Evaluation of comparative antioxidant potential of four cultivars of *Hibiscus rosa-sinensis* L. by HPLC-DPPH method

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ABSTRACT

Introduction: *Hibiscus rosa-sinensis* L., distributed throughout tropics and subtropics, is useful for various purposes like ornamental, religious and therapeutic. Flowers are believed to possess haematinic and hair growth enhancing properties. At times a single plant may exhibit flowers of two different colours, may be a result of chimiral hybridization. It has dazzling cultivar of forms and colours, however the red coloured flowers are said to be best for medicinal use. The plant is reported to possess antioxidant activity. An attempt has been made to study the comparative antioxidant activity, using HPLC-DPPH method, and total phenolic as well as total flavonoid contents of four cultivars i.e. plants with red, pink, white and yellow coloured flowers of *Hibiscus rosa-sinensis*. **Methods:** The methanolic extract of the defatted samples was used for the study. The Total Phenolic content was determined by spectrophotometric method using Folin-Ciocalteu reagent whereas the total flavonoid content was estimated by colorimetric method using aluminium chloride. DPPH free radicals scavenging activity was assessed by HPLC-DPPH method. **Results:** All the cultivars showed presence of considerable amount of phenolic compounds, flavonoids and significant antioxidant activity. A noteworthy variation in phytochemical concentration was observed. The red cultivar revealed highest antioxidant activity, total phenolic and total flavonoid content. **Conclusion:** The result of present study indicated the potential of *Hibiscus rosa-sinensis* flowers as a natural antioxidant. The data revealed significant variations in phytochemical content among its different cultivars and these variations contribute to their studied bioactivity, i.e. *in vitro* antioxidant activity.

Keywords: *Hibiscus rosa-sinensis* L., Antioxidant activity, HPLC-DPPH method, Cultivars comparison.

INTORDUCTION

Hibiscus rosa-sinensis, known as *Japakusum* in Sanskrit, is one of the important medicinal plants occurring throughout India and tropics and subtropical areas of world. This taxon propagates through cutting. Colourful and perennial blossoms of the plant makes it the most ideal ornamental plant which do not require much care and attention of the plant grower. Leaves and flowers are said to be emollient. Flowers are being employed as emmenagogue, sudorific and used to check excessive bleeding in menstruation.[1] They are useful in epilepsy, leprosy, bronchial catarrh and diabetes.[2]

Various parts of the plant have been shown to possess different pharmacological actions. Sharma & Sultana, 2004[3] have reported antiproliferative and anticancer activities of the plant. Roots possess hypolipidemic, antifertility and neuroprotective properties[4–6] whereas leaves exhibited analgesic, antidiabetic, wound healing, and antimutagenic activities.[7–10] Varied pharmacological and biological activities have been reported from the flower of this plant by different workers viz. antihyperlipidemic, wound healing, antimutagenic, anti-anxiety, anticonvulsant, cardioprotective, hypotensive, cholesterol lowering

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and hypoglycaemic.[9–16] Flowers have also shown antibacterial, antioxidant activities as well as inhibitory effects on the alkaline phosphatase enzyme.[17–19] Contradictory actions like hair growth potential[20,21] and hair growth retarding effect have also been reported.[22]

The species has many vibrant colored flowers cultivar; however, red flower is considered more effective for medicinal use.[21] Genetic variations among different cultivars have been thoroughly studied so far to identify the variations[23,24] but nothing has worked out for the phytochemical variations and their potential medicinal efficacy. The present study was aimed to compare the phytochemical content and relative antioxidant activity among four cultivars of *Hibiscus rosa-sinensis* L. flowers with different colours viz. red, pink, white and yellow through a HPLC-DPPH method.

MATERIALS AND METHODS

Collection of plant material

Fresh flowers of the four cultivars of *Hibiscus rosa-sinensis* L. (Family: Malvaceae) were collected (Fig. 1) from the campus of RMD Research and Development Center, Waghaldhara, Valsad (Gujarat) in the month of February and were identified from the Flora of Gujarat.[25] Voucher specimens of red (RMDRDC158), pink (RMDRDC159), white (RMDRDC160) and yellow (RMDRDC161) flowered samples were deposited to the herbaria of RMD Research and Development Centre. Flowers were shed dried, powdered and stored in an airtight container at room temperature for further use.

Chemicals and solvents

All the solvents used for HPLC were of HPLC grade and for others were of AR grade. DPPH was of Sigma, USA while standard Gallic acid and Ascorbic acid were of Lobachemie and Merck respectively.

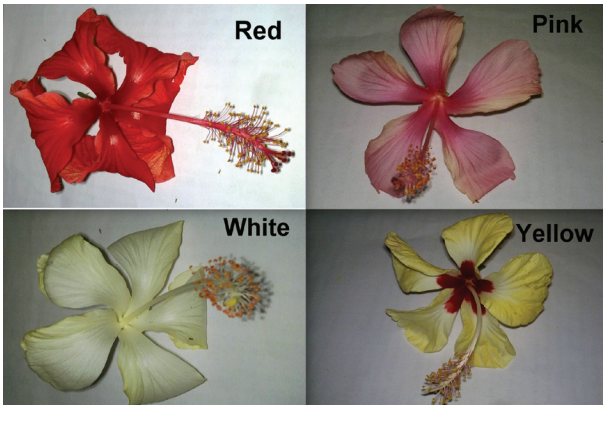

Figure 1. Red, Pink, White and Yellow cultivars of *Hibiscus rosa-sinensis* L. under study.

Extract preparation

100 mg powdered sample was defatted with petroleum ether (60°–80°C) by maceration and the defatted sample was extracted with 20 ml methanol by macerating overnight followed by filtration and making up the volume to 20 ml to obtain a concentration of 5 mg/ml. The extract was used for further study.

Total phenolic content (TPC)

The concentration of phenolics in plant extracts was determined by spectrophotometric method using Folin-Ciocalteu reagent.[26] 0.2 ml extract was diluted to 8.5 ml with distilled water. To this 0.5 ml Folin-Ciocalteu reagent (1:1 dilution made with water) was added. One ml of 20% sodium carbonate solution was added after 3 min and kept for incubation at room temperature. Absorbance was measured at 760 nm, after 60 min against blank. Gallic acid was used as standard and calibration curve was plotted using various concentrations (5–40 μg/ml). Total phenolic content was expressed as mg Gallic acid equivalents per gram of air dried sample. Measurements were done in triplicate (n = 3).

Total flavonoids content (TFC)

Total flavonoid content was estimated by colourimetric method using aluminium chloride. To 0.5 ml extract 2.5 ml distilled water and 3 ml of 5% sodium nitrite solution were added, mixed and allowed to stand for 3 min and 0.3 ml 10% aluminium chloride solution was added. After 6 min 2 ml of 1M NaOH solution was added and final volume was adjusted to 10 ml in each tube. Absorbance was measured at 415 nm after 60 min against blank. Quercetin was used as standard and calibration curve was plotted using various concentrations (100–400 μg). Total flavonoid content was expressed as mg Quercetin equivalents per gram of air dried sample. Measurements were done in triplicate (n = 3).

Antioxidant activity by HPLC-DPPH method

Ability of the plant extract to scavenge DPPH free radicals was assessed by HPLC-DPPH method described by Bhandari *et al.*, 2010 with suitable modifications.[27]

Sample and reagent preparation

DPPH stock solution (1 mM in methanol) was prepared fresh and protected from light. It was further diluted to 0.1 mM with methanol before use.

The stock solution of ascorbic acid (0.1 mg/ml) was prepared in methanol and stored in refrigerator.

Sample solution (0.1 mg/ml) was prepared by diluting stock extract solution (5 mg/ml) with methanol and was used for HPLC analysis.

DPPH scavenging analysis through HPLC

Free radical scavenging activity was evaluated with DPPH stable radical through HPLC. 0.1 ml (1 mg/ml) sample solution was diluted to 1 ml with methanol. To this 2 ml freshly prepared 0.1 mM DPPH solution was added and allowed to react at room temperature (25°C). The reaction mixture was kept in dark to protect from light and after 30 min HPLC analysis was performed.

Chromatography was performed using Waters binary gradient HPLC with PDA detector, on Sunfire C18 RP column (250 × 4.6mm, 5µ) using methanol : water (90:10) as mobile phase in isocratic mode at flow rate of 1 ml min^{-1} and each run time for 10 min, injection volume 20 µl. Monitoring of DPPH peak was done at 517 nm. L-Ascorbic acid was used as positive control. Control was prepared with methanol instead of sample. Difference in the peak area (PA) reduction between the control and sample was used to calculate % inhibition of free radicals using the following equation:

$$\% \text{ inhibition} = [(PA \text{ control} - PA \text{ sample})/PA \text{ control}] \times 100$$

The spectra of DPPH, before and after reaction with the samples/standard, were also recorded.

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation.

RESULTS AND DISCUSSION

Total phenolic content (TPC) and total flavonoid content (TFC)

Literature review has revealed that the flowers of *H. rosa-sinensis* are rich in phenolic compounds and flavonoids.[28] Health benefits of flavonoids are well known. Hence, the total phenolic and total flavonoid content of the samples were determined and expressed in terms of Gallic acid (the standard curve equation: $y = 0.017x + 0.025$, $R^2 = 0.999$) and Quercetin (the standard curve equation: $y = 0.984x + 0.0592$, $R^2 = 0.999$) mg eq./g of air dried sample respectively. The values have been presented in Table 1.

Table 1 Total phenolic and total flavonoid content of *Hibiscus rosa-sinensis* L. flowers

Sample	Total Phenolic content, Gallic acid mg eq./g of air dried sample	Total Flavonoids content, Quercetin mg eq./g of air dried sample
Red cultivar	24.29 ± 0.470	25.50 ± 3.56
Pink cultivar	13.58 ± 0.117	11.75 ± 2.92
White cultivar	10.58 ± 0.058	12.21 ± 2.80
Yellow cultivar	19.56 ± 0.055	14.02 ± 4.92

Values are Mean ± SD., n = 3

Total phenolic contents in the examined cultivars ranged from 10.58 to 24.29 Gallic acid mg eq./g of air dried sample. The red cultivar showed highest concentration of phenols followed by yellow. Pink and white cultivars contain comparatively smaller concentration of phenols.

Total flavonoid contents in the examined cultivars ranged from 11.75 to 25.50 Quercetin mg eq./g of air dried sample. Red cultivar exhibited the highest flavonoids content. Yellow, pink and white cultivars exhibited significantly low flavonoids content as compared to red.

DPPH scavenging activity

The antioxidant activity of different cultivars of *H. rosa-sinensis* was determined using a methanol solution of DPPH reagent. Spectrophotometric method has been used to study anti-oxidant activity of the flowers of *H. rosa-sinensis*, however no reports were found for HPLC-DPPH method.

Optimization of DPPH determination by HPLC-DPPH method in the samples was carried out by using different mobile phases and finally methanol - water (90:10) was selected as mobile phase for the analysis. L-Ascorbic acid was used as positive control. Optimum concentration range of L-ascorbic acid to plot the standard graph of %inhibition of DPPH molecule under prescribed HPLC conditions was determined and the concentration range of 2–10 µg/ml was used for plotting a standard curve ($y = 5.073x + 29.04$, $R^2 = 0.993$).

The antioxidant activity is expressed in terms of percentage of inhibition (%). The activities of the samples were compared with that of standard ascorbic acid.

The chromatograms of the pure DPPH and scavenged DPPH in presence of the extracts have been presented in Figs. 2 and 3 respectively.

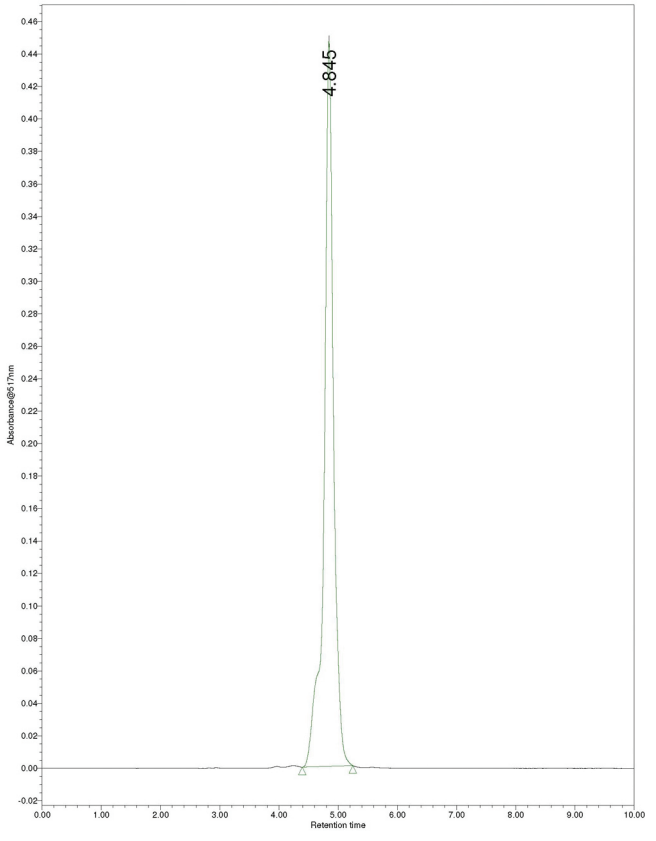

Figure 2. HPLC chromatogram of pure DPPH revealing DPPH peak at Rt 4.845 min.

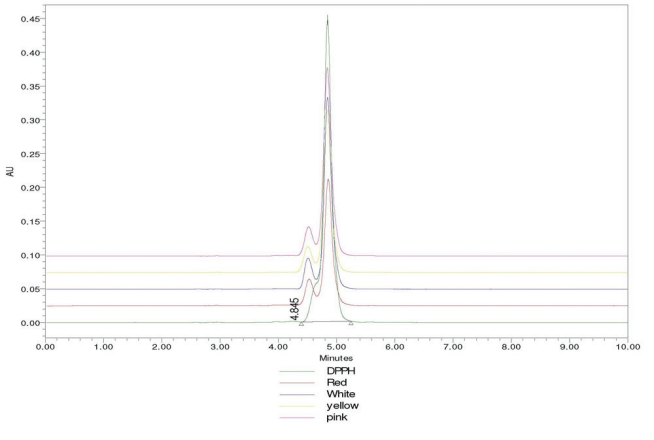

Figure 3. Overlay HPLC chromatogram of DPPH pure and after scavenging in presence of *H. rosa-sinensis* flowers extracts.

The DPPH peak at R_t 4.84 can be seen in all the samples but with varying area. With the increase of DPPH scavenging, the intensity of absorption at 517 nm decreases. The comparative spectra of pure DPPH and scavenged DPPH after reaction with the samples have been presented in Fig. 4. The spectrum of DPPH shows two absorption peaks around 327 and 517 nm. After scavenging the intensity of absorption at 517 nm decreases. Red cultivar

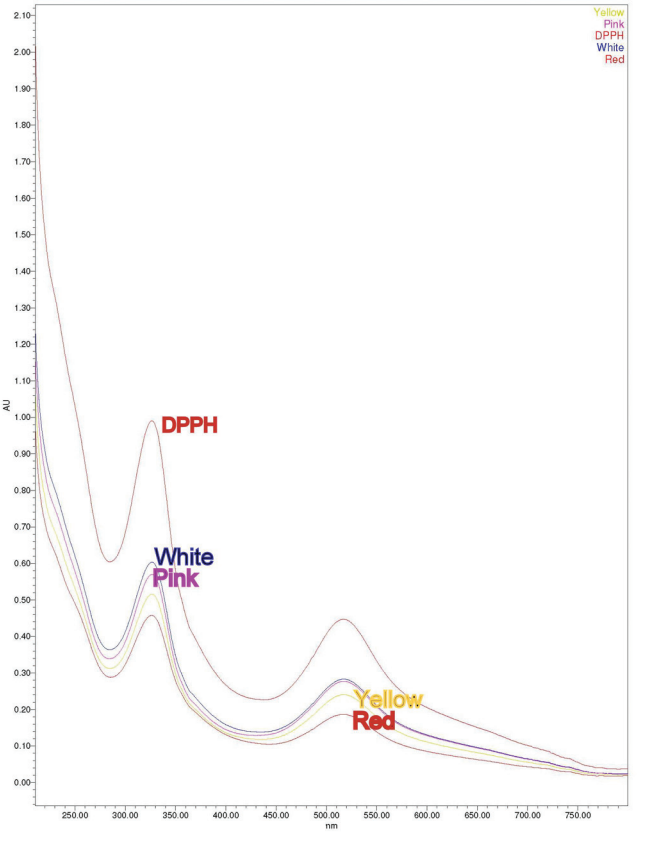

Figure 4. Overlay spectra of pure DPPH and scavenged DPPH by *H. rosa-sinensis* cultivars flowers extracts.

has shown highest peak area reduction of DPPH, indicating highest scavenging activity, followed by yellow, pink and white cultivars respectively.

The % inhibition of free radicals for all the samples as well as standard ascorbic acid was calculated from the area of DPPH in the chromatogram. The data have been presented in Table 2.

The scavenging effect of ascorbic acid, i.e. the positive control, on DPPH radical is 56.38% at 5 µg/ml concentration while that for the samples of 100 µg/ml varies between 30.95 to 55.11%. IC_{50} value for ascorbic acid is 4.13 µg/ml.

Correlation coefficient of Total phenolic content and DPPH scavenging was found to be 0.9441 while that for

Table 2 Free radical scavenging activity of standard and four cultivars of *H. rosa-sinensis*

Standard/Sample	Concentration	% inhibition
Ascorbic acid	5 µg	56.38
Red cultivar	100 µg	55.11
Pink cultivar	100 µg	30.95
White cultivar	100 µg	31.89
Yellow cultivar	100 µg	40.37

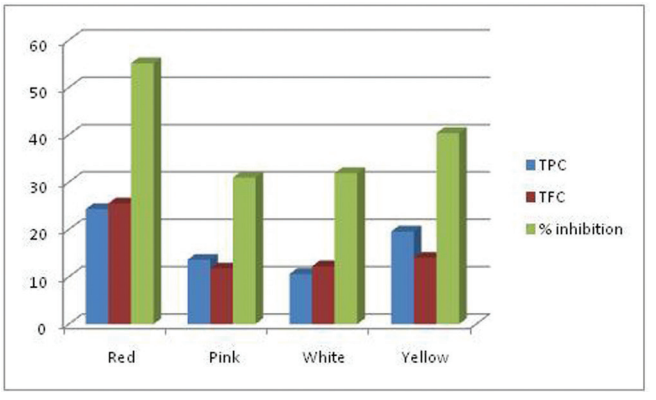

Figure 5. Graphical representation of comparative data of TPC, TFC and DPPH scavenging activity of four *H. rosa-sinensis* cultivars flowers extracts.

total flavonoids content and DPPH scavenging was 0.9717. Comparison of TPC, TFC and DPPH scavenging activity of four *H. rosa-sinensis* cultivars has been presented in Fig. 5.

The data reveals significant difference in the total phenolic and total flavonoids content among examined cultivars. Among the four cultivars, red cultivar is the most superior one as far as total phenolic content, total flavonoid content and antioxidant activity are concerned. Polyphenols and flavonoids are considered to be responsible for anti-oxidant activity.[29] Antioxidant activity is widely used as a parameter to access the biomedicinal value of compounds. As compared to white cultivar the TPC, TFC and %inhibition of DPPH are 129%, 109% and 72.81% respectively more in red cultivar indicating substantial difference in phytochemical contents and biological activity among different cultivars. The data revealed direct relation of total phenolic and total flavonoid content with antioxidant activity.

Genetic variation study carried out with ISSR and isozyme studies also indicated pink flower cultivars as distantly related cultivars with others.[23] Phytochemical analysis and antioxidant activity presently carried out also observed the vast difference among the cultivars under study.

CONCLUSION

The result of present study indicated the potential of *Hibiscus rosa-sinensis* flowers as a natural antioxidant. The data of the present study revealed significant variations in phytochemical content among its different cultivars and these variations contribute to their studied bioactivity, i.e. *in vitro* antioxidant activity. Our study also supports the traditional belief that the red cultivar has better medicinal properties.

The present HPLC-DPPH method is simple and accurate. Moreover, it has advantage over conventional spectrophotometric assay to overcome the shortcomings due to interference of colouring materials in the samples.

The study will help to elucidate the potential therapeutic efficiency based on phytochemical variations, to provide basis for selection of the cultivars to take utmost therapeutic benefits and to prepare a natural pharmaceutical product of high therapeutic value.

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