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EVALUATION OF ANDROGRAPHIS ECHIOIDES FOR ITS PHYTOCHEMICAL AND IN VITRO ANTIOXIDANT PROPERTIES

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ABSTRACT

The aim of the present study was to screen phytochemical derivatives from an Indian medicinal plant *Andrographis echioides* (L.) Nees and to evaluate its antioxidant potential. A preliminary phytochemical screening was carried out in plant extracts using qualitative and quantitative methods. Polar solvents showed presence of tannins, glycosides, proteins and organic acids while non-polar solvents showed presence of steroids, triterpenes and polysaccharides. The study concludes that the methanolic seed extract of plant possesses potent antibacterial property and justifies the need for further investigations and characterization of the bioactive compounds present in it. Based on the antioxidant assay results it was found that methanol extract exhibited better free radical scavenging activity than other extracts. The methanol extract exhibited highest DPPH scavenging activity exhibited best antioxidant activity with an EC_{50} value of 51.98 mg/mL. Among the different extracts, methanol was more effective in all the antioxidant assays i.e. DPPH radical scavenging assay and superoxide scavenging assay.

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INTRODUCTION

Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources. Phytomedicine or phytotherapy or botanical medicine was collectively called as herbal medicine. It refers to herbal materials or herbal preparations that contain plant parts as active ingredients. The lack of scientific evidence in herbal medicines, when compared to that of modern medicine, gradually made herbal medicine to become unpopular among people.

Even though, herbal medicine had a long history of its effective usage, chemical analysis and modification of herbal active molecules to synthetic pharmaceuticals and also the fast therapeutic actions of synthetic drugs altogether added to the disinclination of herbal medicine (Newman and Cragg, 2007)(Parasuram *et al.*, 2014). The importance of plant and their role in nature cannot be over emphasized. Majority of the plants are edible and are composed of different quantity of vitamins, protein or carbohydrates. These components help the body to replace worn out cells or tissues, digest food and combat diseases among other health related problems (Halliwell, 2009). Plant-derived compounds have played an important role in the development of several clinically useful anti-cancer agents. The first agents to advance into clinical use were the vinca alkaloids, vinblastine and vincristine from the *Madagascar periwinkle*, *Catharanthus roseus* (L.) (Apocynaceae) as anticancer agents, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma. India's Council for Scientific and Industrial Research (CSIR) in 2001 had launched a traditional knowledge digital library of remedies and medicinal plants. European patent office had been able to consult the 34 million page, multilingual database for granting patents and also had cancelled or withdrawn 36 applications to patent traditionally known medicinal formulations in just under two years (Rinaldi and shetty. 2015).

Andrographis echioides L. is an annual herb present in throughout South Indian. However, information on the chemical composition and bioactivity of this species is very rare (Govindachari et al., 1965; Shen et al., 2013). The plant from genus Andrographis is used in goiter, liver diseases (Nadkarni et al., 1976), fertility problems, bacterial (Qadre et al.,2009), malarial and fungal disorders (Pandikumar et al., 2005). Leaf juice boiled with coconut oil had controlled the falling and graying of hair (Nirubama and Rubalakshmi, 2014) and anti-inflammatory activity of this plant was thoroughly studied by (Basu, 2009). Recently, *in vitro* antioxidant potential of *A. echioides* was reported (Ruba and Mohan, 2016). In this article, we report a detailed investigation of phytochemicals by qualitative and quantitative, GC-MS analysis and antioxidant activity of whole plant extracts of *A. echioides*. In addition, the phytochemicals especially phenolic contents have been played the relationship between their biological efficacy towards antioxidant activity.

MATERIALS AND METHODS

Chemicals and reagents

Acetic acid, ammonium molybdate, barium chloride, chloroform, 1,1-Diphenyl-2-picryl hydrazyl (DPPH), dragendorff's reagent, DMSO, ethanol, ether, $FeCl_2$, ferric chloride, ferrozine, hydrochloric acid, hydrogen peroxide, magnesium chips, mayer's reagent, methanol, molisch's reagent, Mueller Hinton Agar (MHA), ninhydrin, nutrient broth, nitroblue tetrazolium (NBT), potassium hydroxide, pyridine, phenazine methosulphate (PMS), phosphate buffer, sodium phosphate, sodium nitroprusside, sulfanilamide sulphuric acid were procured from Sigma Aldrich, E-Merck and Hi Media.

Collection and identification

Andrographis echioides were collected from in and around Sivakasi, TN State, India. The plant was identified by Dr. A. Saraswathy, Director, Captain Srinivasan Murthi Institute of Ayurveda and Siddha drug development, Arumbakkam, Chennai-106.

Preparation of plant extract

Healthy and fresh leaves of *Andrographis echioides* were collected from the field and washed thoroughly with running tap water and rinsed in sterile distilled water by following method of (Akowuah et al., 2005). The washed plant materials were shade dried at room temperature for 10 to 15 days. The shade dried plant parts were made into coarse powder and it was extracted with different solvents such as hexane, chloroform, ethyl acetate, methanol and aqueous in a Soxhlet apparatus for 8 to 16 h. The extracts were concentrated using a rotary evaporator (Heidolph laborata, Germany) at various temperature under reduced pressure.

Preliminary screening of phytochemicals

Screening for the presence of active phytochemicals in leaf extracts of *A. echioides* was carried out using the standard method (Harborne, 1998).

Test for alkaloids

For 2 mL of leaf extract, 2 mL of conc. hydrochloric acid and few drops of Mayer's reagent were added. Appearance of green color or white precipitate indicated the presence of alkaloids.

Test for anthraquinones

For 1 mL of leaf extract few drops of 10% ammonia solution was added, appearance of pink color precipitation indicated the presence of anthraquinones.

Test for carbohydrates

For 2 mL of leaf extract, 1 mL of Molisch's reagent and few drops of conc. sulphuric acid were added. Appearance of purple or reddish color indicated the presence of carbohydrates.

Test for flavonoids

For 2 mL of leaf extract, 1 mL of 2 N sodium hydroxide was added. Appearance of yellow color indicated the presence of flavonoids.

Test for glycosides

For 2 mL of leaf extract, 3 mL of chloroform and 10% ammonia solution was added. Formation of pink color indicated the presence of glycosides.

Test for phenols

For 1 mL of the leaf extract, 2 mL of distilled water followed by few drops of 10% ferric chloride was added. Presence of blue or green color indicated the presence of phenols.

Test for quinones

For 1 mL of leaf extract, 1 mL of conc. sulphuric acid was added. Appearance of red color indicated the presence of quinones.

Test for Saponins

For 2 mL of leaf extract, 2 mL of distilled water was added and vortex in a graduated cylinder for 15 minutes. Formation of 1 cm foam layer indicated the presence of saponins.

Test for tannins

To 1 mL of leaf extract, 2 mL of 5% ferric chloride was added. Appearance of dark blue or greenish black indicated the presence of tannins.

Test for Triterpenoids

For 1.5 mL of leaf extract, 1 mL of Libermann-Buchard reagent (acetic anhydride + conc. sulphuric acid) was added. Appearance of blue green color indicated the presence of triterpenoids.

Test for steroids and phytosteroids

For 1 mL of leaf extract equal volume of chloroform and few drops of conc. sulphuric acid were added, appearance of brown ring indicated the presence of steroids and formation of bluish brown ring indicated the presence of phytosteroids (Harborne, 1998).

***In vitro* antioxidant activity**

Free radical scavenging assay using DPPH

The free radical scavenging activity of each sample was determined using the UV/vis spectrophotometer according to the method described by (Leong and Shui, 2002). Briefly, 0.1 mM solution of DPPH in methanol was prepared and the initial absorbance was measured at 517 nm and did not change throughout the period of assay. An aliquot (20-100 µL) of an extract was added to 3 mL of methanolic DPPH solution. Methanol alone served as blank and DPPH in methanol without plant extracts served as positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 517 nm. Radical scavenging activity was calculated as follows:

$$\mathbf{FRSA = [(A_c - A_s) / A_c] \times 100}$$

Where *A*c is the absorbance of the control and *A*s is the absorbance of the tested sample after 30 min.

Superoxide anion radical scavenging assay

The measurement of superoxide anion radical scavenging of different extracts were performed with slight modifications (Sun et al., 2004). The nitro blue tetrazolium (NBT) solution of 1 mL (156 µM), 1 mL of NADH solution (468 µM) and various concentrations of test samples (20–100 µg/mL) were mixed and the reaction started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM) prepared in phosphate buffer (100 mM, pH 7.4). The reaction mixture incubated at 25°C for 5 min and the absorbance was measure at 560 nm.

$$\mathbf{Radical\ scavenging\ activity\ (\%) = [(A_O - A_1) / A_O] \times 100}$$

Where A_O is absorbance of the control, A_1 is the absorbance of test samples

Antibacterial activities - Agar well diffusion

Sterility test of the plant extracts

Plant extracts were tested for growth or contaminants. Pour 1 mL of each extracts on to the sterile Mueller Hinton Agar plates and incubated at 37°C for 24 h. After incubation, plates were observed and absence of growth in the extracts indicated that they were sterile and it could assess for antibacterial activity.

Preparation of inoculum

The human pathogenic bacteria namely *Escherichia coli* (MTCC443), *Bacillus subtilis*, (MTCC 441), *Micrococcus luteus* (MTCC 4698), *Staphylococcus aureus* and *Pseudomonas aeruginosa* (1034) were procured from the Microbial type culture collection (MTCC) center, IMTECH, Chandigarh, India. The obtained bacterial isolates were cultured on nutrient agar and incubated at 37ºC for 24 h. After, they were inoculated into nutrient agar slants and stored at 4ºC. Overnight broth culture of the respective bacterial strains was adjusted to turbidity equivalent to 0.5 McFarland standards.

Antibacterial activity of *A. echioides* was determined using agar well diffusion method (Holder and Boyce, 1994). When the media solidified, 0.1 mL of active growth culture was poured over feeder layer and spread evenly by sterile spreader. The 6 mm diameter well was made by using a sterile cork borer. Each well received different concentration (50, 100 and 150 µg/mL) of crude extract, they were dissolved in 0.4% DMSO (Dimethyl sulfoxide) and were incubated at 37ºC for 48 h, appropriate control was maintained. After incubation, the inhibition zone was measured by millimeter.

RESULTS AND DISCUSSION

Quantification of bioactive constituents of *A. echioides*

The whole plant extract of *A. echioides* was extracted sequentially with different solvents such as petroleum ether, chloroform, ethyl acetate, methanol and aqueous (non-polar to polar) and it was concentrated using a rotary evaporator. Whole plant extracts of *A. echioides* methanolic extract yielded dark brown paste which was 14.35% and in ethyl acetate extract it was 6.25%, respectively. Although, hexane and chloroformic extract yielded 3.80% and 2.50% of bioactive principles, it was also dark brown in texture (Table. 1).

Phytochemical analysis of different solvent extracts of *A. echioides*

Preliminary screening of phytochemicals

Phytochemical screening of whole plant extracts of *A. echioides* revealed the strong presence of various active phytochemicals. The qualitative phytochemical analysis revealed, that the methanolic extract of *A. echioides*, showed the strong presence of alkaloids, anthraquinones, coumarins, flavonoids, phenols and tannins, while ethyl acetate extract revealed abundant presence of steroids and tannins and chloroformic extract contained maximum terpenoids (Table 2). Though, ethyl acetate extract possessed most of the phytochemicals, it lacked glycosides and terpenoids. Comparatively, moderate amounts of phytochemicals were present in petroleum ether and aqueous extracts. The alkaloids, flavonoids, quinones and phenols were present in abundance in the methanolic extract of *A. echioides*, which might provide credence to their antioxidant and antidiabetic activities.

Table 1. Qualitative phytochemical analysis of *A. echioides.*

Phytochemicals	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Aqueous
Carbohydrates	+++	+++	++	+++	+++
Alkaloids	+	++	++	+++	++
Flavonoids	+	+++	+++	+++	++
Phenols	+	++	+++	+++	++
Fatty acids	+	++	+	+++	-
Steroids	+	-	+	+	++
Anthroquinones	+++	++	+++	+++	++
Triterpenoids	++	++	+++	+++	-
Glycosides	++	++	++	+++	++
Tannins	++	-	++	+++	++
Saponins	++	++	++	+++	++

Table 2: Quantitative phytochemicals present in *A. echioides.*

Constituents	Chloroform (mg/g)	Ethyl acetate (mg/g)	Methanol (mg/g)
Alkaloids	4.93	8.09	10.84
Triterpenoids	1.32	4.77	12.56
Steroids	2.78	0.46	4.96
Tannins	8.16	9.17	12.67
Saponins	3.64	1.02	9.36
Flavonoids	2.92	3.90	6.21
Anthraquinones	4.64	4.81	11.93
Proteins	4.22	9.23	10.12
Carbohydrates	6.9	7.5	5.4
Phenols	5.79	8.34	6.23

Quantitative phytochemical analysis of *A. echioides*

Since, the methanolic and ethyl acetate solvent extracts contained maximum of quantity of phytochemicals (as evident from qualitative screening) only those solvents alone were selected for the quantification of secondary metabolites. Among the extracts, the chloroformic extract showed the maximum quantity of flavonoids 12.67, anthraquinones 12.56, quinones 11.93, phenols 6.21 and alkaloids 10.54 mg/g of crude extract (Table. 2).

GC-MS analysis of chloroformic extract of *A. echioides*

The rapid and simple method outlined the active principles from the studied herbs which are responsible for some therapeutic and aromatic effects. Results of the GC-MS analysis of ethanol extract provide 10 major peaks (Table. 3) determining the presence of phytoconstituents with different therapeutic potential.

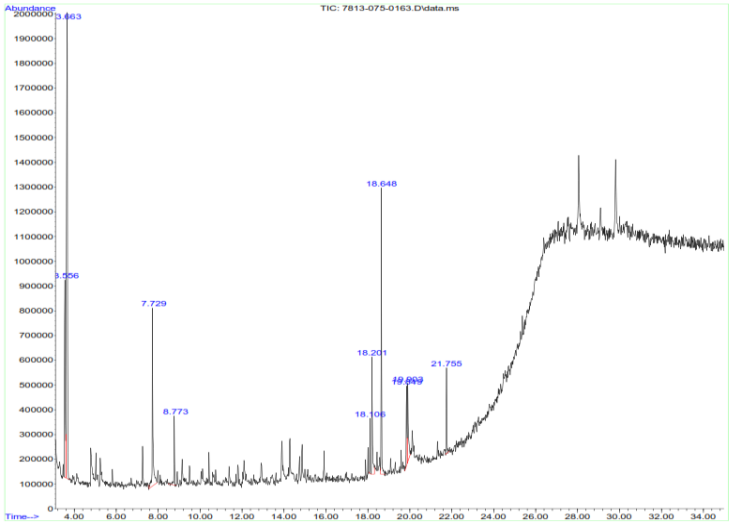

Fig 1. Shows GC of ethyl acetate extract of *A. echioides.*

Table 3. Shows compounds identified in methanolic extract of *A. echioides.*

S. No	RT	Phytocompound(s)	Molecular formula	Mol. weight (g/mol)	Peak area (%)
1.	3.522	Propionic acid	$C_3H_6O_2$	74.07854	11.54
2.	3.668	Benzene	C_6H_6	78.11	38.65
3.	7.734	Glycerin	$C_3H_8O_3$	92.09382	11.47
4.	8.780	Naphthalene	$C_{10}H_8$	128.1705	3.08
5.	18.104	2,6-Dimethoxyamphetamine	$C_{11}H_{17}NO_2$	195.26	2.03
6.	18.205	Indole -3- ethanamine	$C_{10}H_{12}N_2$	160.22	6.67
7.	18.641	1,2-Benzenediamine	$C_6H_8N_2$	108.143	11.98
8.	19.847	9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	294.4721	4.20
9.	19.905	Phenylephrine	$C_9H_{13}NO_2$	167.20502	6.25
10.	21.749	Indole -3- ethanamine	$C_{10}H_{12}N_2$	160.22	4.24

These compounds were identified through mass spectrometry coupled with Gas Chromatography. The mass spectra of these compounds were matched with those found in the NIST/NBS spectral database and the data are given. The GC-MS analysis of methanol extract revealed the presence of 10 bioactive metabolites such as Propionic acid, Benzene, Naphthalene, 2, 6 Dimethoxyamphetamine, Indole-3-amphetamine, Indole-3-ethanalamine, 1,2-Benzenediamine, 9,12-Octadecadienoic acid, Phenylephrine and Indole -3- ethanamine. Among them, the major components were, Benzene (38.65%), 1,2-Benzenediamine (11.98%), Glycerin (11.47%) and Phenylephrine 6.45% (Figure 1; Table 2).

***In vitro* antioxidant activity**

DPPH scavenging activity of different extracts of *A. echioides*

The free radical scavenging activity of root extracts of *A. echioides* such as petroleum ether, chloroform, ethyl acetate, methanol and aqueous were evaluated by their ability to scavenge DPPH radicals and was compared with ascorbic acid standard. Among the different extracts screened for the assay, maximum radical scavenging activity in methanol extract (91.29±0.23% at 100 µg/mL with an EC_{50} value of 49.58 µg/mL followed by ethyl acetate 78.44±0.41% at with an IC_{50} value of 57.40 µg/mL. Chloroform showed 60.04±0.41% radical scavenging 78.10 µg/mL, aqueous extract exhibited the minimum radical scavenging activity of 50.67±1.60 and petroleum ether extract revealed low activity 45.98±0.20 µg/mL respectively, when compared with standard BHT 84.96±0.76% activity with an EC_{50} value of 52.77 µg/mL (Fig. 1).

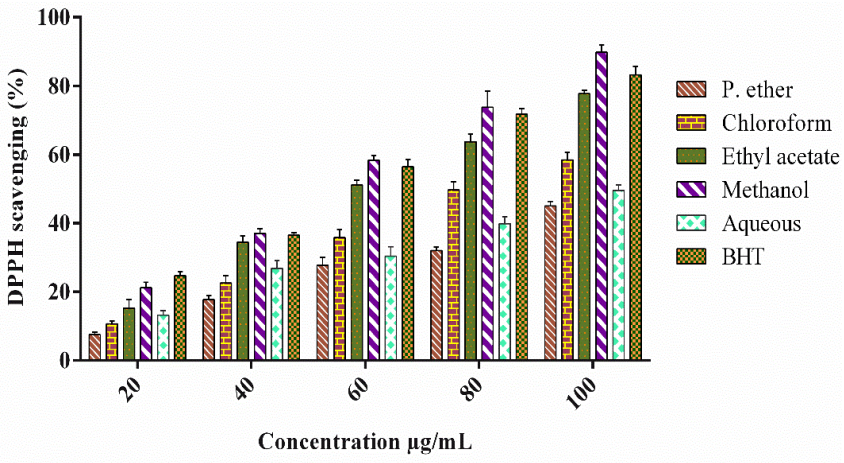

Fig 2. Free radical scavenging of *A. echioides.*

Superoxide radical scavenging activity of different extracts of *A. echioides*

In the present study, all extracts were tested for super oxide anion radical scavenging assay and the concentration ranged from 20-100 µg/mL. Out of the five extracts tried, methanolic extract showed maximum level of inhibition, while the remaining extracts showed varied levels of superoxide anion radical scavenging activity (Table. 6). EC_{50} value of methanolic extract was found to be lower (53.7 µg/ml) while for ethyl acetate extract, it was 77.20 µg/ml, for aqueous extract it was 109.97 µg/ml, for chloroform 104.02 µg/ml and in petroleum ether extract the values were 124.13 µg/ml respectively (Fig. 2). The order of scavenging of different extracts at 100 µg/ml concentration was in the decreasing order, starting from methanol 87.27± 0.48%, ethyl acetate 62.08 ± 0.79%, chloroform 47.49 ± 0.75%, aqueous 45.60 ±1.20% and petroleum ether 41.25 ± 0.39%. In this methanolic extracts (IC_{50} 46.33 µg/ml) behaved as a powerful superoxide anion scavenger that could be tried for therapeutic use against oxidative stress compared with standard Rutin (12.56 µg/ml). The IC_{50} values were obtained after extrapolation through linear regression analysis.

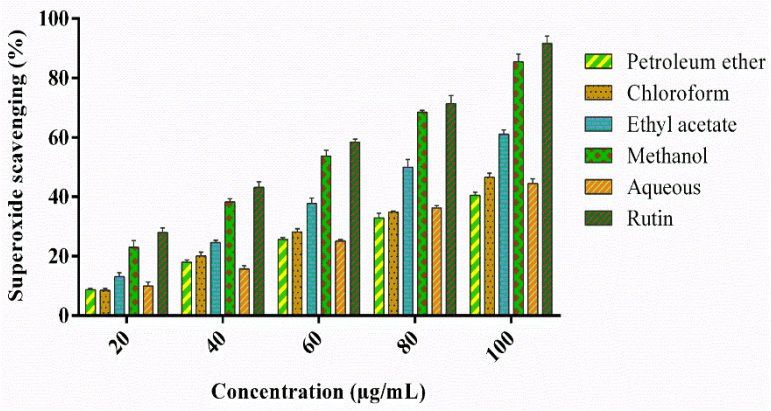

Fig 3. Superoxide scavenging of *A. echioides*.

Sterility of plant extracts

Sterility of the plant extracts of *A. echioides* resulted the absences of growth in different solvent extracts which indicated that they were sterile and it could be used for antibacterial activity.

In vitro antibacterial activity

The antibacterial efficacy of methanolic extract of *A. echioides* showed dose dependent inhibition against human pathogenic bacteria such as *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Staphylococcus aureus* (Table. 1). The most susceptible microorganisms were *S. aureus* (20 mm), *E. coli* (19 mm) while the least susceptible was observed in *M. luteus* (10 mm) with least concentration (150 µg/mL, Table. 3). The results suggested that antibacterial activity of methanolic extract of *A. echioides* is the result of a synergic or additive effect of several compounds present in *A. echioides*.

Table 3. Antibacterial activity of different extracts *Andrographis echioides*.

Extract(s)	Zone of inhibition (mm)														
	B. subtilis			***E. coli***			***M. luteus***			***S. aureus***			***P. aeruginosa***		
	µg/mL														
	50	100	150	50	100	150	50	100	150	50	100	150	50	100	150
Hexane	-	8	11	7	10	12	-	7	8	-	8	11	9	12	13.5
Chloroform	7	9	12	10	14	16	8	10	13	-	12	15	-	-	11
E. acetate	-	10	13	9	12	16	-	8	9	7	8	10	-	8	9
Methanol	11	14	17	9	13	19	7	9	14	10	16	20	7	9	17

DISCUSSION

The phytochemical analysis of the plant indicated various class of molecules in different extracts of the whole plant extracts. The methanolic extract showed the significant presence of diverse class of molecules including terpenoids, flavonoids and tannins, phenols and glycosides. On the other hand, the chloroform extract possessed a good amount of flavonoids and steroids. The petroleum ether extract showed the presence of smaller amount of steroids and flavonoids. Flavonoids have antioxidant activities and they have the property of preventing oxidative cell damage and carcinogenesis. Steroids, abundant in many plants, have been shown to have hypercholesterolemia effects, diuretics and also exhibit anti-leukemic, antipyretic, and derivatives of steroids are active as anticancer and anti-viral agents. Steroids have been reported to stimulate menstrual discharge and diminish secretion of milk (Shahidi and Wanasundara, 1992).

Chlorozotocin is a nitrosourea, *i*t is used in cancer therapy and also used in a cytostatic agent that is used in the investigational treatment of cancers of the stomach, lung, large intestine and pancreas. 2-Furancarboxylic acid is a preservative in industrial use, acting as a bactericide and fungicide and its derivatives also aid in the production of nylons are often used in biomedical research. Chlorozotocin is a cytostatic agent that is used in the investigational treatment of cancers of the stomach, large intestine, pancreas and lung.

Free radicals and other reactive oxygen species are considered to be important causative factors in the development of diseases of ageing such as neurodegenerative diseases, cancer and cardiovascular diseases. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Dehpour et al., 2009). Phytochemicals have long been recognized to possess many properties including anti-oxidant, anti-allergic, anti-inflammatory, anti-viral, anti-proliferative and anti-carcinogenic (Eastwood, 1999) (Bhargav et al., 2013). Presence of free radicals and oxidative stress are implicated in the onset of many diseases like stroke, asthma, cancer, atherosclerosis, diabetes and arthritis. There had been an increasing interest on plant derived antioxidants, because it could protect our body from free radical damage, diabetes and age related disorders (Forstermann, 2010). Reactive oxygen species (ROSs) exist in various forms, including free radicals such as free radicals such as superoxide ions (O_2^-), hydroxyl radicals (OH) and peroxyl (Ooh, ROO) radicals as well as non-free radicals such as hydrogen peroxide (H_2O_2) (Squadriato et al., 1998, Waris et al., 2006).

The search for antimicrobials from natural sources has received much attention to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones (Kelmanson et al., 2000). Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism2These active compounds either might act alone or in combination to inhibit bacterial growth (Ruban and Gajalakshmi, 2012). Many early studies indicated that phenolic compounds prevent many diseases from pathogenic invasion (Al-Dabbas et al., 2006). Cell wall of Gram-positive bacteria are of single layered, whereas the Gram-negative cell wall is multi-layered bounded by an outer cell membrane. Therefore, bacterial intracellular space could be easily hyper acidified and initiated functional disorder of bacterial energy metabolism (Scheffers and Pinho, 2005).

There is growing awareness in correlating the phytochemical components and their biological activities (Fernie et al., 2004). Nowadays efforts were focused on plants because of their usage of historical times and world's population rely on plants for the treatment of infections and noninfectious diseases. However, isolation of individual phytoconstituents and screening for biological activities definitely will give successful results. From this present study, it could be concluded that *A. echioides* contains various bioactive compounds hence, it can be considered as a plant of phytopharmaceutical importance. Therefore, this plants seem to serve as prospective material for advance development of plant-based antioxidant agents. In conclusion, different extracts of *A. echioides* can be very effective antioxidant and it could protect biological systems against the oxidative stress including aging, cancer, diabetes and cardiovascular disorders.

Competing Interests

The authors declare no conflict of interest.

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