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ANALYTICAL STANDARDS FOR THE ROOT TUBERS OF *ACONITUM HETEROPHYLLUM* WALL. EX ROYLE. -A RARE MEDICINAL PLANT OF SIKKIM HIMALAYAN REGION

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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 includes powder microscopy, physico-chemical screening, TLC profile and chromatographic studies. Preliminary phytochemical investigations revealed the presence of primary and secondary metabolites as carbohydrates, phenolics in methanolic and aqueous extract. The alkaloids may be present in chloroform extract .Result also indicated that, petroleum ether and chloroform extracts of *Aconitum heterophyllum* showed presence of steroids and triterpenoids. TLC profiling of all plant extracts also give an idea about the presence of these phytochemicals. R_f (Retention factor) value of different phytochemicals provide valuable clue regarding their polarity and selection of solvents for separation of phytochemicals. Concentrating on results obtained in TLC, the column chromatographic studies were set. The fraction containing search here was flavonoids as, quercetin. Performing TLC of each individual fraction, same R_f value fractions were collected& concentrated during column chromatography.
Key Words: *Aconitum heterophyllum*, phytochemical screening, TLC Profiling, & Retention factor (Rf.), Column chromatography

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INTRODUCTION

The Himalayan endemic *Aconitum heterophyllum* Wall. Ex Royle, Ranunculaceae is the genuine *Ativisha* of Ayurvedic (The Ayurvedic Pharmacopoeia of India, 1999) and *Athividayam* of Siddha literature (Anandakumar A. *et al*, 1982). Charaka described this drug, as per its therapeutic actions, under *Lekhaniya* (tissue scraping action) *Arsoghna* (haemorrhoids-curing), *Sirovirecana* (errhines, nasal therapy) Gana (groups) and *Tikta skandha* (bitter tasting) (Pandeya G. ,1997).)while Sushruta placed it under *Mustadi* and *Vacadi*. *Ativisha* is broadly used for its antipyretic, anti-inflammatory, anti-diarrheal activities and prevention of vomiting, cough and cold (Sharma P.V., 2006). On account of its high demand, *A. heterophyllum* has now become critically endangered (Srivastava N. *et al*, 2004)

Fragmentary pharmacognostic studies have been made earlier on *A. Heterophyllum* (The Ayurvedic Pharmacopoeia of India, 1999; 2001; The Siddha Pharmacopoeia of India ,2008).The phytochemistry of *Ativisha* has been studied extensively for its alkaloid profile (Srivastava N. *et al*, 2004; Csupor D. (2007). Quality standards for *Ativisha* (*A. heterophyllum*) including pharmacognosy and alkaloid content in *Ativisha* (The Ayurvedic Pharmacopoeia of India, 1999).

Due to its high cost as well as unavailabity, the chance for adulterating root tubers of *Aconitum 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 *Aconitum heterophyllum* by physicochemical characterization, TLC fingerprinting and column chromatographic analysis.

MATERIALS AND METHODS

Collection and authentication of samples

Fresh mature underground parts, used as raw drugs, were collected from Sikkim, India in February 2012 and authenticated from NISCAIR, New Delhi, where herbarium voucher specimen has been deposited under the (Ref No. NISCAIR/RHMD/Consult/2014/2410-190). The collected roots were cleaned and shade dried. Fresh samples were used for anatomical studies and dried parts were powdered, sieved and stored in an airtight container for further use.

Powder microscopy

Powder studies were carried out by using reagents and stains like iodine, potassium iodide, ferric chloride, Sudan III, ruthenium red and phloroglucinol with Con. HCl (1:1) (Khandelwal K.R., 2007; Johansen DA, 1940; World Health Organization, Quality control methods for medicinal plants, 2002). Safranin (4%) and toludine blue were used to double stain the transverse sections (Krishnamurthy K.V., 1988). All the reagents of analytical grade were procured from Hi-Media, Mumbai, India. Organoleptic characters like colour, texture, odor and taste were determined for flower powder.

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 (Pharmacopoeia of India 1996; World Health Organization, Quality control methods for medicinal plants, 1998).

Preparation of extracts and preliminary phytochemical analysis

Petroleum ether (40-60^{o}), benzene, chloroform, methanol extract obtained by successive extraction method, and water extract by maceration method. All the extracts were subjected to proximate chemical analysis. The phytochemical analysis was performed using standard procedures (Kokate C.K., 2000; Raaman N., 2006; S. John Adams *et al,* 2013). Dried, powdered (mesh size 85) samples were successively extracted, using a soxhlet apparatus, with solvents of increasing polarity, namely pet. ether, benzene, chloroform, methanol and water at 60-70 ºC for three complete cycles. All extracts were concentrated at 40-45 °C using a rotary evaporator (Rota vapor R-3, Buchi, Switzerland) to 50 ml and tested for presence of various chemicals.

Preparation of extract for Thin Layer Chromatography

87.07 g of powdered roots was extracted with 1.5 liter of chloroform by Soxhlet apparatus for 4 hrs. The AHCE (*Aconitum heterophyllum* chloroform extract) was concentrated on rotary vacuum evaporator (ROTEVA EQUITRON, Mumbai) and further dried in vacuum dryer. 0.73 g yield obtain from 87.07g powdered material (Khandelwal K.R. ,2005).

TLC pattern for AHCE

The *Aconitum heterophyllum* chloroform extract shows presence of triterpenoids in preliminary phytochemical study.

Materials and methods for TLC study (Harborne JB, 1998; Stahl E, 2007).

Plate dimensions	: 15 x 5 cm (length x width).
Plate material	: Glass material
Stationary phase	: Silica gel G slurry prepared in distilled water
Chamber material	: Glass material
Chamber dimensions	: 25 x 10 x 30 cm (length x width x height).
Solvent system	: Chloroform: Methanol **(8:0.6)**
Saturation time	: 30 minutes.
Visualization	: Spray H_2SO_4 (10 % in ethanol)
Treatment after spraying	: Heated in oven at 110^{o}C for 5-10 minutes
Sample	: AHCE
Reference standard	: stigmasterol
Observation	: Phytoserols gives pink color

Column Chromatography (CC) of Chloroform Extract of *Aconitum heterophyllum*

The triterpenoids are soluble in chloroform solvent which was used for the isolation (Patra A. *et al*, 2010). The chloroform solvent, concentrated and approx. 700mg adsorbed on silica gel (60-120 #) separately.

Column : Glass
Dimensions:
Diameter : 20 mm
Length : 550 mm
Stationary phase : Silica gel 60-120 #
Flow rate : 5 ml/min.
Fraction volume : 10-12 ml
Elution mode : Gradient
Elution : Hexane (100 %)
Hexane: Ethyl acetate (95:5)
Hexane: Ethyl acetate (90:10)
Hexane: Ethyl acetate (85:15)
Hexane: Ethyl acetate (80:20)
Hexane: Ethyl acetate (70:30)
Hexane: Ethyl acetate (60:40) Hexane: Ethyl acetate (50:50)
Hexane: Ethyl acetate (40:60)
Hexane: Ethyl acetate (30:70)
Hexane: Ethyl acetate (20:80)
Hexane: Ethyl acetate (10:90)
Ethyl acetate (100)
Ethyl acetate: Methanol (90:10)
Ethyl acetate: Methanol (80:20)
Ethyl acetate: Methanol (70:30)
Ethyl acetate: Methanol (60:40)
No. of fractions collected : 51 for AHCE

RESULTS AND DISCUSSION

Table 1. Physicochemical analysis

S.No	Physical Standard		Results (%W/W)
1	Ash Values (As per Ayurvedic Pharmacopoeia)	Total Ash Acid Insoluble Ash Water Soluble Ash	03.33 ± 0.22 00.57 09.75
2	Extractive values (As per Ayurvedic Pharmacopoeia)	Pet. Ether Water soluble alcohol soluble	03.05 ± 0.07 28.10 ± 0.31 8.00 ± 1.03
3	Moisture content (As per Ayurvedic Pharmacopoeia)	Standard value {NMT 10% W/W}	6.35 ± 0.12

Table 2. Phytochemical analysis showing successive Extractive Values of *Aconitum heterophyllum* root tubers

Extractives	*Aconitum heterophyllum*	
	% w/w	Consistency
Pet. ether (40-60^{o})	0.35	Very Sticky
Benzene	0.51	Sticky
Chloroform	0.80	Sticky
Methanol (95 %)	9.77	Sticky
Water	5.26	Solid

Table 3. Preliminary Phytochemical Investigation of *Aconitum heterophyllum* root tubers

Solvent Extract	Alkaloid	Carbohydrate	Phytoserols	Triterpenoids	Glycoside	Phenolics	Tannins	Proteins	Mucilage
Petroleum ether [40-60°]	-	-	+	-	-	-	-	-	-
Benzene	-	-	+	-	-	-	-	-	-
Chloroform	+	-	+	-	-	-	-	-	-
Methanol	-	+	-	+	+	+	+	-	-
Water	-	+	-	-	+	+	-	+	+

The Methanol extract shows presence of tannins, so the specific tests for tannins for *Aconitum heterophyllum* were performed given in Table 4.

Table 4. Specific Tests of Tannins for *Aconitum heterophyllum*

Tannins	***Aconitum heterophyllum***
	Methanol extract
Gallotannins	+
Ellagitannins	+
Catechin	-

The ethanol and water extract shows presence of glycosides, so the specific tests for glycosides for *Aconitum heterophyllum* were performed given in Table 5.
This results in presence of flavonoids and, saponins in methanolic and aqueous extracts.

Table 5. Specific Tests of Glycosides *Aconitum heterophyllum*

Glycosides	***Aconitum heterophyllum***	
	Methanol extract	**Aqueous extract**
Cardiac	-	-
Antraqinone	-	-
Cyanogenetic	-	-
Coumarin	-	-
Flavonoid	+	+
Saponins	+	+

'+' test is positive; '-' test is negative

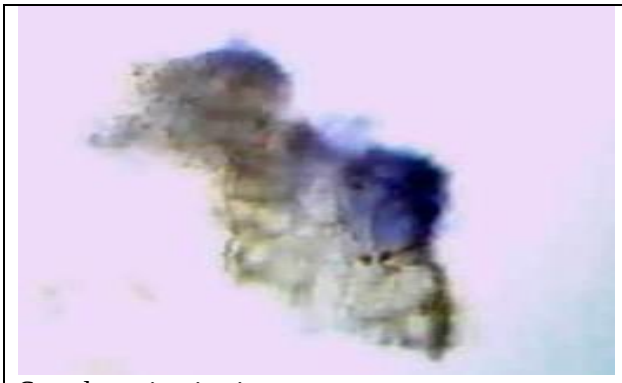
TLC Analysis

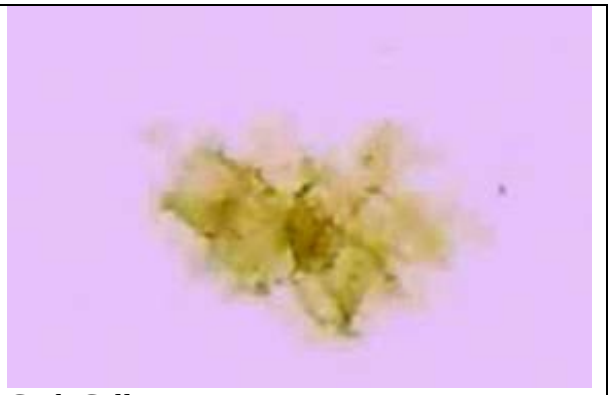
After adjusting the solvent system best results were arises as, R_f value of standard stigmasterol shows 0.78 match with the obtained R_f value of AHCE.

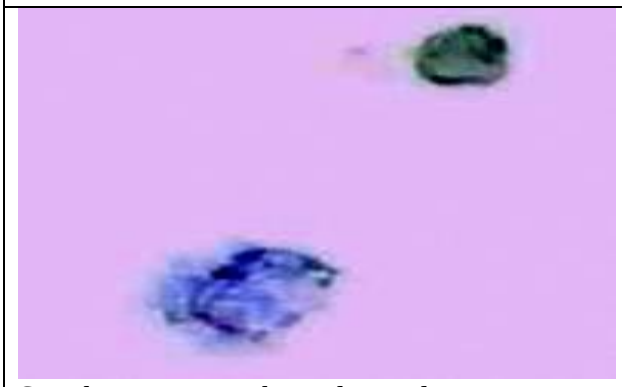
Table 6. TLC Pattern for AHCE

Color (After spraying with 10 % ethanolic H_2SO_4)	**R_f Value**	
	AHCE	**Std Stigmasterol**
Pink	0.17	--
Pale yellow	0.38	--
Brown	0.54	--
Pink	0.68	--
Dark Pink	0.76	0.78
Pink Yellow	0.86	--
Pink	0.87	--

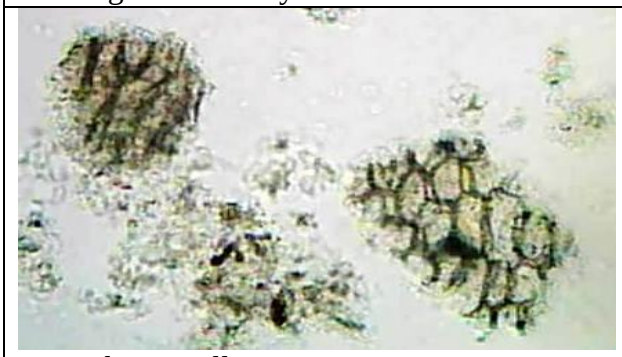
The TLC profile has revealed the presence of seven spots in AHCE after spraying 10 % ethanolic H_2SO_4 acid. The screening was carried out targeting the triterpenoids viz., Stigmasterol.

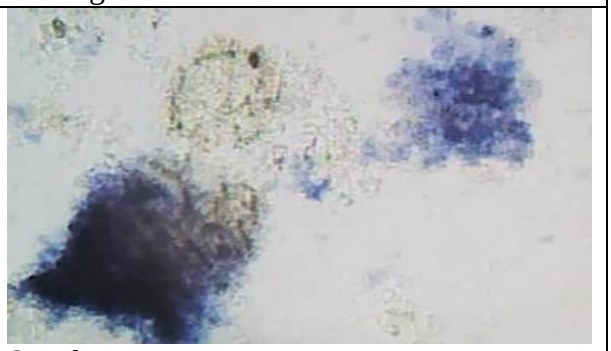

Starch grains in tissue

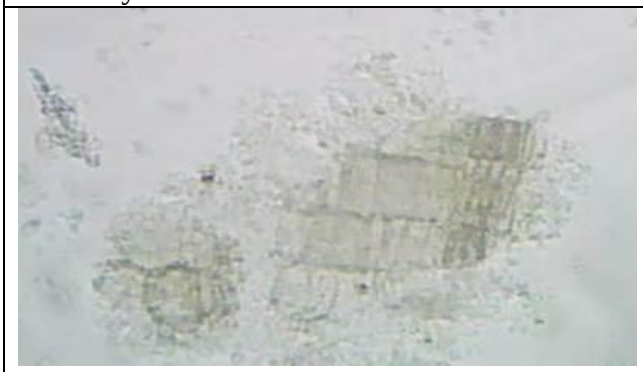

Cork Cells


Starch grains stain by Iodine solution


Phellogen cells


Parenchyma cells


Starch grains in tissue


Cork cells followed by cortex parenchyma cells

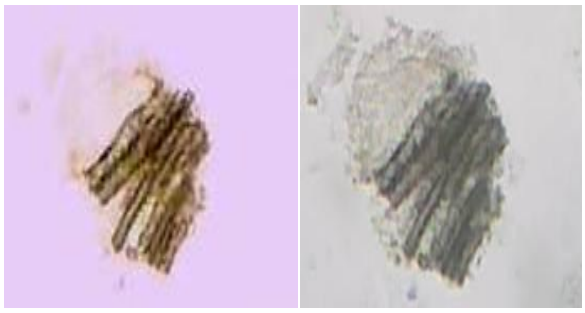
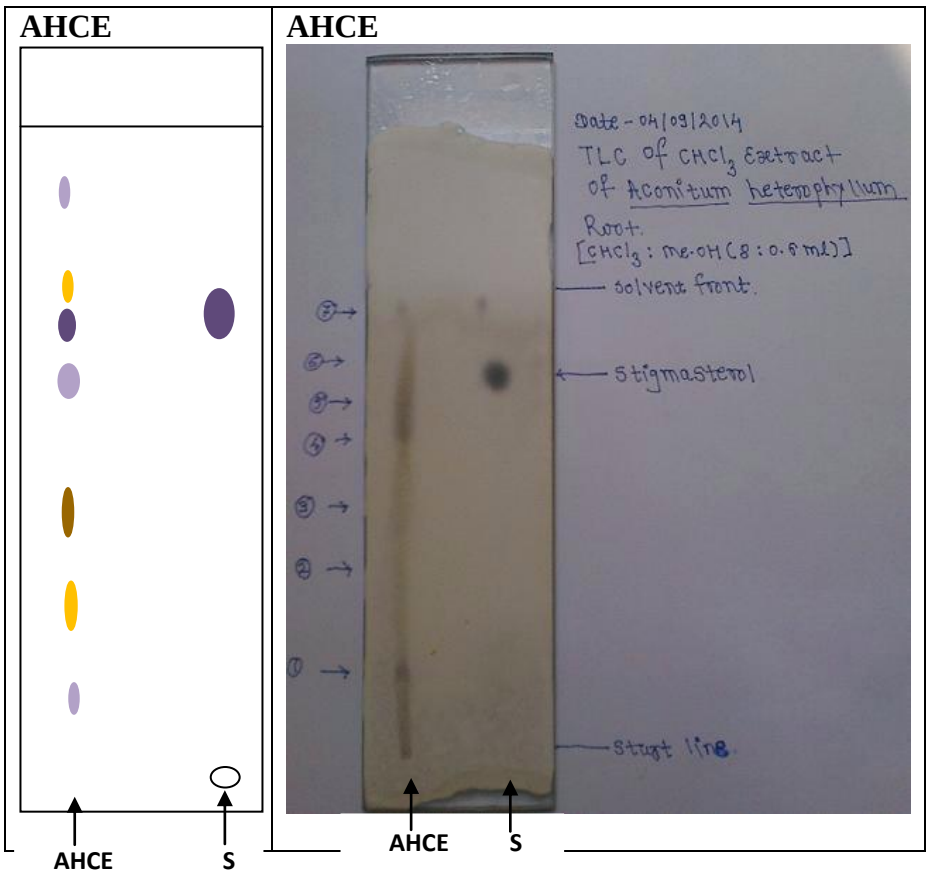

Spiral Xylem vessel stain by phloroglucinol + Conc. HCl (1:1)

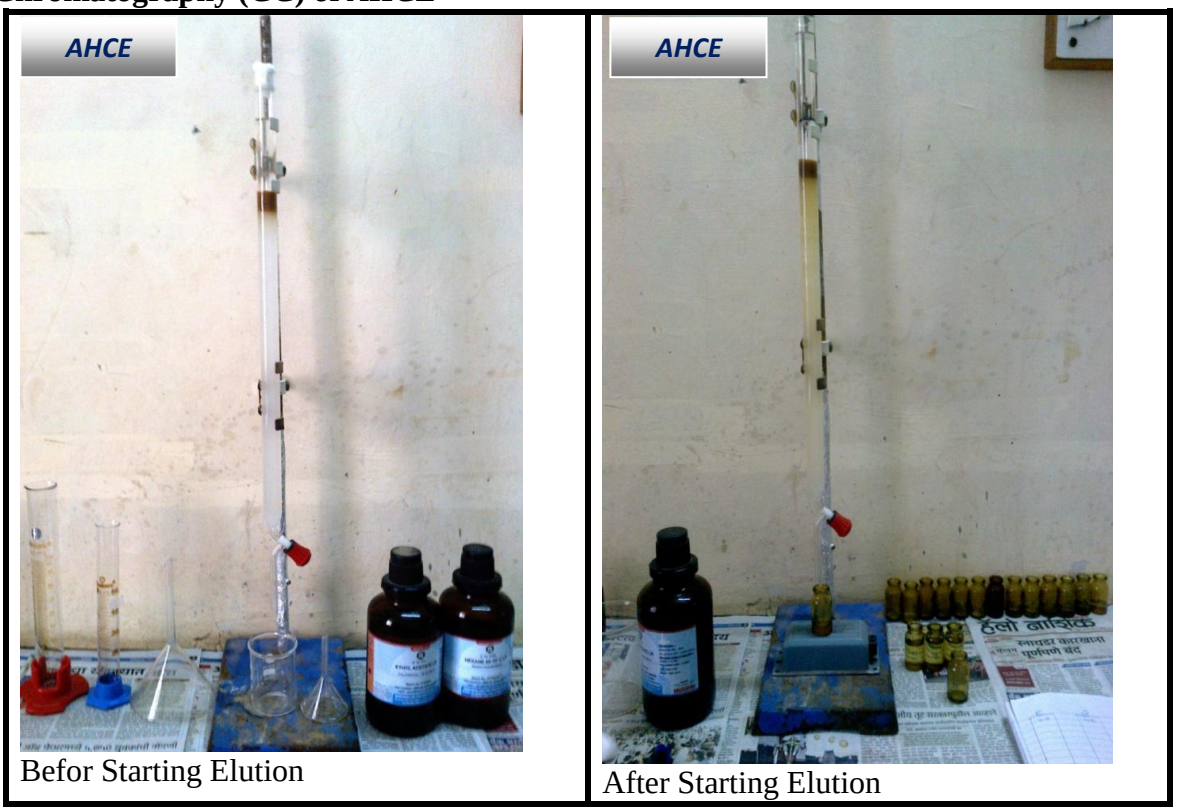
Figure 1. Powder characteristics of *Aconitum heterophyllum*



Abbreviation: AHCE- *Aconitum heterophyllum* chloroform extract; S- Stigmasterol.

Photograph 1. TLC Plate of AHCE after Spraying 10 % Ethanolic H_2SO_4 Acid

Column Chromatography (CC) of *AHCE*



Befor Starting Elution

After Starting Elution

Photograph 2. Column Chromatography of AHCE

Concentrating on results obtained in TLC, the column chromatographic studies were set. The fraction containing search here was triterpenoids as, stigmasterol. Performing TLC of each individual fraction (Table 7.), same R_f value fractions collected, concentrated during column chromatography.

Table 7. TLC Pattern of AHCE Fractions

Fractions	TLC pattern	Qty (mg)
01-05	No any spot	--
06-11	Fixed oil	07.0
12-17	Trace amount mixture	--
18-20	Single Spot	11.3
21-24	Waxy substance	--
25-32	Mixture of 2 compound	17.0
33-40	Mixture of 3 compound	11.0
41-51	Mixture of 3 compound	09.0

Observation

AMACE: In TLC fraction 25 to 32 shows compounds with similar R_f value for solvent system Chloroform: Methanol (8:0.6) followed by 1 % H_2SO_4 spraying then kept at 50^{o}C in oven for 10 min. The fraction 25 to 32 prepared by mixing after evaporation of solvents it gives yellowish white solid mixtures of compounds.

These mixtures of compounds subjected for preparative TLC to obtained single pure compounds.

Preparative TLC

Fractions obtained from column chromatography mostly consist of mixture of 2 components. This requires further fractionation, which can be done by preparative TLC. Plates for preparative TLC are thick in layer as compared to normal TLC plates.

Samples were applied in the form of bands and allowed to run in TLC chambers. After development of the plate, the band separated was scrapped out in strip forms, collected separately and labeled. De-sorbing in appropriate solvent recollected samples and purity was checked by TLC. Further samples were subjected to spectroscopic studies.

Fraction 25 and 32 of AHCE were subjected to preparative TLC in Chloroform: Methanol (9:0.6). From this one pure compound (9mg) was obtained and labeled as; *Aconitum heterophyllum* chloroform fraction (AHC-1).

AHC-1

Description

Color **:** Off white color
Solubility **:** Soluble in chloroform, methanol, ethyl acetate and ethanol
M.P. **:** 175-177^{o}C
Salkowaski Test **:** Red color at the bottom shows presence of Steroids
λ_{max} **:** 250 nm

After studying the physical properties and chemical properties, we can predict that **AHC-1** is **stigmasterol** (Fig 2.).

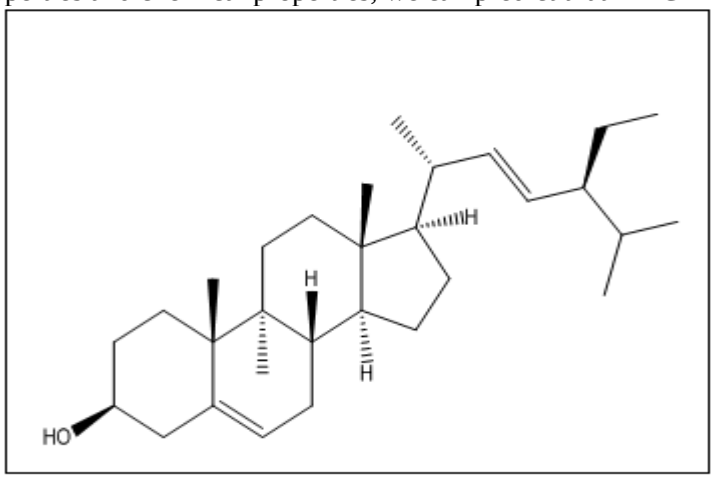


Figure 2. Predicted Structure of AHC-1 as, Stigmasterol

DISCUSSION

When a new drug is to be discovered, qualitative phytochemical analysis is a very important step as it gives information about the presence of any particular primary or secondary metabolite in the extracts of the plant which is having a clinical significance. In any case, if any significant bioactive natural product is present, it is necessary to separate that compound from the mixture of compounds by using suitable chromatographic technique.

The evaluation of a crude drug is an important diagnostic character useful in determining authenticity and identifying adulteration. As there is no phytochemical work recorded on this medicinally potent plant, the present work was undertaken to lay down the standards which could be useful for establishing its authenticity. Pharmacognostic parameters like microscopic features of roots have been studied. Preliminary phytochemical screening reveals the useful findings about chemical nature of drugs. Total ash values and extractive values are useful in identification and authentication of the plant material .Extractive values is useful to evaluate the chemical constituents of crude drug.

Preliminary phytochemical investigations revealed the presence of primary and secondary metabolites as carbohydrates, phenolics in methanolic and aqueous extract. The alkaloids may be present in chloroform extract .Result also indicated that, petroleum ether and chloroform extracts of *Aconitum heterophyllum* showed presence of steroids and triterpenoids.

TLC profiling of extracts gives an impressive result that directing towards the presence of number of phytochemicals. Various phytochemicals give different R_f values indifferent solvent system. This variation in Rf values of the phytochemicals provide a very important clue in understanding of their polarity and also help in selection of appropriate solvent system for separation of pure compounds by column chromatography. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extracts can only be achieved by analyzing the Rf values of compounds in different solvent system. Different R_f values of the compound also reflect an idea about their polarity. This information will help in selection of appropriate solvent system for further separation of compound from these plant extracts.

CONCULUSION

The results obtained in the present investigation indicated *Aconitum heterophyllum* as a rich source of secondary metabolites. These findings suggested that *Aconitum heterophyllum* could be a potential source of natural antioxidant having great importance as therapeutic agent and preventing oxidative stress related degenerative diseases

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