluted (40 °C) with a quaternary solvent manager and a ‘Hot Pocket’ column oven. The mobile phase consisted of water +0.1% formic acid (A) and acetonitrile +0.1% formic acid (B), following a gradient of (min/%A): 0.0/100, 9.91/74, 18.51/35, 18.76/0, 23.76/0, 23.88/100, 26.00/100. For detection, a Q Exactive MS (Thermo Fisher Scientific, Bremen, Germany) was used with heated electrospray ionization (HESI). For quantitative analysis, full scan data were acquired using polarity switching with a mass/charge (m/z) range of 120–1800 and resolving power set at 70 000 at full width at half maximum (FWHM). Data were also recorded using data dependent fragmentation (ddMS\(^2\)) in positive and negative ionization mode to obtain additional structural information. The PDA detector was set to scan from 190 to 800 nm during all analyses. The lowest calibration point included in the calibration curve was used to calculate the limits of quantitation (LOQs). The concentration ranges described by De Paep et al.\(^3\) were also used during the present work. Results regarding concentrations of identified compounds were calculated as µg/mg of extract dry weight.

**Mineral composition.** Freeze-dried pooled extracts were digested in a combination of nitric acid (HNO\(_3\)) and hydrogen peroxide on a hot plate and evaporated until dryness (up to 24 h). Digested samples were diluted in 20 mL of 5% HNO\(_3\) and analysed for mineral content by Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), as described in Pereira et al.\(^6\). Instrumental detection limits were as follows: Ca: 0.04 µg/L, Cd: 1.4 µg/L, Cr: 0.3 µg/L, Cu: 0.5 µg/L, Fe: 1.7 µg/L, K: 0.6 µg/L, Mg: 0.031 mg/L, Mn: 0.1 µg/L, Na: 0.1 µg/L, Ni: 1.1 µg/L, Pb: 2.5 µg/L and Zn: 3.1 µg/L. Results were expressed as mg or µg/g of extract dry weight (DW). Appropriate blanks were also produced and analysed.

**Toxicological evaluation of the samples.** Samples’ toxicity was assessed using murine microglia (N9), murine bone marrow stromal (S17) and human hepatocellular carcinoma (HeptG2) cell lines. The N9 cell line was provided by the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal), S17 and HepG2 cells were delivered by the Centre for Biomedical Research (CBMR, University of Algarve, Portugal). Cell culture was maintained as described in Pereira et al.\(^6\). Cell viability was determined by the MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and results were expressed in terms of cell viability (%).
Biological activities. Antioxidant activity assessed by four radical-based assays. The extracts’ radical scavenging capacity against the DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), NO (nitric oxide) and O₂⁻• (superoxide) radicals was assessed as described in Rodrigues et al. and BHT (butylated hydroxytoluene), ascorbic acid and catechin were used as positive controls. Results were calculated as percentage of antioxidant activity in relation to a control containing ultrapure water or aqueous ethanol, and expressed as IC₅₀ values (mg/mL; half maximal inhibitory concentration, ascertained for extracts with activities higher than 50% at 10 mg/mL).

Antioxidant activity assessed by three metal-related assays. The extracts’ chelating ability towards copper (CCA) and iron (ICA) and their Fe³⁺ reducing capacity (ferric reducing antioxidant power, FRAP) were assessed as described previously. EDTA (ethylenediamine tetraacetic acid) and BHT were used as positive controls. Results were calculated as percentage of antioxidant activity relative to a positive control for FRAP, and in relation to a negative control (ultrapure water/aqueous ethanol) for CCA and ICA, and were expressed as IC₅₀ values (mg/mL).

In vitro anti-diabetic activity: inhibition of microbial and mammalian α-glucosidases. The microbial α-glucosidase enzyme was obtained from the yeast Saccharomyces cerevisiae; rat’s intestine acetone powder was used to obtain a crude enzyme extract as an example of a mammalian-origin α-glucosidase. The extracts’ capacity to inhibit both enzymes was assessed following Kwon et al. and using acarbose as positive control. Results are expressed as IC₅₀ values (mg/mL), calculated as percentage of inhibitory activity in relation to a control (ultrapure water/aqueous ethanol).

In vitro tyrosinase inhibition. The extracts’ ability to inhibit tyrosinase was assessed following Custódio et al., using arbutin as positive control. Results, calculated as percentage of inhibitory activity in relation to a control (ultrapure water/aqueous ethanol), are expressed as IC₅₀ values (mg/mL).

Statistical analysis. Experiments were conducted at least in triplicate and results were expressed as mean ± standard deviation (SD). Significant differences (p < 0.05) were assessed by one-way analysis of variance (ANOVA) followed by Tukey pairwise multiple comparison test or, when parametricity of data did not prevail, Kruskal Wallis one-way analysis of variance on ranks followed by Dunn's test. Statistical analyses were executed using XLStat version 19.4. IC₅₀ values were computed by curve fitting in GraphPad Prism version 6.0c.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
C.G.P., L.C. and L.B. designed the study. S.B. analysed the phytochemical profile of the extracts by LC-PDA-amMS; C.M. performed the toxicological evaluation of the samples; T.F.S. assessed the extracts’ mineral contents; C.G.P. undertook the remaining work. C.G.P. wrote the main manuscript text with the contribution of L.C., S.B., L.P., J.V. and L.B. L.C. and L.B. jointly supervised the work. All authors reviewed and approved the manuscript.

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