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Balsamite major Desf.: Redox properties, antiinflammatory and cytoprotective effects

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ABSTRACT

The chemical composition of leaves of Costmary, Balsamita major Desf., cultivated in Tuscany was investigated. This aromatic plant is an important natural resource of bioactive products, such as flavonoids, triterpenes and carotenoids, which give it remarkable pharmaceutical properties (hepatoprotective, antifermentative, spasmolytic, carminative and antiviral). The aim of the present study was to confirm and better define, with three different methods, the relations between the antioxidant capacity and antiradical activity of an extract of Balsamita major Desf. demonstrated in preliminary research. Moreover two specific in vitro tests were used to evaluate the anti-inflammatory activity of costmary extract on isolated human monocytes. The results show non-toxic anti-inflammatory effects on human monocytes of the active compounds in an aqueous costmary extract. The test used to assess the cytotoxicity of the phytocomplex is based on the brine shrimp Artemia salina L. (Artemiidae), an invertebrate living in brackish and marine ecosystems, virtually no mortality was recorded during the experiment, demonstrating that the costmary extract was not cytotoxic at the tested concentrations (100-500 µl/ml). These relationships have not been reported in the literature for costmary, a rare and forgotten plant. The rediscovery and re-evaluation of aqueous costmary extract for food, cosmetic and pharmacological products could be a good starting point to help science in the prevention of serious human pathologies.In conclusion, costmary cultivation should be increased to favour its use in the cosmetics, food and pharmaceutical fields.

Keywords: *Balsamita major* Desf., aqueous extract, polyphenols, antioxidant capacity, cytotoxicity.

INTRODUCTION

The name costmary comes from the Latin *costus*, an oriental plant whose root was used as a spice and preservative, and Mary, referring to the Virgin Mary. Costmary, *Balsamita major* Desf. (syn.:107 *Chrysanthemum balsamita* L.) Asteraceae, is native to the Orient. Introduced to England in the sixteenth century, it can be found in almost every garden in that country and it has become naturalized in many parts of southern Europe (19); it has been present in Italy since the Middle Ages. A perennial herbaceous plant, either spontaneous or cultivated, it has an erect leafy stem about 2 to 3 feet high, which is grooved and angular. The leaves are alternate, very serrated, 2 to 6 inches long and about 4 inches wide. The plant is conspicuous in August and September by its heads of round, flat, dull yellow flowers growing in clusters, called “buttons”. It is slightly velvety and the fresh and dried leaves have a strong aromatic odour similar to mint.

Known by many different names all over the world, *Balsamita major* (fig. 1) was one of the favourite herbs of Charlemagne, was used to aromatize beer and was highly valued by Dominican friars in Tuscany. Already known by

the Romans and Greeks, it was cited in the Holy Bible among the bitter herbs, later becoming popular in the first part of the IX century. Many therapeutic properties have been attributed to this aromatic plant: digestive-eupeptic, carminative, anti-fermentative, spasmolytic (in particular anti-cough) and diuretic. Since ancient times, it has been used in gastronomy, in sweet alcoholic beverages and in perfumes. Considering the plant's digestive-eupeptic and spasmolytic properties, it was used in folk medicine to cure stomach cramps and bronchial affections and to help during childbirth.

Several natural products such as vitamin C, vitamin E, β-carotene, flavonoids and other phenolic compounds have shown excellent antioxidant activity (24). Antioxidants are endogenous or exogenous substances that combat the state of oxidative stress. The role of polyphenolic compounds in plants is to protect them against UV radiation and oxidative stress (12). However, as demonstrated by many studies (1, 16), the regular dietary consumption of some phenolic compounds can prevent various human pathologies including cardiovascular disease, cancer, atherosclerosis, osteoporosis, allergies, diabetes and neurodegenerative pathologies. Recently, pharmacologists have focused on the development of new drugs based on herbal formulations and medicinal plant extracts used in ancient traditional medicine systems (20).

Many human disorders are related to the pathological situation known as oxidative stress, caused by highly reactive species endogenously produced by the organism (10). This process derives from an imbalance caused by excessive production of oxidant factors (as free radicals) and decreased antioxidant defences, and leads to damage to the organism. Oxidative damage and inflammatory processes are related in many pathologies; moreover, the redox status is a powerful mechanism signalling inflammatory pathways associated with oxidative stress (9).

Figure 1 - *Balsamita major* Desf.

Many serious diseases trigger a situation of chronic oxidative stress due to the presence of high amounts of free radicals such as ROS (Reactive Oxygen Species), O_2^-, RO_2 H_2O_2, etc. In humans, these radicals can be produced during physiological processes, muscular or psychophysical stress and can also be induced by external causes (UV exposure, smoking and environmental pollution). Pathologies activated by these molecules affect various systems: cardiovascular (28), inflammatory (rheumatic arthritis), self-immune and degenerative diseases (Alzheimer and Parkinson).

Most free radicals are inactivated by antioxidant molecules able to produce species more stable and less reactive than the initial ones (10). Synthesized antioxidants are widely used as food and cosmetic ingredients thanks to their protective characteristics. However, they have also shown some negative effects that have shifted attention to natural antioxidant compounds. Many studies on natural antioxidants have demonstrated that their addition to the diet can help prevent inflammatory processes and diseases caused by free radicals and cellular ageing (23). A frequent reaction in inflammatory processes involves the production of mediators (cytokines) to destroy, dilute or inhibit the toxic agent. Cytokines are proteins able to act at very low concentrations, creating high affinity bonds with specific receptors expressed on target cells. Cells involved in chronic inflammatory processes are attracted to the affected area and, once activated, release several factors that prevent damage and tissue fibrosis. Cytokines act to combat, regulate and end inflammatory processes. Nevertheless, in addition to their classical immunological actions, some cytokines (e.g. IL-1) are potent mediators of inflammation reactions in the brain. This cytokine is believed to have a pathogenic role in various diseases, such as i) Alzheimer (8), ii) multiple sclerosis (13), iii) traumatic brain injuries

(22) and various forms of cerebral infectious processes, including AIDS-dementia (5). Cell activation mediated by IL-1 induces the secretion of other cytokines such as interleukin-6 (IL-6) and tumour-necrosis-factor alpha (TNFα) (21).

The aim of the present study was to confirm and better define the relations between the antioxidant capacity and antiradical activity of an extract of *Balsamita major* Desf. supposed in preliminary research (4). Moreover, the results show non-toxic anti-inflammatory effects on human monocytes and on the brine shrimp *Artemia salina* of the active compounds in an aqueous costmary extract.

EXPERIMENTAL SECTION

2.1 Plant extracts

Costmary leaves were collected during blooming, dried in an oven (Instruments PID system) at 70°C for 20 hours, milled and stored in the dark. 5 g of dried plant were extracted several times with water: in total, 5 extractions were carried out in a Soxhlet apparatus for 12 hours at boiling temperature. The final volume was 500 ml.

2.2 Antiradical and antioxidant activities

2.2.1 DPPH radical scavenging assay (RSA).

DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich) is a stable radical that can be reduced by reaction with an antiradical hydrogen-donor compound. This colorimetric reaction is measured with a spectrophotometer (Beckman DU 640) at 517 nm: the DPPH radical colour shifts from violet to yellow. The costmary extract was dissolved in ethanol and diluted as necessary. 1 ml of diluted extract was added to 1 ml of ethanolic DPPH solution ($6.3 \mu 10^{-3}$ M) and mixed. The absorbance was measured every 2 min from 0 to 20 minutes. The standard absorbance was given by 1 ml of ethanol and 1 ml of the DPPH solution, while the zero was given by the absorbance of pure ethanol. The experiments were carried out in triplicate. The RSA of the extract was calculated with the following formula:

$$\% \text{ inhibition} = (A_s - A_b) / A_s \times 100$$

where A_s is the absorbance of the standard solution (t = 0) and A_b the absorbance of the costmary extract (t = 20 min).

2.2.2 ORAC Assay (Oxygen Radical Absorbance Capacity).

The method was adapted from the one described by Cao and Prior (1998) and the instrument was a fluorescence spectrophotometer (Varian Cary Eclipse) (Palo Alto, CA, USA). The sample is added to a free-radical generator (AAPH, 2,2'-azobis(2-aminopropane) dihydrochloride) and the inhibition of the free radical is measured. Fluorescein is used as a target for free radical attack. Free radicals cause conformational changes in the protein structure of fluorescein, leading to dose- and time-dependent fluorescence quenching. The following were added to a quartz cuvette: 2 738 µl fluorescein (25.5 mg/l solution, maintained at 4°C), 37 µl phosphate buffer solution (75 mM, pH 7.4) and 150 µl Trolox standard (Sigma-Aldrich, 100 µM), blank (buffer solution) or sample solution. After incubation at 37°C for 30 min, the addition of 75 µl AAPH solution (86.8 mg/ml in buffer solution and kept in ice) starts the reaction. The exciting λ is 490 nm and the emission λ is 512 nm. Total antioxidant capacity or ORAC unit (µM) is given by the following formula:

$$\text{ORAC unit } (\mu M) = 20 \text{ k } (S_{sample} - S_{blank}) / (S_{Trolox®} - S_{blank}),$$

with k the dilution factor, S_{sample} the under curve area of the sample, S_{blank} the under curve area of the blank and $S_{Trolox™}$ the under curve area of the standard.

2.2.3 Cyclic voltammetry

The cyclic voltammetry analysis was performed using a conventional 3-electrode system, as shown in figure 2. Since the working electrode must have redox inactivity in the potential range needed for the electrochemical experiment, it has to be a gold, platinum, carbon (only if paste, glassy or pyrolytic carbon) or mercury electrode. The auxiliary electrode was a platinum wire, while the reference electrode was Ag-AgCl.

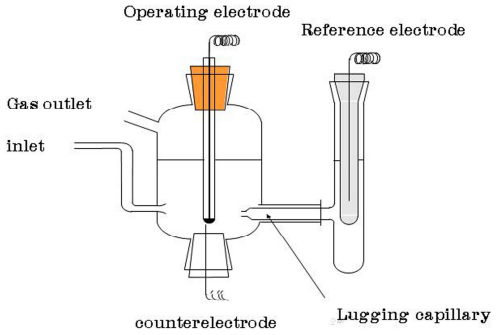

Figure 2 - Cycling voltammetry system

2.3 Total phenolic compounds

The total phenolic content was determined by the Folin-Ciocalteau method (6) using pyrogallic acid (Sigma-Aldrich) as standard. 5 ml of Folin-Ciocalteau solution and 10 ml of Na carbonate anhydrous (50 g/300 ml) were added to 1 ml of standard solution and then diluted to 50 ml with water. After 2-3 hours in the dark, the absorbance of the solution was spectrophotometrically measured at 730 nm. The analysis was carried out in duplicate. The total phenolic content was calculated with the following formula:

$$gl^{-1}\ Pp = (Abs_s - Int)\ /m \times dilution$$

where Abs_s is the absorption of samples at 730 nm after 2 hours, Int the value of the intercept on the x axis of the calibration curve and m the slope of the calibration curve.

2.4 LDH

As lactate dehydrogenase (LDH) is a cytosolic enzyme, the kit measures the cell membrane damage caused by LDH activity. The colorimetric reaction measures the enzymatic activity in the presence of substrate (tetrazolium iodide and lactate), enzymatic cofactor (NAD+) and the enzyme catalysing the inverse reaction (diaphorase): the LDH converts lactic acid into pyruvic acid and NAD+ into NADH. NADH is cofactor of diaphorase, which forms Formazan (red colour) from tetrazolium (yellow colour). The percentage of dead cells is given by comparison of the absorbance of cells incubated without cytotoxic substances and cells incubated with a cell membrane detergent standard (Triton100), which represents 100% dead cells.

2.5 Anti-inflammatory activity: IL-6 assay

A commercial kit (Biosource Human IL-6 CytoSet TM) was used to evaluate the anti-inflammatory activity in a multiwell plate (96 wells). The absorbance was measured at 450 nm with a plate reader (Model 550, Bio Rad Laboratories) (Hercules, CA, USA).

2.6 Cytotoxicity test

The brine shrimp (*Artemia salina*) lethality test was performed according to the method described by Meyer et al. (18) to evaluate the cytotoxicity of the costmary extract. The brine shrimp survival was observed after 24, 96 and 116 hours. The test was carried out in triplicate.

2.7 Determination of thujon

Terpenes were analysed by GC using the method of Raffa and Smalley (25). The analysis was carried out with a Perkin-Elmer GC (AutoSystem XL) (Norwalk, USA) equipped with FID and a Cyclodex-B 30 m × 0,25 mm capillary column (J&W Scientific, CA, USA). The carrier gas was hydrogen. The amounts of single monoterpenes were expressed in percentages of the total amount of monoterpenes. Terpenes were identified by comparing their retention times with standard samples.

RESULTS AND DISCUSSION

The costmary extract solution contained 10 mg plant/ml. The phyto-complex weight was 19.2% of dried leaves. Other authors (14) have reported that the weight of different plant extracts was 20-26% of dried plants. Confirming comparative studies on green tea and other herbs, the total polyphenol (Pp) content was 8.1% of the costmary extract, mainly represented by flavonoids (more than 85%) and phenylpropanoic derivates such as caffeic and chlorogenic acid (about 2%, fig. 3). Tsai et al. (29) showed that rosemary metabolic extract weight was 16,4%,

while Pp content was about 7.5%. Marculescu et al. (17) found the same amounts of the mentioned acids in *Chrysanthemum balsamita* cv. "Carvona".

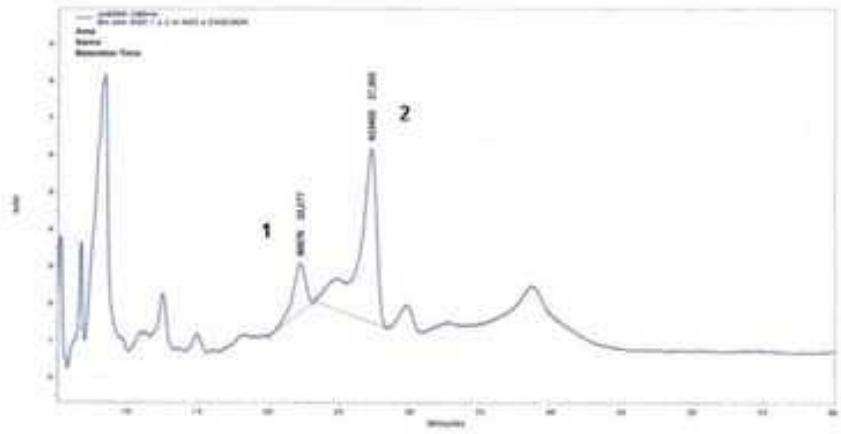

Figure 3 - HPLC analysis: 1-chlorogenic acid; 2-caffeic acid

Phenolic compounds are organic antioxidant molecules and they constitute a large heterogeneous family of secondary metabolites of plant cells. The reaction of an antioxidant with DDPH or AAPH radicals is possible only when it is able to transfer an electron to either of the radicals, and the reduction potential of the antioxidant is such that the reaction is favourable thermodynamically (20). Electrochemical measurements indicated the redox trace of costmary phyto-complex: two main oxidations took place at -135 mV and +14 mV. The aqueous solution of the extract showed only non-reversible oxidation, indicating that it did not have a cyclic redox trace. Redox traces were more evident when HCl was added, and the two oxidation potentials were +331 mV and +568 mV. Cyclic voltammetry traces at different pH are shown in fig. 4. It is important that the oxidation redox traces were in an area very important for biological action, i.e. that of natural antioxidants such as reduced glutathione.

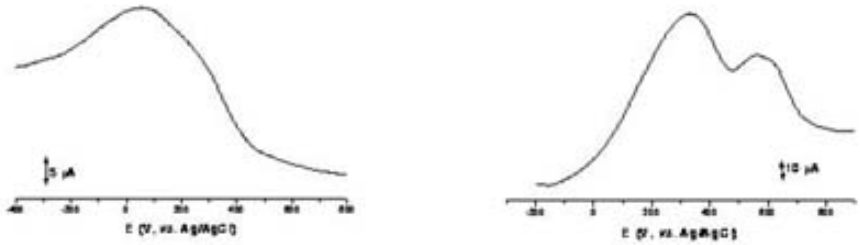

Figure 4 - ESBM: small cell, Ag/AgCl reference electrode, GC (glassy carbon) working electrode

The antiradical activity assay showed that the decrease of DDPH absorbance ended after 8 min and remained constant for the rest of the time (fig. 5). The concentration required to inhibit 50% of DPPH radical activity (IC_{50}) was 2.9 µg Pp / mg aqueous extract (fig. 6). As the costmary Pp content was of the same magnitude as that of other common aromatic plants (sage, rosemary, etc), the IC_{50} was lower than that of those herbs. The study by Su et al.

(27) on several aromatic plants (black peppercorn, oregano, cinnamon, nutmeg and rosehip) showed that methanolic cinnamon extract had the lowest IC_{50} value (10 µl/ml) while black peppercorn extract had the highest value (1.46 mg/ml).

Regarding the ORAC (Oxygen Radical Absorbance Capacity) antioxidant capacity, costmary extract gave a result of 17069.80 µmol TE % g of dried plant. The extract, blank and standard traces are shown in figure 7. As an ORAC unit is expressed in µmol TE/100 g and the world daily dose is between 1200 and 6000 µM of Trolox™ equivalent, 20-30 mg of dried costmary would supply the daily dose (at least 5 000 ORAC units) suggested by the National Research Council (USA). This dose would combat important chronic pathologies and cell ageing.

With respect to the antioxidant capacity of other aromatic plants (e.g. thyme and rosemary) (fig. 8), *Balsamita* can be placed in a medium-high part of the graph, as it is well able to neutralize free radicals.

Cell vitality was tested with the LDH method in human monocytes. Gandhi and Cherian (7) reported that karanja oil has *in vitro* cytotoxicity. LDH released from cells into the supernatant, as a general indicator of cell injury, was chosen as the cytotoxic endpoint; the highest value was found in the red cell when they had a full haemolysis. When monocytes were incubated with Triton, the mortality was 92%. After incubation of the cells with aqueous costmary extract, there was no difference on either LDH production or cell mortality. When bacterial LPS was added, haemolysis affected about 20% of all cells; co-incubation of the monocytes with bacterial LPS and costmary extract resulted in a return to basal values. At the highest concentration tested (0.5 mg/ml), there was no cell membrane damage and no significant mortality with respect to control (fig. 9).

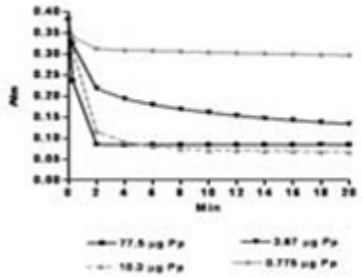

Figure 5 – Antiradical activity of the aqueous costmary extract

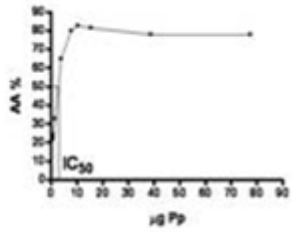

Figure 6 – IC_{50} of the aqueous costmary extract

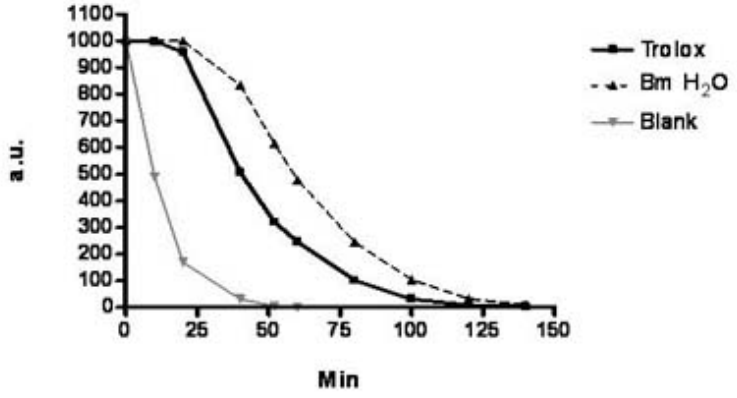

Figure 7 – Oxygen Radical Absorbance Capacity of the aqueous costmary extract.

Two specific in vitro tests were used to evaluate the anti-inflammatory activity of costmary extract on isolated human monocytes. The first assay used bacterial LPS incubated with the cells: this bacterial toxin produces mortality in 30% of cultured cells after 24 hours. The damage caused by the toxin was counteracted by co-incubation of LPS with 0.2 mg/ml *Balsamita major* Desf. extract. The second specific assay used IL-6, an

inflammation mediator produced by monocytes. IL-6 is produced by macrophages, endothelial cells and fibroblasts as a response to IL-1 and TNF. Target cells are B lymphocytes and hepatocytes: in recent years, IL-6 has been demonstrated to have a complementary role to that of IL-1 in acute inflammation. It stimulates hepatocytes to synthesize proteins of the "acute phase": C-reactive protein, fibrinogen, etc. (Lee et al., 2007). In this assay, the basal value of IL-6 was very low while it reached 1 400 ng/mg when monocytes were incubated with LPS. Co-incubation with costmary extract demonstrated that this phytocomplex has a strong concentration-dependent anti-inflammatory activity (fig. 10). The IL-6 level decreased even at a very low phytocomplex concentration (0.1 mg/ml), and reached values comparable to the basal levels at the concentration of 0.5 mg/ml. The inhibitory effect of costmary extract on this pro-inflammatory mediator could be due to the specific dose-dependant activity of flavonoids and hydroxycinnamic components. Therefore, both specific in vitro tests on isolated human monocytes demonstrated that the aqueous costmary extract had high anti-inflammatory activity.

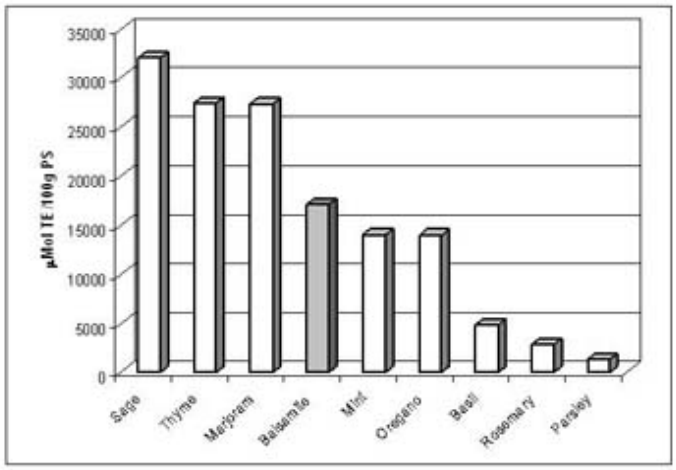

Figure 8 – Comparison of extracts of various aromatic plants.

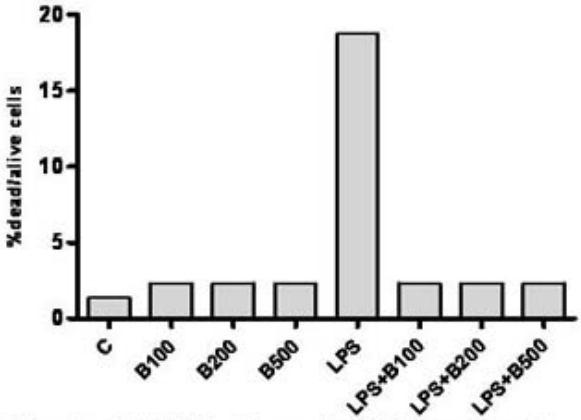

Figure 9 – Cell vitality of monocytes in the presence and absence of bacterial LPS (c: control; B 100, 200 and 500: 100, 200 and 500 µg/ml of balsamite extract).

The test used to assess the cytotoxicity of the phytocomplex is based on the brine shrimp *Artemia salina* L. (Artemiidae), an invertebrate living in brackish and marine ecosystems (26). It plays an important role in the energy flow of the food chain and can be used in laboratory bioassays to determine toxicity through estimation of the median lethal concentration (LC_{50} value), as reported for different toxins and vegetable extracts by Meyer et al. (18). As shown in table 1, virtually no mortality was recorded during the experiment, demonstrating that the costmary extract was not cytotoxic at the tested concentrations (100-500 µl/ml).

Bylaite et al. (2) reported that *Chrysanthemum balsamita* L. essential oil contained 76 terpenic compounds, the main ones being carvon (51.8-68.0%) and thujon (9.0-16.1%). Thujon is a neurotoxin that depresses the central nervous system, increases gastric acidity and causes mental alterations (even leading to psychosis). For this reason, its

presence in food and beverages is regulated by EU norms: maximum 10 mg/kg in alcoholic beverages, 35 mg/kg in bitters. Thujon cannot be added to food without treatment (European Council Directive 88/388/EWG of June 22, 1988). GC analysis showed no significant presence of thujon in the aqueous costmary extract. The unexpectedly strong anti-inflammatory activity of this aqueous extract is made even more important by the lack of the neurotoxin (perhaps due to the type of extraction and the insolubility of terpenes in water).

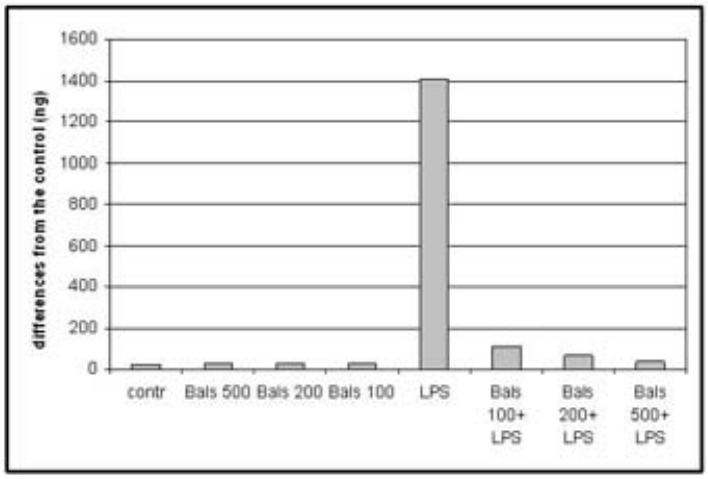

Figure 10 – IL-6 levels in monocyte cultures with costmary extract in the presence and absence of bacterial LPS (contr: control; Bals: 100, 200 and 500: 100, 200 and 500 µg/ml of balsamite extract).

An optimal mix of phytochemical substances, such as natural antioxidants, provides *Balsamita major* Desf. with strong non-reversible antioxidant activity (shown by three different assays) and thus with health benefits for the prevention of chronic human diseases. Moreover, aqueous costmary extract does not contain thujon, a neurotoxic substance that could limit its use. In *in vitro* tests, costmary inhibited the production of specific cytokines involved in inflammatory processes.

Table 1 Mortality at different concentrations of aqueous costmary extract

	Initial nr shrimps	24 h dead shrimps	96 ore dead shrimps	116 ore dead shrimps
Control 1	16	0	0	0
Control 2	16	0	0	0
B.m. 100	16	0	0	0
B.m. 200	16	1	1	1
B.m. 500	16	0	0	0

CONCLUSION

In the light of the growing need to replace synthetic products and to preserve the environment, costmary could be a valid natural alternative to synthetic drugs. *Balsamita major* can be considered an important source of natural compounds that can be used in phytotherapy, and their synergy could be a good basis for greater phytochemical and phytopharmacological development of this plant.

The rediscovery and re-evaluation of aqueous costmary extract for food, cosmetic and pharmacological products could be a good starting point to help science in the prevention of serious human pathologies.

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