The Pharma Innovation Journal 2023; SP-12(8): 1962-1965

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(8): 1962-1965

www.thepharmajournal.com
Received: 09-05-2023
Accepted: 13-07-2023

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Phytochemical and antioxidant assessment in methanolic leaf extract of *Boerhavia diffusa* (Punarnava)

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Abstract
Boerhaavia diffusa holds significant traditional importance due to its considerable therapeutic benefits. A thorough analysis of the plant's phytochemical composition was undertaken to scientifically substantiate its traditional usage. The primary objective of this study was to evaluate the phytochemical constituents and overall antioxidant potential present in the B. *diffusa* leaves extract (methanolic), utilizing well-established experimental procedures. The methanolic extract of *B. diffusa* was subjected to a thorough phytoconstituent screening, revealing the definite presence of both flavonoids and phenols. The findings have demonstrated that the methanolic extract of B. *diffusa* leaves exhibited a total antioxidant activity of 25.61±1.07 µM Fe II equivalents, as determined through the Ferric-Reducing Antioxidant Power Assay. Additionally, the extract displayed a peroxidation inhibition rate of 18.15 ± 0.12% in the Ascorbate-Iron (III)-catalyzed phospholipids peroxidation assessment. The results of this study suggested that the extract of *B. diffusa* leaves (methanolic) has the potential to serve as an important reservoir of natural antioxidants.

Keywords: *Boerhavia diffusa*, phenols, flavonoids, antioxidant, oxidative stress, phytochemicals

1. Introduction
Boerhavia diffusa, widely recognized as Punarnava, is a highly regarded herb in Ayurvedic medicine. Due to its widespread presence worldwide, *Boerhavia diffusa* offers diverse ethno pharmacological applications against gynaecological disorders, hepatic dysfunction, diuretic, cardiovascular, cutaneous diseases, and ophthalmic conditions (Mishra *et al*., 2014) [8]. Its name originates from the combination of two words, "Punar" meaning "once again" or "restoring," and "Nava" meaning "new," "renew," or "young." Therefore, the name itself conveys the essence of rejuvenation associated with Punarnava. The *B. diffusa* plant contains many phytoconstituents such as alkaloids, flavonoids, steroids, Lignins, terpenoids, proteins, carbohydrates and lipids. *Boerhavia diffusa* serves the purpose of maintaining cell viability, facilitating cellular communication, and preserving the structural integrity of cell membranes (Premkumar *et al*., 2010) [10]. Oxidative stress is elaborated as an imbalance between antioxidant and pro-oxidant species and it occurs due to free radicals production and presence of reactive intermediates in a system which then surpass the system's ability to neutralize and banish them (Muthulinga and Chaithanya, 2019) [7]. Based on growing attention on free radical biology, the plant-derived antioxidants are essential to combat against diseases. The antioxidant activity of various components of *Boerhavia diffusa* indicates that the leaves exhibit greater antioxidant activity compared to the roots. Specifically, the aqueous extract of leaves of *B. diffusa* to possess stronger antioxidant properties than the roots (Pereira *et al*., 2009) [9].

2. Materials and Methods
2.1 Extract Preparation
The powdered form of *Boerhavia diffusa* (leaves) was obtained from pharmaceutical firm Aerosols Pharma Ltd., Dehradun. A quantity of 200 grams of *B. diffusa* leaf powder was placed in glass containers and soaked in methanol for 48 hours at room temperature, with regular agitation. The mixture was filtered using a dual-layered muslin cloth, subsequently passing through Whatman No. 1 filter papers. The obtained *B. diffusa* leaf extract was concentrated using a rotary evaporator under reduced pressure at a temperature of 40 °C, resulting in the final methanolic extract.

2.2 Plant extract yield (%) determination
The percentage yield (w/w) of the plant extracts was calculated using the formula:

Yield (%) = $(W_1 \times 100)/W_2$

Where, Where W1 represents the dry weight of the extract after solvent evaporation and W_2 is the weight of the plant powder which was soaked in the solvent i.e. methanol

2.3 Quantitative analysis of extracts for phytochemicals
2.3.1 Total phenols content (TPC)
The extract's total phenolic content (TPC) was assessed, and their effectiveness was detected using the Folin-Ciocalteu reagent technique (Biglari *et al.*, 2008) [2]. For this, 0.4 ml of the plant extract (1 mg/mL) was transferred into a test tube and then distilled water was added followed by addition of Folin-Ciocalteu reagent (1 ml) and then the mixture was gently agitated. Following a one-minute interval, addition of 1.6 ml of sodium carbonate (7.5%) was done and the resulting mixture was left undisturbed for 30 minutes while being intermittently shaken. A linear regression curve depicting dose-response relationships was constructed by using the UV spectrophotometer and analyzing the absorbance readings of gallic acid at 765 nm. The potency of TPC (Total Phenol content) in the *B. diffusa* leaves extract (methanolic) was estimated and recorded as milligrams of gallic acid equivalent per gram of extract dry weight (mg GAE/g).

The total phenolic compounds (TPC) in the obtained plant extract were calculated as:
C = A/B;

Where, C: mg GAE/g extract dry weight;
A: equivalent conc. of gallic acid as derived from standard calibration curve (mg); and
B: plant extract dry weight (g).

2.3.1.1 Total Flavonoids Content (TFC)
The TFC (total flavonoid content) was assessed using modified aluminium chloride ($AlCl_3$) colorimetric assay (Zou *et al*., 2004) [11]. For this 0.5ml (5mg/100ml) of *B. diffusa* leaf extract (methanolic) was mixed in a test tube containing distilled water (2ml) and 5% sodium nitrate (150 µl). It was followed by addition 10% aluminium chloride (150 µl) and 1 M sodium hydroxide (2 ml) after five minutes and then incubation at room temperature for 15 min was done. The absorbance's (OD) of the mixtures prepared were recorded at 510 nm. The total flavonoid content (TFC) was estimated as quercetin equivalents (QE) which was derived from a calibration curve using quercetin as a standard. The calibration curve was generated following a similar procedure, utilizing quercetin solutions in methanol spanning a range of 0 to 120 µg/ml. The total flavonoid content (TFC) in the extract of *B. diffusa* leaves (methanolic) was determined after recording the absorbance and using the calibration curve to convert absorbance into flavonoid concentration. The final result was calculated in terms of milligrams of flavonoid equivalent per gram of dry weight of the sample extract (mg FAE/g) of, using the molecular weight of the quercetin.

2.4 *In vitro* assessment for antioxidant potential of plants
2.4.1 Ferric-Reducing Antioxidant Power Assay (FRAP)
The FRAP assay is versatile and can be readily applied to various extracts of plants In this analysis, the antioxidant activity was assessed by measuring its capacity to reduce ferric (Fe3+) iron to ferrous (Fe2+) iron. The FRAP assay was done using EZAssay™ Antioxidant Activity Estimation Kit (FRAP) by HiMedia. This assessment depends on reduction of Fe^{3+}-chromogen complex into Fe^{2+} at low pH in presence of antioxidant to liberate an intense blue colour. This complex has absorption maximum at 593nm and can be measured spectrophotometric ally it can be recorded at 560 nm. Absorbance is directly propotional to antioxidant activity of the sample. The standard curve was constructed using $FeCl_2$ solution.

2.4.2 Ascorbate-Iron (III)-catalyzed phospholipids peroxidation (AICPP): The method employed to estimate the scavenging power of the sample extract and potencies against hydroxyl radicals was as per by Aruoma *et al.*, (1997) [1]. To prepare the homogenate liposomes, goat liver was combined with 10 mM of phosphate buffered saline (pH 7.4) in a ratio of 1:10 and subjected to sonication in an ice bath. A mixture of liposomes (0.2 ml), PBS (0.5 ml), 1 mM ferric chloride-FeCl3 (0.1 ml), and different volumes (100µl and 200µl) of plant extracts was made and subsequent addition of 0.1 ml ascorbic acid (1 mM) was done. The mixture was then incubated at 37ºC for time period of 60 minutes. Next, 1 ml quantity of trichloroacetic acid (10%) was added and the resulting solution was centrifuged for 10 minutes at 2000 rpm (at room temperature). The resulting supernatant was then mixed with 0.67% of 2-thiobarbituric acid (1ml) in 0.05 M of NaOH (sodium hydroxide), followed by vortexing and then it was heated in a water bath for 20 minutes at 100 ºC. After that distilled water (1 ml) was added to it and the absorbance (OD) was recorded at wavelength of 532 nm. A control sample was prepared which was composed of all the reagents except the extracts. The triplicates of the assay were conducted and the standard reference compound used for this assay was vitamin E.
The formula used for calculation of percentage inhibition activity was as follows:
[(Absorbance (OD) of control – Absorbance (OD) of sample)/Absorbance (OD) of control] × 100%.

3. Results and Discussion
Leaves of *Boerhavia diffusa* yielded 4.5% of methanolic extract. Residues of *Boerhavia diffusa* were dark green in colour. Methanol is most commonly used extraction solvent due to its high polarity it could produce high extraction yields.

3.1 Estimation of Total Phenol content (TPC)
Total phenols in extract of *Boerhavia diffusa* leaves (methanolic) were quantified and then represented as miligrams of GAE (gallic acid equivalent) per gram of the sample (plant) extract. Linearity of gallic acid was maintained in the concentration range of 100 to 600µg/ml. TPC of methanolic extract of *B. diffusa* leaves was quantified as gallic acid equivalent (GAE) (mg GAE/g). The calibration curve of gallic acid was used to get the linear equation for TPC calculation (Linear equation- $Y= 0.001x+0.042$, $R^2 =0.9664$). Gallic acid standard curve is depicted in Figure 1.

Total phenol content (TPC) in *B. diffusa* leaf extract (methanolic) was 21.62 ± 0.74 mg GAE/g. The assessment affirmed that total phenol content and antioxidant potential are correlated which is attributed to the hydroxyl (-OH) groups' scavenging capability. Additionally, it was documented that phenolic compounds function as proficient hydrogen donors, improving their effectiveness as antioxidants (Gagandeep *et al*., 2016) [6].

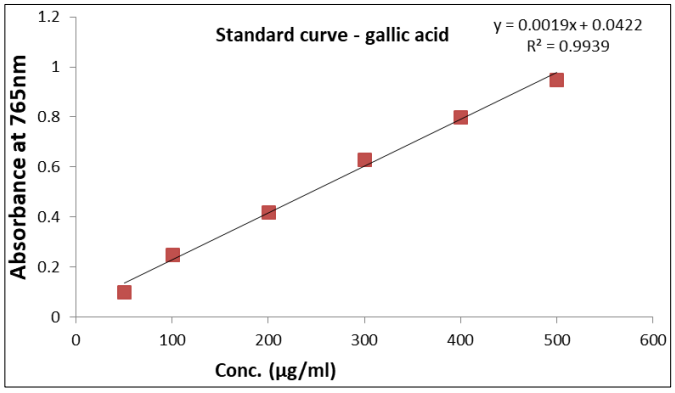

Fig 1: Standard curve - gallic acid for assaying TPC in plant extracts

3.2 Assessment of Total Flavonoid content or TFC

The total flavonoids content (TFC) in methanolic extracts of *Boerhavia diffusa* leaves was quantified and represented as mg of quercetin equivalents (QE) per gram of extract. TFC of extract of *B. diffusa* leaves (methanolic) was estimated as Quercetin Equivalent (QE) (mg/g). Quercetin was used as a standard in TFC estimation and TFC is estimated using a standard calibration curve of quercetin, which was as per equation Y = 0.009x+ 0.034, R^2 =0.990 as shown in Figure 2. Total flavonoid content in *B. diffusa* 18.64 ± 0.84 mg QE/g respectively. Flavonoids function as scavengers of diverse oxidizing agents, such as superoxide anions, hydroxyl radicals, and peroxy radicals. Additionally, they operate as singlet oxygen quencher (Gagandeep *et al*., 2016) [6].

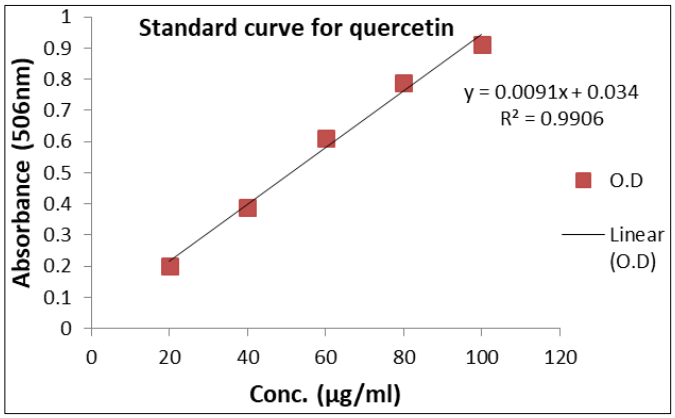

Fig 2: Standard curve of the quercetin for assaying TFC in *B. diffusa* leaves extract

3.3 *In vitro* assessment for estimation of antioxidant potential of plant extract

3.3.1 Ferric-Reducing Antioxidant Power (FRAP) analysis

The capacity of a substance to lower its oxidation potential can be a significant indicator regarding its ability as an antioxidant. A higher absorbance corresponds to a heightened capacity for reduction or antioxidant action within a plant extract. To assess this, the FRAP assay was employed on methanolic extract of *Boerhavia diffusa* leaves. This assay revolves around gauging antioxidant effectiveness by gauging the potential to convert ferric iron (Fe^{3+}) into ferrous (Fe^{2+}) iron. Plotting of standard curve was done using ferric chloride, and the obtained results were quantified as µM of ferrous ion equivalents per gm of dried extract sample weight (y=0.003x + 0.027, R^2 = 0.998). The extract of *Boerhavia diffusa* leaves (methanolic) is a potent reducing agent i.e. 25.61±1.07 µM Fe II iron equivalents.

3.4 Ascorbate-Iron (III)-catalyzed phospholipids peroxidation (AICPP) analysis

In this analysis, the potential of extract to quench the hydroxyl (-OH) radicals produced by ascorbic –Fe (III) which inhibit the production of 2-thiobarbituric acid reactive species (TBARS) was analysed. *Boerhavia diffusa* showed noticeable activity in this regard. Even at a low concentration, *B. diffusa* extract (methanolic) exhibited an inhibitory action on the production of 2-thiobarbituric acid reactive species (TBARS) by consuming hydroxyl (–OH) radicals which were produced by the reaction of iron III (Fe^{3+}) and ascorbate.

Boerhavia diffusa depicted a significant deduction (p<0.05) in its ability to scavenge hydroxyl (-OH) radicals, with an AICPP of 18.15 ± 0.12%. Phospholipids are an important substrate for the analysis of antioxidant potency and hence are appropriate for analyzing the herbal medicines and extracts against membrane lipid peroxidation (Chatterjee and Agarwal, 1988) [3]. The reduction of ferric (Fe^{3+}) into ferrous (Fe^{2+}) and the scavenging of synthetic radicals are commonly regarded as indicators of potential antioxidant activity (Dorman *et al.*, 2003) [4]. In the presence of both ascorbic acid and iron (III), liposomes which are phospholipid-based undergo accelerated peroxidation facilitated by hydroxyl (OH) radicals. This process results in the generation of malondialdehyde and related aldehydes (Esterbauer *et al.*, 1991) [5].

4. Conclusion

India possesses a diverse range of flora that has been harnessed for traditional medicinal practices. These plants owe their medicinal efficacy to the presence of various phytochemical constituents. These bioactive compounds exert therapeutic effects on health. Based on the current investigation, methanolic extract of *B. diffusa* leaves exhibit notable antioxidant capabilities due to its potent phytochemical composition. This plant's methanolic extract is particularly rich in flavonoids and phenols. Consequently, the methanolic extract of *B. diffusa* leaves is a promising reservoir of bioactive agents, holding the potential for the development of natural antioxidant-based remedies aimed at mitigating disorders associated with oxidative stress.

5. Acknowledgment

The authors are thankful to Aerosols Pharmaceuticals Pvt. Ltd., Dehradun (Uttarakhand) for helping in procurement and authentication of plant materials i.e *Boerhavia diffusa* leaf powder.

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