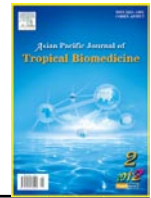




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Physicochemical standardization and evaluation of *in-vitro* antioxidant activity of *Aconitum heterophyllum* Wall

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ABSTRACT

Objective: To evaluate different physicochemical parameters and *in-vitro* antioxidant activity of *Aconitum heterophyllum* (*A. heterophyllum*), having great medicinal value in treatment of diseases associated to nervous system, digestive system, rheumatism, fever and also in case of bacterial, viral and fungal infections. **Methods:** Various physicochemical parameters such as extractive values, haemolytic activity, foaming index, swelling index, heavy metal analysis and pesticide content as per the guidelines of WHO were included. Determination of starch grains, crude fiber, fluorescence powder drug analysis and preliminary phytochemical analysis of root of *A. heterophyllum* were also included. Various phytoconstituents such as total phenols, tannins, flavonoids, flavonols, alkaloids and saponins were quantified in the present study. The ethanolic extract was further screened for *in-vitro* antioxidant activity using different models. **Results:** The result depicted a higher extractive value in case of aqueous extract which decreased as the polarity decreased. The haemolytic activity of *A. heterophyllum* was found to be 212.5 units/g of plant material while chlorinated and phosphate pesticides along with heavy metals were found to be within the standard limits of WHO. The quantitative estimations demonstrated the root to be highly rich in alkaloids while phenols, tannins, flavonoids and saponins were found in less quantity. The *in-vitro* antioxidant study showed a moderate to low activity in all models which may be attributed to low phenolic and flavonoid content. **Conclusion:** The valuable information provided in the study will ascertain the identity and authenticity of *A. heterophyllum* and will help in maintaining quality and purity thus, preventing its adulteration.

1. Introduction

Aconitum heterophyllum (*A. heterophyllum*) Wall commonly known as Atis or Patis belonging to family Ranunculaceae is a perennial herb distributed over temperate parts of western Himalaya extending from Kashmir to Kumaon^[1]. Studies on traditional system of medicine showed that the plant is used in curing hysteria, throat infection, dyspepsia, abdominal pain, diabetes and is considered as a valuable febrifuge nervine tonic especially combating debility after malaria and in hemoplageia^[2]. The plant has shown to contain alkaloids heteratisine, heterophyllisine, heterophylline, heterophyllidine, atidine, isoatisine hetidine, hetsinone, benzoylheteratisine^[3]. *Aconitum* has also shown to exhibit antipyretic, analgesic, anti-fungal, anti-bacterial,

insecticidal, brime shrimp cytotoxic activities and is used to treat diseases of nervous system, digestive system, rheumatism and fever^[4]. Reports have also shown that the plant posses a good anti-viral, antidiarrhoeal and immunostimulant properties^[5]. The alkaloids mesaconitine and 3 acetylaconitine have shown to posses' anti-inflammatory activity^[6].

Even though the plant has a wide range of medicinal importance, but still some physicochemical parameters of the plant remains unexplored. Therefore, present study deals with evaluation of some of these parameters that may act as a beneficial role in standardization and authentication of plant *A. heterophyllum*. In addition, the study also includes heavy metal analysis, determination of some pesticides and *in-vitro* antioxidant activity of root of the plant.

2. Materials and methods

The root of *A. heterophyllum* were obtained from Panakudi,

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Tirunelveli district of Tamilnadu and were authenticated by Prof. V. Chelladurai, Research Botanist (Retd.), Palayamkottai, Tamil Nadu, India. A voucher specimen (COG/AH/13) of the root of plant has been deposited for future reference in Department of Pharmaceutics, Banaras Hindu University, Varanasi (U.P), India.

2.1. Chemicals and reagents

Florisil R grade 60/100 PR was purchased from E. Merck, Mumbai, India. L-ascorbic acid, butylated hydroxy anisole, rutin, 1-diphenyl-2-picryl-hydrazil (DPPH) and ethylenediaminetetraacetic acid (EDTA) were procured from Hi-Media Research Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals and reagents were of analytical grade and were purchased from S.D. fine Chemicals Pvt. Ltd., Mumbai, India. All the spectrophotometric analysis was carried on Shimadzu, Pharmaspec-1700 ultraviolet-visible spectrophotometer.

2.2. Determination of physicochemical parameters

The obtained root of the plant were first dried and then grinded to coarse powder which was further used for evaluation of various physicochemical parameters such as extractive values in different solvents based upon increasing order of polarity, haemolytic activity, foaming index, swelling index and bitterness value as per the guidelines of WHO[7]. Lycopodium spore method as described by Wallis[8] was used to determine the total number of starch grains in the root of *A. heterophyllum*. For determining the crude fiber present in the plant material, it was boiled with 10% nitric acid followed by treating it with 2.5% NaOH[9]. The powdered root were then subjected to fluorescence powder drug analysis which was performed under day light, short UV and long UV according to the methods described by Chase and Pratt[10]. Preliminary phytochemical screening for the presence of various phytoconstituents was carried on different extracts obtained after cold maceration of crude drug in different solvents[9].

2.3. Heavy metal analysis

Heavy metal analysis of the plant material was performed by preparing acid digestion by consecutive treatment with nitric and sulfuric acid which was further treated with ammonium oxalate until sulfur trioxide vapors were developed. The analysis of the heavy metals was carried out as per the procedure described in WHO guidelines using atomic absorption spectrophotometer (Thurmo Fcher AA 303) [7].

2.4. Pesticide content

The pesticide content of the root of *A. heterophyllum* was determined as per the guidelines of WHO[7]. Briefly, add a mixture of 350 mL of acetonitrile: water (65: 35) to 50 g of grinded powdered root which was blended at high speed for 5 min followed by filtering it. The filtrate (250 mL) was

then transferred to a separating funnel to which further 100 mL light petroleum, 10 mL of sodium chloride (40%) and 600 mL of water were added with constant shaking up to 35–45 s. After discarding the aqueous layer the solvent layer was washed twice with 100 mL portions of water to which 15 g of anhydrous sodium sulfate was added with vigorous shaking. The extract was separated and its volume was reduced up to 5 to 10 mL which was allowed to pass through column packed with Florisil R grade 60/100 PR, activated at 650 °C at a rate of not more than 5 mL per min. Three different elutes were obtained after running the column with three different ratios of ether: light petroleum mixture as mobile phase. Elute 1 contained 6% of ether while elute 2 and 3 contained 15% and 50% of ether. The obtained elutes were evaporated to dryness, transferred to a sample holder, and burned in a suitable combustion flask flushed with oxygen. The gases produced during combustion are then absorbed in a suitable solution in the combustion flask (water for chloride and H₂SO₄ in case of phosphate pesticides). For determining the chloride pesticides 15 mL of the solution obtained after combustion was mixed together with 1 mL of ferric ammonium sulfate (0.25 mol/L) and 3 mL of mercuric thiocyanate which was allowed to stand for 10 min after swirling it. Its absorbance was measured at 460 nm using a spectrophotometer. Phosphate pesticides were determined after mixing 7 mL of the solution obtained after combustion to 2.2 mL of sulfuric acid (300 g/L), 0.4 mL of ammonium molybdate (40 g/L) and 0.4 mL of aminonaphtholsulfonic acid followed by swirling it. The absorbance of the solution was measured at 820 nm after heating it at 100 °C for 12 min.

2.5. Quantitative estimations

The powdered root of *A. heterophyllum* (500 g) was then subjected to Soxhlet extraction using ethanol (1.5L) which was continued till the whole plant material was exhausted. The obtained extract was then evaporated and concentrated in a Rota evaporator and was kept in desiccators until use. The total phenolic and tannin contents in the ethanolic extract of root of *A. heterophyllum* was estimated according to the method described by Hagerman *et al*[11]. For determining the total flavonoid and flavonol content in extract the method proposed by Kumaran & Karunakaran[12] was adopted. Total alkaloid content in the root was estimated by gravimetric analysis as proposed by Wagner[13]. Total saponin content in plant material was estimated using diosgenin as a standard by implementing method of Baccou *et al*[14].

2.6. In-vitro antioxidant activity

The ethanolic extract of root of *A. heterophyllum* was then subjected to *in-vitro* antioxidant activity by using various *in-vitro* models which includes determination of total antioxidant capacity, assay of reducing power, free radical scavenging activity, nitric oxide scavenging assay, H₂O₂ scavenging activity and scavenging of hydroxyl radical by deoxyribose method[15–17].

2.6.1. Total antioxidant capacity

Phosphomolybdenum method was used to estimate the total antioxidant capacity of extract. The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH.

2.6.2. Assay of reducing power

Assay of reducing power was carried out by potassium ferricyanide method by using phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (10 g/L). Absorbance was measured at 700 nm. Ascorbic acid was used as standard and phosphate buffer was used as blank solution. Increased absorbance of the reaction mixture indicated stronger reducing power.

2.6.3. Free radical scavenging activity

The free radical scavenging activity of plant extract was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) method by using 100 μ M/mL DPPH in methanol. The absorbance was then measured 30 min later at 517 nm. The free radical scavenging activity was calculated using the following equation: DPPH scavenging effect = $[(1-A_t/A_0) \times 100]$ Where A_0 is the absorbance of the blank and A_t is the absorbance test sample. Then % inhibition was plotted against respective concentrations used and IC_{50} was calculated by using ascorbic acid as control.

2.6.4. Nitric oxide scavenging assay

Nitric oxide scavenging assay was carried out by using sodium nitroprusside in aqueous solution at physiological pH, which spontaneously generates nitric oxide that interacts with oxygen to produce nitrite ions. This can be determined by the use of the Griess Illosvov reaction.

2.6.5. Scavenging of hydrogen peroxide

Scavenging activity of plant extract was evaluated by method described by Jayaprakash et al^[17] by using hydrogen peroxide (20 mM) in phosphate buffered saline (PBS, pH 7.4). The absorbance was measured at 230 nm.

2.6.6. Scavenging of hydroxyl radical by deoxyribose method

Hydroxyl radical scavenging activity of ethanolic extract was measured by treating various concentrations of extract with 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2 , 1 mM L-ascorbic acid, and 30 mM deoxyribose, 1 mL of 2.8% (w/v) trichloroacetic

acid and 1 mL of 1% (w/w) 2-thiobarbituric acid in potassium phosphate buffer (pH 7.4). The colour developed was measured at 532 nm against a blank containing phosphate buffer.

3. Results

3.1. Determination of physicochemical parameters

As per the results obtained the extractive value of the root decreased with reducing order of polarity i.e. water (7.141% w/w), methanol (2.604% w/w), ethanol (2.119% w/w), ethyl acetate (0.256% w/w), chloroform (0.406% w/w), hexane (0.061% w/w), acetone (0.295% w/w) and petroleum ether (0.09% w/w). The haemolytic activity of root of *A. heterophyllum* was found to be 212.5 units/g of powder. The length of the foam of the sample was found to be less than 1 cm therefore; foaming index of root of *A. heterophyllum* is less than 100 while swelling index was found to be 3.233 mL/g and the root had a strong bitter taste. The total number of starch grains present in 1 mg of powder drug was found to be 132129, whereas the crude fiber content of root was found to be 2.55% w/w of plant material. Table 1 represents the fluorescence powder drug analysis of root of *A. heterophyllum*. Preliminary phytochemical screening of different extracts of root is represented in Table 2.

3.2. Heavy metal analysis

Heavy metal contents in medicinal plants depend on climatic factors, plant species, air pollution, and other environmental factors. The root of *A. heterophyllum* showed the presence of arsenic (As) and cadmium (Cd) at a concentration of 0.202 8 and 0.241 6 in ppm. The level of chromium, copper, iron, mercury, nickel and lead were also depicted and were reported to be 1.051 ppm, 1.670 ppm, 2.048 ppm, 0.334 ppm, 45.897 ppm and 5.867 ppm.

3.3. Pesticide content

From the results obtained the chlorinated pesticide in the first and the second elute was found to be 0.281 and 0.336 mg/kg of plant material. The phosphated pesticide present in the

Table 1.
Fluorescence analysis of *A. heterophyllum*.

Test	Day light	Short UV	Long UV
Powder + 1 N NaOH in methanol	Khaki	No fluorescence	Spring green
Powder + 1 N NaOH in water	Coral	No fluorescence	Light green
Powder + 1 N HCL in methanol	Pink	No fluorescence	Yellow green
Powder + 1 N HCL in water	Antique white	No fluorescence	Spring green
Powder + 1 N HNO_3 in methanol	Khaki	No fluorescence	Light green
Powder + 1N HNO_3 in water	Light golden	No fluorescence	Light green
Powder + 5% iodine	Orange red	No fluorescence	No fluorescence
Powder + 5% $FeCl_3$	Gold	No fluorescence	No fluorescence
Powder + 50% KOH	Corn silk	No fluorescence	Green yellow
Powder + 25% ammonia	Light solmsn	Khaki	Yellow green
Powder + picric acid saturated	Yellow	No fluorescence	No fluorescence
Powder + acetic acid	Antique white	No fluorescence	Spring green

plant material from the first and second elute of column was found to be 0.013 9 and 0.017 8 mg/kg of plant material while in case of third elute the quantity of phosphated pesticide was found to be negligible.

was found to be 8.75 mg/g equivalent to gallic acid while total tannin content was estimated to be 19.254 mg/g equivalent to tannic acid. The total flavonoid and flavonol content evaluated were reported to be 3.125 and 0.963 mg/g equivalent to rutin. Total alkaloid and saponin estimated in the plant material were reported to be 2.556% w/w and 6.484 mg/g equivalent to diosgenin.



Figure 1. Rhizomes of *A. heterophyllum*.

3.4. Quantitative estimations

Total phenolic content in the root of *A. heterophyllum*

3.5. In-vitro antioxidant studies

Figure 2 represents the results obtained from different *in-vitro* antioxidant models performed on ethanolic extract of root of *A. heterophyllum*. The total antioxidant capacity was determined using the linear regression equation, expressed as the number of equivalent of ascorbic acid which was found to be $(30.645 \pm 1.846) \mu\text{g/mL}$. The assay of reducing power depicted a very moderate reducing capacity of the extract ($0.133 \pm 0.018) \mu\text{g/mL}$ as compared to standard ascorbic acid ($0.416 \pm 0.007) \mu\text{g/mL}$. The free radical scavenging activity of a particular sample is determined by its capacity to reduce DPPH by donating an electron or hydrogen to DPPH. The free radical scavenging activity of the extract of *A. heterophyllum* using DPPH was found to be very moderate as depicted through a low IC_{50} value (263.191 ± 23.958) compared to ascorbic acid (IC_{50} : 80.813 ± 5.155). Griess reagent used for determining the nitric oxide scavenging activity of extract showed a very low IC_{50} value 506.462 ± 21.264 as compared to rutin. In addition the hydrogen peroxide scavenging potential of extract also demonstrated a very low scavenging potential which was evident through

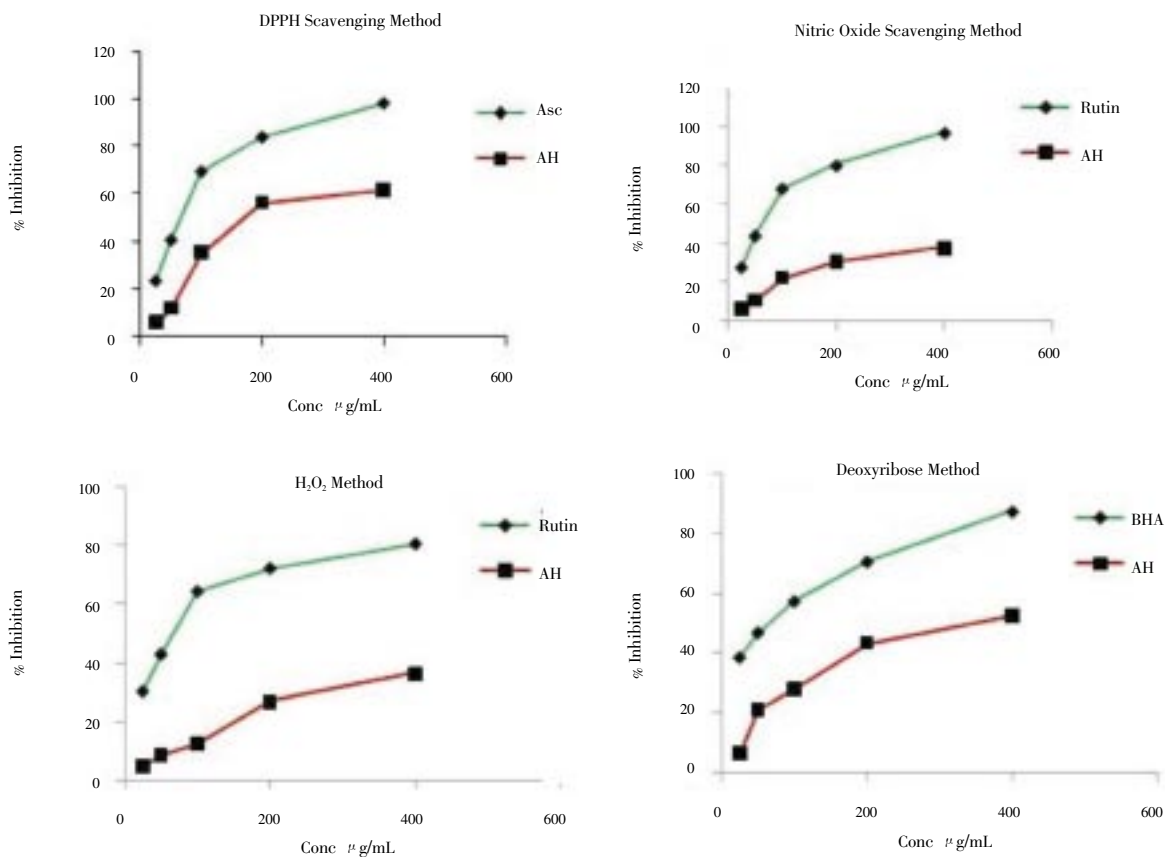


Figure 2. *In-vitro* antioxidant activity of *A. heterophyllum*. Where Asc: ascorbic acid, BHA: butylated hydroxy anisole, AH: *A. heterophyllum*.

Table 2.
Preliminary phytochemical screening of *A. heterophyllum*.

Phytoconstituents	Extract							
	WE	ME	EE	EAE	CE	HE	AE	PEE
Alkaloids	–	+	+	–	+	+	–	+
Steroids	–	+	+	+	+	+	+	+
Anthraquinone glycoside	–	–	–	–	–	–	–	–
Cardiac glycoside	+	+	+	–	–	–	+	–
Cyanogenetic glycoside	–	–	–	–	–	–	–	–
Saponin	+	+	+	–	–	–	–	–
Phenolic compound	+	+	+	–	–	–	–	–
Carbohydrates	+	+	+	–	–	–	–	–
Proteins	+	+	+	–	–	–	–	–
Amino acid	+	+	+	–	–	–	–	–

+: Indicates presence, –: indicates absence. Where: WE: Water extract, ME: Methanolic extract, EE: Ethanolic extract, EAE: Ethyl acetate extract, CE: Chloroform extract, HE: Hexane extract, AE: Acetone extract, PEE: Petroleum ether extract.

a low IC_{50} value (533.413 ± 15.595) as compared to standard rutin (IC_{50} : 84.4546 ± 10.3934). Fenton reaction was used to determine the inhibition potential of hydroxyl radical assessed through a iron (II)–dependent deoxyribose damage assay which demonstrated a moderate scavenging potential of extract with an IC_{50} value of (336.999 ± 10.021) compared to positive control Butylated Hydroxy Anisole (BHA) IC_{50} : (74.5547 ± 3.2124).

4. Discussion

Therapeutic importance of herbal medicine is remarkably increasing since last few decades therefore, it is very essential to obtain a proper quality control profile for various medicinal plant used in traditional system of medicine. Adulteration of these plants may occur due to an improper knowledge regarding the varied geographical conditions, associated problems of different vernacular names, its morphology and microscopy. Thus, to avoid this adulteration and commercialization of particular medicinal plant standardization of that plant is essential for obtaining reproducible standards of that plant[18].

Different physicochemical parameters quantified in the present study include determination of extractive values in different solvents which helps in determining the amount of active chemical constituents present in the plant material. In many medicinal plants swelling index is indicative of its therapeutic or pharmaceutical value based upon the presence of gums, mucilage, pectin and hemicelluloses while foaming index depicts the ability of saponins of that plant materials to form persistent foam[7]. Fluorescence drug analysis have an immense value in qualitative determination of crude drug since, some chemical constituents of plant exhibit fluorescence in the visible range in day light while many others produce fluorescence in ultra violet range (e.g. alkaloids like berberine)[19]. Haemolysis is a processes causing hemoglobin to diffuse in the surrounding medium which is mainly due to the presence of saponins in case of plants. In the present study, even though the plant material showed a low range of saponin still the haemolytic activity observed was high which may be due to the presence of high quantity of alkaloids which have been previously reported to

cause haemolysis[20,21]. Preliminary phytochemical analysis performed in the study gives an idea about the chemical nature of the active constituents present in that extract.

The heavy metals estimated in the present study were found to be in accordance with the prescribed limits of WHO. Heavy metals can accumulate in plants through both foliage and root systems. It is very essential to determine the level of toxic metals in the medicinal plants attributed to the environmental pollutants which may vary depending upon industrial and traffic emissions to the use of agricultural expedients, such as cadmium–containing dung, organic mercury fungicides, and the insecticide lead arsenate. The study also provides valuable information regarding metal which are natural essential components of coenzymes and are important for growth, photosynthesis, and respiration[22]. It is considered that agricultural practice such as spraying and treatment of soils occurring throughout the processes of cultivation, and administration of fumigants during storage may lead to the occurrence of pesticides in plant materials. Thus, it is very important to have at least one laboratory which will provide information regarding the pesticide content of medicinal plants in accordance with the international standards[7]. The results obtained from the above study depicted the presence of chlorinated and phosphated pesticides which were found to be within the normal range.

The phytoconstituents quantified in the present study have a great value in human health care system. Phenols have been reported as an active, quenching of oxygen–derived free radicals by donating hydrogen atom or an electron to the free radicals[23]. Tannins have been reported to possess a strong astringent property causing protein precipitation and is already shown to have anti–bacterial, anti–inflammatory, anti–viral and anti–oxidant activities[24–26]. The property of flavonoids in increasing capillary permeability has been widely used for the treatment of various cardiovascular diseases and is regarded as a agents having potential antioxidant and anti–inflammatory activity[27]. Alkaloids have a wide range of medicinal importance such as in treatment of cancer, malaria, pain, inflammation, parkinsonism, hypertension and number of central nervous system disorders[28].

The ethanolic extract of *A. heterophyllum* in all *in-vitro*

antioxidant models showed a low to moderate antioxidant activity. Total antioxidant capacity and reducing power assay along with different free radical scavenging methods helps us in determining the overall antioxidant potential of a plant. In human body free radicals such as nitric oxide, hydroxyl radical and hydrogen peroxide get bind to DNA nucleotides thus, causing damage to various biological systems which may result in carcinogenesis, mutagenesis, and cytotoxicity. Therefore, plants rich in phenolic, tannins and flavonoid are considered as a potential antioxidant agent as they neutralizes the free radicals via donation of hydrogen atom, quenching of oxygen and by chelation of metals thus, minimizing oxidative stress^[15,16,27,29].

From the above study the physicochemical standards obtained will provide a proper identity and authenticity of *A. heterophyllum* which may help in maintaining its quality and purity and will prevent its adulteration from drug of same or other genus having low potency

Conflict of interest statement

We declare that we have no conflict of interest.

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