Biomedical
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 No (4) Oct-Nov-Dec 2020 Pp 2365-2373

Evaluation of phytochemical, Antioxidant and Reducing Activity in Whole Plant Extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

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ABSTRACT

In the present study, phytochemical screening, antioxidant activity, polyphenolic activity and reducing power of *Andrographis paniculata* plant prepared in different solvents (methanolic, ethanolic and double distilled water) was assessed by different protocols.2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, Hydrogen peroxide (H_2O_2) radical scavenging activity, Polyphenolic contents and reducing activity of the plant was evaluted by modified method. Phytochemical screening of plant showed the presence of carbohydrate, cardiac glycosides, amino acids, flavonoids, alkaloids, phenols, saponins, steroids and tannins. In DPPH free radical and H_2O_2 radical scavenging activity , methanolic extract of plant were most potent in activity with 50% inhibition at 333.34 µg/ml. and 398.12 µg/ml concentration respectively. Total phenolic (309 ± 0.81 mg/g of gallic acid equivalent) and flavonoid content (82.125 ± 0.85 mg/g of rutin equivalent) were maximum in the methanolic extract of plant. High reducing capacity of plant was observed in case of methanolic extract. A significant positive correlation was found between antioxidant activity and polyphenolic content (total phenols and total flavonoids). Moreover, a significant correlation was found between antioxidant activities and reducing potential of plant extract, depicting that reducers are important contributors to antioxidant. The study shows whole plant extract of *A. paniculata* as an important natural source of antioxidants and phytochemicals.

KEY WORDS: ANDROGRAPHIS PANICULATA, WHOLE PLANT EXTRACT, ANTIOXIDANT ACTIVITY, POLYPHENOLIC CONTENT, DPPH FREE RADICAL ACTIVITY, H_2O_2 SCAVENGING ACTIVITY, REDUCING POWER.

INTRODUCTION

Medicinal plant is the future of phytomedicines (plant-derived drugs) and serves as a rich source of food, fibres and drugs. They have been used in folk medicine since ancient times for the prevention and treatment of the numerous diseases as they express a vast array of biological activities. Presently, research is focusing attention on medicinal plants as it is considered as the most sustainable alternative source of antioxidants to supplement the endogenous oxidative stress defense system in humans (Rahman et al., 2012 Banji et al 2028, Engwa 2018, Zwolan et al 2020).

Antioxidants obtained from the plants either in the form of crude extracts or their derived products is very effective to inhibit the destructive processes caused by oxidative stress (Zengin et al., 2011). Oxidative stress generates free radicals in form of reactive oxygen species (ROS) in the human body through aerobic respiration, ionizing radiation and pollution may increase the risk of chronic and degenerative diseases such as cancer, cardiovascular

ARTICLE INFORMATION

*Corresponding Author: nuttyshilpam17@gmail.com
Received 15th Oct 2020 Accepted after revision 30th Dec 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2020 (7.728)

Online Contents Available at: http//www.bbrc.in/
DOI: http://dx.doi.org/10.21786/bbrc/13.4/111

diseases, ageing and atherosclerosis (Valko et al., 2007). The human body generates antioxidant enzymes to neutralize free radicals (Rimbach et al., 2005), a diet rich in edible antioxidants is recommended to assist the human body to protect itself from food borne free radicals. Plants phytochemicals have shown to possess antioxidant properties capable of scavenging free radicals, preventing cellular damages and related diseases via several mechanisms. Hydrogen peroxide (H_2O_2), superoxide ion (O2–) and hydroxide radical (OH–) are considered as most common ROS. Antioxidants are the molecules which stabilize or deactivate free radicals, before they hit targets in living human cells (Nunes et al., 2012 Banji et al 2028, Engwa 2018, Zwolan et al 2020).

Plants contain a wide variety of free radical scavenging molecules, such as anthocyanins, carotenoids, flavonoids, glutathione, vitamins and endogenous metabolites (Zheng and Wang, 2001).The concentration of the phenolic compounds like phenolic acids, flavonoids, anthocyanins and tannins etc. may be related to the antioxidant activity of medicinal plants (Djeridane et al., 2006). Natural antioxidants have gained interest in pharmaceutical research as an alternative for substitution of synthetic substances showing antioxidant activity (Huang et al., 2005). It is mainly due to the fact that natural antioxidants are cost effective, easily available, non-toxic, eco-friendly and sometimes more efficient than synthetic ones. Continuous efforts are required to characterize plants phytochemicals for their antioxidant potentials and mode of action for various therapeutic uses against oxidative stress-related diseases, (Zwolan et al 2020).

Andrographis paniculata (Burm. f.) Wall. ex Nees of Acanthaceae family is commonly known as Kalmegh/ King of Bitter. The plant is gregarious and grows abundantly in moist, shady waste area and dry forests. It is extensively cultivated in southern Asia, some parts of Europe and China. Traditionally it is used for treating common cold, bronchitis, diarrhoea, fever, hypertension, liver disease and sinusitis (Gabrielian et al., 2002) band snake bite (Samy et al., 2008; Premchandran et al., 2011). Major constituents of *A. paniculata* are diterpenoids i.e. andrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide (Pholphana et al., 2013), neoandrographolide, flavonoids and polyphenols reported to possess the most potent hypotensive and vasorelaxing effect. The plant has been reported to exhibit multifarious pharmacological and biological properties (Niranjan et al., 2010) like antibacterial (Mishra et al., 2009), anticancer (Lim et al., 2012), antidiabetic (Akhtar et al., 2006), antifungal (Sule et al., 2012), anti-inflammatory (Chandrasekaran et al., 2010), anti-HIV (Nanduri et al., 2004) and antihepatotoxic (Nagalekshmi et al., 2011).

The plant showed potential therapeutic action in curing liver disorders, common cough and colds in humans (Geethangili et al., 2008). The present study was therefore performed to study the antioxidant and polyphenols of whole plant extract of *A. paniculata* in three different solvents, which may prove to be beneficial against free radical generated disorders. Reducing potential of the plant was evaluated for the first time in methanolic, ethanolic and aqueous extracts derived from the plant.

MATERIAL AND METHODS

Ascorbic acid, 2,2-Diphenyl,1-picryl hydrazyl (DPPH), gallic acid, rutin, trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, aluminium chloride ($AlCl_3$), rutin, sodium potassium tartarate (Na-K tartarate), sodium carbonate (Na_2CO_3) were purchased from Hi-Media Ltd and solvent ethanol and methanol used were of analytical grade and purchased from Merck (Darmstadt, Germany) *A. paniculata* plant was collected from the campus of Banaras Hindu University, Varanasi. The plant was washed under running tap water to remove the soil and dust particles. The plant was authenticated at Botanical Survey of India (BSI), Allahabad. Collection number BHU-173 and voucher number-91924 was given by BSI to plant flora. Whole plant consisting of (root, stem, leaf, seed, flower) was shade dried for one week and kept in an oven at 40-45°C for 24 h, and then grinded in an electrical grinder to make coarse powder. Extraction was done from 20 g of plant powder in 200 ml of solvent by using a soxhlet apparatus for 12 h. Methanol, ethanol and double distilled water were used as extraction solvents for extraction purpose. Extracts were then filtered and dried at 40°C in a rotary evaporator. Extracts were stored at 4°C till use. Percentage yield {PY, expressed in (w/w)} of crude plant extract was calculated by given formula:

$$PY = \frac{\text{Wt of crude extract recovered}}{\text{Wt of powder used}}$$

One gram of each extract was dissolved in 10 ml of respective extraction solvents to obtain a stock solution of concentration 100 mg/ml. Test plant samples were diluted in various concentrations according to the experiments.Phytochemical testing of the plant for various solvent extract was carried using a standard protocol (Harborne, 1973; Sofowora, 1993).

Antioxidant assay through DPPH: The free radical scavenging activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method given by McCune and Johns, (2008) with some modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) was added to 3 ml methanolic solution of DPPH (0.004%) and shaken vigorously. The mixtures were incubated in the dark for 15 min at room temperature. Ascorbic acid was used as standard and methanol served as blank. The solution without sample was served as control. The absorbance of the samples was recorded at 517 nm by using a spectrophotometer (UV1, Thermo Scientific, US). The experiment was expressed as the percent inhibition of free radicals by the sample and was calculated using the following equation:

$\text{DPPH activity}(\%) = \frac{(C-S)}{(C)} \times 100$ (C =Absorbance of control, S = Absorbance of sample).

Hydrogen Peroxide (H_2O_2) Scavenging Assay: The radical scavenging activity of methanolic, ethanolic and aqueous extracts of the plants to scavenge hydrogen peroxide (H_2O_2) was evaluated by the method of Ruch et al., (1989) with slight modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) were added to 2 ml of H_2O_2 (40 mM) prepared in (50 mM, pH-7.4) phosphate buffer. The test samples were incubated for 10 min at room temperature. The absorbance was measured at 230 nm (Thermo Scientific UV 1). Phosphate buffer without H_2O_2 was used for blank and hydrogen peroxide solution without extract served as control. Ascorbic acid was used as a standard. Hydrogen peroxide scavenging activity was calculated by following formula:

$$\text{Hydrogen peroxide scavenging activity}\ (\%) = \frac{(C-T)}{(C)} \times 100$$

Where, C = absorbance of control, T= absorbance of test sample

Total phenolic content (TPC) was measured by Folin-Ciocalteu assay (McDonald et al., 2001). In brief, 0.5 ml Folin reagent (1:10 diluted with DDW) was added to 0.5 ml (200 µg ml-1) PE and finally 4 ml (1M) aqueous sodium carbonate (Na_2CO_3) was added to this reaction mixture and incubated for 15 min at room temperature. Absorbance was recorded at 650 nm. Gallic acid was prepared in methanol and DDW (1:1) and used as standard. Total phenolic content was expressed in terms of gallic acid equivalent (GAE, mg/g of dry mass), which is a common reference compound. Total flavonoid content (TFC) was determined using the method of aluminium chloride ($AlCl_3$) (Chang et al., 2002). The plant extract (1 ml, different concentration) prepared with different solvent (methanol, ethanol and water) was taken in which 100 µl $AlCl_3$ (10% w/v), 100 µl Na-K tartrate and 2.8 ml distilled water were added and kept for 30 min. Finally, the reaction mixture was diluted to 10 ml with double distilled water and the absorbance was measured at 415 nm. The results were expressed as mg rutin (RE)/g plant material.

Reducing potential capacity of methanolic, ethanolic and aqueous plant extract was estimated by the method of Athukorala et al., (2006) with some modifications. In brief, 1ml of PE (50-300 µg/ml) prepared in different solvents were mixed with 2.5 ml of phosphate buffer solution (PBS, 0.2 M, pH- 6.6) and 2.5 ml potassium ferricyanide (30mM). The above reaction mixture was incubated at 50°C for 20 min. After that, 2.5 ml trichloro acetic acid (TCA, 0.6M) was added to the mixture to stop the reaction and centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of supernatant was taken out and mixed with 2.5 ml double distilled water and 0.5 ml ferric chloride ($FeCl_3$) solution. Absorbance was recorded at 700 nm. Ascorbic acid was used as standard.

Statistical analysis: All the above experiments were performed in quadriplate (n=4) and repeated thrice (x=3). Data were analyzed as mean ± SE by applying one way analysis of variance (ANOVA). Tukey's multiple range tests were used for separation of means when ANOVA was significant ($p< 0.001$) (SPSS 16.0; Chicago, IL, USA). IC_{50} was calculated through linear regression analysis. The graphs were drawn in sigma plot 11.0.

RESULTS AND DISCUSSION

Percentage yield of A. paniculata extract was found maximum (22%) in aqueous followed by methanol (18.4%) and ethanol (17.6%) was obtained. The percentage yield of extract differed in various extraction solvents and this may be due to various degrees of solubility of plant materials depending on polarity of solvents. A similar trend was seen in leaves extract of *A. paniculata* (Banji et al., 2018). Our results highlight that methanolic and ethanolic extracts whole plant were enriched in phytochemicals like alkaloids, amino acids, carbohydrate, flavonoids, phenols, saponins, steroids and tannins while aqueous extract shows presence of alkaloids and amino acids only (Table 1). It may be due to poor solubility of these phytochemicals in the aqueous extract.

Table 1. Phytochemical screening of plant in different solvents

Phytochemicals	Test performed	Methanolic extract	Ethanolic extract	Aqueous extract
Carbohydrate	Fehling test	+	+	-
Phenols	Ferric chloride test	+	+	-
Flavonoids	Ammonia test	+	+	-
Alkaloids	Wagner's test	+	+	+
Steroids	Salkowski test	+	+	-
Tannins	Lead acetate test	+	+	-
Saponins	Frothing test	+	+	-
Glycosides	Nitroprusside test	+	-	-
Amino acids	Ninhydrin test	+	+	+

Note: + = Presence; - = Absence of phytochemicals

In the present study, the free radical scavenging ability of the crude methanolic, ethanolic and water extracts were determined through the degree of discoloration of the methanol solution of DPPH (Table 2). In *A. paniculata*, methanolic extract showed higher scavenging activity (IC_{50} = 398.31 µg/ml) than ethanolic (IC_{50} = 404 µg/ml) and aqueous extracts (IC_{50} = 483.29 µg/ml). The present study reveals that the best antioxidant activity in terms of DPPH scavenging strength was displayed by methanol extract followed by ethanol and aqueous extract. The higher antioxidative capacity of methanolic extract followed by ethanolic extract may be explained via the higher content of biologically active substances, such as e.g. polyphenol.

Ethanolic extract was characterized by higher free radical antioxidant activity than water extract in *Argyreia pierreana*, *Matelea denticulata* (Gudise et al., 2019) and *Nigella sativa* (Zwolan et al., 2020). The antioxidant activity of the extract is first estimated based on their capacity to trap free radical DPPH. In the presence of an active free radical scavenger, the absorption vanishes and the resulting discoloration from deep violet to light yellow. The solution fades colour with increase in concentration of antioxidant as electrons are taken up by DPPH radical from the antioxidant (Calliste et al., 2001) of the extract. Ascorbic acid was used as a standard antioxidant as used as a standard to determine the IC50 value of the extract in other plants (Sreekala et al., 2013).

Hydrogen peroxide (H_2O_2) scavenging activity of *A. paniculata* plant was observed higher in methanolic (IC_{50}= 377.074 µg/ml) followed by ethanolic (IC_{50}= 379.06 µg/ml) extract and aqueous extract (IC_{50}= 467.65 µg/ml) (Table-3). H_2O_2 scavenging activity relies upon the phenolic content of the plant extract by donating electrons to H_2O_2, thereby neutralizing it into water. The study suggests that aqueous extract will be required in relatively high concentration to show its effectivenss. The ethanolic extract of the *Aesculus hippocastanum* was capable of scavenging H_2O_2 in a dose dependent manner (Geetha et al., 2013). H_2O_2 radical scavenging activity was also reported from different extracts of *E. prostrata* (Sinha and Raghuwanshi, 2016a).

Table 2. Antioxidant activity of *A. paniculata* by DPPH free radical scavenging method in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	23.89±0.68f	25.77±0.60f	17.07±0.34f	25.12±0.29f
200	30.52±0.63e	35.01±0.23e	24.71±1.9e	39.34±0.20e
300	43.09±0.68d	42.88±0.18d	34.10±0.59d	56.25±0.22d
400	49.92±0.82c	51.58±0.53c	43.69±0.72c	65.15±0.14c
500	59.11±1.04b	58.84±0.12b	51.94±0.51b	86.47±0.38b
600	63.81± 0.49a	63.65±0.19a	60.12±0.80a	95.22±0.32a
IC_{50}	398.31	404.00	483.29	271.47

Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data are significant at p<0.001; a,b,c,d,e,f = different letter shows significant difference between means.

Total phenolic content was reported as mg/g of GAE in reference to standard curve (y=0.001x+0.05, R2=0.997). In A. paniculata plant, maximum TPC (309.00±0.816 mg/g) was found in methanolic extract followed by ethanolic (290.5±1.29 mg/g) and aqueous extracts (189.25±0.957 mg/g) respectively. Total flavonoid content was calculated by standard curve (y=0.0008x+0.198, R2=0.994) and reported as mg/g of RE. A. paniculata plant showed maximum TFC (82.125±0.853 mg/g) in methanolic extract followed by ethanolic (61.375±1.10 mg/g) and aqueous extracts (37.80±0.731 mg/g) (Table 4). Methanol extract of *A. paniculata* shows important antioxidant activity because it contains phenols and flavonoids (Kurzawa et al., 2014). Similar, higher phenolic content in organic solvent has also been reported (Zaman et al., 2011).

Presence of active metabolites like phenol and flavonoid contents in plant extract depend on solvent used (Sulaiman et al., 2011). Phenolic compounds present in plant contain an aromatic ring bearing one or more hydroxyl groups. Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts in form as aglycone and glycosides. It has two benzene rings separated by a propane unit. Their ideal structural chemistry nature helps them to scavenge injurious free radicals such as super oxide and hydroxyl radicals (Younes and Siegers, (1981). Therefore, acting as antioxidants for their scavenging activity (Das and Pereira, 1990) or chelating process, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions (Clavin et al., 2007) and giving protection against cardiovascular disease, certain forms of cancer and age-

related degeneration of cell components. Flavonoids might show higher antioxidant activity in organic solvent due to structure and substitution pattern of hydroxyl group.

The reducing power of the extracts (methanolic, ethanolic and aqueous) of A. paniculata (Fig. 1) plant increased in a concentration dependent manner from lower to higher concentrations. Similar results reported by (Abdallah et al., 2016) in which the reducing power of *Ziziphus mauritiana* extract increased with the increase of their concentrations. Maximum reducing power was observed in the methanolic extract the plant. In reducing potential assay, after the addition of the extract, the yellow colour of the test solution changes from yellowish green to blue. The colour change of sample solution indicates the reducing power of extract of plants. High absorbance shows high reduction potential of the plant. These reducers show their antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Thus, it is concluded that both polyphenolic compounds and reducers present in the extracts are major determinants of antioxidant capacity of extracts.

Table 3. Antioxidant activity of *A.paniculata* by H_2O_2 radical scavenging in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	21.07±0.79f	27.66±0.50f	20.57±0.70f	25.86±0.38f
200	31.68±0.63e	35.63±0.62e	27.88±0.62e	36.80±0.30e
300	43.17±0.56d	43.68±0.60d	36.00±0.34d	48.33±0.31d
400	51.81±0.83c	51.80±0.38c	44.62±0.49c	57.07±0.29c
500	56.69±0.50b	55.67±1.98b	54.75±0.85b	61.87±0.39b
600	62.87±0.78a	63.01±0.49a	59.62±0.87a	74.16±0.38a
IC_{50}	377.07	379.06	467.65	342.56

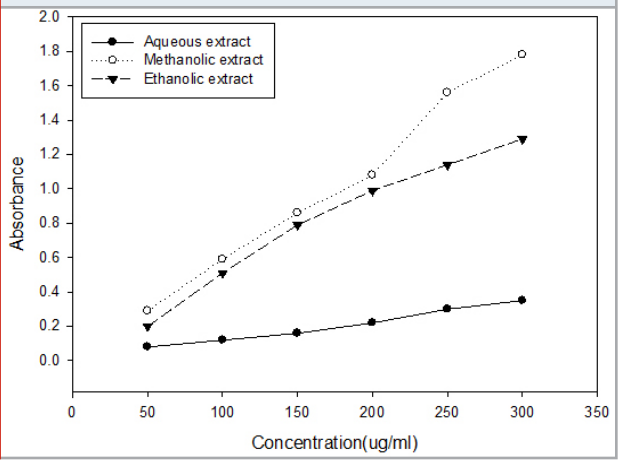
Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data is significant at p <0.001. a,b,c,d,e,f = different letters shows significant difference between means.

Table 4. Total phenolic and flavonoid content of *A. paniculata* in different solvents

Total polyphenolic content (A. paniculata)		
Plant extract	TPC (mg/g GAE)	TFC (mg/g RE)
Methanol	309 ± 0.816a	82.125±0.853a
Ethanol	290.5±1.290b	61.375±1.108b
Aqueous	189.25±0.957c	37.805±0.731c

Data represented as Mean ±SE (n=4); One way ANOVA followed by Tukey's test. All data is significant at p <0.001; a,b,c letters shows significant difference between means.

Figure 1: Reducing potential of *A. paniculata* plant extracts in different solvents



Correlation between total antioxidant activity and reducing power was obtained through linear regression analysis. A significant correlation was found between total antioxidant activities and reducing potential in *A. paniculata* extract (Fig. 2). In, *A. paniculata*, correlation coefficient (R2) between antioxidant activity and reduction potential was (R^2 =0.989) for methanolic, (R^2 =0.992) for ethanolic and (R^2 =0.992) for aqueous extract. Similar studies are seen in *E. prostrata* (Sinha and Raghuwanshi, 2016a) and *Ocimum americanum* (Jaiswal et al., 2019). In our result, there is significant positive correlation between antioxidant activity and reducing power of the plant. Koleva et al., (2002) also reported positive correlation between antioxidant activity and reducing potential.

Correlation between antioxidant activity and polyphenolic compounds: A positive, significant and linear correlation was found between total antioxidant activity and

polyphenolic contents (TPC & TFC) of various extracts. Correlation coefficient (R^2) values of different extracts showed a very close correlation between antioxidant activities and polyphenolic contents (TPC and TFC content). Positive and linear correlation (R^2, ranges from 0.982-0.998) was found in *A. paniculata* in the present experiment (Fig. 3). In the present work, we found a strong correlation between antioxidant activity and total phenolic contents (TPC & TFC). High correlation coefficient ($R^2 \geq 0.946$) values showed close correlation between them. Correlation coefficient (R^2) between antioxidant activity and polyphenolic contents (TPC & TFC) of aqueous and methanolic extracts of Chinese medicinal plant (Cai et al., 2004) and Jordanian plant species (Tawaha et al., 2007) are well reported.

Figure 2: Correlation between antioxidant and reducing potential in (A) Methanolic, (B) Ethanolic and (C) Aqueous extract of *A.paniculata* plants

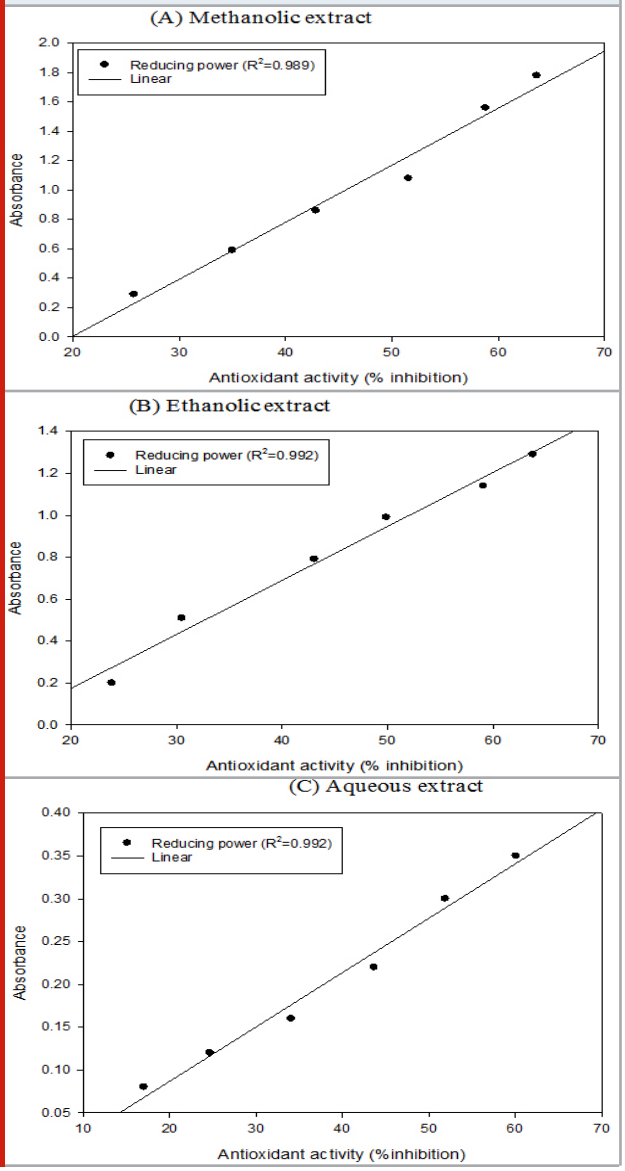
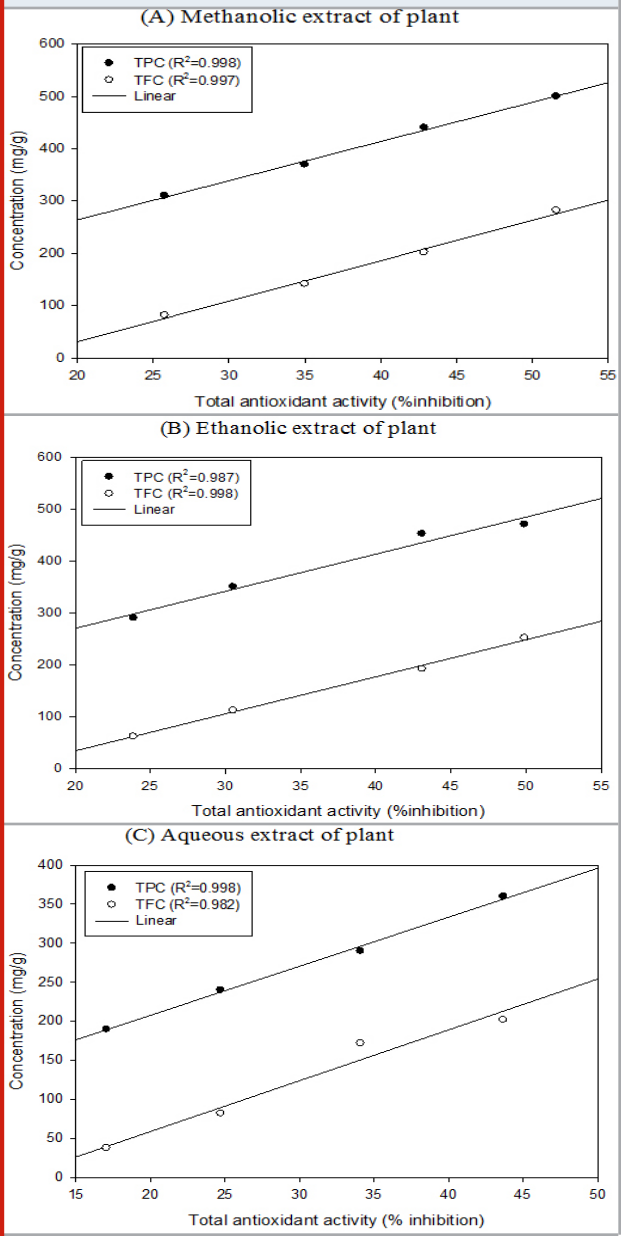


Figure 3: Correlation between antioxidant activity and polyphenols (TPC and TFC) (A) Methanolic (B) Ethanolic (C) Aqueous extract of *A. paniculata*



Phenolic compounds play an important role as antioxidants and a good correlation exists between the concentration of plant phenolics and the total antioxidant capacity (Sinha and Raghuwanshi, 2016a). The phytochemicals present in the plant and food products are generally nontoxic and contain many medicinal properties. Generally, antioxidants and polyphenolic compounds are mutually related with each other for their activities. *A. paniculata* is a good source of phytochemicals like phenolics, flavonoids, antioxidants, alkaloids and tannins etc. These phytochemicals play an important role in promoting pharmaceutical drug preparation and are used for curing various health ailments (Usman and Osuji, 2007).

CONCLUSION

Our study reports that the whole plant extract of *A. paniculata* plant is a rich source of natural antioxidants. The antioxidant property, reducing potential and polyphenolic components like total phenols and flavonoids varied significantly in the different extraction solvent. The organic solvent i.e. methanol and ethanol gave better results than aqueous one. Thus, bioactive compounds present in the extract of this plant may develop into antioxidant agents in the form of plant based drugs that may have applications in human health.

ACKNOWLEDGEMENTS

The authors would like to thank the University Grants Commission for research support.

Conflict of Interests: The authors declare that they have no competing interests.

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