Standardization parameters of leaf and stem bark extracts of *Acronychia pedunculata* (L.) grown in Sri Lanka

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Abstract

Acronychia pedunculata (L.) (*Ankenda*) under family RUTACEAE is widely used in Traditional medicine system. The present study was aimed at the development of quality control parameters for identification and authentication of *A. pedunculata*. Hence, standardization is important in maintaining the purity, quality, safety and efficacy of Ayurveda formulations. Phytochemical, physico-chemical parameters and HPTLC fingerprints of methanol extracts of leaves and stem barks were determined as tools for quality standards. Preliminary phytochemical analysis revealed the presence of carbohydrates, reducing sugars, tannins, saponins, alkaloids, flavonoids, anthranol glycosides, phenols, terpenoids and proteins in both leaf and stem bark extracts. Under physico-chemical parameters; total ash, acid insoluble ash, water soluble ash, loss on drying and extractability in methanol for *A. pedunculata* leaves were 40.38% w/w, 12.64% w/w, 34.42% w/w, 21.80% w/w and 17.84% w/w respectively. Total ash, acid insoluble ash, water soluble ash content, loss on drying and extractability in methanol for *A. pedunculata* stem barks were 20.42% w/w, 8.35% w/w, 25.56% w/w, 15.67% w/w and 13.42% w/w. HPTLC profile of *A. pedunculata* leaf extract showed 10 peaks (R_f values; -0.01, 0.11, 0.32, 0.39, 0.43, 0.57, 0.69, 0.77, 0.85, 0.88) whereas, stem bark extract also showed 10 peaks (R_f values; -0.06, 0.02, 0.06, 0.17, 0.34, 0.48, 0.61, 0.71, 0.82, 0.97) for solvent system; n-hexane: ethyl acetate: chloroform in 2: 6: 2 ratio. The above parameters can be considered as a preliminary tool in detection and contrast of raw materials excluding the counterfeit and substandard raw materials in Ayurveda manufacturing.

Keywords: *Acronychia pedunculata* (L.); high performance thin layer chromatography; phytochemical; physico-chemical; standardization

Introduction

Traditional and complementary medicine which plays a major role in the prevention and management of chronic diseases and disorders is a common practice in use for ages in the majority of population specially in the South East Asian countries[1]. Though there is a long therapeutic history serving a large population worldwide, quality control and quality assurance parameters of Ayurveda system are challenging. Herbal drugs which are used singularly or in combinations contain various chemical components. Therefore, it is difficult to establish their quality control, quality assurance and documentation. In addition, expertise is needed in the guidance of developing national regulations and safety monitoring systems[2]. However, the common difficulties and challenges in the standardization of herbal drugs are lack of information sharing and the lack of safety monitoring methods to evaluate the safety and efficacy of herbal drugs. Furthermore, the development of analytical standardization techniques in herbal drug manufacturing is needed to maintain the quality control and validation of the herbal preparations confirming their identity, quality and purity[1]. Sri Lanka is rich with a vast variety of indigenous medicinal plants which are used in the preparation of herbal products. *A. pedunculata* (L.) (Figure 1) is a commonly used herb belonging to the family RUTACEAE which is called *Ankenda* in Sinhala, claw-flowered laurel in English and *Kattukannior Muttainari* in Tamil[3]. It is a small, evergreen aromatic tree with a pale smooth bark and glabrous branches.

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Leaves are oval in shape with a length of 7.5-12.5 cm in simple, opposite or alternate arrangements.

Flowers are small, loosely arranged and are pale yellowish green in colour. Fruits are globular in shape, 1.2-1.8 cm in length and are indehiscent. Seeds are in all 4 chambers of the fruits[4]. Medicinally important plant parts of this herb are leaves, roots, stem barks and fruits which are used in oral and topical preparations. In addition, the aromatic oils of this plant are used in perfumes. Furthermore, the therapeutic indications of *A. pedunculata* (L.) include; orthopedic conditions, fractures, rheumatism, asthma, diarrhea, tussis, itchy skin, scales, sores, rash, boils, ulcers, swelling, pain, inflammation, cold, cough and disorders of the inflammatory processes. This plant is well-known to have antimicrobial, antifungal, anti-inflammatory, antioxidant, anti-pyretic, anti-tussive, antihemorrhagic, antimutagenic and aphrodisiac properties[3,5]. Since, there is a growing trend in the use of plant herbs in healing many disorders and as they are more preferred than synthetic drug entities; establishment of standardization parameters for these indigenous medicinal plants is advantageous for drug discovery and development[5]. However, the data on scientific validation and justification for the therapeutic uses of *A. pedunculata* (L.) is limited[4]. Hence, this study was conducted to develop quality control standards including; phytochemical profiles, physico-chemical parameters and HPTLC fingerprints of methanol extract of leaves and stem barks of *A. pedunculata* (L.) in order to facilitate the standardization of herbal drug preparations in Ayurveda system of Sri Lanka.

Figure 1: *Acronychia pedunculata* (L.)

Materials and Methods

Materials, chemicals and apparatus used

Materials used for the study include; fresh leaves of *A. pedunculata*, filter papers, ashless filter papers, Sigma Aldrich normal phase TLC plates and reverse phase TLC plates. Chemicals used are; methanol, acetone, hydrochloric acid, potassium mercuric iodide, iodine, potassium iodide, potassium bismuth iodide, picric acid solution, alcoholic alpha-naphthol solution, sodium citrate, sodium carbonate, hydrated copper sulphate, potassium tartrate, sodium hydroxide, ferric chloride, benzene, ammonia solution, sodium nitroprusside, pyridine, sulphuric acid, acetic anhydride, acetic acid, sodium chloride, lead acetate, nitric acid, ninhydrin reagent, ethanol, copper acetate, ethyl acetate, toluene, n-hexane and chloroform of analytical grade. Apparatus used include; electronic balance, hot air oven, grinder, refrigerator, shaker, Soxhlet apparatus, reflux condenser, rotary evaporator, water bath, hot plate, furnace, desiccators, UV lamp and CAMAG REPROSTAR 3.

The matured leaves and stem barks of *A. pedunculata* without any insect or microbial attacks were collected from Western Province, Sri Lanka from July 2019 to March 2020. The plant was authenticated from the National Herbarium, Botanical Garden, Peradeniya, Sri Lanka. Fresh plant materials were washed under running tap water to remove dust and dirt, rinsed with distilled water, air dried, weighed, oven dried until a constant weight was obtained at a temperature below 40 °C and powdered. Leaf and stem bark powders were separately stored in airtight containers for further analysis.

Preparation of leaf and stem bark extracts

Powders of *A. pedunculata* leaves and stem barks were taken separately, accurately weighed (4.0 g) and taken into two conical flasks. 100.0 ml of methanol was added, macerated for 6 hours and kept standing for 18 hours. Extracts were collected after vacuum filtration and concentration by rotary evaporator. They were separately stored in airtight containers under refrigeration for further analysis[4].

Preliminary phytochemical screening of leaf and stem bark extracts

Leaf and stem bark extracts were subjected to preliminary phytochemical screening tests to screen for the available phytoconstituents including: carbohydrates, reducing sugars, tannins, saponins, alkaloids, flavonoids, anthranol glycosides, phenols, terpenoids, steroids, diterpenes, triterpenes and proteins as per Visweswari *et al*., 2013[6].

In Molisch's test, each extract was dissolved separately in distilled water and filtered. Filtrates were treated with 2 drops of alcoholic α-naphthol solution. Violet ring at the junction showed carbohydrates. Fehling's test was carried out to screen for reducing sugars. Fehling's A and B solutions were diluted with distilled water and boiled for 1 minute to obtain a clear blue solution. Each extract was added with 8 drops of the sample. Fehling's solution was added and boiled in a water bath for 5 minutes to each. Brick red precipitate concluded the presence of reducing sugars. Ferric chloride test was conducted to screen for tannins. Filtrate was taken and few drops of 0.1% $FeCl_3$ were added. If a brownish/ greenish black or bluish black colour was seen, the test is positive for tannins. Lead acetate test also screened for tannins. Each extract was mixed with distilled water. Lead acetate was added and shaken. White colour precipitate and a turbid solution were seen, if tannins are present. Foam test was conducted to screen for saponins. Each crude extract (0.5 g) was taken, mixed with 2.0 ml of distilled water and shaken. Formation of foam which persisted for 10 minutes meant that it contained saponins. Mayer's test was conducted to screen for alkaloids. Each extract was dissolved in dilute hydrochloric acid and filtered. Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Yellow colour precipitate was seen if alkaloids were present. Dragendroff's test also screened for alkaloids where each extract was dissolved separately in dilute hydrochloric acid and filtered. Filtrates were treated with Dragendroff's reagent (potassium bismuth iodide). Reddish precipitate showed alkaloids. Lead acetate test was conducted to each extract after treating with few drops of lead acetate solution. Yellow colour precipitate indicated the presence of flavonoids. Modified Borntrager's test screened for anthranol glycosides. Each extract was hydrolyzed with dilute hydrochloric acid and filtered. Filtrate was treated with ferric chloride solution and immersed in boiling water for about 5 minutes. Mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Rose-pink colour in the ammonical layer indicated the presence of anthranol glycosides. Ferric chloride test determined the presence of phenolic compounds. Each was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols. Liebermann-Burchard test was done to screen for steroids and terpenoids. Each extract was added with acetic anhydride and acetic acid. Concentrated sulphuric acid was added slowly. Bluish green solution showed terpenoids while reddish brown solution showed steroids. Copper acetate test was done to each extract. Each was dissolved in distilled water and treated with 3-4 drops of copper acetate solution. Emerald green colour indicated the presence of diterpenes. Salkowski's test was done to each extract by treating with chloroform. Each was filtered and the filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicated the presence of triterpenes. Xanthoproteic test was done by treating each extract with few drops of concentrated nitric acid. Formation of yellow colour indicated the presence of proteins. Ninhydrin test screened for amino acids. Each extract was treated with 0.25% w/v Ninhydrin reagent and boiled for few minutes. Formation of blue colour indicated the presence of amino acids.

Physico-chemical analysis of leaf and stem bark extracts

Physico-chemical parameters including; total ash, acid insoluble ash, water soluble ash, loss on drying and extractability in methanol were determined as per WHO Guidelines[7].

Total ash was determined with powdered crude material (2-4 g) placed in a previously ignited and tared crucible. Sample was spread evenly and ignited at 500-600ºC until it was white. Residue was cooled in a desiccator for 30 minutes and weighed immediately. Content of total ash (mg/g %) was determined[7].

$$\text{Total ash \%} = \frac{\text{Weight of residue (g)}}{\text{Weight of leaf or stem bark powder (g)}} \times 100$$

In order to determine the acid insoluble ash, hydrochloric acid TS (25.0 ml) was added to the crucible with total ash, covered with a watch glass and boiled gently for 5 minutes. Watch glass was rinsed with 5.0 ml of hot distilled water and added to the crucible. Residue was collected after filtration and the filtrate was washed with hot water until it was neutral. Insoluble matter was collected, dried on a hot plate and ignited until a constant weight was obtained. Residue was cooled in a desiccator for 30 minutes and weighed immediately[7].

$$\text{Acid-insoluble ash \%} = \frac{\text{Weight of residue (g)}}{\text{Weight of leaf or stem bark powder (g)}} \times 100$$

Total ash was treated with water (25.0 ml) in the crucible and boiled for 5 minutes. Residue was collected after filtration, washed with hot water and ignited for 15 minutes not exceeding 450ºC to determine the water-soluble ash content[7].

$$\text{Water soluble ash \%} = \frac{\text{Weight of total ash (g)} - \text{weight of residue (g)}}{\text{Weight of leaf and stem bark powder (g)}} \times 100$$

Loss on drying was calculated for *A. pedunculata* leaves and stem barks according to gravimetric determination method as per the WHO Guidelines[7]. Leaves or stem barks were cut or shred without exceeding a thickness of 3 mm. Prepared samples (5 g) were placed in a previously dried and tared flat weighing bottle and dried in hot air oven at 100-105ºC until two consecutive weights were not different by 5 mg[7].

$$\text{Loss on Drying \%} = \frac{\text{Loss of weight (g)}}{\text{Weight of leaf or stem bark material (g)}} \times 100$$

Extractability in methanol was determined for leaves and stem barks of *A. pedunculata*. Each powder weighing 4.0 g was placed in a previously weighed glass-stoppered conical flask. Each was macerated with 100.0 ml of methanol for 6 hours while shaking and was allowed to stand for 18 hours. Each was filtered rapidly and the filtrate (25.0 ml) was transferred to a previously weighed petri dish. Each was dried in hot air oven at 105ºC until a constant weight was obtained, cooled in a desiccator for 30 minutes and weighed[7].

$$\text{Extractability in methanol \%} = \frac{\text{Content of extractable matter (g)}}{\text{Weight of leaf or stem bark powder (g)}} \times 100$$

HPTLC analysis

HPTLC fingerprints were developed for the leaf and stem bark extracts of *A. pedunculata* (L.). Sigma Aldrich normal phase TLC silica gel 60 F_{254} plates (precoated sheets ALUGRAM Xtra SIL, 8.0 cm × 10.0 cm, 0.20 mm thickness) were used. Solvent system (mobile phase) was prepared as n-Hexane: Ethyl Acetate: Chloroform in 2: 6: 2 proportion. The developed plate was air dried to evaporate the solvent system from the plate. The plate was kept in the photo-documentation chamber of CAMAG REPROSTAR 3 and the images were captured under UV light at 254 nm wavelength. The peak area display, peak value table and baseline display were recorded[8,9].

Results

Preliminary phytochemical screening tests revealed the presence of carbohydrates, reducing sugars, tannins, saponins, alkaloids, flavonoids, anthranol glycosides, phenols, terpenoids and proteins in both leaf and stem bark extracts (Table 1).

Table 1: Phytochemical analysis of both leaf and stem bark extracts of *A. pedunculata*

Phytochemicals	Leaves	Stem Barks
Carbohydrates	+ + +	+
Glycosides	+ + +	+
Tannins	+ + +	+
Saponins	+ + +	+
Alkaloids	+ + +	+
Flavonoids	+ + +	+
Phenols	+ + +	+
Terpenoids	+ + +	+

Under physico-chemical parameters; total ash, acid insoluble ash, water soluble ash, loss on drying and extractability in methanol for *A. pedunculata* leaves were determined as 40.38% w/w, 12.64% w/w, 34.42% w/w, 21.80% w/w and 17.84% w/w respectively.

Total ash, acid insoluble ash, water soluble ash, loss on drying and extractability in methanol for *A. pedunculata* stem barks were determined as 20.42% w/w, 8.35% w/w, 25.56% w/w, 15.67% w/w and 13.42% w/w (Table 2).

Table 2: Physico-chemical analysis of both leaf and stem bark extracts of *A. pedunculata*

Parameter	Leaves	Stem bark
Total ash content	40.38% w/w	20.42% w/w
Acid insoluble ash content	12.64% w/w	8.35% w/w
Water soluble ash content	34.42% w/w	25.56% w/w
Loss on Drying	21.80% w/w	15.67% w/w

Discussion

Preliminary phytochemical screening tests revealed the presence of many phytoconstituents in both leaf and stem bark extracts. Plants contain a variety of biologically active compounds called phytochemicals; flavonoids, phenolic compounds, stilbenes, tannins, coumarins, lignans and lignin which are responsible for the therapeutic indications[3]. This study revealed that both *A. pedunculata* leaves and stem barks are rich in phytochemicals which are medicinally important. Results also showed that precise colour changes were obtained for the phytochemical screening tests of leaf extract. This suggests the presence of a high concentration of phytochemicals in the leaf extract than that of the stem bark extract. Physico-chemical parameters were developed as standardization parameters including; ash values, loss on drying and extractability. The study showed that total ash, acid insoluble ash and water soluble ash values were higher in the leaf extract than that of the stem bark extract. Total ash content of the leaf extract was almost twice the value for stem bark extract (Table 2). It was revealed that higher amounts of inorganic substances are present in leaves than the stem barks. Ash values also determine the available earthy matter, inorganic compounds and other impurities in which the values can be used for the detection of adulterated, exhausted or sub-standard materials and is important in quality control and standardization of herbal drugs[2,10].

The present study showed that loss on drying is higher for *A. pedunculata* leaves than that of the stem barks. It also determined that moisture and volatile matter content is higher in the leaves than the stem barks (Table 2). A high level of moisture can encourage the microbial growth, presence of fungi or insects in leaves than that of stem barks; so that they may be subjected to quick deterioration following hydrolysis. The study showed that the extractability in methanol is higher in *A. pedunculata* leaves than the stem barks (Table 2). Therefore, many of the active constituents in leaves had been extracted to methanol than stem barks.

HPTLC techniques are used in the quality assessment of *A. pedunculata*. This method is widely employed in proper identification, authentication and development of quality control and quality assurance aspects of herbal raw materials[11]. HPTLC profile of *A. pedunculata* leaf extract showed 10 peaks at R_f values; -0.01, 0.11, 0.32, 0.39, 0.43, 0.57, 0.69, 0.77, 0.85 and 0.88 (Figure2,3,4). HPTLC profile of *A. pedunculata* stem bark extract also showed 10 peaks at the R_f values; -0.06, 0.02, 0.06, 0.17, 0.34, 0.48, 0.61, 0.71, 0.82 and 0.97 (Figure 5,6,7). HPTLC fingerprints showed that there was a clear separation of the phytoconstituents available in both leaf and stem bark extracts. It was found that different compounds with different R_f values were available in leaf and stem bark extracts. Hence, HPTLC can be used as a tool to contrast *A. pedunculata* with other closely related species and also to contrast leaves with the stem barks of *A. pedunculata*. Further morphological investigations are recommended for *A. pedunculata* leaves and stem barks, since the values obtained for ash analysis are higher. In addition, further studies are required in isolation and detection of active principles of *A. pedunculata* leaves and stem barks; hence the structure-activity relationships and mode of action can be determined.

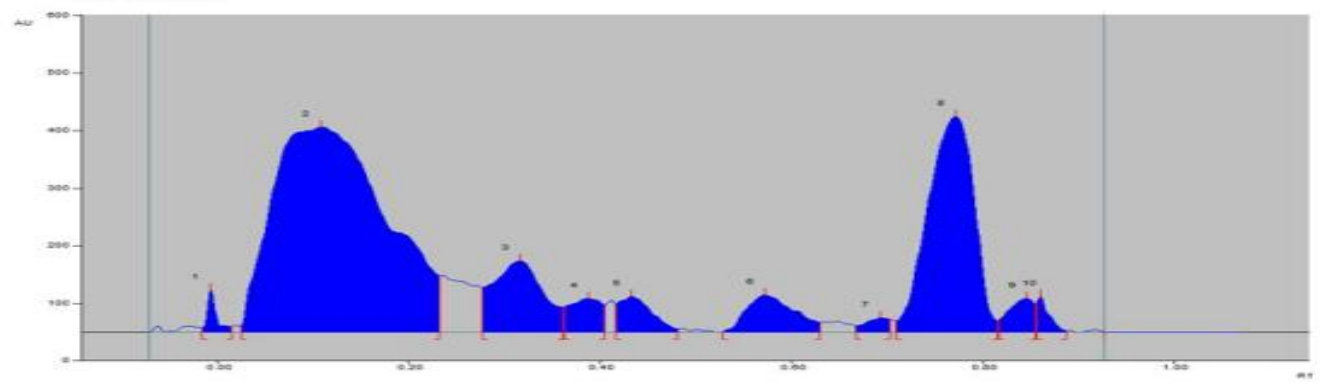

Figure 2: Peak area display for methanol leaf extract of *A. pedunculata*

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned Substance
1	-0.02Rf	7.1AU	-0.01Rf	73.6AU	5.85%	0.01Rf	10.5AU	568.4AU	0.90%	unknown
2	0.02Rf	10.1AU	0.11Rf	356.4AU	28.32%	0.23Rf	93.4AU	34583.6AU	54.53%	unknown
3	0.28Rf	77.9AU	0.32Rf	124.0AU	9.85%	0.36Rf	43.8AU	5264.6AU	8.30%	unknown
4	0.36Rf	44.2AU	0.39Rf	58.4AU	4.64%	0.41Rf	46.8AU	1620.1AU	2.55%	unknown
5	0.42Rf	48.4AU	0.43Rf	61.8AU	4.91%	0.48Rf	5.5AU	1757.1AU	2.77%	unknown
6	0.53Rf	0.1AU	0.57Rf	64.2AU	5.11%	0.63Rf	17.2AU	2634.3AU	4.15%	unknown
7	0.67Rf	11.1AU	0.69Rf	24.8AU	1.97%	0.70Rf	22.6AU	504.6AU	0.80%	unknown
8	0.71Rf	19.6AU	0.77Rf	374.3AU	29.74%	0.82Rf	20.3AU	14615.5AU	23.04%	unknown
9	0.82Rf	20.7AU	0.85Rf	58.7AU	4.66%	0.86Rf	48.6AU	1273.0AU	2.01%	unknown
10	0.88Rf	52.1AU	0.88Rf	62.2AU	4.95%	0.89Rf	2.5AU	604.4AU	0.95%	unknown

Figure 3: Peak value table for methanol leaf extract of *A. pedunculata*

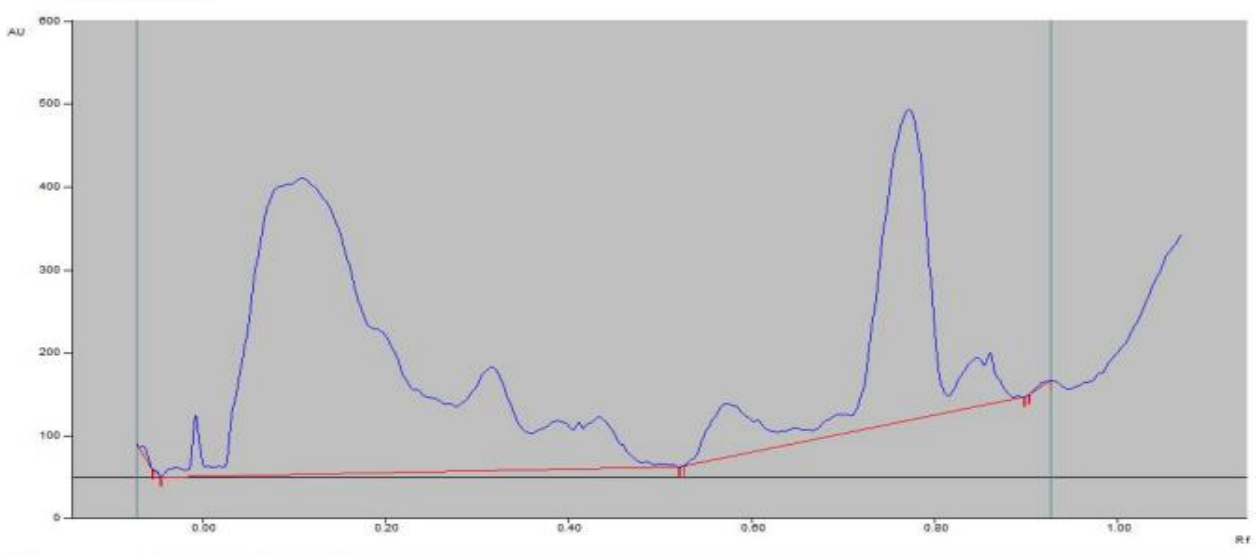

Figure 4: Baseline HPTLC for methanol leaf extract of *A. pedunculata*

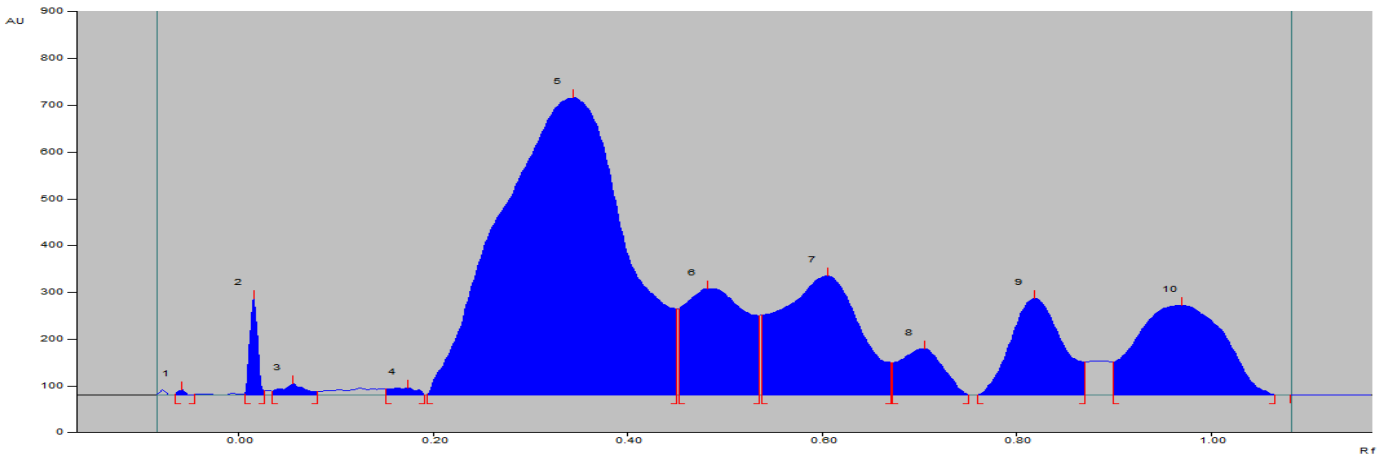

Figure 5: Peak area display for methanol stem bark extract of *A. pedunculata*

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned Substance
1	-0.06Rf	1.6AU	-0.06Rf	11.5AU	0.61%	-0.05Rf	1.3AU	56.5AU	0.05%	unknown
2	0.01Rf	1.8AU	0.02Rf	206.1AU	11.02%	0.03Rf	9.3AU	1099.1AU	1.04%	unknown
3	0.04Rf	8.1AU	0.06Rf	23.3AU	1.25%	0.08Rf	6.8AU	389.3 AU	0.37%	unknown
4	0.15Rf	12.1AU	0.17Rf	15.0AU	0.03%	0.19Rf	0.9AU	301.2 AU	0.28%	unknown
5	0.19Rf	1.7AU	0.34Rf	635.3AU	33.97%	0.45Rf	64.4AU	56468.8AU	53.33%	unknown
6	0.45Rf	185.0AU	0.48Rf	227.1AU	12.14%	0.54Rf	70.0AU	10297.3AU	9.73%	unknown
7	0.54Rf	170.3AU	0.61Rf	254.6AU	13.61%	0.67Rf	69.6AU	14613.5AU	13.50%	unknown
8	0.67Rf	69.5AU	0.71Rf	99.4AU	5.31%	0.75Rf	0.6AU	3077.1AU	2.91%	unknown
9	0.78Rf	0.5AU	0.82Rf	206.6AU	11.05%	0.87Rf	70.2AU	7537.5AU	7.12%	unknown
10	0.90Rf	70.7AU	0.97Rf	191.5AU	10.24%	1.07Rf	0.5AU	12040.8AU	11.37%	unknown

Figure 6: Peak value table for methanol stem bark extract of *A. pedunculata*

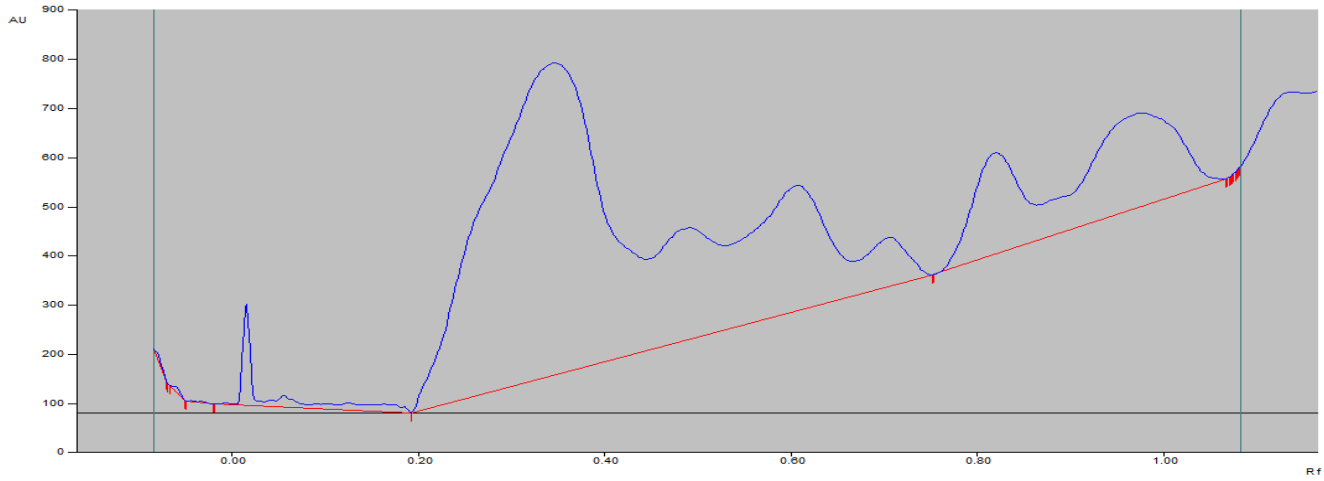

Figure 7: Baseline HPTLC for methanol stem bark extract of *A. pedunculata*

Conclusion

A. pedunculata leaves and stem barks are commonly used in Ayurveda treatments without standardization parameters. Standardization and pharmacognostic parameters are important aspects for the correct identity of a plant raw material[12]. Preliminary phytochemical profiles, physico-chemical standards and HPTLC fingerprints are helpful in the identification and authentication of *A. pedunculata*. Hence, such quality control tools are important in proper raw material handling and manufacturing of safe, quality and efficacious herbal preparations because counterfeit or sub-standard materials can be detected. HPTLC profiles ascertain the contrast between closely related species and among different plant parts of the same species.

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