Pacific Science Review A: Natural Science and Engineering 18 (2016) 138–144

Contents lists available at ScienceDirect

Pacific Science Review A: Natural Science and Engineering

journal homepage: www.journals.elsevier.com/pacific-science-review-a-natural-science-and-engineering/

Chemical and biological evaluation of *Andrographis echioides* leaf extracts collected from the Vellore district in Tamil Nadu, India

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ARTICLE INFO

Article history:
Received 16 June 2016
Received in revised form
1 September 2016
Accepted 26 September 2016
Available online 22 November 2016

Keywords:
A. echioides
Total phenols
Total flavonoids
Total carbohydrate
Antioxidants
Antibacterial

ABSTRACT

The chemical and biological activities of crude extracts of *Andrographis echioides* leaves using petroleum (pet) ether, ethyl acetate, methanol and aqueous solvents were assayed.

The total phenol, flavonoid, protein and carbohydrate contents of *A. echioides* crude extracts were evaluated. The antioxidant activities of the leaf extracts were determined by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), hydrogen peroxide, metal chelating, reducing power and total antioxidant activity assays. In addition, the crude extracts were screened for antibacterial activity (gram-positive and gram-negative bacteria) by disc diffusion method and the minimum inhibitory concentration was determined.

Of the crude extracts tested, the pet ether extract of *A. echioides* contained more total phenols (0.01955 ± 0.0012 mg/g), flavonoids (1.2889 ± 0.3606 mg/g) and proteins (1.6065 ± 0.160 mg/mL). The aqueous extract of *A. echioides* was enriched for carbohydrate (467.6 ± 0.003 mg/mL). The methanol extract of *A. echioides* exhibited potent antioxidant activity IC50 values (1.14 ± 0.06 to 1.15 ± 0.45 μg/mL), as well as antibacterial activity (16 ± 1.527 mm), than the aqueous extract of *A. echioides* (20 ± 1.527 mm).

A. echioides has great potential as a source of anti-cancer compounds due to its high antioxidant content and antibacterial activity.
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1. Introduction

Plants have a long history of use in traditional medicine and represent a nearly limitless source of pharmaceutical compounds. In fact, plants have a large number of bioactive compounds, predominantly secondary metabolites, which are beneficial to humans [1]. Phenols, flavonoids, carbohydrates and proteins commonly found in plants are responsible for the anti-allergenic [2], antioxidant [3], antimicrobial [4], anticancer [5] and anti-inflammatory effects [6] that plants have.

Andrographis echiodes plants are seen mostly in dry places, such as India, Sri Lanka and South Asian countries, and exhibit diuretic [7], analgesic [8], anti-ulcer [9], hepatoprotective [10], and antioxidant [11] activities.

Free radicals are mainly responsible for oxidative disorders including cancer, neurodegeneration and inflammation. So natural antioxidant agents are more focused for use the prevention and treatment of diseases because of its side effect considerations compared to synthetic agents [12]. Anti-microbial studies using natural products rather than synthetic compounds have attracted more researchers to the field because they have fewer side effects when compared to the synthetic compounds [13]. New infectious diseases exist that are expected to be cured at an affordable cost. In this study, we report a different approach for determining the total amount of phenols, flavonoids, carbohydrates and proteins from the *Andrographis echioides* plant, which plays a major role in the resistance against microbial infections because of its few harmful and long term effects [14]. In a previous paper we reported some compounds, based on GC–MS analysis, which may be responsible for these chemical and biological activities in *A. echioides* [15].

2. Materials and methods

2.1. Plant material

The leaves of *A. echioides* were collected in August 2014 from a local field in the district of Vellore, Tamil Nadu, India (coordinates

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Peer review under responsibility of Far Eastern Federal University, Kangnam University, Dalian University of Technology, Kokushikan University.

http://dx.doi.org/10.1016/j.psra.2016.09.016
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12°58′09″N 79°09′21″E). The plant was authenticated by plant anatomy research centre Ref No: PARC/2014/2057. The specimen copy was given by The Flora of Madras Presidency by Gamble IS: 1921, Vol-II, Page no-1051.

2.2. Preparation of extracts

The leaf samples were dried at room temperature and samples were powdered using a mortar and pestle and were extracted using the Soxhlet extraction method (hot continuous percolation). Approximately 40 g of powdered leaves were used for the extraction of fatty acids, waxes and sterols using 300 mL of petroleum ether. The defatted material was extracted using ethyl acetate, methanol and water. The solvents were completely evaporated at 60 °C using a Rotavapor. Chemicals were (AR) were purchased from (SDFL) s d fine – chemical limited, Vellore, Tamilnadu, India. The residues were designated petroleum ether (PE), ethyl acetate (EA), methanol (MeOH) and aqueous (Aqs) extracts, respectively, and stored for analysis at 4 °C [16].

2.3. Chemical evaluation of *A. echioides* leaf extracts

2.3.1. Determination of phenol content

The amount of phenol present in the extracts was determined using a spectrophotometric Foline-Ciocalteau assay with slight modifications [17]. The reaction mixture was prepared using 1 mL of extract and 9 mL of distilled water to which 1 mL of Foline-Ciocalteau phenol reagent was added. After a 5 min incubation, 10 mL of 7% sodium carbonate solution was added to the mixture and the volume was adjusted to 25 mL. Standard concentration (10, 20, 30, 40, 50 μL/mg) of gallic acid were prepared as per the Foline-Ciocalteau assay. The reaction mixture was incubated for 90 min at room temperature after which the absorbance at 765 nm of the standard and sample solutions against a blank solution were determined. Total phenol content was expressed as mg of GAE/g of extract.

2.3.2. Determination of total flavonoid content

The total flavonoid content was evaluated by the colourimetric method with slight modifications [18]. Ten millilitres of sample and of a standard solution of quercetin diluted to 10, 20, 30, 40 and 50 mg/mL) was added to a 10 mL volumetric flask that contained 4 mL of DD water then 0.3 mL of 5% NaNO2 was added to the flask. After 5 min, 0.3 mL of 10% AlCl3 was added. At 6 min 2 mL of 1 M NaOH was added to the mixture followed immediately by 2.4 mL of double distilled water and thoroughly mixed. The absorbance of the mixture (which was pink in colour) was determined at 510 nm. The total flavonoid content of the extracts was expressed in terms of mg/100 g quercetin equivalents (QE).

2.3.3. Determination of total carbohydrate content

The phenol sulfuric acid method was used to estimate the total amount of carbohydrates present in each sample using spectrophotometric analysis [19]. Briefly, 100 mg of sample and the sample and standard were incubated in a water bath for three hours with 5 mL of 2.5 N HCl and were then cooled to room temperature. The reaction was neutralized with solid sodium carbonate until the effervescence ended. Next, the volume was adjusted to 100 mL and the solution was centrifuged. Volumes of working standard (0.2, 0.4, 0.6, 0.8 and 1 mL) were pipetted into a series of test tubes followed by 1 mL of phenol solution and 5 mL of 96% sulfuric acid. The tubes were shaken for 10 min then placed in a water bath at 25–30 °C for 20 min. The absorbance at 490 nm was measured and the amount of total carbohydrate present in the sample solution was evaluated using the standard graph.

2.3.4. Determination of total protein

The total protein content was analysed using spectrophotometric analysis [20]. The reaction mixture was prepared using 5 mL of Biuret reagent and a Biuret reagent blank in different test tubes. Protein standard was prepared using 10 mL of 1.5 mM sodium azide solution and the standard was diluted to 20, 40, 60, 80, and 100 g/L by adding the appropriate amount of water so that the final volume was 10 mL. The reagent and blank series were prepared by adding 100 μL of Biuret reagent and water. For sample preparation, 100 μL of extract was added to the Biuret reagent and the blank (separately) were incubated at room temperature for 30 min. After the incubation the absorbance was measured at 540 nm.

2.4. In vitro antioxidant activities

2.4.1. Scavenging activity on DPPH radical (DPPH method)

The free radical scavenging activity of the crude extracts was measured using a spectrophotometer with slight modifications to the published method [21]. The samples and standard were prepared by the following concentrations 10, 20, 50, 100, and 200 μg mL^{-1} in methanol. A 0.2 mmol L^{-1} solution was diluted with 50 mL of methanol incubated for 30 min at room temperature and the absorbance measured at λ_{max} of 517 nm. The % RSA values were converted to the half maximal inhibitory concentration (IC50) values using a graph plotted between the %RSA values and the concentrations. Ascorbic acid was used as a standard for this method.

$$\%\ \text{RSA} = [1 - (A\text{sample}/A\text{blank})]] \times 100$$

Standard: 4.4 mg DPPH in 100 mL of methanol.
Sample: 1 mL of sample solution mixed with 2 mL of 0.2 mmol L^{-1} DPPH solution
Blank: 1 mL of methanol mixed with 2 mL of 0.2 mmol L^{-1} DPPH solution
mmol L^{-1} DPPH solution.

2.4.2. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined using a previously modified method with slight modifications [22]. Briefly, hydrogen peroxide (2 mM) was prepared in a 50 mM phosphate buffer (pH 7.4) solution, and the plant samples and respective standards were prepared in the range of 100–500 μg/mL. The standard, ascorbic acid, was transferred into the test tubes and the volume brought up to 0.4 mL with 50 mM phosphate buffer (pH 7.4) or methanol. After the addition of 0.6 mL of hydrogen peroxide solution the tubes were vortexed and incubated. A blank was prepared using 50 mM phosphate buffer without hydrogen peroxide and sample solution. The absorbance was measured at 230 nm and the hydrogen peroxide scavenging activity was calculated using the formula:

$$\%\ \text{RSA} = [1 - (A\text{sample}/A\text{blank})] \times 100$$

2.4.3. Metal chelating activity

The chelating activity of the extracts was determined using ferrous ions according to a slightly modified, previously published method [23]. The reaction mixture was prepared using 2 mL of extract dissolved in DMSO at different concentrations (100, 200, 300, 400, and 500 μg/mL) followed by the addition of 50 μL of (2 mM) ferrous chloride and 200 μL (5 mM) of ferrozine solution. The solution was mixed thoroughly and incubated in the dark for 30 min. The absorbance was measured at 562 nm. A 10 mg/mL

solution of ethylene diamine tetra acetic acid (EDTA) in dimethyl sulfoxide (DMSO) was used as a standard.

$$\% \ RSA = [1 - (Asample/Ablank)] \times 100$$

2.4.4. Reducing power assay

The reducing power activity of the extracts was analysed using a slightly modified version of Oyaizu's method [24]. The reaction mixture consisted of different concentrations of extracts (10 mg/mL) 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 mol/L sodium phosphate buffer. The solution was incubated at 50 °C for 30 min, and the reaction was stopped with 2.5 mL of 10% trichloroacetic acid. Then, the sample was centrifuged at 3000 r/min for 10 min and 2.5 mL of the supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The blank contained distilled water and phosphate buffer and the absorbance was measured at 740 nm. An increase in absorbance indicated an increase in the reducing power of the sample. BHT was used as a standard.

2.4.5. Total antioxidant assay

The total antioxidant capacity of the plant extracts was evaluated by the phosphomolybdenum method [25]. Triplicates of 100–500 µL of plant extract sample (plant extract) and standard solution (ascorbic acid) were added to 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated for 90 min at 95 °C. The absorbance was measured at 695 nm against the reagent blank.

2.5. Biological evaluation of leaf extracts from A. echioides

2.5.1. Antibacterial activity

2.5.1.1. Microorganism and culture conditions. The bacterial pathogens used for the study were *Staphylococcus aureus* (MTCC 7405), *Bacillus subtilis* (MTCC 1168) for Gram positive, *Escherichia coli* (MTCC 1886), *and Proteus vulgaris* (MTCC 7299) for Gram negative microorganisms. All the bacterial and fungal pathogens were purchased from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the microbial pathogens were revived from 20% glycerol stocks stored at −80 °C. After warming to room temperature, the bacterial and fungal cultures were cultured for the activity assay in nutrient broth (NB) and Sabourdaud's dextrose broth (SDB) by incubating at 37 °C for 24 h.

2.5.2. Agar well diffusion method

The potential of petroleum ether, ethyl acetate, methanol and aqueous extracts for microbial growth inhibition were determined using the agar well diffusion method [26]. The pre-inoculated bacterial and fungal pathogens were uniformly spread on Muller Hinton Agar (MHA) and Sabourdaud's Dextrose Agar (SDA) using a sterile cotton swab. Wells were made in the media using a sterile cork borer. The extracts were dissolved in 10% DMSO to a final concentration of 1 mg/mL and then 100 µL of extract was transferred into the agar well. The standard (Ciprofloxacin) was used as a positive control and 10% DMSO was used as a solvent control. The bacterial cultures were incubated at 37 °C for 24 h and zones of inhibition were measured in mm after the incubation. The experiment was repeated thrice and average values were recorded for antimicrobial activity.

2.5.3. Minimum inhibitory concentration (MIC) determination

The MIC of the extracts was determined for the test organisms on which leaf extracts showed potent antibacterial activities [27]. The MIC assay is a technique used to determine the lowest concentration of a particular antibiotic needed to kill bacteria and fungi. Briefly, 100 µL of MHB and SDB were added to 1 mL of varying concentrations of the extracts and serially diluted to obtain the following final concentrations of extracts: 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, 3.12 mg/L, 1.56 mg/L, 0.78 mg/L and 0.39 mg/L. Afterwards, 20 µL of the test bacterial organisms, *E. coli*, *B. subtilis*, *P. vulgaris and S. aureus*, were introduced to the 96 well plates. Growth control (cells + broth) and media control (broth only) conditions were also added to the plates. Wells containing bacterial cultures were then incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration (mg/L) of extract showing no visible bacterial and fungal growth. The results were measured at 556 nm using an Enzyme Linked Immunosorbent Assay (ELISA) microplate reader.

3. Results and discussion

3.1. Total phenol content

The total phenolic content of the leaf extracts ranged from 0.1649 to 0.1955 mg GAE/g (Table 1). The pet ether extract had higher phenolic content compared to the ethyl acetate, methanol and aqueous extracts of *A. echioides*.

3.2. Total flavonoid content

The total flavonoid content of the leaf extracts ranged from 0.991 to 1.288 mg QE/g (Table 1). The pet ether extracts contained enriched amounts of flavonoids compared with the ethyl acetate, methanol and aqueous extracts.

3.3. Total carbohydrate content

The total carbohydrate content of the leaf extracts ranged from 0.249 to 0.467 mg/100 mL of glucose (Table 1). The aqueous extracts had the maximum amount of carbohydrate.

3.4. Total protein content

The total protein content of the leaf extracts ranged from 0.804 to 1.606 mg/mL (Table 1). The pet ether extracts had higher protein content compared to the ethyl acetate, methanol and aqueous extracts.

Overall, the pet ether extract had trace amounts of phenols, flavonoids and proteins. Flavanoid and protein concentration was highest in the ethyl acetate extract while carbohydrate and flavonoid content was higher in the methanol extract compared with the aqueous extract.

3.5. In vitro antioxidant assay on A. echioides leaf extracts

3.5.1. Scavenging activity on DPPH radical (DPPH method)

DPPH is a stable free radical widely used to test the ability of extracts to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity of an *A. echioides*. The IC50 values for DPPH scavenging of extracts are summarized with ascorbic acid as a standard (Table 2). The scavenging effects of the different extracts on the DPPH radical were expressed as half maximal inhibitory concentration (IC50). The IC50 values of plant extracts were recorded as 2.40 µg/mL for the pet ether extract, 2.30 µg/mL for the ethyl acetate extract, 1.14 µg/mL for the methanol extract and 4.33 µg/mL for the aqueous extract. Based on these data, the methanol extract had the highest scavenging activity.

Table 1
Total estimation of chemical compounds in *A. echioides* leaf extracts.

S. no	Crude extracts	Phenolics (mg/g)	Flavonoids (mg/g)	Carbohydrate (mg/mL)	Proteins (mg/mL)
1	Pet ether	0.1955 ± 0.0012	1.2889 ± 0.3606	249.8 ± 0.0606	1.6065 ± 0.160
2	Ethyl acetate	0.1669 ± 0.0026	0.991 ± 0.1796	354.4 ± 0.0796	1.2591 ± 0.079
3	Methanol	0.1649 ± 0.0014	1.0906 ± 0.1202	386.3 ± 0.0202	0.6062 ± 0.120
4	Aqueous	0.1779 ± 0.0043	1.1047 ± 0.2003	467.6 ± 0.003	0.8047 ± 0.202

Table 2
IC50 values of *A. echioides* leaf extracts as determined by antioxidant assays.

S. no	Plant extract	DPPH (IC50)	Hydrogen peroxide (IC50)	Metal chelating (IC50)
1	Pet ether extract	2.40 ± 0.12	4.69 ± 0.02	5.64 ± 0.02
2	Ethyl acetate extract	2.30 ± 0.17	2.92 ± 0.06	2.76 ± 0.27
3	Methanol extract	1.14 ± 0.06	1.51 ± 0.04	1.15 ± 0.45
4	Aqueous extract	4.33 ± 0.13	5.70 ± 0.08	6.12 ± 0.72
5	Standard	0.71 ± 002	0.83 ± 0.04	1.02 ± 0.08

3.5.2. Hydrogen peroxide scavenging activity

The radical scavenging property of a compound, which may serve as a significant indicator of its potential antioxidant activity, was evaluated using the H2O2 radical scavenging ability of *A. echioides* and compared with ascorbic acid as a standard (Table 2). The IC50 values of the extracts were recorded as 4.69 μg/mL, 2.92 μg/mL, 1.51 μg/mL and 5.70 μg/mL for pet ether, ethyl acetate, methanol, and aqueous extracts, respectively. The data from this assay also showed that the methanol extract had the highest scavenging activities.

3.5.3. Metal chelating activity

Iron is essential for life as it is required for oxygen transport, respiration and for activity of many enzymes. Chelating agents inhibit lipid peroxidation by stabilizing the transition metals. The metal chelating ability of different *A. echioides* extracts and EDTA standard are presented in Table 2. The IC50 values were 5.64 μg/mL for the pet ether extract, 2.76 μg/mL for the ethyl acetate extract, 1.15 μg/mL for the methanol extract and 6.12 μg/mL for the aqueous extract. These data showed that the methanol extracts had the highest chelating activity.

3.5.4. Reducing power assay

The reducing ability of a compound generally depends on the presence of reductones which exert their antioxidant activity by donating a hydrogen atom and breaking the free radical chain. The reductive abilities of the *A. echioides* extracts analysed and compared with butylated hydroxyl methyl toluene (BHT) are shown in bar diagram 4. As the concentration of the extract increased the reducing power increased. The reducing power of the extracts was 0.0874 μg/mL for the pet ether extract, 0.1063 μg/mL for the ethyl acetate extract, 0.1369 μg/mL for the methanol extract and 0.0861 μg/mL for the aqueous extract. These data show that the methanol extract had good reducing power(Figs. 1–4).

3.5.5. Total antioxidant capacity assay

The total antioxidant capacity (TAC) of *A. echioides* was based on the reduction of Mo(VI) to Mo(V) by the sample extract and subsequent formation of a green phosphate Mo(V) complex at an acidic pH. The assay evaluated both water-soluble and fat-soluble antioxidants (total antioxidant capacity). The results indicated a higher TAC in the *A. echioides* extracts compared with the standard, ascorbic acid (Fig. 5). The total antioxidant capacity increased with increasing concentrations of the extract. The antioxidant capacity was 0.0298 μg/mL for the pet ether extract, 0.0439 μg/mL for the ethyl acetate extract, 0.0947 μg/mL for the methanol extract and 0.0352 μg/mL for the aqueous extract. The methanol extract had the highest total antioxidant capacity of all the *A. echioides* extracts.

3.6. Antibacterial activity

3.6.1. Zone of inhibition using crude extracts of A. echioides by the well diffusion method

The agar well diffusion method is the widely accepted method for the evaluation of the antibacterial activity of samples. A preliminary screening for the antibacterial activity of the extracts

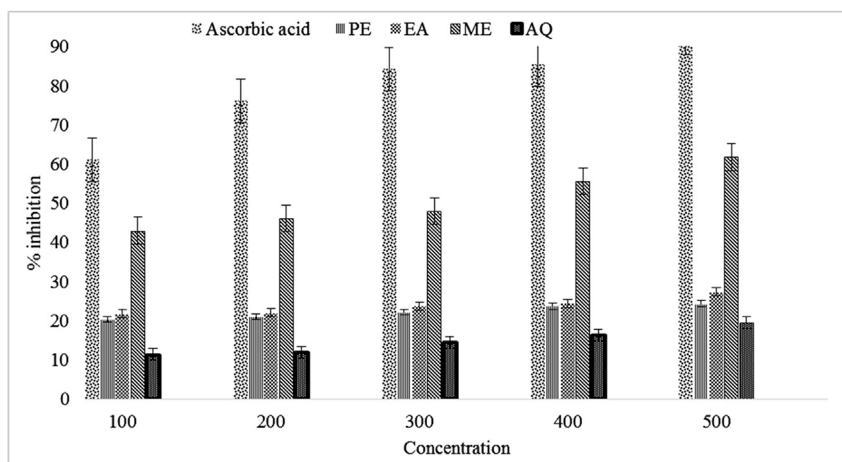

Fig. 1. DPPH assay on *A. echioides* leaf extracts.

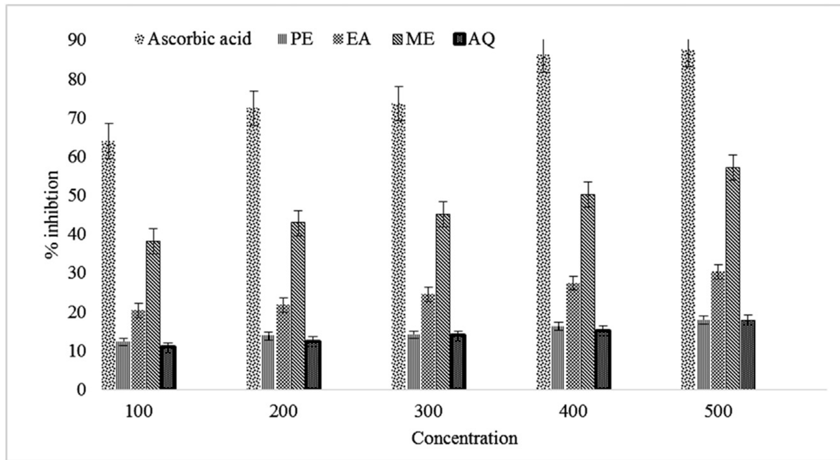

Fig. 2. Results of the hydrogen peroxide assay on *A. echioides* leaf extracts.

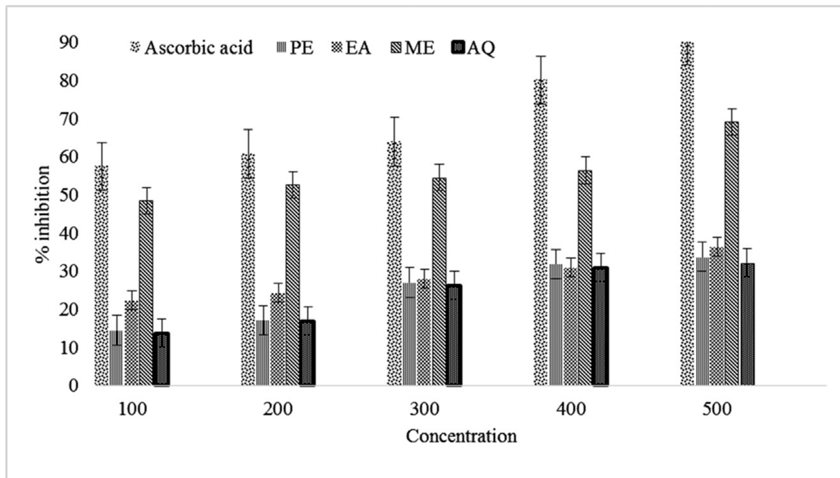

Fig. 3. Percentage inhibition of the metal chelating activity of *A. echioides* leaf extracts *echioides.*

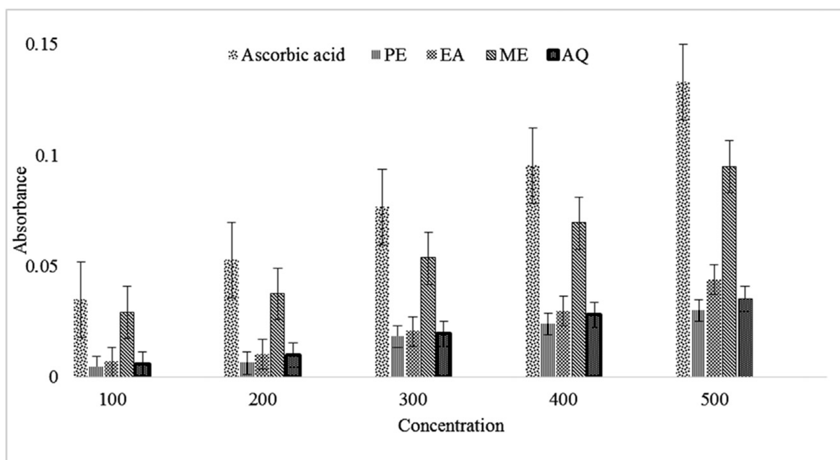

Fig. 4. Reducing power of *A. echioides* leaf extracts.

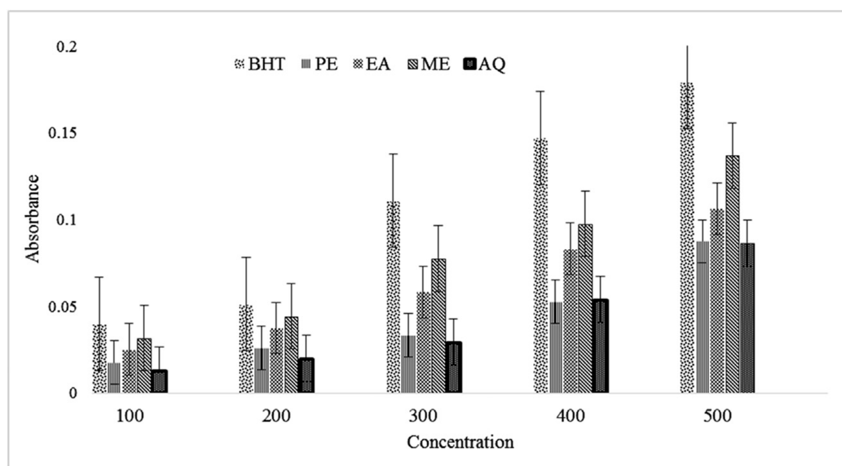

Fig. 5. Total antioxidant capacity of *A. echioides* leaf extracts.

Table 3
Zone of inhibition using crude extracts of *A. echioides* by the well diffusion method.

Pathogens	Methanol (mm)	P. ether (mm)	Water (mm)	Ethyl acetate (mm)	Control (mm)
S. aureus	16 ± 1.527	14 ± 0.577	20 ± 1.527	–	25 ± 2.51
B. subtilis	–	14 ± 1.527	–	–	30 ± 0.577
P. vulgaris	13 ± 0.577	–	–	–	30 ± 1
E. coli	14 ± 1.527	14 ± 1.154	17 ± 2	–	28 ± 1.155

Table 4
MIC values of crude extracts of *A. echioides*.

Pathogens	Methanol μg/ml	Pet ether μg/ml	Ethyl acetate μg/ml	Water μg/ml	Control (mm)
S. aureus	0.79 ± 0.648	0.49 ± 0.052	0.46 ± 0.041	0.73 ± 0.067	0.80 ± 0.011
B. subtilis	0.71 ± 0.047	0.52 ± 0.022	0.52 ± 0.108	0.79 ± 0.065	0.09 ± 0.0237
P. vulgaris	0.68 ± 0.016	0.50 ± 0.065	0.62 ± 0.087	0.73 ± 0.047	0.37 ± 0.0438
E. coli	0.67 ± 0.067	0.48 ± 0.056	0.60 ± 0.076	0.76 ± 0.084	0.70 ± 0.0476

was performed with various strains of gram-positive and negative pathogenic bacterial strains. Of the four extracts three showed moderate amounts of antimicrobial activity. The methanol extract had a diameter of 16–18 mm, the pet ether extract had a diameter of 15–16 mm, the aqueous extract had the highest antibacterial activity with a diameter of 19–22 mm (Table 3).

3.6.2. Minimum inhibitory concentration (MIC) of A. echioides leaf extracts against bacterial pathogens

The MIC values of the *A. echioides* extracts ranged from 50 to 3.12 μg/mL. The most significant inhibition of bacterial growth, with an MIC value of 0.76 ± 0.084/3.12 μg/ml against *E. coli* in aqueous solution was shown by pet ether extracts (Table 4).

4. Conclusions

The results revealed that *A. echioides* is a good source of antioxidants and antimicrobial compounds. The methanol extracts exhibited excellent antioxidant activity and the aqueous extracts showed prominent antimicrobial activity compared with the other crude extracts. The antioxidant activity in the methanol extracts may be due to the presence of diphenylmethane [15]. The pet ether, methanol and aqueous extracts all showed potent antibacterial activity. This antibacterial activity is due to the presence of 1-hexadecene [15]. *A. echioides* could be used as an antioxidant agent as it has high amounts of natural antioxidants. Antioxidant supplements contribute to the protection against cancer and cardiovascular health problems. This research was focused on the isolation and evaluation of anticancer bioactive compounds with high antioxidant and antimicrobial activities.

Acknowledgements

The authors are grateful to VIT University for providing the necessary laboratory facilities and financial support.

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