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Comparative Chemical and Biochemical Analysis of Extracts of Hibiscus sabdariffa

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ABSTRACT

Hibiscus sabdariffa extracts have attracted attention because of potentially useful bioactivity. However, there have been no systematic studies of extraction efficiencies of H. sabdariffa. The nature of extracts used in different studies has varied considerably, making comparisons difficult. Therefore, a systematic study of extracts of H. sabdariffa made with different solvents was carried out using water, methanol, ethyl acetate and hexane in the presence/absence of formic acid, using different extraction times and temperatures. The extracts were analysed for total polyphenol content, antioxidant capacity using DPPH, FRAP and TEAC assays, and specific anthocyanins were determined using HPLC and LC-MS. The results showed the highest antioxidant capacities were obtained by extracting using water, with or without formic acid, for 10 min at 100 °C. These extracts provided the highest concentrations of cyanidin 3-sambubioside and delphinidin 3-sambubioside. It will be important to use extraction conditions giving optimal extraction efficiencies for subsequent bioactivity experiments.

KEYWORDS: Hibiscus sabdariffa; antioxidant capacity; TEAC; DPPH; FRAP; total polyphenols; solvent extraction; anthocyanins; cyanidin 3-sambubioside; delphinidin 3-sambubioside.

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1 INTRODUCTION

The current interest in natural antioxidants from plant sources has become overwhelming, particularly in bioactive antioxidants such as polyphenols and flavonoids. Anthocyanins are a subgroup of flavonoids appearing in plants mostly in the glycoside form as anthocyanidins.

Hibiscus sabdariffa is from the Malvaceae family, described as an annual, bushy plant with a height of up to 2.5 m, characterised by smooth, cylindrical red stems, reddish veins and long, green leaves (Mohamed, Fernandez, Pineda, & Aguilar, 2007). The flowers are borne singly, having different colours yellow or buff with a rose or maroon eye and turn pink during maturation. The calyx is red, consisting of 5 valves, each containing 3 to 4 kidney-shaped light-brown seeds. H. sabdariffa flowers are a rich source of the anthocyanins responsible for red colour (Mohamed et al., 2007).

H. sabdariffa flowers are used to make teas commonly known by many names around the world, such as hibiscus tea, zobo, roselle, red sorrel, sour tea, agua de Jamaica and karkade, (Ali, Al Wabel, & Blunden, 2005; McKay, Chen, Saltzman, & Blumberg, 2010) being particularly very popular in regions such as West Africa and South Asia. Hibiscus flowers have a sour flavour and are commercially available in the form of jams and juices in an increasing number of countries (Ali et al., 2005).

Many different anthocyanins have been reported in tissues of H. sabdariffa such as cyanidin 3-rutinoside, (Maganha, Halmenschlager, Rosa, Henriques, Ramos, & Saffi, 2010) delphinidin 3-sambubioside (Gradinaru, Biliaderis, Kallithraka, Kefalas, & Garcia-Viguera, 2003) cyanidin 3-sambubioside (Ali et al., 2005; Gradinaru et al., 2003; Maganha et al., 2010), cyanidin 3-glucoside and delphinidin 3-glucoside (Ali et al., 2005; Gradinaru et al., 2003). Ali et al. (2005) and Gradinaru et al. (2003) found that aqueous extracts of dried calyces of H. sabdariffa have two predominant anthocyanin compounds (delphinidin 3-sambubioside and cyanidin 3-sambubioside) and two minor compounds (cyanidin 3-

glucoside and delphinidin 3-glucoside). Maganha et al. (2010) reported that delphinidin, delphinidin 3-glucoxyloside, delphinidin 3-monoglucoside and cyanidin 3,5-diglucoside were the major anthocyanins in H. sabdariffa. Figure 1 shows the structure of some of these H. sabdariffa anthocyanins.

H. sabdariffa has been used in folk medicine as a treatment for several diseases (Ali et al., 2005) More recently, it has been reported that H. sabdariffa extracts have bioactive properties that may play a crucial role in preventing chronic diseases such as hypertension (Ali et al., 2005; Maganha et al., 2010; McKay et al., 2010) and in reducing hepatic disease (Ali et al., 2005; Prenesti, Berto, Daniele, & Toso, 2007), cardiovascular disease (Chen et al., 2004; Prenesti et al., 2007), atherosclerosis (Chen et al., 2004) and diabetes (Agoreyo, Agoreyo, & Onuorah, 2008). Extracts have been reported to reduce high cholesterol (Hainida, Ismail, Hashim, Mohd-Esa, & Zakiah, 2008) and may work as anticancer, antimutagenic and antiproliferative agents stimulating the immune system in vivo (Liu et al., 2010). It has also been reported that extracts of H. sabdariffa have antimicrobial properties (Maganha et al., 2010) and increased reproductive function (Amin, Hamza, Kambal, & Daoud, 2008). Maganha et al. (2010) have argued that H. sabdariffa can be utilized to treat other diseases, such as kidney disease and bladder stones. H. sabdariffa has shown potent antioxidant properties in vitro. Several researchers have examined the antioxidant capacity of H. sabdariffa extracts using different solvents to extract and different assays for analysis, but none have carried out a comparative study of extraction or analytical protocols. At present, it is difficult to compare different studies because of the different doses and different extraction protocols used. Furthermore, a wide range of extraction conditions have been used **(Table 1)**.

Each of these studies reported different results for in vitro antioxidant capacity and reported different levels of bioactivity in vivo. Ramirez-Rodrigues, Plaza, Azeredo, Balaban & Marshall (2011) commented on the difficulty of comparing studies due to the variations in

extraction conditions. Therefore, the aim of the present study was to investigate the optimal extraction conditions for different chemical and biochemical analyses.

2 MATERIALS AND METHODS

2.1 Plant material.

Sun-dried flowers of H. sabdariffa were obtained from a local market in Jeddah, Saudi Arabia. The flowers of H. sabdariffa were from the Sudan region. One batch was used for all extractions.

2.2 Chemicals.

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate, phosphate buffered saline solution, gallic acid, Folin Ciocalteu phenol reagent, glacial acetic acid, formic acid, sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) were supplied by Sigma Aldrich Chemical Company Ltd. (Poole, UK). 2, 2-Diphenyl-1-picrylhydrazyl free radical (DPPH), ferric chloride hexahydrate, hydrochloric acid and sodium acetate trihydrate were from Merck Chemicals Ltd. (Darmstadt, Germany). Methanol, ethyl acetate and hexane were obtained from Fisher Scientific Ltd. (Loughborough, UK). All solvents were of analytical grade. Sodium dihydrogen phosphate anhydrous was supplied from BDH Laboratory Supplies (Poole, UK). Cyanidin 3-glucoside, cyanidin 3-sambubioside, delphinidin 3-glucoside and delphinidin 3-sambubioside and cyanidin 3,5-diglucoside were purchased from PhytoLab GmbH & Co. KG (Dutendorfer, Germany).

2.3 Extraction procedures.

Dried flowers of H. sabdariffa were ground to a fine powder using a domestic blender (Kenwood Chef Classic, model KM 330). The dried H. sabdariffa powder (0.1 g) was extracted using solvent (water, methanol, ethyl acetate or hexane; 10 ml) with and without the

addition of 1% (v/v) formic acid solution. The extraction mixtures were each heated at 25 °C, 50 °C or the solvent boiling point for either 3, 5 or 10 min. The final extract was obtained by filtering through a Whatman No. 1 filter paper. Freshly prepared extracts were used each time for analysis without further treatment or storage.

2.4 Determination of total polyphenol content.

Total polyphenol content was evaluated using the Folin assay according to Packer (1999) This method is based on an oxidation-reduction reaction using the ability of the extract to reduce the tungstate-molebdate mixture. The results are expressed as l mg gallic acid equivalents per gram of H. sabdariffa on a dry weight basis.

2.5 Determination of antioxidant activity.

The antioxidant free radical scavenging activity of the H. sabdariffa extracts were measured using the (DPPH) assay described by Aoshima, Hirata, and Ayabe (2007) with minor modifications. Instead of dissolving the DPPH in ethanol, methanol was used. The radical-scavenging activity (%) was calculated from the following equation:

% radical scavenging activity = 100× (absorbance of the control - absorbance of the sample)/absorbance of the control.

The ability to reduce ferric ions (FRAP) was measured using a modified version of the method described by Benzie and Strain (1996) and total equivalent antioxidant capacity (TEAC) was measured according to Huang, Ou, and Prior (2005). Both FRAP and TEAC results were expressed as l mg Trolox equivalents per gram of H. sabdariffa on a dry weight basis.

2.6 Determination of total anthocyanins.

Total monomeric anthocyanins were measured using pH differentials following the method described by Wrolstad (2005) Results are expressed as l mg cyanidin 3-glucoside equivalents per gram of H. sabdariffa on a dry weight basis.

2.7 Quantification of anthocyanins by HPLC.

Reversed-phase high performance liquid chromatography (HPLC) was used to quantify anthocyanin compounds in individual extracts using a liquid chromatograph (Shimadzu LC-20AD), with auto sampler (SIL-20AC) and a UV/VIS detector (SPD-20A). A Gemini C18 column (5µm, 250 mm×4.6 mm) was used (Phenomenex, Macclesfield, Cheshire, UK) and the wavelength of the detector was set at 520 nm. The mobile phase was (A) 0.2% (v/v) formic acid in water, (B) acetonitrile. Gradient elution was used starting with 10% (B) which increased to 20% (0-10 min) and then to 70% (10-11 min). The conditions were held at 70% B for 4 min prior to returning to 10% B (15-16 min) with a final isocratic run of 10% B (16-25 min). The flow rate was 0.8 ml/min and the injection volume was 2.5 µl.

2.8 Determination of anthocyanins by LC-MS.

Liquid chromatography–mass spectrometry was used to confirm the identity of anthocyanins in the extracts using LC with triple quad MS (Agilent Technologies 1200 series 6410). The same column, mobile phases and wavelength were used as for HPLC. The starting mobile phase was 10% B which was increased to 50% (0-8 min) and then to 70% (8-15min). The conditions were held at 70% B for 0.30 min prior to returning to the final isocratic run of 10% B (15.30-25 min). The flow rate was 0.3 ml/min and the injection volume was 1 µl. Nitrogen gas temperature was 35 °C, gas flow 11L/min, Nebulizer 60 psi. Full scan mode was used for the accurate determination of the parent ion MS2 mode used to obtain fragmentation data. The most significant ion in the scan was collected in ion trap, fragmented and the spectrum of the fragments recorded. Co-chromatography was done with standards to identify and confirm the presence in the extracts. The standard and the sample extracts were run in positive mode.

2.9 Statistical analysis.

The Pearson correlation coefficient 2-tailed ($\alpha = 0.01$) and analysis of variance (ANOVA) using Tukey's test ($\alpha = 0.05$) were performed to evaluate the significance of differences between extraction time, temperature and solvents, using IBM SPSS statistic software Version 20 (where $P < 0.05$ and $P < 0.01$ shows significance).

3 RESULTS AND DISCUSSION

Despite significant interest in the bioactive properties of H. sabdariffa there has been no systematic study of the characterisation and efficiency of different solvent extraction procedures.

3.1 Time of extraction.

The study examined the effect of extraction time (3, 5 and 10 min) on the total polyphenol content, the amount of antioxidant capacity and the total monomeric anthocyanin content.

The results (data not shown) revealed that time of extraction resulted in no significant differences between extracts analysed by TEAC assay and for the total anthocyanins determination ($P > 0.05$). For total polyphenol content, a significant difference was found between 3 min and 10 min of extraction ($P = 0.026$), but there was no significant difference between 5 min and 10 min of extraction ($P = 0.872$). Extraction for 10 min was the optimum time. For FRAP assay, there was no significant difference between 3 min and 5 min ($P = 0.916$) but there was a significant difference between both 3 min and 10 min and 5 min and 10 min extraction ($P = 0.014$ and $P = 0.004$ respectively). Both 3 and 5 min extraction time gave optimal results. Finally, DPPH showed no significant difference between 5 and 10 min extraction ($P = 0.870$) but did show a significance between both 3 and 5 min and 3 and 10 min extraction ($P < 0.001$ and $P = 0.002$ respectively). Three min extraction time was optimum.

The study by Ramirez-Rodrigues et al. (2011) concluded that increasing extraction time led to a significant increase in total polyphenol content, antioxidant capacity and amounts of anthocyanins. The present study is in partial agreement with this, however, the antioxidant capacity assays show varying results. When considering all assays, we suggest a 10 min extraction time for H. sabdariffa for optimal extraction of bioactive compounds, which provides a safety margin and certainty for all assays.

3.2 Extraction temperature.

Temperature had an effect on all extractions (carried out for 10 min) measuring DPPH, FRAP, TEAC and total polyphenol content as shown in figure 2. Depending on both the solvent used for extraction and the assay, there were variations in significance. For DPPH and FRAP assays the results showed significant differences between extraction at 25 °C and solvent boiling point ($P < 0001$ for both DPPH and FRAP assays) with solvent boiling point being optimal. However, for some solvents and determinations the optimum yield was seen at 50 °C. For total monomeric anthocyanins significant differences ($P = 0.011$, $P < 0.001$ and $P = 0.02$) were found for yields between all the temperatures tested (25 °C, 50 °C and solvent boiling point) with maximum yield at the solvent boiling point. Extraction at solvent boiling point for 10 min was therefore the optimal condition for extracting dried H. sabdariffa. Prenesti et al. (2007) and Ramirez-Rodrigues et al. (2011) also showed an increased yield of polyphenols at a higher temperature coupled with minimal losses of antioxidant compounds.

3.3 Effect of solvent on the total polyphenol content.

Total polyphenol content was measured for each extract (10 min extraction time) as previously described. Figure 2 (I) shows that the water extracts with and without formic acid, gave the highest yields of total polyphenols (21.67 ± 0.93 and 17.94 ± 0.29 mg/g gallic acid equivalent, respectively). In contrast, the ethyl acetate and hexane extracts with, and without

196 formic acid, resulted in the lowest amounts of polyphenol. At 25 °C and boiling point
197 temperatures the differences between the acidified and non-acidified aqueous extracts were
198 not significant (P = 0.790, P = 0.088) but at 50 °C there was a significant difference between
199 them (P = 0.001). Methanol extracts had lower amounts of total polyphenols in comparison
200 with water extracts by approximately 1.3 fold. Ethyl acetate and hexane extracts resulted in
201 approximately 75% less total polyphenols at all temperatures, compared with aqueous
202 extracts. There was a significant difference between water extracts and other solvents (P
203 <0.05) at all conditions.

204 **3.4 Effect of solvent on the DPPH assay.**

205 Figure 2 (II) shows the percentage of radical scavenging activity of different extracts of dried
206 H. sabdariffa for 10 min extraction time. From figure 2 (II), it can be concluded that water
207 extracts with and without formic acid had the highest percentage of radical scavenging
208 activity relative to other solvents (71.98% ± 5.77 and 69.88% ± 0.66, respectively). There
209 was no significant difference between water extracts with or without formic acid at 25 °C
210 and boiling point (P = 0.737, P = 0.990 P >0.05) while there was a significant difference
211 between water extracts with or without formic acid at 50 °C (P < 0.001). In addition, there
212 was a significant difference between the antioxidant capacity of the water extracts and all
213 other solvents when measured by the DPPH assay (P < 0.001). Ramakrishna, Jayaprakasha,
214 Jena & Singh (2008) measured the antioxidant capacity of ethyl acetate, acetone and
215 methanol extracts of both the calyces and fruit of H. sabdariffa using the DPPH assay. The
216 study found that the methanol extract gave the highest DPPH value in comparison with ethyl
217 acetate and acetone. This is in agreement with the findings from this study, as the methanol
218 extract of H. sabdariffa had a higher percentage of DPPH scavenging activity (34.88% ±
219 3.94) than ethyl acetate (3.10% ± 0.94).

3.5 Effect of solvent on the FRAP assay.

Figure 2 (III) shows the FRAP concentration for extracts of dried H. sabdariffa. Acidified and non-acidified aqueous extracts made at boiling point had the highest FRAP values (38.18 ± 0.64 and 39.14 ± 1.69 mg/g trolox equivalents, respectively), whereas acidified and non-acidified hexane and ethyl acetate extracts made at boiling point had the lowest values (0.03 ± 0.37 and 0.15 ± 0.47 mg/g trolox equivalent, respectively). There was no significant difference between water with or without formic acid at 50 °C and boiling point ($P = 0.932$, $P = 0.795$) and methanol extracts with or without formic acid at 25 °C and 50 °C ($P = 0.998$, $P = 0.986$) respectively. FRAP results showed a significant difference between the antioxidant levels of water extract with and without formic acid compared to ethyl acetate and hexane with and without formic acid ($P < 0.001$).

3.6 Effect of solvent on the TEAC assay.

The TEAC antioxidant capacity values of extracts of H. sabdariffa are shown in Figure 2 (IV). There was a significant difference between the TEAC values for aqueous extracts with and without formic acid at 25° C and 50 ° C ($P < 0.001$) and between the water extracts and all other solvents ($P < 0.001$). The TEAC antioxidant capacity of methanol, ethyl acetate and hexane extracts was not significantly different from the values obtained in the presence of formic acid at 50 ° C and boiling point temperatures.

3.7 Correlation of antioxidant assays and total phenol content.

Statistical results from the present study showed that total phenol content had strong correlation with other assays TEAC values ($R = 0.837$), FRAP ($R = 0.565$) and DPPH ($R = 0.787$). In addition, TEAC values had a positive correlation with both DPPH ($R = 0.787$) and with FRAP values ($R = 0.496$). In addition, there was a significant correlation between FRAP and DPPH ($R = 0.513$). However, DPPH, FRAP and TEAC assays do not give the same results due to their different mechanisms (Fukumoto & Mazza, 2000; Pellegrini et al., 2003).

Pearson correlation coefficient indicates there is a significant correlation between total phenol content and antioxidant assay with high positive correlation.

3.8 Effect of solvent on the total monomeric anthocyanin content.

Figure 3 illustrates the effects of extraction on the total monomeric anthocyanin content (expressed as cyanidin 3-glucoside equivalents) for water and methanol extracts with and without formic acid. Water extracts with and without formic acid had the highest amount of anthocyanin (58.60 ± 0.48, 54.08 ± 1.38 mg/g, respectively), compared with methanol extracts There is no significant difference between acidified and non-acidified solvents at 50 °C ($P = 0.586$ for water, $P = 1$ for methanol; $P > 0.05$, respectively). However, there was a significant difference between water with and without formic acid at 25 °C and solvent boiling point ($P < 0.001$) and a significant difference between methanol with and without formic acid at solvent boiling point ($P = 0.033$, $P < 0.05$). Anthocyanins were undetectable in ethyl acetate and hexane extracts due to difficulties in solubility (data not shown). Water extracts yielded higher amounts of anthocyanins compared to methanol extracts ($P < 0.001$). In addition, the results showed correlation between total monomeric anthocyanins and total phenol content ($R = 0.705$), DPPH ($R = 0.718$) TEAC ($R = 0.781$) and FRAP ($R = 0.630$).

3.9 Determination of anthocyanins in extracts by HPLC.

Delphinidin 3-sambubioside, delphinidin 3-glucoside, cyanidin 3-sambubioside and cyanidin 3-glucoside were identified by HPLC (confirmed by LC-MS) in all water and methanol extracts of H. sabdariffa, with and without formic acid. None of the compounds analysed were detected in ethyl acetate or in hexane with or without formic acid**. Figure 4** shows a chromatogram of the anthocyanins detected in an aqueous extract of H. sabdariffa made at 100 °C for 10 min. Delphinidin and cyanidin 3-sambubioside were the major compounds present and delphinidin and cyanidin 3-glucoside were minor compounds present. Ali et al. (2005) reviewed several studies isolating bioactive compounds from several parts of H.

sabdariffa using different types of Hibiscus species, and also found the most predominant compounds from these extracts were delphinidin and cyanidin 3-sambubioside and delphinidin and cyanidin 3-glucoside. **Table 2** shows the concentrations of these four anthocyanins in the different aqueous and methanolic extracts. Results show that water with and without formic acid extracted the highest amount of the individual anthocyanins.

3.10 Comparison of results to others.

Due to inconsistencies in methodology used in previous studies measuring the antioxidant capacity of H. sabdariffa, it is difficult to compare the results of the present study with other work as each study has used a different method for extraction. Wrolstad, Moyer, Hummer, Finn, and Frei (2002) attributed the variation in the results of different studies to differences in extraction methods, temperatures or other factors, which could affect the enzyme activity and oxidation. Additionally, when studying H. sabdariffa, different cultivars, different locations of cultivation, different climatic conditions and condition of storage will all contribute significantly to the variation in results. Light can also influence the amount of antioxidant activity and the total anthocyanin content (Lajolo, Hassimotto, & Genovese, 2005).

3.11 Optimal extraction conditions.

Using the optimal extraction time of 10 min and the optimal extraction temperature of solvent boiling point, it can been seen that water either with or without formic acid is the best solvent for extraction to maximise the total polyphenols, total anthocyanins and antioxidants extracted from H. sabdariffa. Acidified and non-acidified water extracts gave the highest values in all antioxidant capacity assays, which is in concurrence with Fukumoto et al. (2000). However, there was no significant difference between acidified and non-acidified solvents. These findings are in disagreement with Nollet (2000). They reported that adding acid helps anthocyanins to form the flavylium cation and eliminates degradation, which

295 increases the amount of extracted compounds (Nollet, 2000). Ramakrishna et al. (2008)
296 related the strong antioxidant capacity of H. sabdariffa extracts to the total phenol content.
297 They explained the variance between the antioxidant capacities of extracts as due to the
298 hydrogen-donating ability of the compounds in the extract as different solvents extracted
299 different types of phenol compounds. Fukumoto et al. (2000) reported that antioxidant
300 activity of extracts increased when compounds with 2 to 3 hydroxyl groups in the B ring,
301 increased, since the level of antioxidant capacity is dependent on the number of hydroxyl
302 groups in the compound. Any loss of these groups will mean a reduction in the antioxidant
303 capacity. Our HPLC results show the presence of delphinidin 3-glucoside and delphinidin 3-
304 sambubioside, which have 3 hydroxyl groups in the B ring, and cyanidin 3-glucoside and
305 cyanidin 3-sambubioside, which both have 2 hydroxyl groups in the B ring in acidified and
306 non-acidified water and methanol extracts. We did not detect any of these compounds in
307 acidified and non-acidified ethyl acetate or hexane extracts which would explain the low
308 antioxidant values obtained from these extracts. The present report is the only study to have
309 compared different solvents when determining the antioxidant capacity and anthocyanins
310 present in H. sabdariffa. We used different solvents to identify which solvent would give the
311 maximum antioxidant activity. However, it should be noted that different solvents extract
312 different compounds, which is why antioxidant values vary so using one type of solvent for
313 extraction can underestimate total antioxidant activity (Pellegrini et al., 2003) and no single
314 solvent can optimally extract all antioxidants (Yang et al., 2006). Pellegrini et al. (2003)
315 suggested that solvent extracts with low antioxidant activity may contain other types of
316 compounds such as those with bioactive properties and therefore, they recommend using at
317 least two different solvents for extraction. A study by Orhan, Kartal, Abu-Asaker, Senol,
318 Yilmaz, and Sener (2009) found that some solvents were unable to extract flavonoids from
319 edible plants and exhibited low antioxidant activity. In the present study, ethyl acetate and

hexane extracts had a low total phenol content and low or negative antioxidant capacity in comparison with water and methanol extracts and they did not extract any anthocyanins. This could be due to the polarity of these solvents compared with water and methanol. Some of the assays used are hydrophilic, so the ethyl acetate and hexane when mixed with high polarity solvents will be immiscible, which would affect the mechanism of the assay. Mohd-Esa, Hern, Ismail, and Yee (2010) also explains that if the polarity of the solvent increased it would increase the extractable compounds and extraction yields. This theory justifies our results as the polarity of solvents used in our study in descending order were water>methanol>ethyl acetate>hexane. HPLC results found that H. sabdariffa is an important source of anthocyanins, which may play an important role in the prevention of several diseases. The results of the antioxidant capacity in vitro cannot be related to the in vivo results, due to insufficient information about the bioavailability, absorption and metabolism of anthocyanins. Therefore, further study must be done in vivo.

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420

FIGURE CAPTIONS

Figure 1. Chemical structures for some anthocyanins compounds found in dried H. sabdariffa

Figure 2. Total polyphenol content (I) and antioxidant assays (II-IV) of extracts of H. sabdariffa extracted for 10 min. Values with different letters in each chart are significantly different (Tukey's test, $p<0.05$). Results are triplicates (± SD)

Figure 3. Total monomeric anthocyanins content expressed as cyanidin 3-glucoside equivalents (mg/g of H. sabdariffa) for 10 min at solvent boiling point. Values with different letters are significantly different (Tukey's test, $p< 0.05$). Results are triplicates (± SD)

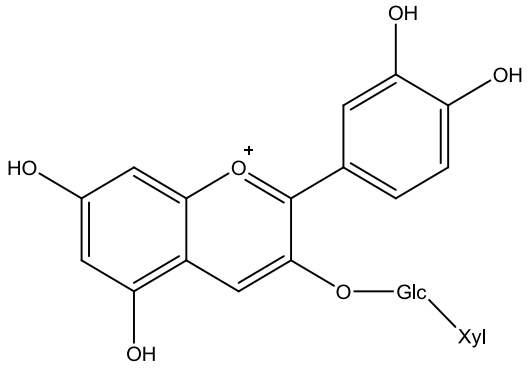
Figure 4: Chromatogram of compounds in an aqueous extract of dried H. sabdariffa (100°C for 10 min). 1: Delphinidin 3-sambubioside (RT=4.5min), 2: Delphinidin 3-glucoside (RT=5.4 min), 3: Cyanidin 3-sambubioside (RT=5.8 min) 4: Cyanidin 3-glucoside (RT=6.8 min).

Table 1. Examples of extraction conditions used in different reported studies on H. sabdariffa (1 g).

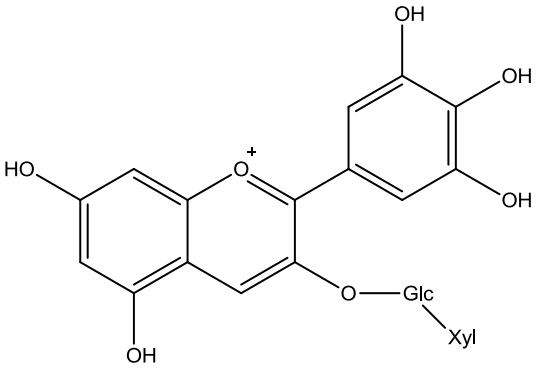
Solvent	Solvent volume (ml)	Time (min)	Temperature (°C)	Study
water	50	60	25	(Wong, et al., 2006)
	100	5	100	(Oboh & Rocha, 2008)
	10	600	25	(Cisse et al., 2009)
Acidified methanol	50	180	64	(Farombi & Fakoya, 2005)
80% methanol	1000	120	25	(Mohd-Esa et al., 2010)
70% ethanol	10	2	Microwave 150 W	(Amin et al, 2008)

Table 2. Concentration of anthocyanins identified by HPLC in extracts of H. sabdariffa at solvent boiling point for 10 min. (n=3).

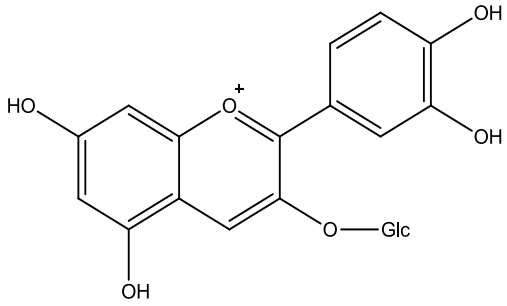
Solvent	Delphinidin 3-sambubioside (mg/g)	Delphinidin 3-glucoside (mg/g)	Cyanidin 3-sambubioside (mg/g)	Cyanidin 3-glucoside (mg/g)
Water	4.11 ± 1.47	0.15 ± 0.01	3.81 ± 1.21	0.46 ± 0.01
Water + Formic Acid	3.687 ± 0.34	0.17 ± 0.04	2.98 ± 0.21	0.52 ± 0.01
Methanol	2.26 ± 0.07	0.19 ± 0.02	1.96 ± 0.03	0.47 ± 0.01
Methanol + Formic Acid	2.41 ± 0.09	0.16 ± 0.02	2.10 ± 0.02	0.47 ± 0.01



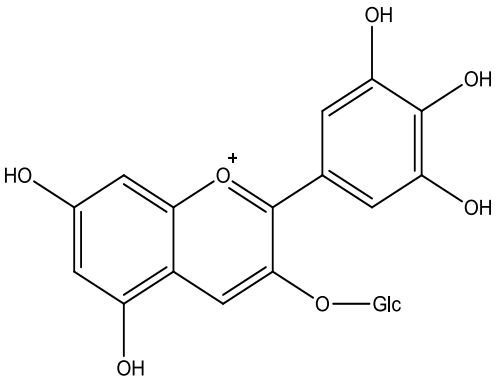
Cyanidin 3-sambubioside



Delphinidin 3-sambubioside



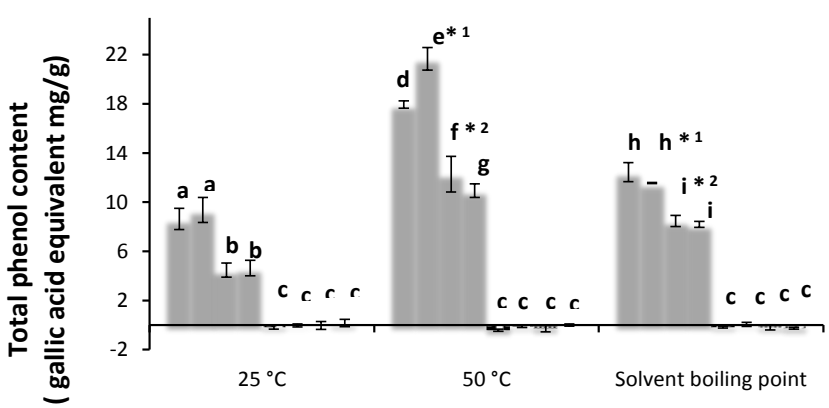
Cyanidin 3-glucoside



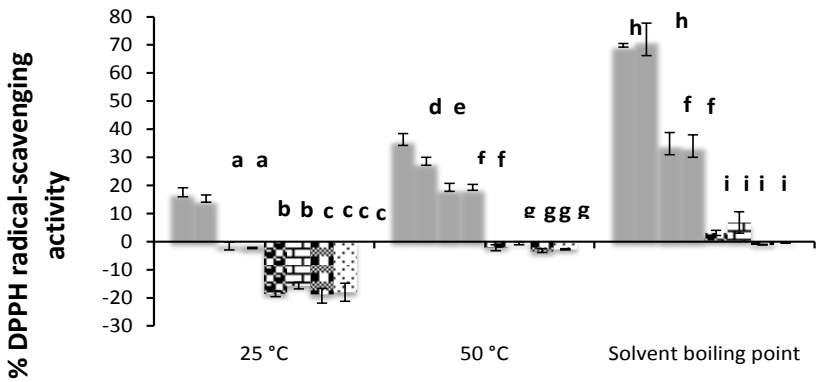
Delphinidin 3-glucoside

Figure 3. Chemical structures for some anthocyanins compounds found in dried H. sabdariffa

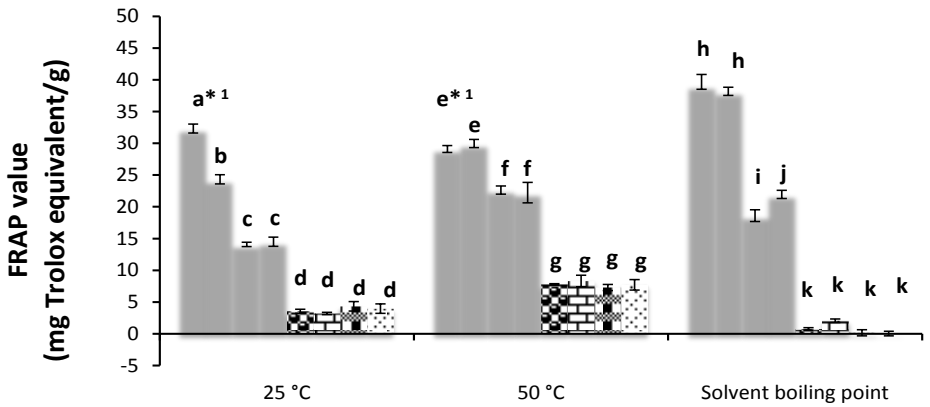
I


Total phenol content
(gallic acid equivalent mg/g)
25 °C
50 °C
Solvent boiling point

II


% DPPH radical-scavenging activity
25 °C
50 °C
Solvent boiling point

III


FRAP value
(mg Trolox equivalent/g)
25 °C
50 °C
Solvent boiling point

IV

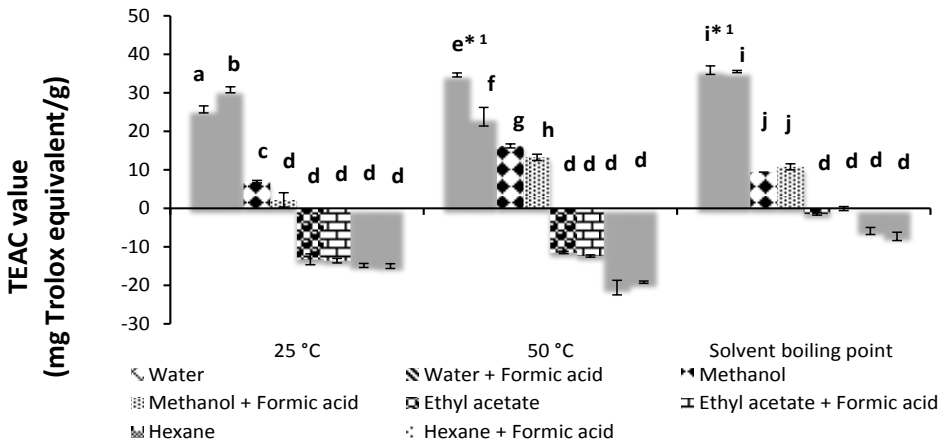
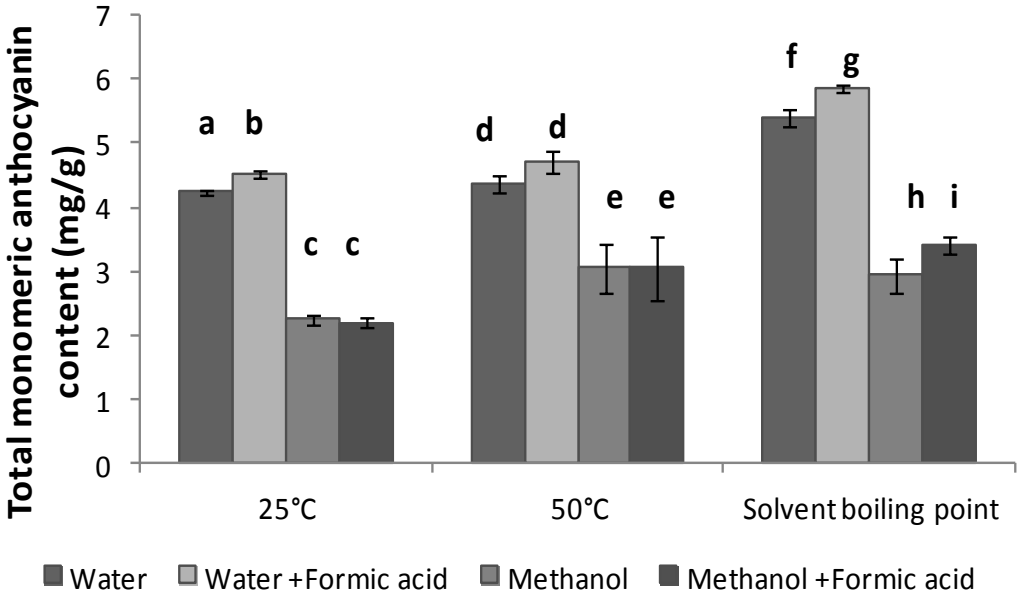

TEAC value
(mg Trolox equivalent/g)
25 °C
50 °C
Solvent boiling point
Water
Water + Formic acid
Methanol
Methanol + Formic acid
Ethyl acetate
Ethyl acetate + Formic acid
Hexane
Hexane + Formic acid

Figure 4. Total polyphenol content (I) and antioxidant assays (II-IV) of extracts of H. sabdariffa extracted for 10 min. Values with different letters in each chart are significantly different (Tukey's test, $p<0.05$). Results are triplicates (± SD)



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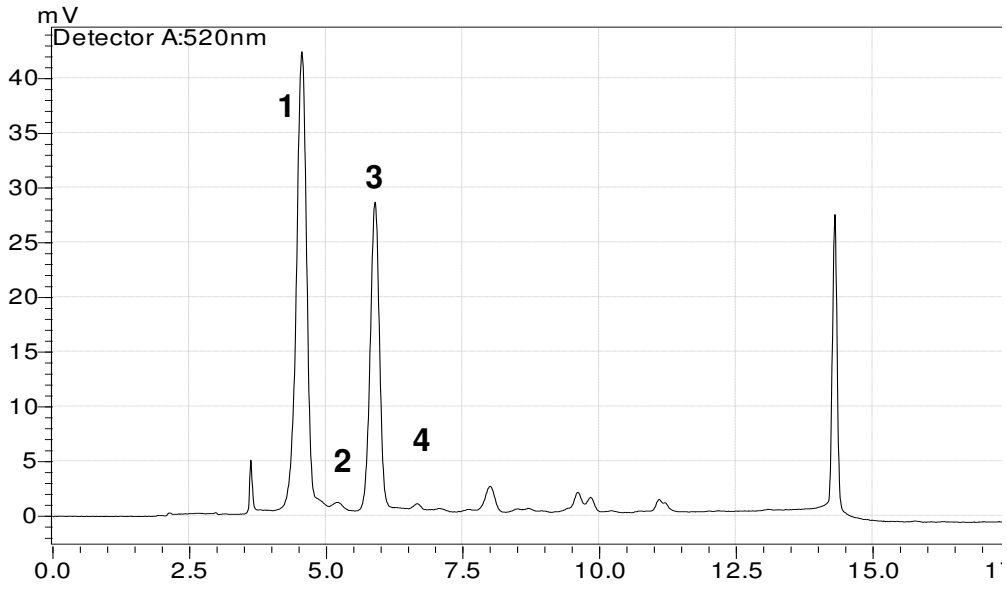

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