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Research Article

PHARMACOGNOSTIC EVALUATION AND ANTIBACTERIAL ACTIVITY OF DRY FRUITS OF *PIPER ATTENUATUM* BUCH-HAM

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ABSTRACT

Objective: The aim of the present work was to perform complete pharmacognostic evaluation of *Piper attenuatum.* Antibacterial activity was performed to explore the possible inhibitory property of dry fruit extract of *Piper attenuatum* against different microorganisms.

Methods: Extensive pharmacognostic evaluation of dry fruits of *P. attenuatum* was performed which includes morphology, microscopy, phytochemical screening, physicochemical parameters (loss on drying, extractive values, ash values and pH) and high performance thin layer chromatography (HPTLC) profile. We also performed antibacterial activity for methanol, ethanol and ethyl acetate extracts of dry fruit powder of *P. attenuatum* by agar diffusion method.

Results: The morphology of fruits of *P. attenuatum* was found similar to that of *Piper nigrum* with less folding on the fruits of *P. attenuatum*. The fruits have bland taste unlike the dry fruits of *P. nigrum* which are pungent. Powder microscopy showed the presence of different cellular structures. Phytochemical screening tests revealed the presence of different chemical constituents like alkaloids, tannins, carbohydrates, steroids and volatile oil. The HPTLC profile of crude sample showed many spots indicating its chemical diversity.

Conclusion: All these tests gave the valuable data which may be helpful for its qualitative identification and further study. Antibacterial activity did not show prominent inhibitory property except at higher concentrations in methanol extract.

Keywords: *Piper attenuatum*, Pharmacognostic evaluation, Antibacterial activity.

INTRODUCTION

The use of herbal intervention is widespread in all regions of the developing world and is rapidly growing in developed countries. The family Piperaceae comprises 12 genera and about 1400 species, mainly found in the tropical region [1]. The genus Piper contains more than 700 species; they grow in tropical and subtropical rain forests. Piper species existing in South India are economically important, as they are closely related to the cultivated black pepper. Several species of Piper such as Piper longum, Piper cubeba, Piper retrtofractum etc. have been used in indigenous system of medicine of India. Piper species have been used in the treatment of several diseases such as venereal diseases, intestine disorders [2] and epilepsy and to prevent conception [3]. The valuable medicinal properties of different plants are due to the presence of several constituents i.e. saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes lactones and terpenoids [4].

Piper attenuatum (Buch-Ham) belongs to the family Piperacea is found in Vishakhapatnam of Andhra Pradesh and Madurai and Tirunnelveli of Tamil Nadu. It is a substitute for black pepper (*P. nigrum*) [5]. Different parts of *P. attenuatum* have been used as herbal medicine for the treatment of headache, muscular pain and have been used as a rubefacient. The wood of plant has been used to treat throat pain.

The root part possesses diuretic activity [6]. Leaves have been used for their wound healing property. The whole plant is reported to contain a rare phytoconstituent crotepoxide, which has been reported to exhibit significant antitumor activity against Lewis lung carcinoma cell lines [7].

Phytochemical studies have shown that the plant contains pipoxide and chlorohydrins which are major chemical components. (−)-galbelgin and a new aliphatic alcohol; 8-hentriacontanol have also been isolated from the leaves of P. attenuatum. Several aristolactams have been reported from the aerial parts of the plant. Roots have been reported to contain alkamides including piperine, piperlonguminine and guineensine. The petroleum extract of stems and leaves of P. attenuatum have been reported to contain a novel long chain alcohol, 14-benzo[1, 3]dioxol-5-yl-tetradecan-2-ol [8]. Recently methanol extract of dry fruits has also been reported to contain antioxidant components with promising activity [9].

MATRIALS AND METHODS

Collection, authentication and preparation of extracts

Dried fruits of *P. attenuatum* were collected from Royapuram, Chennai in the month of August. The authentication of sample was done from National Institute of Science Communication and Information Resources (NISCAIR), Council of Scientific and Industrial Research.

The sample was dried in shade for 4 days and then ground coarsely for extraction. About 150 g of powdered sample was extracted with ethyl acetate, ethanol and methanol separately by hot percolation (Soxhlet extraction) method. The solvent was evaporated; extract was dried and used for further study.

Preliminary evaluation

Morphological features and organoleptic characters like color, odor, taste, shape, size and texture were observed.

Powder microscopy [10]

The dried powder material was passed through sieve 44 and a pinch of powder was cleared with freshly prepared 5 % chloral hydrate solution. It was further treated with phoroglucinol and HCl and mounted on slide with the help of glycerin. The slide was observed under microscope.

Evaluation of physicochemical parameters [11]

Loss on drying

4 g of sample was weighed in a pre-weighed porcelain dish. It was kept in hot air oven at 105 ºC for 5 h. Then it was cooled in desiccators and weighed. The procedure was repeated till the constant weight was obtained. The loss on drying (% W/W) was calculated as (Loss of weight of sample/Wt of sample taken) X 100.

Water/ether soluble extractive value

4 g of air-dried coarsely powdered sample was macerated with 100 ml of respective solvents [distilled water or petroleum ether (40-60)] in a stoppered flask for 24 h, shaken occasionally during the first 6 h and allowed to stand for 18 h. It was filtered rapidly through Whatman filter paper (No.41) taking precaution against the loss of solvent. 25 ml of the filtrate was pipette out into a pre-weighed dry porcelain dish and it was allowed to evaporate at 105 ºC for 5 h. The dish was kept into desiccators for cooling and weighed. Soluble extractive value (% W/W) in respective solvent was calculated as [(Wt of extract) / (25 X Wt of sample taken)] X 100 X 100

Ash values

Ash values are helpful in determining the quality and purity of a crude drug; especially in the powdered form. The object of preparing ash of vegetable drugs is to remove all traces of organic matter which may otherwise interfere in an analytical determination. Crude plant materials on incineration leave an ash which usually consist carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. The total ash of a crude drug reflects the care taken in its preparation. A higher limit of acid insoluble ash imposed, especially in cases where silica may be present or when the calcium oxalate content of the drug is very high. The ash remaining following ignition of medicinal plant material is generally determined by four different methods which measure total ash, acid insoluble ash, water soluble ash and sulphated ash.

Total ash

2 g of the air-dried coarsely powdered sample was taken in a previously ignited and pre-weighed silica crucible. The material was spread uniformly and ignited at 600 ºC until white ash was formed. It was allowed to cool at room temperature in desiccators for 30 min, weighed and percentage of total ash (% W/W) was calculated with respect to the air-dried material as (Wt of ash / Wt of sample taken) X 100.

Acid insoluble ash

The ash obtained in total ash was boiled with 25 ml of 2 M HCl for 5 min. The solution was filtered through ashless filter paper (Whatman, No.41) and then insoluble residue was ignited along with the filter paper in the same silica crucible at 600 ºC until white ash was formed. Then the crucible was allowed to cool in desiccators for 30 min, weighed and the acid insoluble ash (% w/w) was calculated with respect to the air-dried sample as (Wt of ash / Wt of sample taken) X 100.

Foreign organic matter

100 g of sample was weighed and spread uniformly on the white tile without overlapping. The sample was inspected with naked eyes. Foreign organic matters other than plant sample were separated manually. After complete separation, the sample was again weighed. The foreign organic matter (% w/w) was calculated as (Wt of foreign organic matter / Wt of sample) X 100.

Determination of pH

Solutions of pH-4, pH-7 and pH-9.2 were freshly prepared using buffer capsules. The pH meter was calibrated using these buffer solutions. Then the pH of the water extract of powdered sample was taken at room temperature 25º C.

Preliminary phytochemical screening [5]

The crude sample and various extracts were subjected to the various chemical tests for the detection of different class of secondary plant metabolites. The results of these tests are shown in table 2.

HPTLC Profile [12-13]

The spotting was done with the help of Linomat 4 applicator on special HPTLC plate. It was run in an instrument using nitrogen gas to develop pressure. Toluene and acetone (8:2) was used as a mobile phase. Photo-documentation was done by Camag Retrospark 3. Wincats 1.4.3 software was used for conducting the HPTLC study.

The plates were observed under 254 nm and 366 nm. Anisaldehyde and Sulphuric acid and Iodine were used for visualization of the spots. The R_f value of all the spots observed under 366 nm (which showed maximum spots) was calculated.

Antibacterial activity [14]

Cultures of *Staphylococcus aureus* (Gram positive) and *Escherichia coli and Pseudomonas aruginosa* (Gram negative) were prepared in a nutrient broth (without agar) and used for inoculation. Ciprofloxacin was used as a standard and ethyl acetate, ethanol and methanol were used as a blank. Agar diffusion method was used for performing antibacterial activity.

The readymade nutrient agar medium (23 g) was suspended in distilled water (1000 ml) and heated up to boiling until it dissolved completely. The medium and the Petri dishes were autoclaved at pressure of 15 lb/inc^2 for 20 min. Stock solutions of extracts were prepared by dissolving plant extract in solvent. i.e. Ethyl acetate, ethanol and methanol. Different dilutions were made to give the final concentrations of 25, 50, 100, 200 and 500 µg/50 µl in a well. The medium was poured into Petri dishes under aseptic conditions in a laminar flow chamber.

When the medium in the plates solidified, 0.5 ml of 24 h old culture of test organism was inoculated and spread equally by sterile cotton swab. After inoculation, cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup, different concentrations of test solutions as mentioned above were added. A solution of 10 µg/50 µl of ciprofloxacin was also inoculated as a standard. Ethyl acetate, ethanol and methanol were inoculated as blank readings. All plates were incubated at 37^0 C for 24 h. The diameter of zone of inhibition was measured after 24 h.

RESULTS AND DISCUSSION

Pharmacognostical evaluation

Macroscopic characters

The external morphology of *P. attenuatum* Showed globular dry fruits with few striations than *P. nigrum*. The average diameter of dry fruits was found to be 4-6 mm (Figure 1). The fruits were found to be grayish black in color with slight pungent taste with no characteristic odor.

Fig. 1: External morphology of dry fruits of *P. attenuatum*

Powder microscopy

The powder microscopy of *P. attenuatum* Ham Showed the presence of pink colored lignin (Figure 2), fibrous sclereids from the stalk (Figure 3), Perisperm cells containing densely compacted masses of starch granules (Figure 4), multicellular covering trichomes (Figure 5) and isolated sclereids from the outer mesocarp (Figure 6).

Physicochemical parameters

The physico-chemical parameters like loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive and foreign organic matter were determined (Table 1).

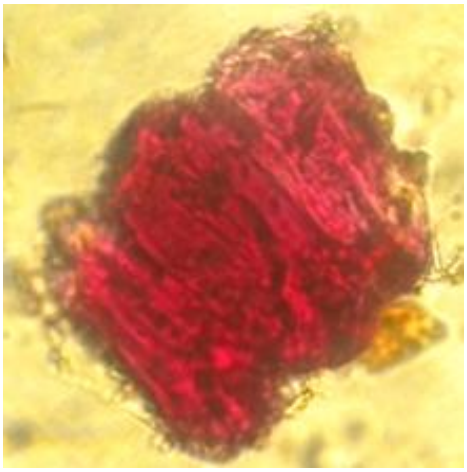

Fig. 2: Pink coloured lignin

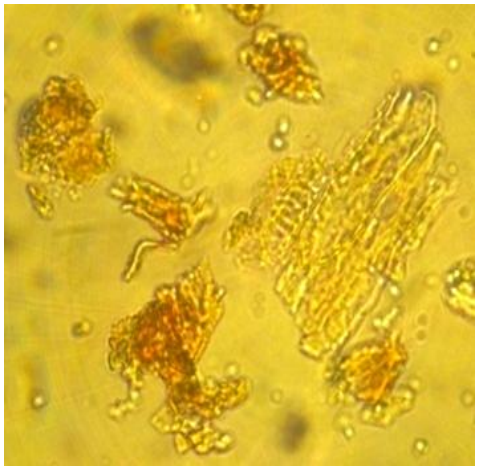

Fig. 3: Fibrous sclereids from the stalk

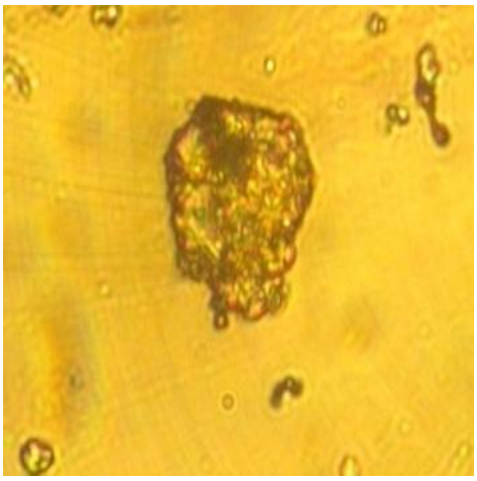

Fig. 4: Perisperm cells containing densely compacted masses of starch granules

Fig. 5: Multicellular covering trichomes

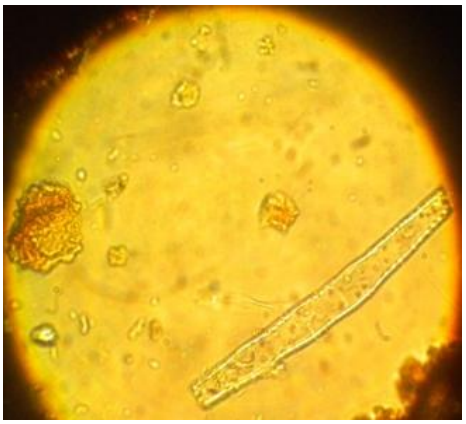

Fig. 6: Isolated sclereids from the outer mesocarp

HPTLC profile of P. attenuatum

The plates showed different spots under the 254 and 366 nm. The plate under 254 nm showed some prominent spots in the middle (Figure 7). The plate at 366 nm showed very bright red colored spots in ethyl acetate and ethanol extract indicating the presence of colored compounds. These spots are very faint in methanol and chloroform extract. Methanol, ethanol and ethyl acetate extracts showed bright fluorescent spots at the lower and middle region of the plate (Figure 8). Plates treated with anisaldehyde and sulphuric acid (used as visualizing agent) showed dark purple colored spots in both ethanol and ethyl acetate extract but very faint purple spots in methanol and chloroform extract (Figure 9). Plates treated with iodine showed dark brown spots at the bottom in methanol and ethanol extracts and at the top and middle part in the ethyl acetate extract (Figure 10). All plates showed a thick unmoved (non-eluted) sample at the bottom indicating the presence of the highly polar components. Methanol, ethyl acetate and ethanol showed many spots throughout the band unlike in chloroform extract which showed only few light spots indicating that the sample is sparingly soluble in chloroform. HPTLC profile of different extracts in toluene and acetone (8:2) gave a fingerprint profile for *P. attenuatum* which showed reproducible results. This may help in qualitative identification of *P. attenuatum.* The R_f value for all spots at 366 nm (showed maximum spots) was calculated (Table 3).

Table 1: Evaluation of physicochemical parameters

Physicochemical parameters	Value
Loss on drying	9.94% w/w
Water soluble extractive	12.4% w/w
Ether soluble extractive	2.66% w/w
Total ash	6.2% w/w
Acid insoluble ash	6.0% w/w
Foreign organic matter	0.98% w/w
PH	6.64 at 21^{0}C

Phytochemical screening

Phytochemical tests were performed to detect the presence of different secondary metabolites (Table 2).

Table 2: Phytochemical screening of dry fruits of *P. attenuatum*

Constituent	Test		Crude drug	Methanol extract	Ethanol extract	Ethyl acetate extract
	a.	Dragondroff	++	--	++	++
Alkaloids	b.	Mayer	++	++	++	++
	c.	Wagner	++	++	++	++
	a.	Salkowski	++	--	++	++
Steroids	b.	Lieberman-Buchard	++	--	++	++
	a.	Lead acetate	--	--	--	--
Flavonoids	b.	Alkaline	--	--	--	--
	c.	Shinoda	--	--	--	--
	a.	Molisch	++	++	--	++
Carbohydrates	b.	Fehling	++	--	--	++
	c.	Benedict	++	--	--	++
Proteins	a.	Xanthoproteic	--	--	--	--
	b.	Warming	--	--	--	--
Amino acids	a.	Ninhydrin	--	--	--	--
Glycosides	a.	Borntrager	--	--	--	--
	b.	MBT	--	--	--	--
Tannins	a.	Ferric chloride	++	--	++	++
Volatile oil	a.	Sudan III	++	++	++	++

++ Positive test, -- Negative test

Table 3: R_f values of HPTLC (366 nm)

Methanol extract		Ethyl acetate extract		Chloroform extract		Ethanol ectract	
Spot	**Rf**	**Spot.**	**Rf**	**Spot**	**Rf**	**Spot**	**Rf**
1	0.15	1	0.07	1	0.58	1	0.15
2	0.30	2	0.15	2	0.70	2	0.23
3	0.43	3	0.30	3	0.89	3	0.38
4	0.46	4	0.4	4	0.90	4	0.46
5	0.53	5	0.53	5	-	5	0.6
6	0.55	6	0.58	6	-	6	0.63
7	0.61	7	0.61	7	-	7	0.76
8	0.72	8	0.73	8	-	8	0.81
9	0.8	9	0.8	9	-	9	0.90
10	0.89	10	0.89	10	-	10	-

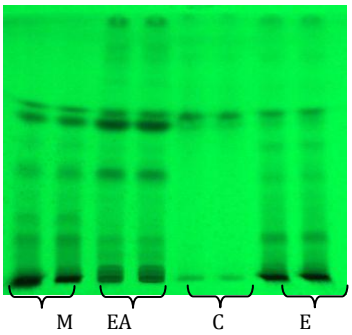

Fig. 7: HPTLC profile under 254 nm

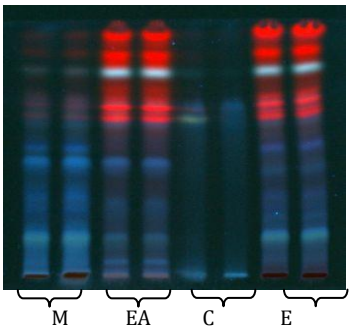

Fig. 8: HPTLC profile under 366 nm

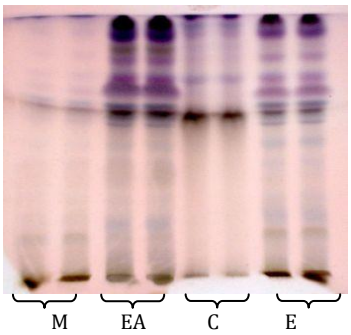

Fig. 9: HPTLC profile after treating with anisaldehyde and sulphuric acid

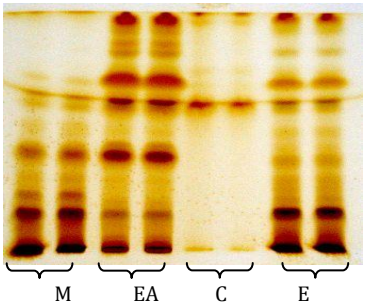

Fig. 10: HPTLC profile after treating with iodine

M = Methanol extract, EA = Ethyl acetate extract, C = Chloroform extract, E= Ethanol extract

Antibacterial activity

All three extracts were screened for their antibacterial activity against *S. aureus, E. coli* and *P. aeroginosa* by agar diffusion method. Methanol extract showed comparatively good inhibition at higher doses at 200 and 500 µg against all organisms (Table 4). Ethanol extract also showed some inhibition against *E. coli* at higher doses (200 and 500 µg). But, ethyl acetate extract failed to show inhibition against any organism.

The methanol extract gave hope for developing a good antibacterial compound by purifying the extract by chromatography and isolating the active constituents.

Table 4: Antibacterial activity

Conc. of Extract	Zone of inhibition (mm)		
	E. coli	*S. aureus*	*P. aeruginosa*
Standard (ciprofloxacin)	28	27	28
Methanol Extract			
25 µg	--	--	--
50 µg	--	--	--
100 µg	--	--	--
200 µg	10	--	--
500 µg	18	8	10
Ethanol Extract			
25 µg	--	--	--
50 µg	--	--	--
100 µg	--	--	--
200 µg	10	--	--
500 µg	15	--	--
Ethyl acetate Extract			
25 µg	--	--	--
50 µg	--	--	--
100 µg	--	--	--
200 µg	--	--	--
500 µg	--	--	--

SUMMARY AND CONCLUSION

The present study gave a complete data of physicochemical parameters like loss on drying, extractive value, ash values and pH. Microscopic evaluation showed different specific characters and cellular organization of the drug. Phytochemical screening of powder sample and different extracts of *P. attenuatum* showed the presence of many secondary metabolites like alkaloids, carbohydrates, tannins, proteins and volatile oil. HPTLC study revealed the polarity nature of the constituents of *P. attenuatum* along with the densities of different spots. The HPTLC showed many spots with different R_f values. All these parameters can be used for identification and authentication of dry fruits of *P. attenuatum* and isolation of different chemical constituents from it. Methanol and ethanol extracts showed comparatively good antibacterial activity at higher doses (200 and 500 µg) against different organisms which could be further studied by isolating the active constituents in pure form by chromatographic techniques.

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