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Antioxidant activities, polyphenolic composition and their correlation analysis on *Hibiscus sabdarifa* L. (sabdariffa) calices

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ABSTRACT

Background & Aim: Extracts of Roselle calyx, possess compounds which could be considered as a great source of natural antioxidants. In this project, antioxidant activities, phenolic compounds content and their correlation analysis on calices of Roselle (Hibiscus sabdariffa) was investigated.
Experimental: Total polyphenolic compound, flavonoids and anthocyanins were determined spectrophotometrically. Catalase and peroxidase activity was estimated based on unit per milligram protein. Total antioxidant capacities of the extract were studied using phosphomolybdate assay, DPPH assay and FRAP method.
Results: The hibiscus calices were rich in phenolic compounds, flavonoids and anthocyanins. Peroxidase and catalase activity was estimated as 3.13 and 0.05 unit mg^{-1} protein, respectively. Calyx extract showed high antioxidant activities specially based on DPPH assay. Correlation analysis was also performed between parameters. There was a positive significant correlation between the levels of polyphenolics and flavonoids, anthocyanins and antioxidant capacity of calices at $P<0.05$.
Recommended applications/industries: Extracts of *H. sabdariffa* L. (sabdariffa) calices are rich in antioxidant compounds including total phenolic compounds, flavonoids content, and anthocyanins. Based on all the method of measuring antioxidant capacities in this project, the calices showed an acceptable antioxidant activity. Thus, *H. sabdariffa* L. (sabdariffa) may be suggested as a potential source of natural antioxidants in food and pharmaceutical industry.

1. Introduction

Antioxidants are compounds with the ability of preventing or delay of the oxidation of the substrate. They are important in the safeguarding of health, and the protection from coronary heart disease and cancer (Khurana *et al.*, 2013). Polyphenols as antioxidants are the most abundant secondary metabolites in plants having redox properties, which allow them to act as

reducing agents, hydrogen donators, and singlet oxygen quenchers. Many plants extract are rich in phenolic substances, usually referred to as polyphenols, which are ubiquitous components of plants and herbs (Khurana *et al.*, 2013; Škrovánková *et al.*, 2012). Polyphenols include several classes of compounds such as phenolic acids, colorful anthocyanins, and simple and complex flavonoids (Balasundram *et al.*, 2006). Several researches have shown that the ingestion of natural extracts containing antioxidants, especially polyphenolic compounds, is associated with lower rates in coronary heart disease, cancer and diabetes probably by protecting cellular processes against oxidation of lipids and proteins owing to the antioxidant potential of polyphenols to capture and react with free radicals (Proestos *et al.*, 2013; Zengin *et al.*, 2011). Studies show that foods containing phenolic compounds have protective effects against many diseases and can be used as anti-mutagenic, anti-bacterial, anti-viral and anti-inflammatory compounds (Mariod *et al.*, 2012).

There is some evidence that shows growing tendency to use phenolic compounds found in natural food sowing to their antioxidant activity (Mariod *et al.*, 2012). Studies showed that the Roselle calices are rich in poly-phenolic compounds and antioxidants which those compounds could be considered as a natural antioxidants source (Yang *et al.*, 2014).

Roselle, *Hibiscus sabdariffa* L. (sabdariffa), belonging to Family Malvaceae is an annual, erect, bushy, herbaceous shrub, with branched, erect, smooth stem. The main product of this crop its persistent calyx (El-Boraie *et al.*, 2009; Yang *et al.*, 2014). The red fresh calices are used to make jam, jelly and drinks (Islam *et al.*, 2008). It is the major component possessing a sour taste that is used as beverage and food colorants (Hirunpanich *et al.*, 2005). Extracts of Roselle calyx, was found to possess compounds which could be considered as a great source of natural antioxidants (Farombi Fakoya, 2005; Mak *et al.*, 2013; Tsai *et al.*, 2002; Tseng *et al.*, 1997).

As antioxidants, Roselle is capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases. Typical phytochemicals that possess antioxidant activity include phenols, phenolic acids (Yang *et al.*, 2014) and their derivatives, flavonoids, phytic acid, anthocyanins (Obouayeba *et al.*, 2014; Tsai *et al.*, 2002) and many sterols. Many studies reported that the plant also contains cyanidin, querecetin, hentriacontane, calcium oxalate, thiamine, riboflavin, niacin, ascorbic, citric, tartaric and oxalic acid (Ali *et al.*, 2005; Salem *et al.*, 2014).

Since studies on the correlation between phenolics compound content and antioxidant activity of Roselle calyx extracts are very limited, the main objective of this study was to investigate some biochemical and phenolics content, antioxidant activity and the correlation of these parameters in Roselle (*Hibiscus sabdariffa* L. var. sabdariffa) calyx.

2. Materials and Methods

2.1. Plant Material

Calices of *Hibiscus sabdariffa* L. var. sabdariffa were collected from Field Laboratory, Agricultural and Natural Resources Recearch Center of Kerman (25°55′ latitude and 53°26′ longitude, elevation 1754 m), in June 2013.

Calices were powdered (mesh size 30), covered with aluminum foil (to avoid exposure to light) and stored at -20º C until analysis.

2.2. Antioxidant compounds and Biochemical analysis

2.2.1. Determination of total phenolic content (TPC)

The amount of total phenolic content in extract was determined according to Gao *et al.* (Gao *et al.*, 2000). 100 µl of sample solution (1mg/mL) were introduced into test tube containing 100 µL of Folin-Ciocalteu's reagent and 2 ml of Na_2CO_3 (7.5%). The final volume was brought up to 5ml with deionized water. After 30 min incubation at room temperature, the absorbance was spectrophotometrically measured at 765 nm (Shimadzu, UV-1800). The total phenolic content was expressed as gallic acid equivalents (GAE) in microgram per gram of extract (µg GAE g^{-1} extract).

2.2.2. Determination of flavonoids content (FC)

The flavonoids content was analyzed as described by Krizek *et al.* (1998). The amount of 0.5 g of fresh weight of the seedlings extracted in 10 ml of acidified ethanol (ethanol: acetic acid, 99:1 (v/v)). The extracts were gently boiled in water bath at 80°C for 10 min, and the absorbance was measured at 330nm using UV-VIS spectrophotometer (Cary 50).

Determination of Anthocyanin content (AC): To measure the total anthocyanin content, 0.02 g of dried

calices were extracted with 4 ml of 1% HCI (w/v) in methanol for 2 d at 3 to 5°C with occasional shaking. The resulting solution was kept in the refrigerator for 24 hours. The absorbance of the extracts, clarified by filtration, was measured at 530 nm (peak of absorption of anthocyanin) and 657 nm (peak of absorption of degradation products of chlorophyll in acidic methanol) against the control (1% methanolic solution of hydrochloric acid) (Mancinelli Rabino, 1985). Anthocyanin content of each extract was expressed as absorbance after the amount calculated from the following equation: $A= A_{530}-(0.25\times A_{657})$ Where, A_{530} and A_{657} denote absorbance of the solution at 530 and 657 nm respectively.

2.2.3. Enzyme extraction

Frozen shoot samples (0.5 g) were homogenized in 2.5 ml of 50mM phosphate buffer (pH 7) containing 1M EDTA, 1mM PMSF, and 1% PVP. The homogenate solution was centrifuged at 20000g at 4°C for 20 min and the clear supernatant was used directly for the assay of enzyme activity and estimation of protein. The supernatant was used for measurement of total soluble protein according to Bradford (1976) using bovine serum albumin as standard (Bradford, 1976).

2.2.4. Determination of antioxidant enzyme activity

Catalase activity was measured as described by Aebi, (1984) with slight modifications. The disappearance of H_2O_2 was monitored at 240 nm absorbance using UV-vis spectrophotometer (Cary 50) for 5 min. Reaction was carried in a final volume of 3 ml of reaction mixture containing 2.78 ml phosphate buffer (pH 7), 0.1 mL enzyme extract and 30µl of 15 mM H_2O_2(Aebi, 1984). Enzyme activity was calculated by using $\varepsilon = 0.28$ mM^{-1} cm^{-1} and expressed as enzyme units per mg protein. One unit of enzyme determines the amount necessary to decompose 1 mmol of H_2O_2 per minutes at 25ºC.

Peroxidase activity was measured by using the method of Plewa *et al*. (1991). Kinetics of the reaction was followed at 470 nm. Guaiacol oxidation (tetraguaiacol formation) was monitored by reading the absorbance at 470 nm at the moment of enzyme extract and after 3 min. Activity was calculated using extinction coefficient 25.5 $mM^{-1}cm^{-1}$ at 470 nm for tetraguaiacol and expressed as units per gram of protein. The enzyme unit in the extract was calculated for the formation of 1 mM tetraguaiacol for 1 minute (Plewa *et al*., 1991).

2.3. Determination of total antioxidant activities

Preparation of the Extract for antioxidant activity: Calices of the plant were dried in the shade and grinded. 1 g of each sample was soaked in 50 mL of 70% methanol and kept at room temperature for 48 hours. Then, the extracts were filtered and the solvent was evaporated on a rotary device at 40°C. The remaining was kept in refrigerator at 4°C for further analysis.

2.3.1. Determination of total antioxidant capacity (TAC) by phosphomolybdenum assay

The total antioxidant capacity of extract was evaluated according to Prieto *et al*. (1999) by phosphomolybdenum method. 0.3 mL of extract (1 mg/ml) solution was mixed with 3 mL reagent solution (6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm against blank (Prieto *et al*., 1999). The antioxidant capacity of extract was evaluated as equivalents ascorbic acid (µg AAE g^{-1} extract).

2.3.2. DPPH radical scavenging capacity (RDSC)

The capacity of the flower extracts to scavenge DPPH radicals (2, 2-diphenyl-1-picrylhydrazyl) was measured based on the method described by Sanchez-Moreno *et al*. (1998) with some modifications. 200 µL of extract and control (methanol at the place of extract) were added to 3 ml of freshly prepared DPPH solution (0.004%) in methanol. The reaction mixture was incubated in the dark at room temperature. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm after 30 min (Sánchez-Moreno *et al*., 1999). The obtained results were expressed as the percentage inhibition of DPPH based on the following formula:

$$\text{Inhibition of DPPH (\%)} = A_{control}-A_{sample}/A_{control} *100$$

Where $A_{control}$ is the absorbance of the DPPH solution without sample extract and A_{sample} is the absorbance of the sample with DPPH solution. The effective concentration of sample required to scavenge DPPH radical by 50% (IC50 value) was obtained by

linear regression analysis of dose-response curve plotting between %inhibition and concentrations.

2.3.3. Ferric reducing antioxidant power (FRAP)

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of extracts (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50º C for 20 min. Then 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (Oyaizu, 1986). Ascorbic acid was used as standards. Ferric reducing power of extracts was expressed as milligram ascorbic acid equivalents (AAE) per gram of extract (mg AA g^{-1} extract).

2.4. Statistical analysis

Data were analyzed using the analysis of variances (ANOVA) followed by Duncan test (SAS v. 9.1). Each data was the mean of three replicates and means evaluation was done at a confidence level of 95%. The correlation between parameters analyzed using Pearson's correlation analysis.

3. Results and discussion

3.1. Antioxidant compounds

Total phenolic contents (TPC) for Roselle calyx extracts, determined by Folin-Ciocalteu assay using gallic acid as calibration standard. The assay is a fast and simple method to rapidly determine the content of phenolics in samples. Total phenolic compounds content were determined as 590.6±15.3 µg GAE g^{-1} extract (Table 1).

Plant phenolics present in plants have received considerable attention because of their potential antioxidant activity. Bergmeier *et al.* (2014) reported the total phenolic content of *H. sabdariffa* ethanolic flower between 66.91 up to 501.2 mg GAE/g extract. Phenolic secondary metabolites play an important role in plant-derived food quality, as they affect quality characteristics such as appearance, flavor and health-promoting properties. Their content in foods is affected by many factors that influence phenolic stability, biosynthesis and degradation. In terms of their biosynthesis the key enzyme phenylalanine ammonia-lyase (PAL) is especially relevant, as it can be induced by different stress (environmental) conditions (Tomás-Barberán Espin, 2001).

Table 1. Polyphenolic compounds an antioxidant enzymes activity of hibiscus calix extract.

TPC µg GAE g^{-1} extract	FC (Abs)	AC (mg g^{-1} FW)	POD activity (U mg^{-1} protein)	CAT activity (U mg^{-1} protein)
590.6±15.3	1.188±0.05	0.132±0.05	3.13±0.5	0.05±0.001

Data show the mean±SD of three replicates. FC: Flavonoid content, TPC: total phenolic compounds, CAT, catalase, POD: peroxidase

Phenolic compounds include a wide diversity of compounds such as flavonoids and anthocyanidins (Mak *et al.*, 2013). Polyphenolic compounds like flavonoids and phenolic acids as well as anthocyanins commonly found in plants have been reported to have multiple biological effects, including an antioxidant activity. The content of flavonoids and anthocyanin are given in W It was reported that organic acids, anthocyanins and flavonoids are the main antioxidant phytochemicals of *H. sabdarifa* (Da-Costa-Rocha *et al.*, 2014).

The results of Obouayeba *et al.* (2014) indicated that Roselle petals contained alkaloids, anthocyanins, flavonoids, saponins, steroid, sterols and tannins which are the main phytochemical groups with biological activities. Flavonoids have high antioxidant properties which are produced as natural secondary metabolites. These compounds are capable of interact and scavenge free radicals (Heim *et al.*, 2002). It was shown that Hibiscus contains polyphenols of the flavonol and flavanol type in simple or polymerized form. The following flavonoids have been also described in Roselle extracts: hibiscitrin (hibiscetin-3-glucoside), sabdaritrin, gossypitrin, gossytrin and other gossypetin glucosides, quercetin and luteolin; as well as chlorogenic acid, protocatechuic acid, pelargonidic acid, eugenol, quercetin, luteolin and the sterols β-sitosterol and ergosterol. The presence of these flavonol glycosides was low, with hibiscitrin being the major compound followed by gossypitrin and sabdaritrin. Kim *et al.* (2013) reported that the levels of active ingredients such as flavonoids are influenced by

climatic conditions in plants. The anthocyanins are a group of flavonoid derivatives and natural pigments present in the dried flowers of *Roselle* and their color varies with pH (Da-Costa-Rocha *et al.*, 2014). Anthocyanin pigments play an important role in improving plant tolerance to stress. Delphinidin and cyanidin-based anthocynins, include delphinidin-3-sambubioside (hibiscin), cyanidin-3-sambubioside (gossypicyanin), cyanidin-3, 5-diglucoside, delphinidin (anthocyanidin) has been found to be main anthocyanins in Roselle. Rezaei and Bagherian (2009) stated that anthocyanin concentration is a function of temperature patterns and planting date, in cotton.

Peroxidases and catalase activity was as 3.13 U mg^{-1} protein and 0.05 U mg^{-1} protein, respectively. POD and CAT as two main antioxidant enzymes convert hydrogen peroxide into water and in the case of catalase to oxygen and water. A high significant correlation was observed between CAT activity and POD activities at $P<0.01$.

Data showed that hibiscus have antioxidant enzymes activity and a significant amount of polyphenols including flavonoids and anthocyanin which may employed as natural antioxidant sources in some industrial processes.

3.2. Antioxidant Activity

In the present study, we measured the potential antioxidant activity of *H. sabdarifa* calices extracts for correlative study with antioxidant compounds. Antioxidant capacity of the methanolic extract was examined using three different assays because of inaccuracy of single universal method. Free radical scavenging properties of methanolic extract was measured by phosphomolybdate assay; DPPH assay and ferric reducing antioxidant power (Table 2).

The phosphomolybdenum assay is a quantitative method and it works based on the reduction of Mo (VI) to Mo(V) in presence of antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acidic pH and at higher temperature. Total antioxidant capacity was reported as ascorbic acid equivalents. The method is utilized for the spectrophotometric quantitation of total antioxidant capacity and employs cost-effective reagents. The value of extract total antioxidant capacity was as 27.70 µg AE g^{-1} extract (Table 2).

Determining antioxidant activity of a sample extract based on the scavenging effects of DPPH radical is one of the routinely employed antioxidant assays (Mak *et al.*, 2013). Methanolic extracts of *H. sabdariffa* exhibited antioxidant activity against DPPH radical in a concentration-dependent manner (Figure 1). IC_{50} value was also calculated from a calibration curve for the extract. The lower IC_{50} value reflects to higher antioxidant activity of plant extracts. IC_{50} value of the extract was determined as 150.06 µg ml^{-1} (Table 2).

Table 2. Antioxidant activities of hibiscus calyx extract

TAC µg AAE g^{-1} extract	IC50 RDSC mg AA g^{-1} extract	FRAP mg AA g^{-1} extract
27±0.7	55.9±0.0	1.93±0.11

Data show the mean±SD of three replicates. TAC: total antioxidant capacity, RDSC: DPPH radical scavenging capacity, FRAP: ferric reducing antioxidant power.

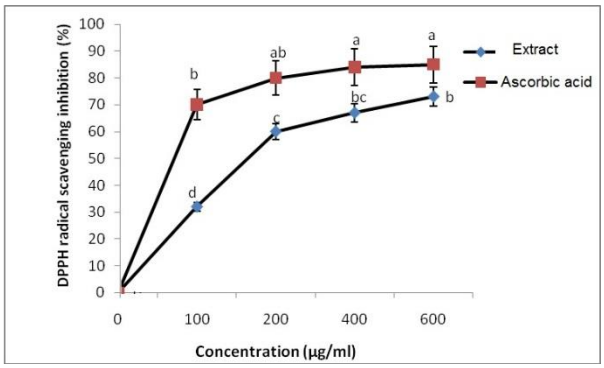

Fig 1. Antioxidant activity of hibiscus calix extracts determined by DPPH assay

Data were presented as Mean±SE (n=3). Same letters on each point-according to Duncan's multiple range test are not significantly different ($P<0.05$). DPPH: 2,2-diphenyl-1-picrylhydrazyl

The bleaching of the DPPH coloration is an indication of the free radical scavenging capacity of the samples. However, in this assay, this activity was about two times lower than that of ascorbic acid (IC50 = 73.9 µg ml^{-1}), used as a standard. In line with this, the total phenolic content of extracts assayed by the Folin–Ciocalteu method revealed that ethanolic extract exhibit high total phenolic content (TPC) (Table 1). The results obtained in this study clearly demonstrate that methanolic extract of hibiscus calyx, has a stronger antioxidant activity by DPPH radical scavenging comparing to total antioxidant capacity based on

reduction of Mo (VI), and ferric reducing antioxidant power of the extract.

Table 3. Pearson`s correlation coefficient of polyphenols and antioxidant activity in Roselle calyx.

	TPC	FC	AC	POD	CAT	TAC	RDSC	FRAP
TPC	1							
FC	0.915*	1						
AC	0.995*	0.872	1					
POD	-0.995*	0.872	-1.000**	1				
CAT	-0.995*	-0.872	-1.000**	1.000**	1			
TAC	0.995*	0.872	1.000**	-1.000**	-1.000**	1		
RDSC	0.996*	.949*	0.982	0.982	0.982	0.982	1	
FRAP	0.989*	0.964	0.971	0.971	0.971	-0.971	0.999*	1

The ferric reducing power of the sample was assayed and the results are shown in Table 2. In FRAP assay, antioxidant capacity is evaluated based on the capability of the sample extracts to reduce ferric tripyridyltriazine (Fe (III)-TPTZ) complexes to ferrous tripyridyltriazine (Fe (II)-TPTZ). As can be seen from the results, the ferric reducing power of methanolic extract was found as 1.93mg AA g^{-1} extract. The correlation between antioxidant activity with their reducing powers of ferric ions have been reported before for certain plant extracts (Maksimović *et al.*, 2005). Previous studies have also proven the effectiveness of the petal extract of *H. sabdariffa* compared with the reference standard antioxidant ascorbic acid using DPPH method. It was reported that the high antioxidant activity observed with extracts of petals in Roselle would be linked to the presence of polyphenolic compounds such as phenolic acids, flavonoids and anthocyanins (Obouayeba *et al.*, 2014).

Based on Pearson correlation analysis (Table 3) there was a positive significant correlation between the levels of TPC and FC, AC, TAC, RDSC and FRAP at $P<0.05$. Therefore, at the higher content of total phenolics, the higher content of flavonoids, anthocyanin and antioxidant activity is expected and it seems that higher radical scavenging activity might be attributed to the presence of high phenolics. In addition to TPC, DPPH radical scavenging capacity of the calyx extract was also correlated to flavonoids content. While ferric reducing antioxidant power showed a significant correlation with anthocyanin content of the extract. In the other word, the increase in anthocyanin content cause increasing power of reducing ferric radicals of the hibiscus calices extract. CAT and POD activity showed a significant negative correlation with total phenolic content, anthocyanin content and total antioxidant capacity of the extract. It was reported that the antioxidant capacity is highly correlated with the polyphenolic compounds contents in plants (Obouayeba *et al.*, 2014). Numerous studies have described a positive correlation between the antioxidant activity and phenolic content in plants (Maksimović *et al.*, 2005; Odabasoglu *et al.*, 2004; Voon *et al.*, 2012). Thus, in this research, rather to catalase and peroxidase activity, the high antioxidant activity observed with extracts of calices in Roselle would be linked to the presence of polyphenolic compounds such as phenolic acids, flavonoids and anthocyanins. In *Monodora myristica* it was shown that antioxidant enzymes activity such as catalase activity is significantly decreased in the control compared to the under stress condition. Therefore during normal condition in Roselle, it seems that antioxidant activities of calyx extract is highly dependent to polyphenolic compounds rather to enzymatic antioxidant system. Lako *et al.* (2007) attributed antioxidant power of fruit, vegetables and other readily available plants to their total polyphenol content, total anthocyanin content and major flavonol and carotenoids. The antioxidant activity of polyphenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Obouayeba *et al.*, 2014).

4. Conclusion

The present study has demonstrated that extract of *H. sabdariffa* L. (sabdariffa) is rich in total phenolic, flavonoid content, and anthocyanins. The species have antioxidant activity based on reduction of Mo(VI), scavenging the DPPH radicals, ferric reducing power. Thus, *H. sabdariffa* L. (sabdariffa) may be suggested as a potential source of natural antioxidants and excellent source of dietary phytochemicals such as anthocyanins, flavonoids and phenolic acids in food and pharmalogical industry.

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6. References

Aebi, H. 1984. Catalase in vitro. *Methods in Enzymology*, 105: 121-26.

Ali, B.H., Wabel, N.A., Blunden, G. 2005. Phytochemical, pharmacological and toxicological aspects of Hibiscus sabdariffa L.: a review. *Phytotherapy Research*, 19: 369-75.

Balasundram, N., Sundram, K., Samman, S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99: 191-203.

Begmeier, D., Berres, P.H.D., Filippi, D., Bilibio, D., Bettiol, V.R., Priamo, W.L. 2014. Extraction of total polyphenols from hibiscus (Hibiscus sabdariffa L.) and waxweed/'sete-sangrias'(Cuphea carthagenensis) and evaluation of their antioxidant potential. *Acta Scientiarum Technology*, 36: 545-51.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-54.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., Heinrich, M. 2014. *Hibiscus sabdariffa* L.–A phytochemical and pharmacological review. *Food Chemistry*, 165: 424-43.

El-Boraie, F., Gaber, A., Abdel-Rahman, G. 2009. Optimizing irrigation schedule to maximize water use efficiency of Hibiscus sabdariffa under Shalatien conditions. *World Journal Agricultural Science*, 5: 504-14.

Farombi, E.O., 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: 1120-28.

Gao, X., Ohlander, M., Jeppsson, N., Björk, L., Trajkovski, V. 2000. Changes in antioxidant effects and their relationship to phytonutrients in fruits of sea buckthorn (*Hippophae rhamnoides* L.) during maturation. *Journal of Agricultural and Food Chemistry*, 48: 1485-90.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry*, 13: 572-84.

Hirunpanich, V., Utaipat, A., Morales, N.P., Bunyapraphatsara, N., Sato, H., Herunsalee, A. et al. 2005. Antioxidant effects of aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn.(Roselle) in vitro using rat low-density lipoprotein (LDL). *Biological and Pharmaceutical Bulletin*, 28: 481-84.

Islam, M., Islam, A., Islam, F.Fakir, M. 2008. Effect of Planting Date on Canopy Characters and Capsule Production in *Hibiscus sabdariffa* (var. sabdariffa) Ground in Boundaries of Rice Field. *Journal of Agronomy for Environment*, 2: 1-6.

Khurana, S., Piche, M., Hollingsworth, A., Venkataraman, K., Tai, T. 2013. Oxidative stress and cardiovascular health: therapeutic potential of polyphenols 1. *Canadian Journal of Physiology and Pharmacology*, 91: 198-212.

Kim, Y.J., Lee, J.-H.Kim, S.-J. 2013. Cultivation characteristics and flavonoid contents of wormwood (Artemisia montana Pamp.). *Journal of Agricultural Chemistry and Environment*, 2: 117.

Lako, J., Trenerry, V.C., Wahlqvist, M., Wattanapenpaiboon, N., Sotheeswaran, S.Premier, R. 2007. Phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Food Chemistry*, 101: 1727-41.

Mak, Y.W., Chuah, L.O., Ahmad, R., Bhat, R. 2013. Antioxidant and antibacterial activities of hibiscus (*Hibiscus rosa-sinensis* L.) and Cassia (*Senna bicapsulari*s L.) flower extracts. *Journal of King Saud University-Science*, 25: 275-82.

Maksimović, Z., Malenčić, Đ., Kovačević, N. 2005. Polyphenol contents and antioxidant activity of Maydis stigma extracts. *Bioresource Technology*, 96: 873-77.

Mancinelli, A.L.Rabino, I. 1985. Effectiveness of intermittent light treatments on anthocyanin synthesis in dark-grown and light-pretreated seedlings. *Plant Physiology*, 78: 203-06.

Mariod, A., Ibrahim, R., Ismail, M.Ismail, N. 2012. Antioxidant activity of phenolic extracts from kenaf (Hibiscus cannabinus) seedcake. *Grasas y Aceites*, 63: 167-74.

Obouayeba, A., Djyh, N., Diabate, S., Djaman, A., N'guessan, J., Kone, M. et al. 2014. Phytochemical and antioxidant activity of Roselle (Hibiscus sabdariffa L.) petal extracts. *Research Journal Of*

Pharmcology Biology Chemistry Science, 5: 1453-65.

Odabasoglu, F., Aslan, A., Cakir, A., Suleyman, H., Karagoz, Y., Halici, M. et al. 2004. Comparison of antioxidant activity and phenolic content of three lichen species. *Phytotherapy Research,* 18: 938-41.

Oyaizu, M. 1986. Studies on products of browning reaction--antioxidative activities of products of browning reaction prepared from glucosamine. *Eiyogaku zasshi= Japanese Journal of Nutrition,*

Plewa, M.J., Smith, S.R., Wagner, E.D. 1991. Diethyldithiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhibiting tobacco cell peroxidase. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 247: 57-64.

Prieto, P., Pineda, M., Aguilar, M. 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry,* 269: 337-41.

Proestos, C., Lytoudi, K., Mavromelanidou, O.K., Zoumpoulakis, P., Sinanoglou, V.J. 2013. Antioxidant capacity of selected plant extracts and their essential oils. *Antioxidants,* 2: 11-22.

Rezaei, M., Bagherian, F. 2009. Influence of planting date and sulfur coating in seed coating solution (SCS) on cotton (Gossypium hirsutum L.) seeds: physiological traits. *Physiology,* 4: 917-23.

Salem, M.Z., Olivares-Pérez, J., Salem, A. 2014. Studies on biological activities and phytochemicals composition of Hibiscus species-A review. *Life Science Journal,* 11:

Sánchez-Moreno, C., Larrauri, J.A., Saura-Calixto, F. 1999. Free radical scavenging capacity of selected red, rose and white wines. *Journal of the Science of Food and Agriculture,* 79: 1301-04.

Škrovánková, S., Mišurcová, L., Machu, L. 2012. 3 Antioxidant Activity and Protecting Health Effects of Common Medicinal Plants. *Advances in Food and Nutrition Research,* 67: 75.

Tomás-Barberán, F.A.Espin, J.C. 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture,* 81: 853-76.

Tsai, P.-J., McIntosh, J., Pearce, P., Camden, B., Jordan, B.R. 2002. Anthocyanin and antioxidant capacity in Roselle (Hibiscus sabdariffa L.) extract. *Food Research International,* 35: 351-56.

Tseng, T.-H., Kao, E.-S., Chu, C.-Y., Chou, F.-P., Wu, H.-W.L., Wang, C.-J. 1997. Protective effects of dried flower extracts of Hibiscus sabdariffa L. against oxidative stress in rat primary hepatocytes. *Food and Chemical Toxicology,* 35: 1159-64.

Voon, H.C., Bhat, R., Rusul, G. 2012. Flower extracts and their essential oils as potential antimicrobial agents for food uses and pharmaceutical applications. *Comprehensive Reviews in Food Science and Food Safety,* 11: 34-55.

Yang, L., Gou, Y., Zhao, T., Zhao, J., Li, F., Zhang, B. et al. 2014. Antioxidant capacity of extracts from calyx fruits of roselle (Hibiscus sabdariffa L.). *African Journal of Biotechnology,* 11: 4063-68.

Zengin, G., Aktumsek, A., Guler, G.O., Cakmak, Y.S.Yildiztugay, E. 2011. Antioxidant properties of methanolic extract and fatty acid composition of Centaurea urvillei DC. subsp. hayekiana Wagenitz. *Records in Natural Products,* 5: 123-32.