Jyoti Vandana Mani et al /J. Pharm. Sci. & Res. Vol. 9(11), 2017, 2033-2041

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Quantitative Study of Total Phenolic Content and *in-vitro* Antioxidant Activity of Aerial Part Extracts of *Plantago major* L.

***Jyoti Vandana Mani[1], Ashok Kumar Gupta[2] and Alok Mukerjee[3]**

[1]*Assistant Professor, Department of Chemistry, SHUATS, Allahabad, Uttar Pradesh, India*
[2] *Professor and Head, Department of Forensic Science, SHUATS, Allahabad, Uttar Pradesh, India*
[3]*Professor and Principal, Department of Pharmacology, United Institute of Pharmacy, Allahabad, Uttar Pradesh, India*

Abstract

Objective

In the modern times the medicinal plants are gaining attention as sources of biologically active substances including antioxidants. The present study investigates the total phenolic content quantitatively and *in-vitro* antioxidant activity of aerial part extracts of *Plantago major* L. by four different antioxidant activity methods

Methods

In this study, *Plantago major* aerial part extracts were screened for presence of bioactive phytochemical constituents, total phenolic content and *in vitro* antioxidant activity. TPC was evaluated by Folin-Ciocalteau assay and *in-vitro* antioxidant activity was conducted using 2,2-diphenyl-1-picrylhydrazyl free radical scavenging, nitric oxide scavenging, hydrogen peroxide radical scavenging and reducing power methods.

Results

Phytochemical screening studies revealed the carbohydrates, tannins, steroids, flavonoids, terpenoids, glycosides, fats and oils, alkaloids, phenols and iridoid glycosides were the main phytochemicals present in aerial part extracts of *P. major*. A significant positive correlation was found between antioxidant activity and TPC in *P. major* aerial part extracts. The *Plantago major* extracts showed concentration dependent radical scavenging and reducing power activities. Among all the extracts methanolic extract showed greater DPPH radical scavenging activity (IC50=139.19µg/ml), NO radical scavenging activity (IC50=124.16 µg/ml), H_2O_2 radical scavenging activity (IC50=148.20µg/ml) and reducing power (0.643 absorbance at 800µg/ml) as well as highest TPC (34.41mgTAE/g of dry weight). BHT used as a standard antioxidant. The order of activity is in the following manner: BHT> methanol extract > ethanol extract> chloroform extract > benzene extract. The antioxidant activity of the methanol extract is close in magnitude and comparable to that of standard antioxidant compound.

Conclusion

The results of present study concluded that among all the extracts, methanolic extract is a rich source of phytochemicals and exhibits highest amount of phenolic compounds and significant antioxidant activity. Therefore *P. major* can be a potential source of natural antioxidant compounds and hence used to prevent diseases related to oxidative processes.

Keywords: *Plantago major*; total phenolic content; 2,2-diphenyl-1-picrylhydrazyl; nitric oxide; hydrogen peroxide; reducing power.

1. INTRODUCTION

There is an improved and increased interest in plants secondary metabolites like polyphenols, because of their therapeutic effects. Polyphenols or phenolic compounds form a large group of secondary compounds including phenolic acids, carotenoids, flavanoids, tannins, tocopherols, flavones glycosides etc. These, phenolic compounds have the property of quenching oxygen-derived free radicals by donating a hydrogen atom or an electron [1]. Phenolic compounds are responsible for antioxidant property. Antioxidants are classified into two major categories, natural and synthetic antioxidant. Plants are prospective source of natural antioxidants. The natural antioxidants are safer and environment friendly than synthetic antioxidants. Plants are a potential source of natural antioxidants which are secondary metabolites of plant, that exhibit a wide range of biological effects like antibacterial, anti-inflammatory, anti-allergic, antihepatotoxic and antithrombotic activities and prevention of cardiovascular diseases. Several synthetic antioxidants, like BHA and BHT, are commercially available, used in foods and have many side effects such as mutagenesis and carcinogenic in human beings [2].

Antioxidants are capable of blocking the effect of the Reactive Oxygen Species (ROS). In living organisms, the imbalance in the production and detoxification of free radicals by the biological system causes oxidative stress. Free radicals are generated by different types of exogenous chemicals and a number of endogenous metabolic processes oxidize the bio molecules leading to cell death and tissue damage. The organism must keep free radicals at relatively low concentrations using different defence systems and antioxidant molecules [3].

Production of high amount of reactive oxygen species overcomes inbuilt antioxidant system and damages the cells, tissues and organs and hence, there is a need to develop new drugs from traditional medicine to protect and support the biological system to avoid serious disorders of liver, cardiac and cancer diseases etc [4].

Medicinal plants occur naturally all over the world and are used in many countries. Either people use them in the form of traditional preparations as pure active forms because of

their known therapeutic qualities and to avoid the side effects of the prescribed medicine. One such plant is *Plantago major*, it is commonly known as broad leaf plantain, greater plantain, plantain, snake weed etc., it is an old medicinal plant known for centuries [5],[6]. *P. major* belongs to the genus Plantago, family Plantaginaceae. *Plantago major L.* is a small perennial plant with height about 15 cm, but the size varies depending on the growth habitats [7] and found effective to cure chronic constipation, digestive disorders, diarrhoea, piles and alleviated problems of kidney, bladder and haemorrhoids. Scientific studies have shown that plantain extract has wide range of biological effects, including wound healing activity [8]-[10], anti-inflammatory and analgesic [11], antioxidant [12], immuno modulating [13], antiviral, antileukemia and anticarcinogenic activity [14],[15]. These medicinal properties of *P. major* due to the presence of biologically active chemical compounds such as polysaccharides, lipids, caffeic acid derivatives, flavonoids, iridoid glycosides, terpenoids, alkaloids and some organic acids [5],[16]. Some studies reported that t he leaves of *P. major* are used for its wound healing property, anti-inflammatory, antioxidant activity, immunoenhancing property[5],[13] and its seeds are used to treat various gastrointestinal disorders. The basic aim of the research was to determine the total phenolic content and *in-vitro* antioxidant activity of aerial part extracts of *P. major* using spectrophotometric methods.

2. MATERIALS AND METHODS

2.1. Collection and Identification of plant material

The plant material was collected from Forest Research Institute (FRI), Dehradun. It was identified and authenticated by Dr. Veena Chandra, Head, Botany Division, FRI, Dehradun, Uttarakhand, India. A voucher specimen has been preserved in laboratory for future reference. The accession number is 164439A/B/C.

2.2. Preparation of Plant Material

The collected plant material was washed thoroughly in water, chopped, air dried at room temperature under shade for two weeks and pulverized in laboratory electric grinder. The powdered plant material was kept in air tight containers for further use.

2.3. Preparation of Plant Extracts

Extraction yield of total phenolic compounds from plant materials are mainly depends on different extraction methods. Besides, the difference in polarities of extracting solvents might influence the solubility of chemical constituents in a sample and its extraction yield. Therefore, the selection of an appropriate solvent system and extraction method is one of the most relevant steps to determine the phenolic and other compounds from a sample. In this study different solvent extracts were prepared by successive hot continuous extraction method. The dried and powdered plant material was weighed and then successively extracted in benzene, chloroform, ethanol and methanol by soxhlet extraction method. Separately the powdered plant material was extracted with water by decoction method. The extraction solvents were selected on the basis of increasing polarity. The obtained extracts were filtered separately and each extract was concentrated by distilling off the solvent and then evaporating it to dry by placing it on a porcelain dishes on electric water bath. The percentage yield of all the extracts was calculated. Then the extracts were subjected to phytochemical screening, total phenolic content and *in-vitro* antioxidant studies.

2.4. Preliminary Phytochemical Screening

The preliminary phytochemcial screening for bioactive compounds was carried out by the standard methods [17]-[19].The phytochemical examinations were performed on the liquid and dried extracts. The preliminary phytochemical screening of *P. major* aerial part extracts was carried out for the presence of steroids, tannins, flavonoids, saponins, alkaloids, carbohydrates, glycosides, proteins, fats and oils etc.

2.5. Estimation of Total Phenolic Content

2.5.1. *Reagents prepared for estimation of total phenolic content:*

i. Folin-Ciocalteu reagent (1N): The commercially available FC reagent was diluted with an equal volume of distilled water and then transferred it in brown bottle and stored in refrigerator at 4^{o}C.

ii. Sodium Carbonate (20%)

iii. Standard Tannic acid solution (0.1 mg/ml)

2.5.2. *Procedure for estimation of total phenolic content*

The concentration of phenolic compounds in different solvent extracts was measured spectrophotometrically using Folin-Ciocalteu reagent method as described by Makkar *et al*., [20].The method for total phenolic content is useful in order to know the efficiency of extraction of phenolics in solvents. The tannic acid was used as standard antioxidant compound and the concentration of total phenolic compounds in the each extract, determined as mg tannic acid equivalents per gram dry extract, was calculated by using the standard curve ($R^2 = 0.990$) as shown in figure 1. The stock solution (1 mg/mL) of each extract was prepared. Suitable aliquots of extracts in different conc. (0.02, 0.05 and 0.1 ml) were taken in test tubes to make up the volume to 1.0 ml with distilled water and 0.5 ml of FC reagent (50%) was added followed by the addition of 2.5 ml of sodium carbonate (20%) solution . The test tubes were vortexed before incubated in the dark for 40 min. Absorbance was read at 725 nm. The same procedure was repeated for the standard solution of tannic acid and the calibration curve was constructed (fig 1). The amount of total phenols as tannic acid equivalent from the calibration curve was calculated and expressed as mg per gram tannic acid equivalent using the following formula: $C=cV/M$ Where C is total content of phenolic compounds in mg/g TAE, c is the concentration of tannic acid established from the calibration curve, V is volume of the extract and M is the weight of extract. All the determinations were performed in triplicate. The FC reagent being sensitive to reducing compounds including polyphenols produced a blue colour upon reaction which was measured spectrophotometrically.

Table 1: Absorbance recorded at 725 nm of tannic acid (different conc.) for calibration curve

Tube	Tannic Acid Sol. (0.1mg/ml) Ml	Tannic Acid (µg)	Distilled water (ml)	FC reagent (ml)	Sodium Carbonate Sol. (ml)	Absorbance at 725 nm
Blank	0.00	0.0	1.00	0.5	2.5	0.0
1.	0.10	10	0.90	0.5	2.5	0.093
2.	0.20	20	0.80	0.5	2.5	0.104
3.	0.40	40	0.60	0.5	2.5	0.177
4.	0.60	60	0.40	0.5	2.5	0.289
5.	0.80	80	0.20	0.5	2.5	0.353
6.	1.00	100	0.00	0.5	2.5	0.438

2.5.3. *Preparation of Standard Calibration Curve*

Tannic acid was used as standard phenolic compound to calculate the amount of total phenols as tannic acid equivalent. 100µg/ml of tannic acid stock solution was prepared. From the stock solution 0.1 to 1.0 ml of aliquots were pipette out into test tubes and make up the volume to 1.0 ml with distilled water. The above same procedure was repeated for the standard solution of tannic acid (Table1) and calibration curve of absorbance vs. concentration was plotted (fig 1).

2.6. **Determination of *in - vitro* Antioxidant Activity**

Antioxidant activity should not be concluded based on a single antioxidant test model. In practice several *in vitro* test procedures are carried out for evaluating antioxidant activities. For present investigation four different methods such DPPH, nitric oxide, hydrogen peroxide scavenging and reducing power method were used to evaluate the antioxidant activity of *P. major* aerial part extracts.

2.6.1. *DPPH Free Radical Scavenging Activity Assay*

Most popularly used 1, 1- dipenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl; DPPH) is a stable radical that has been widely used to evaluate the antioxidant activity of various natural products [21] The DPPH free-radical scavenging activity of the extracts was determined according to the method of Ogunlana and Ogunlana [22]. The hydrogen atom or electron donating activity of the different extract was measured from the bleaching of the purple coloured methanol solution of DPPH. DPPH (1mM) solution was freshly prepared in methanol it was protected from light by covering the test tubes with aluminium foil and kept in the dark at 4°C. The different concentrations 10 to 400 µg/ml (1 mg/ml) of each extract of *P. major* was taken in test tubes and diluted to 4 ml with distilled water. Then 1 ml, 1 mM DPPH was added to each test tube, shaken and left to stand at room temperature for 30 minutes. DPPH was reduced from deep violet to light yellow after 30 minutes of incubation in dark at room temperature. The test was carried out in triplicate and the absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The DPPH solution was used as control and methanol was used as blank. The effect of BHT on DPPH was also assessed for comparison with that of plant extract BHT was used as reference standard and same procedure as in DPPH scavenging experiment was followed for BHT. The percent of scavenging activity (% inhibition) of different extracts of *P. major* and BHT was calculated by the following formula:

$$\text{Scavenging Activity (\%)} = \frac{(A_C - A_S)}{A_C} \times 100$$

Where, A_C is the absorbance of the control reaction and A_s is the absorbance of the sample. The IC50 value was calculated graphically which was the concentration required to inhibit DPPH radical by 50%.

2.6.2. *Nitric Oxide Radical Scavenging Assay*

The aqueous solution of sodium nitroprusside at physiological pH spontaneously generates nitric oxide [23]. Such nitric oxide reacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. The nitric oxide scavengers compete with oxygen leading to decrease formation of nitric oxide. The nitric oxide (NO) radical scavenging activity was determined according to the method reported by Sreejayan and Rao [24]. The different concentrations 10 to 400 µg/ml of *P. major* extracts (1mg/ml) and standard BHT (1mg/ml) was taken in test tubes in triplicates. 3 ml of sodium nitroprusside (10 mM) in 0.2 M phosphate buffer saline (pH7.4) was added in test tubes. All the test tubes were incubated at room temperature for 150 min. From the incubated mixture 1.0 ml was taken out and added into 3 ml of Griess reagent (1% sulphanilamide, and 0.1 % N-1-naphthyl ethylene diamine dihydrocholoride in 2% phosphoric acid) to all the test tubes and incubated at room temperature for 30 min. The absorbance of the chromophore was read at 546 nm against the blank and compared with standard BHT. The same reaction mixture without the extract sample was used as negative control and the test tube with phosphate buffered saline used as blank. The percentage radical scavenging activity of the samples (extracts and standard) was calculated as follows:

$$\text{\% Scavenging Activity} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \times 100$$

The IC50 value was calculated.

2.6.3. *Hydrogen Peroxide Radical Scavenging Assay*

The ability of plant extracts to scavenge the hydrogen peroxide can be estimated according to the method of Ruch *et al*. [25]. 40mM H_2O_2 solution was prepared in phosphate - buffer saline (0.1M, pH 7.4). The concentration of H_2O_2 was determined spectrophotometrically at 230 nm. 10 - 450µg/ml plant extract of 1 mg/ml plant extract stock solution were taken in different test tubes and was diluted in 4ml distilled water. 0.6 ml hydrogen peroxide PBS

solution was added. Absorbance of H_2O_2 was read at 230 nm was determined after 10 minutes against a blank solution containing the phosphate buffer without H_2O_2. BHT was used as a positive control compound and same H_2O_2 scavenging experiment procedure was followed for BHT (1 mg/ml). Absorbance was determined 10 minutes later against a blank solution similar to that above. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using following equation.

% Scavenging (H_2O_2) Activity

$$= \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extracts or standard. The tests were carried out in triplicate. The IC_{50} value was calculated.

2.6.4. *Reducing Power Assay*

In this method, antioxidant molecules forms a coloured complex with $K_3Fe(CN)_6$, $FeCl_3$ and CCl_3COOH which is measured at 700nm. Increase in absorbance of the reaction mixture shows the reducing power of the samples [26]. The ferric reducing power of the extracts was determined according to the method described by Oyaizu [27].The different concentrations 10 to 800µg/ml of plant extracts (1mg/ml) and standard (BHT) was taken in different test tubes was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50^{o}C in a water bath for 20 min. After incubation, the reaction was stopped by adding 0.75 ml of trichloroacetic acid (10%) solution and then centrifuged at 3000 rpm for 10 min. Then 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power and an increase in the antioxidant activity. BHT was used as standard.

2.6.5. *Calculation of 50% Inhibition Concentration (IC_{50})*

The optical density obtained with each concentration of the extract/standard was plotted taking concentration on X-axis and percentage inhibition on Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ standard [28].

2.7. **Statistical analysis**

All experimental work were performed in triplicates. The results were reported as mean± standard deviation (SD). Calibration curve was obtained by plotting percentage inhibition against standard concentration. The IC50 value was calculated from linear regression analysis using Microsoft excel.

3. RESULTS

The phytochemicals from plants can be obtained from different steps. These steps are milling, grinding, homogenization and extraction. Among these steps, extraction is the most important step for obtaining and separating the bioactive compounds from plant materials. The efficacy of extraction is affected by the following conditions such as chemical nature of bioactive compounds, extraction method used, sample particle size, solvent type and the presence of interfering substances. The percentage yield of extraction depends on type of solvent, pH value, temperature and extraction time. Among these parameters extraction solvent and composition of sample are most important [29]. In this study, *P. major* aerial part extracts (benzene, chloroform, ethanol and methanol extracts) were obtained by hot continuous extraction method and aqueous extract was obtained by decoction method. There is an increasing interest in phytochemicals, present in *P. major* because of their potential use in food products and medicines. It has various phytochemicals in its leaves, seeds, stem and roots, which actually have medicinal properties and can also be used as taxonomic markers [5].The preliminary phytochemical screening was done to identify the secondary metabolites present in the aerial part extracts of *P. major*. The chemical tests were carried out for bioactive compounds by the standard methods with slight modifications as described by Trease and Evans, [17]; Sofowora,[18]; Harborne,[19].The phytochemical screening of each tested extract revealed the presence of carbohydrates, tannins, steroids, flavonoids, terpenoids, glycosides, fats and oils, alkaloids, phenols and iridoid glycosides. The maximum phytochemicals were recorded in methanolic extract as compared to other extracts. The percentage yield of each extract was also calculated. The percentage yield of various solvent extracts decreased in the following order: water >methanol >ethanol > benzene > chloroform. The different yields of extracts might be influenced by the polarities of solvents. The percentage extraction yield with water and methanol was highest than other solvents. This shows that the extraction yield increases with increasing polarity of the solvent.

3.1 **Total Phenolic Content**

The phenolic compounds are commonly found in all plants but every plant contains different phenolic combinations and characterization of these compounds is often difficult. Therefore, fast evaluation methods such as spectrophotometric method are commonly used for estimation of total phenolic content or content of particular class of phenolic compounds. Plant extracts are classified as rich in phenolic compounds when their TPC (expressed as GAE) is more than 20 mg/g [30].

The TPC was calculated with the help of the standard curve as shown in figure 1 and from the regression equation: $y=0.004x+0.035$ with $R^2=0.990$ and expressed as mg per gram tannic acid equivalent. The TPC of the aerial part extracts of *P. major* ranged from 2.33 to 34.41mg TAE/g of dry weight (Figure 2). The quantitative screening results showed that the methanolic extract of *P. major* possessed the highest amount of phenolic compounds (34.41mgTAE/g of dry weight) when compared with benzene, chloroform, ethanol and aqueous extracts as shown in the Figure 2. The amount of phenolic compounds in the extracts is depends on the polarity of extraction solvent used. The phenolic compounds are polar in nature therefore they have high solubility in polar solvents, as a result high concentration of these compounds are in polar solvents [31]. The order of extracts (obtained by successive hot continuous extraction method) on the basis of

increasing phenolic content are benzene < chloroform < ethanol < methanol. The recovery, yield and types of phenolics compounds in an extract are influenced by the polarity of extraction solvents [32].

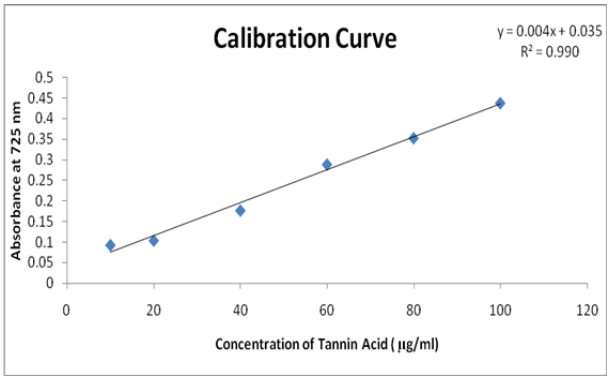

Fig.1: Tannic acid calibration curve

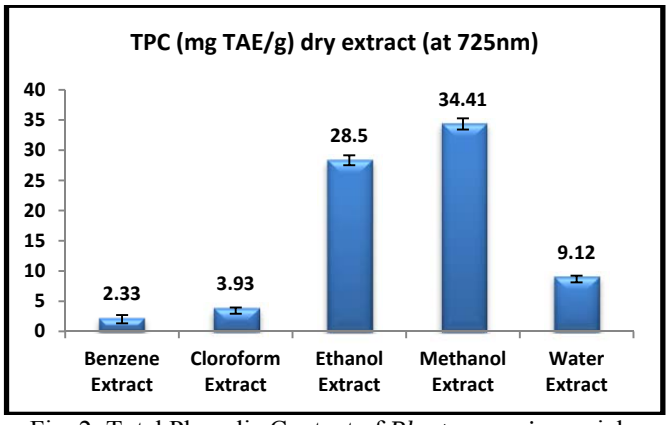

Fig. 2: Total Phenolic Content of *Plantago major* aerial part extracts

Each value shown in the Figure 2 was obtained by calculating the average of three experiments±SD.

3.2. ***In vitro* - antioxidant activity**

In the present study different solvent extracts of aerial part of *P. major* were investigated for antioxidant activity by using four different methods. The results of present showed that all the extracts exhibited antioxidant activity. Among all the extracts, methanolic extract showed significant antioxidant activity then the other extracts.

3.2.1. *DPPH free radical scavenging activity assay*

The DPPH free radical scavenging activity results of *P. major* aerial part extracts and BHT (standard antioxidant) are shown in figure 3.

The effect of antioxidant molecules on DPPH radical scavenging was due to their hydrogen donating ability. The antioxidant activity of plant extracts were calculated according to the percentage inhibition in DPPH assay. BHT was used as the standard antioxidant compound. The DPPH assay results of extracts in figure 3 shows that the methanol extract have better DPPH radical scavenging activity 80.67% at 400µg/ml as compared to ethanol (67.53%), chloroform (51.14%) and benzene (30.29%) extracts at same concentration. The DPPH free radical scavenging activity of methanol extract was compared with BHT (90.12%) at 400µg/ml. The qualitative and quantitative phytochemical screening results have confirmed the presence of polyphenolic compounds, which might be responsible for this activity. The free radical scavenging activity of all tested extracts is concentration dependent, if concentration increases DPPH radical scavenging activity also increases as shown in the figure 3. The IC50 values of polar and nonpolar extracts as given in the table 2 shows the lowest value of IC50 (139.19 µg/ml) for methanol extract which means lower the IC50 value, higher the antioxidant activity whereas ethanol (190.36 µg/ml), chloroform (341.80µg/ml) and benzene(691.93 µg/ml) extracts showed low antioxidant activity as their IC50 value were higher. The BHT has the lowest IC50 (99.95µg/ml). The comparison of IC50 values among extracts provides more understanding about the antioxidant potential of *P. major* extracts.

3.2.2. *Nitric Oxide Radical Scavenging Assay*

Nitric oxide radical scavenging activity results of aerial part extracts of *P. major* and BHT are shown in figure 4.

Nitric oxide radical scavenging activity of tested extracts was carried out at concentration of 10 to 400µg/ml. BHT was used as the standard antioxidant compound. The medicinal plants showed antioxidant activity through competing with oxygen to scavenge the nitrite radical. The percentage radical scavenging results shown in figure 4 reveal that nitric oxide radical scavenging activity increased with an increase in concentration of the tested extracts. Among all the tested extracts methanolic extract was the most potent nitric oxide radical scavenger. The scavenging activity of methanolic extract was 80.86% at 400µg/ml concentration as compared to ethanol (68.26%) chloroform (30.65%) and benzene (20.10%) extracts at same concentration. This activity of methanol extract was compared with BHT (98.69%) at 400µg/ml. The IC50 values of each examined extracts as shown in the table 2 shows the value in increasing order of IC50, methanol (124.16µg/ml), ethanol (201.38µg/ml), chloroform (728.26µg/ml) and benzene (1026.09µg/ml) extracts. The BHT has the lowest IC50 (76.15µg/ml). It is reported that lower the IC50 higher the antioxidant potential of the extracts [12].

3.2.3. *Hydrogen Peroxide Scavenging Activity Assay*

The antioxidant activity of plant extracts was calculated according to the percentage inhibition in H_2O_2 assay. The H_2O_2 scavenging activity results of aerial part extracts of *P. major* are shown in figure 5. The results obtained shows that methanolic extract have better H_2O_2 scavenging activity (83.27%) followed by ethanol (71.05%), chloroform (43.23%) and benzene (32.71%) extracts at 400µg/ml concentration. The scavenging activity of each extract was compared with BHT. The thorough study of chemical nature of the bioactive compound(s) is responsible for antioxidant activity which is still unknown. But preliminary phytochemical screening has confirmed the presence of polyphenolic compounds, which might be responsible for this activity. The H_2O_2 scavenging activity of extracts is concentration dependent, concentration increases activity also increases as shown in the figure 5. The IC50 value of each extract is given in the table 2 shows

the value in increasing order of IC50, methanol (148.20µg/ml), ethanol (198.16µg/ml), chloroform (450.10µg/ml) and benzene (622.66µg/ml) extracts. The BHT has the lowest IC50 (108.90µg/ml). It is reported that lower the IC50 higher the antioxidant potential of the extracts.

3.2.4 *Reducing Power Assay*

In this method, reducing ability of the tested extracts was measured by change of Fe^{3+} to Fe^{2+} form. Among all the extracts methanolic extract had high absorbance values than ethanol, chloroform and benzene extracts as shown in figure 6, this indicates that greater reducing and electron donor ability of methanol extract for breaking chain reaction and giving stable free radicals. Reducing power of all tested extracts and BHT (standard antioxidant) with respect to their absorbance values are shown in figure 6. The maximum absorbance of methanolic extract at 800µg/ml is comparable with BHT at 500µg/ml.

The *in-vitro* antioxidant results revealed that methanolic extract possessed maximum antioxidant potential while benzene extract showed least antioxidant activity in all the assays. When antioxidant activity of methanolic extract was compared with standard BHT, it was found that methanolic extract has lower antioxidant activity than the BHT.

The phytochemical compounds are capable of protecting the human being from the harmful effect of free radicals damage, which causes the oxidative stress. In the present study, the antioxidant potency of examined extracts were evaluated by four different methods, namely DPPH radical scavenging assay, NO scavenging assay, H_2O_2 scavenging assay and FRAP assay. IC50 of DPPH, NO and H_2O_2 scavenging activities of each examined extracts was calculated and compared to IC50 of BHT as shown in table 2.

Table 2: Antioxidant activity of aerial part extracts of *Plantago major*

Extracts	DPPH (IC50) µg/ml	NO (IC50) µg/ml	H_2O_2 (IC50) µg/ml
Benzene	691.93	1026.09	622.66
Chloroform	341.80	728.26	450.10
Ethanol	190.36	201.38	198.16
Methanol	139.19	124.16	148.20
Gallic acid	99.9	76.15	108.90

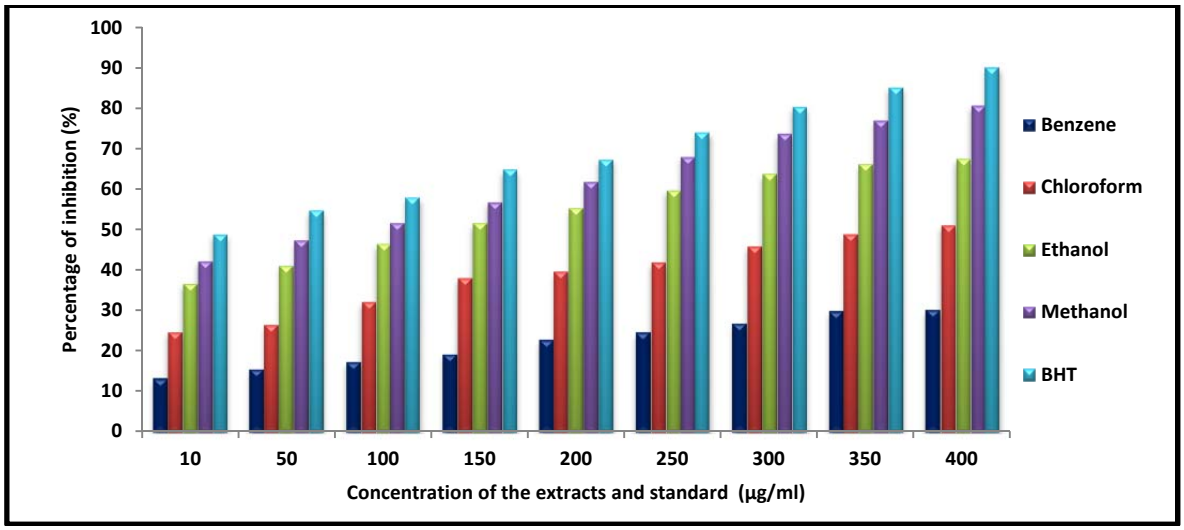

Figure 3: DPPH free radical scavenging activity of aerial part extracts of *p. major* and BHT

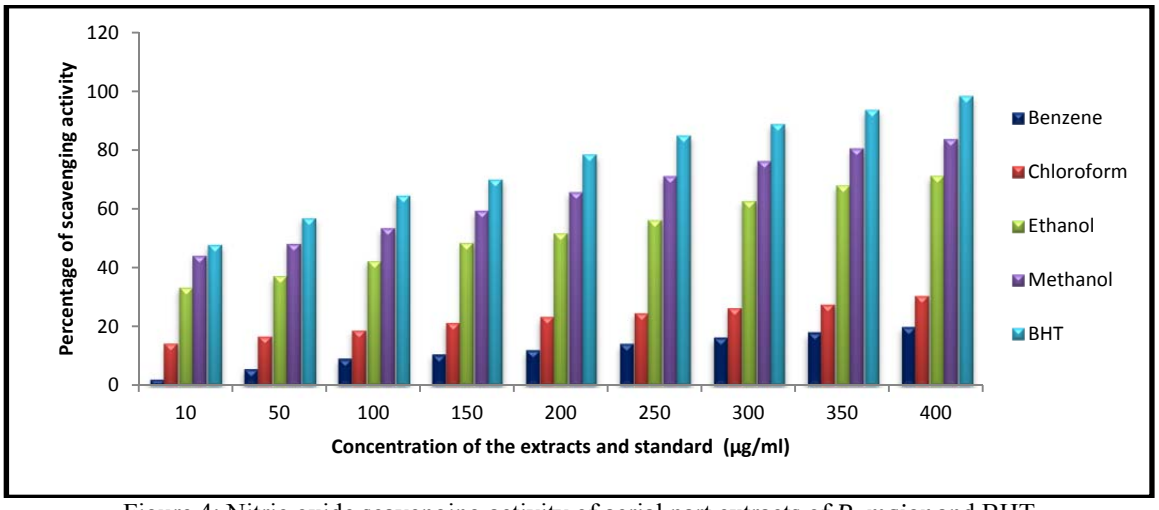

Figure 4: Nitric oxide scavenging activity of aerial part extracts of *P. major* and BHT

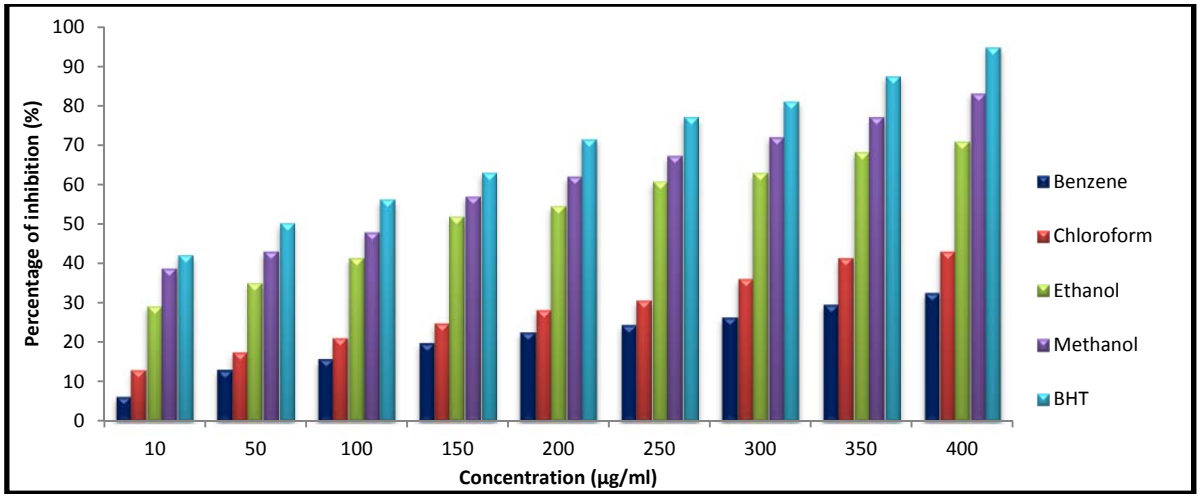

Figure 5: The hydrogen peroxide scavenging activity of aerial part extracts of *P. major* and BHT

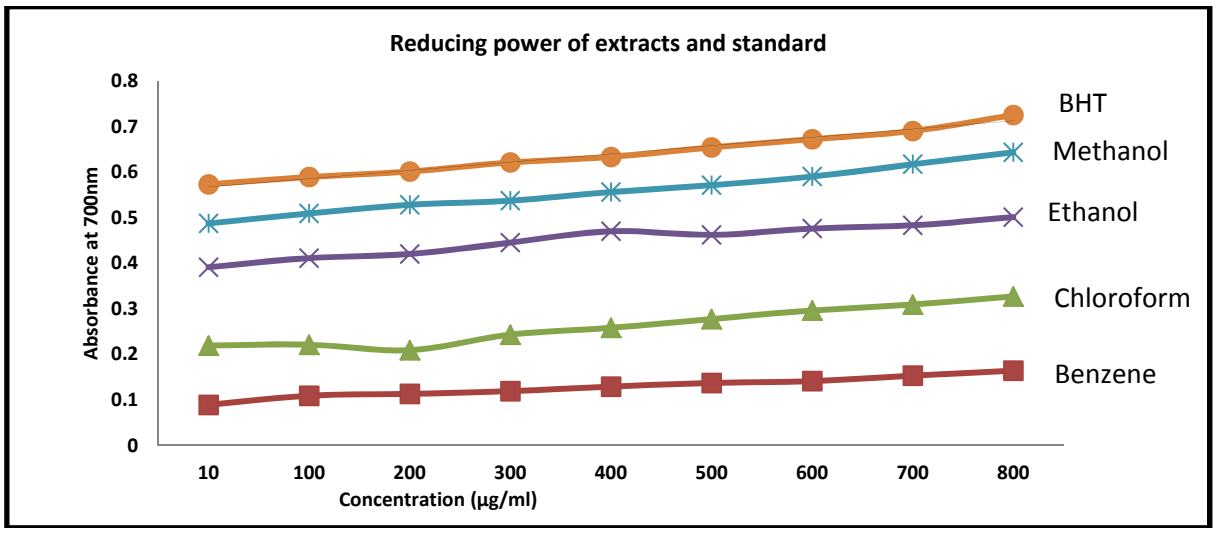

Figure 6: Reducing power of aerial part extracts of *P. major and BHT*

4. DISCUSSION

The TPC gives an idea about the phenolic compounds present in this plant which may be responsible for the pharmacological activities and the reason for their use as a traditional medicine in India and all over the world. The polarity of extraction solvent may influence the number of bioactive compounds obtained from the plant extracts. The results of TPC obtained in this study shows that polar solvents extracted more phenolic compounds than the non polar solvents. But in polar solvents, organic polar solvents (methanol and ethanol) extracted more phenolic compounds. It has been reported that methanol is found to be more desirable to extract the phytoconstituents. Metivier *et al*. reported that methanol was more effective as an extraction solvent for extracting the bioactive compounds from plant extracts and was 20% and 73% more effective than ethanol and water respectively [33]. Generally, the phenolic content of all the extracts were considerably high, which could be a major contributing factor to the strong antioxidant activity of plant extracts. According to Afolayan *et al*., [34], high phenolic content of plant extracts could be responsible for their antioxidant activity. In the present study, the antioxidant activity results were obtained showed that DPPH free radical scavenging activity of methanolic extract was highest than the ethanol, chloroform and benzene extracts. The nitric oxide and H_2O_2 scavenging activity results showed that scavenging activity by NO and H_2O_2 was correlated with the scavenging activity by DPPH assay. The results of present study are related with previous findings of antioxidant activity of *P major*. In ferric reducing power assay the reducing ability of each extract was depending on the electron donating power and ability to quench free radicals or terminate the free radical chain reaction by the formation of more stable products. The reducing agents delay the lipid peroxidation process because they donate a hydrogen atom for breaking the chain reaction which causes membrane lipid damage [35]. The capacity of phenolic compounds to scavenge free radicals may be due to phenolic hydroxyl groups they posses because they transfer hydrogen to free radicals and produce phenoxide radical, which is stabilized. The results of the study showed that among all the solvents, methanol was most efficient solvent for the extraction of polyphenolic compounds. This may be due to better

salvation of antioxidant compounds in aerial part extracts because of the interaction between the polar sites of the antioxidant molecules and methanol solvent.

The results of present study are in agreement with those obtained by Galvez *et al*., [36], Souri *et al.,* [37]; Beara *et al*., [16]. They reported that the phenolic compounds have been the main bioactive compounds present in the methanolic extract of selected Plantago species including *P. major* and are responsible for antioxidant activity of Plantago species. In this perspective Samuelsen, [5] reported that, *P. major* contains bioactive compounds such as flavonoids and phenolic compounds. Kobeasy *et al*., [38] also reported that, among three extracts namely ethanol, cold and hot water extracts, ethanolic extract of *P. major* leaves, had the highest total phenolic content, flavonoid and tannin content which is related to the highest antioxidant activity of ethanol extract of *P. major* leaves. Pourmorad *et al.*, [12] reported that methanolic extract of whole parts of *P. major* showed good radical scavenging activity which was comparable to BHT due to the presence of high amount of phenolic compounds was 31±4 mg of GAE/g of dry weight. The range of values of TPC was higher as reported in literature for *P. major* and also other species of Plantago such as *P. coronopus* and *P. lanceloata* [16].The differences noticed in the values of TPC could be related to different extraction methods or harvesting time and environmental features that can influence the concentration of phenolics in plantains [39].

Several studies reported that polyphenolic compounds may contribute antioxidant properties but some studies also reported that other than polyphenolic compounds may also contribute antioxidant activities. The study conducted by Reina *et al.* [6] concluded that ethanol extract of *Plantago major* and its two bioactive compounds baicalein and aucubin significantly reduced the production of reactive oxygen species by human neutrophils. It is reported in some studies that the natural products possessing antioxidant property shows liver protective activity [40]. Some earlier studies also reported that polar solvent extracts exhibited stronger antioxidant activity than nonpolar solvent extracts. This can be explained that bioactive compounds known for their antioxidant activity such as polyphenolic compounds which are soluble in polar solvents due to their polar nature [41]. Some studies also shows that there is a linear correlation between total phenolic content and antioxidant activity of sample. The results of this study shows that aerial part extracts of *Plantago major* may serve as a potential source of natural antioxidants. Thus this Plantago species can be suggested as a natural source of antioxidants as food and in pharmaceutical companies. Therefore, it can be used as dietary supplement to treat chronic diseases.

CONCLUSIONS

From this study it can be concluded that all the tested extracts showed antioxidant activity but among all the extracts, methanolic extract found out to be rich source of phytochemicals and exhibits highest amount of phenolic compounds and significant antioxidant activity on all examined antioxidant assays (DPPH, NO, H_2O_2 and reducing power). Therefore it can be stated that *P. major* is a potential source of natural antioxidant compounds may be useful for the development of newer and more potent natural antioxidant and can be used to prevent diseases related to oxidative processes. These results might be helpful for researchers and pharma companies for the development of valuable medicines which will be useful for treatment of various oxidative stress related diseases.

ACKNOWLEDGEMENTS

The authors are thankful to Honourable Vice-Chancellor of Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, U.P., India for providing all necessary facilities and Head, Department of Chemistry, SHUATS, Allahabad for providing necessary laboratory facilities to accomplish the experimental work.

ABBREVIATIONS

TPC - Total Phenolic Content
BHT – Butylated Hydroxyl Toluene
BHA – Butylated Hydroxyl Anisole
ROS – Reactive Oxygen Species
TAE – Tannic Acid Equivalent
FC – Folin Ciocalteu
DPPH – 1,1-diphenyl-2 picrylhydrazyl
IC50 – 50% Inhibition Concentration
NO – Nitric Oxide

REFERENCES

[1] Madhu, K., *Asian Journal of Pharmaceutical and Clinical Research*. 2013, 6(2), 38 - 42.
[2] Sadeghi, Z., Valizadeh, J., Shermeh, O. A., Akaberi, M., *Avicenna Journal of Phytomedicine.* 2015, 5(1), 1 - 9.
[3] Bhattacharyya, A., Chattopadhyay, R., Mitra, S., Crowe, S, E., *Physiological Review.* 2014, 94(2), 329 - 354.
[4] Shivasharanappa, K., Londonkar, R., *World Journal of Pharmaceutical Research*. 2014, 3(4), 2106 - 2116.
[5] Samuelsen, A, B., *Journal of Ethnopharmacology.* 2000, 71(1-2), 1 - 21.
[6] Reina, E., Al-Shibani, N., Allam, E., Gregson, K, S., Kowolik, M., Windsor, L, J., *Journal of Traditional and Complementary Medicine*. 2013, 3(4), 268 - 272.
[7] Tarvainen, M., Suomela, J., Kallio, H., Yang, B., *Chromatographia.* 2010, 71(3), 279 - 284.
[8] Mohmood, A, A., Phipps, M, E., *International Journal of Tropical Medicine*. 2006, 1(1), 33 - 35.
[9] Amini, M., Kherad, M., Mehrabani, D., Azarpira, N., Panjehshahin, M, R., Tanideh, N., *Journal of Applied Animal Research*. 2010, 37, 53 - 56.
[10] Zubair, M., Nybom, H., Lindholm, C., Brandner, J, M., Rumpunen, K., *Natural Product Research*. 2015, 30, 622 - 624.
[11] Guillen, M,E,N., Emim, J, A, S., Souccar, C., Lapa, A, J., *International Journal of Pharmacognosy*. 1998, 35(2), 99 - 104.
[12] Pourmorad, F., Hosseinimehr, S. J., Shahabimajd, N., *African Journal of Biotechnology*. 2006, 5(11), 1142 - 1145.
[13] Gomez-Flores, R., Calderon, C, L., Scheibel, L, W., Tamez-Guerra, P., Rodriguez-Padilla, C., Tamez-Guerra, R, *et al., Phytotherapy Research.* 2000, 14(8), 617 - 622.
[14] Chiang, L, C., Chiang, W., Chang, M, Y., Ng, L, T., Lin, C, C., *Antiviral Research.* 2002, 55(1), 53 - 62.
[15] Chiang, L, C., Chiang, W., Chang, M, Y., Lin, C, C., *American Journal of Chinese Medicine.* 2003, 31(2), 225.
[16] Beara, I, N., Lesjak, M, M., Jovin, E, D., Balog, K, J., Anackov, G, T., Orcic, D, Z, *et al*., *Journal of Agricultural and Food Chemistry.* 2009, 57(19), 9268 - 9273.
[17] Trease, G, E., Evans, W, C., *Pharamacognosy*, W, B, Scandars, Company, Ltd, London 1989.

[18] Sofowora, A., *Medicinal plants and Traditional Medicine in Africa*, John, Wily, and Sons, New York 1993.
[19] Harborne, J, B., *Phytochemical methods*: *A guide to modern techniques of Plant Analysis,* 3rd ed. Chapman, and Hall, London 1998.
[20] Makkar, H, P, S., Blummel, M., Borowy, N, K., Becker, K., *Journal of Science and Food Agriculture.* 1993, 61, 161-165.
[21] Wojdylo, A., Oszmianski, J., Czemerys, R., *Food Chemistry*. 2007, 105, 940 - 949.
[22] Ogunlana, O, E., Ogunlana, O, O., *Research Journal of Agriculture and Biological Sciences.* 2008, 4(6), 666 - 671.
[23] Marcocci, L., Maguire, J, J., Droylefaix, M, T., Packer, L., *Biochemical and Biophysical Research Communications*. 1994, 201(2), 748 - 755.
[24] Sreejayan, Rao, M, N, A., *Journal of Pharmacy and Pharmacology*. 1997, 49, 105 - 107.
[25] Ruch, R, J., Cheng, S, J., Klaunig, J, E., *Carcinogenesis*. 1989, 10, 1003 - 1008.
[26] Jayaprakash, G, K., Singh, R, P., Sakariah, K, K., J*ournal of Agricultural and Food Chemistry*. 2001, 55, 1018 - 1022.
[27] Oyaizu, M., *Japanese Journal of Nutrition.* 1986, 44, 307 - 315.
[28] Rao, A., Ahmad, S, D., Sabir, S, M., Awan, S., Shah, A, H., Khan, F, M., et al., *Journal of Medicinal Plants Research.* 2012, 7(4), 155 - 164.
[29] Do, Q, D., Anqkawijaya, A, E., Tran-Nquyen, P, L., Huynh, L, H., Soetaredjo, F, E., Ismadji, S., *et al*., *Journal of Food and Drug Analysis.* 2014, 22(3), 296 - 302.
[30] Pereira, C, G., Custodio, L., Rodrigues, M, J., Neng, N, R., Nogueira, J, M, F., Carlier, J., *et al*., *Brazilian Journal of Biology.* 2017, 77(3), 632 - 641.
[31] Stankovic, M, S., *Kragujevac Journal of Science.* 2011, 33, 63 - 72.
[32] Luque, de, Castro, M, D., Valcarcel, M., Tena, M, T., Analytical Supercritical Fluid Extraction, Butterworth Publishers, Boston 1994.
[33] Metivier, R, P., Francis, F, J., Clydesdale, F, M., *Journal of Food Science*. 1980, 45 (4), 1099 - 1100.
[34] Afolayan, A, J., Jimoh, F, O., Sofidiya, M, O., Koduru, S., Lewu, F, B., *Pharmaceutical Biology*. 2007, 45, 486 - 493.
[35] Loganayaki, N., Siddhuraju, P., Sellamuthu, M., *Journal of Food Science and Technology.* 2013, 50(4): 687 – 695.
[36] Galvez, M., Martin-Cordero, C., Houghton, P, J., Ayuso, M, J., *Journal of Agricultural and Food Chemistry.* 2005, 53(6), 1927 - 1933.
[37] Souri, E., Amin, G., Farsam, H., Barazandeh, Tehrani, M., *DARU Journal of Pharmaceutical Sciences*. 2008, 16(2), 83 - 87.
[38] Kobeasy, M, I., Abdel-Fatah, O, M., El-Salam, S, M, A., Mohamed, Z, E, M., *International Journal of Biodiversity and Conservation*. 2011, 3(3), 83 - 91.
[39] Tamura, Y., Nishibe, S., *Journal of Agriculture and Food Chemistry.* 2002, 50(9), 2514 - 2518.
[40] Hussan, F., Basah, R, H, O., Yusof, M, R, M., Kamaruddin, N, A., Othman, F., *Asian Pacific Journal of Tropical Biomedicine.* 2015, 5(9), 728 – 732.
[41] Iloki-Assanga, S, B., Lewis-Lujan, L, M., Lara-Espinoza, C, L., Gil-Salido, A, A., Fernandez-Angulo, D., Rubio-Pino, J, L., *et al*., *BMC Research Notes.* 2015, 8(396), 1 - 14.