In-vitro antioxidant activity and phytochemical analysis in extracts of *Hibiscus rosa-sinensis* stem and leaves

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ABSTRACT

Free radicals induce numerous diseases by lipid peroxidation, and DNA damage. It has been reported that numerous plant extracts have antioxidant activities to scavenge free radicals. In the present study, the antioxidant properties of crude (aqueous and methanolic) extract of *Hibiscus rosa sinensis* (Malvaceae) were studied in six *in vitro* models viz. radical scavenging activity by DPPH reduction Assay, Scavenging of SO, H_2O_2 and NO, reducing power, FRAP assay. The extract was found to contain large amounts of phenolic compounds and flavonoids. Methanolic extract of *Hibiscus rosa-sinensis* possessed significant antioxidant activity as compare to aqueous extract. These results suggest that hibiscus has potential to develop a new functional dietary agent to treat chronic metabolic diseases, such as diabetes and hyperlipidemia.

Keywords: *Hibiscus rosa-sinensis*, antioxidant capacity, scavenging activity, *in-vitro* models.

INTRODUCTION

Free radicals are chemical species which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Living cells generate free radicals and other reactive oxygen species (ROS) by-products as a result of physiological and biochemical processes.

In recent years, focus on plant research has increased all over the world. Collected evidences showed immense potential of medicinal plants used in various traditional systems, for their biological activities and antioxidant principles.[1]

Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defence system against disease or more accurately, to protect against disease. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more.[2]

Hibiscus rosa sinensis (Malvaceae) is an ornamental plant often planted as a fence or hedge plant. The flowers have been reported to possess anti-implantation and anti spermatogenic activities.[3,4] The extracts of *Hibiscus rosa sinensis* have also been shown a protective effect against the tumour promotion stage of cancer development. The leaves and flowers are observed to be promoters of hair growth and aid in healing of ulcer.[5] Aerial part of *H. rosa sinensis* has calcium channel blocking action.[6]

Total antioxidant activities of plant extracts cannot be evaluated by using one single method, due to the complex composition of phytochemicals as well as of oxidative processes. The present study was designed to determine the phytochemical constituents and *in vitro* antioxidant activity of methanolic and aqueous extracts from leaves

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and stem of *Hibiscus rosa sinensis* through a number of testing methods.

MATERIALS AND METHODS

Plant material and preparation of extracts

Fresh, young leaves and stem of *Hibiscus rosa sinensis* were collected from local area, authenticated, washed and allowed to dry at room temperature. The dried leaves and stem were then ground to fine powder. 30 g of the dry powder was weighed and was used for extract preparation. Extracts for the plant leaves were prepared using both methanol and distilled water. 30 g of the dry powder was ground to a paste in pestle and mortar using 150 ml of the respective solvents and was filtered twice through Whatman filter paper. The resulting filtrate was collected in a beaker and was subjected to evaporation in a Rotary Evaporator for 10 min at 100°C (for aqueous extraction) and 60°C (for methanolic extraction). The extract was suitably diluted for use.

ESTIMATION OF PHYTOCHEMICAL CONSTITUENTS

1. Estimation of total phenol content (TPC)

The total phenol content was determined by Folin-Ciocalteu reagent method.[7] 0.5 ml of extract (1:5 dilution) and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 15 min. 2.5 ml saturated sodium carbonate was added, incubated for 30 min at room temperature and absorbance was measured at 760 nm. The total phenol content was expressed in terms of Gallic acid equivalent (mg/g).[8]

2. Estimation of total flavonoids (TF)

The total flavonoid content was determined by Aluminum chloride method.[10] The reaction mixture (3.0 ml) that comprised of 1.0 ml of extract (1:10 dilution), 0.5 ml of aluminum chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) was incubated at room temperature for 30 min and absorbance was measured at 415 nm. The total flavonoid content was expressed in terms of quercitin equivalent (mg/g).[10]

3. Estimation of sugars

Estimation of sugars in the extract was done by DNSA method.[9] 1 ml of diluted extract was added to 1 ml DNSA, mixed and heated for 5 min. 2 ml of distilled water was added to the mixture and absorbance was measured at 525 nm. Total sugars were expressed in terms of maltose equivalent (mg/g).

4. Estimation of tannins[8]

The tannin content was determined by Folin-Ciocalteu reagent method. 1:10 diluted extract was added to Folin-Ciocalteu reagent (0.5 N), mixed and incubated at room temperature for 15 min. 2.5 ml saturated sodium carbonate was added, incubated for 30 min at room temperature and absorbance measured at 760 nm. Tannins were expressed in terms of tannic acid equivalent (mg/g).

5. Estimation of chlorophyll and carotene[11]

1 g of leaf sample was weighed and was ground in pestle and mortar with 5 ml distilled water to a paste. The contents were transferred to a centrifuge tube and the total volume was made up to 10 ml with distilled water. 0.5 ml from the tube was transferred to a tube containing 4.5 ml of 80% acetone. The contents were centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was measured at the following wavelengths-645, 663, 490, 638 nm and the content of chlorophyll was calculated.

EVALUATION OF ANTIOXIDANT ACTIVITY

1. α, α-diphenyl-β-picryl-hydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity was measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picrylhydrazyl by the method of McCune and Johns.[12] The reaction mixture consisted of 1.0 ml of DPPH in methanol (0.3 mM) and 1.0 ml of the extract. After incubation for 10 min in dark, the absorbance was measured at 517 nm. DPPH scavenging activity was expressed in terms ascorbic acid equivalent (mg/g).[8]

2. Nitric oxide (NO) radical scavenging assay

3.0 ml of sodium nitroprusside in phosphate buffer (10 mM) was added to 2.0 ml of extract (1:200 dilutions). The resulting solution was then incubated at 25°C for 60 min. To 5.0 ml of the incubated sample, 5.0 ml of Griess reagent (1% sulphanilamide, 0.1% naphthyethylene diamine dihydrochloride in 2% H_3PO_3) was added and absorbance of the chromophore formed was measured at 540 nm.[8,13] NO radical scavenging activity was expressed in terms of ascorbic acid equivalent (mg/g).

3. Ferric reducing antioxidant power (FRAP) assay

0.2 ml of the extract (1:20 dilution) was added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20 mM $FeCl_3$. $6H_2O$ solution) and the reaction mixture was incubated at 37°C for 30 min and the increase in absorbance at 593 nm was measured.[8] The antioxidant capacity based on the ability to reduce ferric ions of sample was expressed in terms of ascorbic acid equivalent (mg/g).

4. Estimation of reducing power (RP)

The reducing power was determined by the method of Athukorala.[14] 1.0 ml extract was mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (600 mM) was added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm.[8] RP was expressed in terms of standard ascorbic acid (mg/g).

5. Superoxide anion (SO) radical scavenging assay

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski.[15] The superoxide anion radicals are generated in 3.0 ml of Tris-HCl buffer, containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH solution (0.936 mM), 1.0 ml extract (1:100 dilution) and 0.5 ml Tris-HCl buffer (16 mM, pH 8). The reaction was started by adding 0.5 ml PMS solution to the mixture, incubated at 25°C for 5 min and then the absorbance was measured at 560 nm. Ascorbic acid was used as standard (0.1–0.5 mg/ml.[8] SO anion scavenging activity was expressed in terms of standard equivalent (mg/g).

6. Hydrogen peroxide (H_2O_2) radical scavenging assay

The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch.[16] A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM, pH 7.4) and 2 ml of the solution is added to 1 ml extract (1:20 dilution). The absorbance at 230 nm is determined after 10 mins.[8] H_2O_2 radical scavenging activity was expressed in terms of ascorbic acid equivalent (mg/g).

RESULTS AND DISCUSSION

Antioxidants orchestrate many biologic responses to inflammation and immunity, they function as signalling mechanisms for redox regulation, even minimal levels of oxidative stress is highly sensed and the protective antioxidant mechanism is set into action which is essential for the maintenance of the structural integrity of proteins. In recent years, attention has been focused on the antioxidant properties of plant- derived dietary constituents of food.[17]

Our preliminary phytochemical analysis for *H. rosa sinensis* stem and leaves (Table 1) revealed the presence of high content of phenolics, carbohydrates, flavonoids, and tannins in aqueous and methanolic extracts. Phytochemical studies have revealed the presence of several chemicals, including flavonoids, flavonoid glycosides, hibiscetin, cyanidine, cyanidin glucosides, taraxeryl acetate, β-sitosterol, campesterol, stigmasterol, ergosterol, citric, tartaric and oxalic acids, cyclopropenoids and anthocyanin pigments.[18]

Polyphenol are the major plant compounds with high level of antioxidant activity. This activity is due to their ability to adsorb, neutralize and to quench free radicals.[19] Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid per oxidation.[20]

Total phenolics, flavonoids and tannins in methanolic extract of leaves were highest among all extracts. Since phenolics and flavonoids are responsible for the antioxidant activity, and high amount present in the extract indicates good antioxidant activity. Flavonoids like kaempferol and quercitin have been shown to exhibit the action through effects on membrane permeability, and

Table 1 Phytochemical constituents in *Hibiscus rosa-sinensis* stem and leaf extracts

Chemical constituents	Standard equivalent in methanolic extract (mg/g)		Standard equivalent in aqueous extract (mg/g)	
	Stem	Leaves	Stem	Leaves
Total phenol content	1.4 ± 0.17	2.55 ± 0.15	1.8 ± 0.15	1.45 ± 0.08
Total flavonoids	1.17 ± 0.04	3.0 ± 0.075	1.1 ± 0.11	1.1 ± 0.16
Sugar content	2.25 ± 0.0	1.5 ± 0.0	5.25 ± 0.75	1.0 ± 0.43
Tannin content	0.62 ± 0.02	1.17 ± 0.07	0.72 ± 0.02	0.57 ± 0.07

(The results obtained were expressed as Mean ± S.D. of triplicates)

by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2.[21]

Reductant like Quercitin glycoside, flavonoid glycosides and cynadin glycoside content in the form of reducing agents was highest in *Hibiscus* stem (aqueous) extract. Like phenols, polysaccharides also exhibit varied bio-activities such as antitumor, anticancer, antiviral, antibacterial, antifungal, anticoagulant and immunological activity as reported by Xu et al.[22]

Chlorophyll has been suggested as an effective antioxidant since it scavenges free radicals such as 1, 1-diphenyl-2-picrylhydrazyl.[23] Carotenes have the ability to detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of the photosynthetic complexes by light. In terms of its antioxidant properties carotenoids can protect the photosystems in one of four ways – by reacting with lipid peroxidation products to terminate chain reactions or by scavenging singlet oxygen and dissipating the energy as heat or by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen or by the dissipation of excess excitation energy through the xanthophyll cycle.[24] The total chlorophyll content in hibiscus leaves was found to be 0.017 g/l and the carotene content was 0.166 g/l.

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. It is known that free radical cause auto-oxidation of unsaturated lipids in food.[25] On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of lipid. Ascorbic acid, tartaric acid and oxalic acid are natural antioxidants present in hibiscus extracts.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[27] Aqueous leaf extract displayed striking DPPH radical scavenging activities then methanolic leaf and stem extract (Table 2) that might be attributed to their hydrogen donating ability. Presence of cyclopropenoids, taraxeryl acetate attributes to their reducing capability.

The toxicity and damage caused by NO and O_2 is multiplied as they react to produce reactive peroxynitrile ($ONOO^-$), which leads to serious toxic reactions with bio molecules. In our study the crude aqueous extract of *H. rosa sinensis* showed a remarkable nitric oxide radical scavenging activity (Table 2). It is well documented that

Table 2 Antioxidant activity of *Hibiscus rosa-sinensis* stem and leaf extracts

Tests	Standard equivalent in methanolic extract (mg/g)		Standard equivalent in aqueous extract (mg/g)	
	Stem	Leaves	Stem	Leaves
DPPH scavenging assay	9.75 ± 1.15	11.85 ± 3.01	15.1 ± 4.5	34.5 ± 4.65
No radical scavenging assay	7.2 ± 0.15	6.68 ± 0.12	6.05 ± 0.42	12.3 ± 0.12
Frap assay	11.0 ± 0.31	15.4 ± 0.47	10.9 ± 1.7	6.0 ± 0.3
Reducing power assay	0.3 ± 0.0	2.2 ± 0.07	2.07 ± 0.12	0.82 ± 0.32
So radical scavenging assay	65.78 ± 2.4	67.65 ± 1.89	65 ± 0.46	63.8 ± 1.08
H_2O_2 Radical scavenging assay	32.4 ± 0.3	23.0 ± 0.46	32.9 ± 0.17	32.7 ± 0.3

(The results obtained were expressed as Mean ± S.D. of triplicates)

NO plays a crucial role in the pathogenesis of inflammation where it is secreted as a mediator, this may explain the use of *H. rosa* extract for the treatment of inflammatory diseases.[28,29]

Superoxide anion plays an important role in plant tissues and is involved in the formation of other cell-damaging free radicals.[30] All extract exhibited excellent superoxide anion scavenging activity, which can be correlated with high content of flavonoids like hibiscetin, cyanidine, cyclopropenoides in extracts. Study suggested that the flavonoids may be involved in the dismutation of superoxide anion radical.[31]

Hydrogen peroxide though a weak oxidizing agent is important because of its ability to penetrate biological membranes, once inside the cell it can probably react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.[32] Like superoxide anion, all extract showed excellent H_2O_2 scavenging activity. Leaves and stems contain β-sitsterol, stigmasterol, taraxeryl acetate and three cyclopropane compounds and their derivatives responsible for antioxidant activity.

FRAP assay directly measured antioxidant or reductants in a sample that react with ferric tripyridyltriazine (Fe^{3+} TPTZ) complex and produce colored ferrous tripyridyltriazine (Fe^{2+} TPTZ). The antioxidant ability of hibiscus varied significantly (Table 2) the leaf methanolic extract showed higher FRAP antioxidant activity. The phenolic compounds exhibited reduction properties by acting as

reducing agents, hydrogen donators and singlet oxygen quenchers.[33]

Reducing power of plant extract was reported to be directly correlated with its antioxidant activity and is based on the presence of reductants like Quercetin-3, 5-diglucoside and cyanidin-3-sophoroside-5-glucoside which exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom.[34,35] Reducing power is highest in Hibiscus leaf (methanolic) extract.

It is reported that the antioxidant activity of putative antioxidants have been attributed to various mechanisms among which are: prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging power.[36]

CONCLUSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times. Present study shows that poly-phenols content in the methanolic leaf extracts of *Hibiscus rosa sinensis* is high and these extracts exhibit strong antioxidant activities compared to that of the standard compounds. The results would help to ascertain the potency of the crude extract from *H. rosa sinensis* as potential source of natural antioxidants. It can be used for minimizing or preventing lipid oxidation in pharmaceutical products and retarding the formation of toxic oxidation products. *H. rosa sinensis* has been used for the treatment of a variety of diseases. It is an easily available plant for natural remedies. Further studies on definitive mechanisms of its chemotherapeutic activities and potential effects *in vivo* are warranted.

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