Research Article

Compound Isolation and Purification by Chromatographic Method of Stem Bark of *Anisoptera scaphula* (Roxb.).

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ABSTRACT

Anisoptera scaphula (Roxb.) belonging to the family Dipterocarpaceae, has been studied for isolation of its secondary metabolites and evaluation of biological activities of the extractives, with special emphasis on TLC chromatographic data. Identification and isolation of the active constituents from traditionally used phytotherapy can ensure the health care of the poor people. In addition, herbal drugs could be scientifically modified for better pharmacological activity and to establish safe and effective drugs and the rationality of the present study lies in meeting the challenge of developing herbal medicines, which needs a systematic research on indigenous medicinal plants for the welfare of the humanity. This study will help to elucidate the hidden potential compounds which may be helpful for human being. The stem bark of *A. scaphula* was extracted with organic solvents and the extracts were fractionated with standard chromatographic techniques. A total of five compounds were isolated from the investigated plant. Chromatographic fractionation and purification of the pet-ether extract yielded a triterpene (AS-1), which was purified and identified as stigmasterol**.** The ethyl acetate extract yielded a total of five compounds and Characterization of these isolated compounds by ^{1}H NMR spectrum is underway. Therefore plant materials, especially the traditionally used medicinal plants can be potential sources of chemically interesting and biologically important drug candidate.

Keywords: *Anisoptera scapula* (Roxb.), chromatographic techniques, dipterocarpaceous, isolation, characterization, stigmasterol.

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INTRODUCTION

Anisoptera scapula (Roxb.) **Synonyms:** *Aniso-ptera glabra* Kurz, *Hopea scaphula* (Roxb.), *Scaphula glabra* Parker, *Vatica scaphula* (Roxb.) Dyer, *Hopeoides scaphula* (Roxb.) Cretz. Very tall tree, the girth of bole reaching up to 9 m. twigs glabrous. Dipterocarpaceous plants contain various resveratrol oligomers that exhibit a variety of biological activities, such as antibacterial and antitumor effects. For example vaticanol C, a resveratrol tetramer, exhibits strong cytotoxicity against various tumor cell lines [1]. Flowering: December-April, Fruiting: March-May. Anisoptera is a clearly defined botanical group distinct from other groups in Dipterocarpaceae by the entire calyx tube fused to the seed [2-4]. Sterols are the first steroid isolated from natures and the most common sterol in plants is β-sitosterol. Stigmasterol is closely related to β-sitosterol containing an additional double bond at position 22. Sterols are formed biosynthetically from isopentenyl pyrophosphate and involve the same sequence of reaction as terpenoid biosynthesis, where Squalene is an intermediate. In this present study Stem bark of *Anisoptera scaphula* (Roxb.) extracts were subjected to several chromatographic techniques to isolation & characterization compounds.

MATERIALS AND METHODS

Plant sample of *A. scaphula* was collected from Dhaka in August 2011. A voucher specimen has been deposited in Bangladesh National Herbarium, Dhaka for collection.

Bark of the plant was cut into small pieces and then air-dried for several days. The pieces were then oven dried for 24 hours at considerably low temperature to effect grinding. The plant was then ground into a coarse powder. The air-dried and powdered plant material (200.5 gm) was extracted with petroleum ether, followed by ethyl acetate and finally with methanol. The time duration was of 15 days at room temperature with occasional shaking and stirring for each successive extraction. It was then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then reduced using a Buchii Rotavapor at low temperature and pressure. Thus three types of crude extracts were found:

I. Petroleum ether extract (1.7 gm)
II. Ethyl acetate extract (1.5 gm)
III. Methanol extract (1.00 gm)

Thin layer chromatography (TLC)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available percolated silica gel (kiesel gel 60 PF254) plates are usually used. For initial screening, TLC plates are made on glass plates with silica gel (kiesel gel 60 PF254). A number of glass plates measuring 20 cm x 5 cm are thoroughly washed and dried in an oven. The dried plates are then swabbed with acetone-soaked cotton in order to remove any fatty residue. To make the slurry-required amount of silica gel 60 PF254 and appropriate volume of distilled water (2 ml/gm of silica gel) are mixed in a conical flask and the flask is gently shaken. The slurry is then evenly distributed over the plates using TLC spreader. After air-drying the coated plates are subjected to activation by heating in an oven at 110^0c for 70 minutes [5]. (**Table 1)** shows the amount of silica gel required for preparing plates of varying thicknesses.

Cylindrical glass chamber (TLC tank) with airtight lid is used for the development of chromatoplates. The selected solvent system is poured in sufficient quantity into the tank. A smooth sheet of filter paper is introduced into the tank and allowed to soak in the solvent. The tank is then made airtight and kept for few minutes to saturate the internal atmosphere with the solvent vapor. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%) [6].

Table 1: **Amount of silica gel required for preparing TLC plates of various thicknesses.**

Size (cm x cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20 x 5	0.3	0.9
	0.4	1.2
	0.5	1.5

A small spot of the solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drawn 2 cm below the upper edge of the activated plate, which marks the upper limit of the solvent flow. The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap/lid is placed again. The plate is left for development. When the solvent front reaches up to the given mark, the plate is taken out and air-dried. The properly developed plates are viewed under UV light of various wavelengths as well as treated with suitable reagents to detect the compounds. Preparative thin layer chromatographic technique is routinely used in separating and for final purification of the compounds. The principle of preparative TLC is same as that of TLC. Here larger plates (20 cm x 20 cm) are used. (**Table 2**) shows the amount of silica gel required for preparing plates of varying thicknesses.

The sample to be analyzed is dissolved in a suitable solvent and applied as a narrow uniform band rather than spot. The plates are then developed in an appropriate solvent system previously determined by TLC. In some cases multiple development technique was adopted for improved separation. After development, the plates are allowed to dry and the bands of compounds are visualized under UV light

(254 nm and 366 nm) or with appropriate spray reagents on both edges of the plates. The required bands are scraped from the plates and the compounds are eluted from the silica gel by treating with suitable solvent or solvent mixtures.

Table 2: Amount of silica gel required preparing PTLC plates of various thicknesses

Size (cm x cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20 x 20	0.3	3.6
	0.4	4.8
	0.5	6.0

Visualization/detection of compounds

Detection of compounds in TLC plates is a very important topic in analyzing extractives to isolate pure compounds. The following techniques are used for detecting the compounds in TLC/PTLC plates.

Visual detection

The developed chromatogram is viewed visually to detect the presence of colored compounds.

UV light

The developed and dried plates are observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.

Iodine chamber

The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

Spray reagents

Different types of spray reagents are used depending upon the nature of compounds expected to be present in the fractions or the crude extracts.

a) Vanillin/H2SO4 [5]: 1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100^0C for 10 minutes.

b) Modified Dragendorff's reagent [6]: Modified Dragendorff's reagent was used to detect alkaloids. Some coumarins also give a positive test with modified Dragendorff's reagent. The reagent is prepared by mixing equal parts (v/v) of 1.7 % bismuth subnitrate dissolved in 20 % acetic acid in water and a 40 % aqueous solution of potassium iodide.

c) Ferric chloride/EtOH [7]: Some of the phenolic compounds were detected by spraying the plates with ferric chloride (5% ferric chloride in absolute ethanol) reagent.

d) Perchloric acid reagent [8]: 2% aqueous perchloric acid produces brown spots with steroids after heating at 100^0C for 10 minutes.

e) Potassium permanganate reagent [9]: Only the oxidizable compounds were detected by this reagent. After spraying with the reagent the compound appeared as yellow or pale yellow spot on the colored (color of permanganate) plate.

Determination of rf (retardation factor) values:

Rf value is characteristic of a compound in a specific solvent system. It helps in the identification of compounds. Rf value of a compound can be calculated by the following formula:

$$\text{Rf value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent system}}$$

Investigation of the pet-ether soluble fraction:

The Petroleum ether crude extract was subjected to TLC screening to see the type of compounds present in the extract. 400 mg of the petroleum ether extract was subjected to Column Chromatography (CC) for fractionation. Then the column fractions were analyzed by TLC. The fractions with satisfactory resolution of components were subjected to PTLC to obtain the pure compound.

Column chromatography (cc) of pet-ether soluble fraction:

The column was packed with silica gel (Kieselgel 60, mesh 70-230). Slurry of silica gel was added into a glass column having the length and diameter of 55 cm and 1.1 cm respectively. When sufficient height of the adsorbent bed was obtained, a few hundred millilitre of petroleum ether was run through the column for proper packing

of the column. The sample was prepared by adsorbing 400 mg of petroleum ether extract onto silica gel (Kieselgel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate of increasing polarity, then by ethyl acetate and finally with ethyl acetate and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of petrol extract were listed in **(Table 3)**.

Table 3: Different solvent systems used for the column chromatographic analysis of pet-ether extracts.

Fraction number	Solvent systems	Volume collected (ml)
1	Petroleum ether 100 %	100
2	Petroleum ether - Ethyl acetate (99 : 1)	100
3 to 5	Petroleum ether - Ethyl acetate (97 : 3)	100
6 to 18	Petroleum ether - Ethyl acetate (95 : 5)	300
19 to 31	Petroleum ether - Ethyl acetate (92.5 : 7.5)	300
32 to 43	Petroleum ether - Ethyl acetate (90 : 10)	300
44 to 56	Petroleum ether - Ethyl acetate (85 : 15)	300
57 to 70	Petroleum ether- Ethyl acetate (80 : 20)	400
71 to 86	Petroleum ether- Ethyl acetate (70 : 30)	400
87 to 102	Petroleum ether- Ethyl acetate (50 : 50)	400
103 to 110	Ethyl acetate 100%	200
111 to 115	Ethyl acetate - Methanol (99 :1)	100
116 to 120	Ethyl acetate - Methanol (98 : 2)	100
121 to 125	Ethyl acetate - Methanol (95 : 5)	100

Analysis of column fractions by TLC:

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulphuric acid reagent. A number of compounds were detected, which were purified from the different sub-fractions employing various techniques. The isolated compounds have been summarized in **(Table 4)**.

Table 4: A list of isolated compounds from pet-ether and ethyl acetate soluble fractions

Column Fractions	Mobile phases	Rf value	Amount (mg)	Yield* (%)	Code
57-59 (PE)	Toluene : Ethyl acetate 70 : 30	0.67	5.0	0.003	AS-1
05-06 (EA)	Toluene : Ethyl acetate 88 : 12	0.88	29.5	0.015	AS-3
05-06 (EA)	Toluene : Ethyl acetate 88 : 12	0.53	4.0	0.002	AS-4
05-06 (EA)	Toluene : Ethyl acetate 88 : 12	0.33	5.0	0.003	AS-6
09-11 (EA)	Toluene : Ethyl acetate 88 : 12	0.50	4.0	0.002	AS-8

** Calculated on total amount of plant.*

PE = pet-ether soluble fraction obtained from column chromatography

EA = ethyl acetate soluble fraction obtained from gel chromatography

RESULTS

Isolation of compound AS-1

The fractions 57 to 59 were bulked together as they showed similar TLC feature *i.e.* a distinct spot with same Rf value (0.67) was found in the solvent system Toluene: Ethyl acetate (70:30). It was then subjected to PTLC. White needles of AS-1 were obtained, which was found to be pure by TLC screening.

Isolation of compound AS-3, AS-4 and AS-6

The ethyl acetate fractions 05-06 were mixed together as they showed three distinct spots on TLC in the solvent system Toluene: Ethyl acetate (88:12). It was then subjected to PTLC using the same solvent system. The desired three bands were scrapped and then eluted with distilled ethyl acetate to give AS-3, AS-4 and AS-6.

Isolation of compound AS-8

The ethyl acetate fractions 09-11 were bulked together as they showed similar TLC feature in the solvent system Toluene: Ethyl acetate (88:12). It was then subjected to PTLC using the same solvent system. The desired band was scrapped and then eluted with distilled ethyl acetate to give AS-7 and AS-8.

Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 are summarized in the **(Table 5)**.

Table 5: Properties of the isolated compounds from pet-ether and ethyl acetate soluble fractions.

Isolated Compounds	Physical form	Color	Solubility						Color with Vanillin-H_2SO_4
			Pet-ether	EtOAc	$CHCl_3$	Acetone	MeOH	DMSO	
AS-1	Needle shaped crystal	White	-	-	+	+	+	+	Purple
AS-3	Oily liquid	Pale Yellow	-	P.s	+	+	+	+	Yellow
AS-4	Amorphous Powder	White	-	P.s	+	+	+	+	Blue
AS-6	Amorphous Powder	White	+	+	+	-	-	-	Pink
AS-8	Amorphous Powder	Pale yellow	P.s.	+	+	+	+	-	Yellow

+ indicates completely soluble; p.s. indicates partially soluble; - indicates not soluble

Characterization of AS-1 as STIGMASTEROL (15)

Compound AS-1 was obtained as needle shaped crystals. It was evident as a purple spot on TLC (Silica gel PF_{254}) when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110°C for 5-10 minutes. The R_f value of the compound was 0.67 in toluene-ethyl acetate (70: 30) on Silica gel PF_{254} plate. It was found to be soluble in petroleum ether, methanol and chloroform. Its melting point was 160-164°C which is identical to that observed for stigmasterol (92) [10].

DISCUSSION

From the extractives five pure compounds were isolated applying various chromatographic techniques & this data will help to characterize the isolated compounds for further research and application.

CONCLUSION

Successive chromatographic separation and purification of the pet-ether, ethyl acetate and methanol fraction of *A. scaphula* yielded a total of five compounds. The isolated pure compounds structure elucidation & characterization by ^1H NMR spectrum is underway.

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