



A NEWER APPROACH IN PURIFICATION OF ACETONE EXTRACT OF *PYRUS PASHIA* BUCH.-HAM. ex D. DON STEM BARK

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ABSTRACT

This study reported the purification of acetone extract of *Pyrus pashia* Buch.-Ham. ex D. Don stem bark. The powdered drug was defatted with petroleum ether and then extracted with acetone. The orange brown acetone extract had no odour, showed the presence of desired phytochemicals *i.e.* steroids, tannins, terpenes *etc.* The acetone extract was further subjected to chromatographic purification in separating funnel and one pure compound has been successfully isolated from acetone fraction which was confirmed on the basis of TLC, paper chromatography. UV spectroscopic studies were also performed on isolated compound. This method of purification involving separating funnel was employed for the first time and found to be simple, cost effective and highly efficient for purification of acetone extract of *Pyrus pashia* Buch.-Ham. ex D. Don stem bark.

Keywords: *Pyrus pashia*, TLC, Purification, acetone extract.

INTRODUCTION

Nature has been a potential source of therapeutic agents for thousands of years. An impressive number of modern drugs have been derived from natural sources. Over the last century, a number of top selling drugs have been developed from natural products. Anticancer drug vincristine from *Vinca rosea*, narcotic analgesic morphine from *Papaver somniferum*, antimalarial drug artemisinin from *Artemisia annua*, anticancer drug Taxol from *Taxus brevifolia* and antibiotic penicillins from *Penicillium* sp. are just a few examples. Recently, natural products chemistry has undergone explosive growth due to advances in isolation techniques, synthetic and biosynthetic approaches as well as spectroscopic and chromatographic methods¹.

Various chromatographic techniques for isolation of compounds²⁻⁴: The most important factor that has to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. The general features of the molecule that are helpful to ascertain the isolation process include solubility (hydrophobicity or hydrophilicity), acid-base properties, charge, stability, and molecular size. If isolation of a known compound from the same or a new source is to be done, literature information on the chromatographic behavior of the target compound can be easily obtained, and one can choose the most appropriate method for isolation without any major difficulty. The chromatographic techniques used in the isolation of various types of natural products can be broadly classified into two categories: classical or older and modern.

Classical or older chromatographic techniques include:

- Thin-layer chromatography (TLC)
- Preparative thin-layer chromatography (PTLC)

- Open-column chromatography (CC)
- Flash chromatography (FC)

Modern chromatographic techniques are:

- High-performance thin-layer chromatography (HPTLC)
- Multiflash chromatography
- Vacuum liquid chromatography (VLC)
- Chromatotron
- Solid-phase extraction
- Droplet countercurrent chromatography (DCCC)
- High-performance liquid chromatography (HPLC)
- Hyphenated techniques (e.g., HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR)

Description of plant: The genus *Pyrus* L. is a tree member of subfamily *Maloideae*, family *Rosaceae*. *Pyrus pashia* Buch.-Ham. ex D. Don is a small or medium-sized deciduous tree. Leaves are 5-10 cm long, ovate to broadly lance-shaped, long-pointed, toothed, hairless and shining. Flowers are 2-2.5 cm across, with white obovate petals with darker veins. Fruit is round, 1.3-2.5 cm, dark brown, covered with raised pores and is edible when half-rotten. *Pyrus pashia* Buch.-Ham. ex D. Don is found in the Himalayas, from Afghanistan to South West China and Burma, at altitudes of 750-2700 m. Flowering season is from March-April⁵. *Pyrus* species (Pear) have been known to possess various physiological activities and have possessed various useful compounds including chlorogenic acids, flavan-3-ols and arbutin. These polyphenols have shown antioxidant properties, antimutagenic and anti-carcinogenic effects⁶⁻⁸.



MATERIALS AND METHODS

Collection and authentication of plant: The stem bark was collected in the month of January from Distt. Kangra, Himachal Pradesh, India. The bark was identified and authenticated by Raw Materials Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources, New Delhi, India and voucher specimen number was NISCAIR/RHMD 1691/289. The collected bark was shade dried for 40 days and finally pulverized by into coarse powder. It was stored in a well closed container free from environmental climatic changes or any other contamination till usage for the further studies.

Extraction procedure: The powdered material (200 g) was successively extracted using soxhlet apparatus with petroleum ether (defatted) and acetone. The resultant extracts were concentrated on water bath at 40°C. The extractive value was calculated and the dried extracts of the drug were also evaluated for physical parameters such as consistency, colour, odour *etc.*

Phytochemical evaluation: It involves testing of different extracts of *Pyrus pashia* Buch.-Ham. ex D. Don for their contents of different classes of compounds i.e. Steroids, Flavonoids, Tannins, Saponins *etc.* The methods used for detection of various phytochemicals were followed by qualitative chemical test to give general idea regarding the nature of constituents present in crude drug⁹.

Purification of acetone extract: Purification of acetone extract was achieved in separating funnel as shown in Fig. 1a, 1b with following conditions:

- Adsorbent used: Silica gel 230-400 for column chromatography
- Dimensions of the separating funnel: L- 17 cm
- Length of adsorbent packed: 11 cm
- Rate of elution: 5 drops per min.
- Volume of elute collected: 5 mL

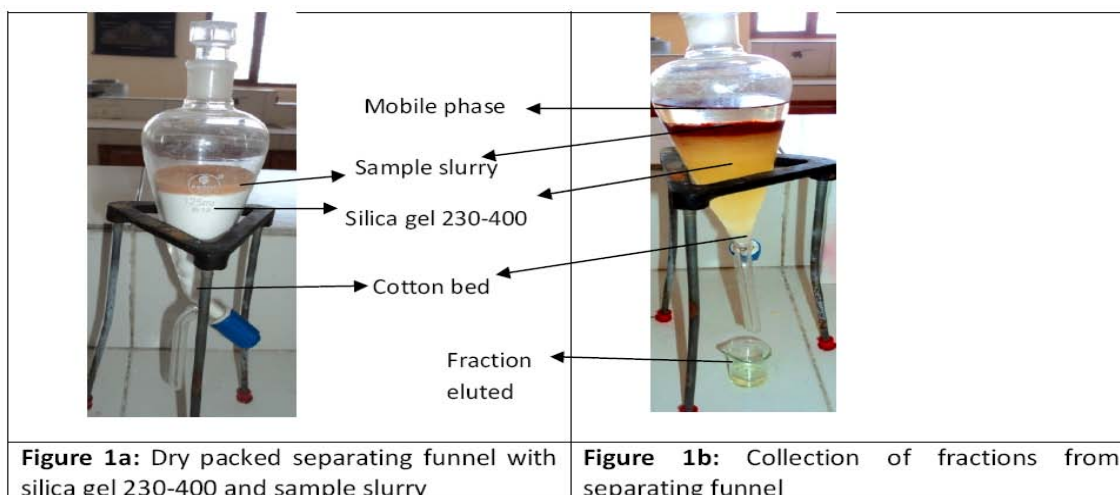


Figure 1a: Dry packed separating funnel with silica gel 230-400 and sample slurry

Figure 1b: Collection of fractions from separating funnel

Preparation of sample slurry: 1 g of acetone fraction was dissolved in appropriate amount of methanol and mixed with 3 g of silica gel 230-400. The slurry was dried with constant stirring in bowl on water bath at 40°C in order to make a free flowing powder. The adsorbed material obtained was then transferred to the separating funnel.

Dry packing of separating funnel: The cotton was fixed at bottom. The silica gel 230-400 was dry packed in to the separating funnel, in appropriate portion, with gentle tapping after each addition in order to ensure uniform packing and to remove air bubbles, if any. Sample slurry was then loaded into separating funnel.

Eluting solvent: Mobile phase was then added slowly and was allowed to run continuously and a small quantity of eluent was allowed to remain at the top of the separating funnel so as to prevent the drying of funnel. Gradient elution *i.e.* EtOAc:CHCl₃:MeOH:H₂O in ratio 12:8:8:2 solvent system was used as eluting solvent. 32 fractions of 5 mL each were collected and the collected fractions

were grouped together according to their homogeneity judged from the TLC and paper chromatographic analysis.

Chromatographic investigation: Chromatographic investigations of acetone extract of *Pyrus pashia* Buch.-Ham. ex D. Don was achieved using thin layer chromatography and paper chromatography.

Thin layer chromatography (TLC): Thin layer chromatography (TLC) is a chromatographic technique used to separate mixtures. It is performed on a sheet of glass which is coated with a thin layer of adsorbent material, usually silica gel. This layer of adsorbent is known as the stationary phase. After the sample is applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate *via* capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Preparation of the plates: The adsorbent used for thin layer chromatography was silica gel G. About 50 g of silica gel G was taken in a 250 mL stoppered conical and about

100 mL of distilled water was added to it. The mixture was shaken vigorously for about 2 min. until it became homogeneous suspension. This suspension was then spread immediately on thin layer chromatographic plates with the help of applicator.

Drying and storage of the plates: The freshly coated plates were then air dried until the transparency of the layer had disappeared. The plates were then stacked in a drying rack and were heated in an oven for 30 min. at 110°C. The activated plates were kept in a desiccator, till required for further use.

Application of the sample: For applying test samples on TLC plate, glass capillaries were used. The spots were applied with the help of a transparent template, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity.

Chromatographic chamber, conditions of saturation and the development of TLC plates: Chromatographic rectangular glass chamber (16.5 cm x 29.5 cm) was used in the experiments. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth sheet of filter paper approximately of 15 x 40 cm size was placed in the chromatographic chamber in a 'U' shape and was allowed to be soaked in the developing solvent. After being thus moistened, the paper was then pressed against the walls of the chamber, so that it adhered to the walls. The chamber was allowed to saturate before use. The experiments were carried out at room temperature in diffused daylight.

Visualization of spots in TLC:

Spots were detected by using both non-destructive and destructive visualisation techniques.

(a) Non-destructive technique involves use of UV light, iodine vapours.

(b) Destructive technique involves use of spraying reagents (Anisaldehyde sulphuric acid and Vanillin-sulphuric acid). Various extract were applied with the help of micro capillary, just 2 cm above from the bottom. The spots were equally sized, dried, and developed and finally the R_f values were also noted¹⁰.

Paper chromatography: Paper chromatography is a method of separating mixtures by using a piece of absorbent paper. In this process, the solution to be

separated is placed on a piece of dry filter paper (stationary phase). A solvent (the moving phase) is allowed to travel across the paper by capillary action. As the solvent front moves, the components of the mixture separate. The components of the mixture that is most soluble in the solvent and least attracted to the paper travel the farthest¹¹. We employed this technique for determining presence of various types of phytoconstituents in acetone extract using Whatman filter paper (Grade 1) of *Pyrus pashia* Buch. Ham. ex D. Don stem bark. Extract is applied to a piece of absorbent paper. Mobile phase (EtOAc:CHCl₃:MeOH:H₂O in ratio 12:8:8:2) is then allowed to move through the applied substance with radially ("wicking" action of the paper disperses the solvent in a particular) and vertically (capillary action) motion.

Visualization of spots in paper chromatography: Spots were detected by using dipping the paper in vanillin-sulphuric acid reagent solution followed by drying in hot air oven at 80°C until spots appeared.

UV spectroscopy: UV determination of isolated compound was done to elucidate the structure of compound isolated. Spectrophotometry is used for both qualitative and quantitative investigations of samples. The wavelength at the maximum of the absorption band will give information about the structure of the molecule or ion and the extent of the absorption is proportional with the amount of the species absorbing the light. Quantitative measurements are based on Beer's Law (also known as "Lambert-Beer Law")¹².

RESULTS AND DISCUSSION

The plant after shade drying was powdered and subjected for the organoleptic characterization. The colour of the powdered drug was found to be brown with no odour and rough texture (Table 1). After this 200 g of powdered drug was weighed and was first defatted in soxhlet apparatus and then extracted with acetone. The extractive value was also calculated. The extract was further examined physically like colour, odour, consistency, etc. as indicated in Table 2.

Qualitative chemical tests were carried out for the petroleum ether and acetone extract of *P. pashia* Buch.-Ham. ex D. Don stem bark. The results of the tests showed the presence of steroids, terpenes and tannins (Table 3).

Table 1: Organoleptic characters of powdered of crude drug

Drug	Nature	Colour	Odour	Texture
<i>Pyrus pashia</i> Buch. Ham. ex D. Don stem bark	Coarse	Brown	No odour	Rough

Table 2: Physical characteristics of extracts of *Pyrus pashia* Buch.-Ham. ex D. Don

Extract	Consistency	Colour	Odour	Extractive value (%w/w)
Petroleum ether	Sticky	Light greenish	Characteristic	0.92
Acetone	Powdered	Orange brown	None	3.49



Table 4: Polarity table for acetone fraction

Solvent system used EtOAc:CHCl ₃ :MeOH:H ₂ O	Fractions	TLC system EtOAc:CHCl ₃ :MeOH:H ₂ O
12:8:2:2	1-5 (I)	12:8:4:2
12:8:4:2	6-11 (II)	12:8:4:2
12:8:6:2	12-18 (III)	12:8:4:2
12:8:8:2	19-25 (IV)	12:8:6:2
12:8:8:2	26-31 (V)	12:8:6:2
Elution with methanol	32 (VI)	12:8:8:2

EtOAc- Ethyl acetate; CHCl₃-Chloroform; MeOH-Methanol

Phytochemical evaluations: The preliminary phytochemical screening of petroleum ether and acetone extract showed the presence of following constituents (Table 3).

Table 3: Preliminary phytochemical screening

Chemical test for	Petroleum ether	Acetone
Alkaloids	-	-
Carbohydrates	-	-
Glycosides	-	-
Flavonoids	-	-
Proteins and amino acids	-	-
Saponins	-	-
Tannins	-	+
Lipids/fats	+	-
Steroids	-	+

Purification of acetone extract: Purification of acetone extract was achieved using separating funnel. Separating funnel was first dry packed in silica gel 230-400 and was gradually eluted with EtOAc:CHCl₃:MeOH:H₂O in ratio 12:8:2:2-12:8:8:2 as indicated in Table 4. Fractions were collected and finally a pure compound had been isolated which was confirmed on the basis of TLC and paper chromatography.

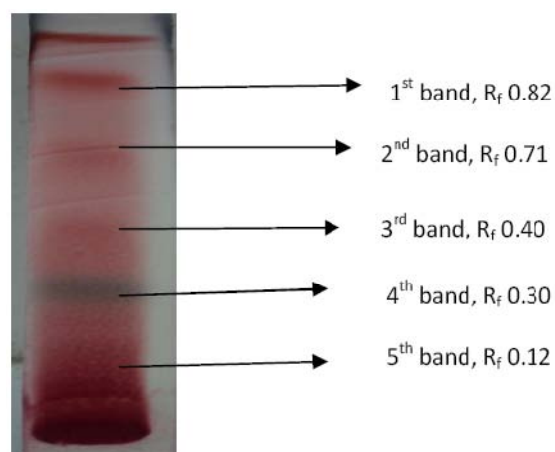
Fraction 6-11 (II) showed a single spot at R_f value (0.57) and fraction 12-31 (III+IV+V) also showed a single spot at same R_f value which was concluded on the basis of TLC and paper chromatography. So these fractions were pooled separately and concentrated, to yield a pure compound coded as **AEPP1**. Physical characteristics of AEPP1 were shown in Table 5 given below.

TLC profiling: TLC of acetone extract in solvent system

EtOAc:CHCl₃:MeOH:H₂O (12:8:8:2) showed five coloured bands; band 1st at R_f 0.82, band 2nd at R_f 0.71, band 3rd at R_f 0.40, band 4th at R_f 0.30, band 5th at R_f 0.12 as shown in figure Fig. 2a. Further TLC profiling of first five fractions of extract showed mixture of compounds (Fig. 2b), later fractions eluted from separating funnel yield a single red coloured spot at same R_f value (Fig. 2c, 2e, 2d, 2e). Finally pooled fractions were compared against parent acetone extract on TLC which revealed pure isolated compound coded as **AEPP1** at R_f 0.82 (Fig. 2f).

Table 5: Physical characteristics of AEPP1

Parameter	Observation
State	Solid
Colour	Light brown
Solubility	Methanol

**Figure 2a:** TLC of acetone extract showing five coloured bands**Fig. 2b:** TLC of acetone extract, fraction (I)**Fig. 2c, 2d:** TLC of fraction (II)**Fig. 2e:** TLC of fraction (III), (IV), (V)

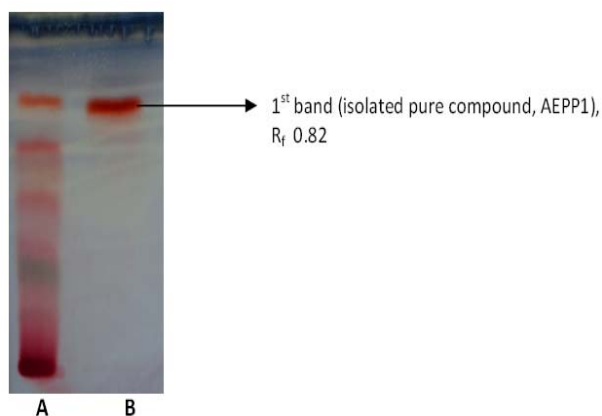


Figure 2f: TLC of acetone extract (A) and isolated pure compound (B)

Paper chromatography: Paper chromatography of acetone extract in solvent system EtOAc:CHCl₃:MeOH:H₂O (12:8:8:2) showed coloured bands as in TLC but found less prominent as compared to TLC and showed different R_f values; band 1st at R_f 0.82, band 2nd at R_f 0.52, band 3rd at R_f 0.22 as showed in Fig. 3a; radial paper chromatography of acetone extract showed red coloured bands at R_f 0.81, R_f 0.49 (Fig. 3b). Further vertical and radial paper chromatography of purified fractions were showed in Fig. 3c, 3d at R_f 0.44, R_f 0.61 respectively.

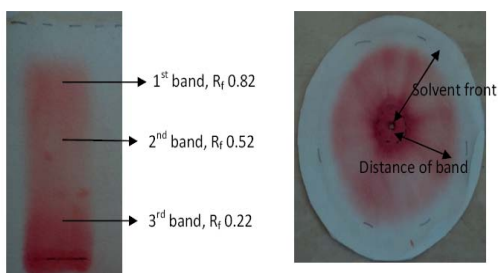


Fig. 3a: Acetone extract vertical paper chromatography

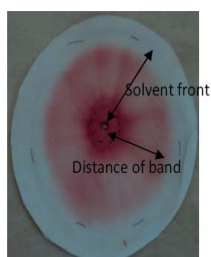


Fig. 3b: Acetone extract radial paper chromatography

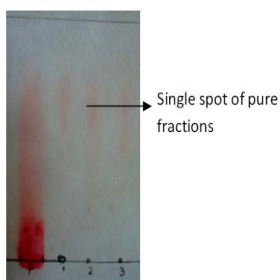


Fig. 3c: Vertical paper chromatography of isolated pure compound

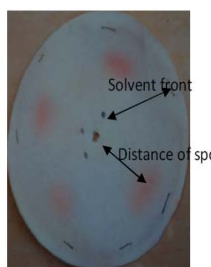


Fig. 3d: Radial paper chromatography of isolated pure compound

Ultraviolet spectroscopy (UV): A Shimadzu 1700, UV/VIS double beam spectrophotometer was used for determination of isolated compound (**AEPP1**). Figure 4 depicted the methanol spectra of **AEPP1** in the region 200-400 nm, showed two major absorption peaks at 205 (Absorbance 0.890) and 280 (Absorbance 0.206) nm.

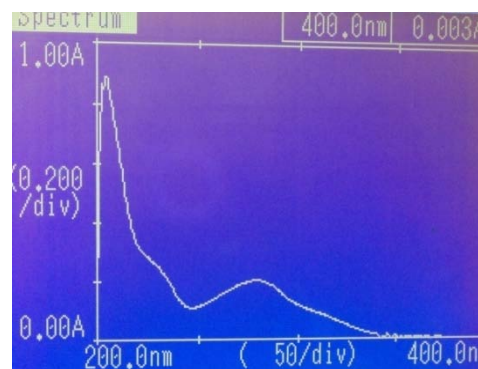


Figure 4: The UV spectra of **AEPP1** in methanol

CONCLUSION

The present investigation reported the purification of acetone extract of *Pyrus pashia* Buch.-Ham. ex D. Don stem bark. An attempt made in separating funnel was employed for the first time to carry out the purification process and a pure compound had been successfully isolated from acetone extract. More detailed study must be required for further characterization of isolated compound.

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