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# QUANTIFICATION OF ANTIOXIDANT POLYPHENOLS FROM *BOENNINGHAUSENIA ALBIFLORA*

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**ABSTRACT**

The aim of this study was to screen various extracts prepared from aerial part and root of *Boenninghausenia albiflora* for their metabolite profiling and *in-vitro* antioxidant activity. Arial part and root were subjected to quantitative screening test for various constituents which revealed the presence of sugar (6.5 and 8.4%), starch (7.0 and 10%), and tannins (37.5 and 36.96 µg tannic acid equivalent /mg dry extract), respectively. The order of total phenolic content was descended in following order: hydro-alcoholic extract of aerial part (HEA) >methanolic extract of aerial part (MEA) >hydro-alcoholic extract of root (HER)>methanolic extract of root (MER). HEA showed an effective scavenging of DPPH radical ($IC_{50}$, 195.9µg/mL) followed by MEA ($IC_{50}$ 243.8 µg/mL). While $IC_{50}$ values for HER and MER were recorded by 370µg/mL and 600µg/mL, respectively. Based on $IC_{50}$ values, the order of antioxidant activity based on β-carotene bleaching of different extracts was found to be MER> MEA > HEA >HER. Data revealed that act as an antioxidant agent due to its free radical scavenging activity. For achieving good separation of phenolic compounds using HPTLC, a mobile phase of toluene: ethyl acetate: formic acid (5:5:1) was used and data revealed the presence of caffeic, ferulic, gallic, and vanillic acids in roots and aerial part extracts of *B. albiflora*. The quantification of phenolic compounds and antioxidant activity in *B. albiflora* have not yet been reported, thus this information can be useful for proper standardization of herbal drug containing *B. albiflora*.

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## INTRODUCTION

*Boenninghausenia albiflora* (Hook) Reichb. ex Meisn., a member of Rutaceae family, is a common weed in hilly areas. Traditionally it is used to treat ticks and lice. According to tribals, dried plant is effective against the bed bugs and dust mites [1] (Ramdev *et al*., 2011). In ethno botanical literature, the aerial as well as the root part has been described as an antiseptic. Leaf has been used to apply on cuts and wounds whereas root powder is being used as antiseptic [2] (Gaur,. 1999)). Some workers also reported this plant as flea repellent [3] (Sood *et al.*, 1966), as well as calcium blocking activity. It showed promising anti-malarial activity against *Plasmodium falsiparum.* [4] (Alam *et al.*, 2011). Earlier workers have investigated *B. albiflora* for its chemical composition and reported various terpenoids, alkaloids, coumarins, acridones and essential oils [5, 6] (Talpatra *et al.*, 1975; Reisch *et al*., 1990).

Reactive oxygen species (ROS) or free radicals continuously produced in our body during cellular metabolism at a constant rate that is in equilibrium with the antioxidant mechanism of our body. However, recently it has been realised that the rate of free radical generation has been increased due to stress physiology, modern stressful life style, and environmental biohazards such as tobacco smoke, radioactive substances, herbicides, pesticides, depleted ozone, ultraviolet rays, etc. [7] (Cadenas., 1997). These reactive oxygen species (ROS) causes oxidative stress, via altering the balance between themselves and antioxidant defence system of the body. Excess generation of ROS are responsible for more than hundreds of diseases including nerve disease, atherosclerosis, arthritis, ischemia and repercussion injury of many tissues, gastritis and cancer [8,9,10] (Su *et al.*, 2007; Wong *et al.*, 2006; Tepe *et al.*, 2007). Antioxidant activity is shown by major inseparable phenolic compounds of plant species acting as metal chelator, reducing agent, hydrogen donors and singlet oxygen quenchers. Recently it has been realised that synthetic antioxidant like butylated hydroxyltoluen (BHT) and butylated hydroxyanisole (BHA) used in food stuffs shown toxicity [11] (Canadanovic-Brunet *et al.*, 2006).

Quantification of major phenolics compound and the antioxidant property of plant are still remaining to investigate. The main objectives of this study were to evaluate and compare total antioxidant capacity using three common antioxidant activity methods such as free radical (DPPH) scavenging, reducing power and β-carotene bleaching. Identification and quantification of major phenolic compounds by HPTLC and determine the relationship between antioxidant activity of reported phenolic compound in plant extracts to confirm that phenolic constituents are responsible for antioxidant activity of the plants and also to explore possibilities of its usage in medicaments.

## MATERIAL AND METHODS

### Plant material

The plant samples i.e. aerial part and roots of *B. albiflora* were collected from Purola (Uttarkashi), Uttrakhand, India in the month of November, 2012. It was identified and authenticated by Dr. AKS Rawat, CSIR-NBRI, Lucknow, India. A voucher specimen has been submitted in institute's herbarium (254036).

### Chemical and reagents

Vanillic acid (purity: 98% w/w), Gallic acid (purity: 98% w/w), Ferulic acid(purity: 98%w/w), and Caffeic acid (purity : 98%w/w),(1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) were procured from Sigma-Aldrich USA, DL-α-Tocopherol acetate (TOCO), Butylated hydroxy toluene (BHT) from Laboratory Rasayan, linoleic acid, β-carotene from MP Biomedicals LLC, Tween-40 were procured from Merck. All the solvents used were of analytical grade from Rankem India.

### Extraction

The fresh plant was collected, thoroughly washed with water to remove all debris, and dried in shade. The dried material was powdered by using electric grinder at 100 mesh size. Extraction was performed by soxhlation process in two steps. Firstly, the powdered drug was defatted under soxhlet assembly using 250 mL of 98% petroleum ether for 6 h. This is followed by 9 hours soxhlation of defatted drug powder by using 250 mL of methanol and 70% hydro-alcoholic solvent separately. The final extracts obtained were passed through Whatman No. 1 filter paper. The filtrates obtained were concentrated under vacuum in a rotary evaporator (Buchi, USA) at 40 °C and stored at 4°C for further use. The crude extracts were obtained by dissolving a known amount of dry extract in 98% methanol and hydro-alcoholic (70%) solvents, respectively to obtain a stock solution of 10 mg/mL. Further dilutions of stock solutions were prepared in respective solvent for HPTLC studies.

### Physicochemical and Phytochemical Studies

Physicochemical and Phytochemical studies like extractive values, total ash, acid insoluble ash, total sugar, starch were calculated from the shade-dried and powdered (60 mesh) plant material [12,13,14] (Anonymous 2004; Anonymous 2007; K. Peach and M. V. Tracy 1955).

### Estimation of total phenol content

Total phenolic content (TPC) was determined using the slightly modified protocol developed by [15] Singh *et al* (2010). Reaction mixture was prepared by adding 100 µL (1mg/mL) plant extract, 500 µL (1 N) folin-Ciocalteau's reagents and 1000 µL sodium carbonate solution (20%). The reaction mixture was mixed properly and left at room temperature for 30 min. The volume of reaction mixture was made up 15 mL with distilled water and absorbance was measured at 725 nm. TPC was expressed as mg of gallic acid equivalent (GAE)/g extract.

**Estimation of total flavonoid content**

A method as described by [15] Singh *et al*. (2010) was used to determine total flavonoid content (TFC). Plant extract prepared in methanol (1mg/mL) was added with 0.3mL of sodium nitrite (5%) solution and mixed well. After 5 minute of interval, 0.3 mL of aluminium chloride ($AlCl_3$, 10%) solution was added. Six minutes of time interval, NaCl was added, mixed well and volume made up to 6.4 mL with distilled water. Optical density was measured at 570 nm and flavonoid content was estimated in mg of quercetin equivalent (QE)/g extract.

**In-vitro antioxidant assays**

**DPPH radical scavenging activity assay**

Antioxidant activity of test plant extract was assayed using DPPH as stable free radicals. The diluted working solution of the extract was prepared in used solvent system. Ascorbic acid was used as the standard ranging from1 to10µg/mL. The 0.002% DPPH solution was prepared in HPLC-grade methanol. 2.9 ml of this solution was mixed with different concentrations of test sample (100 µL) and kept for 20 min at room temperature to complete the reaction and optical density was measured at 517 nm. The optical density was recorded and percent of inhibition was calculated according to the formula given below [16] (Verma *et al*., 2012).

% of inhibition of DPPH activity = (A–B/A) × 100, where, A is optical density of the control and B is optical density of the sample.

**β-carotene assay**

Antioxidant activity of test sample was determined by autoxidation of β-carotene and linoleic acid coupled reaction method [15] (Singh *et al*., 2010). A stock solution of β-carotene was prepared in chloroform (3.2mg/15mL) and 3 mL of this solution was added to 100 mg of linoleic acid and 400 mg of tween-40 emulsion. The chloroform was removed by vacuum pump and 100 mL of distilled water was added to this chloroform free emulsion and mixed thoroughly. An aliquot (5 mL) was mixed with test extract and oxidation of this emulsified reaction mixture was monitored at 470 nm at zero time, incubate in water bath for 60 min at $50^0$C for further absorbance. Control contained only 5 mL aliquot of β-carotene and linoleic acid emulsion and methanol.

**Reducing power assay**

Reducing power (RP) of test samples was determined using a slightly modified ferric reducing-antioxidant power assay [17] (Singh *et al*., 2009). Briefly, plant extract (0.5–2 mg/mL; 1 mL) was mixed with 2.5 mL of phosphate buffer (0.1 M pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at $50^0$C for 20 min. About 2.5 mL of 10% (w/v) trichloro acetic acid solution (TCA) was added to terminate the reaction. The reaction mixture was centrifuged at 5000 rpm for 10 min and upper layer (2.5 mL) was diluted with equal volume of deionised water. Finally, 0.5 mL of 0.1% (w/v) $FeCl_3$ solution was added and after 10 min, the absorbance was measured at 700 nm, against a blank.

**HPTLC analysis**

Air dried powdered sample of test plant (2.0g) in triplicate were extracted separately with 3x10 mL methanol and hydro-alcoholic solvents. The extracts were concentrated under vacuum and re-dissolved in respective solvents, filtered and used for HPTLC analysis. Chromatography was performed on Merk TLC pre-coated silica gel $60GF_{254}$ (20x10 cm) plates. The extracts and standard compounds were applied to the layers as 6 mm-wide bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150nl/s from application syringe. These conditions were kept constant throughout the analysis of samples. Separation of targeted compounds were achieved in a Camag twin trough glass chamber using toluene: ethyl acetate: formic acid (5:5:1) as a mobile phase till proper separation of bands up to 8 cm height. The plate was dried with an air dryer and caffeic, ferulic, gallic and vanillic acids were simultaneously quantified using Camag TLC scanner model 3 equipped with Camag Wincats IV software.

**RESULTS**

**Physicochemical and Phytochemical Studies**

Extractive value of aerial part and root of *B. albiflora* was examined using different solvents viz. alcoholic, hydro alcoholic, and chloroform separately. The obtained results showed highest extractive values in alcohol for root and aerial part to be 16.5 and 16.2% respectively. Total ash contents of root and aerial part of *B. albiflora* were 6.85 and 15.055% whereas acid insoluble ash contents were 3.1 and 5.25% respectively. The content of chemical constituents of *B. albiflora* such as total sugar and starch were estimated. Aerial and root were found to contain 6.5 and 8.4% total sugar and 7.0 and 10% starch, respectively. Aerial part and roots contained 37.5 and 37 µg TAE/mg extract tannic acid, respectively.

**Estimation of total phenol and total flavonoid content**

TPC were found to contain (168.9 and 134.2µg GAE /mg) in alcoholic and hydro-alcoholic extracts of aerial part, while alcoholic and hydro-alcoholic extracts of roots contained (110.9 and 42.9 µg GAE /mg), respectively. Highest TFC content was recorded in alcoholic aerial extract (81.3µg/mg) and root had 42.8µg/mg. While hydro-alcoholic extract of aerial part and roots possessed 78.5 and 33.8 µg/mg respectively. Content of flavonoid was calculated in respect of quercetin equivalent (Table 1).

**Table 1. Quantitative phytochemical analysis.**

| Compounds | Amount | Equivalents | Compounds | Amount | Equivalents |
|---|---|---|---|---|---|
| Sugar(A) | 6.52% | NA | HER | 42.92 | GAE |
| Sugar (R) | 8.38% | NA | MEA | 168.9 | GAE |
| Starch (A) | 6.96% | NA | MER | 134.24 | GAE |
| Starch(R) | 10.2% | NA | (Flavonoids) HEA | 78.53 | QE |
| Tannin (A) | 37.5 | TAE | HER | 33.78 | QE |
| Tannin(R) | 36.96 | TAE | MEA | 81.27 | QE |
| (Phenolics) HEA | 110.95 | GAE | MER | 42.79 | QE |

A: Aerial part; R: Root; HEA: Hydro-alcoholic aerial extract; HER: Hydro-alcoholic root extract; MEA: Methanolic aerial extract; MER: Methanolic extract of root; TAE: Tannic acid equivalent; GAE: Gallic acid equivalent; QE: Quercetin equivalent.

**HPTLC Analysis**

**Calibration and quantification**

The calibration curve of four compounds detected was linear in the concentration range of 1-6 µg/spot and their correlation coefficient ($r^2$) 0.996, 0.999, 0.998 and 0.999 for caffeic, ferulic, gallic and vanillic acids, respectively. A good linear relationship was shown by regression analysis. Development of plate, scanning of spots and their quantification were performed as mentioned earlier. Calibration curve was also constructed (Table 2).

**Table 2 Validation data for the HPTLC method for the estimation of Vanillic acid, Gallic acid, Caffeic acid and Ferullic acid.**

| Parameters | Vanillic acid (%) | Gallic acid (%) | Caffeic acid (%) | Ferulic acid (%) |
|---|---|---|---|---|
| Rf | 0.69±0.005 | 0.48±0.01 | 0.60±0.01 | 0.68 |
| Linearity range | 200-600 ng | 200-600 ng | 200-600 ng | 200-600 ng |
| Regression via area | y=459.753+7.508*x | y=289.893+11.722*x | y=71.668+13.185*x | y=1475.082+14.425*x |
| r | 0.999 | 0.998 | 0.996 | 0.999 |
| Slope | 7.508 | 11.722 | 13.185 | 14.425 |
| Intercept | 459.753 | 289.893 | 71.668 | 1475.082 |
| LOD | 40 ng | 40 ng | 40 ng | 40 |
| LOQ | 100 ng | 100 ng | 100 ng | 100 |

The HPTLC of hydro-alcoholic extract of *B. albiflora* aerial and roots was performed with different phenolic marker compounds at six point calibration curve in which caffeic, ferulic, gallic and vanalic acids were observed and quantified. A densitogram and banding patterns obtained from extracts shows all four marker compounds (Figure 1 a & b).

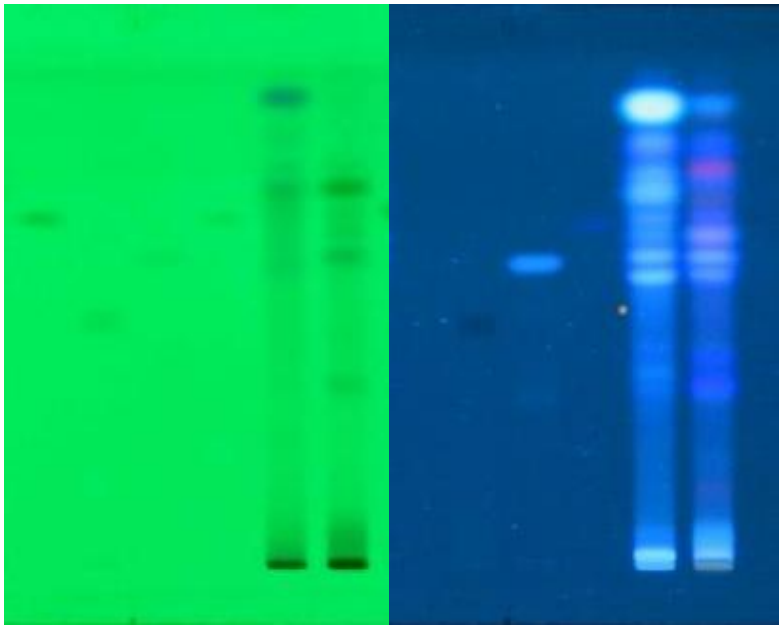


**Figure 1 (a) HPTLC profile of *B. albiflora* methanol fraction with standard Vanillic acid, Gallic acid, Caffeic acid &Ferulic acid at 254 & 366 nm**

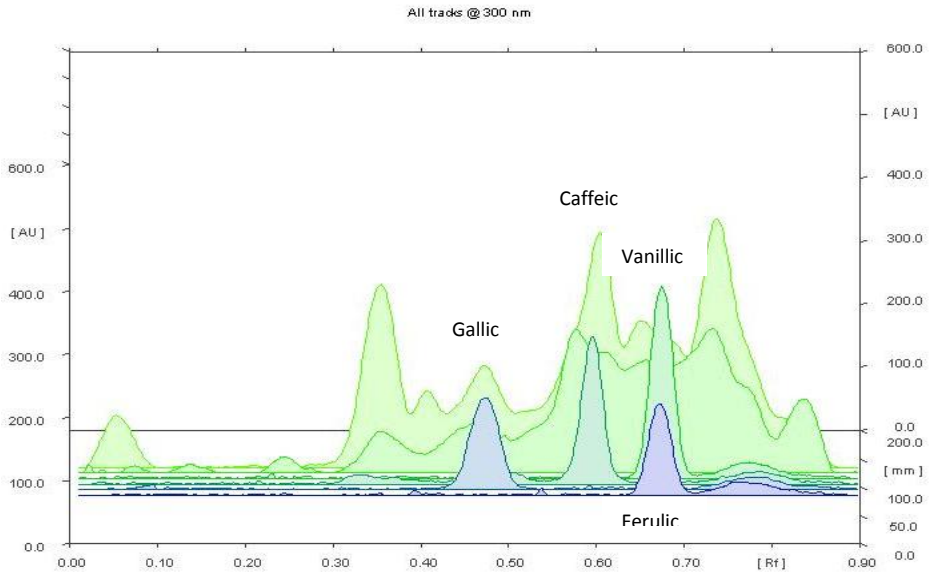


**Figure 1(b) HPTLC densitometric chromatogram of Vanillic acid, Gallic acid, Caffeic acid &Ferulic acid in *B. albiflora* methanolic fractions and standard.**

Caffeic acid was estimated upto 0.007%, ferulic acid 0.012%, gallic acid 0.02%, and vanalic acid 0.004% in methanolic extract of *B. albiflora* roots. Whilst the content of these marker compounds were also estimated in methanolic extract of aerial part which were caffeic acid 0.05%, ferulic acid 0.03%, gallic acid 0.04%,and vanalic acid 0.02% (Table 3).

**Table 3 HPTLC quantification of secondary metabolites in *B. arbiflora* root and aerial samples.**

| Sample | Vanillic acid (%) | Gallic acid (%) | Caffeic acid (%) | Ferulic acid (%) |
|---|---|---|---|---|
| *B. albiflora* root | 0.004 | 0.02 | 0.007 | 0.012 |
| *B. albiflora* aerial | 0.02 | 0.04 | 0.05 | 0.03 |

**Limit of detection (LOD) and quantification (LOQ)**

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted six times and the signal-to-noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of caffeic, ferulic, gallic and vanillic acids until the average responses were approximately 3 or 10 times of the responses for six replicate determinations (Table 2).

**DPPH scavenging activity of *B. albiflora***

DPPH radicals were recorded to scavenge by $H^+$ ions of methanolic and hydro-alcoholic extracts of *B. albiflora*. Percentage inhibitory activity of DPPH by 50% ($IC_{50}$) was used as parameter to measure antioxidant activity. Dose dependant inhibition of DPPH by methanolic and hydro-alcoholic extracts and their different phenolic compounds screened. Hydro-alcoholic extract of aerial part showed an effective scavenging of DPPH radicals ($IC_{50}$ 195.9µg/mL) followed by its methanolic extract ($IC_{50}$243.8 µg/mL). Hydro-alcoholic and methanolic extracts of roots exhibited 50% inhibition for DPPH radicals at 370and 600 µg/mL, respectively (Fig.2).

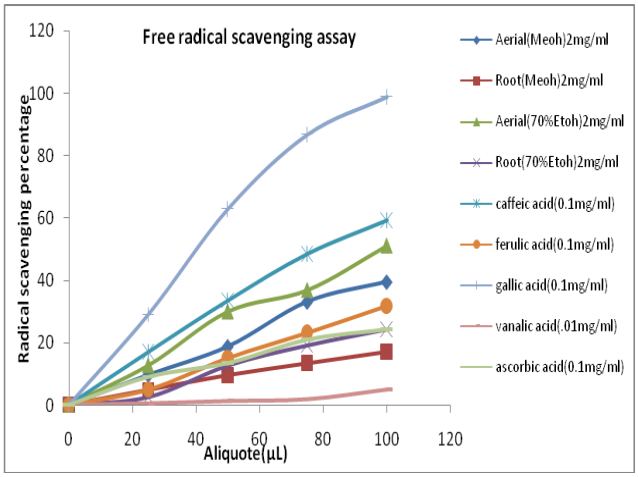


**Figure 2 DPPH radical scavenging rate (%) of different concentrations of methanolic and hydro-alcoholic extracts of *B. albiflora* along with pure tested compounds.**

These results indicate that the hydro-alcoholic extract of *B. albiflora* contains free radical quenchers more than the alcoholic extract alone.

**β-carotene bleaching assay**

Colour degradation of β-carotene, linoleic acid and tween-40 generated emulsions through oxygen free radical produced from reactive oxygen species of oxygenated water which leads to discoloration of β-carotene is the principle of working for this assay. Antioxidants mostly phenolic compounds present in plants inhibit this discoloration. $IC_{50}$ value was determined to know the activity of plant extract compared with butylated hydroxyl toluene (BHT), a reference antioxidant. Results obtained from this study concluded the bleaching inhibition efficiency in terms of antioxidant activity, was maximum in methanolic extract of root. Potency of bleaching inhibition of β-carotene decreases for methanolic extracts of root ($IC_{50}$ 307.2µg/mL) and aerial ($IC_{50}$ 348.6µg/mL) and hydro-alcoholic extracts of aerial ($IC_{50}$ 449.2 µg/mL) and root ($IC_{50}$ 755.4µg/mL) (Fig.3).

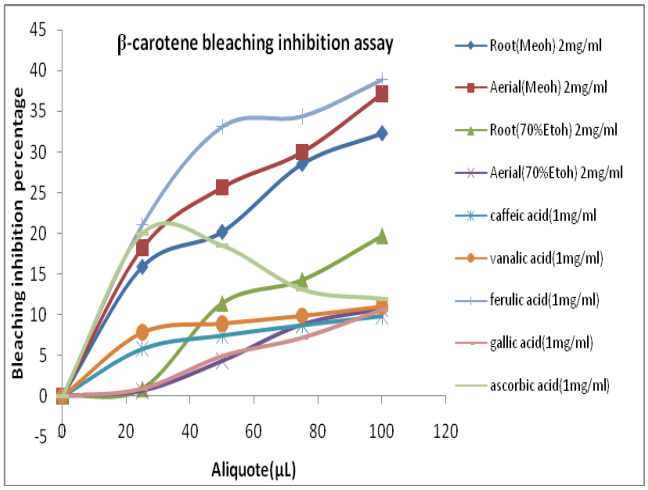


**Figure 3 β-carotene bleaching inhibition rate (%) of various concentration of methanolic and hydro-alcoholic extracts of *B. albiflora* along with pure tested compounds.**

**Reducing power assay**

This method is absorbance based, here the reaction mixture was observed by UV to find the antioxidant activity of plant extract. Alcoholic and hydro-alcoholic extracts were used to examine their reducing power. Hydro-alcoholic extract showed highest activity followed by aerial alcoholic and minimum activity was performed by hydro-alcoholic extract of roots (Fig.4).

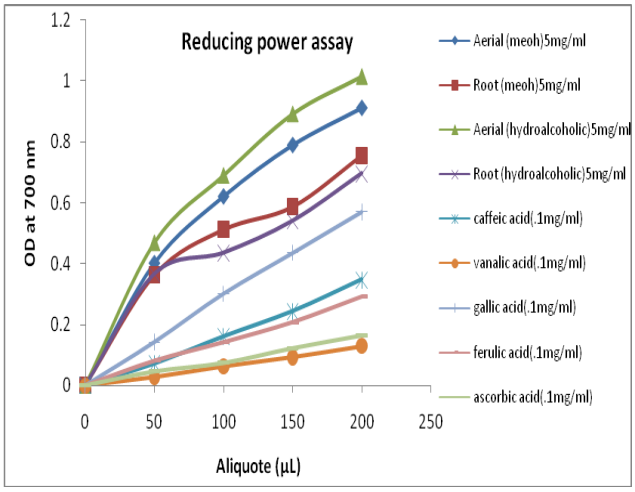


**Figure 4 Ferric reducing ability of different concentrations of *B. albiflora* methanolic and hydro-alcoholic extracts together with tested pure compounds.**

Value was calculated in gallic acid equivalent (GAE) showed 3.2, 2.81, 2.53, and 2.56 µg GAE/mL for aerial hydro-alcoholic extract, aerial alcoholic, root methanolic and root hydro-alcoholic extract respectively. Whereas reducing power assay for identified phenolic compounds such as ferulic, caffeic, and vanillic acids showed 0.081, 0.072, 0.046 and 0.028 µg GAE /mL reducing power, respectively.

**DISCUSSION**

Total ash and Acid insoluble ash contents are useful parameter to designate the quality as well as the purity of herbal medicine. Phenolic compounds are present in significant amount contributes to the antioxidant properties and usefulness of plant in herbal medicine. Radical scavenging activity of *B. albiflora* extracts increases with increasing concentration. 50% ($IC_{50}$) inhibition was observed by 195.94 µg/mL aerial hydro-alcoholic (HEA) extract showed maximum activity in hydro alcoholic extract in comparison with HER (370.75µg/mL) while methanolic extract of aerial part showed 50 % inhibition at (243.85µg/ml) and root (600µg/mL). β-carotene bleaching assay is used to screen antioxidants based on bleaching inhibition of β-carotene caused by reactive oxygen species (ROS), produced from oxidation of linoleic acid. Antioxidants decrease the discoloration of β-carotene which is measured at 470 nm. Inhibition of discoloration increases in accordance with increase in extract concentration, 50% inhibition ($IC_{50}$) was observed at concentration, 307.2µg/mL in MER. MEA and HEA both extract are comparatively less potent. Colour retention totally depends upon the antioxidant potential of plant extract is higher in methanolic extract, concluded the solubility of compounds in methanolic extract responsible for antioxidant activity. Reducing power of extract was measured via reduction of $Fe^{3+}$/ferric

cyanide complex to $Fe^{2+,}$ done by reducing agents (antioxidants) presents in plant extracts. Green colour formed, which darken with increase in concentration of plant extract confer the formation of more $Fe^{2+}$**,** responsible for the colour generation. Hydro-alcoholic extract of aerial part reduces maximum $Fe^{3+}$ (OD, 1.015) using 200 µL sample solution (5mg/mL) succeeded by aerial methanolic extract (OD, 0.912) at same volume and concentration, while root extract in both solvents reported weak. Activity was reported in Gallic acid equivalent (GAE). A comparative data of reducing power (GAE) of pure compounds reported in *B. albiflora* estimated and presented in Figure 3. HPTLC studies of plant extract showed the presence of major phenolic compounds viz. caffeic acid, ferulic acid, vanillic acid and gallic acid which may be responsible for antioxidant activity [18,19] (Rakesh et al., 2010; Kikuzaki et al., 2002). The milder effect may be due to crude nature of extract as compared to pure compounds [20] (Parimala1 et al 2013).The activity somewhat different in different solvents and in different part of plant material showed the importance of extraction and solubility of bioactive compounds in different solvents. The potential scavenging and bleaching ability of phenolics is attributed to the number and position of hydrogen donating hydroxyl groups on the aromatic ring of phenolic molecules [21] (Patt et al., 1990).

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**Abbreviation**

VA = Vanillic acid
GA = Gallic acid
CA = Caffeic acid
FA = Ferulic acid
BgaR = Root of Boenninghausenia albiflora
BgaA = Aerial root of Boenninghausenia albiflora

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