International Journal of Pharma and Bio Sciences V1(2)2010

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

SACHIN UTTAM RAKESH[1]*, PRIYANKA R. PATIL[2], V. R. SALUNKHE[3].

[1] College of Pharmacy, Medha, At- Jawalwadi, Post- Medha, Tal. Jaoli- 415 012, Dist. Satara, State- Maharashtra, India., [2]Department of Chemical Technology, Dr. B. A. Marathwada University, Dist. Aurangabad, Maharashtra, India, 431001., [3]Govt. College of Pharmacy Karad, Vidyanagar, Karad- 415 124, Satara, Maharashtra, India.

Corresponding Author sachinrakesh@rediffmail.com

ABSTRACT

The free radical scavenging potential of the dried flower of plant *Nymphaea stellata* Willd was studied by using different *in vitro* antioxidant models of screening. The hydroalcoholic extract at 500 µg/ml showed maximum scavenging of the *in vitro* riboflavin NBT assay (26.22%), hydroxyl radical scavenging activity (26.06%), DPPH (25.70%) and nitric oxide radicals (25.11%). However, the extract showed only moderate scavenging activity of total antioxidant capacity (15.25%). DPPH- Radical scavenging activity, Scavenging of superoxide radical, Scavenging of nitric oxide radical, Iron chelating activity, Hydroxyl radical scavenging activity, Rapid screening for antioxidant activity, Total antioxidant capacity and Riboflavin NBT method were used as *in- vitro* antioxidant models for screening of antioxidant potential. The extract showed significant scavenging and IC_{50} value as compared to standard Ascorbic acid. Further investigation is necessary to find out such activities using other solvents for extraction.

KEYWORDS: Free radical, Antioxidant, *Nymphaea stellata*, DPPH.

INTRODUCTION

Nymphaea stellata Willd. (Nymphaceae), a medicinal plant has been mentioned for the treatment of liver disorders in Ayurveda, an ancient system of medicine. The leaves, roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and as cardiotonic, emollient, diuretic, narcotic and as an aphrodisiac[1,2]. The plant also has antihepatotoxic[3], antidiabetic[4], antihyperglycaemic and antihyperlipidaemic[5] activities. The flowers of plant contains[6] flavanoids, gallic acid, astragalin, quercetin and kaempferol. And the seeds also contains[7] proteins, pentosan, mucilage etc. But yet the plant has not been subjected to systematic scientific investigation to assets its antioxidant potential. Therefore it was our intention to investigate antioxidant activity of this plant. There are many evidences indicates that free radical are responsible for birth of many disorders like

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

inflammation, atherosclerosis, diabetes, aging and hepatic toxicities.[8,9,10] The free radicals are formed in many metabolic processes and attack to the membrane lipids, generating lipid radicals. These lipid radicals can combine with oxygen producing peroxy radicals[11]. The peroxy radicals can further cause peroxidation of cellular membrane lipids leading to cell necrosis. It leads to a number of severe pathophysiological conditions[12].

It is believed that the herbal products have less side effects and toxicities. As these herbal product contains mixture of various ingredients. After some period, herbal formulations may undergo various changes in chemical composition. Therefore real time stability study at different intervals should be carried out during storage shedule. Antioxidant potential is one of the parameter to observe the stability of herbal product. Free radical scavenger may prevent the oxidative stress by peroxidation, inhibiting free radicals and also by other mechanism can prevent desease[13].

MATERIALS AND METHODS

All chemicals, solvents were of A. R. and procured from Loba chemie Pvt. Ltd., Mumbai. DPPH was obtained from Sigma chemicals, Bangalore, India. The dried flowers of the plant *N. stellata* Willd. were procured from Satara Ayurvedic Arkshala, Satara and authenticated by authorize Botanist, Y. C. College of Science, Karad, Maharashtra. About 300 gm of the powder was taken in a Soxhlet extractor and extracted with hydroalcohol. The solvent recovered by glass distillation. The residue was concentrated, dried and stored in the dessicator for subsequent use of experiments.

Methods:

1. DPPH assay[14]

To 1ml extract of different concentrations, 1 ml solution of 0.1 mM of DPPH (1, 1-diphenyl-2-picryl hydrazyl) was added. An equal amount of methanol and DPPH solution served as control. After 20 min of incubation in the dark, absorbance was measured at 517 nm. The experiment was repeated for three times.

2. Iron chelating activity[15,16]

To 1 ml of each extract was treated with an equivalent amount of reaction mixture which contains 1 ml, 0.05% 0 – phenanthroline in methanol, 2ml ferric chloride (200 mM). The treated compound was incubated at ambient temperature for 10 min and the absorbance of same was measured at 510 nm. The experiment was repeated for three times.

3. Total antioxidant capacity[17]

To 1 ml of extract of different concentrations was treated with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in eppendorf tube. Capped tubes were incubated in thermal block at 95^0 C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm against blank. The experiment was performed in triplicate.

4. Scavenging of superoxide radical by riboflavin-NBT-system[18,19]

The reaction mixture contains 50 mM phosphate buffer, pH 7.6, 20gm riboflavin, 12 mM NBT. Reaction was started by illuminating the test samples of the extract. The absorbance was measured at 590 nm. The experiment was performed in triplicate.

5. Hydroxyl radical scavenging activity[20]

Few mL of extracts of different concentrations were taken in series test tubes and concentrated on water bath. Then, 1 ml of Fe-EDTA, 0.5 ml of EDTA and 1 ml DMSO were added and the reaction was initiated with adding 0.5 ml ascorbic acid to each of the test tubes. Test tubes were capped properly and heated on water bath at 80^0-90^0 c for 15 min. Then the reaction was terminated by addition of ice-cold TCA (17.5% w/v) to all the test tubes and kept aside for 5 min. The formaldehyde formed was determined by adding 3ml Nash reagent (75 gm ammonium acetate, 3 ml

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

glacial acetic acid, 2 ml acetyl acetone was mixed and raised to 1 liter with distilled water). Resulted reaction mixture was kept aside for 15 min for color development. Intensity of yellow color formed was measured spectrophotometrically at 412 nm against blank reagent. The experiment was repeated for three times.

6. Scavenging of nitric oxide radical[21,22]

As per the standard procedure the absorbance of chromophore formed during diazotization of nitric oxide with sulphanilamide, and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was repeated for three times.

7. Reducing power[23]

10 mg of extract in 1 ml distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and pot. ferricyanide (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 20 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and then the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. The activity was compared with std. ascorbic acid. The experiment was repeated for three times.

8. In vitro non-enzymatic haemoglobin glycosylation[24]

In this method, 5 gm% haemoglobin, in 0.01 M phosphate buffer (pH 7.4) was incubated in presence of 2 gm/ 100 ml concentration of glucose for 72 h in order to find out the best condition for haemoglobin glycosylation. The assay was performed by adding 1 ml of glucose solution, 1 ml of haemoglobin solution and 1ml gentamycin (20 mg/100 ml) in 0.01 M phosphate buffer (pH 7.4) with various concentrations of samples. The mixtures were incubated in dark at room temperature. The degree of glycosylation of haemoglobin in presence of different concentration of fractions and their absorbance were measured colorimetrically.

9. Rapid screening for antioxidant compounds by using TLC[25]

To make a semi-quantitative visualization possible, total hydroalcoholic extracts of *N. stellata* was applied on a TLC plate and developed in solvent system consisting of chloroform : ethyl acetate : formic acid (7.5: 6: 0.5 v/v/v). The plate was then dipped in a 0.2 % solution of DPPH in methanol. The yellow colored spots on stationary phase are an indirect measure of antioxidant activity. The experiment was performed in triplicate.

RESULTS

Several concentrations ranging from 100- 500 µg/ml of the hydroalcoholic extract of *N. stellata* was tested for their antioxidant activity on different *in- vitro* models. It is observed that free radical was scavenged by the test compounds in a concentration dependant manner up to the given concentration in all the models.

In all above the method percentage inhibition was calculated by using formula-

$$\% \text{ Radical scavenged} = \frac{(1 - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Where, control is a sample without test material and test is a sample containing the substance to be tested. The antioxidant activity of hydroalcoholic extract of *N. stellata* was evaluated in different in vitro models. The results of various models are summarized in Table 1. Statistical analysis indicated significant antioxidant activity of hydroalcoholic extract tested. Fig. 1 to 8 shows the reductive capabilities of plant extract in term of ascorbic acid equivalent.

The maximum inhibitory concentration (IC_{50}) in all models viz. DPPH assay, scavenging

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

of nitric oxide radical, total antioxidant capacity, superoxide radical by riboflavin – NBT system activity, Iron chelating activity, hydroxyl radical scavenging activity, reducing power, in vitro non enzymatic haemoglobin glycosylation and scavenging of extract were found to be 25.70 µg/ml, 25.11µg/ml, 15.25µg/ml, 26.22 µg/ml, 22.43 µg/ml, 26.06 µg/ml, 23.47 µg/ml and 18.67 µg/ml.

IC_{50} was calculated by using formula:-

$b = \sum x \cdot y / \sum x^2$

$a = \bar{y} - b\,\bar{x}$

$IC_{50} = a + b\,(50)$.

Where, b = regression coefficient of x on y; a = Intercept of the line, x = Concentration in µg/ml; and y = % scavenging; $\bar{x}$ = mean of the concentration; $\bar{y}$ = mean of the % scavenging.

Table 1.
% Scavenging activity by various in vitro antioxidant models

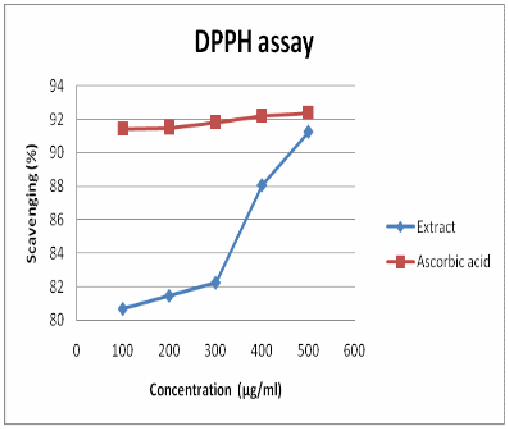
Conc. in µg/ml	DPPH assay		Nitric oxide model		Total antioxidant capacity		Riboflavin NBT method	
	Test	Std.	Test	Std.	Test	Std.	Test	Std.
100	80.68	91.43	67.11	70.43	31.06	51.96	66.80	70.28
200	81.46	91.51	67.23	74.21	54.04	64.04	67.81	70.63
300	82.24	91.82	67.62	77.89	70.63	79.63	69.12	71.59
400	88.08	92.21	67.92	79.85	72.34	84.39	69.27	71.89
500	91.27	92.36	68.21	82.67	73.19	86.49	70.63	72.7

Table 2.

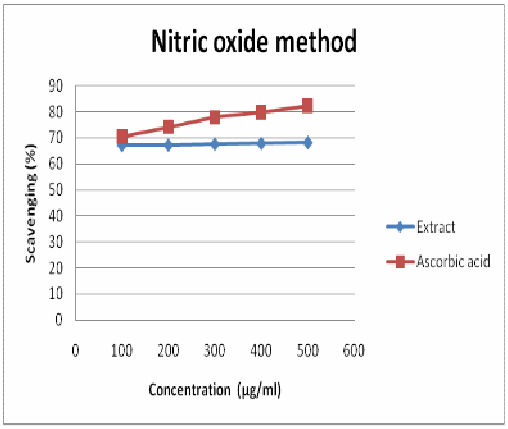
Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

% Scavenging activity by various in vitro antioxidant models

Conc. in µg/ml	Iron chelating method		Hydroxyl radical method		Reducing power method		Hb. Glycosylation assay	
	Test	Std.	Test	Std.	Test	Std.	Test	Std.
100	65.60	70.39	72.61	74.28	74.74	77.02	53.79	58.31
200	66.63	70.62	73.56	74.62	75.43	77.76	55.52	58.75
300	67.19	71.02	73.69	74.95	76.06	77.91	56.52	59.11
400	68.28	71.89	74.11	75.34	76.90	78.39	57.19	59.65
500	69.45	72.50	74.28	75.68	77.39	79.13	57.86	60.23

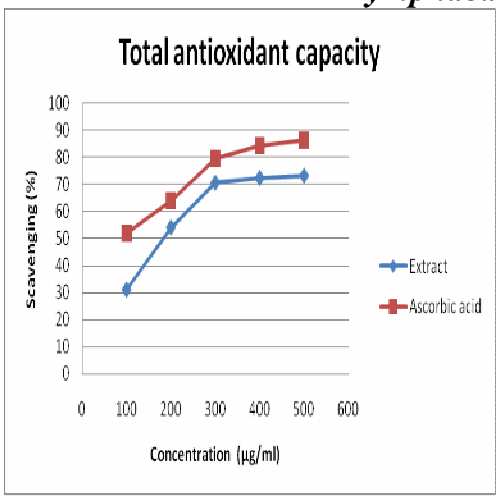


a. DPPH Assay

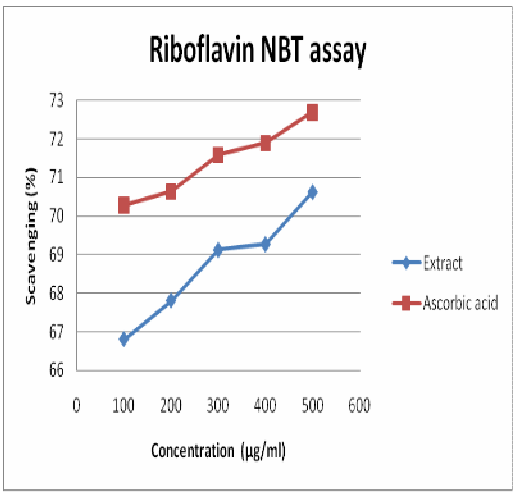


b. Nitric oxide method

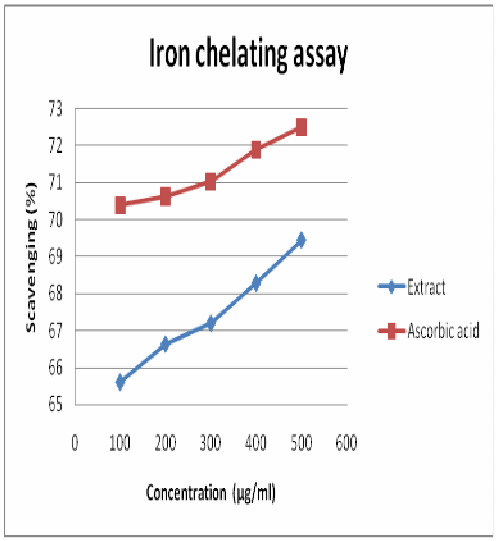
Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.



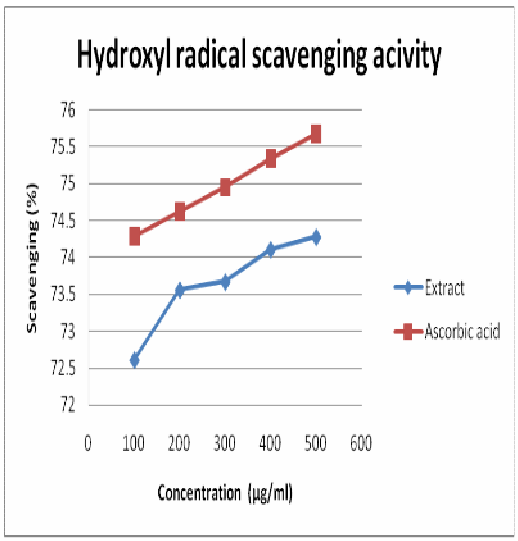
c. Total antioxidant capacity



d. Riblavin NBT asay

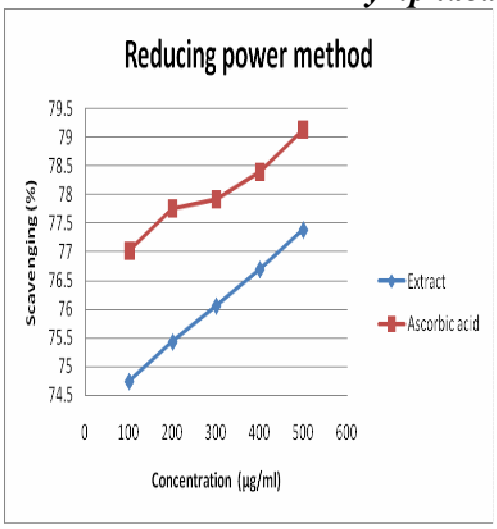


e. Iron chelating assay

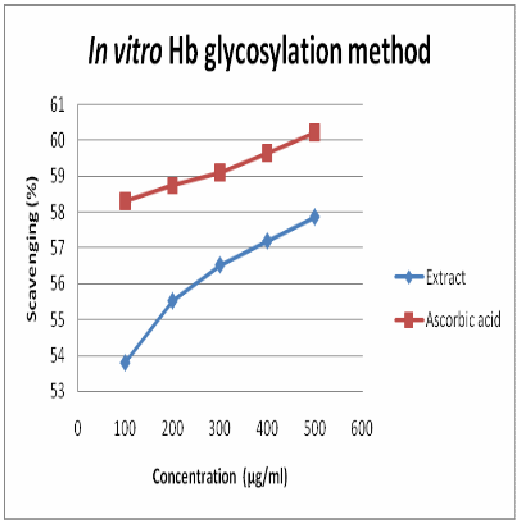


f. Hydroxyl radical scavenging activity

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.



g. Reducing power method



h. *In- vitro* Hb glycosylation method

DISCUSSION

In the present study, we investigated the antioxidant activity of the hydroalcoholic extract of *N. stellata* and the possible mechanism involved, based on the response obtained in the different *in- vitro* models covering major radicals viz., superoxide, hydroxyl and nitric oxide radicals.

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants toward stable radical DPPH. From the present result it may be concluded that, *N. stellata* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in antioxidant principles[15].

The total antioxidant capacity[17] of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrometrically at 695 nm.

The assay of scavenging of superoxide radical was based on the capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in the riboflavin-NBT-system[18, 19]. Superoxide anion which is a reduced form of molecular oxygen has been implicated in initiating oxidation reactions associated with ageing. It plays an important role in the formation of other reactive oxygen species such as H_2O_2, Hydroxyl radical and singlet oxygen, which induced oxidative damage, proteins and DNA. The hydroalcoholic extract was found to be good scavenger of superoxide radical the decrease of absorbance in presence of hydroalcoholic fractions indicates the consumption of superoxide anion in the reaction mixture.

Hydroxyl radicals are most reactive species, initiating the peroxidation of the cell membrane[20]. The lipid radical, thus generate would initiate chain reaction in the presence of oxygen, giving rise to lipid peroxide, which break down to aldehydes such as malondialdehyde, which are known to be mutagenic and carcinogenic.

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases[26]. Nitric oxide is a very unstable species under aerobic condition. It reacts with O_2 to produce stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using Griess reagent. In presence of test compound which is a scavenger the amount of nitrous acid

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

decreases. In the present study, the nitrite produced by incubation of solution of sodium nitroprusside in standard phosphate saline buffer at 25º C was reduced by hydroalcoholic extract of *N. stellata*. This is due to presence of antioxidant principle in the extract which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrites.

The reducing capacity[23] of a compound may serve as a significant indicator of its potential antioxidant activity. For the measurements of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of the hydroalcoholic extract of *N. stellata* using the method of Oyaizu, 1986.

Since non-enzymatic glycosylation of haemoglobin[24] is an oxidation reaction, an oxidation is expected to inhibit this reaction. The degree of glycosylation of haemoglobin in vitro can be measured colorimetrically. The ethanolic extract and its fractions showed potent haemoglobin glycosylation.

Rapid screening for antioxidants by TLC revealed the presence[25] of many constituents with radical scavenging properties in varying proportion in hydroalcoholic fraction. These constituents can be isolated and can be used for future structural elucidation.

CONCLUSION

Thus in the present study antioxidant potential of hydroalcoholic extract of *N. stellata* may be attributed in the presence of flavanoids, phenolic constituent therein. Hence it concludes that, this activity is due to the presence of Gallic acid, Astragalin, Kaempferol and other phenolic moieties present in dried flowers of *N. stellata*.

REFERENCES

1) Nadakarni, K.M. Indian Materia Medica, Vol. I, (3rd Eds) Popular Book Depot, Dhoot Papeshwar: Bombay, 1954, pp. 860.
2) Kirtikar KR, Basu BD. Indian medicinal plants. vol. I Dehradun: International Book Distributors; 1999, pp.114.
3) Manoj R. Bhandarkar, Aqueel Khan. Antihepatotoxic effect of *Nymphaea stellata* willd., against carbon tetrachloride-induced hepatic damage in albino rats, J. Ethnopharmacol 91: 61–64 (2004).
4) Subbulakshmi G, Mridula Naik. Indigenous foods in the treatment of diabetes mellitus, Bombay Hosp J 43: 548-561 (2001).
5) Rajagopal K, Sasikala K. Hypoglycemic and Antihyperglycemic Activity of *Nymphaea stellata* Flowers in Normal and Alloxan Diabetic Rats, Singapore Med J 49 (2): 137 (2008).
6) Kizu H, Tomimori T. Phenolic Constituents from the Flowers of *Nymphaea stellata,* Nat Med 57 (3): 118 (2003).
7) Kapoor, Khan V. P., Raina P. S. H., Farooqui, M. H. Farooqui, M. H. Chemical analysis of seeds from 40 non- leguminous species, Part III., Sci Cult 41: 336 (1975).
8) Marx J L. Oxygen free radicals linked to many diseases, Science 235: 4788, 529 (1987).
9) Pandey S, Sharma, Chaturvedi P and Tripathi B. Protective effect of *R. cordifolia* on lipid peroxide formulation in isolated liver homogenate, Indian J Exp Biol 32: 180 (1994).
10) Malila N, Virtamo J, Virtanen M, et al. Dietary and serum α-tocopherol, β-carotene and retinol, and risk for colorectal cancer in male smokers, Euro J Clin Nut 56: 615 (2002).
11) Geesin J. G., gordan J. S. and Berg R. A. Retinoids affect collagen synthesis through inhibition of ascorbate-induced lipid peroxidation in cultured human dermal fibroblasts, Arch Biochem Biophys 278: 352 (1996).

Free radical scavenging activity of hydroalcoholic extracts of dried flowers of *Nymphaea stellata* Willd.

12) Kappus., H. Sies., Eds. Oxidative Stress, Academic Press: London, 1985 pp. 273.
13) Upadhyay Y. N., Shukla K. P., et al., J. Med. Sci 5: 97 (1964).
14) Sreejayan N and Rao M N A. Free radical scavenging activity of curcuminoids, Drug Res 4: 169 (1996).
15) Benzie I F F and Strain J T. The ferric reducing ability of PLASA (FRAP) as a measure of "antioxidant power". The FRAP assay, Annal Biochem 239: 70 (1996).
16) Benzie I F F and Szeto Y T. Total antioxidant capacity of Teas by the Ferric Reducing/ antioxidant power assay, J Agric Food Chem 47: 633 (1999).
17) Shirwaikar Annie and Govindraju R, et al. Anti-inflammatory activity Lam. Biol Pharm Bull 26: 1424 (2003).
18) Robak J and Gryglewski R J. Flavanoids are scavengers of superoxide anions. Biochem Pharmacol 37: 837 (1998).
19) Beauchamp C and Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylaide gels, Anal Biochem 44: 276 (1971).
20) Halliwell B, Gutteridge J M C and Arnoma O L. The Deoxyribose method: A simple test tube assay for the determination of rate constant for reaction of hydroxyl radical, Anal Biochem 165: 215 (1987).
21) Sreejayan N and Rao M N A. Nitric oxide scavenging by curcuminoids, J Pharm Pharmacol 49: 105 (1997).
22) Shirwaikar Annie and Govindrajau R, et al. Studies on the Antioxidant activities of Desmodium gangeticum, Biol Pharm Bull 26: 1424 (2003).
23) Oyaizu M. Studies on product of browning reaction prepared from glucose amine, Jpn. J. Nutr 44: 307-315 (1986).
24) Yadav SP, Tripathi V, Singh RK, Pandey HP. Antioxidant activity of Cuscuta reflexa Steam, Indian J. Pharm. Sci. 62: 477- 478 (2000).
25) Cuendet M, Hostettmann K and Potterat O. Iridoid glucosides with free radical scavenging properties from Fagraea blumei, Helv Chim Acta. 80: 1144 (1997).
26) Gibanananda Ray and Syed Akhtar Hussain. Oxidants, antioxidants and carcinogenesis, Indian J Exp Biol. 40: 1214 (2002).