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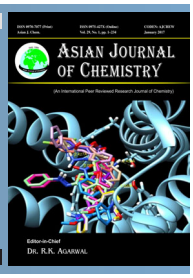

Studies on *in vitro* Antioxidant and Free Radical Scavenging Activities of Ethanol Extract of Whole Plant *Biophytum sensitivum* (Linn.) DC

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The whole plant ethanol extract of *Biophytum sensitivum* Linn. DC (EEBS) was evaluated for its total phenolic content, total flavonoid content, free radical scavenging activities and reducing power. Phytochemical analysis revealed the presence of carbohydrates, alkaloids, steroids, saponins, tannins, phenolic compounds and flavonoids. The total phenolic contents in the extract was estimated by Folin-Ciocalteu reagent and indicated that extract contained 53.55 mg of GA/g of extract. The total flavonoid contents in the ethanol extract of *Biophytum sensitivum* Linn. DC were found to be 153.08 mg of RU/g of extract. The IC_{50} value of DPPH radical, ABTS radical, hydroxyl radical and superoxide radical scavenging activities were found to be 46.34, 42.01, 94.42, 72.12 µg/mL respectively. The IC_{50} value is defined as the concentration that causes a decrease in the initial amount of free radical by 50 %. The IC_{50} value in FRAP assay was found to be 52.12 µg/mL. Ethanol extract of *Biophytum sensitivum* Linn. DC at 80 µg/mL and 100 µg/mL showed higher reducing activity than standard ascorbic acid. The result of the present study showed that ethanol extract of *Biophytum sensitivum* Linn. DC possessed significant antioxidant and free radical scavenging potential and this may be attributed to the presence of bioactive phyto-consitutents like amentoflavone, a bioflavonoid with trace amounts of cupressoflavone, luteolin, isoorientin and isovitexin.

Keywords: *Biophytum sensitivum*, *in vitro* Antioxidant, ROS, Phenolic compounds, DPPH, Flavonoids.

INTRODUCTION

Production of free radical is an integral part of metabolism. These extremely reactive oxygen species (ROS) produced by all aerobic organism are different forms of activated oxygen which include free radicals such as superoxide (O_2^-), hydroxyl (OH^-), nitric oxide (NO), peroxyl (ROO^-), lipid peroxyl radicals (LOO^-) radicals and non-free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^-), ozone (O_3) and lipid peroxide (LOOH), *etc.* [1,2]. These highly unstable free radicals, if produced in excess quantity have deleterious effects by readily reacting with DNA, lipids, proteins and lipoproteins of biological membrane. This leads to large number of complications such as cancer [3], cardiovascular diseases [4], neurodegenerative diseases [5], ulcerative colitis [6], aging [7], inflammation [8], diabetes, liver and kidney diseases by generating oxidative stress [9].

In recent years, research on drugs of plant origin has increased all over the world as herbs are generally measured to be harmless owing to their natural origin [10]. Drugs of plant origin play a vital role in management of various diseases and many plant derived drugs/phytoconsitutents have been proven to be protective against oxidative stress [11-14]. Plants are potential sources of various natural antioxidative compounds mainly phenolics, flavonoids and proanthocyanins that counter act cellular damage due to oxidative stress [15].

Biophytum sensitivum Linn. DC (*B. sensitivum*) (Common names: *Nilaccurunki, Tintaanaalee* in Tamil; *Mukkutti* in Malayalam; *Lajalu, Lajjaalu, Lakshmana* in Hindi) belongs to family oxalidaceae [16]. It is found in wetlands, plains of tropical Africa and Asia, normally grows in shades of trees, at a low and medium altitude and is distributed throughout, Philippines and hotter parts of India, Nepal, Thailand, Malaysia, Indonesia and Sri Lanka [17]. Phytochemical investigation of the extract of *B. sensitivum* had revealed the presence of large amount of phenolic and poly phenolic compounds, saponins, polysaccharides, pectin and essential oil. Main bioactive constituents are bioflavonoids like amentoflavone with trace amounts of cupressoflavone, luteolin, isoorientin and isovitexin [18,19].

It has been used as a traditional folk medicine in various ailments such as stomach ache, asthma, insomnia, convulsions,

cramps, chest complaints, inflammations, tumors, chronic skin diseases, fever, malaria, wounds, diabetes, gonorrhea, tuberculosis, thirst, tumor, burns, snake bite, arthralgia, arthritis, back pain, bursitis, carpal tunnel syndrome, bone spurs, cervical spondylitis, degenerative joint disease, degenerative neck disease, fibromyalgia and kidney stones [20]. Grounded leaves are indicated for diuretic effect, amenorrhea and dysmenorrhea. The flower of this plant is considered as one of the ten sacred plants which are called as '*Dasapushpam*' in tradition and culture of Kerala state in India [21]. Recent pharmacological studies shows that it has antioxidant [22], antibacterial [23], antidiabetic [24], antitumor [25,26], immunomodulation, radioprotective, anti-inflammatory [27] and cardioprotective activity [28].

EXPERIMENTAL

The whole plant, *B. sensitivum* was collected from Shevaroy Hills, Salem District, Tamil Nadu and was taxonomically identified and authenticated by Dr. A. Balasubramanian, Executive Director, ABS Botanical conservation, Research and Training Centre, Kaaripatti, Salem (Dt.), Tamilnadu (Ref. No. AUT/JKK/095).

Preparation of extract: The whole plant was washed and dried in shade for about 3 weeks. Dried plant was coarsely powdered, sieved (mesh size = 40) and stored in air tight container at room temperature. Powdered plant material (500 g) was sequentially extracted with petroleum ether (60-80 °C) for defatting the drug and then with 70 % ethanol by using Soxhlation method. Percentage yield of petroleum ether and ethanol extracts were 4.92 % w/w and 12.54 % w/w, respectively. The solvent extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator [29]. Phytochemical screenings were performed using standard procedures and the data indicated that the whole plant ethanolic extract of *B. sensitivum* (EEBS) showed the presence of phytochemicals such as carbohydrates, alkaloids, steroids and sterols, saponins, proteins, aminoacids, flavonoids, tannins and phenolic compounds [30].

Determination of total phenolic compounds: Total soluble phenolic compounds in ethanolic extract of *B. sensitivum* was determined by using Folin-Ciocalteu reagent according to the method of Singleton and Rossi [31] with a mild modification. Briefly, 1 mL of diluted extract was mixed with 1 mL of 1/10 dilution of reagent in water. After waiting for 5 min, 1 mL of Na_2CO_3 (7.5 % w/v) was added to the sample and were then allowed to stand for 30 min and measured at 743 nm. Gallic acid was used to calculate the standard curve (0.01-0.04 mM) and estimation was carried in triplicate. The results were mean values ± standard deviation and expressed as gallic acid equivalents (GAE) in mg/100 mL.

Determination of total flavonoids: Flavonoid content present in ethanolic extract of *B. sensitivum* was determined using spectrophotometric method described by Quittier *et al.* [32]. 1 mg/mL of ethanolic extract of *B. sensitivum* was mixed with 1 mL of 2 % $AlCl_3$ in ethanol and allowed to stand for 1 h at room temperature. Absorbance was measured using spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for standard solution of rutin and calibration curve was constructed. Based on the measured absorbance the concentration of flavonoid was read (mg/mL) on the calibration line. The content of flavonoids in extract was expressed in terms of rutin equivalent (mg RU/g) of extract.

Determination of ferric reducing antioxidant potential (FRAP assay): The antioxidant capacity was estimated according to the method of Benzie and Strain [33]. The working FRAP reagent was freshly prepared by adding 10 mM of 2,4,6-Tris (2-pyridyl)-1,3,5-triazine (TPTZ) dissolved in 40 mM of HCl, 20 mM of $FeCl_3$ in water and 300 mM of acetate buffer (pH 3.6) in the ratio of 1:1:10. To 900 µL of FRAP reagent add different concentration of sample solution and final volume were made up to 1 mL. After incubation for 5 min at room temperature, the absorbance was measured at 593 nm against ferrous sulphate ($FeSO_4 \cdot 7H_2O$) as standard. The absorbance of samples were compared to $FeSO_4$ standard curve and the FRAP values were expressed as ferrous equivalent.

Determination of DPPH radical scavenging activity: DPPH radical scavenging activity was evaluated according to method of Blois [34]. The ethanolic extract of *B. sensitivum* and standard solution at various concentrations ranging from (10-100 µg/mL) were mixed with 1 mL of freshly prepared 0.3 Mm DPPH ethanol solution and 2 mL of 0.1 M acetate buffer. The resulting solutions were then incubated at room temperature for 30 min and measured calorimetrically at 517 nm. Ascorbic acid was used as positive control. Negative control was without any inhibitor or extract *i.e.*, DPPH solution (1.0 mL, 0.3 Mm) with 1 mL ethanol served as negative control. The percentage of DPPH radical scavenging activity of extract was calculated from decrease in absorbance in comparison with negative control by using formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

where $A_{control}$ was the absorbance of control and A_{test} was the absorbance in presence of extract/standard. Mean value were obtained from triplicate analysis. The antioxidant activity of the extract was expressed as IC_{50}.

Determination of total antioxidant capacity by ABTS radical cation assay: Total antioxidant potential of extract was determined by scavenging of 2,2-azinobis(3-ethyl benzoline-6-sulphonic acid)diammonium radical (ABTS) radical cation based on the procedure described by Re *et al.* [35]. ABTS radical was freshly prepared by adding 5 mL of 4.9 Mm $(NH_4)_2SO_4$ solution to 5 mL of 14 mM ABTS solution and kept for 16 h in dark at room temperature. This solution was diluted with ethanol (99.5 %v/v) to yield an absorbance of 0.70 ± 0.02 at 734 nm. Varying concentration of extracts were allowed to react with 900 µL of ABTS radical solution and the reaction mixture was vortexed for 10 s. After 6 min, the absorbance was recorded at 734 nm. The ABTS scavenging capacity of the extract was compared with that of ascorbic acid which was used as positive control under the same assay condition. Negative control was without any inhibitor or extract. All tests were carried out in triplicate. The extract concentration providing 50 % inhibition (IC_{50}) was obtained by plotting inhibition percentage *versus* extract concentration. Percentage inhibition was calculated from the formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

Determination of hydroxyl radical scavenging activity: The 2-deoxyribose assay was used to determine the scavenging effect of the extract on -OH radical, as reported by Halliwell and Gutteridge [36]. 0.4 mL of extract, at different concentrations, were mixed with deoxy ribose 0.6 mL (1 mM) and made up to 1.6 mL using phosphate buffer. The tubes were then incubated for 10 min then 0.4 mL of 0.2 mM phenyl hydrazine hydrochloride was added. The mixture was incubated for 1 h after adding 1 mL each of 2.8 % TCA and 1 % TBA. The mixture was then heated on a boiling water bath for 10 min and the absorbance was measured at 532 nm. The negative control without any antioxidant was considered 100 % deoxyribose oxidation. Ascorbic acid was taken as the positive control. The percentage hydroxyl radical scavenging activity of extract was determined by comparing with negative control.

Determination of superoxide radical scavenging activity: Super oxide anion radical scavenging assay was measured according to the method described by Nishimiki *et al.* [37]. Various concentration of ethanolic extract of *B. sensitivum* or standard was mixed with about 1 mL of 156 µM of NBT solution in phosphate buffer (100 mM, pH 7.4), 1 mL 468 µM NADH in phosphate buffer (100 mM, pH 7.4) and the reaction was started by adding 100 mL of 60 mM PMS in phosphate buffer (100 mM, pH 7.4). Incubated the reaction mixture for 5 min and the absorbance was measured at 560 nm against the standard solution ascorbic acid. The percentage inhibition was then compared with reference compound.

Reducing power assay: The reducing power activity was estimated according to the method of Oyaizu [38]. Various concentrations of ethanolic extract of *B. sensitivum* in 1 mL of deionized water were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10 % trichloroacetic acid (w/v) were added and then centrifuged at 3000 rpm for 10 min. The upper layer of solution 5 mL was mixed with 5 mL deionized water and 1 mL of 0.1 % $FeCl_3$ and absorbance was measured at 700 nm. Assays were carried out in triplicate and the results were expressed as mean values ± SD. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of reaction mixture indicates increase in reducing power.

Statistical analysis: Results were expressed as mean value ± SD (n = 3). Student's t-test was used for comparison between values of samples and standards. Difference was considered statistically significant when $P < 0.001$ and < 0.05.

RESULTS AND DISCUSSION

Total phenolics: Phenolics are aromatic compounds which exhibit significant antioxidant activity. It acts as singlet oxygen quenchers, reducing agents and hydrogen donators [39]. They have also good metal chelation properties [40]. Polyphenol in the plant extract reacts with specific redox reagent to form blue chromophore constituted by phosphotungistic-phosphomolybdenum complex that can be quantified by visible light spectrophotometry. Maximum absorption depends on the concentration of phenolic compounds [41]. In this study total phenolic content of ethanolic extract of *B. sensitivum* was expressed in terms of gallic acid equivalents. The concentrations of total phenols are expressed as mg of GA/g of extract. The total phenolic contents in the extract *B. sensitivum* were 53.55 of GA/g of extract.

Total flavonoids: Flavonoids are a group of polyphenolic compounds having excellent free radical scavenging activity and protect organism from damage caused by free radical induced oxidative stress. Formation of acid stable complexes with C-4 keto group and either C-3 or C-5 hydroxyl group of flavones and flavanols in addition with *ortho*-dihydroxyl group in the A or B ring of flavonoids form the basis of the estimation [42]. In this study total flavonoid content of ethanolic extract of *B. sensitivum* was expressed in terms of rutin equivalents. The concentrations of total flavonoids are expressed as mg of RU/g of extract. The total flavonoid contents in the extract *B. sensitivum* were 153.08 of RU/g of extract. The major bioactive constituents are bioflavonoids like amentoflavone, cupressoflavone, luteolin, isoorientin and isovitexin and these may be an attributing factor for its pharmacological activities against various diseases related to oxidative stress.

Ferric reducing antioxidant potential assay (FRAP assay): FRAP assay was employed to estimate the reducing ability of antioxidants *in vitro*. The principle of this method is based on the reduction of ferric tripyridyltriazine complex at low pH to its ferrous complex which has an intense blue colour in presence of antioxidants. Reducing ability of antioxidants can be monitored by measuring the absorbance at 593 nm against $FeSO_4 \cdot 7H_2O$, as standard [43,44]. In the current study, ethanolic extract of *B. sensitivum* exhibited reducing ability in a concentration dependent manner and was comparable with that of standard. Ethanol extract of *Biophytum sensitivum* Linn. DC at concentrations from 10-100 µg/mL reduced ferric tripyridyltriazine complex to its ferrous form by 15-69 %. Fig. 1 shows that the IC_{50} value of ethanolic extract of *B. sensitivum* was found to be 52.12 µg/mL (standard 36.23 µg/mL). The results suggested that this reducing ability of extract might be due to the presence of flavonoids or polyphenolic compounds.

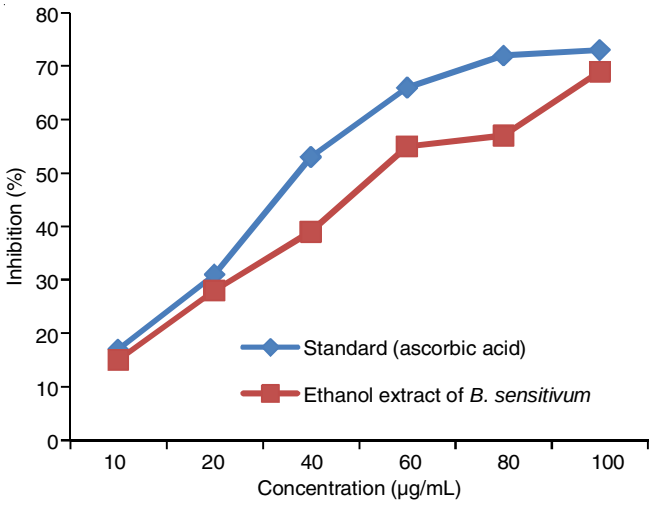

Fig. 1. Ferric reducing antioxidant potential of ethanolic extract of *B. sensitivum*

Inhibition of DPPH radical: Determination of DPPH radical scavenging activity is a rapid, simple, inexpensive and widely used method to measure antioxidant activities. This method is based on the reduction of DPPH, a stable free radical to non-radical form DPPH-H. Free radical DPPH with an odd electron is purple complex, paired off in presence of hydrogen donating antioxidant and decolourized to yellow. Reduction by antioxidant was quantified by measuring the absorbance at 517 nm. More the decolonization more is the reducing ability [45]. Fig. 2 suggested that the IC_{50} value of ethanolic extract of *B. sensitivum* was found to be 46.34 µg/mL and that of ascorbic acid (STD) was 14.12 µg/mL. The ethanolic extract of *B. sensitivum* showed significant scavenging effects on DPPH radical ($P < 0.05$) and this indicated that ethanolic extract of *B. sensitivum* contained sufficient phytochemical constituents capable to donate 'H' to free radical DPPH and reduced to non-free radical DPPH-H.

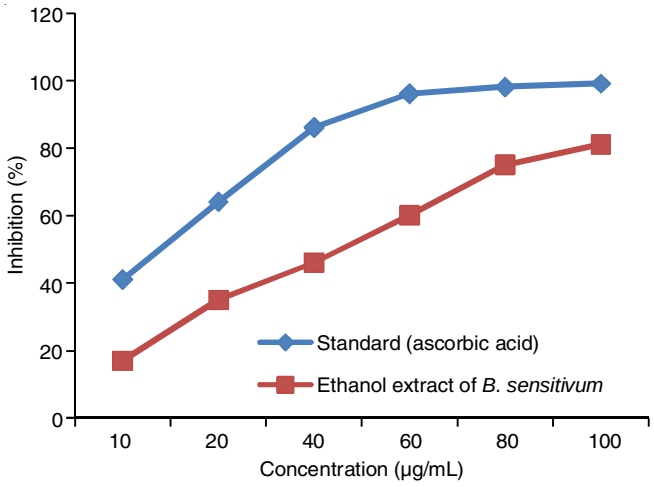

Fig. 2. DPPH radical scavenging activity of ethanolic extract of *B. sensitivum*

Total antioxidant activity: ABTS radical decolourization study is an excellent method to measure the antioxidant activity of phenolic compounds. Blue coloured ABTS radical generated in presence of free radical inducer ammonium persulphate was quenched by antioxidant and change in colour intensity is then quantified by measuring absorbance at 734 nm [35]. Fig. 3 shows the IC_{50} value of ascorbic acid (18.43 µg/mL) and ethanolic extract of *B. sensitivum* (42.01 µg/mL). Ethanolic extract of *Biophytum sensitivum* Linn. DC scavenged ABTS radical in a concentration dependent manner and was comparable with that of ascorbic acid. This antioxidant activity might be due to the presence of electron donating group in ethanolic extract of *B. sensitivum* and the results suggested that the extract can act as a free radical scavenger.

Inhibition of hydroxyl radical: Major reactive oxygen species, hydroxyl radicals ($OH^{\bullet}$) cause oxidation of poly-unsaturated fatty acid (PUFA) and induce severe cellular and tissue damage resulting in carcinogenesis, mutagenesis and cytotoxicity [46,47]. This assay is based upon the determining the degree of deoxyribose degradation, an indicator of thio-barbituric acid-malonaldehyde (TBA-MDA) adduct formation. Hydroxyl radicals are formed in free solution causing degradation of deoxyribose into malonaldehyde, which produces a

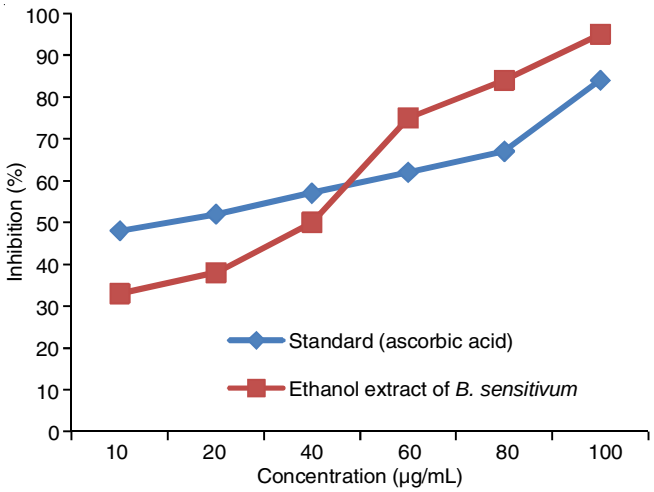

Fig. 3. ABTS radical scavenging activity of ethanolic extract of *B. sensitivum*

pink chromogen on heating with thiobarbituric acid [36]. Decreased absorbance of the reaction mixture indicated increased hydroxyl scavenging activity. As shown in the Fig. 4, the extract inhibited hydroxyl radical induced deoxyribose degradation in a concentration dependent manner. The extract showed significant ($p < 0.05$) hydroxyl radical scavenging activity with an IC_{50} value of 94.42 µg/mL in comparison to that of ascorbic acid (60.31 µg/mL). These results indicate that ethanolic extract of *B. sensitivum* can act as a hydroxyl radical scavenger.

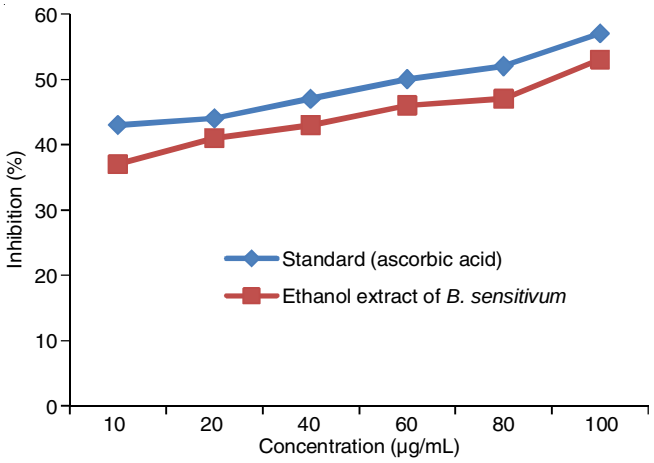

Fig. 4. Hydroxyl radical scavenging activity of ethanolic extract of *B. sensitivum*

Inhibition of superoxide anion radical: Concentration of superoxide anion (O_2^{-}) increases under condition of oxidative stress. In PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT [48]. Fig. 5 illustrated superoxide radical scavenging activity of ethanolic extract of *B. sensitivum* compared with the same dose of ascorbic acid ranging from 10-100 µg/mL. IC_{50} value of ascorbic acid and ethanolic extract of *B. sensitivum* was found to be 23.64 and 72.12 µg/mL, respectively ($p < 0.01$). From the result, it can be concluded that extract possess significant superoxide scavenging activity and this ability depends on the concentration of phenolic compounds and number of hydroxyl group.

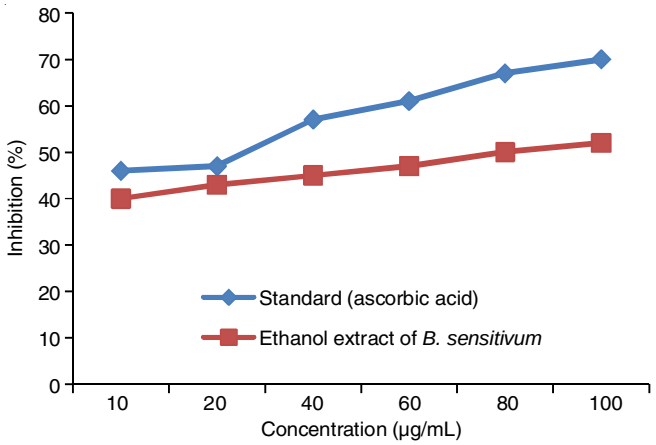

Fig. 5. Superoxide anion radical scavenging activity of ethanolic extract of *B. sensitivum*

Reducing power activity: The reducing power of ethanolic extract of *B. sensitivum* was determined by reduction of Fe^{3+} to Fe^{2+} using the method performed by Oyaizu [38]. Absorbance was measured at 700 nm and the results indicated that the reducing power of ethanolic extract of *B. sensitivum* and standard (ascorbic acid) increased with increase in concentration. Higher absorbance of reaction mixture indicated higher reducing power. The reducing power of extracts and standard are summarized in Fig. 6. The ethanolic extract of *Biophytum sensitivum* Linn. DC at 80 µg/mL and 100 µg/mL showed higher reducing activities than the standard and differences were significant. Reducing power of the extract may be due to its quenching ability of free radical. The presence of reductants in the extract may donate electrons and convert them to more stable product. Hence, the phenolic compounds in extract may contribute towards the antioxidant activity. Previous research have mentioned the significance of polyphenols and flavonoids in the antioxidant activity of different plant extracts [49,50]. Results of this study are in accordance with results published by Guruvayoorappan *et al.* [22], Kalitha *et al.* [51] and Johnsin *et al.* [52].

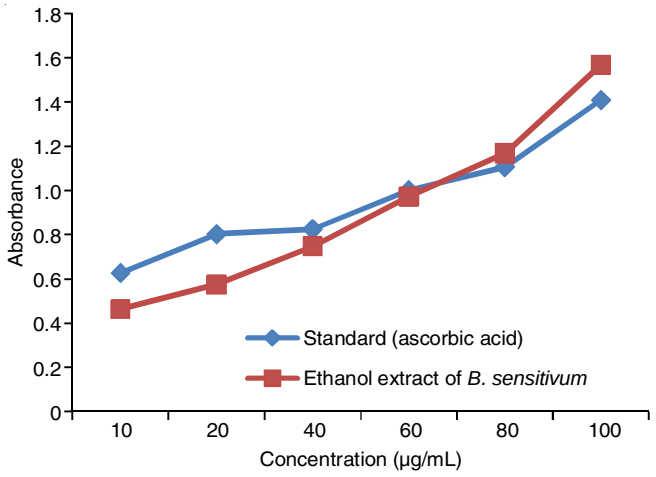

Fig. 6. Reducing power activity of ethanolic extract of *B. sensitivum*

Conclusion

Phytochemical investigation of ethanolic extract of *B. sensitivum* revealed the presence of large amounts of phenolic and polyphenolic compounds, saponin, polysaccharides, pectin and essential oil. The main bioactive constituents were found to be bioflavonoids like amentoflavone with trace amounts of cupressoflavone, luteolin, isoorientin, isovitexin. It has been used as traditionally in the treatment of various diseases. The flower of this plant is considered as one of the ten sacred plants which are called as '*Dasapushpam*' in tradition and culture in Kerala, a state in India. This study showed that this drug possessed significant antioxidant and free radical scavenging activities. Hence, this plant, used alone or in combination with other herbal drugs, may exhibit excellent antioxidant activities to protect body from deleterious effects of free radicals.

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