ANTIOXIDANT, PHENOLICS, PHYTOCHEMICAL, FLUORESCENCE PROFILE OF BAOBARANG (EMBELIA RIBES FRUITS): PROMISING UNANI IMMUNO PROTECTOR

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Abstract. In unani medicine *Embelia ribes* fruits known as Baobarang are used to treat various diseases. Previous studies have shown them to possess Analgesic, Anti-microbial, Anti diabetic, Anti-cancer, anti-inflammatory activity etc. In view of the medicinal importance of *Embelia ribes* here, its fruit extracts in various solvents were evaluated for chemical constituents, total phenolics and in-vitro Antioxidant scavenging capacity by spectrophotometer. The Fluorescence profile of the herb was also generated by treatment with chemical reagents under UV (254 and 365 nm) and day light. The total phenolics in Aqueous, Alcoholic and hydroalcoholic extract were 46.66, 72.33 and 54.83 mg/g Gallic Acid Equivalent respectively and Antioxidant activity was determined using 2,2-diphenyl 1-picrylhydrazyl (DPPH)radical. Hydroalcoholic extract of Baobarang showed maximum scavenging activity with minimum IC50 value. Phytochemical analysis showed the presence of flavonoids, phenols, steroids, tannins, proteins, amino acids, carbohydrates, alkaloids, glycosides, resins and saponins. The presence of phenolics and antioxidant activity accounts for its use as potential herbal natural oxidative stress reducing remedy. The study further provides scientific support to the use of Baobarang as General tonic Anti-ageing and Immuno protective in unani system of medicine.
Keywords: *unani, antioxidant, DPPH, total phenolic content, fluorescence, phytochemicals*

Introduction

Embelia ribes (Baobarang) is a member of Myrsinaceae family. Baobarang is a shrub, branches are long, cylinderical, flexible and bark contains with lenticles. The fruits are reddish black berries and globular in shape (Warrier, 1993). It is also known as false pepper and found throughout India predominantly in Kerala, Tamil Nadu and Maharashtra, in Eastern Ghats and Karnataka in Western Ghats (Annapurna et al., 2013). The plant has been traditionally used to treat intestinal worms, infection of skin, Lung diseases, pneumonia, mouth ulcer, fungus infections, obesity and heart diseases. The Baobarang has been studied for Analgesic activity (Zutshi et al., 1989), Anthelmintic (Tambekar et al., 2009), Anti-bacterial (Antoney et al., 2016), Antioxidant property (Swamy et al., 2007), Anti diabetic (Mahendran et al., 2011), Anticonvulsant (Joshi et al., 2017), Anti-cancer (Swamy et al., 2007) and Chemotherapeutic activity (Joy and Lakshmi, 2010). It has also exhibited Antiproliferative (Bhandari et al., 2002), Antitumor and anti-inflammatory activities (Swamy et al., 2007), Antihyperlipidemic, Antifungal (Singh and Singh, 2001), Antihyperhomocysteinemic (Krishnaswamy and Purushothaman, 1980), Mollusicidal (Vinutha et al., 2007) and Wound healing property (Joy and Lakshmi, 2010). Antifertility activity and Antispermatogenic activity (Swamy

et al., 2007) were also shown by the baobarang extracts. The major component responsible for medicinal properties of dried fruits of Embelia ribes is Embelin (2, 5-dihydoxy-3-undecyl1,4-benzoquinone) and Christeembine (Lal and Mishra, 2013). Thus the present study is aimed to Determine Total Phenolic Content quantitatively and in-vitro Antioxidant potential of various crude extracts in the fruits of Embelia ribes (Baobarang) by spectrophotometer. The phytochemical profile of the test drug to confirm the presence of chemical constituents especially phenols and fluorescence analysis of powdered drug and successive extracts was also carried out to characterize the genuine herbal drug.

Materials and Methods

Selection and validation

The test sample of Baobarang (*Embelia ribes*) was collected fromlocal sources. Baobarang fruits belong to Myrsinaceae family. The drug was properly verified through the Unani literature and then the drug was confirmed in National Institute of Science Communication and Information Resources (NISCAIR, New Delhi). Ref.No. NISCAIR/RHMD/Consult/2018/3258-59-3. The voucher specimen (Voucher No. SC-0221/17) was deposited in the mawalid-e-salasa museum of the Department.

Preparation of crude extract

The coarse dried powder of the drug was subjected to Soxhlet extraction using various solvents in order of increasing polarity, i.e. Petroleum ether, Acetone, Chloroform, Ethyl Acetate and water for 6 hours with each solvent. The extract obtained was subjected to dryness at 40-50°C and was kept under refrigerator till further use. The ethanolic and aqueous extracts were used for the proposed study. Further 50% hydro alcoholic solution (50:50, ethanol and water) was also prepared using reflux method of extraction. After 6 hours of extraction the solution was filtered, the extract was concentrated by evaporating solvent at reduced pressure, the extract obtained was used for the study.

Phytochemical qualitative analysis

The chemical constituents present in the test drug were checked qualitatively following the scheme given by Bhattacharjee and Das (1969). Then analysis of different chemical plant components like alkaloids, glycoside, phenols, flavonoids etc were done through various chemical tests by using the standard methods.

Fluorescence analysis

Fluorescence analysis of powdered drugs

The powdered drug was subjected to Fluorescence analysis for characterization and identification of the crude drug. It was treated with a number of chemical reagents (Kokoski et al., 1958; Chase Jr and Pratt, 1949) and the changes in colour in daylight and on exposure to ultra violet light were observed.

Fluorescence analysis of the soxhlet extractives of the baobarang

Successive extracts obtained by Soxhlet extraction of the test drug using solvents Petroleum ether, diethyl ether, chloroform, ethyl acetate, ethanol and water were also noticed in UV lights using UV cabinet and in daylight.

Quantitative determination of total phenolic content in the Embelia ribes fruits with spectrophotometer

Total phenolics in ethanolic, aqueous and 50% hydro alcoholic extract of test drug was done by Folin Ciocalteau reagent according to the method given by Singleton and Rossi (1965). Calibration curve was recorded by taking 1 ml solutions of 50, 100, 200, 300, 400 and 500 µg/ml of solution of Gallic acid in distilled water , 5.0 ml of Folin Ciocalteau reagent (diluted tentimes) and sodium carbonate solution (4.0 ml; 75g/l). After 30 minutes the absorbance was noted at 765 nm. As did in construction of Calibration Curve same reagents were mixed separately with One ml of ethanolic, aqueous and hydro alcoholic (1000µg/ml) extract and after 1 hour the absorbance of test drug extracts was measured separately for the estimation of total phenolics. Distilled water was used as blank. Three readings were taken for each and every solution for checking the reproducibility and to get accurate result. Results are provided and describe in tables below and Total phenolic content was represented as mg/g Gallic Acid Equivalent.

Determination of antioxidant activity by DPPH radical scavenging activity

A simple, inexpensive and quick method to know the antioxidant potential of any substance is the use of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical. It is used to check the antioxidant activity of foods and also it's ability to act as hydrogen donor or free radical scavengers. DPPH method is a general method to measure the total antioxidant property of the given sample. Here the free radical scavenging capacity of ethanolic, aqueous and 50% hydroalcoholic extracts was measured using the method of Chang et al. (2001). Ascorbic acid (1000 µg/ml) was taken as the standard. The change in the absorbance value of the DPPH solution, as test drug extract added at 517 nm was measured as a criterion of antioxidant capacity. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a purple coloured stable radical powder, which changes to yellow when scavenged. This character is used in DPPH assay to show scavenging activity. The reaction between DPPH free radical and an antioxidant RH is as: (DPPH) + RH = DPPH-H + R. Antioxidant reduces DPPH to DPPH-H, causing a decrease in absorbance with discolouration of the test solution. In the same way DPPH stable free radical reacts with H-donors like phenols, polyphenols and flavonoids present in herbal drug extract. the scavenging potential of the drug extract is directly proportional to the discolouration produced indicating the antioxidant potential in terms of ability to donate hydrogen and stop the free radical chain reaction (Kalim et al., 2010; Brand-Williams et al., 1995).

Preparation of working solutions

DPPH (0.1mM) solution: This reagent was made 2 hour prior to use to ensure all the DPPH has dissolved and stabilised. 394 mg of DPPH was dissolved in 1000 ml methanol in standard flask and this will be our DPPH stock. Now from this 0.1 mM DPPH was prepared in standard flask and kept in refrigerator covered with aluminium foil to protect from the light. *Ascorbic acid stock (1000µg/ml) solution*: 100 mg Ascorbic acid was weighed and made up to a final volume of 100ml using methanol.

Then different concentrations from 10 to 1000µg/ml were made by using stock solution. *Test solutions*: The aqueous, ethanolic and 50% hydro alcoholic extract of the test drug was dissolved in methanol to obtain the 1000µg/ml stock solution. Then from the stock solution 10 to 1000µg/ml solutions were prepared by dilution method and were used for the study. *Working Procedure*: 1.0 ml of DPPH solution (0.1mM in methanol) was added to 2.0 ml of test drug extract solution at different concentration (10-1000µg/ml) in methanol and mixed thoroughly. The reaction flasks were retained for 30 minutes in dark. The absorbance of the above mixture, after 30 minutes was noted at 517 nm using methanol as blank. Decreasing absorbance with increase in concentration of extract indicates increasing free radical scavenging ability. Standard used was ascorbic acid. Experiment was done in triplicate. The percent DPPH radical inhibition can be was calculated using the following Eq. (1):

$$\%\, radical\ scavenging\ activity = \frac{A_{control} - A_{sample}}{A_{control}} \qquad \text{Eq. (1)}$$

Where, $A_{control}$ are the absorbance of DPPH + Solvent; A_{sample} are the absorbance of DPPH + plant extract; and values were represented as mean±S.E.M of three parallel data. The concentration of test drug extract for scavenging 50% of DPPH radicals required is represented by IC_{50} value and can be calculated using the curve showing the percent inhibition Vs concentration of extracts used. The IC_{50} values were compared by one way ANOVA test.

Results and Discussion

Phytochemical qualitative analysis

Qualitative analysis of the test drug Baobarang was accomplished for the determination of phytochemicals present in the crude drug as they are mainly responsible for therapeutic values of herbal drugs. Baobarang contained alkaloids, carbohydrates, glycosides, proteins, flavonoids, sterols, phenols, tannis, saponins, fixed oil and volatile oil (*Table 1*). The presence of phenols and flavonoids was an indication of its expected antioxidant potential.

Table 1. *Qualitative analysis of the phytochemicals present in the Embelia ribes fruits.*

S. No.	Chemical constituents	Test/reagent	Result
1	Alkaloids	Dragendorff's reagent	+ve
		Mayer reagent	+ve
		Wagner reagent	+ve
2	Carbohydrate	Molisch	+ve
		Benedict reagent	+ve
		Fehling solution test	+ve
3	Flavonoids	Mg ribbon test	+ve
		Zinc dust test	+ve
4	Glycosides	NaoH test	+ve
5	Tannins	Ferric Chloride test	+ve
6	Proteins	Biuret test	+ve
		Xanthoproteic test	-ve
7	Starch	Iodine test	-ve
8	Phenols	Lead acetate test	+ve
9	Sterols	Salkowski reaction	+ve
10	Amino acid	Ninhydrin solution test	+ve
11	Resin	Acetic Anhydride	+ve
12	Saponins	Frothing with NaHCO3	+ve
13	Fixed oil and volatile oil	Filter paper test	+ve

Fluorescence analysis of baobarang (Embelia ribes)

Fluorescence analysis under UV light is sometimes very characteristic for a drug. Therefore fluorescence analysis of the crude drug powder and various extracts was carried out by treating them with different chemical reagents and color changes on exposure to UV and day light were observed (*Table 2* and *Table 3*).

***Table 2**. Fluorescence analysis of Baobarang (Embelia ribes).*

S. No.	Powdered drug	Day light	UV short	UV long
1.	Drugs	Brown	Light Green	Black
2.	P. drug +Conc. HNO3	Brown	Light Green	Black
3.	P. drug+ Conc.HCl	Brown	Dark Green	Black
4.	P.drug+ H2SO4	Brown	Light Green	Redish Black
5.	P. drug+ Glacial Acetic Acid + HNO3	Brown	Light Green	Green
6	P. drug +Distilled. H2O	Brown	Light Green	Black
7.	P. drug + NaOH (10%)	Brown	Dark Green	Light Green
8.	P. drug +Dil. HNO3	Brown	Light Green	Green
9.	P. drug +Dil. H2SO4	Brown	Light Green	Black
10.	P. drug + Dil. HCl	Brown	Light Green	Black
11.	P. drug +Dragendorff's reagent	BB/B	Brownish Black	Black
12.	P. drug +Wagner's reagent	Brown	Dark Brown	Dark Brown
13.	P. drug +Benedict's reagent	BB/B	Blackish Brown	Black
14.	P. drug + Fehling's reagent	Black	Black	Black
15.	P. drug +KOH (10%)	Brownish Black	Brownish Black	Brownish Black
16.	P. drug + CuSO4 (5%)	Dark Brown	Dark Brown	Dark Brown
17.	P. drug + Ninhydrin solution (2% in Acetone)	BB/B	BB/B	BB/B
18.	P. drug + Picric Acid	Reddish Brown	Reddish Brown	Dark Brown
19.	P. drug + Lead Acetate (5%)	Brown	Light Green	Brown
20.	P. drug +Acetic Acid + H2SO4	Black	Black	Black
21.	P. drug + Formic Acid	Reddish Black	Reddish Brown	Dark Brown
22.	P. drug + Acetic Acid	Dark Brown	Dark Brown	Dark Brown

Note: P. drug=Powdered Drug; BB/B=Blackish Brown/Black.

***Table 3**. Fluorescence analysis of the successive extracts of Baobarang (Embelia ribes).*

S. No.	Extract	Day light	UV short	UV long
1.	Petroleum Ether	Brown	Light Brown	Blackish Brown
2.	Diethyl Ether	Brown	Dark Brown	Brown
3.	Chloroform	Brown	Brown	Brown
4.	Ethyl Acetate	Light Brown	Brown	Blackish Brown
5.	Ethanol	Brown	Dark Brown	Brown
6.	Aqueous	Light Brown	Light Brown	Light Brown

Quantitative estimation of total phenolic content in the Embelia ribes fruits with spectrophotometer

The presence of phenols in extract of Baobarang was checked first by phytochemical screening qualitatively and were tested positive. After that the total phenolics in alcoholic, hydroalcoholic and aqueous extracts of the test drug was determined by the Folin Ciocalteu reagent as given by Singleton and Rossi (1965) with spectrophotometer at 765nm in mg/g, Gallic acid equivalent (GAE). The total phenolics were calculated using as in the *Figure 1*, respective Y equation of calibration curve was $Y=0.006X+0.2238$ with $R^2=0.9995$. It was found that all the extracts contain phenolic in significant amounts (*Table 4* and *Table 5*; *Figure 1* and *Figure 2*).

***Table 4**. Estimation of total phenolic content (in mg/g gallic acid equivalent): Absorbance recorded for test drug samples at 765nm.*

S. No.	Drug name	Drug concentration	Aqueous extract absorbance	Alcoholic extract absorbance	Hydroalcoholic extract absorbance
1	Boabarang	1000 µg/ml	0.50	0.649	0.552

***Table 5**. Total phenolic content (in mg/g gallic acid equivalent).*

S. No.	Drug name	Aqueous extract	Alcoholic extract	Hydroalcoholic extract
1	Baobarang	46.66	72.33	54.83

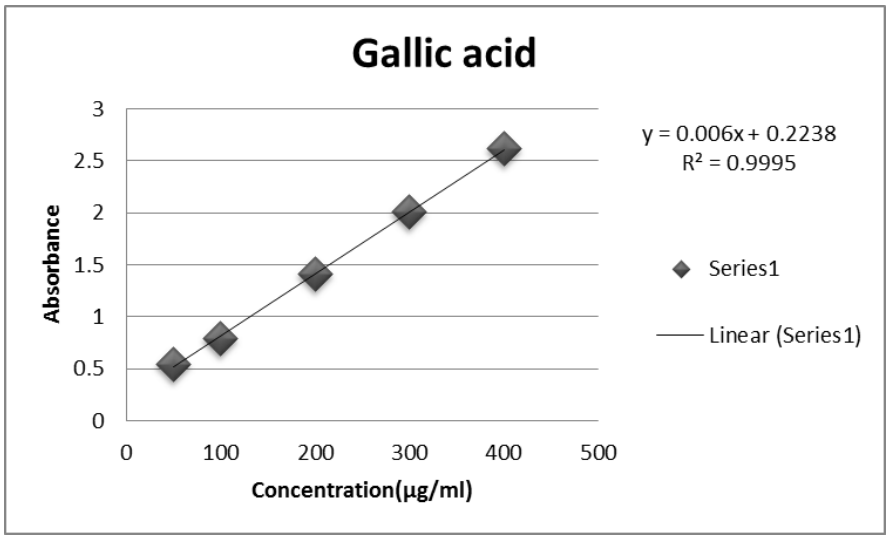

***Figure 1.** Standard curve of Gallic Acid.*

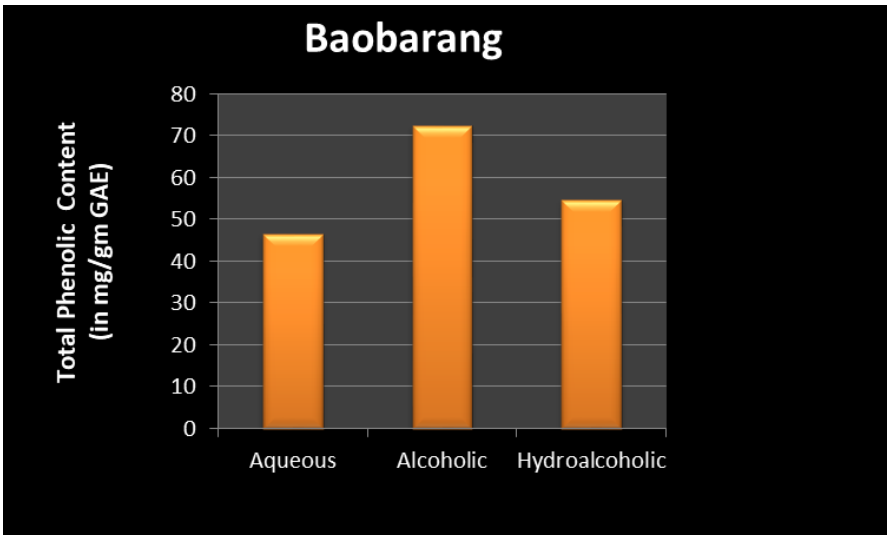

***Figure 2.** Total phenolic content of Boabarang (Embelia ribes).*

Evaluation of antioxidant property by radical scavenging method

The antioxidant capacity of the alcoholic, hydroalcoholic (1:1) and aqueous extract of the baobarang was evaluated by the method of DPPH radical scavenging and compared With the Ascorbic acid as standard. IC_{50} value indicates the drug's extract concentration required to inhibit 50% radicals, was find out. In Alcoholic extract of Baobarang standard curve equation obtained was y=0.138x+45.39 where R^2=0.999, the concentration at 50%inhibition (IC_{50}) was 33.40 (µg/ml) with p<0.0001µg/ml. For Hydroalcoholic extract of Baobarang y=0.373x+40.81 with R^2=0.997, IC_{50} value was found to be 24.63 (µg/ml) with p<0.0001.For Aqueous extract the standard curve equation was found to be y=0.680x+11.17, R^2=0.998, IC_{50}=57.10 (µg/ml) (p<0.0001). Baobarang showed highly significant antioxidant activity. The Hydroalcoholic extract

showed maximum activity among all the three extracts, when compared with ascorbic acid (IC_{50}=14.56 µg/ml) (*Table 6* and *Table 7*; *Figure 3* to *Figure 6*).

***Table 6**. Antioxidant activity of ascorbic acid.*

Equation	R^2 value	IC_{50} (µg/ml)
y=0.320x + 45.34	0.999	14.56

***Table 7**. Antioxidant activity of Baobarang (Embelia ribes).*

S. No.	Extract	Equation	R^2 value	IC_{50} (µg/ml)
1.	Alcoholic	y = 0.138x + 45.39	0.999	33.40
2.	Hydroalcoholic	y = 0.373x + 40.81	0.997	24.63
3.	Aqueous	y = 0.680x + 11.17	0.998	57.10

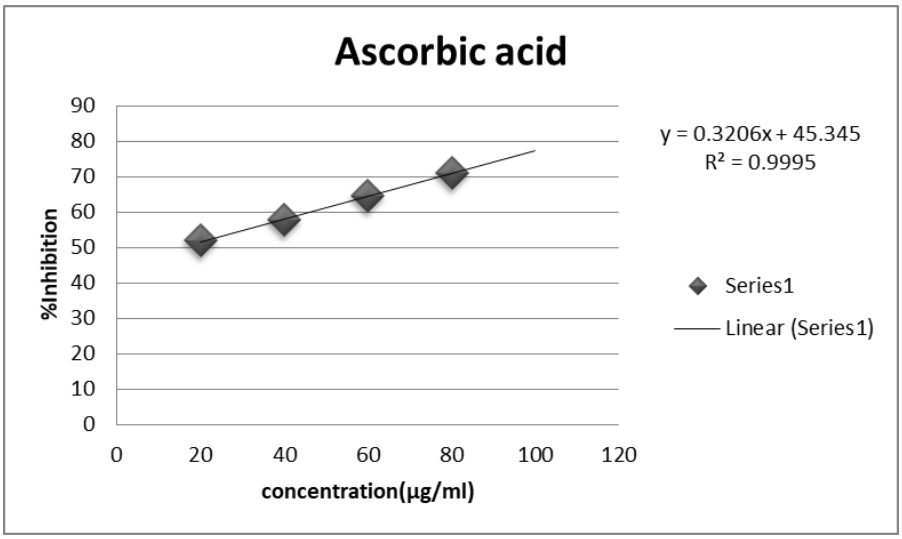

***Figure 3.** Standard curve of Ascorbic Acid.*

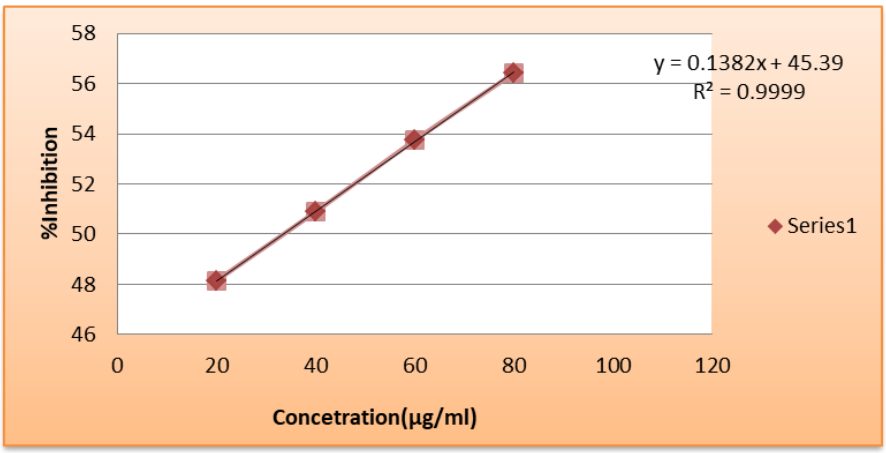

***Figure 4.** DPPH radical scavenging assay of Baobarang Alcoholic extract.*

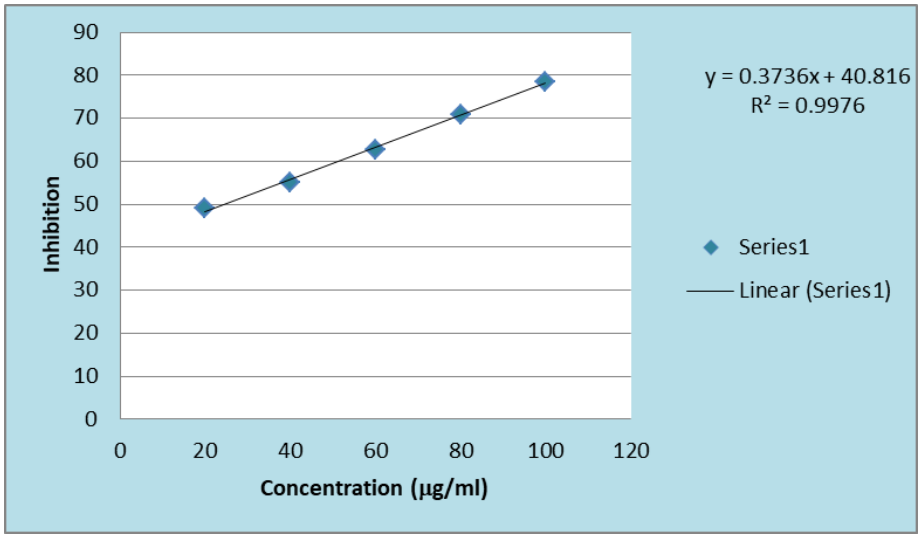

Figure 5. *DPPH radical scavenging assay of Baobarang Hydroalcoholic extract.*

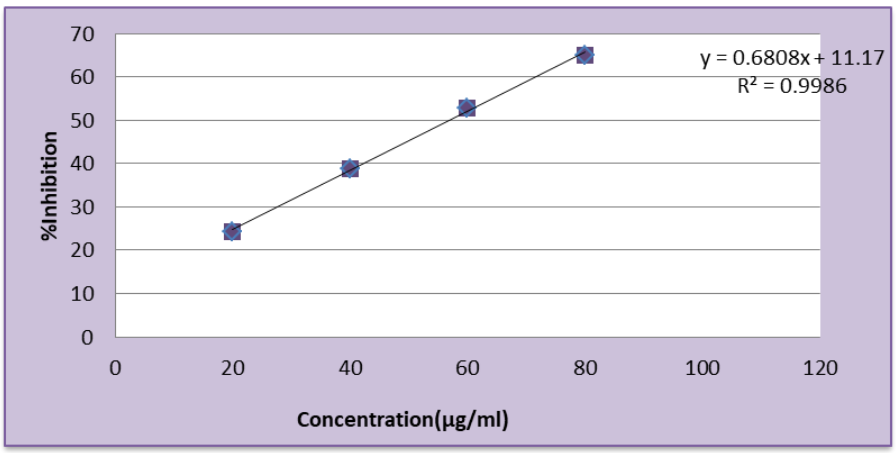

Figure 6. *DPPH radical scavenging assay of Baobarang Aqueous extract.*

In current scenario there is a great demand for plant derived products in the world. There is an increase in the use of these natural products as medicinal products. The concentration of chemical components present in a herbal drug varies in different plants but also varies in various samples of same species. Qualitative examination of the test drug was executed to find out the physiologically active chemical constituents responsible for biological activity of the test drug. Baobarang contained alkaloids, carbohydrates, glycosides, proteins, flavonoids, sterols, phenols, tannis, saponins, fixed oil and volatile oil. When some herbal drugs are exposed to ultraviolet radiations they emit specific colour known as fluorescence and this is due to various chemical constituents present in the plant material. It is one of the important pharmacognostical parameter and in assessing quality of crude drugs. So, powdered drug and successive extracts on exposure to UV light at 254 and 365nm showed characteristic fluorescence patterns (*Table 2* and *Table 3*) that will be helpful in correct identification of baobarang.

Plants have varying levels of total phenolic content. Herbs rich in phenolic compounds and flavonoids showed various biological activities (Saeed et al., 2012; Fecka et al., 2007; Rahman, 2007) like anti-inflammatory, anti-bacterial, anti-allergic, anti-mutagenic, anti- viral etc. So after checking phenols qualitatively, they were estimated by Folin's reagent using spectrophotometry and in Baobarang it was found to

be in order of Alcoholic > Aqueous > Hydroalcoholic extract. Studies from literature Piluzza and Bullitta (2011) as well as Kähkönen et al. (1999) suggested that the plants with the phenolic content have potential for antioxidant activity, thus all the three extracts of the test drug were subjected to antioxidant study. Natural Antioxidants are essentially the plants secondary metabolites inhibiting the harmful effects of free radicals (Piluzza and Bullitta, 2011). All the extracts of Baobarang (Embelia ribes fruits) showed highly significant antioxidant activity. Based on lower IC_{50} value, the hydroalcoholic extract showed maximum activity among all the three extracts, while alcoholic and aqueous extract also showed very good antioxidant activity when compared with Ascorbic acid. It was found that scavenging activity increases with increase in concentration of extract.

Conclusion

The phytochemical and fluorescence profile of *Embelia ribes* will be of help correct identification confirming the purity of genuine drug. The study here suggests that fruits of *Embelia ribes* (Baobarang) possess sufficient antioxidant activity and thus could be used as potential herbal natural antioxidant to cure various diseases related to oxidative stress. The presence of phenolic compounds and antioxidant activity justifies its use as General tonic (Muqawwi-e-Azaaa-e-Raeesah), Anti-ageing (Muharrikehararat-e-Gareeziyah) and Immuno protective (Muhafize-Tabiyat) drug in unani system of medicine.

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Conflict of interest

The authors confirm that there is no conflict of interest involved in this research study.

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