TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF ROSELLE (*Hibiscus sabdariffa*) EXTRACTS

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ABSTRACT

Introduction: Roselle (*Hibiscus sabdariffa*) is widely known for its benefits and medicinal properties. The aims of this study were to determine the total phenolic content (TPC) in *H. sabdariffa* extract, its antioxidant activity and relationship between TPC and its antioxidant scavenging capacity. **Methods:** The TPC value for each extract of free, bound soluble and bound insoluble phenolic acids was determined by using Folin-Ciocalteu assay. The antioxidant scavenging capacity was evaluated by using DPPH assay. **Results:** The results obtained in this study showed that bound soluble phenolic acids (7.63 $\pm$ 0.28 mg GAE/g DW) had the highest TPC value than the free and insoluble phenolic acid extracts (2.78 $\pm$ 0.20 mg GAE/g DW and 3.61 $\pm$ 0.85 mg GAE/g DW, respectively) ($p<0.05$). As for the IC_{50} values, bound soluble phenolic acid extract exhibited the greatest value(0.62%) as compared to the free and bound insoluble phenolic acid extracts (0.90% and 1.14%, respectively). A positive correlation ($R^2 = 0.956$) between three extracts (free, soluble and insoluble phenolic acids) of TPC and antioxidant radical scavenging capacity (DPPH) was obtained. **Conclusion:** It can be concluded that phenolic acids in *H. sabdariffa* extracts have an effect to the antioxidant scavenging capacity particularly in the bound soluble form. This finding proved that *H. sabdariffa* extracts could be a good source of natural antioxidant for food, cosmetic and pharmaceutical industries.

Keywords: *H. sabdariffa*, free phenolic, bound phenolic, TPC

INTRODUCTION

According to Morton (1987), Roselle (*Hibiscus sabdariffa*) is distributed throughout the world especially in Asia and central of America. It is known for its delicacy taste and widely used in various applications including in the diet and as a medicinal plant. The flower and calyces part of Roselle are well-known for its antiseptic, diuretic, antioxidant and antimutagenic properties (Salleh *et al.*, 2002). It was reported by Olvera Garcia et al. (2008) that the extracts from the calyces have various therapeutic

effects either *in vivo* or *in vitro* including anticancer or antioxidants properties (Farombi and Fakoya, 2005). The imbalance of oxidants and antioxidants will lead the body to oxidative stress resulted in damaging of DNA, proteins, unsaturated lipids and other essential molecules (Lobo et al. 2010). Therefore, it is crucial to explore the product of *H. sabdariffa* and determine its bioactive compounds especially on the calyces part as it showed a potential for therapeutic uses and contain flavonoids, phenolic acids and anthocyanins.

Study done by Prasongwatana et al. (2008) proved that the extract of Roselle calyces can be used as an effective treatment for kidney stones disease due to its uricosuric effect. The calyces part of Roselle has been recognized as a source of antioxidant since ancient time and it has been used to treat patient that having hypertension, high fever and gastrointestinal disorder (Mohd Esa et al. 2010). Meanwhile, the research done by Olvera-Garcia et al. (2008), demonstrated that the extract from the calyces has mixture of therapeutic effects either in vivo or in vitro, including anticancer and antioxidant properties.

There are abundance of phenolic compounds occurring in plants and majority of it occurs as esters, soluble conjugate (SPE) (glycoside) and insoluble forms (IPE) (Nardini and Giselli, 2004; Arranz et al. 2009) and very few in free form. The extractable polyphenols that consist of free phenolic acid, ester, glycosides and bound complexes (Naczk and Shahidi, 1989) are classified as soluble compounds that can be extracted by using an aqueous organic solvent. However, non-extractable polyphenol that bound to the cell wall constituents may remain insoluble after being extracted with aqueous organic solvent (Arranz et al. 2009) it shows that different forms of phenolic acids have different forms of extraction condition and its susceptibilities to degrade with one another.

Till date, more studies done on *H. sabdariffa* species were mainly focused on the phenolic content and their antioxidant activity in the free phenolic extract only. Limited information reported on the antioxidant properties in the bound soluble (soluble in the aqueous organic solvent) and insoluble phenolic acid extracts (its residue fraction) of phenolic compound in *H. sabdariffa*. Thus, this proposed research aimed to determine the total phenolic content of *H. sabdariffa* extract in free phenolic and bound-phenolic acids as well as to evaluate the antioxidant properties of both extracts. Furthermore, this research aims to discover if there are any correlation among the total phenolic compound (TPC) and antioxidant properties in Roselle (*H. sabdariffa*).

MATERIALS AND METHODS

Plant Material and Extract Preparation

The fresh *H. sabdariffa* that was bought from the wet market in Kuantan in March 2017 were cleaned with tap water and only the calyces part were taken before it being stored in -80 °C freezer prior to freeze drying process. The samples were freeze dried for 5 days with temperature -50 °C to -54 °C before it being ground into powder by using a blender. The powdered sample was kept in the air tight jar and stored in -80 °C freezer until further usage.

Free Phenolic Acid Extraction

Method for sample extraction was taken from El-Sayed et al. (2012) with slightly modification. The extraction was done in triplicate. Dried ground sample (1 gram) was sonicated with 20 ml of 80 % methanol for 1 hour by using Ultrasonic sonicator at room temperature followed by filtration through filter paper (Whatman no. 1) prior to alkaline and acid hydrolysis treatments. The step was repeated with the balance of the residue. The supernatant was pooled in 50 ml of falcon tube, while the left residue was kept in -20 °C for further usage. All the solvent was desolventized in a rotary evaporator under vacuum

just until a semisolid residue was obtained. The semisolid residue was washed thoroughly 3 times with diethyl ether and the organic phase was pooled in 50 ml falcon tube while the left residue was stored in -20 °C for further usage. Then, the pooled organic phase was evaporated to dryness under atmospheric pressure (850 mbar), dissolved in methanol and labeled as free phenolic extract (FPE).

Bound Soluble Phenolic Acid Extraction

The supernatant obtained from the free phenolic acid extraction was hydrolyzed with 20 ml of– 4 M sodium hydroxide (NaOH) and stored for 2 hours at room temperature. The residue from the free phenolic extraction was then acidified to pH 2 by using 6 M hydrochloric acid (HCl) and extracted 3 times with diethyl ether before the aqueous phase was collected and pooled in 50 ml falcon tube. The organic layer was evaporated to dryness under vacuum (850 mbar). Then the dried residue was dissolved in methanol and labeled as bound soluble phenolic extract (SPE).

Bound Insoluble Phenolic Acid Extraction

The residue of free phenolic acid extraction was hydrolyzed with 20 ml of 4 M NaOH and stored at room temperature for 1 hour. The residue was acidified to pH 2 by using 6 M HCl followed by centrifugation (4379 rpm, 10 minutes) before the supernatant was collected for further usage. The liberated phenolic acid was extracted with hexane to remove free fatty acid and other lipid contaminants. The aqueous layer was then extracted 3 times with diethyl ether (1:1, v/v). The ethyl-ethyl acetate extract was filtered and evaporated to dryness under vacuum (850 mbar). The dry residue was dissolved in absolute methanol and labeled as bound insoluble phenolic extract (IPE).

Total Phenolic Content (TPC) Assay

A method described by Ismail et al. (2010) with a slight modification was used to determine the total phenolic content (TPC) based on Folin-ciocalteu's method. Gallic acid stock solution (1 mg/ml) was used to prepare 10 different concentration of gallic acid ranged from 0.01 mg/ml to 0.10 mg/ml. Briefly, 150 µl of each extract or standard (gallic acid) or blank (methanol) was mixed with 2.5 ml of the Folin-Ciocalteu reagent and 2 ml of sodium carbonate (Na_2CO_3) The mixture was further incubated at room temperature for 60 minutes. The absorbance of the sample was read at 725 nm by using UV/Vis spectrophotometer. Gallic acid with a concentration ranged from 0.01 mg/l to 0.1 mg/l was used as the external standard. The TPC values of the extracts were expressed as mg gallic acid equivalent (GAE)/g (freeze dry weight).

2-Dipenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

DPPH radical scavenging assay was used to determine the antioxidant capacity of the extract based on the method by Ravishankar et al. (2013) with some modification. The extracts were diluted with absolute methanol in 5 different concentration range from 0.05, 0.07, 0.1, 0.2 and 1 mg/ml. Then, 2 ml of DPPH and 0.5 ml of test extract were combined in 96 well plate and incubated at room temperature for 30 minutes. The absorbance was read at 517 nm by using microplate reader. The external standard used in this assay was gallic acid with a concentration ranged from 0.5 mg/l to 64.0 mg/l. Absolute methanol was used as a blank.

Statistical Analysis

Excel (Microsoft Inc.) 2010 software and SPSS version 23 were used in the data analysis. All the experimental measurements were carried out in triplicate and were expressed as mean ± standard deviation. One-way ANOVA test was used to compare the mean value of each plant extract. Meanwhile, post hoc test was used to determine which extracts were significantly different with each other.

RESULTS

Total Phenolic Content (TPC) of *H. sabdariffa*

Figure 1 shows the total phenolic content from three different fractions of phenolic compounds (free, bound soluble and bound insoluble) that were extracted by using different treatment and ranked as followed: bound soluble phenolic extract (SPE) > bound insoluble phenolic extracts (IPE) > free phenolic extracts (FPE).

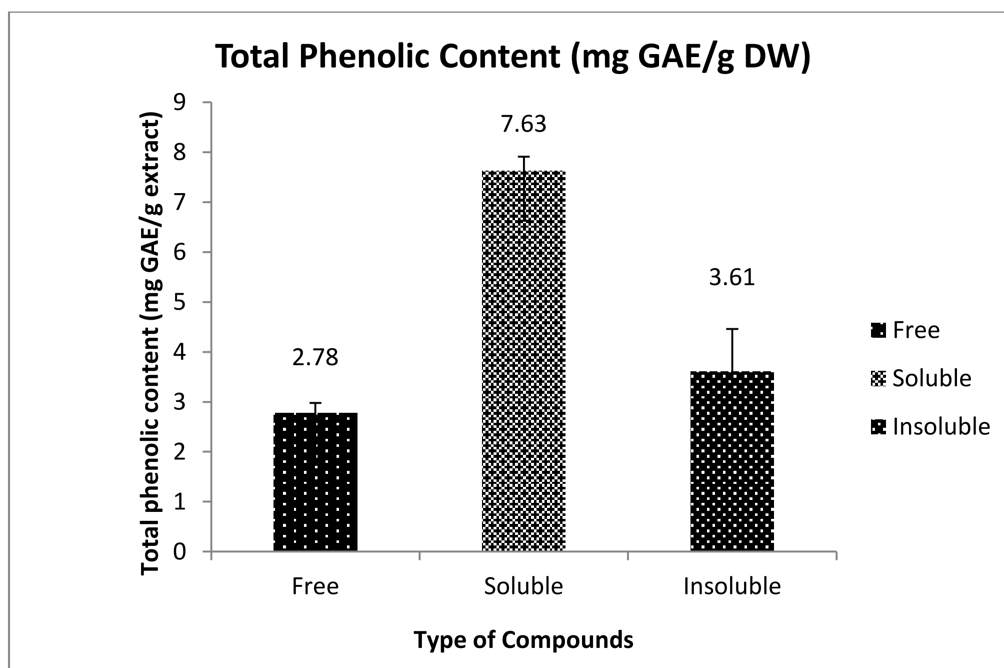

Figure 1
Total Phenolic Content of *H. sabdariffa*

The amount of phenolic acid from different extract were varied (2.78 $\pm$ 0.20 to 7.63 $\pm$ 0.28 mg GAE/g DW), and it can be clearly seen that the extraction of soluble phenolic acid extract showed the highest phenolic content compared with insoluble phenolic acid extract and free phenolic acid extracts. Figure 1 shows that the bound soluble phenolic acid (7.63 $\pm$ 0.28 mg GAE/g DW) had the highest value of TPC followed by bound insoluble phenolic acid and free phenolic acid extracts (3.61 $\pm$ 0.85 mg GAE/g DW and 2.78 $\pm$ 0.200 mg GAE/g DW, respectively) ($p<0.05$). The one way ANOVA revealed that there was significance different ($p<0.05$) observed between the extracts against the total phenolic content. Post hoc comparison by using Tukey's test indicated that there were significant differences between free and soluble; insoluble and soluble extracts as depicted in Table 1.

Table 1

Statistical Post hoc test by using Tukey's test on Total Phenolic Content (TPC) between 3 different extracts (Free, soluble and insoluble)

Test (I)	Test (J)	Significant difference (p<0.05)
Free	Soluble	*0.00
Soluble	Insoluble	*0.00
Insoluble	Free	0.216

*significant difference at p<0.05

Note: Test I and J refer to the further analysis runs in the Tukey's test.

Antioxidant Radical Scavenging capacity of *H. sabdariffa* Extracts by Using DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

In this study, the antioxidant activity of *H. sabdariffa* from free phenolic acid (FPE), bound soluble phenolic acid (SPE) and bound insoluble phenolic acid extracts (IPE) were investigated by using DPPH radical scavenging assay. Gallic acid was used as a standard reference or positive control. A standard curve with concentrations ranged from 0.05 to 1.00 mg/ml with R^2 = 0.998. Table 1 illustrated the percentage radical scavenging capacity (I%) of each phenolic compound extraction where the higher the percentage of radical scavenging capacity, the higher the ability to scavenge free radical, the higher the antioxidant activity that the extract possesses. Results in Figure 2 indicates that all extracts have the ability to exhibit the free radical scavenging activity, where the soluble phenolic acid extract demonstrated the highest percentage of radical scavenging capacity (68.61% ± 0.08) among the extracts followed by insoluble phenolic acid extract (56.18%) and free phenolic acid extract (46.24%) at the concentration of 1 mg/ml).

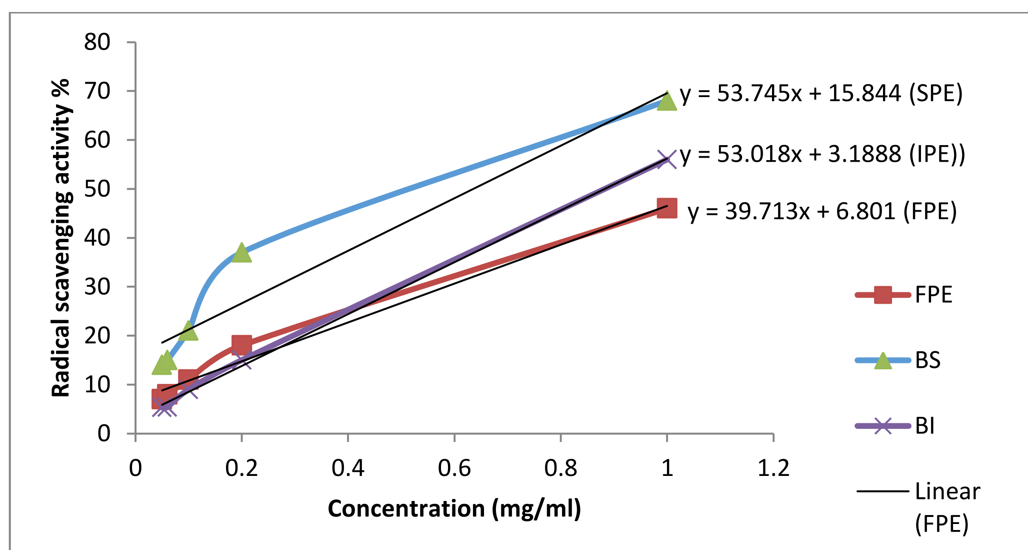

Figure 2

The percentage of radical scavenging capacity (I%) of each extracts in different range of concentration (FPE = free phenolic acid extract; IPE = insoluble phenolic acid extract; SPE = soluble phenolic acid extract)

Table 2 shows the IC_{50} values for all the extracts. IC_{50} value can be defined as the concentration needed to scavenge 50% of DPPH free radical in an antioxidative assay (Rebiai *et al*., 2014). Soluble bound phenolic acid extract showed the highest radical scavenging capacity as compared to free phenolic acid extract (0.62 mg/ml and 1.14 mg/ml, respectively) ($p<0.05$). The lower the IC_{50} value indicates, the higher the antioxidant scavenging capacity that the plant extract possesses. Soluble phenolic acid extract has possessed higher DPPH scavenging ability in comparison with insoluble phenolic acid extract and free phenolic acid extract. One way ANOVA test was conducted to determine the effects of sample extracts on total phenolic content and it is proven that there is a significant difference revealed ($p<0.05$). Post hoc comparison with Tukey's test showed that there is a significant difference between free and soluble extracts. However, there were no significant difference detected between free and insoluble extracts; soluble and insoluble extracts ($p>0.05$). From all the result obtained it can be concluded that the fractions of soluble phenolic extract and free phenolic extract of *H. sabdariffa* have the ability to act as a powerful radical scavenger.

Table 2

IC_{50} of free, soluble and insoluble phenolic acid extract of *H. sabdariffa*

Samples	1st	2nd	3rd	Mean IC_{50}
Free (FPE)	0.82	1.25	1.36	1.14 ± 0.28
Bound Soluble (BPE)	0.63	0.64	0.60	*0.62 ± 0.02
Bound Insoluble (IPE)	0.98	1.02	0.70	0.90 ± 0.17

Results expressed as mean of triplicates ± SD (n=3). * Significance difference at $p < 0.05$

DISCUSSIONS

Total Phenolic Content (TPC) Of *Hibiscus Sabdariffa*

An aqueous methanol (80%) was used to extract free phenolic extract while the alkaline and acid hydrolysis treatments were used to extract soluble and insoluble phenolic acid extracts, respectively. An additional treatment by using hexane was applied to insoluble phenolic extract extracts in order to remove all the fatty acids and other lipid contaminants in *H. sabdariffa* extracts as some of the non-phenolic compound such as sugar, organic acids and fats might present in the extract (Jin Dai and Rusell, 2010). Alcohols was used to extract the free phenolic extract as the study done by Chinedu et al. (2011) claimed that alcohols such as methanol and ethanol are the most effective solvent to extract phenolic acids from *H. sabdariffa* extract as compared to acetone and water. However, the acid and alkaline hydrolysis treatments were used to release bound (soluble and insoluble) phenolic acid due to phenolic physical nature that covalently bound to the cell wall structural component (Wong, 2006). These treatments help to break the ester bond that bind the phenolic acid to the cell wall of the compound and it is the most preferable method to release bound phenolic acids in plant extracts (Stalikas, 2007).

Figure 1 shows that the bound soluble compound (7.63 ± 0.28 mg GAE/g DW) had the highest value of TPC followed by bound insoluble compound and free phenolic compound extracts (3.61 ± 0.85 mg GAE/g DW and 2.78 ± 0.200 mg GAE/g DW, respectively) ($p<0.05$). These results are in agreement with Beatriz et al. (2014), who reported that most of the phytochemical found in fruits and vegetables

especially phenolic acids were either in free phenolic extract or soluble phenolic extract form. Similar results were observed from the work done by Govardhan et al. (2013), who discovered that soluble phenolic extract in *Moringa oleifera* seed flour showed higher TPC compare with free phenolic fraction. The data obtained was also consistent with the study done by Marketa et al. (2008) which found that bound phenolic compound contributed to higher total phenolic content compared to free phenolic extract.

Antioxidant Radical Scavenging capacity of *H. sabdariffa* Extracts by Using DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

The extract sample was diluted with an absolute methanol with the concentration ranged from 0.05 mg/ml to 1 mg/ml. It can be clearly seen that the scavenging capacity was enhanced with the increasing of sample concentration and this is due to the contribution of the TPC in the extract. Similar response was observed in the study done by Chandrasekara and Shahidi (2010); Liyana-Pathirana and Shahidi (2006), they found that bound phenolic compound had higher antioxidant capacity compared to free phenolic compound.

The antioxidant radical scavenging capacities of all phenolic extracts of *H. sabdariffa* from the analysis of I % and IC_{50} in this study were ranked as follow: Bound soluble phenolic extract (SPE) > Bound insoluble phenolic extract (IPE) > Free phenolic extract (FPE). This trend suggested that the ability of the plant extract to act as a powerful radical scavenging activity was influenced by the extraction solvent used to extract the plant. This was supported by Verma et al. (2009), where acid hydrolysis treatment showed highest antioxidant activity compare with the extract without any treatment. Nevertheless, the use of acid hydrolysis treatment can decrease the content of the phenolic acids in the extract (Krygier et al. 1982) but the addition of a metal chelator like (EDTA) during the alkaline hydrolysis treatment can help to prevent the degradation of phenolic acids (Nardini et al. 2002). Other factors might influence the antioxidant activity of the plant extract such as temperature, the concentration of the antioxidant and the pH used during extraction process (Gazzani et al. 1998). However, this study was only focusing on the total phenolic compound (free, soluble and insoluble) that presence in the extracts thus it is not known whether others factor might influence the antioxidant activity of the plant. These reports further affirmed that the total phenolic content does influence the antioxidative capability of the plant extract.

CONCLUSION

The present study reported that TPC in *H. sabdariffa* plays a major role on the antioxidant activity of the plant extracts. It can be clearly seen that bound soluble phenolic compound showed the highest TPC and antioxidant radical scavenging capacity. Meanwhile, bound insoluble phenolic compound exhibited the lowest values in TPC and antioxidant activity. It can be concluded that most of the phenolic acid in *H. sabdariffa* extract was in bound soluble forms. Study discovered that different method of extraction used will give significant difference to the total phenolic content and antioxidant activity of *H. sabdariffa.*

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REFERENCES

Arranz S., Saura-Calixto F., Shaha S. and Kroon P. A. (2009). High Contents Of Non-Extractable Polyphenols In Fruits Suggest That Polyphenol Contents Of Plant Foods Have Been Underestimated. *Journal of Agricultural Food and Chemistry*, **57**, 7298–7303

Beatriz A., Janet A. and Sergio O. (2013). Bound Phenolics in Food, a Review. *Journal of Food Chemistry*, **152**, 46-55.

Chinedu P.A., Ijeoma E., Olusola A., and Ayobami O.A. (2011). Polyphenolic Content and Antioxidant Activity of *Hibiscus sabdariffa* Calyx. *Journal of Medicinal Plant,* **5(5),** 557-566.

El-Sayed M.A, Thin-Miew C., Simarata D., and Iwona R., (2012). Free and Bound Phenolic Acids and Total Phenolics in Black, Blue and Yellow Barley and Their Contribution to Free Radical Scavenging Capacity. *Journal of Cereal Chemistry*, **89(4),** 198-204.

Farombi, E. O. and Fakoya, A., (2005). Free radical scavenging and antigenotoxic activities of natural phenolic compounds in dried flowers of *Hibiscus sabdariffa L. Molecular Nutrition & Food Research,* ***49*(12)**, 1120-1128.

Gazzani, G., Papetti, A., Massolini, G., & Daglia, M. (1998). Anti- And Pro-Oxidant Activity Of Water Soluble Components Of Some Common Diet Vegetables And The Effect Of Thermal Treatment. *Journal of Agricultural and Food Chemistry*, **46**, 4118–4122

Govardhan R S, & Pradeep N & Cheruppanpullil, R. (2013). Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of Moringa oleifera seed flour. Journal of Functional Foods. 5. 1883-1891. 10.1016/j.jff.2013.09.009.

Ismail, M., Mariod, A., Bagalkotkara, G., and Ling, H. S. (2010). Fatty Acid Composition and Antioxidant Activity of Oils from Two Cultivars of Cantaloupe Extracted by Supercritical Fluid Extraction. *Grasas Y Aceites*, **61(1)**, 37-44.

Jin Dai and Rusell J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, **15**, 7313-7352.

Krygier, K., Sosulski, F., & Hogge, L. (1982). Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of Agricultural and Food Chemistry,* **30**, 330–334.

Lobo V., Patil A., Phatak A., and Chandra N. (2010). Free Radicals, Antioxidants And Functional Foods: Impact On Human Health. *Pharmacogn Rev*, **4(8)**, 118-126.

Marketa D., Luis F. G., Pavel D., Zuzana S., Manuela M. M. and Aquiles A. B. (2008). Antioxidant Properties of Free, Soluble Ester and Insoluble-Bound Phenolic Compound in Different Barley Varieties and Corresponding Malts. *The Institute of Brewing and Distilling*, **114(1),** 27-33.

Nardini, M., Cirillo, E., Natella, F., Mencarelli, D., Comisso A, and Scaccini C. (2002). Detection of Bound Phenolic Acids: Prevention By Ascorbic Acid And Ethylenediaminetetraacetic Acid Of Degradation Of Phenolic Acids During Alkaline Hydrolysis. *Journal of Food Chemistry*, **(79)**, 119-124.

Morton J. F., (1987). Roselle (*Hibiscus sabdariff) a,* fruits of warm climate. *Climate Resources System*, 281-286.

Nardini, M. and Ghiselli, A. (2004). Determination Of Free And Bound Phenolic Acids In Beer. *Food Chemistry*, ***84*,** 137–143.

Mohd Esa N., F., Shin Hern, Ismail A. & Yee Lye C. (2010). Antioxidant Activity In Different Parts Of Roselle (Hibiscus Sabdariffa L.) Extracts And Potential Exploitation Of The Seeds. *Journal of Food Chemistry*, 1055-1060.

Olvera-Garcia, V., Castano-Tostado, E., Rezendiz-Lopez, R. I., Reynoso-Camacho, R., Gonzalez De Mejia, E., Elizondo, G. and Loarca-Pina, G., (2008). *Hibiscus sabdariffa* extracts inhibit the mutagenecity in microsuspension assay and the proliferation of Hela cells. *Journal of Food Science*, **73(5).**

Prasongwatana, V., Woottisin, S., Sriboonlue, P., & Kukongviriyapan, V. (2008). Uricosuric effect of Roselle (Hibiscus sabdariffa) in normal and renal-stone former subject. *Journal of Ethnopharmacology*, **117(3),** 491–495.

Ravishankar, K., Ahalya, B., and Priyabandhavi, P. (2013). Evaluation of In-Vitro Antioxidant Activity of *Annona muricata* Bark. *International Journal of Pharmaceutical, Chemical and Biological Sciences*, **3(2),** 406-410.

Rebiai, A., Lanez, T., and Belfar, M. L. (2014). Total polyphenol contents, radical scavenging and cyclic voltammetry of Algerian propolis. *International Journal of Pharmacy and Pharmaceutical Sciences,* **6(1),** 396-400.

Salleh N., Runnie I., Roach D., Mohamed S. and Abeywardena Y. (2002). Inhibition Of Low-Density Lipoprotein Oxidation And Up-Regulation Of Low-Density
Lipoprotein Receptor In Hepg2 Cells By Tropical Plant Extracts. *Journal of Agricultural Food Chemistry*, **50**, 3693-3697.

Stalikas, C. D. (2007). Extraction, Separation, And Detection Methods For Phenolic Acids and Flavonoids. *Journal of Separation Science*, **30(18),** 3268–3295.

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic Acid Composition and Antioxidant Capacity of Acid and Alkali Hydrolysed Wheat Bran Fractions. *Food Chemistry*, ***116*(4),** 947–954.

Wong, D. W. S. (2006). Feruloyl Esterase: A Key Enzyme In Biomass Degradation. *Applied Biochemistry and Biotechnology,* **133(2),** 87–112.