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Phytochemical composition and *in vitro* antioxidant activity of *Achyranthes aspera* Linn (Amaranthaceae) leaf extracts

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The leaves of *Achyranthes aspera* Linn (Amaranthaceae) were screened for the presence of its phytochemical composition, antioxidant, antimicrobial and h**a**emolytic activities. Estimation of total phenolic content was performed by Folin-Ciocalteau reagent method. Antioxidant activity of methanol and aqueous extracts was screened by 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay and ferric ion reduction assay. Antimicrobial activity was performed by well diffusion method and h**a**emolytic activity was performed by spectroscopic method. *A. aspera* leaves showed the presence of carbohydrates, phenolic compounds, Oil and fats, saponins, flavonoids, alkaloids and tannins as major phytochemical groups. Both the extracts exhibited significant antioxidant activity and presence of high level of phenolic compounds, whereas both extracts exhibit low antimicrobial and h**a**emolytic activity. The extracts were studied for their ability to protect pBR322 DNA from damage by Ultra Violate rays induced photolysis of hydrogen peroxide; both extracts protect the DNA quite effectively. Fourier Transform Infrared spectroscopic analysis of the leaves powder showed the presence of –OH group for phenolic compounds and UV-Visible spectroscopic analysis exhibits the presence of flavonol derivatives, carotenoids and b-cryptoxanthin epoxide as major phenolic compounds. In conclusion *A. aspera* leaves possess high antioxidant activity and can be explored as a source of natural antioxidant compounds.

Key words: Phytochemical analysis, phenolic compounds, antimicrobial, h**a**emolytic, photolysis

Introduction

Oxidation is a normal physiological and metabolic process in the cell. During the process, approximately 5% oxygen gets reduced to the oxygen based free radicals, includes superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals (Halliwell and Gutteridge, 1989). These free radicals are collectively known as Reactive Oxygen Species (ROS). Free radicals formed

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during metabolism are electrically charged and react with nucleic acids, mitochondria, proteins and enzymes and resulting in their damage (Halliwell, 1996; Morrissey and O'Brien, 1998). The antioxidant defense system protects the cells against the free radicals. The antioxidant defense mechanism is affected by age, diet and health condition of individual (Yu, 1994). When formation of free radicals overtakes the antioxidant defense system, the free radicals start attacking the cell and resulting in several physiological disorders like Alzheimer's disease, cancer, atherosclerosis, diabetes, liver cirrhosis and rheumatism (Frankel *et al*., 1993; Goodwin and Brodwick, 1995).

Achyranthes aspera (Amaranthaceae) is a habitat of Asia, South America and Africa. In traditional medicinal system, *A. aspera* is known for diuretic, hepatoprotective and emmenagogue properties and used to cure several diseases viz., malarial fever, dysentery, asthma, hypertension and diabetics. Most recently, *A. aspera* is widely studied for its medicinal properties and reported to have immunostimulatory properties (Rao *et al*., 2002), wound healing activity (Edwin *et al*., 2008), antioxidant activity, haemolytic activity (Priya *et al*., 2010), anti-inflammatory (Kumar *et al*., 2009), antibacterial activity (Alam *et al*., 2009) and antifungal activity (Elumalai *et al*., 2009).

The aim of this study was to investigate the *A. aspera* leaves for its phytochemical composition, DPPH radical scavenging activity, reducing power activity, DNA damage inhibition efficiency, antimicrobial activity and haemolytic activity. We have also characterized the phenolic compounds by UV visible and FTIR analysis.

Material and methods

Plant material

A. aspera plant was collected from the natural population growing in the Seshachalam Forest Area, Chittoor district, Andhra Pradesh, India, during December 2009. The plant sample was carried to the Molecular and Microbiology Research Laboratory, VIT University. The taxonomic identification of the plant was made by Prof. V Palanichamy, Plant Biotechnology Division, VIT University, Vellore. Voucher specimens were maintained in our laboratory for further references (Accession number: AS/VIT/MMRL/30.12.2009-1).

Processing of plant

Fresh and mature leaves of *A. aspera* were collected and washed thoroughly in distilled water and shade dried at room temperature. Dried leaves

were powdered uniformly using a mechanical grinder. The powder was extracted in methanol and distilled water separately using a Soxhlet apparatus. These extracts were concentrated at 40˚C under reduced pressure (72 mbar) in rotary evaporator and dried using lyophilizer. Dried extracts were collected in air tight container and stored at 4°C until for further use.

Phytochemical screening

Phytochemical screening of the leaves of *A. aspera* was carried out by using the standard protocols for the presence of carbohydrates, proteins, phenolic compounds, oil and fats, saponins, flavonoids, alkaloids and tannins (Harborne, 1973).

Estimation of total phenolic content

Total phenolic content of the aqueous and methanol extract of *A. aspera* was determined using the Folin-Ciocalteau reagent method (Guha *et al*., 2010). The crude aqueous and methanolic extracts were diluted to obtain different concentrations (125, 250, 500 and 1000 µg). Fifty µl of each extract was mixed with 2.5 ml of Folin- Ciocalteau reagent (1/10 dilution in distilled water) and 2 ml of 7.5% Na_2CO_3 (w/v in distilled water). The mixture was incubated at 45˚C for 15 min. The absorbance was measured at 765 nm using a UV–Visible spectrophotometer. Na_2CO_3 solution (2 ml of 7.5% Na_2CO_3 in 2.55 ml of distilled water) was used as blank. The results were expressed as Gallic acid equivalence in µg. Each experiment was performed in triplicates at each concentration.

DPPH radical scavenging activity

The plant extracts were diluted to make 10, 20, 40, 60, 80 and 100 µg/ml dilutions. Two millilitres of each dilution was mixed with 1 ml of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20˚C for 40 min. Absorbance was measured at 517 nm using a UV–Visible spectrophotometer with methanol as blank (Guha *et al*., 2010). Each experiment was performed in triplicates at each concentration.

The percentage scavenging of DPPH was calculated according to the following formula:

% DPPH Radical scavenging= $[(A_c-A_t)/A_c] \times 100$

Where, A_c is the absorbance of the control

A_t is the absorbance of test

Reducing power activity

The reducing power of methanol and aqueous extracts of *A. aspera* was determined by ferric reducing power assay (Oyaizu, 1986). 1 ml volume of plant extracts at different concentrations (125, 250, 500 and 1000 µg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1 % Potassium Ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50˚C for 20 min. A volume of 2.5 ml of Trichloroacetic acid (10%) was added to the mixture, and was centrifuged at 3000 rpm for 10 min in a cooling centrifuge. 2.5 ml of the supernatant was mixed with equal volume of distilled water and 0.5 ml $FeCl_3$ (0.1%). Absorbance was measured at 700 nm using a UV–Visible spectrophotometer. Ascorbic acid was used as positive control. Higher absorbance of the reaction mixture indicated greater reductive potential. Each experiment was performed in triplicates at each concentration.

DNA damage inhibition efficiency

DNA damage inhibition by methanol and aqueous extracts of *A. aspera* leaf extracts were tested by photolysing H_2O_2 by UV radiation in presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA (Rajkumar *et al*., 2010). A total of 1 µl aliquots of pBR322 (200 µg/ml) were taken in three microcentrifuge tubes. A quantity of 50 µg of methanolic and aqueous extracts was added to the tubes separately. The remaining tube was left untreated as the irradiated controls. An amount of 4 µl of 3% H_2O_2 was added to all the tubes which were then placed directly on the surface of a UV transilluminator (300 nm) for 10 min at room temperature. After irradiation, 4 µl of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added. The samples in all the tubes were analyzed by gel electrophoresis on a 1% agarose gel (containing ethidium bromide) in TBE buffer (pH 8). Untreated non-irradiated pBR322 plasmid (C) was run along with untreated UV-irradiated plasmid DNA (C_R), methanol extract-treated UV-irradiated samples (A_M) and aqueous extract-treated UV-irradiated sample (A_A).

Antimicrobial activity

Test microorganism

Staphylococcus aureus ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922.

Positive and negative control

Vancomycin (30 µg/disc) was used as positive control for *B. cereus*, Cotrimoxazole (23.75 µg/disc) for *K. pneumoniae*, Ciproflaxocin (5 µg/disc) for *S. aureus,* Chloromphenicol (30 µg/disc) for *E. coli* and Piperacillin (100 µg/disc) for *P. aeruginosa*. Sterilized distilled water was used as negative control.

Antibacterial assay

Antibacterial activity of the crude extracts was determined by the agar well diffusion method (Kumar *et al.,* 2010a). Test organisms were inoculated on Mueller Hinton agar by using sterilized cotton swabs. Agar surface was bored by using sterilized gel borer to make wells. A volume of 100 µl of the test extracts (1000 µg/ml) and 100 µl of sterilized distilled water (negative control) were poured in the separate wells. The standard antibiotic discs were placed on the agar surface as positive control. Plates were incubated at 37°C for 24 hours. Each experiment was performed in triplicates.

Haemolytic activity

H**a**emolytic activity was performed by spectroscopic method (Yang *et al.,* 2005). Five milliliters of blood was collected from a healthy individual (blood group O positive) and erythrocytes were collected after repeated washing in sterile normal saline (0.89% w/v NaCl, pH 7.2). The cells were resuspended in normal saline to 0.5%. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml of the plant extracts (125, 250, 500 and 1000 µg/ml concentrations in saline). The mixtures were incubated for 30 min at 37˚C. The mixture was centrifuged at 1500 rpm for 10 min in a cooling centrifuge. The free hemoglobin in the supernatants was measured in UV-Visible spectrophotometer at 540 nm. Saline and distilled water were used as minimal and maximal haemolytic controls. Each experiment was performed in triplicates for each concentration.

The percentage h**a**emolysis was calculated according to the following formula:
% h**a**emolysis= $[A_t–A_n/A_c–A_n] \times 100$
Where, A_t is the absorbance of test sample, A_n is absorbance of the negative control, A_c is the absorbance of the positive control

FT-IR analysis of the plant sample

The dried leaves of *A. aspera* were grounded into fine powder and 2 mg of the sample was mixed with 200 mg KBr (FT-IR grade) and pressed to make into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm^{-1} in FT-IR spectroscopy (Ramamurthy and Kannan, 2007; Kumar *et al*., 2010b).

UV-Visible spectroscopic analysis

Presence of phenolic compounds was confirmed by UV- Visible spectroscopic analysis. One ml of crude extracts were taken and absorbance was measured by using UV–visible spectrophotometer between 200-600 nm (Mattha¨us, 2002).

Statistical analysis

The values of DPPH radical scavenging activity, reducing power activity, antimicrobial activity and h**a**emolytic activity of the aqueous and methanol extract of *A. aspera* leaves are expressed as mean ± standard deviation of the response of three replicates per sample. Results were analyzed using Microsoft Excel 2007.

Results and discussion

A. aspera is an erect pubescent herb, obovate, with opposite short petioled, obovate, softly tomentose leaves and terminal spikes with small greenish-white sessile deflexed flowers. The plant is found in Asia, South America, and Africa (Daniel, 2006). In India, the *A. aspera* found throughout the country, especially in hilly regions. The plant is used by traditional healers to cure several physiological disorders in humans. Leaves of *A. aspera* are reported to contain phenolic compounds in it and as phenolic compounds are good natural antioxidant, so this study was designed to quantify total phenolic content and antioxidant potential of *A. aspera* leaves. Phytochemical screening, antimicrobial and h**a**emolytic activity was also performed.

Percentage yield

Ten grams of dried leaf powder of *A. aspera* was extracted in methanol and water separately using a soxhlet extractor. The yield of aqueous extract (3.77 %) was higher compared to methanol extract (2.09 %).

Phytochemical screening

Phytochemical screening of the *A. aspera* suggests the presence of major phytochemicals in the plant (Table 1). Methanol extract showed the presence of carbohydrates, phenolic compounds, oil and fats, saponins, flavonoids, alkaloids and tannins, whereas, aqueous extract contained phenolic compounds, saponins, flavonoids and tannins as major phytochemical groups (Table 1). Danial (2006) reported the presence of polysaccharides, ecdysterone (harmone), achyranthine, betaine (Alkaliods), vanillic acid, syringic acid, *p*-coumaric acid (phenolic acids), saponin A, saponin B (saponins), protein and carbohydrates in *A. aspera*. Presence of phenolic compounds in the plant suggests the potential use of *A. aspera* as a source of antioxidant compounds (Harborne, 1973).

Table 1. Phytochemical analysis of *A. aspera* leaves

Phytochemicals	***A. aspera***	
	ME	*AE*
Carbhohydrates	+	-
Proteins	-	-
Phenolic compounds	+	+
Oil and fats	+	-
Saponins	+	+
Flavonoids	+	+
Alkaloids	+	-
Tannins	+	+

ME: methanol extract, AE: aqueous extract, +: present, -: not present

Total phenolic content

Phenolic compounds are the key phytochemicals with high free radical scavenging activity. It has generated a great interest among the scientists for the development of natural antioxidant compounds from plants. Phenolic compounds also possess anti-mutagenic and anti-tumor activities (Othman *et al*., 2007). In the current work, the phenolic content of the *A. aspera* extracts was measured (Figure 1). The methanol extract of *A. aspera* showed higher amount of phenolic compounds compared to aqueous extract. The concentration of the phenolic compounds was increased with increase in the dose. The results are described as Gallic acid equivalents (GAE).

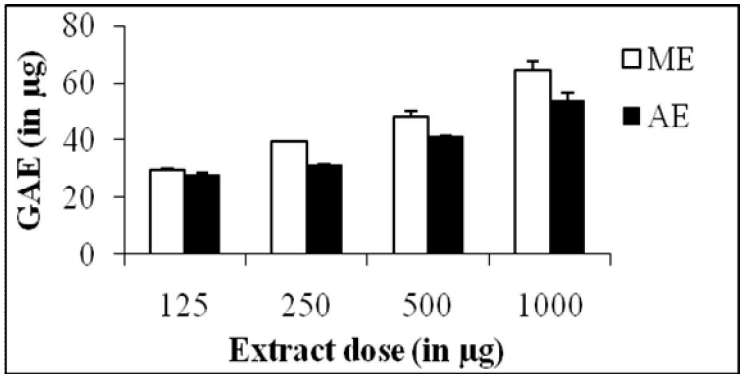

Fig. 1. Total Phenolic content in varying concentrations of *A. aspera* leaves methanol (ME) and aqueous (AE) extracts. Data is represented as mean ± SD (n = 3)

DPPH radical scavenging

DPPH is stable free radical with deep purple color, after receiving proton from a proton donor such as phenolic compounds, it loses its chromophore and became yellow (Sanchez-Moreno *et al*., 1999). Methanol extract showed high DPPH radical scavenging activity compared to aqueous extract (Figure 2). The DPPH radical scavenging potential of aqueous and methanol extracts followed the dose dependent pattern. Priya *et al*. (2010) reported the DPPH radical scavenging activity of *A. aspera* stem, whereas Nehete *et al*. (2009) reported the DPPH radical scavenging activity of leaves and root.

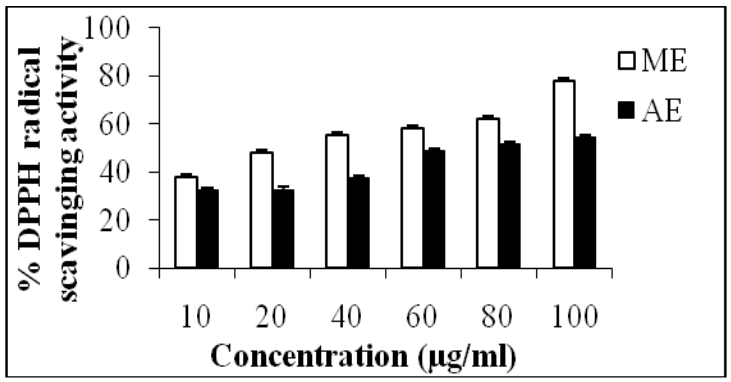

Fig. 2. DPPH radical scavenging activity of the *A. aspera* leaves methanol (ME) and aqueous (AE) extracts. Data is represented as mean ± SD (n = 3)

Reducing power

Reducing power experiment is a good reflector of antioxidant activity of the plant. The plant having high reducing power generally reported to carry

high antioxidant potential too. In this experiment, Ferric ions are reduced to ferrous ions with the color of the reaction mixture changes from yellow to bluish green. The results for ferric reducing power activity of *A. aspera* leaves extract comparision with ascorbic acid are reported in Figure 3. Aqueous extract showed high reducing power than that of methanol extract. Reducing power potential of both extracts increase with the dose, however, both extracts exhibits low reducing power than that of ascorbic acid.

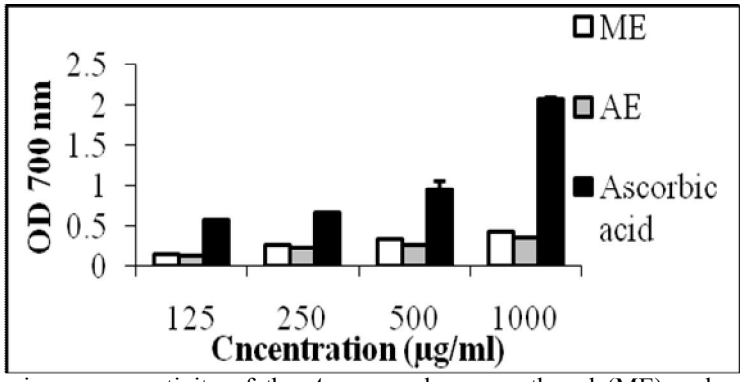

Fig. 3. Reducing power activity of the *A. aspera* leaves methanol (ME) and aqueous (AE) extracts. Data is represented as mean ± SD (n = 3)

DNA Damage Inhibition Efficiency

Figure 4 shows the electrophoretic pattern of pBR322 DNA following UV-photolysis of H_2O_2 in absence (in controls C and CR) and presence (in samples A_M and A_A) of the extracts. Control pBR322 (C) showed a bands on agarose gel electrophoresis. UV-photolysis of H_2O_2 in C_R damaged the entire DNA (no bands visible). Both aqueous and methanol extracts displayed considerable protective activity and indicate the DNA band. The results infer that UV-photolysed H_2O_2 (3%) treatment of pBR322 obliterated the entire DNA (in C_R), while 50 mg of aqueous and methanol extracts protected against DNA damage.

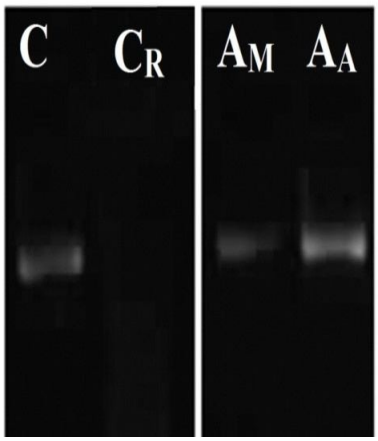

Fig. 4. Effect of *A. aspera* leaf extracts on the protection of plasmid DNA (pBR322) against oxidative damage caused by UV-photolysed H_2O_2. C=untreated non-irradiated DNA (control), CR=untreated UV-irradiated DNA (control), A_M=UV-irradiated and methanolic extract treated, A_A=UV-irradiated and aqueous extract treated

Antimicrobial activity

Antimicrobial activity of *A. aspera* aqueous and methanol extracts was screened against *B. subtilis, S. aureus, K. pneumoniae*, *E. coli* and *P. aeruginosa*. The results of the zone of inhibition are summarized in Table 2. The methanol extract showed the inhibitory effect against *B. subtilis* (13.3±0.57 mm) and *S. aureus* (6.3±1.5 mm) with relative percentage inhibition (Kumar *et al.,* 2010) 90.25 and 4.66% respectively, with compare to positive control. Aqueous extract did not show any inhibitory effect against any one of the test organisms. Another studies on antimicrobial screening of different parts of *A. aspera* revealed its poor antibacterial activity and broad spectrum antifungal activity (Alam *et al*., 2009; Elumalai *et al*., 2009).

Table 2. Antimicrobial activity of *A. aspera* leaves

Test organisms	**Inhibition zone diameter (mm)**			
	ME	AE	PC	NC
B. subtilis	13.3±0.57	-	14.0±1.0	-
E. coli	-	-	24.6±0.57	-
K. pneumoniae	-	-	18.6±2.08	-
P. aeruginosa	-	-	23.0±1.73	-
S. aureus	6.3±1.5	-	29.3±0.57	-

ME: methanol extract, AE: aqueous extract, PC: positive control, NC: negative control
All values represent the mean±standard deviation (n = 3 test).

Haemolytic activity

H**a**emolytic activity of the *A. aspera* leaf extracts were screened against normal human erythrocytes. Both extracts showed very low h**a**emolytic effect. However the h**a**emolytic percentage increased with increase in dose. Methanol extract exhibited more h**a**emolytic effect than that of aqueous extract. The low h**a**emolytic effect of both the extracts suggests the less toxicity of the plant (Figure 5).

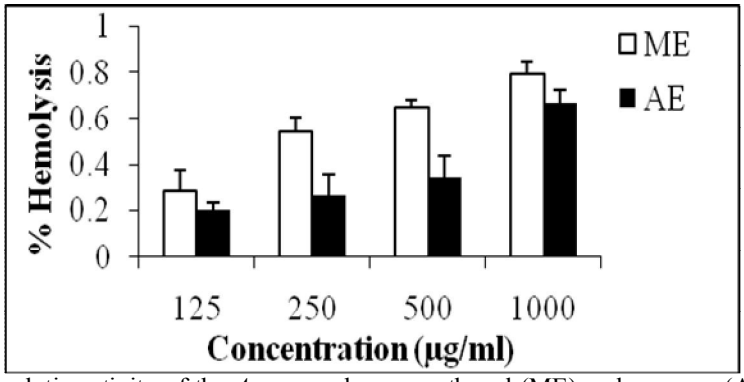

Fig. 5. Haemolytic activity of the *A. aspera* leaves methanol (ME) and aqueous (AE) extracts. Data is represented as mean ± SD (n = 3)

FT-IR analysis of the plant sample

The FTIR spectrum of leaves of *A. aspera* is reported in Figure 6A. Absorption band at 3438.03 cm^{-1} represent –OH group of phenolic compounds. It confirms the presence of phenolic compounds in the plant. The presence of other functional groups in *A. aspera* are listed in Table 3.

Table 3. Assignment of FT-IR absorption bands in the spectra of *A. aspera*

Absorption frequency (cm^{-1})	Tentative assignment
665.58	C-O-O, P-O-C bonding (aromatics) phosphate
774.90	CH out of plane bending (carbohydrate)
1076.89	C-O Stretching of polysaccharides,
1323.64	C=H deformations of $–CH_2$ or $–CH_3$ groups (lignin) in aliphatics
1383.49	C=H deformations of $–CH_2$ or $–CH_3$ groups (lignin) in aliphatics
1628.58	C=O Carbonyl Stretching (Secondary amides)
2927.55	Aliphatic $–CH_3$ and $–CH_2$ Stretching (chlorophyll)
3438.03	–OH group for phenols

UV-Visible spectroscopic analysis

Presence of phenolic compounds was further confirmed by UV- Visible spectroscopic analysis. Phenolic compounds exhibit two major absorption bands in the ultraviolet/visible region: a first band in the range between 320 and 380 nm and a second band in the 250 to 285 nm range (Mattha¨us 2002). The UV- Visible spectrum (200-600 nm) of leaves of *A. aspera* methanol and aqueous extracts is reported in Figure 6B. The absorption peaks at 230 (aqueous extract), 420 and 470 nm (methanol extract), characteristic of flavone/flavonol derivatives, carotenoids and b-cryptoxanthin epoxide respectively (Marcheix, 1990; Britton, 1991; Wanasundara *et al*., 1994; Guerra *et al*., 2005).

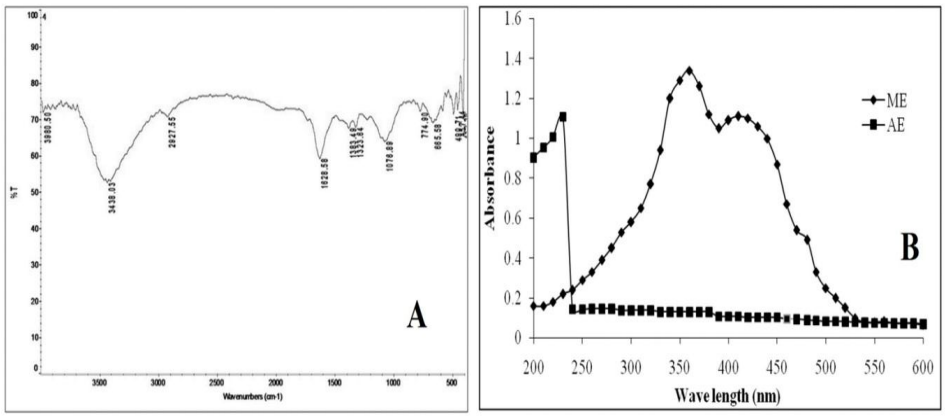

Fig. 6. Spectroscopic analysis. (A) FTIR spectrum of the leaves of *A. aspera,* (B) UV-Visible spectrum of *A. aspera* methanol (ME) and aqueous extract (AE)

The results obtained in the study represented that the aqueous and methanolic extracts of *A. aspera* leaves contain a variety of phytochemical compounds, which can effectively prevent free radical mediated cell damage by free radicals scavenging activity and thus can be used as a potent source of natural antioxidant compounds. Further, the phenolic compounds can be characterized by reverse phase HPLC analysis. This report also indicates the low antibacterial and h**a**emolytic effect of *A. aspera*. With all these results, we can conclude that *A. aspera* can be used as a source of safe and natural antioxidant compounds.

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