Evaluation of Free Radical Scavenging Potential of Different Bioactive Fractions Present in Boerhavia diffusa Linn. Root Extract: An in-vitro Approach

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors MK, MHA, AIF and PA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JA, Badruddeen and AS managed the analyses of the study. Author AS managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Aim and Objectives: Boerhavia diffusa (B. diffusa) frequently known as punarnava is specifically used to replenish the body. The present work was designed to evaluate the scavenging potential of its bioactive constituents.

Materials and Methods: The different fractions of B. diffusa root methanolic extract were examined for phenolic, flavonoids contents, DPPH free radical and Nitric oxide scavenging activities. Further antioxidant activity was evaluated by ABTS free radical scavenging method and also from the reducing potential scavenging activity. The total phenolic content in different fractions...
1. INTRODUCTION

In traditional system, medicinal plants are used as significant source of natural antioxidants. Medicinal plants have the bioactive compounds to perform the antioxidants activities. The chemical constituents or raw extract of medicinal plants have efficient potential to neutralize the free radical which is generated during the oxidation process [1]. Herbal medicines have various functions due to the occurrence of numerous bioactive phyto-constituents. They have the ability to inhibit free radical oxygen species generation and to scavenge free radicals; this may contribute to the beneficial health effects in our body [2]. Free radicals are essentials for aerobic process and metabolism because of its high reactivity with very short half-life. It damages many biochemical compounds present in human body such as DNA/RNA, carbohydrates, proteins, lipids (saturated and unsaturated), proteins, micro and macronutrients such as alpha and beta carotene, lycopene, vitamins A, B6, and B12 [3]. Many harmful diseases are linked to free radicals species [4], the reactive oxygen species circulating with blood stream in the body reacts with the electron of other molecules and effect metabolic process of body. That leads to damage of many vital organs and the possible cause of diseases like rheumatoid arthritis, adult respiratory distress syndromes, cancer, ischemia, aging etc [5].

Tribal communities are totally dependent on herbal medicine for their medication and use them against different acute and chronic diseases [6]. The medicinal plants are economical and effective due to the anti-corrosive activity [7] of different phyto-constituents that are used as a better and effective therapy [8,9].

_Boerhaavia diffusa_ Linn. (Nyctaginaceae) is a perennial creeping herbaceous plant, also known as hogweed, grow anywhere and it may be cultivated in fields [10]. It is mainly found throughout the waste land of India specially in tropical and subtropical regions in the world [11]. It contain an abundant phyto-constituents mainly as alkaloids, glycoside, others are flavonoids, saponins respectively [12]. Whole plant and its roots are used in Ayurvedic and Unani system of medicine for the treatment of diabetes, stress, dyspepsia enlargement of spleen, and also used in congestive heart failure and bacterial infections [13-15]. The plants are rich source of vitamins, minerals, proteins and carbohydrates. In Punjab province, the plant is beneficial in eye infection, and dropsical swellings in Maharashtra region. The fluid of the leaves is used in decreasing the bile juice [16], and the roots are usually used as infusion for internal swelling, urinary disease and in diabetes [17]. It contains quinolizidine alkaloids, potassium salts [16] and boeravinones G and H alkaloids, which inhibits breast cancer resistant proteins [18]. The leaves juice used for the treatment of disorders related to seminiferous tubules by increasing the number of germinal cell, reduce the sperm counts with increase percentage of tail and head abnormalities [19]. A novel research reported its anti-proliferative activity against a variety of tumour cell lines [20].

In this work the scavenging potential of bioactive constituents from _B. diffusa_ root was evaluated.
The total ethanolic extract and its fractions in chloroform, ethyl acetate, n-butanol, and ethanol were carried out to find the antioxidant scavenging activities.

2. METHODS

2.1 Chemicals and Regents

Ascorbic acid, ferrous sulphate, aluminium chloride (AlCl₃), potassium ferricyanide, sulphanilamide, phosphoric acid, naphthylene diamine dihydrochloride, sodium nitrite (NaNO₂) and rutin were purchased from Qualigens Fine Chemicals, Mumbai, India. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and Folin Ciocalteu reagent were purchased from Sigma-Aldrich, USA. Sodium carbonate (NaCO₃) and gallic acid were obtained from Merck Chemicals (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2 Collection of the Plant Material and Authentication

The crude roots of the plant were collected from the local market of Aminabad, Lucknow India. It was identified by the taxonomist Dr. Muhammad Arif (Department of Pharmacy, Kursi Road, Integral University, Lucknow). A voucher sample of the plant was submitted in herbarium for further reference.

2.3 Extraction

The B. diffusa roots were pulverized and macerated with methanol for 3 days [21]. The methanolic extract was filtered with Whatmann filter paper no. 1 for 3 times at an interval of one day and extracts were made viscous at a low temperature on a rotary evaporator so that sticky mass of the extract was obtained. The dried methanolic extract was then fractionated by using the solvent according to polarity in ascending order (chloroform< ethyl acetate <n-butanol<ethanol). The filtrate was concentrated up to dryness by the mean of rotary evaporator under reduced pressure at 40°C. The dried extracts were stored at 4°C until further used [22].

2.4 Estimation of Total Phenolic Content

The modified Folin-Ciocalteu method was adopted for the estimation of total phenolic contents from the different fractions of B. diffusa root extracts [23] and absorbance was measured at 765 nm. The phenolic (µg/mL) content was calculated by using the calibration curve equation.

2.5 Estimation of Total Flavonoids Content

The total flavonoids concentration was determined by Ordon et al. [24] method and absorbance was measured at 420 nm. The calculation of flavonoids (µg/mL) content was done by using the calibration curve.

2.6 Determination of Antioxidant Activity Using the 1,1-Diphenyl-2-Picrylhydrazyl Free Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl method was done by Liyana & Shahidi [25]. For the determination of free radical DPPH scavenging activity of B. diffusa extract; 0.135 mM DPPH solution in 1 ml of ethanol was mixed with the extract and kept on vortex to be mixed thoroughly. In this there is a change by reacting with a hydrogen donor and minimise the equivalent hydrazine and measured at 490 nm. The scavenging of DPPH was calculated as per the below formula:

\[
\% \text{ Inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where A₀= absorbance of the control (blank) and A₁ = absorbance with extract.

2.7 Determination of Antioxidant Activity Using the Nitric Oxide (NO) Scavenging Activity

NO free radical scavenging activity was carried out according to the Sreejayan Roa method, incubated the different fractions at 30°C for 5h [26]. 0.5 mL of incubated fractions was separated and added 0.5 mL of Griess reagent and absorbance was taken at 546 nm.

2.8 Determination of Antioxidant Activity Using the (2,2’-Azino-Bis-3-Ethylbenzthiazoline-6-Sulphonic Acid (ABTS) Free Radical Scavenging Method

The ABTS free radical scavenging activity was carried out according to the Gardeli et al., method. The two solutions were mixed with a ratio of 1:1 and kept in the dark area for 24–48 hours. ABTS solution was diluted with aqueous methanol in a ratio of 1:25. 20 µL of diluted
aqueous methanolic plant extract was mixed with 2 mL of ABTS\(^+\) solution, and the mixture was kept at a standard temperature of 30 °C. The absorbance was measured after a time interval of 0, 5, and 10 min at 734 nm [27].

The percentage of inhibition of ABTS\(^+\) was calculated using the following formula:

\[
\text{% Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

\(A_0 = \) blank, \(A_1 = \) sample

2.9 Determination of Antioxidant Activity Using the Reducing Potential Scavenging Activity

According Khalid and Siddiqui, 2012 method, performed the reducing potential by transformation of \(\text{Fe}^{3+}\) to \(\text{Fe}^{2+}\) in the presence of the fractions [28]. After adding the reagents, the absorbance was measured at 700 nm.

2.10 Statistical Analysis

The experimental results were expressed as mean ± standard error of mean (SEM) of three replicates.

3. RESULTS AND DISCUSSION

A simple, convenient maceration method was adopted for extraction of \(B.\) diffusa roots. Since it was done at relatively low temperature, the nature of bioactive constituent was not changed. The plant extract having ability for quenching of free radical molecule [29]. The solvent evaporation was done below its boiling point to keep it safe and remains the nature of bioactive constituents in an unchanged form. The methanolic extract was fractionated by different solvents viz. ethanol, ethyl acetate, \(n\)-butanol, and chloroform. Then further these fractions were selected for the estimation of various activities.

3.1 Estimation of Phenolics and Flavonoids Contents

The results are shown in Table 1, the absorbance (0.84) was observed at a concentration 134 ± 2.71 µg/mL. It was equivalent to ascorbic acid. The 0.83 absorbance was observed at 250 nm and the flavonoids content of these fractions were found as chloroform fraction (16.91 ± 2.74 µg/mL), ethyl acetate fraction (29.67 ± 2.83 µg/mL), \(n\)-butanol fraction (31.68 ± 1.72 µg/mL) and ethanol fraction (41.93 ± 3.92 µg/mL) that was equivalent to rutin (400 µg/mL).

| S. No. | Fractions   | Total Phenolic Content (µg/mL) | Total Flavonoid Content (µg/mL) |
|--------|-------------|--------------------------------|---------------------------------|
| 1      | Chloroform  | 33.71 ± 2.31                   | 16.91 ± 2.74                    |
| 2      | Ethyl Acetate | 52.97 ± 3.72                  | 29.67 ± 2.83                    |
| 3      | \(n\)-butanol | 77.38 ± 3.96                  | 31.68 ± 1.72                    |
| 4      | Ethanol     | 134.97 ± 2.71                  | 41.93 ± 3.91                    |
Fig. 1. Percentage inhibition (I%) of the DPPH free radical scavenging of the fractions extract of *B. diffusa* root

Fig. 1. Percentage inhibition (I%) of nitrous oxide scavenging activity of the fractions of *B. diffusa* root extract
3.5 Reducing Potential Activity

The ethanolic fraction showed highest free radical scavenging potential means have the maximum absorbance value as 0.293 nm at 140 mg/mL. The other fractions like chloroform, ethyl acetate and n-butanol showed values as 0.197 nm, 0.206 nm and 0.219 nm respectively (Fig. 4).
3.6 Discussion

The reducing potential of these fractions was due to presence of hydroxyl ions in the phenolic and flavonoids compounds in the fractions [30]. They play important key role as scavengers for free radicals is emphasized in some previous works [31]. The plant extract shows presence of total phenolic component and flavonoids in all fractions but the ethanolic fraction have higher amount of them.

DPPH scavenging method is a stable and widely used method to assess the quenching of free radical molecules in many natural products [32]. The scavenging potential of the fractions was significantly reduced than that of ascorbic acid. However it was also evident that the fractions had proton-donating ability therefore it could act as a free radical inhibitor may be as a primary antioxidant [33]. The fractions react with DPPH molecule which changed colour of DPPH from purple to a slightly yellowish (more stable form of 1,1-diphenyl-2-picrylhydrazine) due to the donation of hydrogen atom to DPPH which is present in the compounds (fractions) [34]. Overall the discoloration of the extract indicated the bioactive molecules have free radicals scavenging potentials. The bleaching of DPPH absorption is representative of the activity of tested compounds to scavenge the free radicals. Hence, present work indicated that this fraction may be useful for treating free radical related pathological damage.

Different fractions of methanolic extract of B. diffusa root have the capability to block the formation of free radical molecule in a dose dependent manner. NO radical is a potent intermediary of physiological processes mainly responsible for smooth muscle relaxation. It inhibited platelet aggregation and regulate the toxicity. It is a diffusible free radical which has an effective molecule in biological systems including vasodilatation, antimicrobial and antitumor activities [35]. Although nitric oxide free radicals are involved in the defence mechanism, the overproduction of these free radicals contributes to the pathogenesis of some inflammatory diseases [36]. In general the results so obtained indicated that the compounds available in the root extract were able to inhibit nitric oxide and it may be a scientific evidence for the indigenous system in inflammatory conditions.

Moderate to weak antioxidant activity by ABTS method was shown by some medicinal plant extracts [37]. Since ABTS method is used in both organic and inorganic solvent system as compared to other scavenging activity assay therefore both hydrophilic and lipophilic free radical scavenging activity can be better determined by this method. It is based on the ability of antioxidants to reduce the ABTS radical cation [38].

The ethanolic fractions of B. diffusa root extract reduced Fe$^{3+}$ to Fe$^{2+}$. It served as a marker of its potential scavenging ability. The donated hydrogen atom broke the free radical chain in the system [39]. There may be a variety of mechanisms involved such as blocked the chain initiation, binding of ion catalysts, break down of peroxides, reducing ability and radical scavenging. The B. diffusa root constituted with polyphenolic compounds that may have reducing ability. The ability of extract to reduce iron suggests that it may have electron donors compounds, those can react with free radicals to convert them to more stable products and may terminate radical chain reaction. Reducing power assay showed positive correlation between reducing power and phenolic content in B. diffusa roots fractions. Rice-Evans et al. reported that phenolic compounds have redox properties; therefore they acted as reducing agents, hydrogen donors, and singlet oxygen quenchers. Overall redox potential of phenolic compounds played an important role in determining antioxidant potential.

4. CONCLUSION

It can be concluded that all fractions of B. diffusa root extract have phenolic and flavonoids components which have free radical scavenging activity or DPPH, nitric oxide, ABTS and Fe$^{3+}$ reduction or antioxidant activities. The ethanolic fraction has better antioxidant potential than other different fractions of B. diffusa root extract. Possibly it is due to the presence of highest phenolic and flavonoids content present in the plant. Hence, present work indicated that these fractions can be useful for treating free radical related pathological damages and further can be clinically tested for their different pharmacological action.

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It is not applicable.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

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