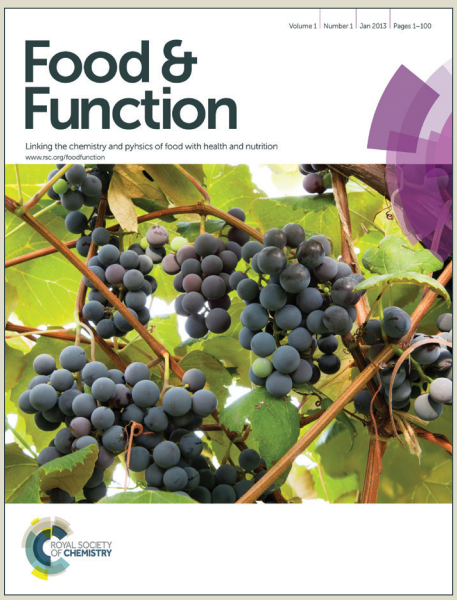
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

New insights on the effects of formulation type and compositional mixtures over the antioxidant and cytotoxic activities of dietary supplements based-on hepatoprotective plants

Carla Pereira[a], João C.M. Barreira[a], Ricardo C. Calhelha[a,b], Maria João R.P. Queiroz[b], Lillian Barros[a], Isabel C.F.R. Ferreira[a,*]

[a]Centro de Investigação de Montanha (CIMO), ESA, Instituto Politécnico de Bragança, Campus de Santa Apolónia, apartado 1172, 5301-855 Bragança, Portugal

[b]Centro de Química, Universidade do Minho, Campus de Gualtar 4710-057 Braga, Portugal

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

Abstract

Artichoke (A), borututu (B) and milk thistle (M) are included in several supplements to provide beneficial effects. Different formulations (infusions, pills and syrups), as also different proportions of A, B and M (1:1:1, 2:1:1, 1:2:1, 1:1:2) within each formulation were assayed to optimize the desired benefits. The antioxidant activity, anti-hepatocellular carcinoma activity, hepatotoxicity and bioactive compounds contents were evaluated. Syrups tended to be the formulation with highest antioxidant activity and total phenolics and flavonoids content; otherwise, pills were the worst formulation. In what concerns A:B:M ratios, the results did not reveal so pronounced differences. None of the assayed mixtures resulted to be toxic (up to the maximum assayed dose) for liver primary cells (PLP2), but some samples, especially infusions, showed toxicity for the hepatocellular carcinoma cell line (HepG2). With no exception, the mixtures for all formulations gave synergistic effects in antioxidant activity, when compared to the activity of single plants.

Keywords: Dietary supplements; Formulation; Compositional mixture; Bioactivity; Citotoxicity; Synergism

Introduction

Artichoke (*Cynara scolymus* L.), borututu (*Cochlospermum angolensis* Welw.) and milk thistle (*Silybum marianum* (L.) Gaertn) are medicinal plants with numerous pharmacological effects, such as antioxidant and hepatoprotective activities, as described in different studies;[1-3] borututu is also recognized as an antimalarial herb,[4] while milk thistle prevent spleen and gallbladder disorders,[5] and artichoke leaves are used for the treatment of dyspepsia and diabetes.[6]

Phytochemicals are very prone to variations depending on the plant material (that changes within phenological cycle), harvest, drying and storage conditions.[7] The genetic, cultural and environmental factors that explain this variability make their use rather challenging and frequently problematic because the active principles are diverse and may be unknown.[7,8] In some cases, these effects might even be harmful; for instance, the leaf extracts of artichoke caused chromosomal instability and cytotoxicity in hamster ovary cells,[9] while milk thistle extracts, at 15 μg/mL, showed toxicity against the activity of hepatic P450 cytochrome.[10]

The consumption of supplements to provide the beneficial effects of certain plants has raised several controversial questions, such as those pointed out by Halliwell, who stated that "we cannot just pull out one or two individual molecules and expect pills containing high doses of them to protect us",[11] suggesting the whole herbal medicine as one active ingredient, *i.e.*, a set of multi-component parts self-organized into an indivisible whole.[12] Nonetheless, there is an increasing number of formulations based on these plants due to their therapeutic applications, namely infusions, pills, capsules, ampoules, syrups, among others.

The bioactivity of the most consumed forms (infusions, pills, and syrups) of borututu, milk thistle, and artichoke was assessed by our research group and these formulations

revealed not only antioxidant and hepatoprotective effects, but also synergism between the three plants in syrups.[13,14] Thus, given the importance of the studied plants in the treatment of liver diseases and the availability of so many formulations, it seems very pertinent to find the better way to achieve the desired benefits from these herbs depending on the kind of formulation, the plant present on it, or even the percentage of each plant in formulations containing the three mentioned plants. To deepen that question, in the present work we investigated the antioxidant and anti-hepatocellular carcinoma activities of twelve mixtures with four different proportions of artichoke, borututu and milk thistle, and different formulations of each plant (infusions, pills and syrups).

Experimental

Samples and samples preparation

Cynara scolymus L. (artichoke; A), *Cochlospermum angolensis* Welw. (borututu; B) and *Silybum marianum* (L.) Gaertn (milk thistle; M) were obtained from an herbalist shop in Bragança (Portugal), as dry material for infusions (leaves, plant and bark, respectively), pills (based-on plant and roots, in the case of B), and syrups (containing 100% of A, 10% of B roots, and 2.3% of M). Each sample was used as recommended in the label: the infusions were prepared from the dry material and further lyophilized, the pills were powdered and the syrups were directly used.

Each formulation (infusion, pill and syrup) of A, B and M, respectively, was mixed in different proportions: 1:1:1; 2:1:1; 1:2:1; and 1:1:2 (*m/m/m*), and further dissolved in distilled water to a final concentration of 6 mg/mL. The twelve stock solutions (four mixtures of infusions, four mixtures of pills and four mixtures of syrups) were

successively diluted and submitted to an evaluation of antioxidant activity, anti-hepatocellular carcinoma activity and hepatotoxicity.

Standards and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Gallic acid, catechin, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA) and Tris were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM (Dulbecco's Modified Eagle Medium) were from Hyclone (Logan, UT, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Antioxidant activity

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. 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. 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. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour 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.[15] 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.

Bioactive compounds content

Total phenolics were estimated by Folin-Ciocalteu colorimetric assay according to procedures previously described[16] and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

Total flavonoids were determined by a colorimetric assay using aluminum trichloride, following procedures previously reported;[16] the results were expressed as mg of (+)-catechin equivalents (CE) per g of extract.

Anti-hepatocellular carcinoma activity and hepatotoxicity

The anti-hepatocellular carcinoma activity was evaluated using HepG2, which is the most widely used tumor cell line and generally regarded as a good hepatocellular carcinoma model. HepG2 cells were routinely maintained as adherent cell cultures in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, at 37 ºC, in a humidified air incubator containing 5% CO_2. The cell line was plated at 1.0×10^4 cells/well in 96-well plates. Sulforhodamine B assay was performed according to a procedure previously described by the authors.[17]

For hepatotoxicity evaluation, a cell culture was prepared from a porcine liver obtained from a local slaughter house, according to a procedure established by the authors;[17] it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

Classification of additive, synergistic or antagonistic effects

Theoretical values for antioxidant and anti-hepatocellular carcinoma activities of the mixtures were calculated as weighted mean experimental EC_{50} or GI_{50} values of the individual samples[14] and considering additive contributions of individual species in each percentage; for instance, mixture 2:1:1: $EC_{50} = EC_{50}A\times0.5 + EC_{50}B\times0.25 + EC_{50}M\times0.25$.

The classification in additive (AD), synergistic (SN) or antagonistic (negative synergistic; AN) effects was performed as follow: AD: theoretical and experimental values reveal differences lower than 5%; SN: experimental values are more than 5% lower than theoretical values; AN: experimental values are more than 5% higher than theoretical values. For each case, the percentage was calculated as follows: [(experimental value – theoretical value)/experimental value]*100.

Statistical analysis

All statistical tests were performed at a 5% significance level, using SPSS (v.20) software. For each formulation (F) and A:B:M ratio (R), three samples were analyzed, with all the assays being also carried out in triplicate. The results are expressed as mean value±standard deviation (SD).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors F and R. In this case, when a statistically significant interaction (F×R) is detected, the two factors should be evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction is verified, means might be compared using, for instance, Tukey's honestly significant difference (HSD) multiple comparison test.

Furthermore, a linear discriminant analysis (LDA) was used to compare the effect of F and R on antioxidant activity and extracted bioactive compounds. A stepwise technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination processes, where the inclusion of a new variable is preceded by ensuring that all variables selected previously remain significant.[18,19] With this approach, it is possible to identify the significant variables obtained for each sample. To verify the significance of canonical discriminant functions, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

Results and Discussion

Antioxidant properties and cytotoxicity for hepatocellular tumor cell line and liver primary cells

There has been an intensive scientific effort to validate the effectiveness of herbal formulations, since the preparation of dietary supplements/nutraceuticals and some pharmaceutical products are based on the extraction of bioactive compounds from natural products.[20] This scientific validation is often supported by evaluating the antioxidant activity of plant derived products, as a preliminary approach. Herein, four different assays were used: DPPH scavenging activity, reducing power (assessed by Ferricyanide/Prussian blue assay), β-carotene bleaching inhibition and TBARS formation inhibition. In addition, total phenolics and total flavonoids were also determined, bearing in mind that the antioxidant activity is often correlated with the contents in phenolic compounds.[21] Also, HepG2 human cell line was used to assess anti-hepatocellular carcinoma activity, while a primary culture of porcine liver cells was established to evaluate hepatotoxicity. In fact, since some potential effects of compounds naturally present in plants are difficult to anticipate, the assessment of the safety of a plant extract used as a food or a medicine by the population is completely mandatory.[9]

Three plant species, namely artichoke (A), borututu (B) and milk thistle (M), which are commonly present in nutraceutical formulations/dietary supplements, were selected due to their availability in different formulations. Besides aiming studying the influence of the formulation type in the bioactivity and phenolic compounds content, this study was designed also to evaluate supposed differences resulting from using different percentages of the plant species in each formulation.

The effects of formulation type (F) and A:B:M ratio (R) were evaluated by fixing one of the factors; *i.e.*, the results are presented as the mean of each F, comprising values for

all R in those conditions, as well as the mean of each R, containing the results for all the corresponding F. Accordingly, the standard deviation values should not be looked up as a simple measure of assays repeatability, since they reflect results from assays performed in different conditions.

As it can be seen in **Table 1**, each factor showed a significant effect *per se*, but the interaction among factors (F×R) was also a significant ($p<0.001$) source of variation for all parameters, indicating a strong interaction between the formulation and the percentages of each plant in the prepared mixtures. Therefore, although the least squares means are presented, the results for multiple comparisons became meaningless. Nevertheless, from the analysis of the plots of the estimated margins means (**Figures 1** and **2**), some particular tendencies can be observed. For instance, pill formulation gave lower antioxidant activity in all antioxidant assays (DPPH scavenging activity: EC_{50} = 1.2 mg/mL; reducing power: EC_{50} = 0.4 mg/mL; β-carotene bleaching inhibition: EC_{50} = 2 mg/mL; TBARS formation inhibition: EC_{50} = 0.3 mg/mL) and also lower contents in total phenolics (69 mg GAE/g) and total flavonoids (5 mg CE/g) contents; syrups and infusions presented similar antioxidant activity levels, except for reducing power (lower on infusion), but total phenolics (469 mg GAE/g) and total flavonoids (78 mg CE/g) contents were higher in syrups. In what concerns A:B:M ratios, the results did not reveal so pronounced differences, except for the lower DPPH scavenging activity (EC_{50} = 1.0 mg/mL), β-carotene bleaching inhibition (EC_{50} = 2 mg/mL) and TBARS formation inhibition (EC_{50} = 0.2 mg/mL) in mixtures 2:1:1, 1:1:2 and 1:1:1, respectively.

Besides the pointed out differences, the assayed mixtures and formulations proved to have higher antioxidant activity than previously assayed formulations, namely syrups with a A:B:M ratio of 1:1:2.35, except in the case of β-carotene bleaching inhibition, to

which the results were similar.[14] A similar result was obtained for the anti-hepatocellular carcinoma activity, which demonstrated to be higher in the present report when compared to the results obtained from infusion, pills or syrups based on a single species or in mixtures different than those assayed herein. This antitumor activity was especially high in infusions (1:1:1, GI_{50} = 24 µg/mL; 2:1:1, GI_{50} = 49 µg/mL; 1:2:1, GI_{50} = 63 µg/mL; 1:1:2, GI_{50} = 67 µg/mL). None of the samples showed hepatotoxicity (GI_{50} > 400 µg/mL, in all cases), which represents an important result considering the need of obtaining innocuous formulations.

Additive, synergistic or antagonistic effects

When comparing with the antioxidant activity and bioactive compounds content of each plant *per se*, the results obtained for the present mixtures and formulations are close to those reported for borututu, which is, by far, the plant with most active derived products among the three assayed species.[13] As a consequence, the possibility of having a synergistic effect within the prepared mixtures was raised. This hypothesis was mathematically verified by calculating the simple mean (for 1:1:1 mixture), or the weighted mean (in all remaining mixtures). The results of these calculations are indicated as theoretical values (**Table 2**); regarding the anti-hepatocellular carcinoma activity, the GI_{50} values higher than 400 µg/mL (the maximum assayed concentration) were included as being 400, since this is precisely the value that most hinder the possible synergistic effect; *i.e.*, if a given mixture shows synergistic effect when considering the GI_{50} value of a determined plant (or plants) as being 400 µg/mL instead of the non-obtained (higher) experimental value, than the effect resulting from including the real experimental value, would certainly be synergistic. With no exception, the mixtures of all formulations gave synergistic effects in antioxidant activity. In fact, the

highest activity of mixtures when compared to the individual plants was previously observed in formulations containing fennel, lemon-verbena and spearmint.[22] Nevertheless, the mixtures 2:1:1 and 1:1:2, for pills and syrups, and also mixture 1:2:1, for syrups, did not result in a synergistic effect in what regards the anti-hepatocellular carcinoma activity on HepG2. The hepatotoxicity, as evaluated on PLP2 cells, was always lower in the mixtures, when compared to the activity of single plants,[13,14] which represents also a good result considering the previously stated objective of obtaining non-toxic mixed formulations.

Linear discriminant analysis of antioxidant properties

In order to have a complete perspective about the effect of F and R on the antioxidant activity and bioactive compounds amounts, two linear discriminant analysis were applied (the anti-hepatocellular carcinoma activity and hepatotoxicity results were not included, since there were some cases with GI_{50} > 400 μg/mL that could not be included). The significant independent variables (results for antioxidant activity assays and bioactive compound contents) were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

In the case of F effect, 2 significant functions were defined (plotted in **Figure 3**), which included 100.0% of the observed variance (first, 58.2%; second, 41.8%). As it can be observed, the tested groups (infusion, pill and syrup) were completely individualized (shadowed ellipses). Function 1 was primarily correlated to TBARS formation inhibition, DPPH scavenging inhibition and β-carotene bleaching inhibition, which were much lower in pill formulation. Actually, this function separated mainly pills from the remaining formulations, as confirmed by the means of canonical variance (MCV:

infusion, -4.684; pill, 5.212; syrup, -0.529. Function 2, by its side, was more correlated to reducing power (lower in syrup), total phenolics and total flavonoids (in higher quantities in syrups). Accordingly, as it can be seen in the vertical axis, function 2 clearly separated syrup formulation (MCV: infusion, -2.806; pill, -2.031; syrup, 4.837). All samples were correctly classified, either for original grouped cases, as well as for cross-validated grouped cases.

Regarding A:B:M ratio, the discriminant model selected 3 significant functions (**Figure 4**), which included 100.0% of the observed variance (function 1: 65.3%, function 2: 20.9%, function 3: 13.8%). In this case, the tested groups (1:1:1, 2:1:1, 1:2:1 and 1:1:2) were not completely individualized, indicating that the differences determined in the antioxidant activity assays and bioactive compounds contents were not enough to discriminate the tested groups. The classification performance allowed 65% of correctly classified samples (sensitivity) and 64% of overall specificity within the leave-one-out cross-validation procedure (**Table 3**). Despite all variables were kept in the final analysis, it became obvious that the differences verified for the assayed ratios were not as significant as it would be necessary to obtain individualized groups. This can be clearly observed in **Figure 4**, in which several overlapping markers confirm the similarity among the assayed mixtures of artichoke, borututu and milk thistle.

Conclusions

Overall, the interaction among F and R was significant in all cases, indicating that the effects caused by each assayed formulation are related to the used proportion of each plant. Even so, syrups tended to be the formulation with highest antioxidant activity and the higher contents in total phenolics and flavonoids; this was specially verified when the mixture 1:1:2 was used, as it can be concluded from the estimated marginal mean

plots. On the other hand, pills were the worst formulation, independently of the used mixture. In what concerns artichoke:borututu:milk thistle ratios, the results did not reveal so observable differences. The higher influence of F in comparison with R was clearly highlighted by the LDA outputs. In addition, the effects of each factor were significantly different, since the correlations among discriminant functions and selected variables were different within each statistical test. The obtained outputs confirmed the significant differences among infusions, pills and syrups, showing also that the artichoke:borututu:milk thistle ratios used in the mixtures had much lower effects in the antioxidant activity assays and bioactive compounds contents.

With no exception, the mixtures for all formulations gave synergistic effects for antioxidant activity assays, and also in several assays regarding hepatocellular carcinoma toxicity, when compared to the activity of single plants. Moreover, none of the samples showed toxicity for liver primary cells.

The obtained results might be helpful to define the best formulation and mixing proportions to be used in the preparation of non-toxic products derived from artichoke, borututu and milk thistle.

Competing interests

The authors declare no competing financial interest.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) for financial support to CIMO (strategic project PEst-OE/AGR/UI0690/2011). J.C.M. Barreira and R.C. Calhelha thank FCT, POPH-QREN and FSE for their grants

(SFRH/BPD/68344/2010 and SFRH/BPD/72802/2010, respectively). L. Barros thanks "Compromisso para a Ciência 2008" for her contract.

References

1 V. Lattanzio, P.A. Kroon, V. Linsalata, A. Cardinali, J. Funct. Foods, 2009, I, 131-144.

2 Shaker E, Mahmoud, Mnaa S. Food Chem Toxicol., 2010, 48, 803-806.

3 J.R.A. Silva, A.S. Ramos, M. Machado, D.F. Moura, Z. Neto, M.M. Canto-Cavalheiro, P. Figueiredo, V.E. Rosario, A.C.F. Amaral, D. Lopes, Memórias do Instituto Oswaldo Cruz 2011, 106, 142-158.

4 W. Presber, D.K. Herrman, B. Hegenscheid, Angewandte Parasitol, 1991, 32, 7-9.

5 F. Rainone, Am. Fam. Phys., 2005, 72, 1285-1292.

6 I. Koubaa, M. Damak, A. McKillop, M. Simmonds, Fitoterapia, 1999, 70, 212-213.

7 O.F. Kunle, H.O. Egharevba, O.P. Ahmadu, Int. J. Biodiv. Conserv, 2012, 4, 101-112.

8 R. Bauer, Drug Inf. J., 1998, 32, 101-110.

9 L.V. Jacociunas, H.H.R. Andrade, M. Lehmann, B.R.R. Abreu, A.B.F. Ferraz, J. Silva, I. Grivicich, R.R. Dihl, Food Chem. Toxicol., 2013, 55, 56-59.

10 J. Doehmer, G. Weiss, G.P. McGregor, K. Appel, Toxicol. In Vitro, 2011, 25, 21-27.

11 B. Halliwell, Nutr. Rev., 2012, 70:257-265.

12 J.W. Wu, L.C. Lin, T.W. Tsai, J. Ethnopharmacol., 2009, 121, 185-193.

13 C. Pereira, R.C. Calhelha, L. Barros, I.C.F.R. Ferreira, Ind. Crops Prod., 2013, 49, 61-65.

14 C. Pereira, R.C. Calhelha, L. Barros, M.J.R.P. Queiroz, I.C.F.R. Ferreira, Ind. Crops Prod., 2014, 52, 709-713.

15 A. Martins, L. Barros, A.M. Carvalho, C. Santos-Buelga, I.P. Fernandes, F. Barreiro, I.C.F.R. Ferreira, Food & Function, 2014. Doi: 10.1039/C3FO60721F

16 Pereira C, Barros L, Vilas-Boas M, Ferreira ICFR (2013b) Int J Food Sci Nutr 64:230-234.

17 D.S. Stojković, J. Petrovic, F. Reis, L. Barros, J. Glamocilija, A. Ciric, I.C.F.R. Ferreira, M. Sokovic, Food & Function, 2014. Doi:10.1039/C4FO00113C

18 A. López, P. García, A. Garrido, Food Chem., 2008, 106, 369-378.

19 J. Maroco, Edições Sílabo, Lisboa, Portugal, 2003.

20 J. Dai, R.J. Mumper, Molecules, 2010, 15, 7313-7352.

21 N. Razali, S. Mat-Junit, A.F. Abdul-Muthalib, S. Subramaniam, A. Abdul-Aziz, Food Chem., 2012, 131, 441-448.

22 R. Guimarães, L. Barros, A.M. Carvalho, I.C.F.R. Ferreira, Phytother. Res., 2011, 25, 1209-1214.

Table 1. *In vitro* antioxidant properties (EC_{50}, mg/mL) and bioactive compounds content for the different formulations prepared from artichoke (A), borututu (B) and milk thistle (M). The results are presented the as mean±SD[a].

		Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)	DPPH scavenging activity	Reducing power	β-Carotene bleaching inhibition	TBARS formation inhibition
Formulation (F)	infusion	148±19	34±4	0.4±0.1	0.2±0.1	0.15±0.03	0.06±0.01
	pill	69±11	5±1	1.2±0.4	0.4±0.1	2±1	0.3±0.1
	syrup	469±164	78±32	0.3±0.2	0.06±0.02	0.10±0.02	0.03±0.01
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A:B:M ratio (R)	1:1:1	232±178	38±33	0.6±0.2	0.2±0.1	0.3±0.3	0.2±0.2
	2:1:1	172±112	31±20	1.0±0.5	0.3±0.2	1±1	0.1±0.1
	1:2:1	198±121	31±20	0.5±0.4	0.2±0.1	1±1	0.1±0.1
	1:1:2	312±301	55±54	0.6±0.4	0.3±0.2	2±2	0.1±0.1
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
F×R	*p*-value (n=108)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

EC_{50}- extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay (reducing power).

Table 2. Theoretical[a] *versus* experimental values of antioxidant EC_{50} (mg/mL) and antiproliferative GI_{50} (μg/mL) activities of different mixtures and formulations.

Bioactivity	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect
	1:1:1			2:1:1			1:2:1			1:1:2		
						Infusion						
DPPH scavenging activity	1.56	0.38±0.02	SN	1.72	0.42±0.04	SN	1.22	0.25±0.02	SN	1.80	0.48±0.03	SN
Reducing power	1.22	0.16±0.01	SN	1.38	0.30±0.02	SN	0.97	0.18±0.01	SN	1.36	0.32±0.02	SN
β-carotene bleaching inhibition	1.21	0.11±0.01	SN	1.34	0.16±0.01	SN	1.07	0.13±0.01	SN	1.26	0.18±0.01	SN
TBARS inhibition	0.17	0.06±0.01	SN	0.16	0.06±0.01	SN	0.13	0.04±0.01	SN	0.22	0.07±0.01	SN
HepG2 (hepatocellular carcinoma)	199.37	24±1	SN	162.55	49±8	SN	186.05	63±7	SN	249.53	67±2	SN
						Pill						
DPPH scavenging activity	6.46	0.85±0.05	SN	7.43	1.85±0.03	SN	5.22	1.07±0.03	SN	6.94	1.12±0.04	SN
Reducing power	1.31	0.38±0.02	SN	1.56	0.49±0.02	SN	1.14	0.35±0.03	SN	1.27	0.43±0.02	SN
β-carotene bleaching inhibition	7.70	0.77±0.04	SN	5.84	2.35±0.05	SN	7.24	1.82±0.05	SN	10.27	4.19±0.05	SN
TBARS inhibition	0.86	0.43±0.02	SN	1.03	0.29±0.01	SN	0.74	0.17±0.01	SN	0.85	0.25±0.02	SN
HepG2 (hepatocellular carcinoma)	400	360±14	SN	400	>400	AD	400	340±2	SN	400	>400	AD
						Syrup						
DPPH scavenging activity	74.89	0.43±0.02	SN	113.05	0.66±0.05	SN	57.07	0.18±0.02	SN	56.81	0.11±0.01	SN
Reducing power	24.51	0.05±0.01	SN	36.85	0.08±0.01	SN	18.83	0.08±0.01	SN	18.58	0.03±0.01	SN
β-carotene bleaching inhibition	5.91	0.10±0.01	SN	8.82	0.13±0.01	SN	4.61	0.12±0.01	SN	4.48	0.07±0.01	SN
TBARS inhibition	7.08	0.03±0.01	SN	10.68	0.04±0.01	SN	5.40	0.04±0.01	SN	5.38	0.02±0.01	SN
HepG2 (hepatocellular carcinoma)	360.16	317± 12	SN	370.12	>400	AN	370.12	>400	AN	340.24	>400	AN

[a]The theoretical values were obtained considering summative contributions of the individual species. A- Additive effect: theoretical and experimental EC_{50}/GI_{50} values reveal differences below 5%. S- Synergistic effect: experimental EC_{50}/GI_{50} values are more than 5% lower than theoretical values. AN - antagonist effect: experimental EC_{50} values are more than 5% higher than theoretical values.

Table 3. Contingency matrix obtained using LDA based on antioxidant activity and bioactive compounds content in different artichoke:borututu: milk thistle ratios.

	Predicted group membership				Total	Sensitivity (%)
	1:1:1	2:1:1	1:2:1	1:1:2		
1:1:1	19	7	1	0	27	70
2:1:1	9	9	0	9	27	33
1:2:1	6	0	21	0	27	78
1:1:2	0	0	6	21	27	78
Total	34	16	28	30	108	65
Specificity (%)	56	56	75	70	64	

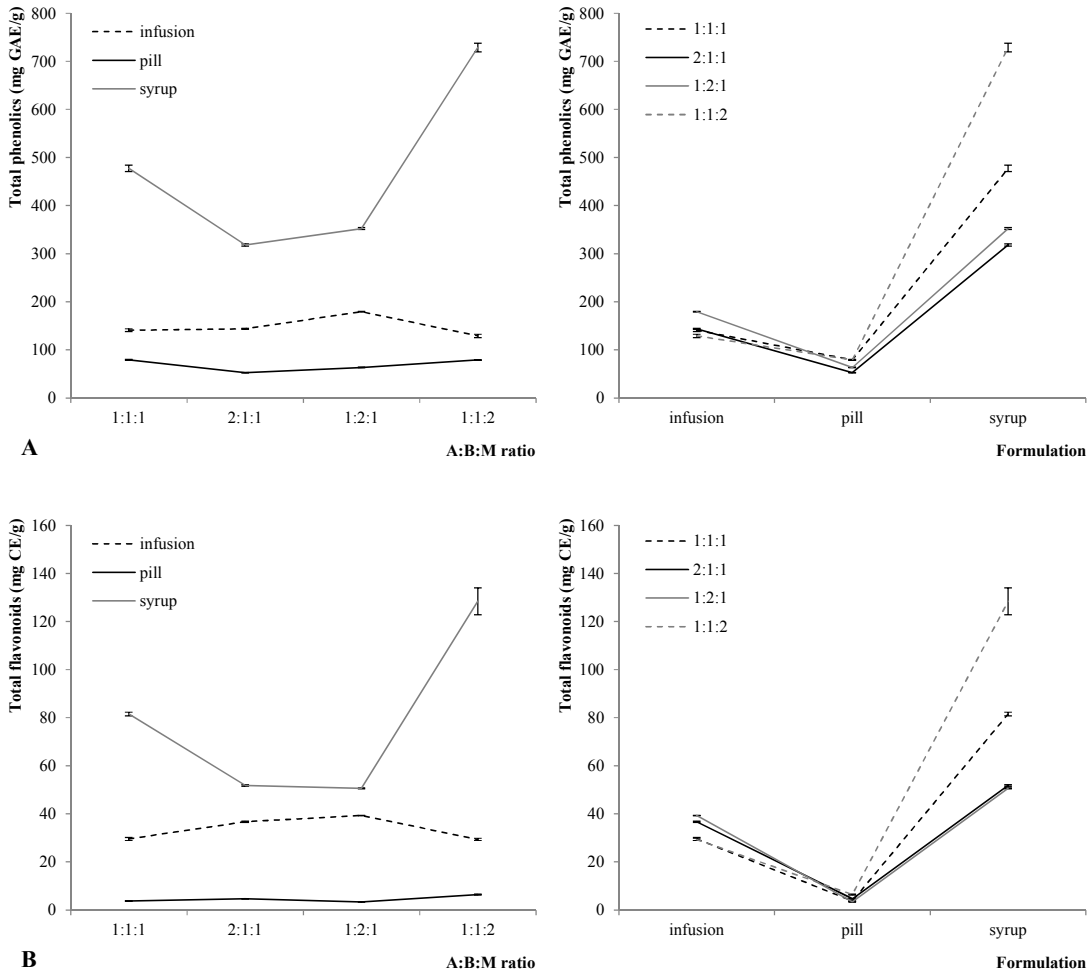
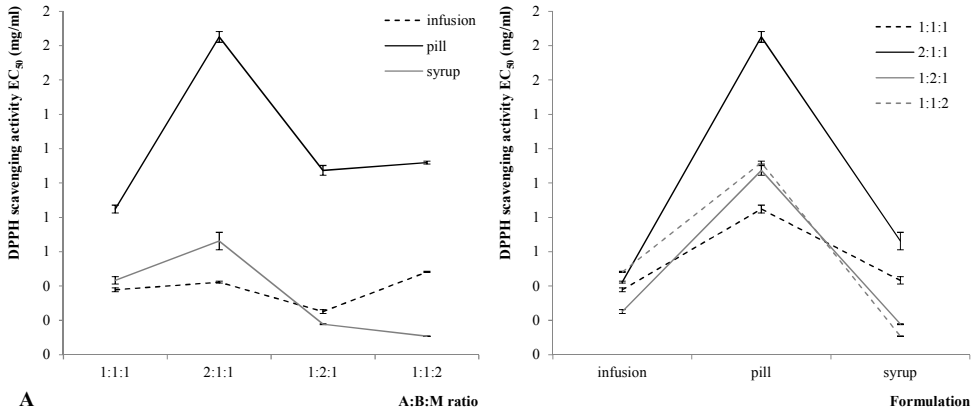
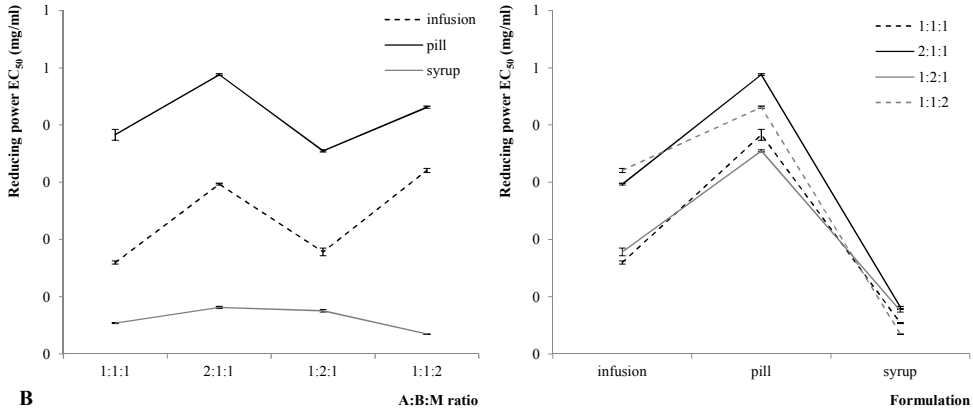


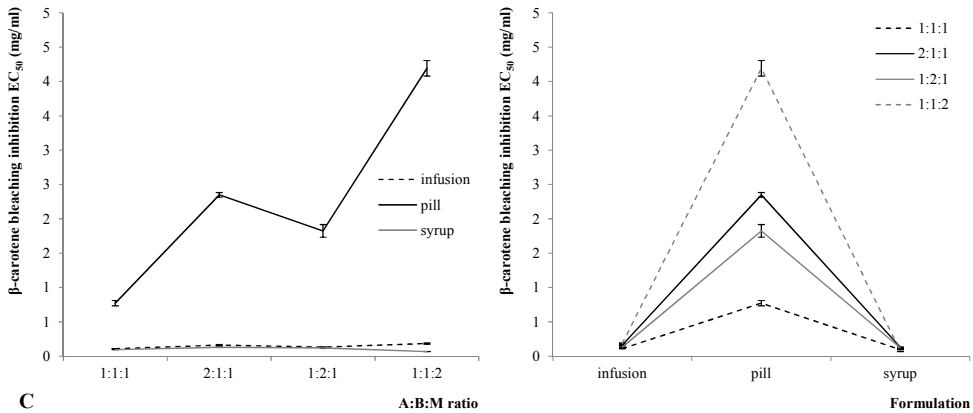
Figure 1. Interactions between formulation (F) and artichoke:borututu:milk thistle ratio (R) effects on bioactive compounds content. Total phenolics (A), total flavonoids (B).



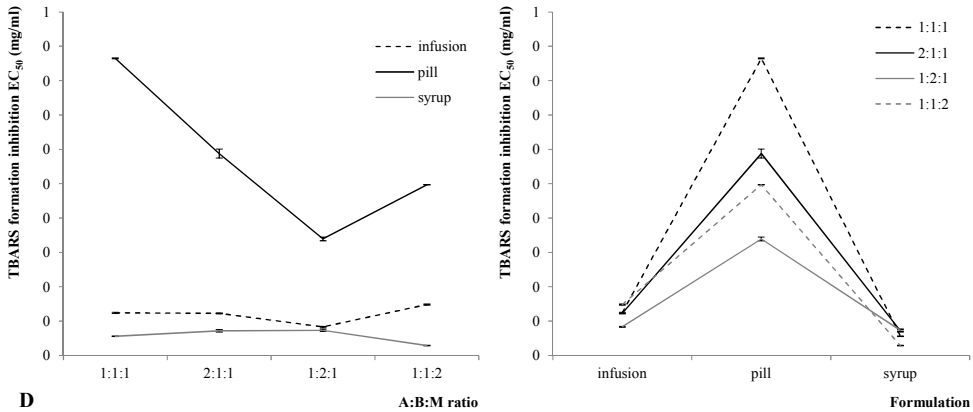
A



B



C



D

Figure 2. Interactions between formulation (F) and artichoke:borututu:milk thistle ratio (R) on the antioxidant activity. DPPH scavenging activity (A), reducing power assay (B), β-carotene bleaching inhibition (C), TBARS formation inhibition (D).

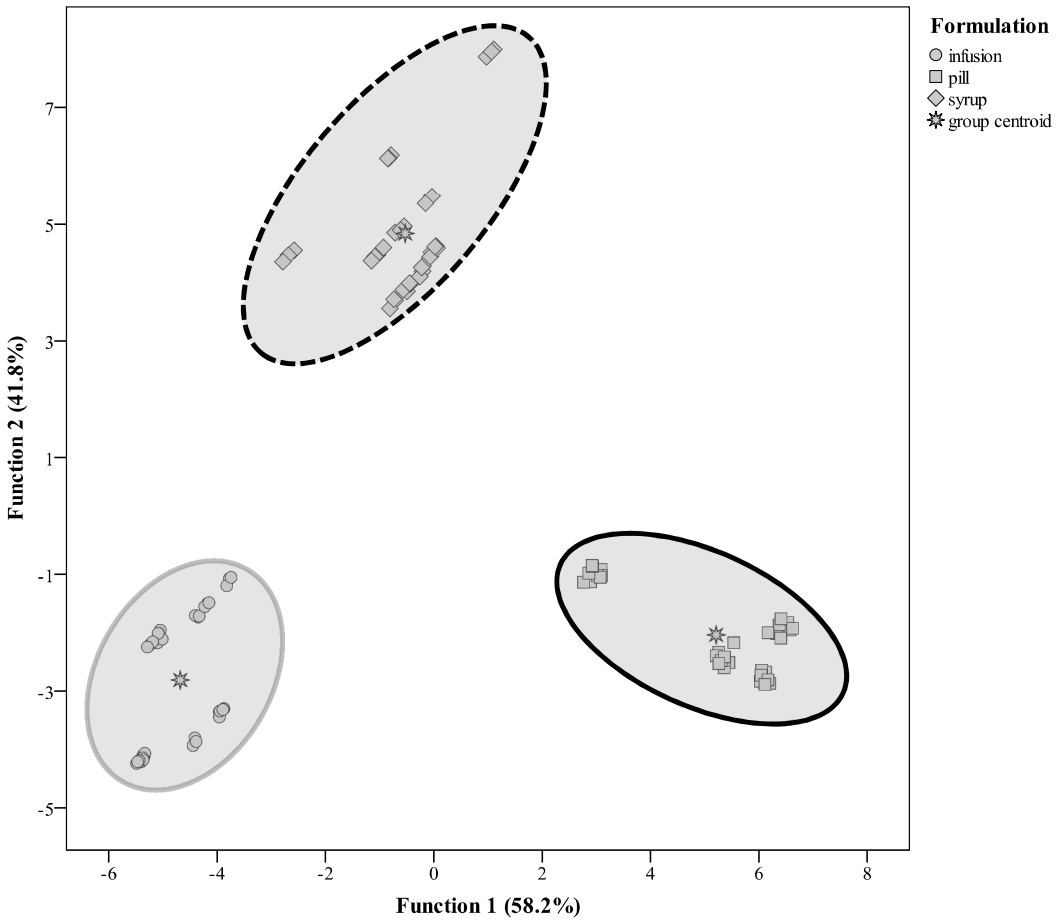


Figure 3. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds content and antioxidant activity results according with formulation.

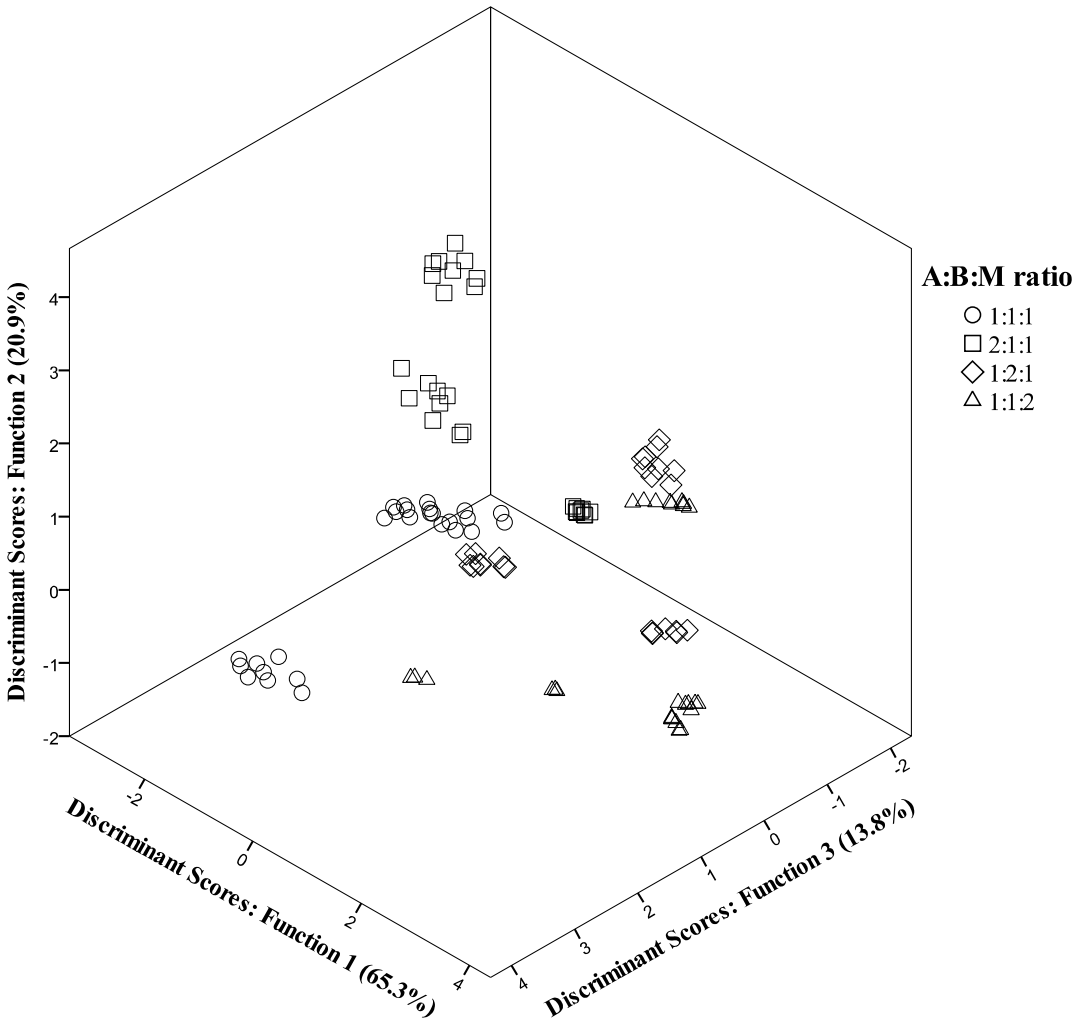


Figure 4. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds content and antioxidant activity results according with artichoke:borututu:milk thistle ratio.