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EVALUATION OF ANTIOXIDANT ACTIVITY OF *ARISTOLOCHIA KRYSAGATHRA* (ARISTOLOCHIACEAE)- AN IMPORTANT MEDICINAL HERB

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ABSTRACT

Antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of the whole plant of *Aristolochia krysagathra* have been tested using various antioxidant model systems viz, DPPH, hydroxyl, superoxide, ABTS and reducing power. Methanol extract of *Aristolochia krysagathra* is found to possess higher DPPH hydroxyl and superoxide radical scavenging activity. Methanol and ethanol extracts of *Aristolochia krysagathra* exhibited highest ABTS radical cation scavenging activity. Methanol extract of whole plant of *Aristolochia krysagathra* showed the highest reducing ability. This study indicates significant free radical scavenging potential of *Aristolochia krysagathra* whole plant which can be exploited for the treatment of various free radical mediated ailments.

Keywords: *Aristolochia krysagathra,* Antioxidant activity, Methanol, ABTS.

INTRODUCTION

Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called "Free radicals". Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction[1]. Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. Free radicals are electrically changed molecules i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves[2]. Antioxidants are capable of stabilizing or deactivating free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systematic health and wellbeing. Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen containing molecules including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochloride radical and various lipid peroxides[3]. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage.

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. Many plant derived substances, collectively termed "Phytonutrients" or "Phytochemicals" are becoming increasingly known for their antioxidant activity. Recently, various phytochemicals and their effect on health, especially the suppression of active oxygen species by natural antioxidants from herbs have been intensively studied[4,5].

The genus *Aristolochia* finds a prominent place in different Indian Systems of Medicine. The different ethnic communities in India have used different species of *Aristolochia* in the treatment of various

human ailments[6-8]. Kanikkar tribals of Kalakad-Mundanthurai Tiger Reserve Sanctuary, Tamil Nadu, boiled the equal quantity of fresh root and leaves of *Aristolochia krysagathra* in coconut oil for about 15-20 minutes over a low flame. The oil is filtered after cooling and applied on the head once in a day as the treatment of rheumatism. The therapy is used to reduce excessive heat of the body[8]. However, no data are available in the literature on the antioxidant activity of whole plant of *A. krysagathra*. Therefore, in the present investigation to examine the antioxidant activities of various extract of whole plant of *A. krysagathra* through various *in vitro* models.

MATERIALS AND METHODS

The whole plant of *Aristolochia krysagathra* Sivaranjan and Pradeep were collected from the natural forest of Kalakad-Mundanthurai Tiger Reserve Forest, Western Ghats, Tirunelveli, Tamil Nadu, India. The plant was identified with help of local flora and authenticated in Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu.

Preparation of Plant extract

The whole plant of *Aristolochia krysagathra* were dried in shade, and then coarsely powdered separately in a willy mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered though Whatman No.41 filter paper. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of Total Phenolics

Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described[9] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (diluted ten-fold) and 4mL (75g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of Flavonoids

The total flavonoid content was determined according to Eom *et al*[10]. An aliquot of 0.5 mL of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3mL of 80% methanol was added to make 5mL volume. The mixture was vortexted and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H[11].

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method[11]. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50,100,200,400 & 800µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell[12]. Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA , 0.01mL of $FeCl_3$**,** 0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50, 100, 200, 400 & 800µg/mL) dissolved in distilled water,0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37^0C

for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al* [13]. The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400&800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^{o}C for 5 min and the absorbance was measured at 560nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al*[14]. ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha[15]. 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50^{o}C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^{o}C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULTS

Total phenolics and total flavonoid content

The total phenolic content and total flavonoid content of the ethanol extract of *A. krysagathra* whole plant were found to be 1.24g100g^{-1} and 3.25g100g^{-1} respectively.

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. krysagathra* whole plant was shown in Fig 1. The scavenging effect increases with the concentration of standard and samples. Among the solvent tested, methanol extract of *A. krysagathra* whole plant exhibited highest DPPH radical scavenging activity. At 800µg/ml concentration, methanol extract of *A. krysagathra* possessed 113.94% scavenging activity on DPPH.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. krysagathra* whole plant was shown in Fig 2. Methanol extract showed very potent activity. At 800µg/ml concentration, methanol extract of *A. krysagathra* possessed 124.19% scavenging activity on hydroxyl radical.

Superoxide radical scavenging activity

The whole plant of *A. krysagathra* extract was subjected to the superoxide radical scavenging assay and the results were shown in Fig 3. It indicates that methanol extract of *A. krysagathra* whole plant (800µg/mL) exhibited the maximum hydroxyl radical scavenging activity.

ABTS radical cation scavenging activity

The whole plant of *A. krysagathra* extract was subjected to the ABTS radical cation scavenging activity and the results were shown in Fig 4. The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800µg/ml concentration, methanol and

ethanol extract of *A. krysagathra* whole plant possessed 112.16 % and 97.28% scavenging activity on ABTS which is higher than the standard trolox whose scavenging activity is 94.27%.

Reducing Power

Figure 5 showed the reducing power ability of different solvent extracts of *A. krysagathra* whole plant compared to ascorbic acid. Absorbance of the extract was increased when the concentration increased. A higher absorbance indicates a higher reducing power. Among the solvent tested, methanol extract exhibited higher reducing activity.

IC_{50} values

IC_{50} values of Petroleum ether extract of *A. krysagathra* whole plant and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 22.38µg/mL and 20.83µg/mL; 21.12µg/mL and 20.96µg/mL; 21.84µg/mL and 22.54µg/mL and 19.85g/mL and 21.65µg/mL respectively. IC_{50} values of Benzene extract of *A. krysagathra* whole plant and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 21.07µg/mL and 20.83µg/mL; 19.49µg/mL and 20.96µg/mL; 18.64µg/mL and 22.54µg/mL and 20.25µg/mL and 21.65µg/mL respectively. IC_{50} values of Ethyl acetate extract of *A. krysagathra* whole plant and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 20.11µg/mL and 20.83µg/mL; 19.02µg/mL and 20.96µg/mL; 21.07µg/mL and 22.54µg/mL and 16.73µg/mL and 21.65µg/mL respectively. IC_{50} values of Methanol extract of *A. krysagathra* whole plant and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 23.96µg/mL and 20.83µg/mL; 23.73µg/mL and 20.96µg/mL; 26.29µg/mL and 22.54µg/mL and 23.17µg/mL and 21.65µg/mL respectively. IC_{50} values of Ethanol extract of *A. krysagathra* whole plant and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 21.57µg/mL and 20.83µg/mL; 21.37µg/mL and 20.96µg/mL; 23.48µg/mL and 22.54µg/mL and 21.97µg/mL and 21.65µg/mL respectively (Table 1).

DISCUSSION

Phenolics have been considered classic defence compounds for protecting plants from herbivores, ever since plant secondary metabolites were suggested to have evolved for that reason. In contrast to these compounds, it has been suggested that the main role of many plants phenolics may be to protect leaves from photodamage, not herbivores; they can achieve this by acting as antioxidants; and their levels may vary with environmental conditions in order to counteract this potential photodamage[16]. Phenolics are an important class of secondary plant metabolites possessing an impressive array of pharmacological activity with excellent radical scavenging ability. The antioxidant activities of phenolics are due to their redox properties. The phenol moiety (hydroxyl group on aromatic ring) helps them to work as reducing agents, hydrogen donors and singlet oxygen quenchers[17,18]. Phenolics compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributors to the antioxidant activity of vegetables, fruits or medicinal plants. There compounds have been effective in many health related properties such as anticancer, antiviral, anti-inflammatory activities, effects on capillary fragility and ability to inhibit human platelet aggregation[19]. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activities[20].

Degenerative diseases such as cancers, cardiovascular diseases, osteoporosis and degenerative diseases are associated with aging. Oxidative damage to cell components, DNA, proteins and lipids accumulates with age and contributes to the degeneration of the somatic cells and to the pathogenesis of these diseases. Antioxidants present in food can help limits this damage by acting directly on relative oxygen species or by stimulating endogenous defense systems. The phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components[21]. The antioxidant potency of polyphenols has been evaluated *in vitro* by measuring their ability to trap free radicals and reduce other chemicals. If it is well established that polyphenol ingestion results in an increase of the plasma antioxidant capacity, there is still some incertainties about its efficiency to enhance the protection of cellular components, such as lipids or DNA, against oxidative stressing humans. In humans, the consumption of antioxidants or of fruits and vegetables has been associated with reduced levels of oxidative damage to lymphocytic DNA[22].

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant of *A. krysagathra* were investigated in the present study by DPPH, hydroxyl, superoxide and

ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox.

DPPH is one of the stable free radicals which are commercially available and widely been used for evaluating scavenging activity of antioxidant standards and herbs/ plant extracts[23]. DPPH radical which is of violet color, accepts an electron or hydrogen atom from the antioxidant compounds and is converted into a colourless or somewhat yellow diamagnetic DPPH molecule[24]. Among the solvent tested, methanol extract of whole plant of *A. krysagathra* exhibited more DPPH radical scavenging activity.

The hydroxyl radical is one of representative reactive oxygen species generated in the body. These radicals are produced through various biological reactions; one of the common reactions is the Iron (II)-based Fenton reaction[25]. Though the DPPH sacavenging model is a useful indicator, it has some limitations. Therefore the extracts ability to sacvenge hydroxyl radical was assessed. Among the solvent tested, methanol extract of whole plant of *A. krysagathra* possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid.

In cellular oxidation reactions, superoxide radical is normally formed first and its effect can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Superoxide anion radical actively participate in the initiation of lipid oxidation. Oxidation of unsaturated fatty acids in biological membranes leads to formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids and eventual destruction of membrane lipids, which produce breakdown products[26]. The present study showed potent superoxide radical scavenging activity of *A. krysagathra* whole plant extracts. The whole plant methanol extracts showed potent superoxide radical scavenging activity with IC_{50} values 26.29 µg/mL compared to ascorbic acid 22.54 µg/mL respectively.

The basic principle underlying the ABTS decolorization assay is that ABTS on reaction with $K_2S_2O_8$ forms a greenish blue radical cation. Standard and sample antioxidants that are able to transfer an electron to ABTS radical scavenge the color of the solution proportionate to their amount. The extent of scavenging depends both upon the concentration of antioxidant and time duration for the reaction. In the present study, whole plant methanol and ethanol extracts of *A. krysagathra* were fast and effective scavengers of ABTS radical and this activity was higher than that of trolox standard. Proton radical scavenging is an important attribute of antioxidants. ABTS a protonated radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals[27].

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the antioxidant activity of antioxidants have been attributed to various mechanisms, among which are prevention of chain initation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging antioxidant activity[28]. In the present study, increase in absorbance of the reaction mixture indicates the reductive capabilities of whole plant extracts of *A. krysagathra* in concentration dependent manner when compared to the standard ascorbic acid.

In conclusion, the present study provides the evidence that the methanol extract of *A. krysagathra* whole plant, which contains phenolic and flavonoids. These *in vitro* assays demonstrate that this plant extract is an important source of natural antioxidant which may be preventive against oxidative stresses. This is the first report on the antioxidant properly of this plant. Therefore, further studies should be carried out to isolate active principles having antioxidant properties.

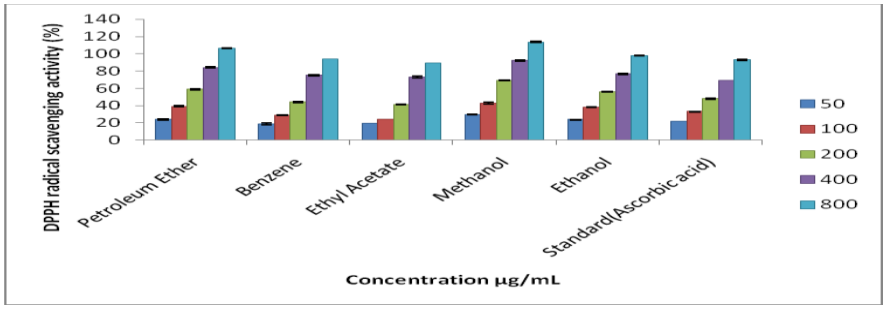

Figure 1: DPPH radical scavenging activity of different extracts of *Aristolochia krysagathra*

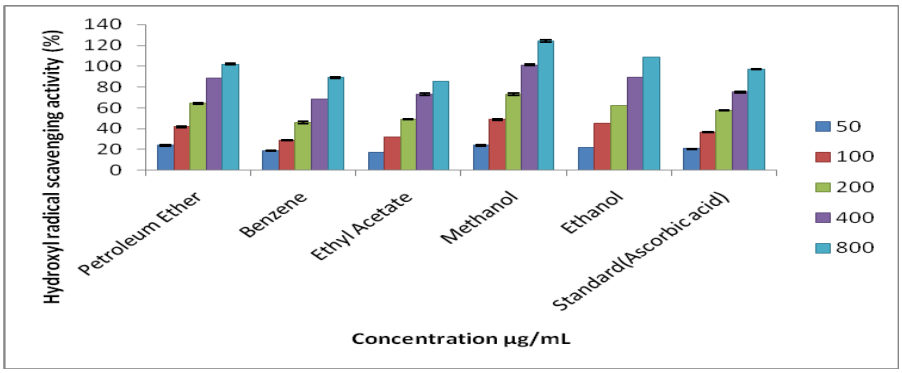

Figure 2: Hydroxyl radical scavenging activity of different extracts of *Aristolochia krysagathra*

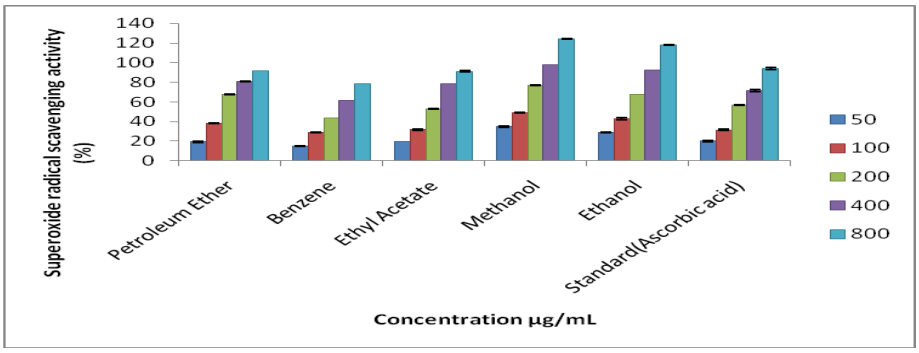

Figure 3: Superoxide radical scavenging activity of different extracts of *Aristolochia krysagathra*

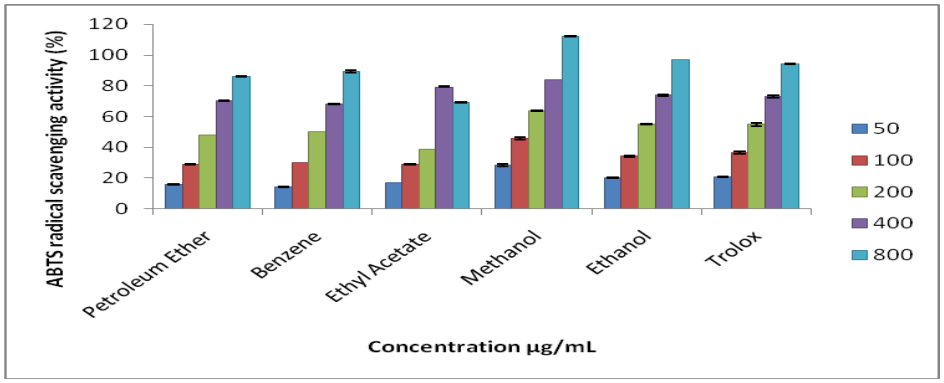

Figure 4: ABTS radical scavenging activity of different extracts of *Aristolochia krysagathra*

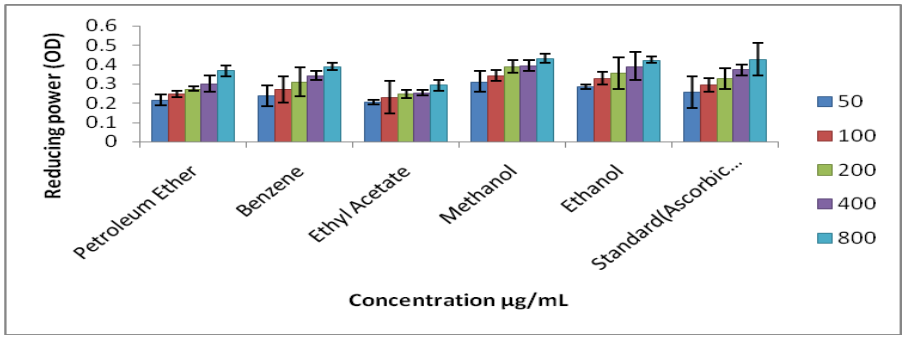

Figure 5: Reducing power ability of different extracts of *Aristolochia krysagathra*

Table 1: IC_{50} values of different solvent extracts of whole plant of *Aristolochia krysagathra*[a]

Solvent	IC_{50} (µg/mL)			
	DPPH	**Hydroxyl**	**Superoxide**	**ABTS**
Petroleum ether	22.38	21.12	21.84	19.85
Benzene	21.07	19.49	18.64	20.25
Ethyl acetate	20.11	19.02	21.07	16.73
Methanol	23.96	23.73	26.29	23.17
Ethanol	21.57	21.37	23.48	21.97
Ascorbic acid	20.83	20.96	22.54	-
Trolox	-	-	-	21.65

All the values are mean by triplicate determines*

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