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Research Article

AN *IN VITRO* STUDY OF ANTIOXIDANT CAPACITY AND RADICAL SCAVENGING EFFECT OF *SPINACIA OLERACEA* LEAF EXTRACT

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ABSTRACT

Objective: The study was carried out to evaluate the preliminary phytochemical screening and antioxidant activity of ethanolic extract of *Spinacia oleracea* (SO).

Methods: The leaves of SO were shade dried, and the extract was prepared using solvent ethanol by Soxhlet extraction method. The preliminary phytochemical screening was carried out on the leaf extract of the plant. The total phenolic content and total flavonoids were estimated using Folin-Ciocalteu's and aluminum chloride reagents, respectively. Antioxidant activities were studied using 1,1-diphenyl-2-picrylhydrazyl, nitric oxide, hydrogen radical, lipid peroxidation, and phosphomolybdenum radical scavenging assays.

Results: The preliminary phytochemical analysis revealed the presence of bioactive constituents such as phenols, alkaloids, flavonoids, saponins, and glycosides. As SO is a rich source of different bioactive component, it contains a considerable amount of flavonoids and phenols. The different antioxidant assays proved that spinach is one of the best antioxidants with its ability to scavenge different radicals that generate oxidative stress.

Conclusion: The observed activity may be associated with bioactive components such as phenols and flavonoids present in the leaf extracts and could have greater importance as nootropic plant in oxidative stress-related degenerative diseases such as Alzheimer and dementia.

Keywords: *Spinacia oleracea*, Antioxidant activity, 1,1-Diphenyl-2-picrylhydrazyl, Saponins, Flavonoids, Phenols, Anti-Alzheimer activity.

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INTRODUCTION

The oldest form of health care known to humanity is the use of herbs as medicine. It has been used in all cultures throughout the history. In ancient time, tribal people methodically collected information on herbs and developed well-defined herbal pharmacopeias. There is physical evidence of the use of herbal remedies. In the 20th century, much of the pharmacopeia of scientific medicine was derived from the herbal knowledge of the native people. Plants or part of a plant that has been converted into phyto-pharmaceuticals using simple processes involving harvesting, drying, and storage is known as "herbal drug." In addition to the definition, it also includes other crude products derived from plants, which no longer show any organic structure, such as essential oils, fatty oils, resins, and gums [1,2].

The substances that neutralize free radicals or their actions are known as "antioxidants." Every cell is having adequate protective mechanisms to fight against any harmful effects of free radicals such as glutathione peroxidase, glutathione reductase, thioredoxin, thiols, and disulfide bonding. These are buffering systems in every cell. Vitamin E and C, carotenoids, flavonoids, and related polyphenols are essential nutrient which is responsible for preventing the propagation of free radical reactions in all cell membranes in the human body. Nowadays, the demand for natural drug products which produces antioxidants is increasing day by day.

"Oxidative stress" is a concept that defines the relationship between free radical and a disease. The generation of prooxidants in the form of reactive oxygen species and reactive nitrogen species which are present in healthy human body when gets exposed to adverse physicochemical, environmental, or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, and overnutrition the favorable conditions for prooxidants occurs by shifting the delicate balance between prooxidants and antioxidative mechanism in body which further results in the formation of "oxidative stress."

Natural compounds which are derived from dietary sources provide a large number of antioxidants. Tea is also rich sources of antioxidants. Catechin is one of the most active constituents present in it which acts as a potent antioxidant. Apart from the dietary sources, a number of Indian medicinal plants are also a rich source of antioxidants. [3,4] Superoxide dismutase, catalase, glutathione peroxidase, or non-enzymatic compounds, such as uric acid, bilirubin, albumin, and metallothioneins, are the enzymes but known for their endogenous antioxidant activity. Exogenous antioxidants came into existence when the endogenous factors were not able to control oxidative stress causing agents. Examples of exogenous antioxidants are Vitamin E, Vitamin C, β-carotene, Vitamin E, flavonoids, mineral Se, Vitamin D, and Vitamin K_3 [5].

Plants are one of the most important sources of medicines. The medicinal plants are rich in secondary metabolites (which are potential sources of drugs) and essential oils of therapeutic importance. The important advantages claimed for the therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective, and their easy availability. *Spinacia oleracea* Linn. (SO) is a plant having medicinal property native to central and southwestern Asia. It is cultivated for the sake of its succulent leaves and was introduced in Europe in the 15th century. It is the favorite food among Indians in the winter season [6].

SO is plant known as spinach is one of the rich sources of nutrients. It is widely cultivated all over the world having different pharmacological activities. Therefore, the aim of the present study is to determine different phytoconstituents of SO and its *in vitro* antioxidant activity to correlate its medicinal or pharmacological activity due to the presence of abundant phytoconstituents [7].

MATERIALS AND METHODS

Instrument

UV Spectrophotometer JASCO V-730.

Software: Spectra manager.

Version 2.13.00.

Collection of plant material

The fresh leaves of SO were collected from different regions of Pune (Maharashtra, India). The plant material was authenticated by the Botanical Survey of India, Pune.

Preparation of ethanolic extracts

The leaves of SO were washed under running tap water. The leaves were shade dried, and then, they were minced using a mixer grinder. 50 g of coarse powder was weighed for the Soxhlet extraction procedure. 50 g extract was placed in a thimble of muslin cloth. The thimble was mounted on a round bottom flask which was placed on heating mantle. Around 250 ml solvent, i.e., ethanol was added to the thimble to complete one and half manual cycle of Soxhlet. Condenser was placed over the Soxhlet extractor, and soxhletion process was started at 55°C. Until complete discoloration of the solvent in the syphon tube, i.e., up to seven syphon cycles, the process was carried out. The extract was removed, and the solvent was evaporated with the help of rotary evaporator. A semisolid extract obtained was weighed, and the percentage yield was calculated.

Methods

Phytochemical screening

The ethanolic leaf extract was tested for the presence of phytoconstituents such as flavonoids, alkaloids, glycosides, phenolics, and saponins using various tests [8].

Determination of total phenolic content

Principle

The Folin–Ciocalteu's assay has been used as a measure of total phenolic content. The mechanism is based on oxidation-reduction reaction. In the original Folin–Ciocalteu's assay, the carbonate is used for pH adjustment and the end point of the reaction was attained after 120 min at room temperature [9-11].

Procedure

It has been reported by a number of methods [9-13], but the procedure was referred from reference [12,13]. Folin–Ciocalteu's reagent was used for the estimation of phenolic content. 1 ml Folin reagent and 0.2 ml extract concentration were added and kept at room temperature for 5 min, and then, 0.8 ml of 2% sodium carbonate was added to it. Then, volume was made up by distilled water up to 10 ml and kept for incubation at room temperature for 2 h, and the absorbance was taken at 760 nm against blank as ethanol. Gallic acid was used a standard. Total phenolic content was expressed in µg of gallic acid equivalent of dry plant material [12,13].

Total flavonoid content

Principle

Total flavonoid content was determined by aluminum chloride ($AlCl_3$) colorimetric method. In this method, $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid-labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids [14].

Procedure

2% $AlCl_3$ hexahydrate ($6H_2O$) was prepared (2 g in 100 ml ethanol). Aliquots of 1.5 ml of each extract dilutions were added into an equal volume of 2% $AlCl_3.6H_2O$. The mixture mixed properly and absorbance was taken at 367 nm after 10 min of incubation at room temperature. Quercetin was used as a standard for calibration curve. Linear equation was plotted. Flavonoid content expressed in mg of quercetin equivalent by dry weight of spinach [15].

In vitro antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Principle

DPPH assay performed with some modifications in Brand-Williams protocols. Delocalization of spare electron over the molecule gives stability to DPPH radical, and thus, it prevents dimer formation. This radical is used in DPPH radical scavenging capacity assay to quantify the ability of antioxidants to quench the DPPH radical. When the radical form reduced to its non-radical form stable organic nitrogen-centered free radical, then the dark purple color of DPPH gets lost to a pink color which when reduced to its non-radical form by antioxidants which becomes colorless. In model system for the investigating the scavenging activities of several natural compounds, DPPH radical is widely used. When the DPPH radical is scavenged, the absorbance decreases at wavelength 517 nm with a change in color of the reaction mixture from purple to yellow [16,17].

Procedure

The stock solution of DPPH was prepared by dissolving 8 mg in 100 ml ethanol. The extract dilutions were prepared from the 1000 ppm solution in series of volumetric flasks to obtain appropriate range. 1 ml from of the extract dilutions was added to the previously prepared volumetric flasks containing 1 ml of DPPH into each and kept it for 20 min at the dark place. 2 ml of ethanol was added into it after 20 min. It was mixed properly by vortexing it. The absorbance was measured at 517 nm against ethanol. Quercetin was used as a standard. The percentage of inhibition was calculated against blank.

$$I\ \% = A_{blank} - A_{sample}/A_{blank} * 100$$

Where A_{blank} is the absorbance of control reaction (containing all reagents except the test compound) [18,19].

Nitric oxide scavenging assay

Principle

It is a diazotization reaction. Sodium nitroprusside generates nitric oxide in phosphate buffer at physiological pH which interacts with oxygen to produce nitrite ions which were measured by using Griess reaction reagent at wavelength 540 nm.

Procedure

Phosphate buffer (pH 7.8) was prepared. 10 mM sodium nitroprusside was dissolved in phosphate buffer. 3 ml of the above solution was added to 2 ml of extract and reference (quercetin) with different concentrations. Incubated the resulting solution at 25°C for 1 h. Similar procedure was repeated for blank sample which served as control. To 5 ml of the incubated mixture, add 0.5 ml of Griess reagent. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and coupling with α-naphthyl ethylenediamine was measured at 540 nm. Activity is measured in the terms of percentage inhibition [20].

Reducing power assay

Principle

The total antioxidant activity can be quantified by the ferric reducing antioxidant power assay. The flavonoids and phenolic acids possess strong antioxidant activity due to which they form the complex with metal atoms, particularly iron and copper. The method is based on the principle of increase in the absorbance of the reaction mixtures, as absorbance increases, it indicates an increase in antioxidant activity. The antioxidant compound present in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid, and ferric chloride, which was measured at 700 nm by UV-spectrophotometer.

Procedure

2.5 ml of extract sample was mixed with an equal volume of phosphate buffer and 1% potassium ferricyanide and was placed in a water bath at 50°C for 20 min. Then, it was cooled rapidly, and then, 2.5 ml 10% trichloroacetic acid was added. The resultant solution was vortexed and centrifuged at 3000 rpm for 10 min. 5 ml supernatant was mixed with an equal volume of water and 0.1% 1 ml ferric chloride. It was further incubated at room temperature for 10 min, and then, absorbance was taken at 700 nm. The reducing property was checked against quercetin used as a standard.

A high absorbance indicates the stronger reducing power [21].

Hydroxyl radical scavenging activity

Principle

Hydroxyl radical scavenging assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of plant sample. In this assay, quantification of the 2-deoxyribose degradation product is done, by its condensation with thiobarbituric acid. The reaction carried out between ascorbic acid-iron-ethylenediaminetetraacetic acid (EDTA) model which is responsible for the formation of hydroxyl radical. The reaction was performed in two conditions: In the presence of EDTA to determine OH scavenging capacity and in the absence of EDTA to determine its metal chelation property [22].

Procedure

Reaction mixture (Phosphate buffer [pH 7.4,100 µl], extract concentration [500 µl], 1.04 mM EDTA [200 µl], 1:1 v/v Ferric Chloride [200 µl],1.0 mM hydrogen peroxide [100 µl],1and mM ascorbic acid [100 µl]) was prepared and incubated for 1 h at 37°C. Then, 1 ml 2.8% trichloroacetic acid and 1% thiobarbituric acid were added to the mixture. The mixture was heated at 100°C for about 15 min and then cooled. After cooling, absorbance was taken at 532 nm. Thiourea was used as standard as it is a good scavenger of hydroxyl radical [23-25].

Lipid peroxidation inhibition assay

Principle

The assessment of lipid peroxidation involves thiobarbituric acid reactivity. The thiobarbituric acid involved for boiling the test sample with acetic acid so as to generate pink pigment. Free radical formed in this reaction ruptures the lipid bilayer to form malonialdehyde as a secondary product. Pink-colored product shows maximum absorbance at 532 nm when two molecules of thiobarbituric acid react with one molecule of malondialdehyde and this reaction is called thiobarbituric acid assay [26].

Procedure

Egg yolk homogenate was prepared and was used as a lipid source, and free radicals were produced by $FeSO_4$. 0.5 ml egg yolk homogenate (10% in distilled water, v/v) was added to the 0.1 ml of extract, and the volume was made up to 1 ml by distilled water. 0.05 ml $FeSO_4$ (0.07M) was incubated at 37°C for 30 min to induce lipid peroxidation. Then, 1.5 ml acetic acid (20%) and 1.5 ml thiobarbituric acid (8%) were added to the above mixture. The mixture was vortexed for 5 min and then heated to 95°C for 1 h. Cooled and 1 ml butanol was added to it. Then, it was centrifuged at 3000 rpm for 10 min. Pink-colored product obtained showed maximum absorbance at 532 nm. The same procedure was repeated for ascorbic acid which was used as a standard [27].

Phosphomolybdenum reduction assay

Principle

The assay is based on the reduction of Mo (VI) to Mo (V) by the methanol extract of leaves of SO and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1 ml of various concentrations (5–150 µg/ml) of extract was added to 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. Greenish-blue color was produced. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer. Ascorbic acid was used as a reference compound [28].

Procedure

1 ml of various concentrations of extract added with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer**.** Ascorbic acid was used as a standard [29].

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening carried out on the ethanolic extract of SO revealed the presence of various bioactive ingredients such as alkaloids, glycosides, saponins, phenols, and flavonoids. It is documented that these phytoconstituents may have several pharmacological activities (Table 1).

Total phenolic content

The total phenolic content of the ethanolic extracts of SO was measured by using Folin–Ciocalteu reagent, and the results were expressed in terms of µg of gallic acid equivalent (the standard curve equation y = 0.093x - 0.0403, R^2 = 0.995). Phenolic compounds may contribute directly to antioxidant action. The total phenolic content found was 11.25% of "gallic acid equivalent" per mg extract in ethanol.

The total flavonoid content

The total flavonoid content of the extracts was expressed in terms of quercetin equivalent (the standard curve equation y = 0.0998x - 0.0236 R^2 = 0.9871).

The total flavonoid content of extract was 3.51% "quercetin equivalent" per mg plant extract.

***In vitro* antioxidant activity**

DPPH radical scavenging activity

DPPH assay of ethanolic extract of SO revealed the presence of antioxidant potential. The percentage of inhibition was observed in all the concentrations of the plant extract. The free radicals were trapped and scavenged by the plant extract in a concentration-dependent manner. The percentage inhibition of scavenging activities, i.e., inhibitory concentration (IC_{50}) value of ethanolic extract of SO for DPPH shown at 50 µg/ml concentration. Polyphenolic compounds in the plant are responsible for showing the antioxidant activity (Table 2).

It indicates that amount of elements contributing to DPPH radical scavenging activity is equal to 31.11% calculated in terms of quercetin.

Table 1: Phytochemical tests and results of the ethanolic leaf extract of *Spinacia oleracea*

S. No	Phytoconstituents	Chemical reagents	Results
1	Alkaloids	Mayer's reagent	+
		Dragendorff's reagent	+
		Hager's reagent	+
		Wagner's reagent	+
2	Glycoside	Baljet test	–
		Legal's test	+
		Keller–Killiani test	+
		Liebermann's test	+
3	Saponins	Foam test	+
		Hemolysis test	+
4	Flavonoids	Shinoda test	+
5	Phenolic components	Ferric chloride test	+

+Represents that compound is present, –represents that compound is absent

Nitric oxide scavenging assay

Ethanolic extract of SO caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50}, i.e., IC_{50} of 60 µg/ml which gave 56.66% inhibition. Quercetin was used as a reference compound, and its E50 value is 0.2 µg/ml (Table 3).

Reducing power assay

Fe3+ was transformed to Fe2+ in the presence of an ethanolic extract of SO and the reference compound quercetin to measure the reductive capability. At 10 µg/ml, the absorbances of the plant extract and quercetin were almost the same. The results of reducing power assay are provided in Table 4. In the assay, the absorbance is increased with increasing the concentration of the extracts indicating the presence of constituents with reducing power (Table 4).

Hydroxyl radical scavenging assay

Hydroxyl radical-mediated deoxyribose degradation in Fe3+EDTA-ascorbic acid-H2O2 reaction mixture was done in this assay. The results are shown in Table 5. The IC_{50} values of the ethanolic extract of SO for scavenging hydroxyl radicals are 250 µg/ml in the presence of EDTA with maximum inhibition at a concentration of 2000 µg/ml and IC_{50} i.e., IC_{50} value of standard 100 µg/ml in the presence and absence of EDTA with maximum inhibition at a concentration of 500 µg/ml (Table 5).

The results revealed that the amount of elements contributing to hydroxyl radical scavenging activity is equal to 35.39% calculated in terms of thiourea.

Lipid peroxidation inhibition assay

The result of anti-lipoperoxidation free radicals of the ethanolic extract of SO to prevent peroxidation showed that trapping potential for LPO radicals with IC_{50}, i.e., IC_{50} is shown by concentration 1500 µg/ml. Oxidative stress in cells and tissues can be best checked by its lipid peroxidation assay, a well-established mechanism both in plants and animals (Table 6).

Phosphomolybdenum reduction assay

The absorbance of SO extract was found to be 0.1624 at a concentration of 100 µg/ml and 0.2124 at 150 µg/ml. The reducing power calculated by this method indicates that the content of reducing agents is 28.05% in terms of ascorbic acid (Table 7).

Table 2: DPPH scavenging activity of extract and quercetin

S. No.	Concentration of ethanolic extract of SO (µg/ml)	% inhibition by extract*	Concentration of quercetin (µg/ml)	% inhibition by quercetin*
1	40	47.83	5	47.28
2	50	56.71	10	49.76
3	60	57.44	15	50.66
4	70	57.57	20	55.83
5	80	58.02	25	59.74
6	90	58.02	30	61.06
7	100	60.55	35	65.01

*Average of two readings. SO: *Spinacia oleracea*, DPPH: 1,1-Diphenyl-2-picrylhydrazyl

Table 3: Nitric oxide scavenging activity of extract and quercetin

S. No	Concentration of ethanolic extract of *Spinacia oleracea* (µg/ml)	% inhibition by extract*	Concentration of quercetin (µg/ml)	% inhibition by quercetin*
1	20	0.187107	0.1	42.58799
2	40	34.95535	0.2	52.6773
3	60	56.6678	1	63.21786
4	80	71.97491	5	80.92962
5	100	93.35472	10	94.16308

*Average of two readings

Table 4: Observations of reducing power assay

S. No.	Concentration of ethanolic extract of *Spinacia oleracea* (µg/ml)	Absorbance of ethanolic extract of *Spinacia oleracea* at 700 nm*	Concentration of quercetin (µg/ml)	Absorbance of quercetin at 700 nm*
1	0.1	0.0880	10	0.2070
2	0.5	0.1040	40	0.2574
3	1	0.1840	80	0.3196
4	5	0.2024	120	0.3946
5	10	0.3083	160	0.4641
6	15	0.4252	200	0.5523

*Average of two readings

Table 5: Observations after addition of EDTA and without addition of EDTA

S. No	Concentration of thiourea (µg/ml)	% inhibition after addition of EDTA	% inhibition without addition of EDTA	Concentration of extract of *Spinacia oleracea* (µg/ml)	% inhibition after addition of EDTA	% inhibition without addition of EDTA
1	100	54.26	47.27	750	87.83	85.58
2	200	76.38	56.39	1000	92.68	95.46
3	300	81.59	97.40	1500	98.75	98.06
4	500	98.49	98.49	2000	99.56	99.64

*Average of two readings. EDTA: Ethylenediaminetetraacetic acid

Table 6: Observation of lipid peroxidation inhibition assay

Sr. No	Concentration of ascorbic acid (µg/ml)	% inhibition	Concentration of extract of *Spinacia oleracea* (µg/ml)	% inhibition
1	100	36.03	500	33.11
2	500	43.59	1000	35.57
3	1000	59.19	1500	49.25
4	1500	65.74	2000	57.87

*Average of two readings

Table 7: Observations for phosphomolybdenum reduction assay

S. No	Concentration of ascorbic acid (µg/ml)	Absorbance at 695 nm
1	5	0.1189
2	10	0.1643
3	50	0.2265
4	100	0.2674
5	150	0.3093

*Average of two readings

CONCLUSION

Preliminary phytochemical investigation of the ethanolic extract of SO revealed the presence of saponins, flavonoids, tannins, triterpenoids, and alkaloids. The systematic literature collection, pertaining to this investigation, indicates that the plant phenolics constitute major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is worthwhile to determine their total amount in the plants chosen for the study. The content of total phenolics in the extracts was determined using the Folin–Ciocalteau assay and quantified as gallic acid equivalents. The total phenolic content was found to be 11.25 % of "gallic acid equivalent" per mg extract in ethanol. The total flavonoid content was determined by incorporating the $AlCl_3$ method using quercetin as the standard. The total flavonoid content was found to be 3.51% "quercetin equivalent" per mg plant extract in ethanol. There is strong evidence, indicating that flavonoids are capable of modulating cellular and molecular processes involved in memory and learning. Flavonoids have been shown to modulate critical neuronal signaling pathways involved in the processes of memory and, therefore, are likely to affect synaptic plasticity and long-term potentiation mechanisms. Various antioxidant assays based on colorimetric reactions were performed, for example, DPPH assay, nitric oxide scavenging assay, reducing power assay, hydroxyl radical scavenging assay, lipid peroxidation assay, and phosphomolybdenum reduction assay. The results confirm the antioxidant potential of extract.

AUTHORS' CONTRIBUTIONS

All the authors have contributed equally to the manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest.

REFERENCES

1. Oluyemisi FK, Omoregie HE, Ochogu PA. Standardization of herbal medicines - a review. Int J Biodiverse Conserve 2012;4:101-12.
2. Dande PR, Sharma GM, Sharma RM, Chakraborthy GS. Pharmacognostical studies of leaves of *Spinacia Oleracea* Linn. Int J Pharm Sci Res 2010;1:41-6.
3. Pal SK, Shukla Y. Herbal medicine: Current status and the future. Asian Pac J Cancer Prev 2003;4:281-8.
4. Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD, *et al.* Free radicals and antioxidants in human health: Current status and future prospects. J Assoc Physicians India 2004;52:794-804.
5. Rasheed A, Marka V, Yalavarthy PD. A study on antioxidant activity of some commonly used spices in India. IJLBPR 2013;2:145-50.
6. Tyagi R, Sharma G, Jasuja ND, Menghani E. Indian medicinal plants as an effective antimicrobial agent. J Citation Reports 2016;3:69-71.
7. Jiraungkoorskul W. Review of neuro-nutrition used as anti-alzheimer plant, spinach, *Spinacia oleracea*. Pharmacogn Rev 2016;10:105-8.
8. Khandelwal KR. Practical Pharmacognocy Techniques and Experiments. 22nd ed. Pune: Nirali Prakashan; 2012. p. 25, 4-6.
9. Singleton VL, Rossi JR. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 1965;16:144-58.
10. Sharma AK, Gangwar M, Chaturvedi AP, Sinha AS, Tripathi YB. Comparative analysis of phenolic and flavonoid content of *Jatropha curcas* Linn. Plant Arch 2012;12:823-6.
11. Meda A, Lamien CS, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasanhoney, as well as the irradical scavenging activity. Food Chem 2005;91:571-7.
12. Adedopo AA, Jimoh FO, Folayan JA, Masika PJ. Antioxidant properties of methanol extracts of leaves and stems of *Celtis* Africana. Rec Nat Prod 2009;3:23-31.
13. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem 2003;51:609-14.
14. Banerjee S, Adak K, Adak M, Ghosh S, Chatterjee A. Effect of some anti-nutritional factors on the bioavailability of minerals along with the study of chemical constituents and antioxidant property in *Typhonium trilobatum* and *Spinacia oleracea*. Chem Sci Rev and Lett 2015;4:429-39.
15. Lamaison JL, Carret A. Teneurs en principaux flavonoids des fleurs de *Crataegus monogyna* Jacq et de *Crataegus laevigata* (Piret DC) en fonction de la vegetation. Plantes Medicinales et Phytotherapie 1990;25:12-6.
16. Dixitha M, Santhy KS. Antioxidant and anti-clastogenic potential of *Piper longum* L. Int J Appl Pharm 2015;7:11-4.
17. Ansari AQ, Ahmed SA, Waheed MA, Sayyed JA. Extraction and determination of antioxidant activity of *Withania somnifera* dunal. Pelagia research library. Eur J Exp Biol 2013;3:502-7.
18. Janaki A, Kaleena PK, Phytochemical screening, antioxidant and antibacterial activities of *Millingtonia hortensis* (L). Int J Curr Pharm Res 2017;9:162-7.
19. Mensor LL, Menezes FS, Leitão GG, Reis AS, dos Santos TC, Coube CS, *et al.* Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 2001;15:127-30.
20. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR, *et al.* Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131-8.
21. Kajaria DK, Gangwar M, Sharma AK, Tripathi YB, Tripathi JS, Tiwari S, *et al.* Evaluation of *in vitro* antioxidant capacity and reducing potential of polyherbal drug- Bhāraṅgyādi. Anc Sci Life 2012;32:24-8.
22. Mandal S, Hazra B, Sarkar R, Biswas S, Mandal N. Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia crista* leaf. Evid Based Complement Alternat Med 2011;2011:173768.
23. Aruoma OI, Halliwell B. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. Biochem J 1987;248:973-6.
24. Kunchandy E, Rao MN. Oxygen radical scavenging activity of curcumin. Int J Pharm 1990;58 Suppl 3:237-40.
25. Alam MN, Bristi NJ, Rafiquzzaman M. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharm J 2013;21:143-52.
26. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 1990;9:515-40.
27. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
28. Narayanan R, Chandrasekaran S, Sooriamuthu S, Jerrine J, Raaman N. Antioxidant activites and phytochemical analysis of methanol extract of leaves of *Hypericum hookerianum*. Int J Pharm Pharm Sci 2014;6:456-60.
29. Umesh TG. *In vitro* antioxidant potential, free radical scavenging and cytotoxic activity of *Simarouba gluaca* Leaves. Int J Pharm Pharm Sci 2014;7:411-6.