Scientific validation of synergistic antioxidant effects in commercialized mixtures of *Cymbopogon citratus* and *Pterospartum tridentatum* or *Gomphrena globosa* for infusions preparation

Custódio Lobo Roriz,[1] Lillian Barros,[1,*] Ana Maria Carvalho[1],Celestino Santos-Buelga,[2] Isabel C.F.R. Ferreira[1,*]

[1]Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.

[2]GIP-USAL, Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405; email: lillian@ipb.pt; telephone +351-273-303903; fax +351-273-325405).

Running title: Synergistic antioxidant effects of commercialized plant mixtures

Abstract

Pterospartum tridentatum (L.) Willk*., Gomphrena globosa* L. and *Cymbopogon citratus* (DC) Stapf. are examples of medicinal plants with antioxidant properties by their own, but that can be improved when mixed. In the present work, the antioxidant activity and phenolic compounds were determined in the infusions prepared from the individual plants, and from mixtures of these plants in different proportions. *P. tridentatum* > *C. citratus* > *G. globosa* was the order observed for antioxidant efficacy, which can be related to their different composition in phenolic compounds. Synergism was the main effect observed among the tested mixtures, mainly for the infusions prepared from the plants in proportion 40%:60% (either *P. tridentatum* and *C. citratus*; or *G. globosa* and *C. citratus*). The infusion obtained with 40% of *P. tridentatum* and 60% of *C. citratus* gave the highest antioxidant properties. The present study validates the commercialization of the studied plants combined in specific proportions.

Keywords: Pterospartum tridentatum; *Gomphrena globosa*; *Cymbopogon citratus*; Infusions; Synergism; Antioxidants; Phenolic compounds

1. Introduction

Nowadays, the therapeutic effects of plants are very important for human health, as the World Health Organization (WHO) estimates that 60% of the total human population still treats primary health care problems with traditional remedies based mainly on phytotherapy (WHO, 2014). Furthermore, contemporary dietary programs generally recommend specific medicinal and aromatic plants as functional foods (foods that provide health benefits beyond normal physiological nutritional requirements) (Gonçalves, Gomes, Costa & Romano, 2013).

Tea and herbal infusions are examples of those foods, being prepared with fresh or dried flowers, leaves, seeds, or roots, generally by pouring boiling water over the plant parts and letting them steep for a few minutes. Herbal infusions are considered rich in phenolic compounds recognized for their beneficial effects on human health (Costa et al., 2012). Phenolic compounds comprise flavonoids, phenolic acids, and tannins, among others. Some applications proposed for natural phenolic compounds are based on their antioxidant activity against reactive species involved in aging and in chronic, autoimmune, inflammatory, coronary and degenerative diseases (Ruiz & Romero, 2001).

Studies involving the evaluation of synergistic effects of combined plants are emerging and seem to highlight the potential of the mixtures when compared with the isolated plant (Pereira, Calhelha, Barros, Queiroz & Ferreira, 2014). Synergy assessment has become a key area in phytomedicine research in recent years, in order to find a scientific rationale for the centuries-old, often-observed therapeutic superiority of many multidrug combinations in traditional medicine over single constituents (Wagner & Ulrich-Merzenich, 2009). As herbal extracts consist of complex mixtures of major compounds, concomitant agents and other substances, the complex multi-component nature of

medicinal herbs may serve as a valuable resource due to its potential treatment effects by synergy (Yang et al., 2014). Synergistic interactions between the components of individual or mixtures of herbs are a vital part of their therapeutic efficacy (Williamson, 2001). In fact, many leading researchers have advocated using combination approaches to pursue the optimum therapeutic efficacy and to improve the patient's overall health status (Yang et al., 2014).

Pterospartum tridentatum (L.) Willk. is traditionally used to treat affections of the nervous, cardiovascular, digestive and urinary systems (Novais, Santos, Mendes & Pinto-Gomes, 2004), and some researchers had already studied its infusion regarding antioxidant capacity (Paulo et al., 2008; Gonçalves et al., 2013). *Gomphrena globosa* L. is commonly consumed for the treatment of several respiratory inflammatory conditions and was previously characterized regarding its phenolic compounds (Silva et al., 2012; Zhu et al., 2013). *Cymbopogon citratus* (DC.) Stapf. infusion is used, in Portuguese traditional medicine, to treat mainly digestive system problems (Novais et al., 2004), and the antioxidant capacity of the infusion was also studied (Cheel, Theoduloz, Rodríguez & Schmeda-Hirschmann, 2005; Francisco et al., 2013).

In a previous study, our research group described the antioxidant activity and phenolic composition in methanol/water extracts (80:20, v/v) of the mentioned species (Roriz, Barros, Carvalho, Santos-Buelga & Ferreira, 2014), but to our knowledge, there are no studies on mixtures of those plants. This is a very interesting topic as there are available commercial mixtures (dry material for infusions preparation) of *P. tridentatum* and *C. citratus*, as also *G. globosa* and *C. citratus*, in specific proportions (40:60%), for infusions preparation. Therefore, in the present work, we intend to validate those combinations by assessing the synergistic effects regarding antioxidant properties and compounds.

2. Material and Methods

2.1. Samples and samples preparation

Plant material of *Pterospartum tridentatum* (L.) Willk., *Gomphrena globosa* L. and *Cymbopogon citratus* (DC) Stapf. was purchased from Ervital, a Portuguese certificated company from Castro Daire (Portugal). *P. tridentatum* flowers were wild gathered in spring 2012 (respecting plant phenology and abundance). The other studied species were grown, also in 2012, with organic farming methods. The parts used were the flowers of *G. globosa* and leaves of *C. citratus*. Harvested plant parts were processed using in-storage and low temperature drying methods (solar heated air, average daily temperature around 30-32 ºC in shade conditions and controlled relative humidity). Botanical identification was confirmed by Ana Maria Carvalho, responsible of the medicinal plant collection of the Herbarium of the Escola Superior Agrária (BRESA), of the Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). Samples for analysis were prepared by mixing dried and powdered (20 mesh) plant materials, in the following proportions: *P. tridentatum* (25%) + *C. citratus* (75%) and *P. tridentatum* (40%) + *C. citratus* (60%); *G. globosa* (25%) + *C. citratus* (75%) and *G. globosa* (40%) + *C. citratus* (60%). Individual samples of each plant species were also analysed.

For infusions preparation, each individual sample or mixture (1 g) was added to 250 mL of boiling distilled water, left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen and lyophilized. The lyophilized infusions were re-dissolved in distilled water, to obtain stock solutions of 10 mg/mL.

For each species, three samples were used and all the assays were carried out in triplicate.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were from Extrasynthèse (Genay, France). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Analysis of phenolic compounds in the infusions

Phenolic compounds were analysed by High-Performance Liquid Chromatography (HPLC, Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Roriz et al., 2014). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS, API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-Vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in mg per g of lyophilized infusion.

Betacyanins. Each sample (1 g) was extracted with 30 mL of methanol containing 0.5% trifluoroacetic acid (TFA), and filtered through a Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and betalain/betacyanin pigments were further eluted with 5 mL of methanol:water (80:20, *v/v*) containing 0.1% TFA. The extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-µm disposable LC filter disk for HPLC analysis. Betacyanins were determined by HPLC as previously described by the authors (Roriz et al., 2014). Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and in a MS connected to the HPLC system via the DAD cell outlet. The betacyanins were tentatively identified by comparing their UV-Vis and mass spectra with available data information reported in the literature. The quantification was performed using a calibration curve of gomphrenin (isolated in our laboratory). The results were expressed in mg per g of lyophilized infusion.

2.4. Evaluation of antioxidant activity in the infusions

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Barros et al., 2013). Reducing power was evaluated

by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above (Barros et al., 2013). Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100 (Barros et al., 2013). Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2013). The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

2.5. Classification of additive, synergistic or antagonistic effects

Theoretical values for antioxidant activity of the mixtures were calculated as weighted mean experimental EC_{50} values of the individual samples and considering additive contributions of individual species in each percentage; for instance, *P. tridentatum* (25%) + *C. citratus* (75%) EC_{50} = EC_{50} *P. tridentatum* × 0.25 + EC_{50} *C. citratus* × 0.75. The classification in additive (AD), synergistic (SN) or antagonistic (negative synergistic; AN) effects was performed as follow: AD: EC_{50} theoretical and experimental values reveal differences lower than 10%; SN: EC_{50} experimental values are more than 10% lower than theoretical values; AN: EC_{50} experimental values are more than 10% higher than theoretical values. The limit of 10% was chosen taking into account the coefficients of variation obtained in the replications of each antioxidant

activity assay. It should be noted that lower EC_{50} values mean greater antioxidant activity.

3. Results and Discussion

3.1. Phenolic profiles

Phenolic compound profiles of the infusion extracts of *P. tridentatum* (Pt), *G. globosa* (Gg) and *C. citratus* (Cc) are shown in **Figure 1**. Data (retention time, λmax in the visible region, molecular ion and main fragment ions observed in MS^2) obtained by HPLC-DAD-ESI/MS analysis regarding phenolic compounds and betacyanins identification and individual quantification in the dry extracts of the infusions are presented in **Tables 1-3.**

All the compounds found in the infusions were already described and tentatively identified in a previous study carried out by our research group, but using methanolic extracts of the plants (Roriz et al., 2014). Nevertheless, it should be highlighted that some of the compounds previously identified in the methanolic extracts could not be found in the infusions studied herein, as the heating process inherent to infusion could destroy them (Samaniego-Sánchez et al., 2011). In particular, 7-*O*-methylgenistein (prunetin) that was described for *P. tridentatum* methanolic extract (Roriz et al., 2014), was not observed in its infusion (**Table 1**). Dihydroflavonol *C*-derivatives (namely peak 1^{Pt}, 43.04 mg/g of infusion) were also the major compounds found in *P. tridentatum* infusions (**Table 1**). The phenolic profile of infusions prepared from wild *P. tridentatum* was already described by some authors (Vitor et al., 2004; Paulo et al., 2008); nevertheless, the results described herein for a commercial sample are more detailed (with a higher number of identified compounds, also indicating the concentration found for each compound- qualitative and quantitative analyses).

Regarding *G. globosa* (**Table 2, Figure 1B and C**), the qualitative profile in phenolic compounds of the infusion was similar to the one previously described for methanolic extract (Roriz et al., 2014) and for other infusions of the plant (Silva et al., 2012). The composition in betacyanins was also similar to the one described by Cai, Sun and Corke (2001) for an infusion prepared from a wild sample. Flavonoids continued to be the main phenolic compounds found in *G. globosa,* being kaempferol 3-*O*-rutinoside (peak 12^{Gg}, 0.89 mg/g of infusion) the main flavonol. Otherwise, gomphrenin III isomers were also the major betacyanins found (**Table 2, Figure 1C**).

trans p-Coumaric acid and luteolin, previously found in *C. citratus* methanolic extract (Roriz et al., 2014), were not observed in the infusion (**Table 3, Figure 1D**). Otherwise, compound $2a^{Cc}$, corresponding to the *cis* isomer of caffeic acid, was only detected in the infusion due to the heat treatment applied. The identity of that compound was established based on the observation that hydroxycinnamoyl *cis* derivatives are expected to elute before the corresponding *trans* ones, as previously checked in our laboratory (Barros, Dueñas, Carvalho, Ferreira & Santos-Buelga, 2012). Flavonoids were also the major group found in the infusion of *C. citatus*, being luteolin 2''-*O*-deoxyhexosyl-6-*C*-hexoside (peak 6^{Cc}, 13.12 µg/g dw) the main compound (**Table 3**). The phenolic characterization described in the present study was more detailed than the ones described by other authors (Figueirinha, Paranhos, Pérez-Alonso, Santos-Buelga & Batista, 2008; Figueirinha, Cruz, Francisco, Lopes & Batista, 2010; Marques & Farah, 2009; Port's, Chisté, Godoy, & Prado, 2013).

3.3. Antioxidant activity of the infusions prepared from individual and mixed samples

Herbal companies have been preparing mixtures of plants to consume as infusions with improved organoleptic parameters, in particular flavor. In fact, despite some healthy properties of *P. tridentatum* and *G. globosa* (as mentioned in the Introduction section),

their flavor is unpleasant for the majority of consumers. Therefore, the strategy is to add a pleasant flavored plant, such as *C. citratus*, to achieve a mixture with suitable organoleptic properties according to consumers' preferences. Furthermore, these mixtures can provide beneficial effects taking advantage on the synergism between plants, besides the improvement of flavor. In this perspective, there are available commercial mixtures (dry material for infusions preparation) of *P. tridentatum* + *C. citratus* and *G. globosa* + *C. citratus* in 40:60% proportion that demands scientific studies in order to validate these percentages of combination. We also studied other possibilities increasing the percentage of *C. citratus* to 75%, as it would not be recommended to increase the other species (*P. tridentatum* and *G. globosa* over 40%) due to the previous mentioned organoleptic and sensorial reasons.

The infusions prepared from individual or mixed plants in different proportions gave high antioxidant activity namely, free radicals scavenging activity, reducing power, and lipid peroxidation inhibition (**Table 4**). The order of antioxidant efficacy of the individual samples in the four *in vitro* assays was *P. tridentatum* > *C. citratus* > *G. globosa*. *Pterospartum tridentatum* and *G. globosa* infusions showed, in general, higher antioxidant activity than methanolic extracts, while the opposite was observed for *C. citratus* (Roriz et al., 2014). Particularly, *P. tridentatum* showed higher DPPH scavenging activity than the infusions prepared from other commonly consumed herbs such as *Camellia sinensis* (L.) kuntze (EC_{50}=250 μg/mL) (Pereira, Barros, Vilas-Boas, & Ferreira, 2013), *Matricaria recutita* L. (EC_{50}=395 μg/mL) (Guimarães et al., 2013) and *Melissa officinalis* L. (EC_{50}=190 μg/mL) (Dias, Barros, Sousa, & Ferreira, 2012).

The highest activity observed for *P. tridentatum* can be related with the presence of dihydroflavonol and isoflavone derivatives. Indeed, the antioxidant properties of isoflavones and their role in the protection against diseases development have been

described (Ferguson et al., 2014). The antioxidant activity of *C. citratus* infusion could be attributed to the high concentration of flavonoids, especially apigenin and luteolin derivatives. Apigenin and luteolin, commonly found in different fruits, vegetables and herbs, have demonstrated to possess high antioxidant ability by scavenging free radicals (Galati, Moridani, Chan & O'Brien, 2001). Finally, *G. globosa* infusion that showed the lowest antioxidant potential, gave also the lowest concentration in phenolic compounds.

Regarding infusions prepared with *P. tridentatum* and *C. citratus* in different proportions (25:75% or 40:60%), only synergistic effects were observed (**Table 4**), although the mixture with 40% of *P. tridentatum* and 60% of *C. citratus* showed greater antioxidant properties. This could be related with the higher percentage of *P. tridentatum,* the individual plant with the highest antioxidant activity.

For the infusion prepared with 25% of *G. globosa* and 75% of *C. citratus,* the antagonistic effects predominated (reducing power and TBARS inhibition); an additive effect was observed for free radicals scavenging activity and synergism was only obtained for β-carotene bleaching inhibition (**Table 4**). Regarding the infusion prepared with 40% of *G. globosa* and 60% of *C. citratus,* synergistic effects were observed for free radicals scavenging activity and lipid peroxidation inhibition, but an antagonist effect was obtained for reducing power (**Table 4**).

The phenolic compounds were further analysed in the infusions prepared from the two mixtures that showed the highest antioxidant activity: *P. tridentatum* (40%) + *C. citratus* (60%) and *G. globosa* (40%) + *C. citratus* (60%) (**Table 5)**. In *P. tridentatum* (40%) + *C. citratus* (60%) infusion it can be observed a prevalence of the profile of *P. tridentatum*, even though this species is present in lower proportion (40%). This could be explained by the fact of *P. tridentatum* being the species with the highest concentration of phenolic compounds, mainly dihydroflavonol and isoflavone

derivatives, that are also present in high concentration in this mixture. The opposite occurs for the mixture *G. globosa* (40%) + *C. citratus* (60%); in this case, the predominant profile is from *C. citratus* (60%), presenting this species a higher phenolic content in comparison with *G. gombosa*. The phenolic profile obtained in the infusions prepared from those mixtures could also explain their highest antioxidant activity.

Overall, *P. tridentatum* infusion displayed the highest antioxidant activity, followed by *C. citratus* and, finally, *G. globosa*. The dissimilarity in antioxidant potential is certainly related with their different composition in phenolic compounds. Synergism was the main effect observed among the tested mixtures, being more evident in the infusions prepared with the plants in proportion 40%:60% (either *P. tridentatum* and *C. citratus*; or *G. globosa* and *C. citratus*). The present study validates the combination of the studied plants in specific proportions to be commercialized for infusions preparation.

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Figure Legends

Figure 1. HPLC phenolic profiles of the infusions of (A)- *P. tridentatum* (recorded at 280 nm); (B)- *G. globosa* (recorded at 370 nm); (C)- *G. globosa* betacyanin profile (recorded at 520 nm) and (D)- *C. citratus* (recorded at 370 nm).

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in infusion extracts of *P. tridentatum* (^{Pt}) (mean ± SD).

Peak	Rt (min)	λmax (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
1^{Pt}	5.0	290,sh340	465	Dihydroquercetin 6-*C*-hesoxide	43.04 ± 0.65
2^{Pt}	6.1	294,sh346	465	Dihydroquercetin 6-*C*-hesoxide	2.74 ± 0.17
3^{Pt}	7.7	290,sh340	479	Myricetin-6-*C*-glucoside	12.87 ± 0.01
4^{Pt}	18.7	356	609	Quercetin deoxyhexosyl-hexoside	0.77 ± 0.01
5^{Pt}	18.9	356	609	Quercetin-3-*O*-rutinoside	2.10 ± 0.13
6^{Pt}	19.8	354	463	Quercetin-3-*O*-glucoside (isoquercitrin)	11.85 ± 0.05
7^{Pt}	20.2	354	463	Quercetin *O*-hexoside	5.81 ± 0.07
8^{Pt}	21.2	262,sh312	431	Genistein 7-*O*-glucoside (genistin)	3.53 ± 0.11
9^{Pt}	22.4	262,sh308	461	5,5'-Dihydroxy-3'-methoxy-isoflavone-7-*O*-β-glucoside	2.25 ± 0.05
10^{Pt}	24.6	260,sh332	431	Genistein-8-*C*-glucoside	1.28 ± 0.03
11^{Pt}	26.0	256,sh322	505	Methylbiochanin A/methylprunetin *O*-hexoside	1.65 ± 0.06
12^{Pt}	27.8	262,sh332	269	Genistein	3.08 ± 0.04
13^{Pt}	28.7	262,sh336	341	Methylbiochanin A/methylprunetin derivative	0.97 ± 0.05
14^{Pt}	29.0	252,sh328	607	Biochanin A *O*-hexoside-*O*-hexoside	0.72 ± 0.07
15^{Pt}	31.4	260,sh340	445	Biochanin A 7-*O*-glucoside (sissotrin)	0.60 ± 0.02
16^{Pt}	33.1	260,sh338	649	Biochanin A *O*-acetylhexoside-O-hexoside	0.31 ± 0.02
17^{Pt}	34.5	264,sh338	491	Biochanin A *O*-hexoside	4.79 ± 0.03
18^{Pt}	37.7	260,sh332	283	4'-*O*- Methylgenistein (biochanin A)	9.52 ± 0.00
19^{Pt}	38.6	262,sh334	283	7-*O*-Methylgenistein (prunetin)	nd
20^{Pt}	39.5	262,sh338	299	7-*O*-methylorobol	1.45 ± 0.03
21^{Pt}	41.0	264,sh290	297	Methylbiochanin A/ methylprunetin	0.58 ± 0.01
				Total flavonols	33.40 ± 0.28
				Total dihydroflavonols	45.78 ± 1.42
				Total isoflavone	30.72 ± 0.38
				Total flavonoids	109.90 ± 1.32

nd- not detected

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds and betacyanins in infusion extracts of *G. globosa* (^{Gg}) (mean ± SD).

Peak	Rt (min)	λmax (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
1^{Gg}	11.0	326	355	*cis*-Ferulic acid hexoside	0.37 ± 0.03
2^{Gg}	12.6	326	355	*trans*-Ferulic acid hexoside	0.52 ± 0.01
3^{Gg}	15.5	309	163	*cis-p*-Coumaric acid	0.09 ± 0.01
4^{Gg}	16.4	310	163	*trans-p*-Coumaric acid	0.41 ± 0.01
5^{Gg}	18.1	324	193	*cis*-Ferulic acid	0.20 ± 0.02
6^{Gg}	18.3	358	595	Quercetin 3-*O*-(6-pentosyl)-hexoside	0.08 ± 0.02
7^{Gg}	18.6	324	193	*trans*-Ferulic acid	0.92 ± 0.01
8^{Gg}	19.0	358	609	Quercetin 3-*O*-rutinoside	0.66 ± 0.01
9^{Gg}	19.2	346	725	Kaempferol 3-*O*-(2-pentosyl, 6-*O*-rhamnosyl)-hexoside	0.56 ± 0.06
10^{Gg}	19.9	358	463	Quercetin 3-*O*-glucoside	0.10 ± 0.01
11^{Gg}	21.1	350	579	Kaempferol 3-*O*-(2-pentosyl)-hexoside	0.19 ± 0.02
12^{Gg}	22.4	348	593	Kaempferol 3-*O*-rutinoside	0.89 ± 0.04
13^{Gg}	23.5	352	623	Isorhamnetin 3-*O*-rutinoside	tr
14^{Gg}	23.9	350	447	Kaempferol 3-*O*-glucoside	0.31 ± 0.05
15^{Gg}	24.9	354	477	Isorhamnetin 3-*O*-glucoside	tr
16^{Gg}	26.3	350	489	Kaempferol *O*-acetylhexoside	0.11 ± 0.03
17^{Gg}	31.9	340	639	Quercetin *O*-glucuronide-*O*-hexoside	0.04 ± 0.01
18^{Gg}	32.2	276,342	475	Gomphrenol 3-*O*-hexoside	0.20 ± 0.01
19^{Gg}	33.7	278,342	517	Gomphrenol 3-*O*-(6-acetyl)-hexoside	0.39 ± 0.04
20^{Gg}	34.6	352	593	Kaempferol 3-*O*-(2-rhamnosyl)-hexoside	0.10 ± 0.01
21^{Gg}	35.0	348	623	Kaempferol *O*-glucuronide-*O*-hexoside	0.12 ± 0.03
				Total phenolic acids	2.51 ± 0.01
				Total flavonoids	3.77 ± 0.32
				Total phenolic compounds	6.28 ± 0.31
Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
22^{Gg}	27.3	550	697	Gomphrenin II	tr
23^{Gg}	29.2	550	697	Gomphrenin II	tr
24^{Gg}	30.0	550	727	Gomphrenin III	0.21±0.01
25^{Gg}	32.0	550	697	Isogomphrenin II	0.01±0.00

26[Gg]	33.4	548	727	Isogomphrenin III	0.35±0.01
27[Gg]	34.9	516	683	17-Descarboxy-amaranthin	tr
				Total betacyanins	0.57±0.02

tr- traces

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in infusion extracts of *C. citratus* (^{Cc}) (mean ± SD).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Tentative Identification	Quantification (mg/g inf)
1^{Cc}	7.9	326	353	5-*O*-Caffeoylquinic acid	1.21 ± 0.05
2^{Cc}	10.3	326	179	Caffeic acid	0.27 ± 0.01
$2a^{Cc}$	10.8	324	179	Caffeic acid	0.77 ± 0.01
3^{Cc}	12.3	350	579	Luteolin 6-*C*-hexosyl-8-*C*-pentoside	0.70 ± 0.03
4^{Cc}	14.6	336	563	Apigenin 6-*C*-pentosyl-8-*C*-hexoside	1.76 ± 0.03
5^{Cc}	15.0	350	563	Apigenin 6-*C*-pentosyl-8-*C*-hexoside	0.53 ± 0.01
6^{Cc}	15.6	352	593	Luteolin 2''-*O*-deoxyhexosyl-6-*C*-glucoside	13.12 ± 0.03
7^{Cc}	16.0	352	447	Luteolin 6-*C*-glucoside	0.35 ± 0.01
8^{Cc}	16.1	350	549	Luteolin 6-*C*-pentosyl-8-*C*-pentoside	1.75 ± 0.01
9^{Cc}	17.0	310	163	*trans*-*p*-Coumaric acid	nd
10^{Cc}	17.6	356	549	Luteolin 6-*C*-pentosyl-8-*C*-pentoside	0.17 ± 0.03
11^{Cc}	18.6	328	577	Apigenin 2''-*O*-deoxyhexosyl-*C*-hexoside	0.99 ± 0.01
12^{Cc}	20.3	348	593	Luteolin 7-*O*-neohesperoside	5.31 ± 0.26
13^{Cc}	20.5	350	447	Luteolin 7-*O*-glucoside	2.07 ± 0.15
14^{Cc}	21.6	352	563	Luteolin 2''-*O*-deoxyhexosyl-*C*-pentoside	6.21 ± 0.36
15^{Cc}	22.0	350	417	Luteolin 6-*C*-pentoside	0.24 ± 0.02
16^{Cc}	24.9	350	575	Luteolin 2''-*O*-deoxyosyl-6-*C*-(6-deoxy-*pento*-hexos-ulosyl)	4.19 ± 0.47
17^{Cc}	27.4	352	577	Methyl-luteolin 2''-*O*- deoxyhexosyl-6-*C*-hexoside.	0.28 ± 0.02
18^{Cc}	34.4	350	285	Luteolin	nd
				Total phenolic acids	2.24 ± 0.07
				Total flavonoids	37.66 ± 1.07
				Total phenolic compounds	39.90 ± 1.14

nd- not detected

Table 4. Theoretical *versus* experimental EC_{50} values of antioxidant activity of the infusion extracts prepared from *P. tridentatum* and *C. citratus* or *G. globosa* and *C. citratus* combined in different proportions (mean ± SD).

	Individual samples		*P. tridentatum* (25%) + *C. citratus* (75%)			*P. tridentatum* (40%) + *C. citratus* (60%)		
EC_{50} values (µg/mL)	*P. tridentatum*	*C. citratus*	Theoretical value	Experimental value	Effect	Theoretical value	Experimental value	Effect
DPPH scavenging activity	50±1	1231±8	936±3	663±19	S (29%)	759±2	67±2	S (91%)
Reducing power	105±2	762±10	598±4	474±10	S (21%)	499±3	181±3	S (64%)
β-carotene bleaching inhibition	266±25	1510±79	1199±33	235±9	S (80%)	1013±29	209±18	S (79%)
TBARS inhibition	93±4	428±14	345±6	261±19	S (24%)	294±5	147±4	S (50%)
	Individual samples		*G. globosa* (25%) + *C. citratus* (75%)			*G. globosa* (40%) + *C. citratus* (60%)		
EC_{50} values (µg/mL)	*G. globosa*	*C. citratus*	Theoretical value	Experimental value	Effect	Theoretical value	Experimental value	Effect
DPPH scavenging activity	4305±74	1231±8	1999±24	1927±67	A (4%)	2460±34	1651±23	S (33%)
Reducing power	916±7	762±10	968±12	1029±20	AN (28%)	957±11	942±13	AN (14%)
β-carotene bleaching inhibition	4079±31	1510±79	2152±34	797±30	S (63%)	2538±30	505±45	S (80%)
TBARS inhibition	2496±5	428±14	989±7	1126±16	AN (19%)	1291±6	1005±64	S (20%)

The theoretical values were obtained considering summative contributions of the individual species. A- Additive effect: theoretical and experimental EC_{50} values reveal differences below 10%. S- Synergistic effect: experimental EC_{50} values are more than 10% lower than theoretical values. AN - antagonist effect: experimental EC_{50} values are more than 10% higher than theoretical values.

Table 5. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in combined infusion extracts of *P. tridentatum* (^{Pt}) and *C. citratus* (^{Cc}) or *C. citratus* and *G. globosa* (^{Gg}) (mean ± SD).

Peak	Rt (min)	λmax (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
				P. tridentatum (40%) + *C. citratus* (60%)	
1^{Pt}	4.8	290,sh340	465	Dihydroquercetin 6-*C*-hesoxide	28.73±0.16
2^{Pt}	6.0	292,sh346	465	Dihydroquercetin 6-*C*-hesoxide	2.48±0.01
3^{Pt}	7.4	290,sh340	479	Myricetin-6-*C*-glucoside	16.38±0.04
2^{Cc}	10.8	326	179	Caffeic acid	1.14±0.02
3^{Cc}	13.2	354	579	Luteolin 6-*C*-hexosyl-8-*C*-pentoside	0.53±0.04
6^{Cc}	15.3	352	593	Luteolin 2''-*O*-deoxyhexosyl-6-*C*-glucoside	1.90±0.10
9^{Cc}	16.6	310	163	*trans*-*p*-Coumaric acid	3.28±0.04
4^{Pt}	18.6	356	609	Quercetin deoxyhexosyl-hexoside	0.74±0.03
5^{Pt}	18.8	356	609	Quercetin-3-*O*-rutinoside	2.60±0.03
6^{Pt}	19.8	354	463	Quercetin-3-*O*-glucoside (isoquercitrin)	8.16±0.02
7^{Pt}	20.2	352	463	Quercetin *O*-hexoside	4.41±0.12
8^{Pt}	21.1	260,sh312	431	Genistein 7-*O*-glucoside (genistin)	3.28±0.24
14^{Cc}	21.6	352	563	Luteolin 2''-*O*-deoxyhexosyl-*C*-pentoside	0.96±0.02
9^{Pt}	22.3	262,sh308	461	5.5'-Dihydroxy-3'-methoxy-isoflavone-7-*O*-β-glucoside	2.98±0.06
10^{Pt}	24.6	260,sh332	431	Genistein-8-*C*-glucoside	1.61±0.13
16^{Cc}	24.8	350	575	Luteolin 2''-*O*-deoxyosyl-6-*C*-(6-deoxy-*pento*-hexos-ulosyl)	0.97±0.01
11^{Pt}	26.0	256,sh322	505	Methylbiochanin A/methylprunetin *O*-hexoside	2.62±0.02
17^{Cc}	27.2	356	577	Methyl-luteolin 2''-*O*- deoxyhexosyl-6-*C*-hexoside.	0.30±0.02
12^{Pt}	27.8	260,sh332	269	Genistein	2.82±0.04
13^{Pt}	28.6	262,sh336	341	Methylbiochanin A/methylprunetin derivative	1.55±0.10
14^{Pt}	29.0	252,sh328	607	Biochanin A *O*-hexoside-*O*-hexoside	1.08±0.11
15^{Pt}	31.7	258,sh340	445	Biochanin A 7-*O*-glucoside (sissotrin)	0.99±0.11
16^{Pt}	33.2	260,sh338	649	Biochanin A *O*-acetylhexoside-O-hexoside	0.49±0.02
17^{Pt}	34.8	260,sh338	491	Biochanin A *O*-hexoside	3.05±0.10
18^{Pt}	37.8	260,sh332	283	4'-*O*- Methylgenistein (biochanin A)	8.81±0.36
20^{Pt}	38.8	262,sh338	299	7-*O*-methylorobol	1.29±0.02
21^{Pt}	39.7	264,sh290	297	Methylbiochanin A/ methylprunetin	1.05±0.09
				Total phenolic acids	4.41±0.02
				Total flavonoids	99.77±0.92
				Total phenolic compounds	104.18±0.94
				G. globosa (40%) + *C. citratus* (60%)	
Peak	Rt (min)	λmax (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
1^{Cc}	7.9	324	353	5-*O*-Caffeoylquinic acid	1.32±0.15
2^{Cc}	10.3	326	179	*cis* Caffeic acid	0.94±0.05
$2a^{Cc}$	10.8	324	179	*trans* Caffeic acid	2.15±0.01

2[Gg]	12.8	324	355	*trans*-Ferulic acid hexoside	0.60±0.06
4[Cc]	14.6	332	563	Apigenin 6-*C*-pentosyl-8-*C*-hexoside	0.63±0.04
5[Cc]	15.0	350	563	Apigenin 6-*C*-pentosyl-8-*C*-hexoside	0.55±0.01
6[Cc]	15.6	350	593	Luteolin 2''-*O*-deoxyhexosyl-6-*C*-glucoside	3.88±0.02
7[Cc]	16.0	350	447	Luteolin 6-*C*-glucoside	1.12±0.03
8[Cc]	16.1	350	549	Luteolin 6-*C*-pentosyl-8-*C*-pentoside	2.70±0.19
4[Gg]/9 [Cc]	16.7	310	163	*trans*-*p*-Coumaric acid	4.39±0.15
11[Cc]	18.6	350	577	Apigenin 2''-*O*-deoxyhexosyl-*C*-hexoside	0.37±0.05
7[Gg]	19.0	326	193	*trans*-Ferulic acid	1.73±0.08
8[Gg]	19.1	358	609	Quercetin 3-*O*-rutinoside	0.23±0.03
10[Gg]	20.0	354	463	Quercetin 3-*O*-glucoside	0.24±0.01
12[Cc]	20.5	348	593	Luteolin 7-*O*-neohesperoside	1.79±0.12
13[Cc]	20.8	350	447	Luteolin 7-*O*-glucoside	0.35±0.03
14[Cc]	21.7	348	563	Luteolin 2''-*O*-deoxyhexosyl-*C*-pentoside	1.77±0.06
14[Gg]	22.5	348	447	Kaempferol 3-*O*-glucoside	0.48±0.01
16[Cc]	24.9	352	575	Luteolin 2''-*O*-deoxyosyl-6-*C*-(6-deoxy-*pento*-hexos-ulosyl)	1.07±0.02
17[Cc]	27.4	350	577	Methyl-luteolin 2''-*O*- deoxyhexosyl-6-*C*-hexoside	0.37±0.01
				Total phenolic acids	11.13±0.27
				Total flavonoids	15.56±0.74
				Total phenolic compounds	26.69±1.00

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	Tentative identification	Quantification (mg/g inf)
22[Gg]	27.1	550	697	Gomphrenin II	tr
23[Gg]	28.9	550	697	Gomphrenin II	tr
24[Gg]	29.8	550	727	Gomphrenin III	0.08±0.01
25[Gg]	31.9	550	697	Isogomphrenin II	tr
26[Gg]	33.2	548	727	Isogomphrenin III	0.09±0.01
27[Gg]	34.7	516	683	17-Descarboxy-amaranthin	tr
				Total betacyanins	0.17±0.02

tr- traces