WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 5, 121-133. Research Article ISSN 2277– 7105

PHYTOCHEMICAL INVESTIGATION, ANTIOXIDANT ACTIVITY AND ANTIHELMINTIC ACTIVITY OF *MIKANIA MICRANTHA* LEAVES

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Article Received on
21 Feb 2015,

Revised on 15 March 2015,
Accepted on 08 April 2015

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ABSTRACT

In the present research investigation we extracted the powdered leaves of *Mikania micrantha* by Soxhletion method using different solvents. Then extracts were subjected to preliminary phytochemical investigation. Phytochemical screening of methanolic extract revealed the presence of alkaloids, flavonoids, reducing sugars, saponins, phenolic compounds and tannins, amino acids and proteins whereas petroleum ether extracts revealed only saponins, chloroform extract revealed alkaloids and saponins and water extracts revealed saponins and amino acid & proteins. The proximate analysis was carried out for the leaves powder. The total ash value was 5.31%, acid insoluble ash value was 4.77%, and water-soluble ash value was 4.15%. The materials were subjected to successive extraction with solvents. The solvents used were petroleum ether, chloroform, methanol and water in the ascending order of polarity. The total phenolic content was found 45.9 mg GAE/g extract. The IC_{50} values based on the DPPH for methanolic extract (41.8 µg/ml) showed lower than standard ascorbic acid (129.9 µg/ml) and BHA (153.1 µg/ml). The presence of phenolic compounds was mainly found in this extract and could be attributable to the observed high antiradical properties of this extract. The methanolic extract of *Mikania micrantha* has mild antihelmentic activity.

KEYWORDS: *M. micrantha*, Proximate analysis, Phytochemical screening, Antioxidant, DPPH.

INTRODUCTION

Phytochemistry is the name given to the study of the chemistry of plants. Like animals, plants produce a wide variety of chemical compounds, called metabolites, as part of their normal life processes. These compounds perform different functions. For example, some enable plants to store energy in the form of sugar, whilst others are protective against disease or predators. Plants and plant-based medicines are the basis of many of the modern pharmaceuticals we use today for our various ailments.[1] The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids and phenolic compounds. Free radicals are chemical species, which contain one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Free radicals are generated as part of the body's normal metabolic process and play a dual role in our body as both deleterious and beneficial species. Excess production of reactive oxygen species (ROS) and/or a decrease in antioxidant levels may lead to the tissue damage and different diseases. Antioxidant plays a major role in protecting our body from disease by reducing the oxidative damage to cellular component caused by ROS. Recent investigations suggest that the plant origin antioxidants with free radical scavenging properties may have great therapeutic importance in free radical mediated diseases like diabetes, cancer, neurodegenerative disease, cardiovascular diseases, aging, gastrointestinal diseases. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effect; while relatively plant based medicines confer fewer side effects than the synthetic drug in some instances.[2] *Mikania micrantha* is a tropical plant in the Asteraceae; known as Bitter Vine or Climbing Hemp Vine or American Rope. It is also sometimes called Mile-a-Minute Vine. It is used to heal cuts and stop minor external bleeding in Fiji but its medicinal properties are still yet to be fully discovered. It is also a very popular local antiseptic medicine in Bangladesh.

The aim and objective of the current study was to investigate the chemical groups present, evaluate the possible antioxidant activity and antihelmintic activity of petroleum ether, chloroform, methanol and aqueous extracts of Bangladeshi *Mikania micrantha* leaves to justify its use in traditional treatments.

MATERIALS AND METHODS

Collection, Preparation of the Sample

The leaves of *Mikania micrantha* were collected from the campus of Noakhali Science & Technology University, Sonapur, Noakhali, Bangladesh. The leaves were washed properly and air dried for several days. The dried leaves were then oven dried for 24 hours at considerably low temperature not exceeding 50 ^{o}C. The oven dried leaves were then ground into coarse powder using high capacity grinding machine and were used for different investigation.

Proximate Analysis

Proximate analysis of a substance constitutes different classes of nutrients present in the samples such as moisture, ash, acid insoluble ash, water soluble ash content.

Determination of Moisture Content

Accurately weighed 5 gms of powdered of *Mikania micrantha* leaves were taken in a crucible. It was kept in a hot air oven at 105 – 110 ˚C, until free from moisture. The percentage of moisture content was then calculated with reference to the air-dried sample.

Determination of Total Ash Value

Accurately weighed 5 gms of powdered *Mikania micrantha* leaves were taken in a dried silica crucible. It was incinerated at 450 ˚C temperature, until free from carbon and then cooled. The weight of ash was taken and the percentage of it was calculated with reference to the air-dried sample.

Determination of Acid Insoluble Ash Value

The total ash obtained was boiled for 5 minutes with 25 ml of 2 N HCl, filtered and the insoluble matter was collected on ashless filter paper. Then, it was washed with hot water, ignited in silica crucible for 15 minutes at temperature not exceeding 450 ˚C, cooled and weighed the obtained residue. The percentage of acid insoluble ash was calculated with reference to the air-dried sample.

Determination of Water Soluble Ash Value

The total ash obtained was boiled with 25 ml of water for few minutes, filtered and the insoluble matter was collected on ashless filter paper. Then, it was washed with hot water, ignited in silica crucible for 15 minutes at temperature not exceeding 450˚C, cooled and

weighed the obtained residue. The difference in weight represents the water soluble ash. Finally, the percentage of water soluble ash was calculated with reference to the air-dried sample.

Sequential Extraction

The method is based on the extraction of active constituents present in the drug using various solvents ranging from non-polar to polar. The solvents used are petroleum ether, chloroform, methanol and water. The successive solvent extraction procedure was adopted for the preparation of various extracts of *Mikania micrantha*. The materials were subjected to successive extraction with solvents in their ascending order of polarity (non-polar to polar). In this process, the substance which is soluble in a solvent with particular range of polarity was extracted in the solvent and remaining marc further extracted with next solvent. The powder (200 gm) was extracted sequentially for 8 hours in petroleum ether, chloroform and methanol using a Soxhlet apparatus. After methanol extraction, the remaining dried marc was extracted with water to get water extract. For the preparation of aqueous extract, the above dried marc was macerated for 3 days with distilled water and the residue was removed by filtration and filtrate was concentrated to obtain aqueous extract. All the extracts were concentrated with a rotary evaporator and dried using oven dryer at 35-40 ˚C. Dried extracts were stored for further use.

Preliminary Phytochemical Screening

Phytochemical screening of different extracts for the presence of alkaloids, flavonoids, reducing sugars, saponins, phenolic compounds & tannins, proteins & amino acids were carried out.

Test for alkaloids: 0.4 gms of extracts were dissolved individually in 8 ml of 1% hydrochloric acid and filtered. The filtrates were tested carefully with (a) few drops of potassium mercuric iodide (Mayer's reagent) and (b) potassium bismuth (Dragendroff's reagent). Turbidity or precipitation with either of these reagents was taken as evidence for existence of alkaloids.

Test for flavonoids: 50 mg of extracts were suspended individually in 100 ml of distilled water to get the filtrate. 5 ml of dilute ammonia solution was added to 10 ml of filtrate followed by few drops of concentrated H_2SO_4. Presence of flavonoids was confirmed by yellow colouration.

Test for reducing sugars: Extracts were dissolved individually in 5ml of distilled water and filtered. The filtrates were used to test the presence of carbohydrates. (a) Filtrate was treated with Benedict's reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars. (b) Filtrate was hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solutions. A red precipitate was formed which indicated the presence of carbohydrates.

Test for saponins: The extracts were diluted with 20 ml of distilled water separately and further shaken for 15 min in a graduated cylinder. A layer of foam measuring about 1 cm was formed which indicated the presence of saponins.

Test for phenolic compounds & tannins: 50 mg of extracts were boiled in 20 ml of distilled water separately and filtered. A few drops of 0.1% $FeCl_3$ was added in filtrate and observed for colour change; brownish green or a blue-black colouration was taken as evidence for the presence of phenolic compounds and tannins.

Test for proteins & amino acids: The extract was treated with 1 ml of 10% sodium hydroxide solution and heated. A drop of 0.7% copper sulphate solution to the above mixtures was added. The formation, of purplish violet color indicated the presence of proteins.

Antioxidant Activity

In order to investigate the antioxidant properties of the examined extracts, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power assay, total antioxidant capacity, total phenolic content and reduction of ferric ions by ortho-phenanthroline color method were performed.

DPPH Radical Scavenging Activity: The free radical scavenging activity of the extracts were measured *in vitro* by 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier.[3,4] The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20 °C until required. A 3 ml aliquot of this solution was mixed with 100 µl of the sample at various concentrations (31.25-500 µg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The

scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Control absorbance } (A_0) - \text{Sample Absorbance } (A)}{\text{Control absorbance } (A_0)} \times 100$$

Reducing Power Assay: The reducing power was based on Fe (III) to Fe (II) transformation in the presence of the solvent extracts.[5] The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Various concentrations of the sample (2 ml) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50 °C for 20 min followed by addition of 2 ml of trichloroacetic acid (100 mg/l). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2 ml from each of the mixture earlier mentioned was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates a higher reducing power.

Total Antioxidant Capacity: The total antioxidant capacity of the fractions was determined by phosphomolybdate method using ascorbic acid as a standard.[6] An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 35°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A typical blank contained 1 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. Ascorbic acid was used as standard. The antioxidant capacity was estimated using following formula:

$$\text{Antioxidant effect (\%)} = \frac{\text{Control absorbance } (A_0) - \text{Sample Absorbance } (A)}{\text{Control absorbance } (A_0)} \times 100$$

Total Phenolic Content

The concentration of phenolics in plant extracts was determined using spectrophotometric method.[7] Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were

thereafter incubated in a thermostat at 45^{o}C for 45 min. The absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Reduction of Ferric Ions by *ortho*-phenanthroline Color Method: A reaction mixture containing 1 ml *ortho*-Phenanthroline (5 mg in 10 ml methanol), 2 ml ferric chloride 0.2 mM (3.24 mg in 100 ml distilled water) and 2 ml of various concentrations of the extracts was incubated at ambient temperature for 10 min, then the absorbance was measured at 510 nm. Ascorbic acid and gallic acid were used as reference standards.

Antihelmintic Assay

The antihelmintic activity was evaluated on adult earthworm *Pheritima posthuma*. It resembles anatomically and physiologically with the intestinal round worm parasite of human being. The method of Mathew *et al.* and Dash *et al.* was followed for the screening. Five groups of approximately equal size Bangladeshi earthworms consisting of six earthworms in each group were released in 50 ml of desired formulation. Each group was treated with one of the following: albendazole (10 mg/ml) and extract (50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml, 10 mg/ml) in normal saline containing 1% gum acacia. Observations were made for the paralysis time and subsequently for death time. Paralysis was said to occur when the worms did not revive even in normal saline. The worms were said to be dead when they lost their motility followed by fading away of their body colors.

RESULTS AND DISCUSSION

Proximate Analysis

The leaves of *Mikania micrantha* was subjected to evaluate its moisture content, total ash, acid insoluble ash and water soluble ash value (Table 1). Proximate analysis is a method for the quantitative analysis of different macronutrients. The air dried *Mikania micrantha* leaves contain 9.71% moisture. The low moisture content of the leaf would hinder the growth of microorganism and storage life would be high.[8] Moreover, knowing the moisture content of a substance helps to determine if that substance is suitable for a specific use. The ash content of 5.23% indicates that the leaf is comparatively rich in mineral elements. Ash contains

inorganic radicals like phosphates, carbonates and silicates of sodium, potassium, magnesium, calcium etc. Acid and water soluble ash determination is important because it indicates the quality and purity of a crude drug.

Table 1: Moisture content and ash value of *Mikania micrantha* leaves.

Moisture content	Ash value		
	Total ash	Acid insoluble ash	Water soluble ash
9.71 %	5.23 %	4.77 %	4.15%

Preliminary Phytochemical Screening

Secondary metabolites are very important for the plant. Phytochemical screening of methanolic extract revealed the presence of alkaloids, flavonoids, reducing sugars, saponins, phenolic compounds and tannins, amino acids and proteins whereas petroleum ether extracts revealed only saponins, chloroform extract revealed alkaloids and saponins and water extracts revealed saponins and amino acids & proteins (Table 2).

Table 2: Qualitative chemical analysis of different solvent extracts of *Mikania micrantha* leaves.

Phytochemical composition	Results of different extracts			
	Petroleum ether	Chloroform	Methanol	Water
Alkaloid	–	+	+	–
Reducing sugar	–	–	+	–
Flavonoids	–	–	+	–
Saponins	+	+	+	+
Phenolic compounds and tannins	–	–	+	–
Amino acids and proteins	–	–	+	+

Antioxidant activity

DPPH Radical Scavenging Activity: Figure 1 and table 3 shows that the scavenging effects of extracts on DPPH radical and were in the following order: methanolic > chloroform > water > petroleum ether extracts. The IC_{50} value of scavenging DPPH radicals for the methanol extracts was 41.8 µg/ml. Though the antioxidant potential of extract was found to be lower than those of ascorbic acid (129.9 µg/ml) and BHT (153.1 µg/ml), the study revealed that methanolic extract have prominent antioxidant activity; the presence of phenolic compounds are mainly found in this extract and could be attributable to the observed high antiradical properties of this extract.

The electron donation ability of natural products can be measured by DPPH radical purple coloured solution bleaching. The method is based on scavenging of DPPH through the

addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test.[9] In the present study among all the extracts tested, methanolic extract showed significantly higher inhibition percentage and positively correlated with total phenolic content. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

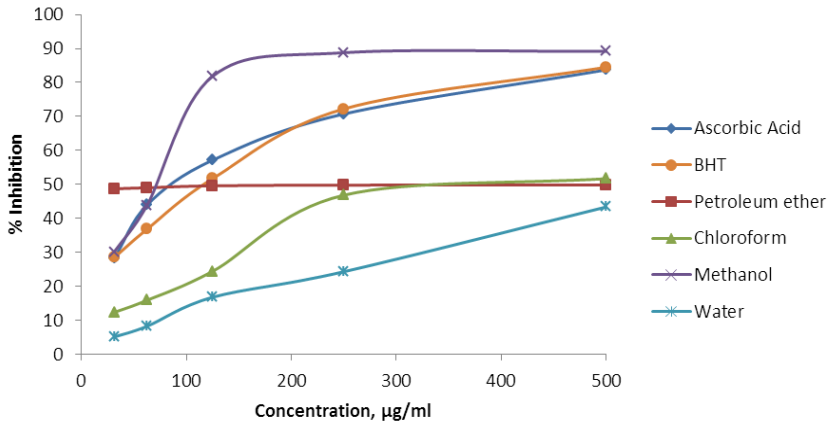

Figure 1: DPPH radical scavenging activity of different extracts of *Mikania micrantha* and standard.

Table 3: DPPH radical scavenging activity of extracts and standard antioxidants.

Concentration (µg/ml)	*Mikania micrantha*				Standard	
	Petroleum ether	Chloroform	Methanol	Water	Ascorbic Acid	BHT
500	49.85± .023	51.69± .010	89.19± .030	43.35± .030	83.73± .030	84.52± .030
250	49.77± .011	46.83±.076	88.79± .063	24.3± .063	70.73± .063	72.22± .063
125	49.58± .002	24.41± .037	81.85± .006	16.77± .006	57.14± .006	51.69± .006
62.5	49.01± .015	15.89± .035	43.85± .013	8.23± .013	43.95± .013	36.81± .013
31.25	48.71± .007	12.3± .038	30.15± .012	5.06± .012	28.17± .012	28.47± .012
IC_{50} Value	**482.9**	**420.0**	**41.8**	**575.7**	**129.9**	**153.1**

Reducing Power Assay: Figure 2 shows the dose response curves for the reducing powers of all extracts (31.25-1000 µg/ml) from *Mikania micrantha*. Methanolic extract showed (1.016± 0.013) significant reducing power comparing to standard BHT (1.4785±0.001). Petroleum ether and chloroform showed weak (0.584±0.077 and 0.841±0.005) reducing power.

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

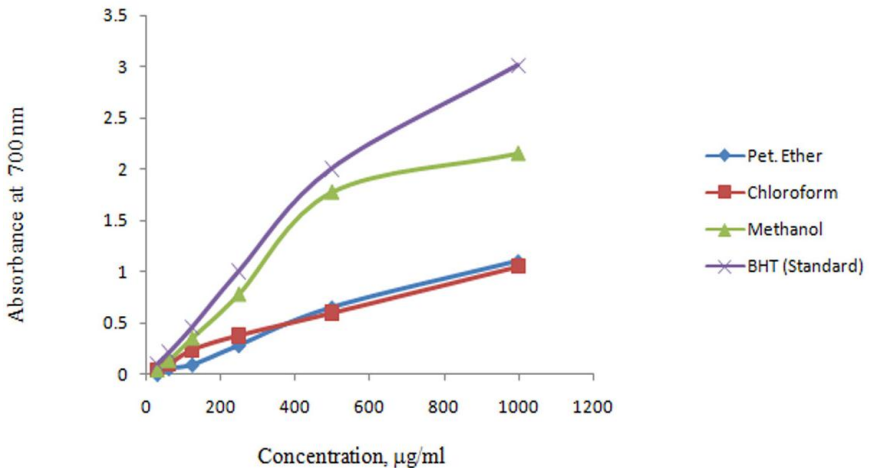

Figure 3: Reducing power assay of different extracts of *Mikania micrantha* with standard antioxidant.

Total Antioxidant Capacity: The phosphomolybdate method is quantitative, since the total antioxidant capacity is expressed as ascorbic acid equivalents. The antioxidant capacity of various solvent extracts of *Mikania micrantha* were found to decrease in this order: methanol (429 AAE/g) > petroleum ether (193 AAE/g) > chloroform (138 AAE/g) > water (33 AAE/g). Strong antioxidant activity of methanol extract statistically similar to ascorbic acid indicates strong antioxidants in this exracts and these could be attributable to the presence of phenolic compounds.

The antioxidant capacity of the extracts were measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 765 nm. The present study demonstrated that methanol extract

exhibited the highest antioxidant capacity for phosphomolybdate reduction. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants.[10, 11]

Total Phenolic Content

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. According to the recent reports, a highly positive relationship between total phenols and antioxidant activity was found in many plant species [12]. In the preliminary phytochemical investigation, only methanolic extracts showed the presence of phenols. That's why, methanolic extract was tested for total phenolic content and it was 45.9 mg of GA/g of extract.

Reduction of Ferric Ions by *ortho*-Phenanthroline Color Method: Ortho-substituted phenolic compounds may exert pro-oxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with ferric ion which get disrupted in the presence of chelating agents. The extracts interfered with the formation of ferrous-*o*-phenanthroline complex, thereby suggesting that the extract has metal chelating activity. The chloroform extract and petroleum ether extract showed appreciable antioxidant activity and the values (1.168 and 0.791) are significantly higher than the standard antioxidants gallic acid and ascorbic acid (0.627 and 0.210) at 500 µg/ml. The methanol and water extracts (0.299 and 0.257) also contributed fairly outstanding antioxidant activity at 500 µg/ml.

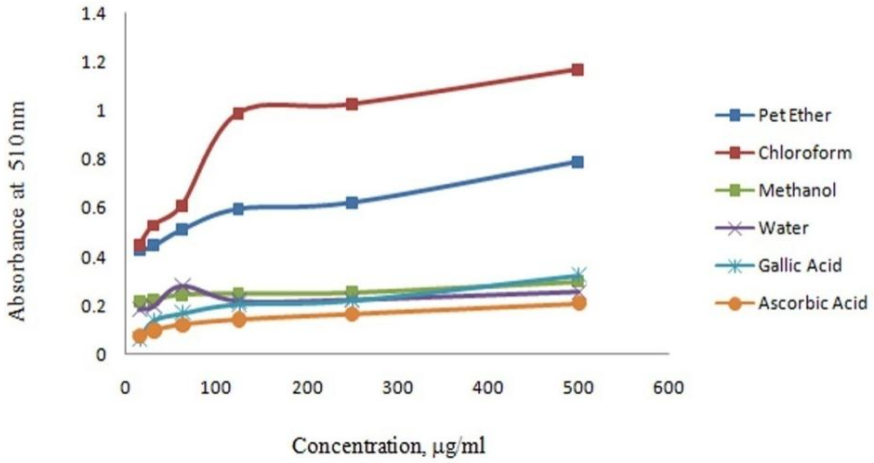

Figure 6: Antioxidants activity of different extracts of *Mikania micrantha* with standard by Reduction of Ferric Ions by Ortho-phenanthroline Color method.

Antihelmintic Assay

The methanolic extract of *Mikania micrantha* leaves was subjected for antihelmintic activity. At 10, 20, 30, 40 and 50 mg/ml concentrations paralysis were observed respectively at 144, 123, 104, 92 and 83 min and death at 159, 143, 124, 112 and 101 min post-exposure. It is clear that the methanolic extract of *Mikania micrantha* have mild antihelmintic activity compare with standard albendazole (for 10 mg/ml paralysis time 56 min and death time 77 min). It showed highest antihelmintic activity on 50 mg/ml concentration.

CONCLUSION

In light of the results of present study, it can be concluded that the plant extracts possess moderate to good antioxidant activity comparable to that of standard drugs BHT, Ascorbic acid and Gallic acid, which led us to the inference that the plant extract may contain bioactive compounds which may aid ongoing anticancer drug discovery from floristic resources. Hence, further studies are suggested to be undertaken to pinpoint the exact compound(s) and to better understand the mechanism of such actions scientifically. This will emphasize on the isolation and characterization of active principles responsible for these activities of *Mikania micrantha* leaves.

ACKNOWLEDGEMENTS

The authors are thankful to the department of Applied Chemistry and Chemical Engineering, Noakhali Science and Technology University, Bangladesh for giving us laboratory facilities for the research work. The research is mainly funded by Higher Education Quality Enhancement Project (HEQEP) supported by University Grants Commission (UGC) of Bangladesh and World Bank.

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