

In Vitro Antioxidant Activity of Aqueous and Alcoholic Extracts of Polyherbal Formulation Consisting of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. Stem Bark Assessed in Free Radical Scavenging Assays

Mradu Gupta*, Nandita Karmakar, Saswati Sasmal

Institute of Post Graduate Ayurvedic Education and Research, 294/3/1, A. P. C. Road, Kolkata, India – 700009

Received: 30th Dec, 16; Revised: 25th Jan, 17; Accepted: 16th Feb, 17; Available Online: 25th February, 2017

ABSTRACT

In vitro antioxidant free radical scavenging capacities of aqueous and alcoholic extracts of herbal drug containing stem bark of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. were evaluated. IC₅₀ (µg/ml) of alcoholic, aqueous extract and Ascorbic Acid was found to be 41.529 ±10.135, 39.654 ±4.022 and 17.511 ±1.17 in Hydrogen Peroxide Radical Scavenging, 1825 ±122, 1035 ±090 and 207 ±006 in DPPH radical scavenging, and 42.024 ±16.816, 49.926 ±16.240 and 5.503 ±0.545 during FRAP assay. Similarly, during ABTS radical scavenging, IC₅₀ (µg/ml) was assessed as 28.088 ±5.618, 23.731 ±4.870 and 6.728 ±0.213 for alcoholic, aqueous extract and Trolox respectively. Alcoholic & Aqueous extracts exhibit high antioxidant activity possibly due to higher phenolic & flavonoid content.

Keywords: antioxidant, ABTS, DPPH, Ferric reducing, medicinal plant, radical scavenging.

INTRODUCTION

Plants have always been a common source of food and medicines, either in the form of traditional preparations or as pure active principles¹. Most of the observed therapeutic effects of plants have been linked to their potent antioxidant activity. In fact, antioxidant activity based healing of diseases or maintenance of a healthy lifestyle could be the scientific basis of traditional herbal medicines such as those used in Ayurveda². It has been suggested that free radicals are involved in the pathology of more than 50 human diseases, including aging³.

Antioxidants are molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage. Antioxidants exist both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment. Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H₂O₂) and then to water, in a multi-step process in presence of co-factors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Normal biochemical reactions, increased exposure to the environment and higher levels of dietary xenobiotics result in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are responsible for the oxidative stress in different pathophysiological conditions. Cellular constituents of our body are altered in oxidative stress conditions, resulting in various disease states. The oxidative stress can be effectively neutralized by enhancing cellular defenses in the form of antioxidants.

Reactive oxygen species (ROS), including superoxide radicals ($\bullet O_2^-$), hydrogen peroxide (H₂O₂), hydroxyl radicals ($\bullet OH$), and singlet oxygen (1O_2), are generated as byproducts of normal metabolism⁴⁻⁵.

To counteract the deleterious effects of ROS, phenolic compounds which are naturally distributed in plants are very effective⁶. The food-derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention because they are known to function as chemo-preventive agents against oxidative damage. Polyphenols are considered to be plant chemical defenses against pathogens and herbivores, and these compounds can exert detrimental effects in a multitude of ways⁷. Polyphenols have many favourable effects on human health, such as the inhibition of the low density proteins oxidization⁸. They also have anti-inflammatory activity and anti-carcinogenic properties⁹.

The research formulation has been prepared by adding equal amounts of dried parts of the stem bark of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. because these two plants have been used since ancient times in the Ayurvedic system of medicine and elaborated in ancient texts such as Charak Samhita (Chikitsa Sthanam) as a astringent, haemostatic, anti-inflammatory substance and useful in arresting the excessive abnormal vaginal discharge, in the form of single drug and in combined polyherbal form¹⁰. This is a new herbal formulation which has not been evaluated till now although it is likely to exhibit sustained and significant antioxidant action due to the synergistic effect of the phenolic and flavonoidic

*Author for Correspondence: mradu_gupta@hotmail.com

compounds present in this research drug and the pharmacological properties of its constituent herbs.

Ficus glomerata Roxb. or Cluster Fig which belongs to the Moraceae family is a moderate sized spreading lactiferous tree without much prominent aerial roots found throughout India. It is commonly known as Udumbar or Gular in Hindi language and its fruits are eaten by villagers. Its leaves are dark green, ovate or elliptical while the fruits contain receptacles 2-5 cm. in diameter, sub-globose, smooth. When ripe, the fruits are orange, dull reddish and having pleasant smell. The stem bark is grayish green, soft surface and uneven 0.5-1.8 cm. thick. On rubbing it, white papery flakes come out from the outer surface; the inner surface is light brown, fracture fibrous, taste mucilaginous. The stem bark, fruits, leaves and latex of this plant have been used since ancient time as mentioned in the Ayurvedic text book for treatment of dysentery, diarrhoea, astringent, toothache, wound healing, stomach-ache, vaginal disorders, menorrhagia, haemoptysis, diabetes, piles and glandular swelling, etc. The roots of the plant are used in dysentery, pectoral complications, and diabetes, and also applied in inflammatory glandular enlargement, mumps, and hydrophobia. The latex is externally applied on wounds to decrease inflammation, pain, and edema, and promote its healing. The Phytochemical compounds isolated from the stem bark are leucocyanidin-3-o-B-glucopyranoside, leucopelarogonidin 3-O-a- L-rhamnopyranoside, B-sitosterol, stigmasterol, tetracyclic triterpene- gluanol acetate and tiglic acid. The reported pharmacological properties of the different plant parts are hypo-glycaemic, antiulcer, antioxidant, wound-healing, anti-inflammatory, anti-diarrhoeal, antibacterial, antifungal, antipyretic and antidiuretic¹⁰⁻¹³.

Symplocos racemosa Roxb. belonging to the family Symplocaceae is distributed throughout North Eastern India, up to 2,500 ft., from the terai of Kumaon to Assam and Pegu, Chota Nagpur and Burma. Also known as Lodhra, it is a small evergreen tree with stem up to 6 m height and 15 cm in diameter. The leaves are dark green above, orbicular, elliptic oblong, coriaceous and glabrous above; the flowers are white, turning yellow, fragrant, in axillary, simple or compound racemes; the drupes are purplish black, sub-cylindrical, smooth and 1-3 seeded. Its stem Bark is useful in bowel complaints such as diarrhea, dysentery, in dropsy, eye disease, liver complaints, wound healing, excessive vaginal discharge, menstrual problems, fevers, ulcers, scorpion-string, etc. The bark is often employed in the preparation of plasters and is supposed to promote maturation or resolution of stagnant tumors. A decoction of the bark or wood is used as gargle for giving firmness to spongy and bleeding gums and relaxed uvula. It is one of the constituents of a plaster used to promote maturation of boils and other malignant growths¹⁰⁻¹².

The phytochemical investigation of the n-butanol soluble fraction of the bark of stem of *Symplocos racemosa* Roxb yielded two phenolic glycosides of salirepin series namely symplocuronic acid and symplocoside while salirepin has also been isolated from this plant. The alcohol extract of stem bark indicated the presence of carbohydrate,

glycoside, saponin and terpenoid & alkaloid and ether extract indicated the presence of glycoside, phytosterol and steroid. The pharmacological activities of its stem bark are antibacterial, anti-inflammatory, antiulcer, anti-tumor, antimicrobial and antioxidant¹⁴⁻¹⁵.

During the present study, the aqueous and alcoholic extracts of the research formulation were initially assessed for their phenolic and flavonoid contents. Thereafter, they were screened for their antioxidant capacities using in vitro standard procedures namely ferric reducing antioxidant power (FRAP), ABTS bleaching assay (α TEAC), DPPH assay and peroxy radical scavenging assay strategies where plants are generally assessed for their function as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators, after which they are classified as primary (chain-breaking) and secondary (preventive) antioxidants. Primary antioxidants act by donating a hydrogen atom, while secondary antioxidants function via binding of metal ions capable of catalyzing oxidative processes and scavenging oxygen, absorbing UV radiation, inhibiting enzymes or decomposing hydroperoxides¹⁶.

The objective of the research was to assess the medicinal potential of this formulation, especially its antioxidant potential in the form of free radical scavenging activity, using scientific techniques and thus justify its traditional and folklore use as a substantial and reliable herbal formulation.

MATERIALS AND METHODS

Plant materials

The stem bark of *Symplocos racemosa* Roxb. and *Ficus glomerata* Roxb. were purchased from crude drug supplier of Katwa Chowrasta, Burdwan district for the preparation of herbal rejuvenator drug and the plant samples were authenticated by the Research Officer, Botanical Survey of India, Howrah, India (Ref. No. BSI/CNH/SF/Tech./2016).

Chemicals

Sodium phosphate was obtained from Sarabhai M. Chemicals Limited, Shantisadan, Ahmedabad, Gujarat, India and Ammonium Molybdate was obtained from GFS chemicals, United States. Quercetin was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Gallic acid, Ascorbic acid, Potassium persulphate, Potassium ferricyanide and Trichloro acetic acid were obtained from Nice Chemicals Pvt. Ltd. Kochi, Kerala, India. Potassium Sodium Tartrate, Aluminium chloride, Folin-Ciocalteu's reagent, sodium carbonate, Sulphuric acid, Hydrogen peroxide, Ferric Chloride (FeCl₃), Di-Sodium hydrogen Phosphate and Butylated hydroxytoluene (BHT) were obtained from Merck Specialties Pvt. Ltd., Mumbai, India. 2, 2- azinobis (3 ethyl-benzothiazoline-6-sulfonic acid) [ABTS] was obtained from Roche diagnostics, Mannheim, Germany while 6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from MP Biomedicals, France.

Preparation of Extracts

The stem bark of *Symplocos racemosa* Roxb. and *Ficus glomerata* Roxb. were taken in equal quantity by weight, washed, sun dried and crushed to particle size of 40 mesh. This coarse powder was sequentially extracted with petroleum ether (60 °C -80 °C), chloroform, acetone, ethanol and water using soxhlet apparatus. These extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments. The aqueous and alcoholic (ethanol) extracts of the herbal rejuvenator drug were used in the study.

Analysis of Phytochemical constituents

Systematic analysis using standard methods was done for ascertaining the physical parameters such as moisture content, ash value & extractive value and also for ascertaining the presence of different phyto-chemical constituents such as alkaloids, amino-acids, reducing sugars, tannins, saponins, anthroquinones, steroids, terpenoids, flavonoids and salicyclates¹⁷.

Total Phenol Content

Total phenol content was determined using the Folin-Ciocalteu reagent. To 0.5 ml aliquot of dried aqueous extract, 2.5 ml of Folin- Ciocalteu's reagent (10 %) and 2 ml of 7.5% sodium carbonate were added. The absorbance was read after 30 min incubation period at room temperature at 760 nm colorimetrically. A standard calibration plot was generated at 760 nm using known different concentrations of Gallic acid (100, 200, 300, 400, and 500 µg/ml). The concentrations of phenol in the test samples were calculated from the calibration plot. Total phenolic content was expressed as mg Gallic Acid Equivalents (GAE). All determinations were performed in triplicates and the results were expressed as mg Gallic acid equivalents per gm sample extract¹⁸⁻¹⁹.

Total Flavonoid Content

The Aluminum chloride [AlCl₃] method was used to determine the Total Flavonoid Content. An aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 1% aluminum chloride and 0.1 ml of potassium acetate solution (1 M). In the mixture, 2.8 ml of distilled water was added to bring up the total volume to 5 ml. The test solution was shaken vigorously and Absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using different and known concentrations of Quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg Quercetin equivalent/gm of sample²⁰⁻²¹.

Total Antioxidant Capacity by Phosphomolybdate Assay

The total antioxidant capacity was assessed using the method of Prieto et al. (1999)²². 0.3 ml of alcoholic and aqueous extracts of the research drug were combined with 3 ml of reagent solution (0.6 M Sulfuric acid, 28 mM Sodium phosphate & 4 mM Ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Thereafter, the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer against blank after cooling to room

temperature. Alcohol (0.3 ml) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gm equivalents of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid (7.5, 15, 30, 60 & 150 µg/ml) with Alcohol.

Hydrogen Peroxide Radical Scavenging

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Keser et al. (2012)²³. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Alcoholic and Aqueous extracts (24.94, 49.75 and 74.44 µg/ml) were added to hydrogen peroxide solution (0.6 ml, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined spectro-photometrically by UV-VIS (Shimadzu UV 2450) 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide and compared with ascorbic acid (12.42, 24.69, 36.81 and 48.78 and 74.44 µg/ml), the reference compound. The percentage of hydrogen peroxide scavenging of extracts and standard compounds were calculated using the methods outlined by Basniwal et al, 2009 and Nishaa et al, 2012²⁴⁻²⁵.

% scavenged [H₂O₂] =

$$\frac{\text{The Abs of Control} - \text{The Abs in presence of}}{\text{The Abs of Control}} \times 100$$

DPPH radical scavenging assay

DPPH [1, 1-di-phenyl-2-picryl hydrazyl] is a stable free radical with purple color, the intensity of which is measured spectrophotometrically at 517 nm wavelength. Antioxidants reduce DPPH to 1, 1-diphenyl-2-picryl hydrazine, a colorless compound.

The DPPH 0.1 mM solution in ethanol was prepared. This solution (3 ml) was added to 1 ml. of alcoholic and aqueous extracts at different concentrations (0.5, 1.0 & 2.0 mg/ml). The mixture was shaken vigorously and allowed to stand at room temp in dark for 30 min. and thereafter the absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu UV 2450)²⁶⁻²⁷. Lower absorbance of the reaction mixture indicated higher free radical activity. Ascorbic acid was used as standard compound due to its strong reducing power and weak metal-chelating ability with different concentrations (0.050, 0.100, 0.150 and 0.200 mg/ml) and experiment was done in triplicate. The IC₅₀ value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using inhibition curve. Linear graph of concentration vs. percentage inhibition was prepared using various concentrations of Ascorbic acid and IC₅₀ values were calculated.

The percent DPPH scavenging effect was calculated by using the following equation:

% inhibition =

$$\frac{\text{Blank} - \text{Extract or Standard}}{\text{Blank}} \times 100$$

where Blank was the absorbance of the control and the absorbance in the presence of the sample was denoted by Extract or Standard.

ABTS radical scavenging assay

Table 1: Estimation of Total Flavonoid Content and Total Phenol Content.

Tests (TFC & TPC)	Alcoholic Extract	Aqueous Extract
Flavonoid content (μg Quercetin equivalent / mg of extract) following the standard curve ($R^2 = 0.999$)	74.76	59.14
Phenol content (μg Gallic acid equivalent / mg of extract) following the standard curve ($R^2 = 0.997$)	220.00	225.67

Table 2: Estimation of Total Antioxidant Capacity.

Research Sample	Concentration (mg/ml)	Antioxidant Content ($\mu\text{g}/\text{mg}$) equivalent to Ascorbic Acid
Alcoholic Extract	0.1	126.06 \pm 4.77
	0.2	166.72 \pm 10.27
	0.3	189.13 \pm 14.91
Aqueous Extract	0.1	69.33 \pm 0.67
	0.2	86.50 \pm 3.62
	0.3	99.82 \pm 5.94

For ABTS (2, 2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay, the working solution was prepared by mixing equal quantities of 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution and allowing them to react for 12 hours at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol to obtain an absorbance of 0.7 to 0.8 units at 734 nm using the spectrophotometer (UV-Vis Shimadzu UV 2450). Fresh ABTS solution was prepared for each assay and used as Blank²⁸⁻²⁹.

Alcoholic and aqueous extracts with different concentrations (200, 300 & 400 $\mu\text{g}/\text{ml}$) were allowed to react with 2.94 ml of the ABTS solution for 6 min in a dark condition. Then the absorbance was measured at 734 nm using the spectrophotometer. Results were expressed in mM Trolox equivalents (TE)/g fresh mass. Trolox (water-soluble analog of vitamin E) in different concentrations (2.70, 4.05, 8.10 & 10.80 $\mu\text{g}/\text{ml}$) was used as a standard and the same procedure was used. Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve. IC₅₀ value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined.

Scavenging ability relative to the reaction control (without plant extract) was calculated by using the formula:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract/standard}}}{\text{Abs}_{\text{control}}} \times 100$$

Where Abs_{control} is the absorbance of ABTS radical in Blank & Abs_{extract/standard} is the absorbance of an ABTS radical solution mixed with extract/ sample.

Ferric reducing antioxidant power assay (FRAP)

The modified Oyaizu method (1986)³⁰ was used to determine the Ferric reducing antioxidant power. Substances which have reduction potential react with potassium ferricyanide to form potassium ferrocyanide, which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm.

The different concentrations of sample alcoholic and aqueous extracts (300, 600 & 900 $\mu\text{g}/\text{ml}$) were mixed with 1 ml of sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixture was incubated at 50° C for 20 min. After that, 1 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (1.5 ml) was mixed with 1.5 ml deionized water and 0.1 ml of 0.1% of ferric chloride, kept for 10 min and the absorbance was measured at 700 nm by UV-VIS Shimadzu UV 2450³¹⁻³². Higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as mean value \pm standard deviation. The extract concentration providing 0.5 of absorbance (EC₅₀) was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid (4, 8, 12, 16, 20 $\mu\text{g}/\text{ml}$) was used as standard.

Increase in reducing power (%)

$$= \frac{\text{Abs}_{\text{test}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{blank}}} \times 100$$

Where Abs_{test} is absorbance of test solution and Abs_{blank} is absorbance of blank. Here reduction of Fe [(CN)₆]₃ to Fe [(CN)₆]₂ leads to the formation of the intense Perl's Prussian blue complex which gives strong absorbance at 700 nm with increasing reducing power.

Statistical analysis

The data generated for each mouse was considered for calculation of Mean \pm S.E.M. for different groups. Statistical evaluation of data was done following Students' t-test. A difference was considered significant at $p \leq 0.05$.

RESULTS

Analysis of phytochemical constituents

Evaluation of the physical parameters of herbal rejuvenator drug indicated that while the moisture content was 8.2 % w/w, the extractive value of aqueous extract was 1.44 % w/w. The total ash content was 12.30 % w/w, the acid insoluble ash was 0.77 % w/w and water soluble ash was 10.66 % w/w. Among phytochemical constituents, alkaloids, flavonoids, tannins and carbohydrates were found to be present in the aqueous and alcohol extract of the polyherbal rejuvenator drug.

Total flavonoid content (TFC) & total phenol content (TPC)

The total flavonoid content (TFC) and total phenol content (TPC) was calculated from the absorbance calibration curve generated with different concentrations of Quercetin and Gallic acid respectively which is shown in table 1.

Total Antioxidant Capacity by Phosphomolybdate Assay

The total antioxidant capacity (TAC) was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic pH with a maximal absorption at 695 nm. It was expressed as the number of equivalents of ascorbic acid as a reference standard in the form of Standard curve for Ascorbic acid with which plant extracts are compared. Total antioxidant capacity of the alcoholic & aqueous extracts of the research drug in different concentrations of 0.1 mg/ml, 0.2 mg/ml and 0.3 mg/ml expressed as microgram equivalents of ascorbic acid were evaluated to be 126.06 ± 4.77 , 166.72 ± 10.27 & 189.13 ± 14.91 and 69.33 ± 0.67 , 86.50 ± 3.62 & 99.82 ± 5.94 respectively as shown in table 2.

Hydrogen Peroxide Radical Scavenging

The HPS activity has usually been determined by following the rate of H_2O_2 consumption in an incubation system (H_2O_2 + scavenger) using the classical UV-method. The results of the hydrogen peroxide scavenging test of the alcoholic and aqueous extracts of the research drug in comparison with the standard (ascorbic acid) at 230 nm is shown in table 3. The percentage of inhibition against different concentrations of both extracts of the research drug as well as ascorbic acid was used to plot the standard curve which was used to calculate the IC_{50} ($\mu\text{g/ml}$) of each sample which was determined as 41.529 ± 10.14 , 39.654 ± 4.022 and 17.511 ± 1.17 for alcoholic extract, aqueous extract and Ascorbic Acid respectively.

DPPH radical scavenging assay

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow-colored diphenyl-picryl hydrazine at 517 nm. The results are expressed as the IC_{50} value (the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%). The results of the DPPH radical scavenging test of alcoholic and aqueous extracts in comparison with the standard (ascorbic acid) are shown in table 4. The percentages of inhibition against different concentrations of both extracts of the research drug as well as ascorbic acid were used to plot the standard curve which was used to calculate the IC_{50} ($\mu\text{g/ml}$) of each sample which was determined as 1.825 ± 0.122 in alcoholic extract, 1.035 ± 0.09 in aqueous extract of herbal rejuvenator drug and 0.207 ± 0.006 in Ascorbic Acid respectively.

ABTS radical scavenging assay

In this assay, ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and Vitamin C. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically. This assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay.

The results of the ABTS radical scavenging test of the alcoholic and aqueous extracts of the herbal rejuvenator drug in comparison with the standard (Trolox) are shown

in table 5. The percentage of inhibition against different concentrations of both extracts of the research drug as well as Trolox as Standard was used to plot the standard curve. This curve was used to calculate the IC_{50} ($\mu\text{g/ml}$) of each sample which was determined as 28.088 ± 5.618 in alcoholic extract, 23.731 ± 4.870 in aqueous extract of herbal rejuvenator drug and 6.728 ± 0.213 in Trolox respectively. It is also observed that inhibition percentage values go on increasing with increases in concentration of research plant extracts in the assay mixture.

Ferric reducing antioxidant power (FRAP) Assay

In this assay the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each compound. The presence of radicals (i.e. antioxidants) causes the conversion of the Fe^{3+} / ferricyanide complex used in this method to the ferrous form indicated by the formation of pearls Prussian blue at 700 nm.

The results of the Ferric Reducing Power Assay test of the alcoholic and aqueous extracts in comparison with the standard (ascorbic acid) at 700 nm is shown in table 5. The percentage of inhibition against different concentrations of both extracts of the research drug as well as ascorbic acid was used to plot the standard curve which was used to calculate the IC_{50} ($\mu\text{g/ml}$) of each sample, which was determined as 42.024 ± 16.816 , 49.926 ± 16.240 and 5.503 ± 0.545 for alcoholic extract, aqueous extract of herbal rejuvenator drug and Ascorbic Acid respectively. It is also observed that inhibition percentage values go on increasing with enhancements in concentration of research plant extracts in the assay mixture.

DISCUSSION

The total phenolic content of the alcoholic root extract was $220.00 \mu\text{g}$ Gallic acid equivalents/mg while it was 225.67 for the aqueous extract. Similarly, the total flavonoid content was assessed as 74.76 and $59.14 \mu\text{g}$ Quercetin equivalent /mg for these two extracts respectively. Thus, the Alcoholic extract of the research drug shows more Quercetin equivalent and Gallic acid equivalent content indicating both higher Flavonoids and higher Phenols than its aqueous extract. Phenolic compounds have redox properties, which allow them to act as antioxidants³³. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity in vitro and also act as antioxidants in vivo. Plants rich in secondary metabolites, including phenolics, flavonoids and carotenoids, have antioxidant activity due to their redox properties and chemical structures³⁴⁻³⁵. The estimation of Total Antioxidant Capacity during the study indicated higher Antioxidant content (expressed as ascorbic acid equivalent) in the alcoholic extracts over the different concentrations (0.1, 0.2 & 0.3 mg/ml) as compared to the aqueous extract,

Table 3: Hydrogen peroxide scavenging activity.

Research Sample	Concentration ($\mu\text{g/ml}$)	% of Inhibition	IC ₅₀ ($\mu\text{g/ml}$) from standard curve
Alcoholic Extract	12.42	33.195 \pm 8.649	41.529 \pm 10.135 (R ² =0.999)
	24.69	38.873 \pm 7.097	
	36.81	44.229 \pm 4.854	
Aqueous Extract	12.42	33.437 \pm 1.627	39.654 \pm 4.022 (R ² =1)
	24.69	41.097 \pm 2.124	
	36.81	48.654 \pm 2.162	
Ascorbic Acid as Standard	12.42	25.350 \pm 3.37	17.511 \pm 1.17 (R ² =0.996)
	24.69	82.036 \pm 7.05	
	36.81	136.020 \pm 4.81	
	48.78	177.579 \pm 4.90	

Values are represented as Mean \pm SEM.

Table 4: DPPH radical scavenging activity.

Research Sample	Concentration (mg/ml)	% of Inhibition	IC ₅₀ (mg/ml) from standard curve
Alcoholic Extract	0.25	10.621 \pm 4.448	1.825 \pm 0.122 (R ² =0.999)
	0.50	16.776 \pm 4.108	
	1.00	29.739 \pm 1.961	
Aqueous Extract	0.25	48.747 \pm 0.144	1.035 \pm 0.09 (R ² =0.995)
	0.50	49.237 \pm 0.0545	
	1.00	50.000 \pm 0.163	
Ascorbic Acid as Standard	0.05	0.545 \pm 0.144	0.207 \pm 0.006 (R ² =0.99992)
	0.10	16.721 \pm 1.911	
	0.15	32.952 \pm 0.964	
	0.20	47.386 \pm 2.047	

Values are represented as Mean \pm SEM.

which is in consonance with the presence of higher levels of Phenolics and Flavonoids in the alcoholic extract. Examination of the IC₅₀ values obtained during Hydrogen Peroxide Radical Scavenging test indicate that when compared with the standard drug ascorbic acid, more than twice amount of concentrations of the aqueous and alcoholic extracts of the research drug are required for scavenging 50 % Hydrogen peroxide. The free radical scavenging effect in respect of Hydrogen Peroxide was found higher in the aqueous extract than in the alcoholic extract at 12.42, 24.69 and 36.81 $\mu\text{g/ml}$ concentrations of the research drug. However, the radical scavenging effect at 24.69 and 36.81 $\mu\text{g/ml}$ concentrations for both aqueous and alcoholic extracts of the research drug was quite low in comparison to the standard drug ascorbic acid.

The high phenolic and flavonoid content is responsible for the bioactivity of these crude extracts. Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases. Hydrogen peroxide (H₂O₂), a biologically relevant, non-radical oxidizing species, may be formed in tissues through oxidative processes, but there has been limited information regarding its scavenging by polyphenolic antioxidants. Among reactive oxygen species (ROS), hydrogen peroxide (H₂O₂) is a relatively stable, non-radical oxidant, which can diffuse across biological membranes. It is produced by 2-electron reduction of molecular oxygen or by dismutation of the superoxide anion radical³⁶⁻³⁸.

Ascorbic acid or Vitamin C has been used as the standard in this research since it is a water-soluble free radical scavenger. Moreover, it regenerates vitamin E in cell membranes in combination with compounds capable of donating reducing equivalents. Vitamin C changes to the ascorbate radical by donating an electron to the lipid radical in order to terminate the lipid peroxidation chain reaction³⁹. The research plant extracts reduce the DPPH radical to corresponding hydrazine when they react with hydrogen donors. It is noticed that aqueous and alcoholic extracts of the research drug are required in higher concentrations than the ascorbic acid standard for performing antioxidant activity measured by assessing the concentration required for 50 % scavenging of the DPPH radical, i.e. IC₅₀ ($\mu\text{g/ml}$). The aqueous extract exhibits higher scavenging activity at various concentrations during the DPPH assay test as compared to the alcoholic extract.

The free radical scavenging effect assessed as percentage inhibition was found to be 50.000 in the aqueous extract at 1.0 mg/ml concentration as compared to 29.739 at same concentration in the alcoholic extract. It is also observed that inhibition percentage increases commensurate with increases in concentration of research plant extracts in the assay mixture during DPPH test.

An antioxidant has the ability to donate a hydrogen atom that will quench the stable free radical which is associated with a change in absorption which can be followed calorimetrically. The pre-formed radical mono-cation of (ABTS^{•+}) is generated by oxidation of ABTS with potassium per-sulfate and is reduced in the presence of

Table 5: Percentage of ABTS radical scavenging activity using Trolox.

Research Sample	Concentration ($\mu\text{g/ml}$)	% of Inhibition	IC ₅₀ ($\mu\text{g/ml}$) from standard curve
Alcoholic Extract	20	45.093 \pm 3.262	28.088 \pm 5.618 (R ² =0.998)
	40	56.937 \pm 3.617	
	60	70.643 \pm 3.410	
Aqueous Extract	20	48.816 \pm 1.736	23.731 \pm 4.870 (R ² =0.999)
	40	56.895 \pm 2.868	
	60	64.721 \pm 3.334	
Trolox as Standard	2.70	16.487 \pm 3.022	6.728 \pm 0.213 (R ² =0.999)
	4.05	27.073 \pm 2.664	
	8.10	62.193 \pm 1.601	
	10.8	83.248 \pm 0.695	

Values are represented as Mean \pm SEM.

Table 6: Results of FRAP Essay.

Research Samples	Concentration ($\mu\text{g/ml}$)	% of Inhibition	IC ₅₀ ($\mu\text{g/ml}$) from standard curve
Alcoholic Extract	75	91.968 \pm 24.734	42.024 \pm 16.816 (R ² =0.999)
	150	172.289 \pm 54.315	
	300	340.161 \pm 138.249	
Aqueous Extract	75	61.765 \pm 8.985	49.926 \pm 16.240 (R ² =0.998)
	150	109.314 \pm 7.418	
	300	191.667 \pm 3.214	
Ascorbic Acid as Standard	4	42.681 \pm 2.681	5.503 \pm 0.545 (R ² =0.994)
	8	76.934 \pm 15.283	
	12	106.019 \pm 25.768	
	16	147.570 \pm 37.717	
	20	189.878 \pm 53.945	

Values are represented as Mean \pm SEM.

hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction are taken into account when determining the antioxidant activity. It is observed that for any particular level of antioxidant activity, higher concentrations of aqueous and alcoholic extracts of the research drug are required than the standard ascorbic acid. This phenomenon is clearly observed when measuring the antioxidant activity for scavenging 50% ABTS radicals as evaluated in the form of IC₅₀ ($\mu\text{g/ml}$). The free radical scavenging effect measured as percentage inhibition in case of the stable ABTS was found to be 70.643 in the alcoholic extract at 60 $\mu\text{g/ml}$ concentration as compared to 64.721 in aqueous extract at the same concentration of the herbal rejuvenator drug. The standard drug Trolox showed high inhibition at very low values of concentration in comparison to both the aqueous and alcoholic extracts. Since Trolox is a water-soluble analog of vitamin E, it is used as an antioxidant like vitamin E in biological or biochemical applications to reduce oxidative stress or damage.

FRAP is an electron transfer based total antioxidant assay, also called redox linked colorimetric method. There is an increase in absorbance at a pre specified wavelength as an antioxidant reacts with chromogenic reagent (Fe (II))⁴⁰. The percentage of inhibition at 150 $\mu\text{g/ml}$ concentrations was found to be 172.289 in case of alcoholic and 109.314 for aqueous extract, while it was 340.161% and 191.667% in case of alcoholic and aqueous extracts respectively at

300 $\mu\text{g/ml}$ concentration. Thus, the alcoholic extract of the research drug consistently showed higher inhibition and, therefore, higher antioxidant activity during this study. The percentage inhibition as well as the IC₅₀ values clearly indicate that the standard (ascorbic acid) showed very low IC₅₀ levels indicating its very high antioxidant capacity in comparison with both the alcoholic and aqueous extracts during this test.

The obtained IC₅₀ values in respect of the two extracts as well as the standard drug using the various techniques discussed above have been shown in fig. 1. It is evident from these findings that aqueous extract shows higher level of antioxidant activity as compared to the alcoholic extract in all the methods of analysis except FRAP assay.

CONCLUSION

The evaluation of in vitro antioxidant activity of the aqueous and alcoholic extracts of the research drug showed substantial and significant free radical scavenging activity during the study in both the extracts which is compared to that of the standard drug. This significant pronounced antioxidant activity of alcoholic and aqueous extracts may be due to the presence of higher concentrations of phenolic compounds and flavonoids in them. This in vitro assessment of the antioxidant properties of the aqueous and alcoholic extracts of polyherbal formulation consisting of the stem bark of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. validates and confirms the free radical

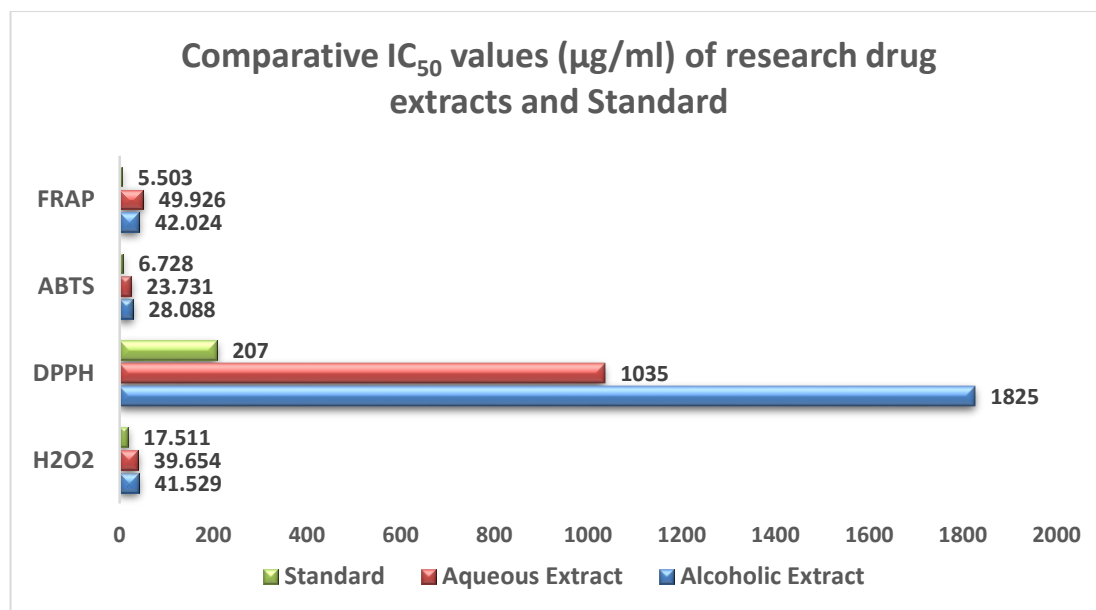


Figure 1: Comparative IC₅₀ values of research drug extracts and Standard drug.

scavenging activity as described in the ancient texts of Ayurvedic system of medicine.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

ACKNOWLEDGEMENTS

This research work was supported by grants from the Indian Council of Medical Research, Government of India (Sanction Letter No. 59/1/2011/BMS/TRM dated 12.12.2013) and the authors are grateful for this support. We are thankful to Dr. Tapan Seal, Senior Scientific Officer, Botanical survey of India, Howrah, Kolkata for identification and other scientific help during the research work.

REFERENCES

- Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. Bull. World Health Organ. 1985; 63: 965-981.
- Hegde MV, Patil S, Bhalerao S A. Philosophy for integration of ayurveda with modern medicine: A biochemist's perspective. Curr. Sci. 2008; 95: 721-722.
- Halliwell B. Drug antioxidant effects. Drugs 1991; 42: 569-605.
- Wang SY, Jiao H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. J. Agric. Food. Chem. 2000, 48 (11): 5677-5684.
- Živković J, Zeković Z, Mujić I, Vidović S, Cvetković D, Lepojević Ž, Nikolić G, Trutić N. Scavenging Capacity of Superoxide Radical and Screening of Antimicrobial Activity of *Castanea sativa* Mill. Extracts. Czech. J. Food. Sci. 2010; 28 (1): 61-68.
- Chung KT, Wong TY, Huang YW, Lin Y. Tannins and human health: a review. Crit. Rev. Food Sci. Nutr. 1998; 38: 421-464.
- Bernays EA, Driver GC, Bilgener M. Herbivores and plant tannins. Adv. Ecol. Res. 1989; 19: 263-302.
- Frankel, EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet. 1993; 341 (8852): 1103-1104.
- Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates SE. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res. 1999; 59 (1): 8-13.
- Shastri SN. *Charak Samhita (Chikitsa Sthanam)*, Chapter 3; Chaukhamba Bharati Academy: Varanasi, India, 1988, 47.
- Sharma PV. *Introduction to Dravyaguna (Indian Pharmacology)*, vol. 2; Chaukhamba Orientalia: Varanasi, India, 1995, 616, 666.
- Sharma PC, Yelne MB, Dennis TJ. Database on medicinal plants used in Ayurveda, Central Council for Research in Ayurveda & Siddha, Department of Indian system of medicine, Govt. of India, New Delhi, 2001, vol. 3, 76-87, 166.
- Joshi U and Mohini U. Evaluation of Antioxidant Activity of Aqueous Extract Bark of *Ficus Glomerata*, Research Journal of Pharmacy and Technology, 2008; 1(4): 537-538.
- Devmurari VP. Antibacterial Evaluation and Phytochemical Screening of *Symplocos racemosa* Roxb, International Journal of PharmTech Research, 2010; 2(2): 1359-1363..
- Ahmad VU, Abbasi MA, Hussain H, Akhtar MN, Farooq U, Fatima N, Choudhary MI. Phenolic glycosides from *Symplocos racemosa*: natural inhibitors of phosphodiesterase I., Phytochemistry, 2003; 63(2): 217-220.
- Kasote DM. Flaxseed phenolics as natural antioxidants. International Food Research Journal, 2013; 20 (1): 27-34.

17. Furniss BS, Hannaford AJ, Smith PWG, Tatchell AR. *Vogel's Textbook of Practical Organic Chemistry*, 5th Edition; Addison Wesley Longman Inc. 1989, 1205.
18. Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii*. *Journal of Taibah University for Science*, 2015; 9: 449-454.
19. Harborne JB. *Phytochemical Methods: A guide to Modern Technique of Plant Analysis*, 2nd edition, Chapman and Hall: London, 1984.
20. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* 2002; 10: 178-182.
21. Usha Sri B, Ibrahim M, Mahendra Kumar CB. Antioxidant activity and total flavonoids content of different parts of *Azadirachta indica* A. Juss. *Journal of Medicinal Plants Research*, 2012; 6 (46): 5737-5742.
22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application of vitamin E. *Anal Biochem* 1999; 269 (2): 337-341.
23. Keser S, Celik S, Turkoglu S, Yilmaz O, Turkoglu I. Hydrogen Peroxide Radical Scavenging and Total Antioxidant Activity of Hawthorn. *Chemistry Journal* 2012; 02 (1): 9-12.
24. Basniwal PK, Suthar M, Rathore GS, Gupta R, Kumar V, Pareek A, Jain D. In-vitro antioxidant activity of hot aqueous extract of *Helicteres isora* Linn. fruits. *Natural Product Radiance*, 2009; 8 (5): 483-87.
25. Nishaa S, Vishnupriya M, Sasikumar JM, Hephzibah PC, Gopalakrishnan VK. Antioxidant activity of ethanolic extract of *Maranta arundinacea* L. tuberous rhizomes. *Asian Journal of Pharmaceutical and Clinical Research* 2012; 5 (4): 85-88.
26. Anil KV, Babul D, Rama T. Evaluation of Anti-ulcerogenic properties from the root of *Flemingia strobilifera*. *Journal of Basic and Clinical Pharmacy*, 2011; 2 (1): 33-39.
27. Özcelik B, Lee JH, Min DB. Effects of light, oxygen and pH on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method to evaluate antioxidants. *J. Food Sci.* 2003; 68: 487-490.
28. Tupe RS, Kemse NG, Khaire AA. Evaluation of Antioxidant potentials and Total Phenolic contents of Selected Indian Herbs powder extracts. *Indian Food Research journal* 2013; 20 (3): 1053-63.
29. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 1999; 26: 1231-1237.
30. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn. J. Nutr.* 1986; 44: 307-315.
31. Hemalatha S, Lalitha P, Arulpriya P. Anti-oxidant activities of the extracts of the aerial roots of *Pothos aurea* (Linden ex Andre). *Der. Pharma. Chemica.* 2010; 2 (6): 84-89.
32. Aparadh VT, Naik VV, Karadge BA. Antioxidative Properties (TPC, DPPH, FRAP, Metal Chelating Ability, Reducing Power and Tac) within some Cleome Species. *Ann. Bot. (Roma).* 2012; 2: 49-56.
33. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun OT. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat. Res. Fundam. Mol.* 2005; 579: 200-213.
34. Geetha S, Sai-Ram M, Mongia SS, Singh V, Ilavazhagan G., et al. Evaluation of antioxidant activity of leaf extract of sea buckthorn (*Hippophae rhamnoides* L.) on chromium (VI) induced oxidative stress in albino rats. *J. Ethnopharmacol.* 2003; 87: 247-251.
35. Shimoi K, Masuda S, Shen B, Furugori M, Kinze N. Radioprotective effects of antioxidative plant flavonoids in mice. *Mutat. Res. Fund. Mol.* 1996; 350: 153-161.
36. Shukla S, Mehta A, Bajpai KV, Shukla S. In vitro antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. *Food Chem. Toxicol.* 2009; 47: 2338-2343.
37. Bravo L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr. Rev.* 1998; 56: 317-333.
38. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: location and functional significance. *Plant Sci.* 2012; 196: 67-76.
39. Nimse SB, Pal DK. Free radicals, natural antioxidants, and their reaction mechanisms, DOI: 10.1039/C4RA13315C (Review Article) *RSC Adv.* 2015; 5: 27986-28006.
40. Everest A, Ozturk E. Focusing on the ethnobotanical uses of plants in Mersin and Adana provinces (Turkey). *Journal of Ethnobiology and Ethnomedicine* 2005; 1(6). DOI: 10.1186/1746-4269-1-6.