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Original Article

Quantification of Total Phenolic Content, HPLC Analysis of Flavonoids and Assessment of Antioxidant and Anti-haemolytic Activities of *Hibiscus rosa-sinensis* L. Flowers *in vitro*

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ABSTRACT

Objective: *Hibiscus rosa sinensis* Linn. is widely available and employed by practitioners of natural health for variety of ailments. The present study aimed to investigate total phenolic content (TPC), analysis of flavonoid constituents using HPLC and assessment of antioxidant and anti-haemolytic activities of *Hibiscus rosa-sinensis* L. flowers *in vitro*
Methods: In this study, free radical scavenging activity of *Hibiscus rosa-sinensis* L. flowers extract was evaluated using 1,1-diphenyl-2-picrylhydrazyl assay (DPPH). Total phenolic compounds and flavonoids were determined using Folin- Ciocalteu method and HPLC respectively. The flower extract at various concentrations was subsequently incubated with erythrocytes and analysed for hydrogen peroxide induced haemolysis and lipid peroxidation as indices of erythrocyte damage.
Results: Results of the study revealed that the flower extract tested exhibited excellent antioxidant, hydrogen peroxide and superoxide radicals scavenging activities with IC_{50} values of 28.41±1.7, 36.69±2.3 and 32.32±2.5 µg mL^{-1}, respectively. The contents of four types of flavonoids, namely rutin, quercetin, kaempferol and myricetin in methanol extract were found to be 4104.0, 7.6, 361.9 and 50.7 µg g^{-1}. Rutin was identified as major flavonoid of the *Hibiscus* flower and reported for the first time. The extract also significantly reduced hydrogen peroxide induced haemolysis and lipid peroxidation *in vitro*.
Conclusion: In conclusion, the findings show that *Hibiscus rosa-sinensis* L. flowers possess significant antioxidant and anti-haemolytic activities. The study further revealed that phenolics and flavonoids may be the main contributors to the antioxidant and anti-haemolytic activities exhibited by the *Hibiscus rosa-sinensis*. Thus, the flowers have great potential to be used in the development of functional ingredients/foods that are currently in demand for the health benefits associated with their use.
Keywords: *Hibiscus rosa-sinensis* flower, Total phenolics, Flavonoids, Rutin, Antioxidant, Anti-haemolytic

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1. INTRODUCTION

Traditional knowledge of medicinal plants has given clues to the discovery of valuable drugs and forms an integral part of complementary and alternative medicine (CAM). Traditional medicinal plants are often cheaper, locally available and easily consumable, raw or as simple medicinal preparations. According to World Health Organization ~80% of the earth inhabitants rely on traditional medicine for their primary health care needs and most of this therapy involves the use of plant extracts or their active components [1,2]. Recently, there has been a renaissance of interest and use of medicinal plant products. Various medicinal plants have been identified and studied using modern scientific approaches and the results revealed the potential of medicinal plants in the field of pharmacology[3].

The herb *Hibiscus rosa-sinensis* Linn [Malvaceae] is a glabrous shrub widely cultivated in the tropics and subtropics as an ornamental plant in gardens and has several forms with varying colours of flowers. In medicine, however, the red flowered variety is preferred. The flowers of *Hibiscus* have been reported in the ancient Indian medicinal literature with beneficial effects in various ailments[4]. In South Asian traditional medicine, various parts of the plant are used in the preparation of a variety of foods. The flowers are considered to be aphrodisiac, emollient and emmenagogic and are used in bronchial catarrh, diarrhoea and fertility control[5]. The flowers contain substantial quantities of flavonoids, proanthocyanidins, anthocyanins [6], β-carotene[7] which are associated anticonvulsant[8], antioxidant [9]anti diabetic [10],antipyretic, analgesic, spasmolytic[11] and anticancer[12-15]activities. The acidic polysaccharides possess wound healing and immunomodulating properties [16]. However, there is a lack of information concerning the total phenolic content, flavonoids profile and anti haemolytic effect of *Hibiscus rosa-sinensis* L.

Based on the ancient practices, traditional uses and efficacy of this flower, present study was designed i) to determine the total phenolic content, ii) HPLC analysis of four types of flavonoids, *viz.* kaempferol, myricetin quercetin and rutin in methanol extract, iii) *in vitro* antioxidant capacity and iv) Evaluation of the anti haemolytic effect of *Hibiscus rosa-sinensis* flower extract (HFE) on hydrogen peroxide induced haemolysis and lipid peroxidation *in vitro.*

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The chemicals nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), standard flavonoids of USP quercetin, kaempferol, rutin and myricetin (HPLC standards) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid and Gallic acid were purchased from Hi Media, India. All other chemicals and solvents used were of analytical grade and highest purity.

2.2 Collection of Plant material and preparation of extract

The flowers of *Hibiscus rosa-sinensis* (Family: Malvaceae; wild-type, red flowered variety, single petals) were collected from a local garden in southern part of India (Kanchepuram District, Tamil Nadu, India) and the pharmacognostic authentication was done by Siddha Central Research Institute, Department of AYUSH, Ministry of Health and Family Welfare, Government of India, Chennai-600 106. The flowers were cleaned; shade dried and then powdered using a laboratory scale mill. Powdered flower (50 g) was extracted three times with 500 mL of 80% methanol (MeOH)/H_2O while being macerated at room temperature for 24 h each time. The extracts were filtered under vacuum using Buchner funnel lined up with Whatman No.1 filter paper. The solvent was eliminated under vacuum using a rotary evaporator at 40 °C. The extracts were used for analysis of TPC, HPLC identification of flavonoids, antioxidant and anti-haemolytic activities.

2.3 Quantification of total phenolic content

Total phenolic contents were determined using Folin-Ciocalteu reagent as described by Liu et al., and Cai et al., with slight modifications [17,18]. The HFE (200 µL) was mixed with 1.5 mL of Folin-Ciocalteu reagent (1:10 diluted with distilled water) and allowed to stand at room temperature for 5 min. The reaction was then neutralised with saturated sodium carbonate solution (1.5 mL, 75 g L^{-1}) and allowed to stand for 2 h in the dark at room temperature. The absorbance of the blue colour developed was measured at 760 nm using a UV-Visible spectrophotometer (SICAN 2310/ Incarp, Japan). Total phenolics were quantified by calibration curve obtained from measuring the absorbance of the known concentrations of gallic acid standard solutions. The results were expressed as milligram of gallic acid equivalent (GAE) per gram extract (mg GAE g^{-1}).

2.4 HPLC analysis of Flavonoids

The flavonoid components of HFE were determined by HPLC analysis using a SHIMADZU, (SPD M20A/LC-20AT**)** system with a diode array detector. The HPLC method employed a 4.6 mm × 25cm column that contains packing L1. The HFE was filtered through a 0.45 µm filter disc and separately injected equal volumes (about 20 µL) of each of the standard solutions of USP quercetin, kaemferol, rutin and myricetin and the sample solution. The flow rate was about 1.5 mL per minute. The chromatography was monitored at 274 nm and UV spectra were collected to confirm peak purity.

2.5 Determination of DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the extract, as well as positive control Vitamin C (ascorbic acid) and BHT (butylated hydroxytoluene) was measured using the method of Shimada et al. and Yang et al., [19,20] but slightly modified as follows: Different concentrations (10, 20, 40, 80 and 160 µg mL^{-1}) of extract solution (2 mL) was mixed with 1mL of methanolic solution containing DPPH radicals, with a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and maintained for 30 min in dark. After 30 minutes, absorbance at 517 nm was measured against methanol Spectrophotometrically (SICAN 2310/ Incarp, Japan). Controls containing methanol instead of the sample and blank containing methanol instead of DPPH solution were also measured. Positive controls Ascorbic acid and BHT were used for comparative purpose. The inhibition of the DPPH radical by the samples was calculated according to the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{1 - (\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of control}} \times 100$$

2.6 Assay of H_2O_2 and superoxide($O_2^{\bullet-}$) radical scavenging activity

The ability of methanolic extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al. [21]. A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (0.1M, pH 7.4). Extracts samples (10, 20, 40, 80 and 160 µg mL^{-1}) in 80% methanol were added to a hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of both extracts and standard compounds was determined using the formula:

$$\% \text{ Scavenged } H_2O_2 = ([Ac - As] / Ac) \times 100$$

where Ac is Absorbance of the control, and As is absorbance of the sample or standard (BHA).

The assay for superoxide anion radical scavenging activity was based on a riboflavin-light-NBT system [22]. The reaction mixture contained 0.5mL of 50 mM phosphate buffer (pH 7.6), 0.3 mL riboflavin (50 mM), 0.25mL PMS (20 mM), and 0.1mL NBT (0.5 mM), prior to the addition of 1mL methanolic extract solution. Reaction was started by illuminating the reaction mixture with different concentrations of the methanolic extracts using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. The absorbance of the control was determined by replacing the sample with methanol. Butylated hydroxyanisole (BHA) was used as a control. The percent inhibition of superoxide anion generation was calculated using the following formula,

$$\text{Scavenging activity (\%)} = ([Ac - As] / Ac) \times 100$$

where Ac is absorbance of the control, and As is absorbance of the sample or standard (BHA)

The IC_{50} values were calculated using Lagrange Interpolation Calculator $p(x)$

$$= y_1 \frac{(x - x_2)(x - x_3)\ldots(x - x_n)}{(x_1 - x_2)(x_1 - x_3)\ldots(x_1 - x_n)} + y_2 \frac{(x - x_1)(x - x_3)\ldots(x - x_n)}{(x_2 - x_1)(x_2 - x_3)\ldots(x_2 - x_n)}$$

$$+ \cdots + y_n \frac{(x - x_1)(x - x_2)\ldots(x - x_{n-1})}{(x_n - x_1)(x_n - x_2)\ldots(x_n - x_{n-1})}$$

2.7. Anti haemolytic study in vitro

2.7.1. Collection of blood and isolation of red blood cells (RBC)

Venous blood samples were collected from healthy volunteers (20-22 years of age) after obtaining informed consent and transferred to heparinised tubes. The blood samples were centrifuged at 4 ºC for 10 minutes at 1000 g to remove plasma and buffy coat. The red blood cells were washed thrice with 0.2 M phosphate buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$ and 5 mM glucose, pH 7.4) and a 5 % V/V RBC suspension in PBS (pH 7.4) was used for in vitro studies.

2.7.2. In vitro haemolysis study

The extent of haemolysis induced by addition of hydrogen peroxide was determined by the method of Grinberg et al. [23]. Briefly, aliquots of 2.0 ml of RBC suspension were delivered into the test tubes followed by addition of 1.0 mL of hydrogen peroxide (100 µM) in order to induce haemolysis. The extract (0.25, 0.50, 0.75, 1.00 and 2.00 mg/mL) was added and the contents were swirled gently and incubated for 150 minutes at 37 ºC under aerobic conditions. After incubation, the tubes were centrifuged at 1,000 g for 10 min and the extent of haemolysis was measured spectrophotometrically at 540 nm by comparing the extracellular haemoglobin content of the aliquots with that of a fully haemolysed reference sample, which was prepared in the same way except that H_2O_2 solution was replaced by 1% Triton X solution. RBC suspension exposed to H_2O_2 (without the extract) was also tested for haemolysis. Percentage haemolysis was calculated according to the following equation:

$$\text{PercentageHaemolysis} = \frac{\text{A (Hb Absorbance)}}{\text{B (Hb 100\% Absorbance)}} \times 100$$

where A is the absorbance of the sample aliquot at 540 nm and B is that of the fully haemolysed reference sample at 540 nm.

The retarding effect of HFE on H_2O_2 induced oxidative haemolysis of RBCs was obtained according to the following equation:

$$\text{Percentage Retardation of Haemolysis} = (A - B)/A \times 100$$

where A- % hemolysis of H_2O_2 induced RBC; B-% hemolysis of HFE treated RBC

2.7.3. Measurement of RBC lipid peroxidation

The effect of HFE on hydrogen peroxide-induced lipid peroxidation in RBC was determined by the method of Tedesco et al. [24]. Aliquots of saline washed RBCs (2.0 mL) and one mL of 100 µM H_2O_2 were incubated with varying concentrations (0.25, 0.50, 0.75, 1.00 and 2.00 mg mL^{-1}) of HFE for 1 hour at 37 ºC under aerobic conditions. Control samples without the extract were also incubated. After the requisite time of incubation, the mixtures were treated with 10% TCA (trichloro acetic acid) and then centrifuged. Aliquot of 0.5 ml of thiobarbituric acid (TBA) (1% TBA in 0.05 M sodium hydroxide) was added to the supernatants removed from the tubes. The mixtures were boiled for 1 hour, cooled, and the absorbance at 535 nm was determined. A standard curve was prepared using 1,1,3',3' tetra methoxypropane. Lipid peroxidation levels were expressed as nmoles thiobarbituric acid reactive substances/mgHb (nmol TBARS/ mgHb). Haemoglobin content was measured by the method of Drabkin and Austin [25].

2.8 Analysis of data

All determinations of antioxidant capacity by DPPH, H_2O_2 and superoxide scavenging assays, anti haemolytic, lipid peroxidation and measurements of TPC were conducted in triplicate. The reported value for each sample was calculated as the mean of three measurements and expressed as mean± SD. Where appropriate, data were analysed using (SPSS software package, version 16) analysis of variance (ANOVA). Values of p <0.05 were considered as significant.

3. RESULTS

3.1 Total phenolic content

In recent years, attention has been focused on the antioxidant properties of plant-derived dietary constituents of food. Phenolics and flavonoids are secondary metabolites widely distributed in plants. They have numerous biological and pharmacological properties that could potentially afford protection against chronic diseases [26,27]. In the present study the phenolics content of HFE was found to be 42.38 ± 2.66 mg gallic acid equivalent (GAE) per gram of extract (Table 1).

Table 1: Percent yield of methanolic extract and total phenolics in methanolic extract of *Hibiscus rosa -sinensis* L. flowers

Yield of Methanolic extract (%)[a]	Total phenol content (mg gallic acid equivalent /g)[a]
17.24 ± 2.36	42.38 ± 2.66

[a] Data expressed as the mean ± standard deviation. (n=3)

3.2 HPLC analysis of Flavonoids

In the present study, the representative flavonoids viz. quercetin, kaempferol, rutin and myricetin present in Hibiscus flower extract (HFE) were analysed using High Performance Liquid Chromatography (HPLC). The results showed that the tested plant extract possessed rutin as a major flavonoid constituent (4104 µg/g) and quercetin, kaempferol and myricetin concentrations were found to be 7.6 µg/g, 361.9 µg/g and 50.7 µg/g, respectively. The retention times, peak area (including standards) and concentrations of various flavonoids identified in this study are depicted in the Figures 1A, 1B and Table 2

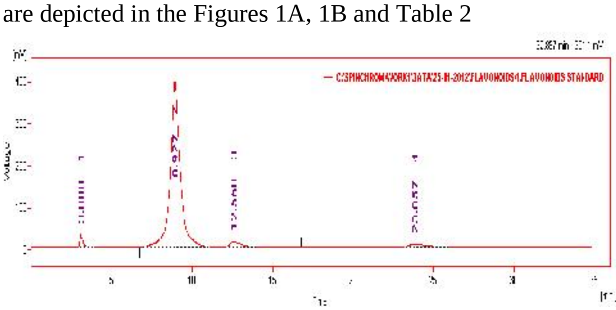

Fig 1A: High-performance liquid chromatogram of four kinds of standard flavonoids. Peaks: 1- Rutin (retention time 3.080); 2- Quercetin (retention time 8.927); 3- Kaempferol (retention time 12.56) and 4- myricetin (retention time 23.857).

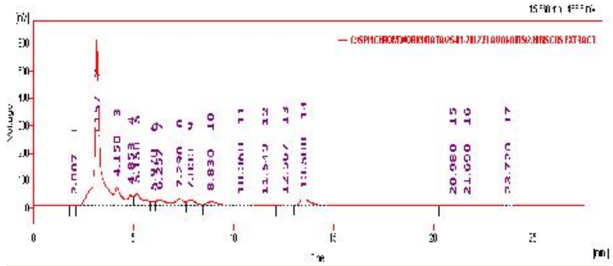

Fig 1B: High-performance liquid chromatogram of HFE. Peaks: 1- Rutin (retention time 3.080); 2- Quercetin (retention time 8.927); 3- Kaempferol (retention time 12.56) and 4- myricetin (retention time 23.857).

Table 2: The retention times, peak area (including standards) and concentrations of various flavonoids identified in *Hibiscus rosa- sinensis* flowers extract.

	Retention times		Peak area		Concentration	% of each
Flavonoids	Standard	Sample	Standard	Sample	(µg g^{-1})	flavonoid
Rutin	3.080	3.157	465.820	11308.772	4104.00	90.71
Quercetin	8.927	8.830	18009.483	814.718	7.60	0.17
Kaempferol	12.560	12.567	841.105	180.028	361.90	7.80
Myricetin	23.857	23.720	1043.843	313.316	50.70	1.12
Total					4524.20	

3.3 DPPH radical-scavenging activity

The DPPH radical-scavenging assay is a commonly used method for evaluating the ability of plant extracts to scavenge free radicals generated from DPPH reagent [28]. The extent of the reaction depends on the hydrogen-donating ability of the antioxidant. As can be seen in Table 3, the scavenging activity of the extract tested was concentration-dependent. The methanol extract, which contained the highest amount of total phenolics and flavonoids, showed a significant effect in inhibiting DPPH, reaching 78.9±2.6% at a concentration of 80 µg/mL, which was lower than the free radical-scavenging activity of reference compounds (ascorbic acid and BHT). The IC_{50} (50% inhibitory concentration) value of the extract was found to be 28.41±1.7 µg/mL.

Table 3 :Free radical scavenging effects of methanolic extract of *Hibiscus rosa-sinensis* flowers on DPPH

L- ascorbic acid[a]		Butylated hydroxytoluene[b]		*H. rosa sinensis* Extract	
Concentration (µg mL^{-1})	Percent inhibition	Concentration (µg mL^{-1})	Percent inhibition	Concentration (µg mL^{-1})	Percent inhibition
1.25	18.3±0.9	1.25	20.8±1.3	10	26.3±2.1
2.5	28.6±1.2	2.5	31.4±1.7	20	37.4±1.6
5.0	55.6±2.9	5.0	53.2±1.6	40	61.7±2.4
10.0	94.6±3.1	10.0	96.8±2.6	80	78.9±2.6
20.0	95.7±2.8	20.0	98.2±2.7	160	94.2±2.8

[a,b] Reference compounds

Data expressed as the mean ± standard deviation. (n=3)

3.4 Hydrogen peroxide and superoxide radical scavenging Assay

HFE was capable of scavenging hydrogen peroxide in a concentration dependent manner. The extract significantly scavenged up to 78.7±3.2% hydrogen peroxide at a concentration of 80 µg/mL (Table 4).

Table 4 :Hydrogen peroxide scavenging activity of methanolic extract of *Hibiscus rosa- sinensis* flowers compared with a reference compound (BHA)

Butylated hydroxyanisole (BHA)[a]		*H. rosa sinensis* Extract	
Concentration ($\mu g\ mL^{-1}$)	Percent inhibition	Concentration ($\mu g\ mL^{-1}$)	Percent inhibition
1.25	21.8±1.9	10	23.3±2.3
2.5	31.4±1.8	20	34.2±2.8
5.0	58.2±2.3	40	53.8±2.4
10.0	96.8±2.9	80	78.7±3.2
20.0	98.2±3.1	160	92.8±3.3

[a] A reference compound
Data expressed as the mean ± standard deviation. (n=3)

The ability of the extract to scavenge superoxide was compared with that of a reference compound BHA and presented in Figures 2A and 2B, respectively. The percentage inhibition of superoxide anion generation by the methanol extract was comparable to that of BHA. For example, the IC_{50} values of the methanol extract was found to be 32.32±2.5 μg/mL.

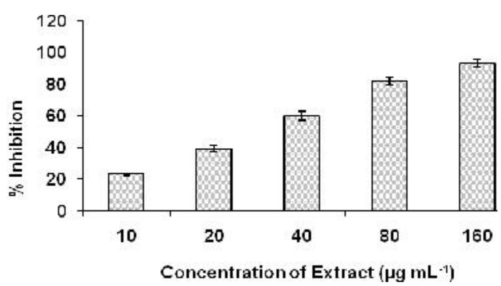

Fig 2A: Effect of varying concentrations of *Hibiscus rosa-sinensis* flower extract on superoxide scavenging activity. Values shown are mean ± standard deviation.

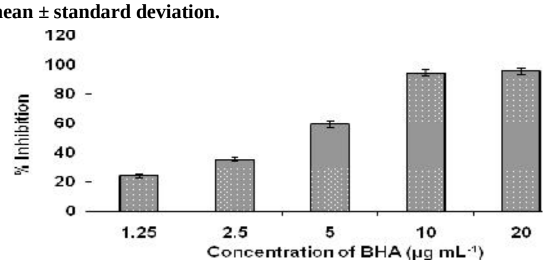

Fig 2B: Superoxide scavenging activity of reference compound Butylated hydroxyanisole (BHA). Values shown are mean ± standard deviation.

The IC_{50} values of the extract for DPPH, hydrogen peroxide and superoxide radical scavenging were calculated using Lagrange Interpolation Calculator and were presented in Table 5.

Table 5: EC_{50} values of reference compounds and methanolic extract of *Hibiscus rosa-sinensis* flowers using three various antioxidant assays

Parameter	% DPPH scavenging			% H_2O_2 scavenging		% Inhibition of $O^{2\cdot-}$	
	Ascorbic acid	BHT	Extract	BHA	Extract	BHA	Extract
IC_{50} value[a]	8.54±0.7	6.93±0.62	8.41±1.7	6.72±1.13	6.69±2.3	8.62±0.8	32.32±2.5

Data expressed as the mean ± standard deviation. (n=3)

[a] IC_{50} value was determined to be the effective concentrations at which DPPH, hydrogen peroxide and superoxide were scavenged by 50%, respectively. The EC_{50} value was obtained by Lagrange Interpolation Calculator.

3.5 Anti haemolytic study

The ability of the HFE to inhibit hydrogen peroxide induced erythrocyte haemolysis and lipid peroxidation was as well concentration dependent and depicted in the Table 6 and Figure 3 respectively. It was found out that when red blood cells were treated with H_2O_2 along with the extract, marked reduction in the haemolysis was observed than that of cells treated with the H_2O_2 alone. When cells were treated with the extract alone, no haemolysis was obtained explaining the non toxic behaviour of the extracts on erythrocytes. In case of lipid peroxidation, simultaneous addition of plant extract to H_2O_2 treated cell decreased the extent of lipid peroxidation.

Table 6: Effect of varying concentrations of *Hibiscus rosa-sinensis* extract on hydrogen peroxide-induced haemolysis of human erythrocytes.

Concentration of Extract mg mL^{-1}	% haemolysis of RBC treated with		% haemolysis of RBC treated with H_2O_2 only	% retardation of haemolysis
	HFE only	HFE+ H_2O_2		
0.25	18.3 ± 1.6	56.8 ± 3.7		11.62 ± 2.6
0.50	16.6 ± 1.5	53.6 ± 4.8		18.28 ± 3.1
0.75	17.5 ± 1.4	51.6 ± 4.6	63.4 ± 4.6	22.86 ± 3.6
1.00	16.4 ± 1.3	46.3 ± 3.9		36.93 ± 4.2
2.00	15.2 ± 1.7	44.5 ± 4.2		42.47 ± 4.4

Data expressed as the mean ± standard deviation. (n=3).

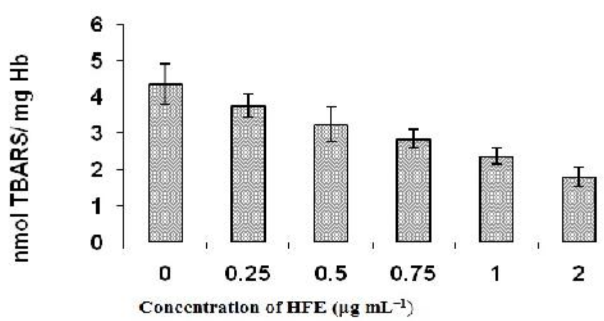

Fig 3 : Effects of varying amounts Hibiscus *rosa-sinensis* flower extract on hydrogen peroxide-induced lipid peroxidation of erythrocytes. Values shown are mean ± standard deviation.

4. DISCUSSION

The world is rich with natural and unique medicinal plants. Medicinal plants are now getting more attention than ever because they have potential of myriad benefits to mankind, especially in the line of medicine and pharmacological.

4.1 Total Phenolics

Phenolic compounds have been proved to be responsible for the antioxidant activity. In the present study the phenolic content of HFE was found to be 42.38 ± 2.66 mg gallic acid equivalent (GAE) per gram extract.

The most important phytochemicals in plant foods are phenolics. These phenolic compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. Moreover, their bioactivities may be related to their ability to inhibit lipoxygenase and scavenge free radicals [29,30]. Probably the most important natural phenolics are flavonoids, which contain hydroxyl functional groups, because of their broad spectrum of chemical and biological activities, responsible for antioxidant effect of the plants [31]. So, the true antioxidant potential is often more accurately revealed by expressing antioxidant activity in terms of phenolics and flavonoids content.Therefore, in this study, the obtained level of phenolics and flavonoid in HFE may be a sign to suggest that the extract may possess antioxidant activity. Our suggestion is in close agreement with previous reports that there is a strong correlation between the total phenolic and flavonoids content and antioxidant activity of extract from plant [18,32].

4.2 HPLC analysis of Flavonoids

Flavonoids are a class of secondary plant phenolics with powerful antioxidant activities. The HPLC analysis of HFE revealed the presence of rutin (peak 2), quercetin (peak 10), kaempferol (peak 13), myricetin (peak 17) and other flavonoids compounds as demonstrated by the numerous surrounding peaks (1,3-9,11,12 and 14-16) in sample chromatogram (Figure 1B). Individual flavonoids concentrations varied greatly between types and rutin was predominant in the extract. In terms of flavonoid composition, rutin, quercetin, kaempferol and myricetin accounted for 90.7 %, 0.17%, 7.85% and 1.12% respectively. The most important classes of phytochemicals in plants are phenolics and there are more than 8000 phenolic phytochemicals [33]. The flavonoids represent about one-half of the 8000 or so recognized phenols and are molecules responsible for the colour of fruit and flowers [34]. One of the more interesting findings in this study regarding the phytochemical content of Hibiscus flowers is that they are relatively high in the flavonols (i.e. rutin). Flavonoids exhibit a wide range of biochemical and pharmacological effects including anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic action, and anti-allergic effects [34,35,36]. Quercetin inhibited oxidation and cytotoxicity of low-density lipoprotein *in vitro* [37]and can reduce risk for coronary heart disease and cancer [38]. An *in vitro* oxidation model showed quercetin, myricetin,and rutin being more powerful antioxidants than the traditional vitamins [33].

Thus, the high content of phenolics and flavonoids may be the main contributors to the antioxidant and anti-haemolytic activities exhibited by the extract.

4.3 DPPH radical-scavenging activity

DPPH is a stable nitrogen-centred free radical, and its colour changes from violet to yellow when are reduced by either the process of hydrogen or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [39].

The methanol extract, which contained the highest amount of total phenolics and flavonoids, showed a significant effect in inhibiting DPPH, reaching 78.9±2.6% at a concentration of 80 µg mL^{-1}. The methanol extract provided a higher IC_{50} than the reference compounds viz. ascorbic acid and BHT. It has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of the sample [40]. The results obtained suggested that some components within the methanol fraction were significantly strong radical scavengers.

4.4 H_2O_2 and Superoxide scavenging Activities

HFE was capable of scavenging hydrogen peroxide in a concentration dependent manner. The extract significantly scavenged up to 78.7±3.2% hydrogen peroxide radicals at a concentration of 80 µg mL^{-1}. Moreover, the superoxide scavenging ability of the HFE at 80 µg mL^{-1} was found to be 81.9 ±2.6 % that of BHA reference was found to be 94.3 ± 2.4 at the concentration of 10 µg mL^{-1}.

It is known that free radical cause auto-oxidation of unsaturated lipids in food [41,42]. 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 [43]. As shown in Table 5, free radical scavenging ability of the HFE was found to decrease in the order: DPPH > superoxide > hydrogen peroxide. Superoxide anion radical is not only one of the strongest reactive oxygen species among the generated free radicals but also a precursor to other active free radicals such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which play an important role in the oxidative damage in proteins, lipids and nucleic acids and thereby inducing tissue damage [44, 45]. Ak and Gulcin have reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical *in vitro* [46]. An up to date study also suggested that the flavonoids may involve the dismutation of superoxide anion radical *in vivo* [47]. Our results showed that HFE inhibited the superoxide radicals in the reaction mixture gradually, in a concentration dependent manner. This scavenging activity of the extract was comparable to that of standard antioxidant BHA suggesting that Hibiscus is a potent scavenger of superoxide. For some extent, hydrogen peroxide itself is not very reactive but has the ability to penetrate biological membranes and it may be toxic to cell because it may give rise to hydroxyl radical which mediates oxidative DNA damage [48]. Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolics, which donate electron to H_2O_2, thus reducing it to water [49]. The scavenging of hydrogen peroxide and superoxide anion radical by HFE was due to the anti-oxidative power of HFE that gained from its phenolic and flavonoids content.

4.5 Anti haemolytic Study

The in vitro oxidative hemolysis of human red blood cells (RBC) was used as a model to study the free radical induced damage of biological membranes and the inhibitory effect of natural antioxidants [50]. The protective effect of HFE on RBC haemolysis was evaluated by oxidative stress induced experimentally using H_2O_2. It was found out that when red blood cells were treated with H_2O_2 along with the extract, marked reduction in the haemolysis and lipid peroxidation was observed than that of cells treated with the H_2O_2 alone.

RBCs are directly exposed to molecular oxygen, have high polyunsaturated fatty acid content in their membranes, and a high cellular concentration of haemoglobin. This makes the RBCs particularly susceptible to oxidative damage. The haemoglobin released from erythrocytes is potentially dangerous because in reacting with H_2O_2 it is not only converted to oxidized forms, but the free hemoglobin exposed to H_2O_2 causes heme degradation with the release of iron ions [51]. H_2O_2 can initiate the formation of free radicals in the presence of iron, described by the Haber–Weiss reaction, and converts the polyunsaturated fatty acids to radicals, which propagate a chain reaction of lipid peroxidation in the presence of oxygen.

Polyphenolic flavonoids are the possible candidates that might explain the antihaemolytic activity of the extract. The data presented in this study show that Hibiscus polyphenol rich extract behave as potent scavenger of reactive oxygen species

5. CONCLUSION

The results of this study indicated that the flowers of *Hibiscus rosa-sinensis* possessed abundant phenolic (42.38 ± 2.66 mg gallic acid equivalent (GAE) per gram) content and exhibited excellent antioxidant activities. HPLC analysis of HFE revealed the presence of rutin, quercetin, kaempferol, myricetin and other flavonoids compounds. The major flavonoid in the extract was identified as rutin. The protective effect of HFE on RBC haemolysis was evaluated by oxidative stress induced experimentally using H_2O_2. In fact, when intact human RBCs were incubated with polyphenol rich extract, a strong protective effect against hydrogen peroxide induced haemolysis and lipid peroxidation was observed. The results of the present study would help to ascertain the potency of the extract from *Hibiscus rosa-sinensis* as potential source of natural antioxidants. It can be used for minimizing or preventing lipid oxidation, slow down the formation of toxic oxidation products and prolonging the shelf life of food and pharmaceuticals. Therefore, the extract from *Hibiscus rosa-sinensis* is worthy of further studies on definitive mechanisms of its therapeutic activities and potential effects *in vivo* are needed.

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2 picrylhydrazyl; NBT,nitrobluetetrazolium; PMS, phenazine methosulfate (PMS), $IC_{50,}$ 50% inhibitory concentration; GAE, gallic acid equivalent; TBARS, thiobarbituric acid reactive substances.

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