Antioxidant activity and phenolic content of water extracts of *Borago officinalis* L.: influence of plant part and cooking procedures.

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ABSTRACT

Borage (*Borago officinalis* L.*)* water extracts were prepared from raw stems and leaves and from cooked (boiled and steamed) stems. Antioxidant activity (AA) was determined by ABTS and DPPH after their respective calibration with Trolox as standard and expressing results of both assays as μg Trolox/g fresh plant. Also total phenolic compounds (TPC) were determined for each extract. Values for DPPH and ABTS in leaf extracts were similar and approximately 3.5 fold the values obtained for stem extracts. The high antioxidant activity of leaf extracts might be attributed to the high amount of phenolic compounds (2.36 mg GAE/g fresh plant for leaves and 0.57 mg GAE/g fresh plant for stems). Boiling significantly decreased antioxidant activity (51-52 % decrease) and total phenolic compounds (67 % decrease). Steaming caused no significant effect on the antioxidant activity values, whereas total phenolic compounds showed approximately half of the decrease found for boiling (35 %). Borage water extracts, and particularly, those of their by-products (leaves) showed great antioxidant activity, that could potentially be used for different applications in food industry.

Keywords: ABTS, boiling, borage, DPPH, steaming.

INTRODUCTION

Borage (*Borago officinalis* L.) is an herbaceous plant that belongs to the *Boraginaceae* family, consumed in many Mediterranean countries, and considered in some of them a highly appreciated luxury product because of its characteristic taste. The edible part of the plant are the stems, which are cooked prior to consumption, whereas the leaves are discarded. Borage seeds are traditionally known by its oil, that has demonstrated certain beneficial effects associated to the high content of γ–linolenic acid (KHAN and SHAHIDI, 2000). However, the non-lipidic fraction of borage has received less attention and a little information is available about it. Defatted borage seeds were subjected to ethanol extractions to evaluate the presence of antioxidant phenolic compounds, which were further evaluated in a meat model system (WETTASINGHE and SHAHIDI, 1999). BANDONIENE and MURKOVIC (2002) analyzed the presence of radical scavenging compounds in crude methanolic extracts of borage leaves and observed a high radical quenching ability due to a great extent to the presence of rosmarinic acid, among other phenolic compounds.

Antioxidant compounds provide protection against harmful free radicals and have been strongly associated with reduced risk of chronic diseases, such as cardiovascular disease, cancer, diabetes, Alzheimer´s disease, cataracts and age related functional decline, in addition to other health benefits (GUNDGAARD *et al.*, 2003). Vegetable waste materials have been successfully explored as sources of natural antioxidants because, like other desired compounds, are at higher concentrations in the residuals (SCHEIBER *et al.*, 2001; OKONOGI *et al*., 2007). In relation to borage leaves, a previous work (BANDONIENE *et al*., 2002) demonstrated for the first time a strong antioxidant activity of their crude acetone extracts.

The information on antioxidant components, antioxidant activity and their changes during cooking is still limited (ZHANG and HAMAUZU, 2004). In general it is known that thermal processing leads to losses in the bioactive compounds of vegetable products due to their unstability to heat. However, when water is used as the heat transfer medium, different and sometimes contradictory effects have been seen on antioxidant activity of vegetables. Nevertheless, it is very important to find the best way to preserve the contents of bioactive compounds and the antioxidant activities of processed vegetables (GORINSTEIN *et al.*, 2009).

The total antioxidant capacity of a given food is not the sum of each single compound, but may result from the integrated and synergistic action of different compounds (DANESI and BORDONI, 2008). No single assay can be considered a "total antioxidant capacity assay" even though it could be performed both in an aqueous solution and in a lipophilic environment (PRIOR *et al*., 2005). Different methods have been used to perform DPPH or ABTS assays in vegetable products, which make the comparison of results among different papers difficult. Furthermore, different ways of expressing concentrations complicate the comparison of results (dry or fresh weight, molar or mass units, etc). For DPPH, results can be shown as the remaining DPPH, (PRIOR *et al*., 2005) % Inhibition (PESCHEL *et al.*, 2006), IC_{50} (concentration that causes a decrease in the initial DPPH concentration by 50%), (OKONOGI *et al*., 2007) or as the antiradical efficiency $AE = 1/(IC_{50}*T_{IC})$ (SANCHEZ-MORENO *et al*., 1998). ABTS results are usually expressed as Trolox equivalents (either μM or μg) per amount of sample, (RIVERO-PEREZ *et al*., 2007) using either absorbance values at a certain time or also measuring the area under the curve resulting from the absorbance decrease during that time (PEREZ-JIMENEZ and SAURA-CALIXTO 2006; RE *et al*., 1999). Trolox, a water soluble analogue of vitamin E, has been the choice as a reference

standard in most cases due to its effectiveness in both lipophilic and hydrophilic systems (NENADIS *et al*., 2007). A more objective comparison of results could be possible by applying the same interpretation procedure with the same common standard and unified standardization procedure (STRATIL *et al*., 2006).

The objective of this paper was to evaluate the antioxidant activity and total phenolic content of different parts of borage (stems and leaves) and to study the modification induced by different cooking treatments in the edible part of this plant. Furthermore, a standardization procedure for expressing these results has been attempted.

MATERIAL AND METHODS

Reactives

ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt), DPPH (2,2-Diphenyl-1-picryl-hydrazyl) and Folin-Ciocalteu‘s reagent, were purchased from SIGMA-ALDRICH Chemie GmbH (Steinheim, Germany), Trolox (6-hydroxy-2,5,7,8 tetramethylchromon-2-carboxylic acid) was purchased from SIGMA-ALDRICH Chemie GmbH (Steinheim, Germany), Gallic acid (Gallic acid 1-hydrate) and methanol from PANREAC Quimica SAU (Barcelona, Spain) and ethanol from OPPAC, S.A. (Noain, Spain).

Vegetable material and sample preparation

5 kg of Borage (*Borago officinalis* L.) were purchased fresh from a local supermarket. Plants were separated into leaves and stems and they were cleaned, washed, and comminuted separately. Moisture content of raw samples was measured according to Official Method AOAC. Water extraction procedure was carried out as follows: 100 g of raw leaves were weighed and added to 100 ml of distilled water,

preheated at 96ºC. The mixture was subjected to sonication during 30 minutes at room temperature, and filtered with a metallic mesh. The extraction process was repeated with another 100 ml of distilled water, and both extracts were joined and adjusted with distilled water to a final volume of 250 ml. The same procedure was applied for obtaining the raw stem extracts, using Whatman 3 as the filter system instead of the metallic mesh.

Two different cooking processes were applied to borage stems. a) 100 g of fresh cut stems were boiled with 1000 ml of distilled water for 30 minutes at 96ºC, to simulate domestic processing. b) 100 g of fresh stems were submitted to steam cooking procedure with a conventional steam pot using 1000 ml of distilled water for 30 minutes at 96ºC. The cooked samples were subsequently subjected to an extraction procedure using the same method explained for raw stem samples.

Each extract was diluted with distilled water to obtain different solutions within a range of concentrations from 0.0009 to 0.4 g of fresh plant/ml, which were subsequently used for the evaluation of the antioxidant activity and the total phenolic content. All extractions were performed in duplicate.

Determination of Total phenolic content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method (SINGLETON and ROSSI, 1965). Dilutions of borage extracts ranging from 0.004 to 0.4 g/ml were chosen in order to obtain readings within the standard calibration curve made from dilutions between 0.005 and 2 mg of gallic acid. The reaction mixture was composed of 0.1 ml of suitably diluted sample, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu´s reagent, and 1.5 ml of 20% sodium carbonate anhydrous solution (added 2 minutes after the Folin-Ciocalteu´s reagent).

After the initial mixing the tubes were allowed to stand at room temperature for 2 hours in the dark. The optical density of the blue-colored resulting solution was measured at 765 nm using a Lambda 5-UV-VIS spectrophotometer (Perkin Elmer, Paris, France). The total phenolic content was expressed as mg of gallic acid equivalents (mg GAE)/g fresh plant, using the corresponding calibration curve and taking into account the concentration of the diluted extracts. Absorbance measurements were made in duplicate for each diluted solution.

DPPH method

The DPPH assay was performed according to the method of BLOIS (1958) with some modifications. Briefly, a DPPH solution of approximately 20 mg/ml was prepared in methanol and subsequently diluted to obtain an absorbance of 0.8 at 516 nm (working solution). 2 ml of diluted water extracts of borage of different concentrations (0.0009 g/ml - 0.2 g/ml) were allowed to react with 2 ml of DPPH working solution during 30 minutes in the dark, at room temperature. A control sample was prepared with 2 ml of methanol. The final absorbance of the reaction mixture was measured at 516 nm Lambda 5 UV-VIS Spectrophotometer (Perkin Elmer, Paris, France). The radical scavenging capacity of each dilution was calculated as percent of inhibition (% I), calculated according to the formula:

$$\% I = (Abs_{control} - Abs_{sample})/Abs_{control} * 100$$

Where $Abs_{control}$ was the absorbance of the control after 30 minutes of reaction and Abs_{sample} was the absorbance of the sample after 30 minutes of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1-200 µg/ml) was used for calculating the antioxidant capacity in µg

Trolox/ml. Results were finally expressed as µg Trolox/g fresh plant. Absorbance measurements were made in duplicate for each diluted solution.

ABTS method

For ABTS assay, the procedure described by RE *et al*. (1999) with some modifications was used. Briefly, the $ABTS^{\cdot+}$ chromogenic radical was generated by a chemical reaction mixing an aqueous solution of ABTS with $K_2S_2O_4$ (140 mM) to reach a 7 mM final concentration of ABTS. The mixture was kept in the dark for 12-16 hours at room temperature (stock solution). Before use, 1 ml of $ABTS^{\cdot+}$ stock solution was diluted with ethanol 50 % to an absorbance of 0.70 (+/-0.02) at 741 nm (working solution). 3 ml of $ABTS^{\cdot+}$ working solution was allowed to react with 300 µl of suitably diluted water borage extracts (0.0009 g/ml - 0.2 g/ml) or control (ethanol-50%) during 6 minutes, and absorbance was measured at 741 nm (Lambda 5 UV-VIS Spectrophotometer, Perkin Elmer, Paris, France). The decrease in absorbance was recorded as percent of inhibition (% I) and was calculated according to the formula:

$$\% I = (Abs_{control} - Abs_{sample})/Abs_{control} * 100$$

Where $Abs_{control}$ was the absorbance of the control after 6 minutes of reaction and Abs_{sample} was the absorbance of the sample after 6 minutes of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1-60 µg/ml) was used for calculating the antioxidant capacity. Results were finally expressed as µg Trolox/g fresh plant. Absorbance measurements were made in duplicate for each diluted solution.

Statistical analysis

Differences between raw, boiled and steamed stems were evaluated by One way ANOVA and Tukey b Post hoc test was applied when appropriate. A Student t test was

used ($p>0.05$) to evaluate differences between raw samples (leaves and stems). A Pearson correlation test was performed to study the correlation between phenolic compounds and both ABTS and DPPH. Also the correlation between ABTS and DPPH was evaluated (SPSS 15.0, Inc., Chicago, IL, U.S.A.).

RESULTS and DISCUSSION

Methodology (ABTS versus DPPH)

Calibration curves were built for both ABTS and DPPH assays using Trolox as the reference compound (Figure 1 a, b) with the objective of further expressing results of antioxidant activity of samples using the same units for both methods. Within the range of concentrations of Trolox that nearly reached a 100% of % I, ABTS fitted to a linear model (R^2=0.999), whereas DPPH better fitted to an exponential model (R^2=0.994).

Increasing concentrations of different diluted borage extracts (g fresh plant/ml) were subjected to both assays, plotting the results of % I. vs. concentration (Figures 2 and 3). As occurred with Trolox, ABTS fitted to a linear model and DPPH to an exponential model. These % I were transformed into their corresponding μg Trolox/ml after the application of the requisite calibration equations (shown in Figure 1 a, b). Subsequently, for each diluted extract and taking into account the concentration (g fresh plant/ml) the antioxidant activity was given as μg Trolox/g fresh plant. After a careful revision of these data, only values resulting from percentages of inhibition between 20% and 80% were considered representative of each extract and were chosen for calculation of the mean final value expressed in μg Trolox/g fresh plant. RE *et al*. (1999) also selected this range for ABTS evaluation of different antioxidant solutions. At the concentrations tested in this study, ABTS results in general did not reach 80% and for

DPPH results, concentration of samples that resulted in % I higher than 80% gave no reproducible results and were consequently not taken into account for further calculation of the mean antioxidant activity of the borage extracts. To our knowledge, this is the first time that this approach has been used in order to obtain the antioxidant activity value of a vegetable extract. Most papers in the literature only evaluate one or two concentrations of extracts in order to make comparisons with fixed amounts of standard compounds.

Applying these methodologies, the analysis of different borage extracts revealed similar values of μg Trolox/g fresh plant for ABTS and DPPH ($p>0.05$), showing low coefficients of variation in both cases (Table 1). In wines, results for both tests, expressed in mM Trolox, gave higher values for ABTS than for DPPH, which was attributed to the capacity of ABTS to measure lipophilic and hydrophilic activity, whereas DPPH seems to be more specific for lipophilic antioxidants (RIVERO-PEREZ *et al*., 2007). However, THAIPONG *et al*. (2006) found comparable data for ABTS and DPPH in guava methanol extracts, pointing also to the fact that the solvent used for extractions seems to play a critical role in the viability of comparisons between tests, as reported by VENSKUTONIS *et al*. (2007) as well. STRATIL *et al*. (2006) obtained nearly the same results for TEAC and DPPH and about 50% higher values for FRAP method when testing antioxidant activity of fruits and cereals. In that paper, significant correlations between total phenolic compounds and total antioxidant activity of vegetables measured by different methods were found. In our study, although some discrepancies were found in the decrease of TPC and AA as a consequence of steaming application, high correlations were found between each one of the AA parameters and TPC (R^2=0.971 for ABTS and TPC; R^2=0.979 for DPPH and TPC). Also good correlation values were obtained between DPPH and ABTS results (R^2=0.985).

Antioxidant activity: leaves and stems

As shown in Table 1, raw borage leaf extracts showed significantly higher antioxidant activity than raw stem extracts ($p<0.05$). Values for both DPPH and ABTS in leaf extracts were 1236.71 μg Trolox/g fresh plant and 1128.38 μg Trolox/g fresh plant respectively, approximately 3.5 fold the values obtained for stem extracts (342.34 μg Trolox/g fresh plant and 324.53 μg Trolox/g fresh plant). These results suggest that, as borage leaves are not used for human consumption and thus are usually discarded before cooking, they might potentially be a by product used as a good source of bioactive compounds. BANDONIENE *et al.* (2002) showed that a borage leaves acetone extract was efficient to reduce the oxidation rate of rapeseed oil at 80 ºC in terms of peroxides. PYO *et al*. (2004) also found significant higher antioxidant activity in leaves of Swiss chard than in their stems, although in that case both parts of the plant are consumed. Other vegetables have also shown significant differences in the antioxidant activity and the levels of phenolics between leaves and stems or other parts of the plants (PESCHEL *et al*., 2006).

The high antioxidant activity of leaf extracts found in this paper might be attributed to the high amount of phenolic compounds (2.36 mg GAE/g fresh plant for leaves and 0.57 mg GAE/g fresh plant for stems) (Figure 4). Although an overestimation of total phenolic compounds has been described when they are analyzed by the Folin-Ciocalteu method due to the interference with some reducing components like ascorbic acid and sugars (PADDA and PICHA, 2007) a linear correlation between radical scavenging activity and polyphenolic concentration has been found in different vegetables and fruits (KAHKONEN *et al*., 1999; MEZADRI *et al*., 2008). This correlation was also found in this paper, specially in raw extracts, so it could be concluded that the antioxidant activity of each extract might be mostly related to their

concentration of phenolic hydroxyl group, as also reported by PYO *et al*. (2004). TURKMEN *et al*. (2005) found total phenolic content of different fresh vegetables (pepper, squash, green beans, peas, leek broccoli and spinach) ranged from 183.2 to 1344.7 mg GAE/100 g on dry weight basis, so 3 fold lower levels than those found for borage extracts in this work (602 mg GAE/100 g dry weight for stems and 4262 mg GAE/100 g dry weight for leaves).

A great number of studies show phenolic content of different vegetables in mg GAE/g or 100 g (WU *et al*., 2004; ZHANG and HAMAUZU, 2004; PATTAHAMAKANOKPORN *et al*., 2008). In these studies different mixtures of solvents were used, being acetone/water/acetic acid the one who showed the highest values for fresh broccoli (337 mg GAE/100 g fresh plant) (PYO *et al*., 2004). The rest of results in those papers did not reach 236 mg GAE/100 g fresh plant, result obtained for water leaves extracts of borage in this work.

LLORACH *et al.* (2003) evaluated water extracts of cauliflower by-products as a source of antioxidant phenolics obtaining 1.8 g total phenolic compounds per kg of cauliflower by-product. Water extracts show similar total phenolic content than other conventional solvent extracts used with by-products (PESCHEL *et al*., 1999), with the advantages of an easier manipulation, a lower cost and a safer extraction process. PESCHEL *et al.* (1999) found that the total phenolic content of extracts from different vegetables obtained with organic solvents were: 48.6, 251.4 and 514.2 mg GAE/g dry extract, for apple, golden rod and artichoke, respectively. However, these authors pointed out that more nutritional studies are needed to determine the necessary level of extracts to observe potential beneficial effects.

Cooking effect

Clear differences in the antioxidant activity and phenolic compounds were observed depending on the type of thermal process applied to borage stems. When stems were boiled, both ABTS and DPPH showed a significant decrease of around 51-52% (Table 1), and total phenolic compounds decreased about 67% (Figure 4). It could be concluded that boiling caused an important reduction in the potential health benefits associated with the antioxidant compounds present in fresh borage. Probably the water used in this type of cooking was enriched with, at least, part of these compounds by dilution effect. This aspect was confirmed by GLISZCZYNSKA-SWIGLO *et al*. (2006) in a similar work carried out with broccoli. These authors also found that cooking in water significantly decreased most of the health promoting compounds of broccoli and they confirmed that the losses of both vitamin C and polyphenols were mainly due to their leaching into the cooking water.

When steam cooking of borage stems was applied, no effect was observed in the antioxidant activity values. Both ABTS and DPPH showed similar results to those obtained for raw stems (Table 1). Total phenolic compounds showed a decrease with this treatment, but it was only around 35 %, approximately half of the decrease found for boiling (67 %) (Figure 4). This milder heating treatment, with no leaching-effect, preserved a higher antioxidant value and phenolic content on borage edible portions than traditional boiling. Nevertheless, in both cooking methods, the relative loss of phenolic compounds in the treated samples compared to the fresh ones, was more evident than that observed for the antioxidant activity. TURKMEN *et al*. (2005) also found a different behavior for phenolic compounds and antioxidant activity depending on the vegetables analyzed and cooking treatments applied. Some authors suggest the possibility of formation of novel antioxidant compounds (e.g. Maillard reaction products) during heat treatment (NICOLI *et al*., 1999).

The available bibliography shows that the effect of thermal treatment on the potential antioxidant activity of vegetables is different depending on several factors such as vegetable species, intensity and modality of the treatment. CHOI et al. (2006) analyzing the influence of heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake mushroom extracts found that they increased as heating temperature and time increased. These results were in agreement to those found by DEWANTO *et al.* (2002), who showed an enhanced nutritional value of tomatoes submitted to thermal processing as a consequence of an increase of the bio-accessible lycopene content and total antioxidant activity.

On the contrary, some works developed with broccoli and cauliflower showed detrimental effect of heating treatments (blanching and cooking) in polyphenols and antioxidant activity in comparison with the raw products (GEBCZYNSKI and KMIECIK, 2007). ZHANG and HAMAUZU (2004) also found that antioxidant components (including phenolic compounds) and antioxidant activity in broccoli were heavily lost during conventional and microwave cooking. RACCHI *et al*. (2002) found different effects of boiling on antiradical activity of water soluble components depending on the type of vegetables. ROY *et al*. (2007) testing the effect of thermal treatment on the water-soluble fraction of different vegetables concluded that normal cooking temperatures (75-100 ºC, 10-30 min) detrimentally affected phenolic content as well as antiradical and antiproliferative activities of juice from most of the vegetables tested, however mild heating (50 ºC, 10-30 min) preserved 80-100 % of phenolic content.

All these findings confirm that more research is needed to determine the effects of cooking on total phenolics, as stated by RICKMAN *et al*. (2007) in a recent review of the nutritional value of different fresh and processed fruits and vegetables.

In summary, borage water extracts, and particularly, those of their by-products (leaves) showed great antioxidant activity, that could potentially be used for different applications in the food industry. Steam cooking of borage resulted in a better technology compared to traditional boiling in order to preserve the antioxidant activity and total phenolic content of this vegetable.

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Table 1. Antioxidant activity measured by ABTS and DPPH.

		Antioxidant activity		
		ABTS (µgT/g fresh plant)	**DPPH (µgT/g fresh plant)**	**Student t test**
Raw	**Leaves**	1128.38±52.23^{A} (4.63)	1236.71±117.19^{A} (9.47)	ns
	Stems	324.53±27.97^{Bb} (8.62)	342.34±29.51^{Bb} (8.62)	ns
Cooked stems	**Boiled**	158.21±7.72^{a} (4.88)	164.60±14.81^{a} (8.99)	ns
	Steamed	368.19±30.04^{b} (8.16)	341.19±36.96^{b} (10.83)	ns

Results are expressed as Means ± Standard deviation (Coefficient of Variation).

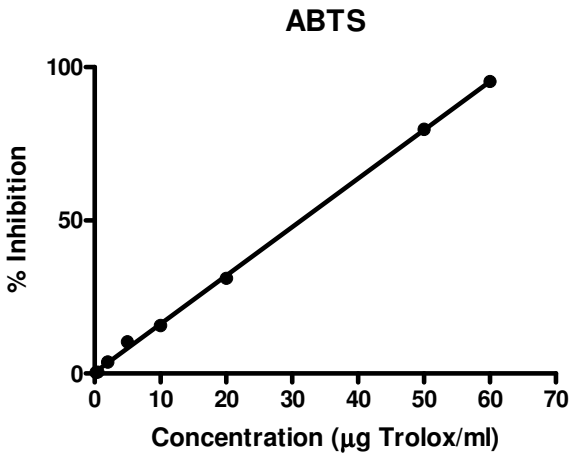
^{AB} In the same column for raw samples denote significant differences between leaves and stems (student t test) ($p<0.05$)

^{ab} In the same column for stems denote significant differences (1 way ANOVA), among raw, boiled and steamed samples.

ns: not significant ($p>0.05$) differences between DPPH and ABTS values.

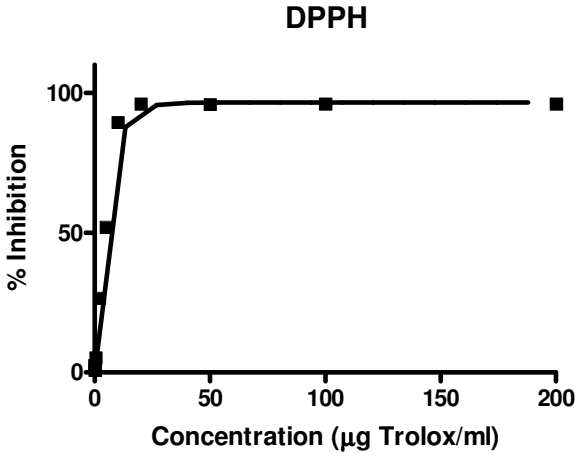
Figure 1. Calibration curves for ABTS and DPPH assays using Trolox as the reference compound.

a)



$Y=1.581X + 0.4287$ $R^2=0.999$

b)



$Y=96.47*(1-e^{(-0.1745*X)})$ $R^2=0.994$

Figure 2. ABTS % Inhibition vs Extract concentration (g fresh plant/ml)

ABTS

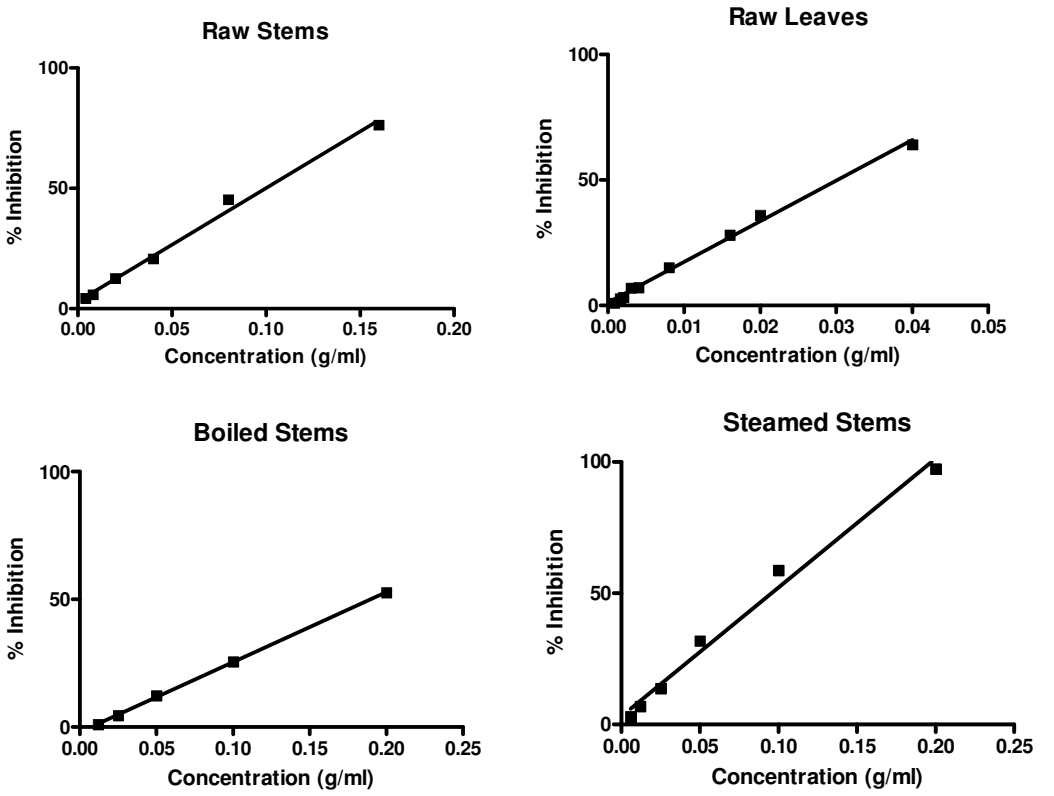


Figure 3. DPPH % Inhibition vs Extract concentration (g fresh plant/ml)

DPPH

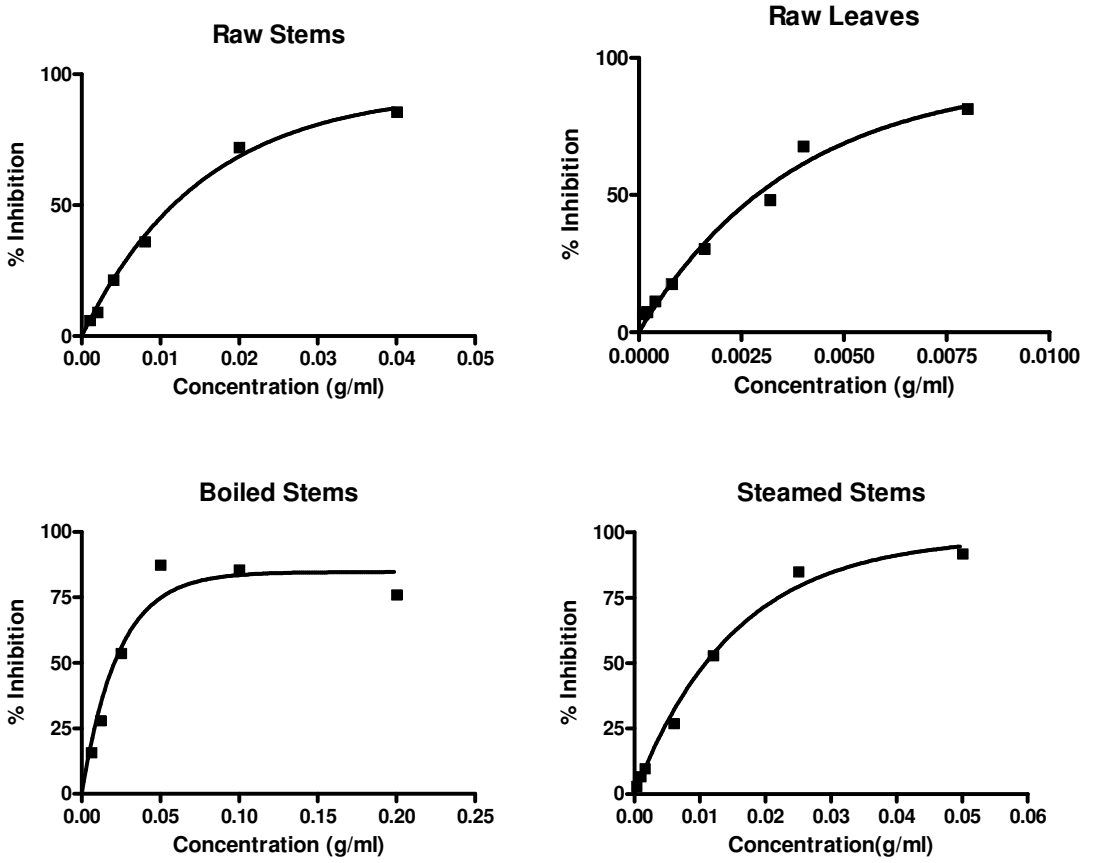
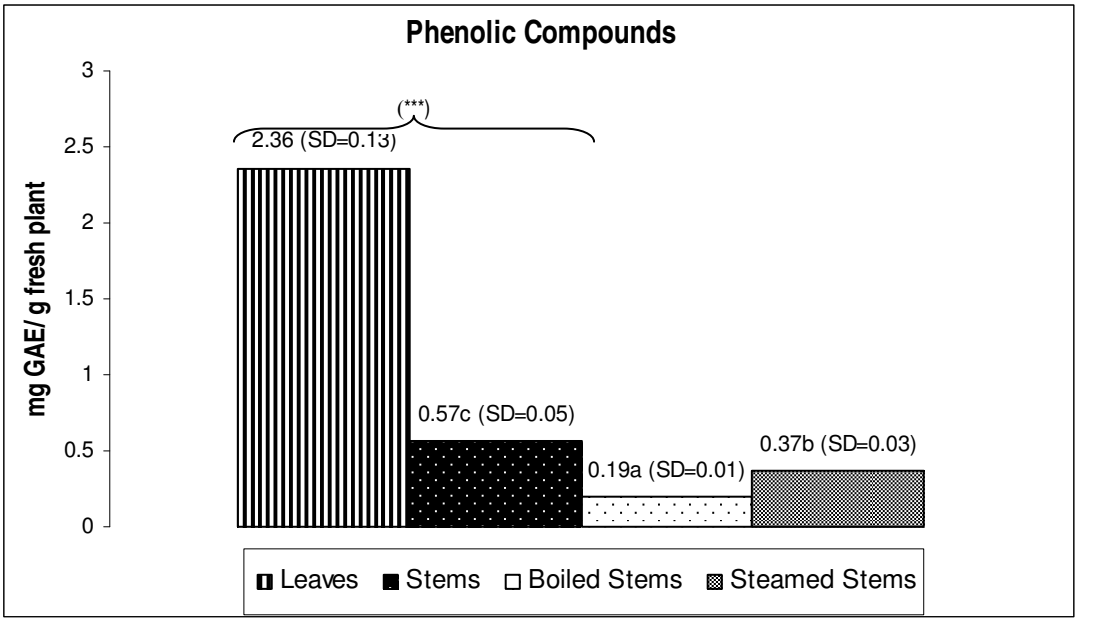


Figure 4. Phenolic compounds (mg GAE/g fresh plant)



Different small letters denote significant differences (one way ANOVA) among raw, boiled and steamed stems.

***Student t test ($p<0.01$): significant differences between leaves and stems