Analytical standards for the root tubers of Ativisha - *Aconitum heterophyllum* Wall. ex Royle

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Abstract- Standardization of herbal drugs is the need of the hour as the use and practice of traditional herbal drugs has increased tremendously. The main objective of the present study is to standardize the root tubers of *Aconitum heterophyllum* as per pharmacopoeial testing protocol which include powder microscopy, physico-chemical screening, HPTLC fingerprinting and GC-MS analysis. Preliminary phytochemical tests indicate the presence of alkaloids, sugars, flavonoids, steroids, quinones and tannins. HPTLC profiling of the ethanol extract using Toluene/ Ethyl acetate (8: 1) as mobile phase revealed the presence of phytochemicals with different R_f values. The GC-MS analysis of the diethyl ether fraction showed the presence of 39 compounds of which 21 were identified.

Index Terms- *Aconitum heterophyllum*, GC-MS analysis, HPTLC Fingerprinting, Powder microscopy.

I. INTRODUCTION

Aconitum heterophyllum commonly called Atis is an important endangered medicinal herb seen in the temperate regions and the alpine regions of Himalayas. The tuberous roots of *A. heterophyllum* are highly medicinal and widely used in Ayurvedic system of medicine. In *Charaka Samhitha* it has been recommended for treating obesity, piles, stomach disorders etc.. In *Sushrutha Samhitha* it was suggested as a remedy against diarrhea. *Aconitum heterophyllum* has been extensively studied for its alkaloid profile (Pelletier *et al.*, 1968; Nisar *et al.*, 2009). Pharmacological studies on the root tubers of *A. heterophyllum* revealed its anti-inflammtory (Verma *et al*., 2010), anti-diarrheal (Prasad *et al*., 2012), anti-helminthic (Pattewar *et al*., 2012) and antihyperlipidemic (Subash and Augustine, 2012) properties.

Due to its high cost as well as unavailabity, the chance for adulterating root tubers of *A. heterophyllum* with substandard products is high. Thus to avoid adulteration, standardization of this valuable herbal drug is the need of the hour. In the present study an attempt has been made to standardize the original and authenticated root tubers of *A. heterophyllum* by physicochemical characterization, HPTLC fingerprinting and GC-MS analysis.

II. MATERIALS AND METHODS

Sample Collection

Root tubers of *Aconitum heterophyllum were* collected from the Raw drug division, Aryavaidyasala, Kottakkal, Kerala and were authenticated by Dr. P. B. Benil, Associate Professor, Department of Agadatantra, VPSV Ayurveda College, Kottakkal, Kerala. The rhizomes were washed, shade dried, coarse powdered and stored at -20^{o}C until further analyses.

Powder microscopy

To study the microscopic characteristics, a pinch of powdered drug was warmed with few drops of chloral hydrate on a microscopic slide, mounted in glycerine and examined under Zeiss AXIO trinocular microscope.

Evaluation of Physical Constants

Physical constants have a major role in identification and purity determination of crude drugs. In the present study, physical constants such as total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive values were evaluated as per standard protocols (Anonymous, 1998).

Elemental analysis

One gram of powdered drug sample was taken into a pre weighed crucible and kept in muffle furnace overnight at 500^{0}C. The ash obtained was wetted with few drops of water and added 1 ml of conc. HNO_3. Excess HNO_3 was evaporated on a hot plate set at 100^{o}C and kept the crucible again to furnace for one hour at 500^{o}C. Ash was then dissolved in 10 ml conc. HCL, filtered to a volumetric flask and made up to 100 ml with distilled water. Later read the concentration by aspirating sample as well as standard solution in Atomic Absorption Spectrophotometer (Parker Elmer- Pinnacle 900H).

Preparation of extract

Weighed quantity of coarse powdered drug was soaked in ethanol/water (1:1) in a percolator for 24 hrs. The soluble portion was filtered through a filter paper and dried on water bath in a weighed evaporating dish. The extracts were dried under vacuum and stored in desiccator until use for further analyses/successive extraction.

Qualitative Phytochemical Tests

The hydro-alcoholic extract was mixed with silica gel for column chromatography and extracted successively in a Soxhlet extractor using solvents such as Petroleum Ether, Chloroform, Ethyl Acetate and Ethanol in the increasing order of polarity. The extracts were completely dried under vacuum. These successive extracts were tested for various phytochemicals (Harborne, 1998; Raman, 2006).

HPTLC Fingerprinting

The hydro-alcoholic extract was mixed with silica gel for column chromatography and extracted by maceration with ethanol. The extract was made up to 50 ml in a volumetric flask. Five and ten microlitre of the ethanolic extract was applied on a pre-coated silica gel F_{254} on aluminum plates to a band width of 7 mm using CAMAG Linomat 5 TLC applicator. The plate was then developed in CAMAG twin-trough chamber using Toluene/ Ethyl acetate (8: 1) as mobile phase. The R_f values were

determined by photodocumentation performed using CAMAG photo-documentation chamber and the plates were scanned under 254 nm, 366 nm and 620 nm after derivatisation using CAMAG Scanner (Sethi, 1996).

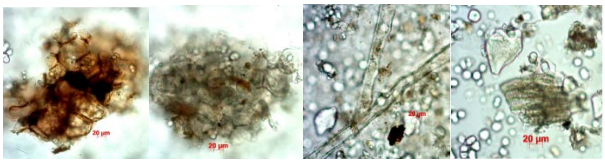
GC-MS Analysis

The hydro-alcoholic extract was mixed with silica gel for column chromatography and extracted by maceration with diethyl ether. The extract was made up to 10 ml in a volumetric flask and analyzed for composition by GC-MS. The study was carried out on a 5975C Agilent system equipped with a DB-5ms Agilent fused silica capillary column (30 × 0.25 mm ID; film thickness: 0.25 µm), operating in electron impact mode at 70 eV.

III. Results and Discussion

Powder Microscopy

The dried root tuber powder of *A. heterophyllum* under microscope shows, overlapping layers of polygonal cells of cork in surface view and the same in longitudinally cut mode with underlying cortical parenchyma containing starch; compact cortical parenchyma cells which are slightly thick walled having no intercellular space but with lot of starch grains; fragments of bundled short simple pitted xylem vessels and tracheids; group of few or isolated squarish to elliptical sclereids with lateral wall thickening having simple pits all over the surface; few fragments of comparatively larger scalariform vessels; lot of parenchyma fragments with round to oval shaped simple and mostly 2 to 3 compound starch grains scattered throughout the powder (Fig. 1).



(a) (b) (c) (d)

Figure 1: Powder microscopy of *A. heterophyllum* rhizome

(a) Cork cells in surface view **(b)** Cortical Parenchyma Starch
(c) Thin-walled fibres **(d)** Scalariform vessels

Physicochemical analysis

In the present study, physical constants such as total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive values were evaluated (Table 1). The amount of macro and micro elements analyzed in the rhizome of *A. heterophyllum* is given in Table 2. The macronutrients - Na and K were analyzed and it was revealed that potassium content is much higher in than sodium. The concentration of heavy metals such as Pb, Cu, Mn, Cr and Fe was also and found to be in permissible limits. The essential heavy metals such as Cu, Zn, Fe and Mn play many biochemical and physiological functions in plants (Nagajyothi *et al*., 2010).

Table 1: Physicochemical parameters of the rhizome of *A. heterophyllum*

Parameters	Results (n=3 % w/w)
Foreign matter	Nil
Total Ash	2.04
Acid insoluble Ash	0.40
Water soluble Ash	0.94
Alcohol soluble extractive	5.06
Water soluble extractive	24.80

Table 2: Concentration of various elements in the rhizome of *A. heterophyllum*

Elements	Concentration (mg/Kg)
Na	32.4
K	4757
Mn	11.7
Cr	1.7
Pb	1.2
Cu	5.2
Fe	216
Zn	15.1

Qualitative Phytochemical Screening

The petroleum ether and chloroform extracts of the tubers of *Aconitum heterophyllum* showed the presence of alkaloids, sugars and steroids while sugars, flavonoids and steroids were seen in ethyl acetate and methanol extracts. The ethyl acetate extract also contain quinones and tannins were seen in the methanol extract. The extractive yield was maximum in methanol followed by petroleum ether (Table 3).

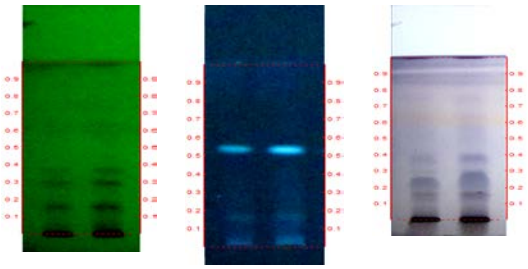
Table 3: Preliminary phytochemical tests of *A. heterophyllum* successive extracts

Test	Petroleum ether	Chloroform	Ethyl Acetate	Methanol
Alkaloid	+	+	–	–
Carbohydrate	+	+	+	+
Carboxylic acid	–	–	–	–
Coumarins	–	–	–	–
Flavanoids	–	–	+	+
Phenol	–	–	–	–
Quinone	–	–	+	–
Resins	–	–	–	–
Steroid	+	+	+	+
Saponins	–	–	–	+
Tannin	–	–	–	+
Terpenoid	–	–	–	–

HPTLC Fingerprinting

The methanol extract of the rhizome of *A. heterophyllum* was subjected to HPTLC analysis using solvent system Toluene – Ethyl acetate (8:1) as mobile phase and the R_f values and colour of the spots were recorded (Table 4). Even though two concentrations, 5 and 10 microlitres of the extract were used, the result was made based on the 10 microlitre of the sample as the number of spots observed optimum at this concentration. The

HPTLC images shown in Fig. 2 indicate that all sample constituents were clearly separated without any tailing and diffuseness. TLC photo-documentation revealed presence of many phytoconstituents with different R_f values (Table 4) while densitometric scan of the plates showed numerous bands under 254 nm, 366nm and 620 nm. On photodocumentation there were 6 spots under 254 nm, 7 spots under 366 nm and 10 spots under 620 nm post-derivatisation with vanillin sulphuric acid spray reagent (Figure 2). Densitometric scan at 254 nm (Figure 3) revealed 10 peaks corresponding to 10 different compounds in the ethanol extract with R_f 0.14 (32.19%), 0.30 (23.24%), 0.39 (18.69%), 0.61 (8.52%) and 0.69 (8.17%) being the major peaks. Densitometric scan at 366 nm (Figure 4) showed 8 peaks, and peaks with R_f 0.04 (14.52%), 0.12 (8.08%), 0.30 (8.20%) and 0.56 (50.69%) were the major peaks detected. Densitometric scan at 620 nm (Figure 5) showed 8 peaks, peaks with R_f 0.14 (23.86%), 0.26 (20.29%), 0.37 (19.47%) and 0.52 (14.58%) being the major ones. Taking the advantage of its low-cost, flexible mobile phase composition, and easy post-derivatization, HPTLC is still keeping active and alive in herbal medicines quality control programmes (Upton, 2010).



Track 1– 5 µl Track 2- 10 µl

Figure 2: HPTLC photo documentation of ethanol extract of *A. heterophyllum*

Table 4: R_f values of ethanol extract of root tuber of *A. heterophyllum* (10 µl)

At 254 nm	At 366 nm	After derivatisation
0.06(L Green)	0.06(F L Violet)	0.06(L Violet)
-	-	0.08(L Violet)
0.16(Green)	0.16(F Violet)	0.16(D Brown)
-	0.24(F L Violet)	-
0.29(Green)	0.29(F L Violet)	0.29(Violet)
-	0.33(F L Violet)	-
0.37(L Green)	-	-
-	-	0.39(Violet)
-	0.41(F L Violet)	-
0.45(L Green)	-	-
-	-	0.49(L Violet)
-	-	0.52(L Violet)
-	0.54(F Aqua)	-
-	-	0.58(L Violet)
0.64(L Green)	-	0.64(L Violet)
-	-	0.75(L Violet)

***F-Fluorescent, L-Light, D-Dark**

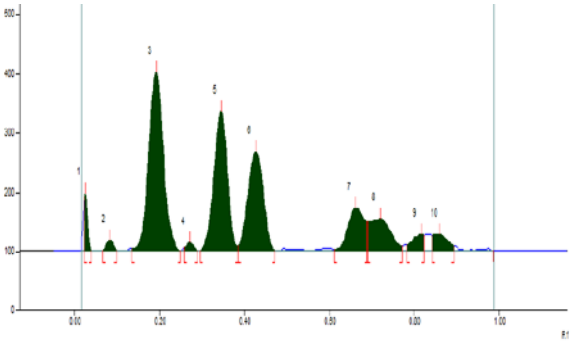


Figure 3: Densitometric scan of ethanol extract of *A. heterophyllum* at 254 nm

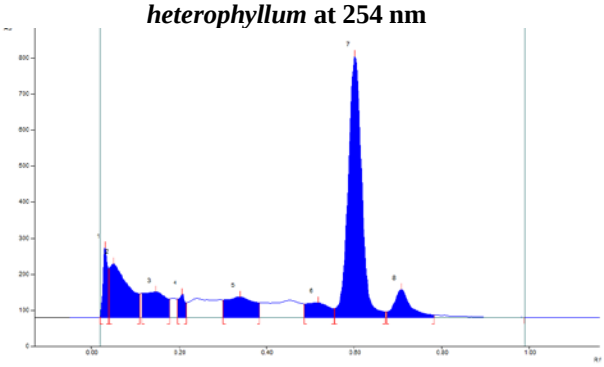


Figure 4: Densitometric scan of ethanol extract of root tuber of *A. heterophyllum* at 366 nm

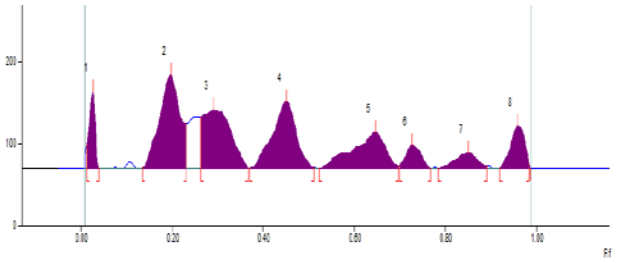


Figure 5: Densitometric scan of ethanol extract of root tuber of *A. heterophyllum* after derivatisation

GC-MS Analysis

GC-MS chromatogram of the diethyl ether extract of the tubers of *A. heterophyllum* showed 39 peaks indicating the presence of as much as compounds and were presented in Table 5. Of the 39 compounds, 21 compounds got matched with the library and were identified. Certain constituents even though exhibited high percentage composition but were not able to identify, as their mass fragmentation showed similarity less than 80%. The results revealed that Oleic Acid (16.93%) was the major component followed by Thymol, β-Asarone, n-Hexadecanoic acid, Octadecanoicacid, 2(3H)-Naphthalenone, 3-Cyclohexen-1-carboxaldehyde, 1H-Cycloprop[e] azulene and Caryophyllene oxide. β-Asarone was reported as a future effective therapeutic drug to manage cognitive impairment associated with neurological disorders such as Alzheimer's disease (Geng et al., 2010). Nonacosane is a straight chain hydrocarbon having antibacterial, antihypertensive, Angiotensin AT-II receptor antagonistic and saluretic activity (Mihailovi, 2011).

Table 5: List of phytochemicals identified by GC-MS analysis of diethyl ether extract of root tubers of *A. heterophyllum*

Peak	RT	% Area	Name
1	11.142	4.51	Thymol*
2	11.824	0.54	Copaene*
3	12.055	0.65	3H-3a,7-Methanoazulene*
4	12.137	0.85	Caryophyllene*
5	12.393	1.31	Benzene*
6	12.549	0.92	Naphthalene*
7	12.656	0.66	Cyclohexene*
8	12.693	0.52	Naphthalene*
9	12.806	0.98	1,6,10-Dodecatrien-3-ol*
10	13.050	4.47	β.-Asarone*
11	13.106	1.74	Caryophyllene oxide*
12	13.250	1.95	3-Cyclohexen-1-carboxaldehyde*
13	13.281	0.57	-
14	13.331	1.21	2,4a-Methanonaphthalen-7(4aH)-one*
15	13.375	1.80	-
16	13.475	1.94	1H-Cycloprop[e]azulene*
17	13.532	1.63	-
18	13.563	1.71	-
19	13.688	0.62	-
20	13.738	2.67	2(3H)-Naphthalenone*
21	13.857	0.82	-
22	13.932	0.69	7-Isopropenyl-1,4 a-dimethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one*
23	14.019	0.94	-
24	14.070	0.50	-
25	14.138	1.20	-
26	14.664	2.69	-
27	14.720	4.16	n-Hexadecanoic acid *
28	14.858	0.53	-
29	15.540	16.93	Oleic Acid*
30	15.621	4.10	Octadecanoic acid*
31	15.871	0.74	-
32	16.384	0.97	-
33	16.622	6.07	-
34	17.504	0.66	-
35	18.611	0.83	-
36	19.743	1.25	Octacosane*
37	20.619	2.87	-
39	20.875	1.30	Nonacosane*

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