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Contributed Paper

RBC Haemolysis Prevention and Antioxidant Activity of *Barleria prionitis*

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ABSTRACT

Rural people across various regions of India uses leaf juice as well as they chew leaves of *Barleria prionitis* in treatment of oral ailments. Some studies pointed involvement of free radicals in oral ailments like aphthous ulcers. In this study the total phenolics and flavanoids were quantified in the extracts. The free radical scavenging activity was evaluated using in-vitro antioxidant assays, along with RBC haemolysis prevention activity. Results of FTC and TBA method suggested higher antioxidant potential of ethanolic extract than aqueous extract, thus further evaluation was carried out on ethanolic extract. Results of antioxidant activity and erythrocyte haemolysis prevention assay suggested significant potency (P value< 0.05) for ethanolic extract of the herb as compared to vitamin C. IC_{50} values for EBP and vitamin C were calculated by linear regression and found to be 622.90 µg/ml and 309.65 µg/ml (H_2O_2 radical scavenging), 505.98 µg/ml and 263.67 µg/ml (DPPH radical scavenging) and 646.93 µg/ml and 333.56 µg/ml (RBC haemolysis prevention). Based on findings of this study, we conclude the usefulness of *Barleria prionitis* in treatment of oral ailments may be due to its antioxidant potential.

Keywords: *Barleria prionitis*, TBA method, FTC method, H_2O_2 radical scavenging, DPPH radical scavenging, RBC haemolysis prevention

1. INTRODUCTION

Plants, since time immemorial are being used for their medicinal values. According to WHO, development of modern drugs depends vitally upon traditional medicinal plants that have been used for their medicinal properties [1-2]. *Barleria prionitis* (BP) Linn, commonly known as vajradanti being used for treatment various ailments by rural population across India. Leaves are chewed or juices of the leaves are used against toothache, gum ailments, dental troubles and pyorrhea and mouth ulcers [2-5]. Oral ailments like recurrent aphthous ulcers, gingivitis, pyorrhea, gum ailments, dental troubles are related to various contributing factors like allergies, genetic predisposition, hormonal influences, hematologic abnormalities, immunologic factors, infectious agents, nutritional deficiencies, smoking cessation, stress, and trauma. These factors have a

direct or indirect potential for disturbing the equilibrium between oxidant and antioxidant systems in humans. The formation of free radicals could be accelerated and toxic reactions could occur once this equilibrium is disturbed. Reactive oxygen radicals are the most common free radicals present in biological systems. Oxidative stress occurs when intracellular concentrations of free radicals increase over the physiological values, which results in cell damage by lipid peroxidation, DNA and protein damage, enzyme oxidation, and stimulation of pro inflammatory cytokine release[6-7]. Literature survey reveals that although this plant and its extracts were evaluated for a number of pharmacological activities, not much of the work has been reported towards its usefulness in oral ailment like aphthous ulcers.

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves with infloresence of BP were collected from tribal region of Amravati (Maharashtra) in the month of October 2014 and were authenticated by Dr. Mrs. Ranjana Mishra, Professor and Head of Department of Botany, Durg Science College, Durg (Chhattisgarh). Leaves collected from the herbs, dried in shade, powered and used for further work.

2.2 Extraction of Plant Material

The powdered plant materials are subjected to de-fatting by petroleum ether. Plant materials thus obtained are further subjected to hot continuous percolation and cold maceration to get ethanolic extracts and aqueous extracts respectively. Extracts are then treated with dichloromethane and ethyl acetate subsequently; in order achieve complete removal of fatty substances from the plant material and designated as EBP (Ethanolic extract of *B.prionitis*), and ABP (Aqueous extract of *B. prionitis*). Rural people across various regions of India uses leaf juice as well as they chew leaves of *Barleria prionitis* in treatment of oral ailments. In the context of traditional uses of the herbs and involvement of free radicals in etiology of oral ailments, we decided to study free radical and RBC haemolysis prevention activity of aqueous and ethanolic extracts of the herb.

2.3 Chemicals and Reagents

Chemicals like Folin's-Ciocalteu reagent, Diphenyl Picryl Hydrazine (DPPH), Gallic acid and Quercetin were procured from Sigma–Aldrich chemicals. Petroleum ether, Dichloromethane, Ethyl acetate, Sodium carbonate, Aluminum chloride, Potassium acetate, Linoleic acid, Phosphate Buffer (pH 7.4), Ammonium thiocyanate, Ferrous chloride, Trichloroacetic acid, Thiobarbituric acid, Ascorbic acid, Hydrogen peroxide, Hydrochloric acid, Sodium citrate, Phosphate buffer saline (pH 7.4), Sodium dihydrogen phosphate, Disodium hydrogen phosphate and Sodium chloride (All from Molychem) were purchased through m/s, Ideal Chemicals, Raipur. Blood sample, was collected from the male goat at local slaughter house.

2.4 Preliminary Phytochemical Screening

Phytochemical screening was carried out to identify the secondary metabolites of EBP and ABP [8].

2.5 Total Phenolic Content

The Total Phenolic content of aqueous and ethanolic extract of *Barleria prionitis was* determined by using Folin's-Ciocalteu reagent and Gallic acid as a calibration standard [9-10]. The total phenolic content of the BP *leaf* extracts was determined by using

Gallic acid equivalence (GAE).The dry extracts were diluted in ethanol in the concentration of mg/ml of the samples and 1ml was transferred to a 10 ml volumetric flasks, to which 0.5ml Folin's-Ciocalteu reagent was added. After one minute, 1.5ml of 20% (w/v) Na_2CO_3 was added and the volume made up to 10 ml with distilled water. The reaction mixture incubated at 25 °C for one hour and the absorbance was measured at 760nm and compared with a prepared Gallic acid calibration curve.

2.6 Determination of Total Flavanoid Content

The total flavanoid content was determined by aluminum chloride method [10-11]. The reaction mixture (2 ml) composed of 1 ml extract (mg/ml), 0.5 ml of 2% aluminum chloride in ethanol and 0.5 ml of potassium acetate (120 mM) was incubated at room temperature for 30 minutes and absorbance was measured at 510 nm. Quercetin was used as calibration standard. The total flavanoid content was expressed as Quercetin equivalent.

2.7 Antioxidant Activity

2.7.1 Ferric thiocyanate (FTC) method

The activity was carried out as per the method given by Kikuzaki and Nakatani [12-13]. A mixture containing 4 mg of the sample in four ml of 99.5% ethanol (final concentration 0.02%). 4.1ml of 2.52% linoleic acid in 99% ethanol, Eight ml of 0.05M phosphate buffer (pH 7.4) and 3.9 ml of water was placed in a vial with screw cap and then placed in an incubator at 40 °C in the dark. To 0.1 ml of this mixture, 9.7 ml of 75% ethanol (v/v) and 0.1 ml of 30% ammonium thiocyanate was added. Three minutes later the addition of 0.1ml of 0.02M ferrous chloride in 3.5% hydrochloric acid was added to reaction mixture; (the absorbance of red color indicated the antioxidant activity) was measured at 500 nm for every 24 hours until the absorbance of the control reached a maximum. The control and the standard were subjected to the same procedures as the sample except that for the control, only the solvent was used, and for the standard 4 mg of the sample was replaced by 1 mg of Vitamin C.

2.7.2 Thiobarbituric acid (TBA) method

The activity was determined as per the method given by Ottoleneghi [13-14]. TBA method used for evaluating the extent of lipid peroxidation. At low pH, and high temperature (100 °C), melonaldehyde binds TBA to form a red complex that can be measure at 532 nm. 2 ml of 20% trichloroacetic acid and 2 ml of 0.67% TBA solutions were added to 2 ml of the mixtures containing the sample prepared in the FTC method. This mixture was kept in water bath (100 °C) for 10 minutes and after cooling to room temperature, was centrifuged at 3000 rpm for 20 minutes. The percent antioxidant activity was based on the absorbance of the supernatant at 532 nm on the final day of the assay.

Percentage of antioxidant activity calculated by following formula for both FTC and TBA methods.

Percentage of antioxidant activity = [Ac -At/ Ac] × 100

Where Ac= Absorbance of control; At = Absorbance of test

2.7.3 Scavenging of hydrogen peroxide radicals

The hydrogen peroxide radical scavenging activity was assessed as per Ruch et al method with slight modifications [15-17]. 30 mg of each extracts and the standard (ascorbic acid) were accurately

weighed and separately dissolved in 10 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions. A solution of H_2O_2 (20 mM) was prepared in PBS (pH 7.4). Various concentrations of 1 ml of the extracts or standards in methanol were added to 2 ml of H_2O_2 solutions in PBS. The absorbance was measured at 230 nm, after 10 min against a blank solution that contained extracts in PBS without H_2O_2. The percentage of inhibition was calculated based on the formula:

% of inhibition = $(A_1-A_2)/A_1 \times 100$

Where, A_1 = Absorbance of the H_2O_2 and A_2 = Absorbance of the reaction mixture with extract.

2.7.4 DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by using ethanolic extract of BP leaves. The extract was mixed with 3.0 ml DPPH (0.5 mM/ L) in methanol, the resultant absorbance was recorded at 517 nm after 30 minute incubation at 37 °C [17-20]. The percentage of scavenging activity was derived using the following formula,

% of inhibition = $(A_1 - A_2)/A_1 \times 100$

Where, A_1 = Absorbance of DPPH

A_2 = Absorbance of the reaction mixture with extract (DPPH with Sample)

2.8 RBC Haemolysis Prevention Assay

2.8.1 Preparation of erythrocytes

Blood sample (100 ml) was collected from male goat in citrated container. The blood was collected from the animal at slaughter house. 10 ml of the collected blood sample was centrifuged at 30 rpm for 10 minutes. Erythrocytes were separated from the plasma and were washed three times by centrifugation (30 rpm, 10 min) with 10 ml of 10 mM phosphate buffer saline (pH 7.4, prepared by mixing 10 mM of NaH_2PO_4, Na_2HPO_4 and 125 mM of NaCl in 1 liter of distilled water). The supernatant was carefully removed with each wash. Washed erythrocytes were stored at 4 °C and used within 6 hours for the haemolysis assay.

2.8.2 In vitro Haemolysis Prevention Assay

This assay was carried out using the modified protocol originally described by Rafat et al. [21]. The volume of the test samples and erythrocyte suspension adjusted to 1 ml by adding PBS and the mixture was pre-treated at 37 °C for 40 min. Oxidative stress was then induced by 100 µM concentrations of H_2O_2 and incubated at 37 °C for 3 h. After the incubation time, the mixture was centrifuged at 1200 rpm for 5 minutes; the supernatant collected and absorbance was measured at 540 nm. Ascorbic acid was used as the positive control, while the non pre - treated erythrocyte was used as the negative control in this study. Complete erythrocyte haemolysis was acquired by using H_2O_2 and labeled as 100% haemolysis, and haemolysis of other samples were stated as a percentage of this value. The % erythrocyte haemolysis prevention was calculated as-

% Erythrocyte haemolysis prevention = $[(A_{H_2O_2} - A_{Sample}) / A_{H_2O_2}] \times 100$

$A_{H_2O_2}$ = Absorbance of H_2O_2 induced oxidative stress in control group.

A_{Sample} = Absorbance of test group extract/ ascorbic acid.

2.9 Statistical Analysis

Results for hydrogen peroxide radical scavenging activity, DPPH radical scavenging activity and erythrocyte haemolysis prevention assay were analyzed by two-way ANOVA (Graph Pad Prism version 5.03). Further analysis was carried out using paired t test.

P value less than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

ABP revealed the presence of phytochemicals like carbohydrates, proteins, amino acids, flavanoids, tannins and glycosides. EBP revealed the presence of phytochemicals like Carbohydrates, proteins, amino acids, alkaloids, flavanoids, tannins, glycosides, Anthraquinones, terpenoids and steroids.

3.2 Total Phenolic Content

The Gallic acid equivalence curve was obtained (Figure 1). Total phenolic content of ABP and EBP were obtained by using the equation Y= 0.003+0.021, R^2=0.009. The total phenolic content for ABP and EBP was found to be 177.667 and 524.332 µg/ml respectively.

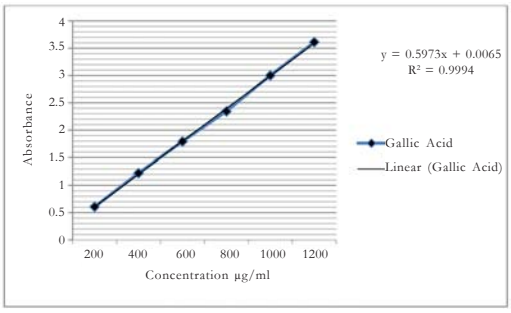

Figure 1. Gallic acid equivalence curve.

3.3 Total Flavanoid Content

The Quercetin equivalence curve was obtained (Figure 2). Total flavanoid content for ABP and EBP was calculated using the equation Y= 0.002 X - 0.020, R^2= 0.997. The total flavanoid content for ABP and EBP was found to be 161 and 434.5 µg/ml respectively.

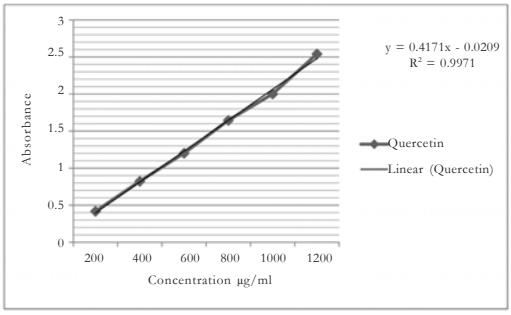

Figure 2. Quercetin equivalence curve.

3.4 Antioxidant Activity- FTC Method

The absorbance values were high for a control group from Day 1 to Day 9 indicates increased peroxide formation and no antioxidant activity. In case of test extracts and standard the absorbance values were also observed to be increasing but when compared with a control group these were less, thus indicating hindrance caused due to presence of test extracts and standard for peroxide formation. Ethanolic extract of herb was found to be having higher antioxidant potential as calculated on day 9 (Table 1).

3.5 Antioxidant Activity-TBA Method

TBA method is used to evaluate the extent of lipid peroxidation. The activity was carried on day 10 with samples prepared for FTC method. Control shows high absorbance value when compared to extracts, and standard indicates the antioxidant activity of extracts and standard. Ethanolic extract of studied herb was found to be having significant antioxidant potential when compared to standard (Table 2).

Table 1. Absorbance values and % antioxidant activity by FTC method.

Samples	Absorbance (Day 1-9)									% antioxidant activity
		2	3	4	5	6	7	8	9	
ABP	0.15	0.16	0.234	0.25	0.26	0.279	0.305	0.377	0.455	24.04
EBP	0.068	0.082	0.102	0.125	0.145	0.169	0.184	0.2	0.215	64.1
Control	0.178	0.19	0.268	0.283	0.311	0.356	0.45	0.599	0.599	0
Vit C	0.0068	0.008	0.00934	0.0125	0.0175	0.054	0.07	0.099	0.119	80.13

Table 2. Absorbance values and %antioxidant activity by TBA method.

Sample	Absorbance	% Antioxidant Activity
ABP	0.136	20.46
EBP	0.045	73.68
Control	0.171	0
Vitamin C	0.021	87.71

Note: For further in vitro evaluation (Hydrogen peroxide, DPPH radical scavenging activity and erythrocyte haemolysis prevention assay) ethanolic extract was used.

3.6 Hydrogen Peroxide Radical Scavenging Activity

There is increasing evidences that, H_2O_2, either directly or indirectly, OH^+ can act as a messenger molecule in the synthesis and activation of several inflammatory mediators. Figure 3 shows significant antioxidant activity of EBP ($P < 0.05$) in comparison to that of standard vitamin C. Gradual increment in percent inhibition can be observed with increasing concentration of the extract. IC_{50} value for EBP and vitamin C were calculated by non-linear regression and found to be 622.90 µg/ml and 309.649 µg/ml respectively.

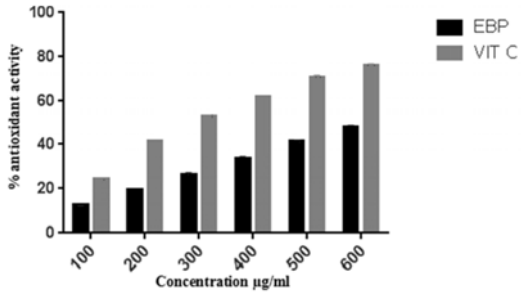

Figure 3. Antioxidant Activity- Hydrogen Peroxide Radical Scavenging Activity.

3.7 DPPH Radical Scavenging Activity

Figure 4 shows DPPH radical scavenging activity of EBP compared with Standard vitamin C. EBP shows promising percent inhibition ($P < 0.05$) which is comparable to that of vitamin C. IC_{50} value for EBP and vitamin C found to be 505.98 µg/ml and 263.6692 µg/ml respectively.

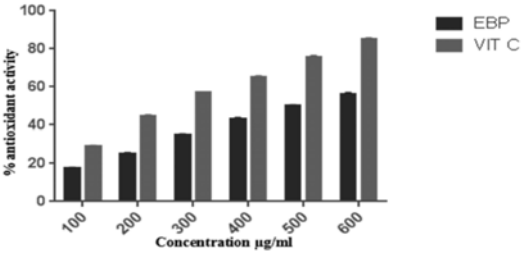

Figure 4. Antioxidant Activity- DPPH Radical Scavenging Activity.

3.8 RBC Haemolysis Prevention Assay

RBC haemolysis prevention assay demonstrated the effectiveness of ethanolic extract where it displayed no significant lysis of normal RBC cells compared to the control. In comparison to vitamin C the percent RBC haemolysis prevention was found to be much lower but significant ($P < 0.05$).Concentrations to achieve 50% RBC haemolysis prevention was calculated by non-linear regression and found to be 643.93 µg/ml and 333.56 µg/ml respectively for EBP and vitamin C (Figure 5).

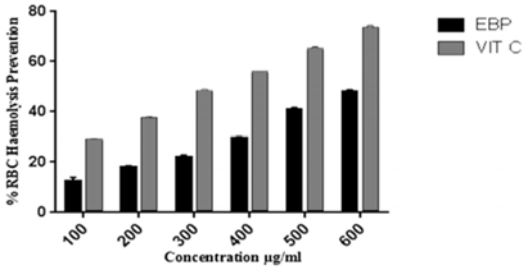

Figure 5. Erythrocyte Haemolysis Prevention Activity.

Aphthous ulcers mostly known as mouth ulcers are very common with incidence rate of 17 to 50%. Recurrent aphthous ulcers (RAU) are characterized by recurrent painful recurring ulcerations of oral mucosa [22]. Therapeutic treatments of mouth ulcer include use of anti-inflammatory agents, immunomodulatory agents, antibiotics and others. The study was undertaken to authenticate the use of *Barleria prionitis* in treatment of oral ailments such as gingivitis, stomatitis and mouth ulcer by rural people across some regions of India. Some studies pointed involvement of free radicals not only in cancer or stroke-related diseases but also reactive oxygen species (ROS) leads to damages related to gingival tissues, periodontal ligaments and alveolar bone. This occurs due to an increase in free radical production and defect in total antioxidant activity of saliva [6-7, 23-25]. The phenolic compounds are directly related to antioxidant potential [12]. Flavanoids are one of the most widespread and diversified group of natural compounds. The antioxidant properties of flavanoids are due to several different mechanisms like scavenging of free radicals, chelation of metal ions and inhibition of enzymes responsible for free radical generation. Depending on their structure, flavanoids are able to scavenge practically all known ROS [13]. The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation [12]. Subsequently, at a later stage of lipid oxidation, peroxide decomposes to form carbonyl compounds that are measured by the TBA method [14]. There is increasing evidences that, H_2O_2, either directly or indirectly, OH^- can act as a messenger molecule in the synthesis and activation of several inflammatory mediators. More ever the presence of hydrogen peroxide in cell culture may cause oxidative damage to DNA. A very slow reactive agent of the hydrogen peroxide sometimes may cause cell death due to production of reactive hydroxyl radicals within the cells [17, 26]. The good antioxidant potential was shown by EBP to scavenge hydrazine radical produce by DPPH. Human RBCs are oxygen carriers with high polyunsaturated fatty acid content on their membranes and high cellular concentration of hemoglobin are particularly exposed to oxidative damage. The hemoglobin released from erythrocytes is potentially dangerous because in reacting with H_2O_2 it is converted into oxidized forms: methaemoglobin (met-Hb) and ferryl hemoglobin, which are powerful promoters of oxidative processes [27]. Moreover, the free haemoglobin exposed to H_2O_2 causes haem degradation with the release of iron ions catalytically active in initiating free radical reaction and

lipid peroxidation [28]. These processes are involved in normal aging and in pathologic conditions [29]. Thus, RBC haemolysis prevention assay demonstrated the effectiveness of test samples where it displayed no significant lysis of normal RBC cells compared to the control. The test samples established to be an effective antioxidant by protecting RBCs from membrane damage due to increasing H_2O_2-induced oxidative stress. Further evaluation by H_2O_2 radical scavenging and DPPH radical scavenging activity suggests antioxidant potential of ethanolic extract of *Barleria prionitis* leaves. *Barleria prionitis* is reported to have chemical constituents to name few are Scutellarein-7-rhamnosyl glucoside, Barlerin, Acetyl Barlerin, luteolin-7-O-β-D-glucoside, shanziside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetyl shanziside methyl ester, Lupilinoside 7-methoxy diderroside, 1, 8-dihydroxy-2, 7- dimethyl 3, 6-dimethoxy anthraquinone and 1, 3, 6, 8- tetra methoxy- 2, 7-dimethyl anthraquinone [2-5]. It will be interesting to relate the current findings of the herbs to the phytoconnstituents for which further study is ongoing. *Barleria* prionitis reported to have antimicrobial activity against oral diseases causing pathogens [30-31]. Although the herb has been reported earlier for its antioxidant potential [32-33] and our results are similar. This study is first of its kind to relate the free radical scavenging potential of the extracts of the herb with oral ailments like aphthous ulcers. In the context of the literature published earlier, we also conclude about the free radical scavenging activity of the aqueous and ethanolic extracts of *Barleria prionitis* with ethanolic extract being more active than aqueous extract.

4. CONCLUSION

Defatted extract of leaves revealed the presence of secondary metabolites like alkaloids, tannins, phenolics, flavanoids, anthraquinone, steroids etc. The total phenolic content in ethanolic extract was found to be higher than aqueous extract. The antioxidant activity evaluated by FTC and TBA method suggest more effectiveness of ethanolic extract than aqueous extract. Further, the evaluation with in vitro antioxidant assays like H_2O_2 radical and DPPH radical scavenging activity establishes the effectiveness of ethanolic extract. Also, for the first time we are reporting the RBC haemolysis prevention activity of the ethanolic extract of the *Barleria prionitis*. Thus, we conclude the usefulness of *Barleria prionitis* in treatment of oral ailments, may be due to its antioxidant potential.

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