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# The comparative preliminary phytochemical investigation, TLC analysis and antioxidant activity of different solvent extracts of *Boerhavia diffusa* Linn.

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ABSTRACT

The Plant *Boerhaavia diffusa* Linn. is a herbaceous member of the family Nyctaginaceae which is widely distributed in the tropics and subtropics. The present study is aimed to investigate the phytochemicals present in ethanol, methanol, chloroform, aqueous, hexane and petroleum ether extracts obtained from *Boerhaavia diffusa* Linn. and TLC profiling. The plant material was extracted with various solvents based on their polarity by the process of soxhlet extraction method. Preliminary phytochemical analysis was performed by different qualitative methods. Preliminary phytochemical analysis of the extracts revealed the presence of carbohydrates, tannins, alkaloids, saponins and phenolic compounds. The solvent system selected for the best results of TLC was chloroform and methanol of the ratio of 9:1 and 8:2. The study will provide referential information for the correct identification of the bioactive compounds and a suitable solvent system for separation of those compounds from the boerhavia diffusa Linn. The different solvent extracts were further screened for *in-vitro* antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, Nitric oxide, superoxide free radical scavenging, FRAP and hydrogen peroxide methods. Taken together, our results suggested that methanolic extract of *Boerhaavia diffusa* Linn. might contain potential antioxidant compounds.

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## INTRODUCTION

Nature is always a golden sign to show the prominent phenomena of coexistence. Natural products from plants, animals and minerals are the basis for treating human diseases. Medicinal plants are presently in demand, and their acceptance is increasing progressively. In ancient Persia, plants were commonly used as a drug and disinfectant and aromatic agent. The use of medicinal plants for the treatment of diseases dates back to the history of human life, that is, since human beings have sought a tool in their environment to recover from a disease, the use of plants was their only choice of treatment (Fatemeh Jamshidi-Kia *et al.,* 2018). The genus Boerhaavia L. (Family: Nyctaginaceae) consists of 40 tropical, and subtropical species found growing wild in different terrestrial habitats, ranging from managed grasslands, wastelands, agroecosystems to large forest gaps. The plants grow vigorously as weeds in tropical and subtropical regions such as India, Brazil, Africa, Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, USA, Iran and in several countries of the Middle East. B. diffusa L. was described as Punarnava (meaning one that rejuvenates the old body) in the Atharvaveda (Pranati Nayak, M., Thirunavoukkarasu., 2016). Secondary metabolites are produced by plants

mainly as products of primary metabolism and as part of the defence mechanisms of plants.

Phytochemicals such as, alkaloids, tannins and flavonoids are examples of secondary metabolites produced by plants, from which the plants are thought to get their healing properties. Phenolic compounds have been associated with antioxidant activity due to their free radical scavenging activities. The presence of an excess of oxygen in the human body has some negative effects as it can trigger radical chain reactions in the presence of reactive species. This can cause health problems, such as ageing and cell destruction. Antioxidants have been found to be the solution to this problem as they interrupt these chain reactions to form radicals that can easily be removed from the human body, thereby generally improving health, assisting cell rejuvenation, cancer prevention and cardiovascular diseases prevention. Thus it is important to investigate the antioxidant potential.

A considerable number of publications have been reported on the phytochemistry of *Boerhavia diffusa Linn.* Limited studies on the whole plant have been directed at the evaluation of its traditional medicinal applications. No reports are available on the phytochemical analysis and antioxidant activities of the different solvent extracts of *Boerhavia diffusa Linn.* The study was therefore aimed at determining suitable solvents for extraction of phytochemicals, phytochemical screening using standard methods, TLC analysis and antioxidant activity of *Boerhavia diffusa Linn.* Our results provide a basis for future studies on isolation, identification and characterisation of active compounds with potential applications in drug development (Kayini Chigayo., *et al.,* 2016).

## MATERIALS AND METHODS

**Procurement of Experimental Plant:** The experimental plant *Boerhavia diffusa Linn.* was collected from Samanthipuram, Arcot, Vellore district. Healthy and mature plant was carefully chosen for sampling. The plant parts were brought to the laboratory in sterilized bags, and optimum temperature and moisture were maintained. The plant was authenticated by Prof. P.Jayaraman, Director, Plant anatomy research centre, Chennai.

**Chemicals:** All chemicals used in the study were of analytical reagent grade and highest quality available and were purchased from reliable firms and institutes.

**Sequential Extraction of *Boerhavia diffusa Linn.:*** The plant material was allowed to shade dry at room temperature for 2 weeks characterized by complete discolouration of leaves and removal of moisture. The plant material was then ground and subjected to sequential extraction using the solvents Petroleum ether, Hexane, Chloroform, Ethanol, Methanol and deionised water. Soxhlet method is used for extraction process. The yield of the extract was calculated by using the formula;

Percentage yield = (Weight of extract obtained/ Weight of plant material used for extraction) * 100

**Preliminary Phytochemical Screening of Successive Extracts of *Boerhavia diffusa Linn.***

i. **PHYTOCHEMICAL SCREENING:** Small amount of the extract/fraction was dissolved in a required solvent and filtered. The filtrate was subjected to the following tests:
ii. **CARBOHYDRATE:** Benedict's test: To 0.5 ml of filtrate 0.5ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes. A reddish brown precipitate indicates the presence of reducing sugar[6].
iii. **PROTEIN:** Millon's Test: To 2 ml of filtrate, few drops of Millon's reagent were added. A white precipitate indicates the presence of proteins [5].
iv. **ALKALOID:** Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids (R.S. Sawant and A.G. Godghate 2013).
v. **TANNIN:** 1 g of each powdered sample was separately boiled with 20 ml distilled water for five minutes in a water bath and was filtered while hot.1 ml of cool filtrate was distilled to 5 ml with distilled water and a few drops (2- 3) of 10 % ferric chloride were observed for any formation of precipitates and any colour change. A bluish-black or brownish-green precipitate indicated the presence of tannins (R.S. Sawant and A.G. Godghate 2013).
vi. **PHENOL:** To the extract, add 1 ml of 1% of FeCl and 1 ml of $K_3$ (Fe (CN) $_6$ (Potassium Ferricyanide). The appearance of fresh radish blue colour indicates the presence of polyphenols [7].
vii. **FLAVONOIDS:** Shinoda Test: To the extract, add a few fragments of Magnesium ribbon and concentrated hydrochloric acid. After a few minutes appearance of red to the pink colour presence of flavonoids.
viii. **TERPENOIDS/TRITERPENOIDS:** Salkowski Test: To the extract few drops of concentrated $H_2SO_4$ and 2ml chloroform and shaken then allow standing, the appearance of golden yellow colour indicates the presence of triterpenes (Nilanjana D, *et al.,* 2013).
ix. **STEROID:** Bubble test: To the 1ml extract, add 5 ml of distilled water shake vigorously. Formation of foam indicates the presence of steroids. To the extract, 10 ml chloroform and conc.$H_2SO_4$ added to form the upper layer as

red colour and the bottom layer as yellow with green fluorescence for the presence of steroids.

x. **SAPONIN:** Foam Test: Small amount of extract add little quantity of water. If foam produced on shaking persists for 10 minutes, indicates the presence of Saponins.

xi. **VOLATILE OIL:** To the extract, 1ml of 0.1M NaOH solution and 1% aqueous HCl was added to form white precipitate for the presence of volatile oil (Harborne JB. 1983).

xii. **BALSAMS:** To the extract, add 10ml of 90% ethanol and filter. Add two drops of 10% alcoholic ferric chloride solution to form a dark green coloured solution for the presence of balsams (Harborne JB. 1983).

xiii. **RESINS:** Dissolve the extract in acetone and pour the solution in distilled water. Turbidity indicates the presence of resins (Harborne JB. 1983).

xiv. **COUMARINS:** Place the extract in the test tube, cover it with filter paper moistened with 1 N NaOH and was kept in a water bath for 10 min. After removing the filter paper, examine under UV light, yellow fluorescence indicates the presence of Coumarins (K.N.V. Rao, *et al.,* 2015).

xv. **PHLOBATANNIN:** Precipitate test: To the extract, 1% HCl added and was boiled to form red precipitate for the presence of phlorotannin (Manjulika Y, *et al.,* 2014).

**Chromatographic profiling**

TLC plates were prepared by using Silica Gel as an adsorbent. Silica Gel-G (15 gm) was mixed with 30 ml of distilled water to make a slurry. The slurry was immediately poured on to the plates. Plates were then allowed to air dry for one hour, and the layer was fixed by drying at 100°C for one and a half hours. Using a micropipette, about 10 µl of extracts were loaded gradually over the plate and air dried. The plates were developed by using two different solvent systems. The solvent system consist of Chloroform: Methanol (9:1 and 8:2). The Rf values of methanolic leaf extract in two different solvent system were calculated by using the following formula (Dutta Jayashree 2013).

Rf = Distance travelled by the solute (cm)/ Distance travelled by the solvent (cm)

**Invitro antioxidant activity**

**DPPH free radical scavenging assay**

**Procedure:** The ability of the extracts to annihilate the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was investigated by the method described by (Blois 1958). A stock solution of the compound was prepared to the concentration of 10 mg/ml. Different concentration of the extract (100, 200, 300, 400 & 500 µg) of the sample was added, at an equal volume to a methanolic solution of DPPH (0.1mM, 1.5 mL). The reaction mixture is incubated for 30min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid was used as a standard control. The annihilation activity of free radicals was calculated in % inhibition according to the following formula (Blois, Marsden.S., 1958).

% of Inhibition = (A of control – A of Test)/A of control * 100

**Nitric Oxide Scavenging Assay**

NO generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci et al. (1994). Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate buffered saline (pH 7.3), with the compound (25, 50, 75,100, 200 & 250 µg) at different concentrations, was incubated at 25°C for 180 min in front of a visible polychromatic light source (25W tungsten lamp). The NO radical thus generated interacted with oxygen to produce the nitrite ion (NO.) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediaminedihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the plant extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. Each experiment was carried out at least three times and the data presented as an average of three independent determinations (Parul R, *et al.,* 2013).

**Total Antioxidant activity- FRAP assay**

**Procedure:** Ferric reducing antioxidant power (FRAP) A modified method of Benzie and Strain (1996) was adopted for the FRAP assay. The different concentration of standard or sample extract (50, 100, 150, 200, 250 µg) was mixed with 300 µl of ferric–TPTZreagent [prepared by mixing 300 mM acetate buffer, Ph 3.6, 10 mM TPTZ in 40 mMHCl, and 20 mM $FeCl_3$6H2Oat a ratio of 10:1:1 (v/v/v)]. The mixture was incubated at 37 ºC, and the absorbance readings were taken at 593 nm after 4 min. Results were expressed in mM Fe(II)/g dry mass using a calibration curve of a freshly prepared ferrous-sulfate solution (Ahmed T *et al.,* 2015). FRAP value of Ascorbic acid.

Calculating formula: [A(Sample Final)-A(Sample Initial)/A(Std Final)-A(Std initial)]×2
A-Absorbance; Std-Standard

**Hydrogen Peroxide scavenging assay**

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.,* (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (8500 II, Bio- Crom GmbH, Zurich, Switzerland). Extracts (200–1000 µg) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing in phosphate buffer without hydrogen peroxide (Sroka Z. 2003). The percentage of scavenging of hydrogen peroxide of extract and standard was calculated using the following equation:

% of Inhibition = (A of control – A of Test)/A of control * 100

**Superoxide scavenging activity**

Measurement of superoxide radical scavenging activity was done by using standard method (Nishikimi*et al,* 1972). The superoxide anions generated by phenazine methosulfate (PMN)/nicotinamid-adenine-dinucleotidphosphate, reduced form (NADPH) system, were detected by the reaction with 2,2'-di-p-nitrophenyl)-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (nitro blue tetrazolium – NBT). A stock solution of plant extracts and Quercitin (standard) was prepared for the concentration of 1mg/ml. The reaction mixture contained 1ml of Nitro blue tetrazolium (NBT) solution (312µM prepared in phosphate buffer, pH-7.4), 1ml of Nicotinamide adenine dinucleotide (NAD) solution (936 µM prepared in phosphate buffer, pH-7.4) and samples at different concentration (200, 600 and 1000 µg/mL) obtained from stock solution were added and finally the reaction was accelerated by adding 100µl phenazine methosulfate (PMS) solution (120 µM prepared in phosphate buffer, pH-7.4). The reaction was incubated at 25°C for 5 minutes, and absorbance was measured at 560nm against the corresponding blank solutions. Blank consists of all the reagents, except for the extract or standard solution is substituted with water (Venkatachalam T *et al.,* 2013). The annihilation activity of free radicals was calculated in % inhibition according to the following relation:

Inhibition % = (Absorbance of control – Absorbance of sample) / Absorbance of control * 100

**Statistical analysis**

Statistical analysis was carried out by One-way analysis of variance (ANOVA) test using a statistical package program (SPSS 10.0), and the significance of the difference between means was determined by Duncan's multiple range test at ($P$<0.05) significant level. The analysis was carried out in triplicate and mean ± SD of three parallel measurements.

**RESULT AND DISCUSSION**

**Extraction yield of sequential extraction of *Boerhavia diffusa* Linn.**

The yield of crude extracts from a whole plant extract of *Boerhavia diffusa Linn.*, obtained by Soxhlet method using a different type of solvents, were calculated and the results were shown in Table 1. The recovery percentage of extractable compounds of whole plant extract of *Boerhavia diffusa Linn.* is ranged from 2.3 to 8.6, respectively. The results revealed that the highest yield extracts were obtained by aqueous and methanol for whole plant extract of *Boerhavia diffusa Linn.*, respectively. Significant differences were observed among extraction yields obtaining by methanol compared to other solvent extracts of *Boerhavia diffusa Linn.* This difference may be attributable to the higher solubility of extractable bioactive components such as carbohydrates and proteins in than in other solvent extracts. The variation in the yields of extracts could be attributed to the difference in solvent polarities used which also plays a key role in increasing the solubility of phytochemical compounds. Differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarity. In fact, six solvents with different polarity were used, and they can be arranged as follows (starting from more low dielectric constant values): Petroleum ether, hexane, chloroform, ethanol, methanol, water. This change appears to be related not only to the difference in the polarity of extracts of the components but also to the solvents used, which also plays a vital role in increasing the solubility of phytochemical compounds.

Therefore, this result confirmed the effect of solvent extraction and the plant on the yield extraction and consequently confirms the richness of this plant in polar substances.

**Effect of extracting solvent on the phytochemical contents of the various extract**

The solubility of the phenolic compounds was mostly influenced by the nature of the solvent used and their polarity. The recovery of phytochemicals from the plant could be influenced by dielectric

**Table 1: Yield of extracts from sequential extraction of Boerhavia diffusa Linn.**

| Solvent | Polarity Index (*P*) | Extraction Yield |
|---|---|---|
| Petroleum Ether | 0.1 | 3.4% |
| Hexane | 0.1 | 2.3% |
| Chloroform | 4.1 | 4.1% |
| Ethanol | 4.3 | 5.9% |
| Methanol | 5.1 | 7.3% |
| Water | 10.2 | 8.6% |

**Table 2: Phytochemical profile of sequential extracts of Boerhavia diffusa Linn.**

| S.No | Phytochemical Tests | Samples | | | | | |
|---|---|---|---|---|---|---|---|
| | | Petroleum Ether extract | Hexane extract | Chloroform extract | Ethanol extract | Methanol extract | Aqueous extract |
| 1 | Carbohydrates test | - | - | + | + | + | + |
| 2 | Tannins test | - | - | - | - | + | - |
| 3 | Saponins test | + | + | - | - | - | - |
| 4 | Flavonoids test | + | - | + | - | + | - |
| 5 | Alkaloid test | - | - | - | - | + | - |
| 6 | Quinones test | + | + | + | - | + | + |
| 7 | Glycosides test | - | - | - | - | - | - |
| 8 | Cardiac glycosides test | - | - | - | - | - | - |
| 9 | Terpenoids test | + | + | - | - | - | - |
| 10 | Phenols test | - | - | - | - | - | - |
| 11 | Coumarins test | + | - | + | - | + | + |
| 12 | Steroids &Phytosteroids | - | - | - | - | + | + |
| 13 | Phlobatannins test | - | - | - | - | - | - |
| 14 | Anthraquinones test | - | - | - | - | + | - |

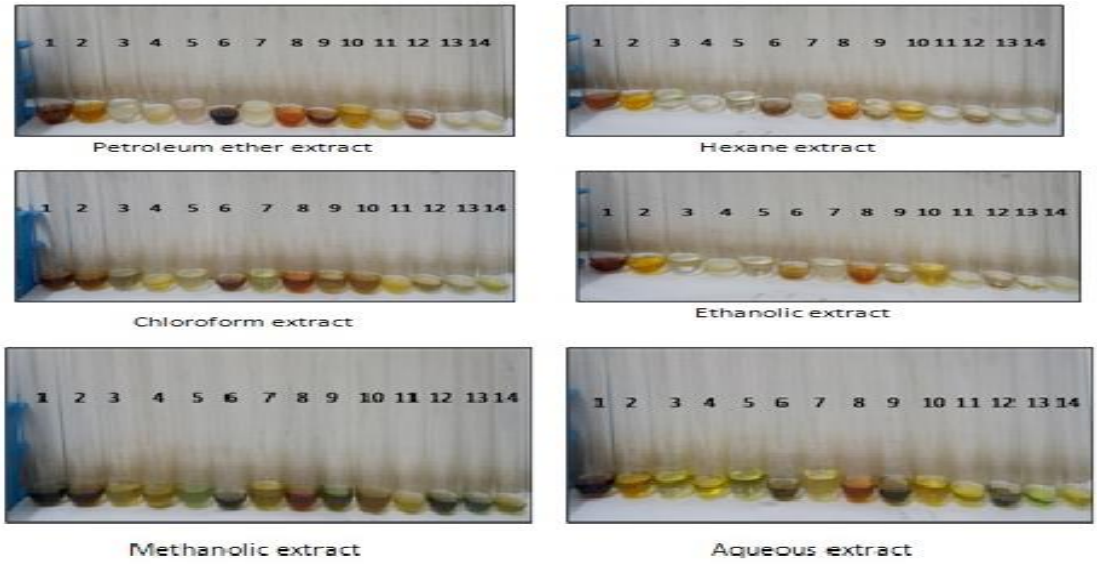


**Figure 1: The details of secondary metabolites present in sequential extraction of Boerhavia diffusa Linn.**

constant, the chemical structure of organic solvents, and as well as chemical properties of plant phytochemicals. The presence of secondary metabolites of different solvent extract from *Boerhavia diffusa Linn.* was given in Table 2.

The study revealed that from the different solvent extracts of *Boerhaavia diffusa Linn.*, methanolic extract shows the maximum presence of secondary metabolites. The results presented in this work confirmed that methanol was the best solvent to extract phytochemicals compounds such as phenolic compounds, cardiac glycosides and anthraquinone.

It should be pointed too that there is a significant difference between phytochemical contents of the different extracts. Therefore, the *Boerhavia diffusa Linn.* extracts demonstrated that the solubility of phytochemicals compounds is not only dependent on the type of solvent used but also on the plant part. Phytochemical constituents have been reported to be associated with antioxidative action in biological systems. In light of the obtained results, *Boerhavia diffusa Linn.* is a good source of phytochemical compounds such as phenolics, cardiac glycoside and alkaloid that could be used in foods, cosmetics or pharmaceutical products.

**TLC profiling of sequential extract of *Boerhavia diffusa* Linn.**

TLC of all sequential extracts of *Boerhavia diffusa Linn.* obtained by sequential extraction methods was carried out to confirm its nature by analysing. Pharmaceutical preparations derived from natural sources often contain compounds that contribute to the antioxidant activity apparently will account for the protection against degenerative diseases. In the present study, preliminary phytochemical analysis of the different solvent extracts of *Boerhavia diffusa Linn.* was carried out to detect the active constituents such as alkaloids, flavanoid and phenols. The details of the TLC profile of sequential extracts of *Boerhavia diffusa Linn.* are presented in table 3 and 4. TLC of pet ether extract of *Boerhavia diffusa Linn.* revealed the presence of 2 compounds having Rf values of 0.83,0.47 respectively when a solvent phase of Chloroform: Methanol (9:1) was used. In Chloroform: Methanol (8:1) one spots were obtained having Rf of 0.93. TLC of ethanolic and aqueous extract of *boerhavia diffusa Linn.* revealed the absence of secondary metabolites in a solvent phase of Chloroform: Methanol (9:1. 8:1) proportion. TLC of methanolic extract of *boerhavia diffusa Linn.* revealed the presence of 2 compounds having Rf values of 0.17,0.37 respectively when a solvent phase of Chloroform: Methanol (9:1) was used. In Chloroform: Methanol (8:1) two spots were obtained having Rf of 018 and o.55. TLC of hexane extract of *boerhavia diffusa Linn.* revealed the presence of 2 compounds having Rf values of 0.83,0.97 when a solvent phase of Chloroform: Methanol (9:1) was used. In Chloroform: Methanol (8:1) one spots were obtained having Rf of 0.95. TLC of the chloroform extract of *boerhavia diffusa Linn.* revealed the presence of 2 compounds having Rf values of 0.62,0.82 when a solvent phase of Chloroform: Methanol (9:1) was used. In Chloroform: Methanol (8:1) two spots were obtained having Rf of 0.82 and 0.93.

***In vitro* antioxidant assays**

***In vitro* antioxidant profile of sequential extracts of *Boerhavia diffusa Linn.* by DPPH Method**

Free radicals produced in the body are partly associated with the aetiology of cancers and other chronic diseases. Therefore, determining the radical scavenging effect of antioxidants in this *Boerhavia diffusa Linn.* plant is important. Antioxidant activities of the *Boerhavia diffusa Linn.* extracts by the different solvents were analysed by measuring DPPH radical scavenging activities. DPPH assay is widely used for evaluating antioxidant activities in a short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. The results on DPPH radical scavenging activities of the methanol, ethanol, aqueous, petroleum ether, hexane and chloroform extracts and the positive control reference standard ascorbic acid is shown in Table 5. According to the results, as the $IC_{50}$ value decreases, the antioxidant activity increases. The $IC_{50}$ value of each extract was compared with ascorbic acid. DPPH radical scavenging activity of the methanolic extract was found to be higher than that of other extracts.

**Free radical scavenging activity by Nitric oxide method**

Nitric oxide inhibitors have been shown to have beneficial effects on inflammation and tissue damage seen in inflammatory diseases. The highest measurable activity was found in the ethanol extracts of *Boerhavia diffusa Linn.* Comparably, it surpassed the performance of standard antioxidants such as quercetin. Noticeably, the chloroform extract of *Boerhavia diffusa Linn.* contributed very low potential for radical scavenging with the IC50 value of 40.24 g/mL [Table 6] compare to other solvent extracts.

**Antioxidant activity of *Boerhavia diffusa Linn.* of different solvent extract using FRAP assay**

FRAP assay is commonly used to study the antioxidant capacity of plant materials. Among the transition metals, iron is known as the most important

**Table 3: Rf values of sequential extracts of *Boerhavia diffusa* Linn. (9:1, v/v; Direct, UV-254nm & 10% Ferric chloride)**

| Sample Name | Spot No. | 9:1, v/v - Direct | 9:1, v/v-UV-254nm | 9:1, v/v –10% Ferric chloride |
|---|---|---|---|---|
| Ethanolic Extract | - | - | - | - |
| n-Hexane Extract | 1 | 0.83 | 0.95 | 0.98 |
| | 2 | 0.97 | - | |
| Chloroform Extract | 1 | 0.62 | 0.97 | 0.63 |
| | 2 | 0.82 | - | 0.98 |
| | 3 | 0.97 | - | |
| Methanolic Extract | 1 | - | 0.17 | 0.17 |
| | 2 | - | - | 0.37 |
| Petroleum Ether Extract | 1 | 0.83 | 0.98 | 0.98 |
| | 2 | 0.98 | - | - |
| Aqueous Extract | - | - | - | - |

**Table 4: Rf values of (8:2, v/v; UV 254nm, Wagner's Reagent) sequential extracts of *Boerhavia diffusa* Linn.**

| Sample Name | Spot No. | 8:2 - v/v –UV 254 nm | 8:2 - v/v - Wagner's reagent |
|---|---|---|---|
| Ethanolic Extract | 1 | 0.93 | 0.95 |
| n-Hexane Extract | 1 | 0.95 | 0.96 |
| Chloroform Extract | 1 | 0.82 | 0.96 |
| | 2 | 0.93 | - |
| Methanolic Extract | 1 | 0.35 | 0.18 |
| | 2 | 0.55 | 0.55 |
| Petroleum Ether Extract | 1 | 0.93 | 0.95 |
| Aqueous Extract | - | - | - |

**Table 5: Antioxidant analysis by DPPH assay**

| Sample No. | Sample name | Percentage Inhibition Concentration (µg/mL) | | | | |
|---|---|---|---|---|---|---|
| | | 100 | 200 | 300 | 400 | 500 |
| 1 | Petroleum Ether | 18.67 | 23.97 | 28.98 | 38.40 | 44.17 |
| 2 | Hexane | 37.56 | 41.08 | 43.06 | 44.94 | 48.25 |
| 3 | Chloroform | 31.86 | 35.15 | 37.00 | 38.76 | 41.86 |
| 4 | Ethanol | 32.52 | 35.87 | 37.74 | 39.53 | 42.68 |
| 5 | Methanol | 38.95 | 42.92 | 44.97 | 56.08 | 58.78 |
| 6 | Aqueous | 40.76 | 43.11 | 45.86 | 57.96 | 63.02 |

lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron can stimulate lipid peroxidation by the Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, which can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The simple and reliable FRAP assay measures the reducing potential of an antioxidant reacting with a ferric-TPTZ (Fe(III)-TPTZ) complex and producing a coloured ferrous-TPTZ (Fe(II)-TPTZ) complex by a reductant at a low pH (about 3.6). The ferric-TPTZ complex can be monitored at 593 nm. A higher absorbance power indicates a higher ferric reducing power. In the present study, the FRAP values for different solvent extracts are shown in Table 7 and figure 7. The methanolic extract registered significantly higher ($P$<0.05) ferric reducing antioxidant activity than the chloroform, ethanol, hexane, aqueous and petroleum ether.

**Hydrogen peroxide radical scavenging activity**

The scavenging effect of different fractions of *Boerhavia diffusa Linn.* on hydrogen peroxide was concentration-dependent (25 - 500 µg/ml) as shown in table 8 and figure 8. TLE displayed strong $H_2O_2$ scavenging activity compared with the standard, ascorbic acid. The scavenging activities of different solvent extracts of *Boerhavia diffusa Linn.* were

presented in Table 8 and figure 8. The scavenging activity for hydrogen peroxide of various solvent extracts from *Boerhavia diffusa Linn.* was in the order of methanol, ethanol, petroleum ether, aqueous, chloroform and hexane.

**Superoxide scavenging assay of sequential extracts of *Boerhavia diffusa Linn.***

The results of our Superoxide radical study revealed that methanolic extract has an effective capacity of scavenging for superoxide radical and correlated with total flavonoid content thus suggesting its antioxidant potential.

**Table 6: Antioxidant analysis by NO assay**

| | Percentage Inhibition | | | | |
|---|---|---|---|---|---|
| | Concentration | | | | |
| Sample | 100µg/mL | 200µg/mL | 300µg/mL | 400µg/mL | 500µg/mL |
| Petroleum Ether | 12.86 | 18.13 | 22.33 | 26.96 | 45.39 |
| Hexane | 9.13 | 15.40 | 22.75 | 29.84 | 42.38 |
| Chloroform | 8.77 | 13.75 | 21.85 | 34.10 | 40.24 |
| Ethanol | 16.62 | 23.58 | 45.51 | 61.38 | 68.53 |
| Methanol | 18.26 | 22.78 | 29.29 | 42.27 | 59.64 |
| Aqueous | 21.84 | 25.76 | 29.72 | 47.52 | 55.43 |

**Table 7: the FRAP reducing the potential of the extracts**

| Concentration (µg/mL) | Reducing potential | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | Pet. Ether | Hexane | Chloroform | Methanol | Ethanol | Aqueous |
| 100 | 0.9034 | 1.0437 | 1.2944 | 0.4929 | 0.3036 | 0.3144 |
| 200 | 0.1288 | 0.6480 | 0.2964 | 0.1200 | 0.1634 | 0.1523 |
| 300 | 1.3287 | 1.4930 | 0.5122 | 0.7713 | 0.6383 | 0.4325 |
| 400 | 1.0062 | 0.7738 | 0.9822 | 0.6352 | 1.1681 | 0.4407 |
| 500 | 0.0312 | 0.5661 | 1.5128 | 1.7294 | 0.7450 | 0.2951 |

**Table 8: H2O2 reducing potential of the extracts**

| Sample no | Sample name | Percentage Inhibition | | | | |
|---|---|---|---|---|---|---|
| | | Concentration (µg/mL) | | | | |
| 1 | Petroleum Ether | 23.48 | 31.27 | 38.25 | 48.69 | 50.10 |
| 2 | Hexane | 25.31 | 28.83 | 34.36 | 38.30 | 44.78 |
| 3 | Chloroform | 30.36 | 31.73 | 34.91 | 37.70 | 46.43 |
| 4 | Ethanol | 38.29 | 47.13 | 54.98 | 61.47 | 63.17 |
| 5 | Methanol | 34.02 | 37.83 | 55.87 | 61.87 | 65.43 |
| 6 | Aqueous | 21.41 | 30.62 | 42.48 | 49.05 | 49.66 |

**Table 9: superoxide scavenging assay of different solvent extracts**

| Concentration (µg/mL) | Mean percentage Inhibition | | | |
|---|---|---|---|---|
| | Hexane extract | Ethyl acetate extract | Methanol extract | Ascorbic acid |
| 100 | 11.70 | 7.82 | 26.49 | 74.69 |
| 200 | 13.67 | 9.35 | 38.78 | 77.56 |
| 400 | 14.76 | 11.56 | 42.34 | 78.62 |
| 600 | 16.05 | 12.73 | 46.40 | 79.89 |
| 1000 | 21.97 | 18.01 | 61.23 | 86.29 |


**Figure 2: TLC Profiling photographs of different solvent extracts of (9:1, v/v; Direct, UV-254nm & 10% Ferric chloride) *Boerhavia diffusa* Linn.**

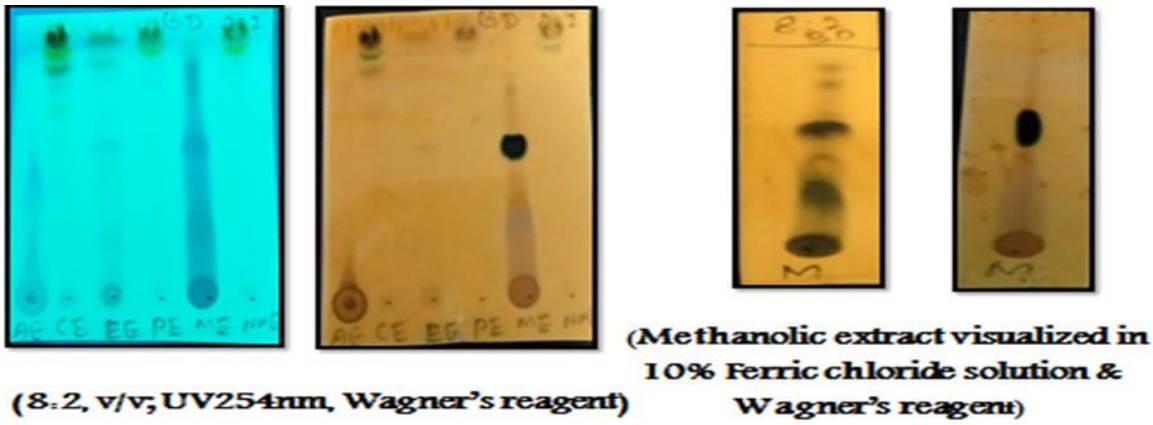


**Figure 3: TLC Profiling of (8:2, v/v; UV254nm, Wagner's reagent) sequential extracts of *Boerhavia diffusa* Linn.**

## DISCUSSION

For the pharmacological as well as the pathological discovery of novel drugs, the essential information's regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts (Talukdar AD, *et al.,* 2010; Paliwal R, *et al.,* 2011). In the present study, qualitative tests for all five extracts showed significant indication about the presence of metabolites. Alkaloids, saponin, polyphenols and cardiac glycosides were found to be present in the all the sequential extracts of *Boerhavia diffusa Linn.* while flavanoids and pholobatannins were present in very low amounts in the extracts. These findings of phytochemicals were good enough to reflect its importance. Compare to all other extracts, a methanolic extract having more secondary metabolites.

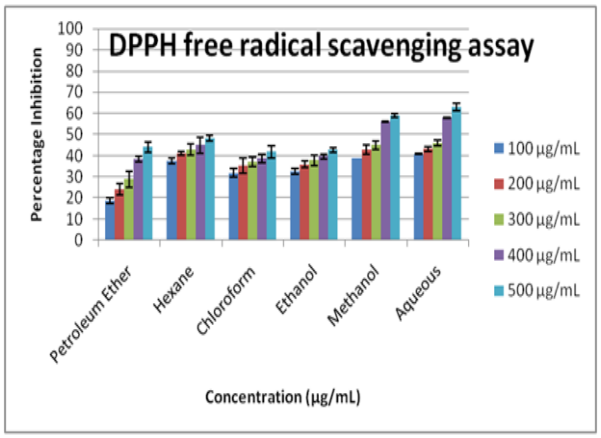


**Figure 4: DPPH free radical scavenging activity of the selected extracts**

TLC profiling of all 6 extracts gives an impressive result that directing towards the presence of a number of phytochemicals. Various phytochemicals give different Rf values in the different solvent system. This variation in Rf values of the phytochemicals provides a very important clue in the understanding of their polarity and also helps in the selection of an appropriate solvent system for separation of pure compounds by column chromatography. A mixture of solvents with variable polarity in different ratio can be used for separation of the pure compound from plant extract. The selection of an appropriate solvent system for particular plant extracts can only be achieved by analysing the Rf values of compounds in the different solvent system (Sharma V, *et al.,* 2011). Different Rf values of the compound also reflect an idea about their polarity. This information will help in the selection of an appropriate solvent system for further separation of the compound from these plant extracts.

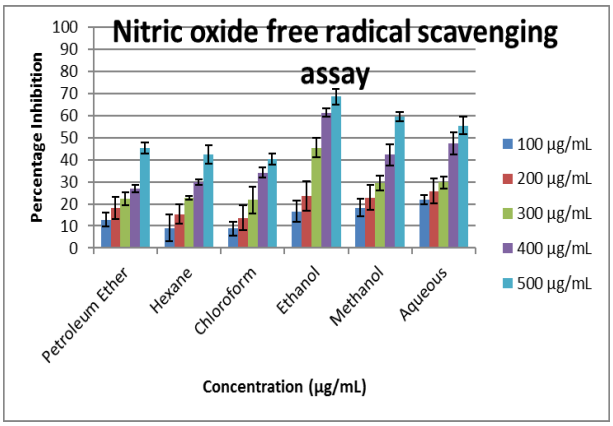


**Figure 5: The nitric oxide scavenging activity of the extracts**

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo*. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms (Nunes PX, *et al.,* 2012).

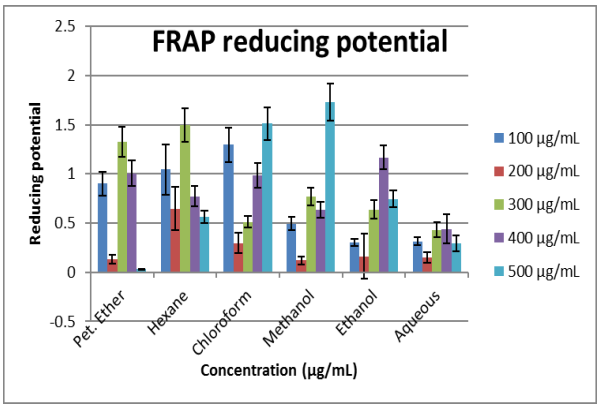


**Figure 6: The FRAP reducing the potential of the extracts**

The electron donation ability of natural products can be measured by 2,2′-diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Fadzai Boora 2014). In the present study among all the fractions tested, methanolic extract showed a significantly higher inhibition percentage and positively correlated with total phenolic content. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

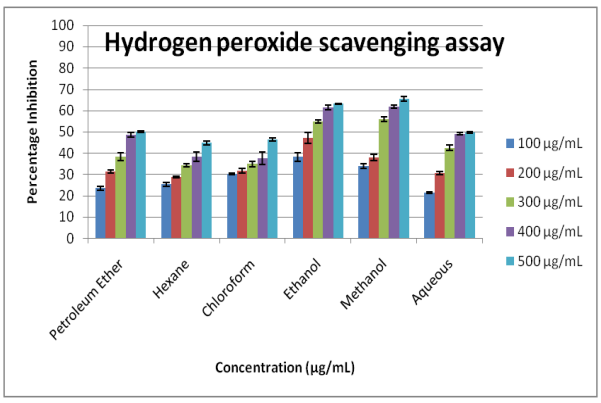


**Figure 7: The $H_2O_2$ reducing potential of the extracts**

Nitric oxide (NO) and reactive nitrogen species (RNS) are free radicals that are derived from the interaction of NO with oxygen or reactive oxygen species. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals such as superoxide. NO is synthesized by three isoforms of the enzyme nitric oxide synthase (NOS), endothelial NOS, neuronal NOS, and inducible NOS (iNOS). Nitric oxide (NO) is generated from amino acid L-arginine by the enzymes in the vascular endothelial cells, certain neuronal cells, and phagocytes. Low concentrations of NO are sufficient in most cases to effect the physiological functions of the radical. NO is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation, and antimicrobial and antitumor activities. Chronic exposure to nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis. The toxicity of NO increases greatly when it reacts with the superoxide radical, forming the highly reactive peroxynitrite anion (ONOO−). Nitric oxide has been shown to be directly scavenged by flavonoids. The result of NO study revealed that ethanolic extract having potential free radical scavenging activity compared to other extracts.

In reducing power assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the $Fe^{3+}$/ferricyanide complex to the ferrous form. Therefore, $Fe^{2+}$ can be monitored by absorbance measurement at 700 nm. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain (Gordon MH. 1990). Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the methanolic solvent extracts caused their reduction of $Fe^{3+}$/ ferricyanide complex to the ferrous form and thus proved the reducing power.

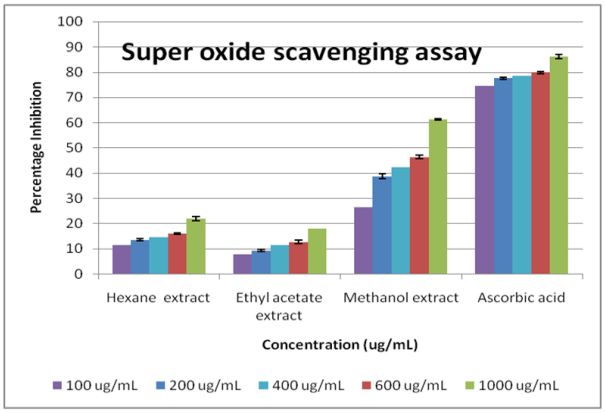


**Figure 8: The superoxide scavenging assay of different solvent extracts**

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food (Gulcin I, *et al.,* 2005). $H_2O_2$ is rapidly decomposed into oxygen and water, and this may produce hydroxyl radicals (·OH) that

can initiate lipid peroxidation and cause DNA damage (Sahreen S, *et al.,* 2011). The methanolic fraction of *boerhavia diffusa Linn.* efficiently scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralising it into the water.

Superoxide radical is considered a major biological source of reactive oxygen species (Alves CQ, *et al.,* 2010). Although superoxide anion is a weak oxidant, it gives rise to a generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer AS, Isaksen A 1995). The results of our study revealed methanolic extract has an effective capacity of scavenging for superoxide radical and correlated with total flavonoid content thus suggesting its antioxidant potential.

Hence, compare to other extracts, methanolic extracts can be used as natural sources of antioxidants as they could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age-associated oxidative stress-related degenerative diseases. They also have potential application in industry as natural antioxidants that could be used as food additives to prevent food deterioration as synthetic antioxidants that are available are associated with a lot of side effects.

## CONCLUSION

In conclusion, methanolic extract of *Boerhavia diffusa Linn.* possessed good antioxidant activities. Although further research is necessary, our study shows that supplementation with this plant extract could at least help in preventing or decreasing the damages caused by oxidative stress. Based on these data, additional studies are needed to characterise the bioactive compounds responsible for the antioxidative activities in *Boerhavia diffusa Linn.* Therefore, the study suggests that the whole plant might be a potential source of natural antioxidants.

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