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Searching for new sources of innovative products for the food industry within halophyte aromatic plants: *In vitro* antioxidant activity and phenolic and mineral contents of infusions and decoctions of *Crithmum maritimum* L.

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Running Title: Antioxidant and chemical characterization of sea fennel

Abbreviations: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ANOVA: one-way analysis of variance; BHT: butylated hydroxytoluene; CA: coumaric acid; CAE: caffeic acid equivalents; CCA: copper chelating activity; CE: catechin equivalents; CGA: chlorogenic acid; CTC: condensed tannin content; DMACA: 4-dimethylaminocinnamaldehyde; DPPH: 1,1-diphenyl-2picrylhydrazyl; DW: dry weight; FA: ferulic acid; FRAP: ferric reducing antioxidant power; GAE: gallic acid equivalents; HAD: Hydroxycinnamic acid derivatives; HepG2: human hepatocellular carcinoma cells; HNO_3: nitric acid ; ICA: iron chelating activity; LOQ: limit of quantitation; MP-AES: Microwave Plasma-Atomic Emission Spectrometer; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N9: murine microglia cells; NCGA: neochlorogenic acid; NO: nitric oxide; QE: quercetin equivalents; RE: rutin equivalents; ROS: reactive oxygen species; RSA: radical scavenging activity; S17: murine bone marrow stromal cells; SD: standard deviation; SH-SY5Y: human neuroblastoma cells; TFC: total flavonoid content; TPC: total polyphenolic content.

Abstract

Aromatic halophyte plants are an outstanding source of bioactive compounds and natural products with potential use in the food industry. This work reports the *in vitro* antioxidant activity, toxicity, polyphenolic profile and mineral contents of infusions and decoctions from stems, leaves and flowers of *Crithmum maritimum* L., an aromatic and edible maritime halophyte (sea fennel). *Aspalathus linearis* (Burm.f.) Dahlg. (rooibos) herbal tea was used as a reference. Sea fennel's tisanes, particularly from leaves, were rich in phenolic compounds and five of them (*p*-hydroxybenzoic and ferulic acids, epicatechin, pyrocatechol and 4-hydroxybenzaldehyde) were here described in *C. maritimum* for the first time. Chlorogenic acid was the dominant phenolic determined. Na was the most abundant mineral in all tisanes followed by Ca and Mg in leaves' tisanes and K in flowers. Sea fennel's samples had a similar antioxidant activity than those from *A. linearis*, and had no significant toxicity towards four different mammalian cell lines. Altogether, our results suggest that sea fennel can be a source of products and / or molecules for the food industry with antioxidant properties and minerals in the form, for example, of innovative health-promoting herbal beverages.

Keywords: sea fennel, herbal beverages, oxidative stress, phenolic profile, minerals

1. Introduction

Crithmum maritimum L., commonly known as sea fennel or rock samphire, is an aromatic, edible and medicinal halophyte common in marine coastal ecosystems along the European and North-African Atlantic, Mediterranean and Black Sea (Atia et al. 2011; Castroviejo et al. 2003;). Sea fennel belongs to the same family (Apiaceae) as parsley and celery and has interesting sensory attributes: a slight salty taste with notes of celery, common fennel and peel of green citrus, followed by a strong aftertaste (Renna and Gonnella 2012). In fact, it is traditionally used in countries such as Italy or Greece as an ingredient in salads, soups, sauces, as pickle or spice and is acknowledged as a rich source of minerals and vitamin C (Castroviejo et al. 2003; Franke 1982; Renna and Gonnella 2012). Sea fennel has also folk therapeutic uses as an appetizer, tonic, purgative, carminative, anthelmintic or to prevent scurvy. Moreover, infusions and decoctions of the plant's aerial parts are used as diuretic, to treat renal and urinary complaints, digestive disorders, colic and inflammation of the urinary tract and prostate (Atia et al. 2011; Cornara et al. 2009; Franke 1982).

Sea fennel is an important local resource in many coastal populations of the Mediterranean since ancient times; the first European farmers made it part of their diet and nowadays it is still consumed in many areas across Europe (Atia et al. 2011; Franke 1982). Considering a growing world population coupled to global climate change, it is imperative to find alternative food resources that can overcome the threat of soil salinization for agriculture. In this sense, edible halophytes like sea fennel that can be cultivated in marine-influenced environments may be potential alternative crops. And, so far, few salt-tolerant plants have had their potential explored although halophyte products have recently draw some attention in food markets throughout the world (Atia et al. 2011; Buhmann and Papenbrock 2013; Ventura and Sagi 2013). For example, *Salicornia* species are currently trending in the gourmet food market and sea buckhorn (*Hippophae rhamnoides* L.) is sold in specialty stores

as constituent in functional beverages or as herbal tea (Barreira et al. 2017; Gruenwald 2009; Ventura and Sagi 2013).

Herbal teas (tisanes made from plants other than *Camellia sinensis* L.) are worldwide popular beverages with a multitude of attributed health benefits (Patel 2013; Pohl et al. 2016). Such is the case of rooibos tea (*Aspalathus linearis*) promoted for its high antioxidant potential (Joubert and de Beer 2011). The health benefits of herbal beverages are mostly related to their high polyphenolic content and they are reported as a great source of these bioactive phytochemicals in our diet, as well as a potential mineral source (Gruenwald 2009; Pohl et al. 2016). Phenolic compounds have recognized antioxidant properties and, given that oxidative stress is an underlying cause for several degenerative diseases, they can have beneficial outcomes in some health challenges like diabetes or neurodegenerative disorders (Lu and Yen 2015; Sindhi et al. 2013). Medicinal and aromatic halophyte plants, such as sea fennel, combine a pleasant taste to potential health benefits and can be explored as sources of innovative bioactive compounds and / or products for the food industry as, for example, herbal beverages (Gruenwald 2009). Such an approach on medicinal plants to unveil their functional properties and constituents, and explore their application as food products has been made with different plant species, as for example *Lycium barbarum* L., *Schisandra chinensis* (Turcz.) Baill and *Euphorbia denticulata* Lam. (Zengin et al. 2017; Mocan et al. 2016, 2017).

Research regarding sea fennel involves mainly organic extracts and *in vitro* studies report antioxidant and antimicrobial activities along with different groups of bioactive molecules, like phenolic acids and flavonoids (Atia et al. 2011; Buhmann and Papenbrock 2013). Nonetheless, infusions made with flower tops and stalks of sea fennel collected in Croatia exhibited a high *in vitro* antioxidant activity and were rich in phenolic compounds (Siracusa et al., 2011). In this context this work reports, for the first time, a comparative evaluation of the *in vitro* antioxidant potential (using eight complementary assays) and the

polyphenolic profile and mineral content of infusions and decoctions made with stems, leaves and flowers of sea fennel collected in the Alentejo coast of Portugal. We also report a preliminary *in vitro* toxicological evaluation using mammalian cells. The rooibos herbal tea was used as a comparison since it is one of the most consumed tea beverages worldwide.

2. Materials and methods

2.1 Plant collection

Crithmum maritimum L. plants were collected in Alentejo coast in Aljezur beach, (37°20'30.7"N 8°51'06.0"W) in August of 2013. The botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) performed the taxonomical classification. The Marbiotech laboratory keeps an herbarium with a voucher specimen (voucher code MBH33). Plants were divided in organs, namely stems, leaves and flowers, which were oven dried at 50°C until complete dryness, milled and stored until use at -20°C. Dried leaves of rooibos tea (*Aspalathus linearis* (Burm.f.) Dahlg., produced in Cape Town, South Africa) were bought in a regional supermarket, milled and stored at -20ºC.

2.2 Extracts preparation: "cup-of-tea" infusions and decoctions

Water extracts were prepared to equal a cup-of-tea: 1 g of dried plant material for 200 mL of ultrapure water. To prepare infusions, the biomass was immersed in boiling water for 5 min; for decoctions, the biomass was boiled in water for 5 min. Extracts were filtered (Whatman nº 4) and aliquots stored at -20ºC until use; some were freeze-dried for yield determination, high performance liquid chromatography (HPLC) and mineral analysis.

2.3 Phytochemical composition of the extracts

Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content

TPC was estimated by the Folin-Ciocalteau method, measuring the absorbance at 725 nm and using gallic acid as a standard. Results were presented as milligrams of gallic acid equivalents per cup-of-tea (mg GAE/200mL). TFC was assessed by the aluminium chloride colorimetric assay with the absorbance measured at 510 nm and rutin used as standard. Results were calculated as rutin equivalents per cup-of-tea (mg RE/200mL). CTC was determined by the assay 4-dimethylaminocinnamaldehyde (DMACA); the absorbance was measured at 640 nm using catechin as standard. Results were presented as catechin equivalents per cup-of-tea (mg CE/200mL). All methods are described in Rodrigues et al. (2015).

2.4 Hydroxycinnamic acid derivatives (HAD) and flavonols content

HAD and flavonols were determined by the method reported in Rodrigues et al. (2015). Absorbances were measured at 320 nm and 360 nm, using caffeic acid and quercetin as standards, to estimate HAD and flavonols, respectively. Results were calculated as standard equivalents per cup-of-tea (CAE and QE, respectively; mg/200mL).

2.5 Phenolic composition by high performance liquid chromatography – diode array detection (HPLC–DAD)

Freeze-dried extracts were dissolved at a concentration of 10 mg/mL in ultrapure water and analysed by HPLC–DAD according to the method and equipment already described by Rodrigues et al. (2015). Concentration of the several compounds were calculated using calibration curves prepared individually for each commercial standard dissolved in methanol (4-hydroxybenzaldehyde, apigenin, catechin hydrate, epicatechin, epigallocatechin, epigallocatechin gallate, pyrocatechol, quercetin, and caffeic, caffeoyquinic, chlorogenic, coumaric, ferulic, gallic, gentisic, *p*-hydroxybenzoic, neochlorogenic, rosmarinic, salicylic,

syringic and vanillic acids) and diluted to the required concentrations in ultrapure water. Results were calculated as mg per cup-of-tea (mg/200mL) based on the extracts' yield.

2.6 Mineral composition

Freeze-dried extracts were analysed for mineral content by Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), after dry ashing the samples for 8h, ash dissolution in hot nitric acid (HNO_3) and in hydrogen peroxide followed by sample dilution in 5% HNO_3. Working standards of different concentrations were prepared from certified standard solutions; for analytical quality assurance results were corrected by subtracting a blank from the analysed metal concentrations and samples were analysed in triplicate. Quantification wavelengths and calibration curves were selected to obtain the highest signal ratio and the lowest interference for the target elements. Spiking-and-recovery readings were carried out to assess validity of the results. Instrumental detection limits were: 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/cup-of-tea (mg/200mL) based on the extracts' yield.

2.7 Toxicological evaluation of the samples

Murine microglia (N9) cell line was provided by the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal); murine bone marrow stromal (S17) and human hepatocellular carcinoma (HepG2) cell lines were obtained from the Centre for Biomedical Research (CBMR, University of Algarve, Portugal); human neuroblastoma (SH-SY5Y) cell line was obtained from Barcelona Science Park, Spain. RPMI-1640 culture medium was used to maintain N9 cells, while DMEM medium was used

for HepG2, S17 and SH-SY5Y cells; both mediums were supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine (2 mM) and 1% penicillin (50 U/mL) / streptomycin (50 μg/mL). Cells were grown in an incubator in humidified atmosphere at 37ºC and 5% CO_2. Extracts' toxicity was assessed following Rodrigues et al. (2016). Briefly, S17 and HepG2 cells were plated at an initial density of 5 x 10^3 cells/well while N9 and SH-SY5Y cells where seeded at 1x0^4 cells/well, in 96-well plates. Freeze-dried extracts at 100 μg/mL were directly dissolved in culture medium and applied for 72 h; cells incubated with only culture medium were used as negative control and hydrogen peroxide (H_2O_2) was used as positive control for cell toxicity. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (absorbance at 590 nm) and results were expressed in terms of % cell viability.

2.8 Antioxidant activity

2.8.1 Determination of antioxidant activity by five radical-based assays

The scavenging capacity of the aqueous extracts against the radicals DPPH (1,1-diphenyl-2picrylhydrazyl), NO (nitric oxide), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), $O_2^{\bullet-}$ (superoxide) and $OH^{\bullet}$ (hydroxyl) was assessed according to Rodrigues et al. (2015, 2016) using butylated hydroxytoluene (BHT), catechin or ascorbic acid as positive controls. Results were calculated relative to a control containing ultrapure water, as percentage of antioxidant activity in a cup-of-tea.

2.8.2 Determination of antioxidant activity by three metal-related methods

The extracts' copper and iron chelating activities (CCA and ICA, respectively) and their ability to reduce Fe^{3+} (ferric reducing antioxidant power - FRAP) were evaluated as described previously (Rodrigues et al. 2015) using BHT and ethylenediamine tetraacetic acid (EDTA) as positive controls. Results were presented as percentage of antioxidant activity in a cup-of-

tea, relative to a positive control for FRAP and to a negative control (ultrapure water) for CCA and ICA.

2.9 Statistical analysis

Results were expressed as mean ± standard deviation (SD) and experiments were conducted at least in triplicate. Significant differences ($p < 0.05$) were assessed by one-way analysis of variance (ANOVA) or Kruskal Wallis one-way analysis of variance on ranks when parametricity of data did not prevail. If significant, the pairwise multiple comparison tests Tukey or Dunn's were applied. Statistical analyses were performed using XLStat2014®.

3. Results and Discussion

3.1 Phytochemical analysis

Herbal teas are important sources of polyphenolics in human diet as these compounds are among the most widely occurring secondary metabolites in plants (Balasundram et al. 2006). However, characterization of phenolic groups is difficult due to the different polyphenolic mixtures in each plant. In this sense, fast-screening spectrophotometric methods are the widespread approach when assessing total phenolic or phenolic-groups content in plant extracts (Dai and Mumper 2010). Tisanes from *C. maritimum* were assessed spectrophotometrically for their total contents in different phenolic groups and results are summarized on Table 1. *Aspalathus linearis* (rooibos) herbal tea was used as comparison because it is also a tisane and is a greatly consumed tea beverage. Rooibos tisanes had higher TPC than sea fennel's samples, which could be expected since the former is reported as rich in polyphenolic compounds (McKay and Blumberg 2007). The CTC of rooibos samples was also higher than in those of sea fennel, but since rooibos is known as a low tannin tea compared to green or black teas (from *C. sinensis*; Joubert and de Beer 2011), sea fennel's

infusions and decoctions can be considered of comparatively low tannin content. Nevertheless, TFC, HAD and flavonols contents in rooibos tisanes was similar or even lower than those from sea fennel's leaves extracts. Amongst the sea fennel's organs, leaves' infusions and decoctions had the highest levels of all phenolic groups analysed except CTC, followed by flowers tisanes and, lastly, the stems' extracts with the lowest content. CTC was equally low in all of sea fennel tisanes (0.0 - 0.96 mg/cup-of-tea), which can be deemed positive in terms of flavouring since these compounds are associated with an astringent and unpleasant taste. Working with the same species Houta et al. (2011) also assessed the phenolic contents between different organs but reported higher TPC, TFC and CTC in stems rather than in leaves or flowers; however, those authors used methanolic extracts and studies have already showed that solvent and extraction method can greatly influence results (Buhmann and Papenbrock 2013). Houta et al. (2011) reported TPC in stems, leaves and flowers between 9 – 14 mg GAE/g dry extract and Meot-Duros and Magné (2009) reported it in bulk aerial organs' methanolic extracts between 10 – 33 mg GAE/g dry extract; they considered that sea fennel had relatively high phenolic content when compared to other crop species as for example spinach and broccoli. These values are lower than those measured in the present study when considering the extraction yields obtained and the TPC per cup-of-tea of sea fennel's organs (see Table 1). Nevertheless, it should be mentioned that phytochemical content can vary according to species provenance, as confirmed by Jallali et al. (2014), since intra-species variables affect biosynthesis of secondary metabolites in plants. Hence, *C. maritimum*'s herbal teas can be considered of comparatively good polyphenolic content, particularly leaves' tisanes, and a potentially good source of these bioactive phytochemicals.

The phenolic profile of infusions and decoctions from the organs of sea fennel was further investigated by HPLC–DAD aiming to identify the individual phenolic compounds. Results (mg/200mL, i.e., mg/cup-of-tea, calculated based on the extraction yields) are

presented in Table 2 and Figure 1. Ten polyphenolic compounds were identified and quantified in the sea fennel beverages from which *p*-hydroxybenzoic acid, ferulic acid, epicatechin, pyrocatechol and 4-hydroxybenzaldehyde are, to the best of our knowledge, here firstly described in *C. maritimum*. Amid the sea fennel's organs, leaves' herbal teas had consistently higher levels of all the phenolics detected, which is in agreement with the leaves' highest values of phenolic groups (TPC, TFC, HAD, flavonols, Table 1). Chlorogenic acid (CGA) was the dominant phenolic compound in all extracts, reaching more than 8 mg/cup-of-tea in leaves' tisanes and around 3.5 and 2.5 mg/cup-of-tea in flowers and stems samples, respectively. Other reports also found CGA as the major phenolic in sea fennel extracts (Meot-Duros and Magné 2009; Nabet et al. 2016; Siracusa et al. 2011), and associate its high levels to an antioxidant protection against the oxidative stress endured by plants exposed to such stressful environments (Meot-Duros and Magné 2009). Those values correspond to 8 – 17 mg CGA/g extract dw (dry weight) considering the extraction yields (Table 1) and are within the range determined by Meot-Duros and Magné (2009). According to these authors, the sea fennel is among the highest CGA-containing species within the Apiaceae family; accordingly, decoctions from common fennel *Foeniculum vulgare* had lower CGA content (4.54 mg/g; Caleja et al. 2015) than that presently determined in sea fennel's herbal teas. Hence, sea fennel's water extracts, especially from leaves, can be a valuable alternative source of CGA for the food industry. According to Santana-Gálvez et al. (2017) this phytochemical is a promising nutraceutical and food additive attending to its multifunctional properties. In fact, CGA has several reported biological activities including antioxidant, antimicrobial and anti-carcinogenic along with hypoglycaemic, hypolipidaemic and hypotensive properties (Meng et al. 2013; Onakpoya et al. 2015; Santana-Gálvez et al. 2017).

Other main compounds determined in sea fennel's beverages (>1 mg/cup-of-tea; Table 2) were neochlorogenic acid (NCGA) and cryptochlorogenic acid (CCGA) in leaves' tisanes;

they were also preferentially detected in stems and flowers' extracts (0.3 – 0.6 mg/cup-of-tea). NCGA and various other caffeoylquinic acids have already been identified in sea fennel extracts at analogous concentrations (Nabet et al. 2016; Siracusa et al. 2011). They are associated with the strong antioxidant activity of several vegetables (Shahidi and Ambigaipalan 2015) and with some health promoting effects, as for example the modulation of glucose and lipid metabolism and reduction of blood pressure (Onakpoya et al. 2015). Epicatechin was also a main compound in leaves' tisanes (0.8 – 1.2 mg/cup-of-tea) and determined in the other samples at lower concentrations (0.2 – 0.3 mg/cup-of-tea, not detected in stems' infusion). This flavanol was not found reported in literature for sea fennel but common fennel's decoction showed lower values (0.43 mg/g; Caleja et al. 2015). Epicatechin has various beneficial properties described such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic and cardio-protective, among others (Shay et al. 2015). Also preferentially detected in sea fennel's herbal teas was ferulic acid (FA) in leaves' tisanes (0.6 – 0.8 mg/cup-of-tea), determined in lower amounts in roots and flowers' extracts (0.2 – 0.3 mg/cup-of-tea). This phenolic acid was also not found described in literature for sea fennel nor was it detected in common fennel's decoction (Caleja et al. 2015); however, FA in *F. vulgare* methanolic extracts represented 3.5% of total phenolics (Roby et al. 2013), a lower ratio than that presently found (4.3 – 5.1%). Besides is potent antioxidant activity, FA has many recognized bioactivities among which anti-diabetic, anti-inflammatory, anti-carcinogenic and cardio-protective (Kumar and Pruthi 2014). Other phenolics detected at noteworthy concentrations (0.3 – 0.4 mg/cup-of-tea) were coumaric acid (CA) in leaves and flowers' samples and pyrocatechol in leaves' herbal teas. CA has been detected in sea fennel but not quantified (Jallali et al. 2012) while pyrocatechol was not found described in literature for this species. The remaining phenolics were detected in minor amounts or below the quantitation limit (LOQ, Table 2). Overall, the phenolics identified in sea fennel's extracts,

described in literature as interesting natural bioactive compounds, help explain some of the plant's medicinal uses and highlight the potential use of sea fennel infusions and decoctions as a source of bioactive molecules / products, namely beverages with health promoting potential.

Despite being important sources of polyphenolic compounds (Balasundram et al. 2006), herbal teas can also be an excellent source of other components such as minerals (Ajuwon et al. 2015; Pohl et al. 2016). In this sense, tisanes from sea fennel's organs were analysed for mineral content and results are summarized on Table 3. Sodium was the most abundant element in sea fennel's beverages (19.8 – 49.3 mg/cup-of-tea), particularly in those from leaves, being higher than the values detected in several herbal teas from commonly used plants used, such as *Cymbopogon citratus* (DC.) Stapf. (lemongrass), *Matricaria chamomilla* L. (chamomile) or *Zingiber officinale* Roscoe (ginger) (Pohl et al. 2016). Na is an essential nutrient, however its recommended daily intake should not exceed 2000 mg (WHO 2012a). Considering that a cup-of-tea from sea fennel's organs contains no more than 49 mg of Na it is a reasonably safe beverage to include in a daily diet. The other macro-elements were also rather abundant in sea fennel's tisanes with levels of Ca (2.93 – 19.9 mg/cup-of-tea) and Mg (1.69 – 5.55 mg/cup-of-tea) being higher in leaves' extracts and K values (2.18 – 22.6 mg/cup-of-tea) higher in flowers' samples. Among microelements, the sea fennel's beverages had similar values of Fe (11.0 – 27.3 μg/cup-of-tea), Mn (2.53 – 17.4 μg/cup-of-tea) and Zn (11.3 – 35.9 μg/cup-of-tea), higher in leaves' decoction for Fe and Mn. According to a compilation of minerals in tisanes from numerous plants, herbal beverages can be deemed good sources of many elements like Na, Ca, K, Mg, Fe, Mn and Zn (Pohl et al. 2016). Of the adult-daily recommended intakes for Ca (1000 – 1300 mg/day), Mg (190 – 260 mg/day), K (3510 mg/day), Fe (9.1 – 58.5 mg/day), Zn (3 – 14 mg/day) (WHO/FAO 2004, WHO 2012b) and Mn (5 – 5.5 mg/day; NHMRC 2006) a cup-of-tea from sea fennel's organs can supply

between 0.2% – 2% of Ca, 0.7% – 2.9% of Mg, 0.06% – 0.6% of K, 0.02% – 0.3% of Fe, 0.08% – 1.2% of Zn and 0.05% – 0.3% of Mn. In this sense sea fennel infusions and decoctions may be considered a mineral supplementary source, just like most herbal teas usually are (Pohl et al. 2016). Moreover, the levels of potentially toxic minerals like Cu, Cr, Ni, Pb and Cd, when detected, were below legislated values for plants (Pb: 0.3 mg/kg wet weight; Cd: 0.2 mg/kg wet weight; maximum levels in finished herbal products are not regulated) pointing to the safe consumption of sea fennel's tisanes (EC Regulation 1881/2006).

3.2 Toxicological evaluation

The toxicity of plant extracts, herbal beverages in particular, must be determined if its safety for human consumption is to be established. Preliminary toxicity screenings are usually assessed through *in vitro* models using different mammalian cell lines to test for cytotoxicity, which delivers quick and reliable results and reduces *in vivo* testing (Rodrigues et al., 2016; Saad, et al. 2006). In this study, *C. maritimum*'s tisanes were subjected to a preliminary toxicological evaluation using four different mammalian cell lines, together with *A. linearis* extracts for comparison, and cellular viability is presented in Figure 2. Sea fennel's extracts had low toxicity with cell viability values always higher than 90% for all cell lines. Rooibos samples had moderate toxicity towards non-tumoural cells (N9 and S17), with viability values (57% – 66%) lower than those obtained for the sea fennel beverages (> 90%). Samples from both species did not exhibited toxicity against tumoural cells SH-SY5Y and HepG2, since cellular viabilities after applying sea fennel's and rooibos extracts were similar or higher than 90%. As a preliminary toxicological assessment, these results suggest that sea fennel's infusions and decoctions can be considered as non-toxic beverages especially when compared to those obtained for the commercial rooibos tisanes. To the best of our knowledge no

toxicological studies of sea fennel extracts are reported but this plant's large use for nutritional and culinary purposes points to its safe consumption (Atia et al. 2011; Renna and Gonnella 2012).

3.3 Biological activities: *in vitro* antioxidant properties

Nowadays, natural antioxidants such as food products and /or herbal beverages are in high demand in the market (Sindhi et al 2013). In fact, consumers are aware of the potential benefits of natural products and are willing to spend more on nutrition and supplements. In this work, the antioxidant potential of "cups-of-tea" from sea fennel's organs was assessed by eight methods targeting radical scavenging activity (RSA) and metal-related potential (Table 4). Results were compared with those obtained with rooibos herbal tea which has well documented antioxidant properties (Ajuwon et al. 2015; Joubert and de Beer 2011). All extracts from sea fennel were more active against the hydroxyl radical ($OH^{\bullet}$; 44.1 – 54.4%) than rooibos herbal teas (18.8 – 25.6%) and all except roots infusions had the same RSA towards DPPH (83.5 – 88.0% activity) as the rooibos samples (84.6%). The NO scavenging capacity of the sea fennel's decoctions from leaves and flowers (58.6% and 57.5%) also matched rooibos tisane's activity (58.6 – 59.5%) and samples from sea fennel's leaves (86.6 – 88.0%) were as effective against the ABTS radical as rooibos extracts (92.7 – 92.9%). Flowers tisanes were slightly less active than those from leaves towards NO but still had around 80% activity. Moreover, herbal teas from sea fennel's leaves and flowers matched the rooibos beverage capacity to reduce iron (FRAP; 96.1 – 100%) and matched or surpassed its copper chelating activity, although being moderate (30.7 – 38.2%). The capacity to chelate iron, while also moderate, was similar between sea fennel's stems and flowers infusions (36.0 – 36-5%) and rooibos decoction (41.8%), and between the remaining sea fennel's extracts and rooibos infusion. Cups-of-tea from sea fennel's organs had approximately 80% of capacity to

scavenge the superoxide radical ($O_2^{\bullet-}$), although they were less effective than rooibos (aprox. 84%). Furthermore, although less active in metal-related potential, sea fennel and rooibos extracts were at least as efficient as the positive controls in radical-scavenging activity.

As can be deduced from our results, the antioxidant capacity of the sea fennel's herbal teas from leaves and flowers were overall as effective as those of rooibos tisanes. Additionally, among the sea fennel's organs, leaves and flowers extracts had the highest scavenging capacity, FRAP and copper chelating activity. Similarly, Houta et al. (2011) reported a higher scavenging activity towards DPPH in sea fennel methanolic extracts from leaves, followed by flowers and stems with the lowest RSA. Nevertheless, high antioxidant activity has already been described in sea fennel undifferentiated aerial extracts (Houta et al. 2011; Jallali et al. 2014; Meot-Duros and Magné 2009; Romojaro et al. 2013; Siracusa et al. 2011). Our results confirm the sea fennel's *in vitro* antioxidant potential, mostly of its leaves and flowers, and thus show that beverages made from this plant's organs may be useful in preventing oxidative-stress related diseases much like the famous rooibos herbal tea is reported to be (Ajuwon et al. 2105).

The antioxidant activity of plant extracts is closely associated to their phenolic content (Dai and Mumper 2010) and, in fact, infusions and decoctions from leaves were consistently the extracts with the higher amounts of all phenolic groups except CTC (Table 1), followed by flower's tisanes. This suggests that polyphenols may be the major contributors to the antioxidant capacity of these sea fennel's extracts, an association confirmed by numerous previous studies attesting to the phenolics role as antioxidants in plants, particularly in halophytes (Ksouri et al. 2012). In fact, the environmental stress factors that salt-tolerant species like sea fennel endure influences their phenolic content and related antioxidant activity (Buhmann and Papenbrock 2013). Moreover, the amount of individual phenolics

(Table 2) can also contribute to the stronger antioxidant activities in leaves and flowers tisanes since most of these phytochemicals were determined in higher amounts in these organ's beverages. For example, the main component detected, chlorogenic acid, is an antioxidant compound (Meng et al. 2013) already linked to the sea fennel's strong radical scavenging ability (Meot-Duros and Magné 2009). Some of the other phenolics determined in higher amounts in leaves and flowers tisanes may have also contributed through addictive and / or synergistic effects, namely NCGA and CCGA, which are known antioxidants (Shahidi and Ambigaipalan 2015). Additionally, sea fennel's leaves beverages had higher levels of all the phenolics detected, which can account for the slightly higher ABTS radical scavenging activity of these tisanes.

Phenolics are recognized powerful antioxidants and plant-products like herbal teas are an important dietary source of these phytochemicals (Gruenwald, 2009; Ksouri et al., 2012). The intake of antioxidants is associated with the prevention or amelioration of oxidative stress-related diseases, as for example neurodegenerative disorders, cardiovascular dysfunction, diabetes and cancer, and their consumption has become a strategy to address such health challenges (Sindhi et al. 2013; Lu and Yen 2015). Thus, the estimated antioxidant capacity and phytochemical contents of the extracts from sea fennel's organs suggest that sea fennel's herbal teas, particularly from leaves and flowers, can be an alternative source for natural antioxidants with possible health benefits beyond its nutritional role in terms of minerals. Herbal teas are popular beverages consumed for their pleasant taste and therapeutic properties (Pohl et al. 2016) and the use of *C. maritimum* as an herbal beverage may have commercial potential. It could well follow the example of *A. linearis*: the rooibos plant had no commercial value until its potential was recognized and nowadays it is highly valued in the food industry and a worldwide consumed herbal tea (Joubert and de Beer 2011).

4. Conclusion

From our results it is clear that infusions and decoctions made from *C. maritimum* leaves and flowers have a high polyphenolic content, a strong antioxidant potential, an interesting mineral profile and can be considered as non-toxic beverages in view of the preliminary toxicological assessment with *in vitro* models. Thus, sea fennel's leaves and flowers herbal teas could be a potential source of bioactive molecules and / or products for the food industry, as for example antioxidants and minerals.

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Table 1. Yields (g extract/200mL) and phenolic contents (mg/cup-of-tea) in infusions and decoctions from stems, leaves and flowers of *C. maritimum* and from *A. linearis* (rooibos)

Plant	Organ	Extract	Yields	TPC[1]	TFC[2]	CTC[3]	HAD[4]	Flavonols[5]
C. maritimum	Stems	Infusion	0.302	12.4 ± 0.36^{e}	22.9 ± 0.69^{e}	0.0^{c}	8.97 ± 0.58^{f}	4.60 ± 0.40^{e}
		Decoction	0.319	12.8 ± 0.92^{e}	27.1 ± 1.15^{e}	0.63 ± 0.15^{c}	11.2 ± 0.56^{e}	5.96 ± 0.48^{d}
	Leaves	Infusion	0.478	33.7 ± 0.91^{c}	54.4 ± 6.67^{bc}	0.96 ± 0.25^{c}	25.3 ± 0.67^{a}	15.7 ± 0.44^{a}
		Decoction	0.500	35.3 ± 2.98^{c}	57.2 ± 6.42^{b}	0.63 ± 0.15^{c}	25.2 ± 1.44^{a}	16.2 ± 0.99^{a}
	Flowers	Infusion	0.404	21.2 ± 0.17^{d}	48.0 ± 2.07^{cd}	0.26 ± 0.00^{c}	21.1 ± 0.71^{b}	10.2 ± 0.41^{c}
		Decoction	0.389	22.6 ± 0.99^{d}	40.7 ± 3.55^{d}	0.0^{c}	18.6 ± 1.27^{c}	9.46 ± 0.75^{c}
A. linearis		Infusion		43.1 ± 3.39^{b}	52.7 ± 5.41^{bc}	11.8 ± 2.82^{b}	12.0 ± 0.75^{e}	12.7 ± 0.60^{b}
		Decoction		51.3 ± 1.08^{a}	66.7 ± 2.81^{a}	13.2 ± 1.37^{a}	14.3 ± 0.25^{d}	15.5 ± 0.45^{a}

Data represent the mean ± SD ($n \geq 6$). In each column, different letters mean significant differences ($p < 0.05$).

[1]TPC: total polyphenol content, mg GAE/200mL, GAE: gallic acid equivalents

[2]TFC total flavonoid content; mg RE/200mL, RE: rutin equivalents

[3]CTC: condensed tannin content, mg CE/200mL, CE: catechin equivalents

[4]HAD hydroxycinnamic acid derivatives, mg CAE/200mL, CAE: caffeic acid equivalent

[5]mg QE/200mL, QE: quercetin equivalents

Table 2. HPLC–DAD analysis of the phenolic profile (mg/cup-of-tea) of infusions and decoctions from stems, leaves and flowers of *C. maritimum*.

			Stems		**Leaves**		**Flowers**	
Peak nº	**RT (min)**	**Compound (Peak)**	Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
Phenolic acids								
Hydroxybenzoic acids								
1	1.5	Gallic acid	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	4.4	*p*-Hydroxybenzoic acid	0.06	0.06	0.15	0.17	0.04	0.05
Hydroxycinnamic acids								
3	2.8	Neochlorogenic acid	0.45	0.53	1.47	1.73	0.34	0.43
4	7.4	Cryptochlorogenic acid	0.53	0.63	1.59	1.83	0.50	0.64
5	7.8	Chlorogenic acid	2.43	2.42	8.24	8.67	3.33	3.66
6	11.6	Coumaric acid	0.09	0.12	0.36	0.38	0.29	0.31
7	13.0	Ferulic acid	0.15	0.18	0.57	0.77	0.23	0.28
Flavonoids								
Flavanols								
8	10.5	Epicatechin	-	0.35	0.84	1.16	0.26	0.35
Other polyphenols								
9	2.5	Pyrocatechol	0.10	0.10	0.31	0.33	0.08	0.08
10	5.0	4-Hydroxybenzaldehyde	0.01	0.01	0.05	0.06	<LOQ	<LOQ
		TOTAL	**3.82**	**4.41**	**13.57**	**15.12**	**5.06**	**5.80**

RT – retention times; LOQ = 0.01 mg compound/g extract dw.

Table 3. Mineral content of infusions and decoctions (mg or µg /cup-of-tea) from stems, leaves and flowers of *C. maritimum.*

		Stems		Leaves		Flowers	
	Mineral	Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
	Macro-elements (mg/cup-of-tea)						
Essential elements	**Na**	33.3 ± 0.32^{b}	32.7 ± 2.80^{b}	40.1 ± 5.44^{ab}	49.3 ± 0.20^{a}	21.9 ± 1.00^{c}	19.8 ± 0.35^{c}
	Ca	2.93 ± 0.01^{b}	3.10 ± 0.14^{b}	17.8 ± 1.93^{a}	19.9 ± 0.51^{a}	4.13 ± 0.15^{b}	4.11 ± 0.19^{b}
	K	3.40 ± 0.18^{cd}	3.64 ± 0.02^{c}	2.18 ± 0.25^{d}	4.58 ± 0.46^{c}	22.6 ± 0.26^{a}	19.3 ± 0.60^{b}
	Mg	1.69 ± 0.03^{c}	1.81 ± 0.00^{bc}	5.03 ± 0.40^{a}	5.55 ± 0.08^{a}	2.40 ± 0.03^{b}	2.41 ± 0.04^{b}
	Micro and trace-elements (µg/cup-of-tea)						
	Fe	18.6 ± 0.78^{b}	15.6 ± 3.12^{bc}	18.9 ± 2.30^{b}	27.3 ± 0.41^{a}	11.0 ± 0.49^{c}	16.0 ± 2.25^{bc}
	Mn	2.62 ± 0.01^{d}	2.53 ± 0.12^{d}	12.7 ± 0.17^{b}	17.4 ± 0.38^{a}	4.00 ± 0.23^{c}	4.28 ± 0.07^{c}
	Zn	35.9 ± 1.46^{a}	17.4 ± 9.57^{a}	12.3 ± 0.20^{a}	11.3 ± 0.27^{a}	12.8 ± 0.74^{a}	28.3 ± 17.0^{a}
	Cu	5.00 ± 0.15^{ab}	3.81 ± 1.00^{ab}	3.39 ± 0.24^{b}	5.47 ± 0.11^{a}	4.26 ± 0.13^{ab}	4.02 ± 0.02^{ab}
	Cr	0.16 ± 0.07^{a}	0.20 ± 0.00^{a}	0.61 ± 0.49^{a}	0.49 ± 0.12^{a}	0.27 ± 0.16^{a}	0.22 ± 0.02^{a}
	Ni	< LOD	< LOD	< LOD	0.44 ± 0.00	< LOD	< LOD
Non-essential elements	**Pb**	< LOD	< LOD	< LOD	2.66 ± 0.00	< LOD	< LOD
	Cd	0.52 ± 0.00^{a}	< LOD	0.22 ± 0.00^{a}	< LOD	< LOD	< LOD

Data represent the mean ± SD (n = 3). In each row different letters mean significant differences (p<0.05).

LOD: Cd, 0.08 µg/cup-of-tea; Ni, 0.06 µg/cup-of-tea; Pb, 0.15 µg/cup-of-tea.

Table 4. Radical scavenging on DPPH, ABTS, NO, $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals, ferric reducing antioxidant power (FRAP) and metal-chelating activities on copper (CCA) and iron (ICA) of infusions and decoctions from stems, leaves and flowers of *C. maritimum* and *A. linearis* (rooibos). Results are expressed as antioxidant activity (% activity in a cup-of-tea).

Plant/ compound	**Antioxidant activity (%)**									
	Organ	**Extract**	**DPPH**	**NO**	**ABTS**	**$O_2^{\bullet-}$**	**$OH^{\bullet}$**	**FRAP**	**CCA**	**ICA**
C. maritimum	Stems	Infusion	79.4 ± 5.28^{c}	45.1 ± 0.69^{d}	62.1 ± 7.83^{d}	52.1 ± 4.82^{c}	46.1 ± 5.11^{bc}	88.3 ± 0.67^{c}	25.6 ± 2.46^{de}	36.0 ± 4.79^{bcd}
		Decoction	83.5 ± 1.43^{abc}	54.5 ± 2.22^{c}	71.3 ± 4.7^{c}	55.7 ± 3.23^{c}	54.4 ± 4.49^{b}	85.4 ± 1.28^{c}	22.0 ± 3.64^{e}	17.7 ± 3.96^{f}
	Leaves	Infusion	86.5 ± 0.95^{a}	37.0 ± 1.44^{e}	88.0 ± 2.97^{ab}	76.9 ± 1.46^{b}	44.1 ± 5.05^{c}	98.6 ± 3.40^{ab}	34.0 ± 2.25^{bc}	31.1 ± 3.87^{cde}
		Decoction	86.0 ± 4.34^{ab}	58.6 ± 1.07^{b}	86.6 ± 3.67^{ab}	76.6 ± 1.15^{b}	48.9 ± 4.39^{bc}	98.8 ± 2.99^{ab}	38.2 ± 2.69^{b}	26.0 ± 7.67^{ef}
	Flowers	Infusion	88.0 ± 0.16^{a}	15.1 ± 1.12^{f}	80.3 ± 6.23^{b}	78.2 ± 1.51^{b}	48.0 ± 4.69^{bc}	96.1 ± 0.81^{b}	37.8 ± 3.51^{b}	36.5 ± 5.51^{bc}
		Decoction	87.3 ± 0.98^{a}	57.5 ± 0.77^{b}	82.6 ± 6.03^{b}	77.5 ± 0.87^{b}	53.2 ± 3.39^{b}	100 ± 0.00^{a}	30.7 ± 2.86^{cd}	30.1 ± 6.17^{cde}
A. linearis		Infusion	84.6 ± 0.41^{ab}	58.6 ± 0.69^{b}	92.7 ± 0.85^{a}	84.4 ± 0.24^{a}	18.8 ± 2.40^{d}	98.1 ± 2.00^{ab}	26.6 ± 3.52^{de}	26.2 ± 1.70^{def}
		Decoction	84.6 ± 0.51^{ab}	59.5 ± 1.29^{b}	92.9 ± 0.63^{a}	84.9 ± 0.15^{a}	25.6 ± 1.40^{d}	100 ± 0.00^{a}	28.6 ± 3.73^{cd}	41.8 ± 4.29^{b}
BHT*			81.7 ± 1.65^{bc}		93.4 ± 0.26^{a}			-		
Ascorbic acid*				90.6 ± 1.35^{a}						
Catechin*						75.2 ± 2.83^{b}	84.4 ± 9.31^{a}			
EDTA*									94.6 ± 0.36^{a}	99.7 ± 0.15^{a}

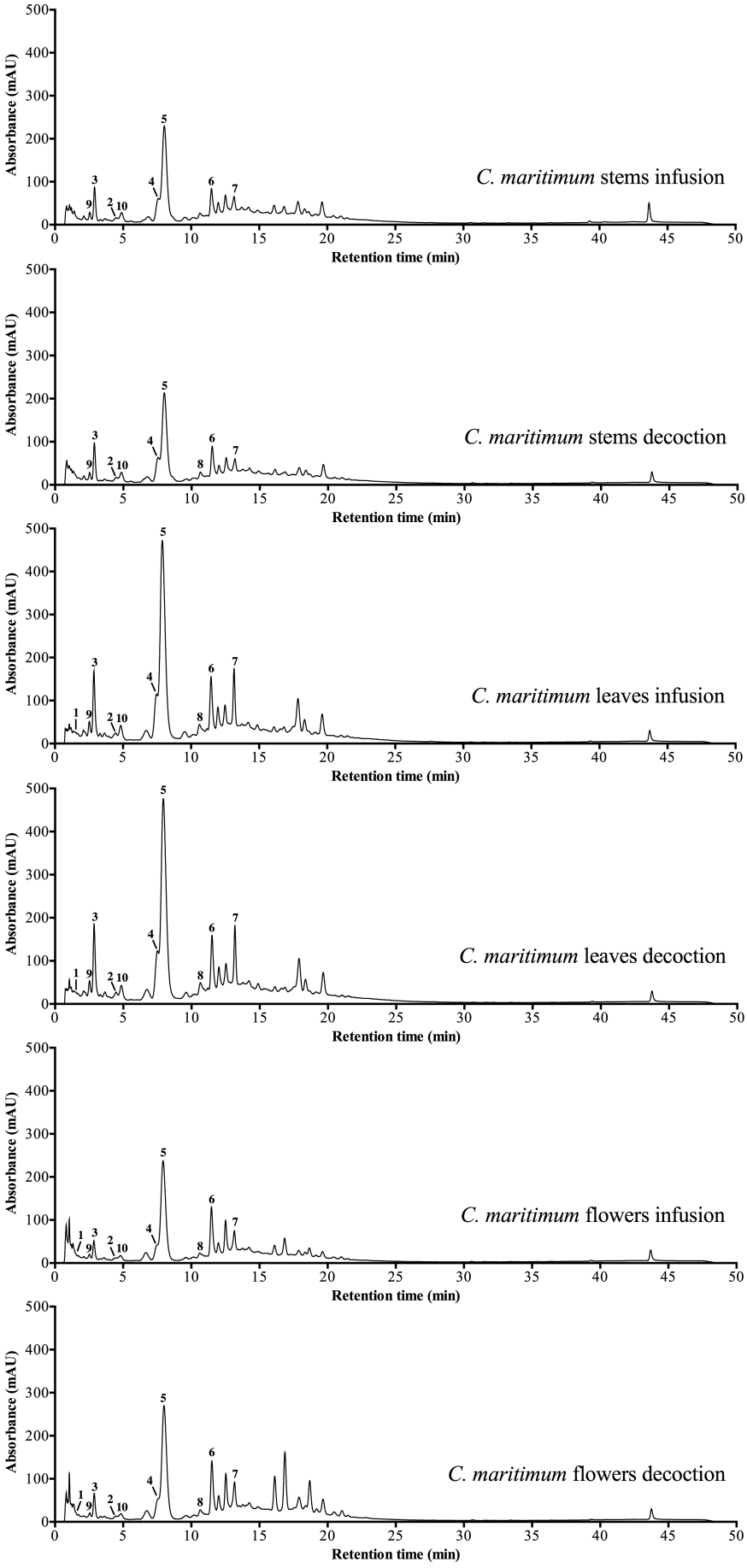
Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each column different letters mean significant differences ($p<0.05$).

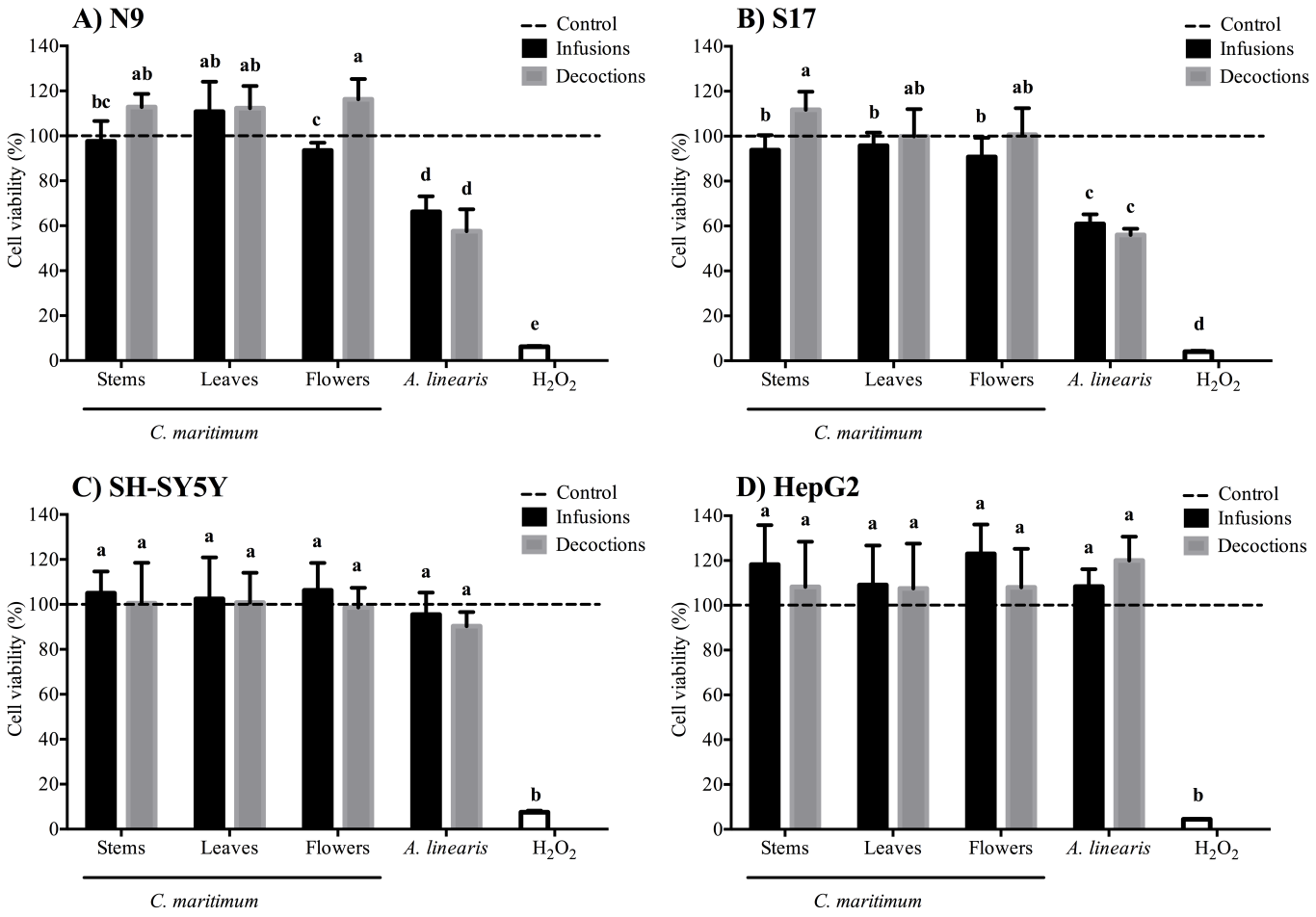
*Positive controls tested at 1 mg/mL (BHT, catechin and EDTA) or 10 mg/mL (ascorbic acid).

Figure legends

Figure 1. HPLC–DAD analysis (280 nm) of phenolic compounds in infusions and decoctions from *C. maritimum* organs. Peak numbers refer to the compounds in Table 2.

Figure 2. Toxicity of infusions and decoctions, applied at the concentration of 100 μg/mL (extract dw) from *C. maritimum* organs and *A. linearis* (rooibos) on mammalian cell lines: A) N9, B) S17, C) SH-SY5Y and D) HepG2. Cells treated only with cell culture medium were used as controls; H_2O_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 ($p < 0.05$).


Absorbance (mAU)
Retention time (min)
C. maritimum stems infusion
C. maritimum stems decoction
C. maritimum leaves infusion
C. maritimum leaves decoction
C. maritimum flowers infusion
C. maritimum flowers decoction


A) N9
B) S17
C) SH-SY5Y
D) HepG2
Control
Infusions
Decoctions
Cell viability (%)
Stems
Leaves
Flowers
A. linearis
H2O2
C. maritimum

Highlights

- *C. maritimum*'s tisanes, particularly from leaves, were rich in phenolic compounds
- Chlorogenic acid was the dominant phenolic determined.
- Na was the most abundant mineral followed by Ca and Mg in leaves and K in flowers
- Sea fennel's leaves and flowers tisanes were as antioxidant as rooibos herbal tea
- *C. maritimum* can be a source of products and / or molecules for the food industry