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Analytical standards of fruits of Bhallataka- *Semecarpus anacardium* Linn.

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ABSTRACT

Standardization of herbal drugs is the need of the hour as the use and practice of traditional herbal drugs and their formulations has increased tremendously. In the present study, an attempt has been made to standardize the fruits of Bhallataka as per pharmacopoeial testing protocol which include powder microscopy, physico-chemical screening, HPTLC fingerprinting and GC-MS analysis. Preliminary phytochemical tests indicate the presence of sugars, flavonoids, steroids, saponins, quinones and tannins. HPTLC profiling of the ethanol extract using Toluene/ Ethyl acetate (8: 1) as mobile phase revealed the presence of phytochemicals with different Rf values. The GC-MS analysis of the diethyl ether fraction showed the presence of 11 compounds of which five were identified.

Keywords: Bhallataka, GC-MS, HPTLC, Physicochemical, Powder microscopy.

INTRODUCTION

Standardization, the process of prescribing a set of standards or inherent characteristics, definitive qualitative and quantitative values that carry an assurance of efficacy, safety and reproducibility is essential to confirm the quality of the herbal drugs in composition and repeatability of the therapeutic value in the clinical settings [1]. Specific standards have to be carried out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular drug [2]. Bhallathaka, an important drug used in Ayurveda and Siddha systems of medicine has its source in *Semecarpus anacardium* Linn. belonging to family Anacardiaceae. In *Charaka Samhitha*, *S. anacardium* has been classified as a promoter of digestion, corrective of excessive urination, curative of obstinate skin diseases and has been prescribed for counter poisoning. In *Sushrutha Samhitha*, plant nut preparations have been recommended for the treatment of intestinal parasites, fever, jaundice, excessive menstruation, ulcers, obesity, uterine and vaginal discharges [3]. A lot of phytopharmaceuticals from different parts of *S. anacardium* have been isolated and reported. Bhilwanols, phenolic compounds, biflavonoids, sterols and glycosides are the important chemical constituents reported from this plant. The pericarp of the fruit of this plant contains a bitter and powerful astringent principle which is used as a substitute for marking ink, thus called marking nut tree. The crushed pericarp on extraction with acetone gives dark coloured oil which on distillation gives light yellow oil, semecarpol, a monophenol and golden yellow oil, bhilawanol [4]. Other studies on the phytochemistry of this plant revealed the occurrence of a variety of flavonoids such as tetrahydroamentoflavone, nallaflavonone, semecarpetin and anacardioflavonone along with other phenolic compounds such as bhilawanols and anacardic acids [5].] In this study standardization of fruits of *Semecarpus anacardium* Linn. was carried out by performing physicochemical, preliminary phytochemical, HPTLC and GC-MS analysis.

MATERIALS AND METHODS

Sample Collection

Fruits of *S. anacardium* were collected from Pathanamthitta district, Kerala and were authenticated by Dr. P. B. Benil, Associate Professor, Department of Agadatantra, VPSV Ayurveda College, Kottakkal, Kerala. The fruits were cleaned, shade dried, coarse powdered (Fig. 1) and stored at -20^{o}C until further analyses.

Powder microscopy

To study the microscopic characteristics, a pinch of powder was warmed with few drops of chloral hydrate

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on a microscopic slide and mounted in glycerine. Characters were observed under microscope and diagnostic characters were photographed using Zeiss AXIO trinocular microscope attached with Zeiss AxioCam camera under bright field light.

Figure 1: Dried and powdered fruits of *S. anacardium*

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 protocol [6, 7].

Elemental analysis

One gram of finely powdered sample was taken into a pre weighed crucible and kept in muffle furnace overnight at 500^{0}C. The ash obtained was wetted with few drops of water and added 1 ml of conc. HNO_3. Excess HNO_3 was evaporated on a hot plate set at 100^{o}C and kept the crucible again to furnace for one hour at 500^{o}C. Ash was then dissolved in 10 ml conc. HCL, filtered to a volumetric flask and made up to 100 ml with distilled water. Later read the concentration by aspirating sample as well as standard solution in Atomic Absorption Spectrophotometer (Parker Elmer- Pinnacle 900H). Concentration of elements is expressed as the mean value mg/kg of dry weight.

Preparation of hydro-alcoholic extract

Weighed quantity of coarse powders was soaked in ethanol (99.9%) /water (1:1) in a percolator for 24 hrs. The soluble portion was filtered through a filter paper and dried on water bath in a weighed evaporating dish. The extracts were dried under vacuum and stored in desiccator until use for further analyses/successive extraction.

Qualitative Phytochemical Tests

Hydro-alcoholic extract prepared as per 2.5 was mixed with silica gel for column chromatography and extracted successively in a Soxhlet extractor using solvents such as Petroleum Ether, Chloroform, Ethyl Acetate and Ethanol in the increasing order of polarity. The extract was concentrated by distillation and solvents were removed by evaporation on a water bath. The extracts were completely dried under vacuum. The percentage of dried extracts with reference to the sample taken was recorded. These successive extracts were tested for phytochemicals [8, 9].

HPTLC Fingerprinting

One gram of hydro-alcoholic extract prepared as per 2.5 was mixed with silica gel for column chromatography and extracted by maceration with ethanol. the extract was made up to 50 ml in a volumetric flask. Five and ten microlitre of the ethanolic extract was applied on a pre-coated silica gel F_{254} on aluminum plates to a band width of 7 mm using CAMAG Linomat 5 TLC applicator. The plate was then developed in CAMAG twin-trough chamber using Toluene/ Ethyl acetate (8: 1) as mobile phase. The Rf values were determined by photodocumentation performed using CAMAG photo-documentation chamber and the plates were scanned under 254 nm, 366 nm and 620 nm after derivatisation using CAMAG Scanner [10].

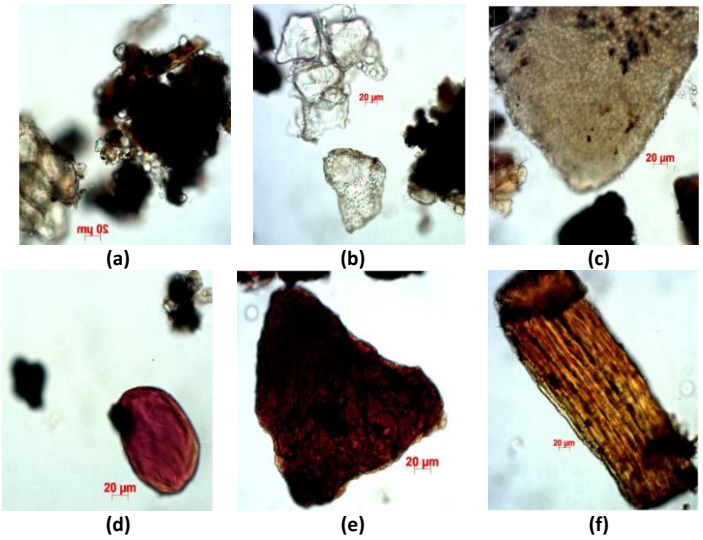
GC-MS Analysis

One gram of hydro-alcoholic extract prepared as per 2.5 was mixed with silica gel for column chromatography and extracted by maceration with diethyl ether. the extract was made up to 10 ml in a volumetric flask and analyzed for composition by GC-MS. The study was carried out on a 5975C Agilent system equipped with a DB-5ms Agilent fused silica capillary column (30 × 0.25 mm ID; film thickness: 0.25 µm), operating in electron impact mode at 70 eV. To identify the compounds, the extract was assigned for comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with the published literature. National Institute of Standards and Technology library sources (NIST II) were used for matching the identified compounds from the sample.

RESULTS AND DISCUSSION

Powder Microscopy

Microscopic powder study of the fruit powder of *S. anacardium* shows diagnostic characters like parenchyma with dark brown contents; many hyaline pitted parenchyma cells; polygonal thin walled cells of epidermis of cotyledon in surface view; thick-walled cells of epicarp in surface view having coloured contents deposited on their lateral walls; cells of cotyledon/endosperm with lot of aleuronic grains dispersing from ruptured cells; few reddish brown coloured resin cells; mesocarp parenchyma without clear cut visibility of cell walls due to the presence of reddish brown content; fragments of bundles of thin and thick-walled fibres; fragments of columnar palisade cells of testa arranged adjacently like pillars; few elongated to dumb-bell shaped pitted sclereids; and fragments of vascular elements having narrow spiral vessels (Fig. 2).



(a) Parenchyma with contents; **(b)** Pitted parenchyma; **(c)** Cells of cotyledon; **(d)** Resin cell; **(e)** Mesocarp parenchyma; **(f)** Thin-walled fibre bundle

Figure 2: Powder microscopy of *S. anacardium* fruit powder

Physico-chemical Analysis

The total ash indicating total inorganic content was found to be 7.60 and acid insoluble part of total ash was found to be 1.27. The low acid-insoluble ash value shows that a very small amount of the inorganic component is insoluble in acid. It indicates that adulteration by substances, such as silica is very less, and may also affect the amount of the component absorbed in the gastrointestinal canal when taken orally [11]. The Ethanol and water soluble secondary metabolites were

found to be 21.00 and 6.01 % w/w respectively (Table. 1). Extractive values are used to determine the amount of active constituents in given amount of medicinal plants, which provides preliminary information about the drug. Higher alcohol-soluble extractive value implies that ethanol is a better solvent of extraction than water.

Table 1: Physicochemical parameters of fruits of *S. anacardium*

Parameters	Results (n=3 % w/w)
Foreign matter	Nil
Total Ash	7.60
Acid insoluble Ash	1.27
Water soluble Ash	0.60
Alcohol soluble extractive	21.00
Water soluble extractive	6.01

Elemental Analysis

The macronutrients - Na and K were analyzed and the results showed that the concentration of potassium is very much higher than sodium. The concentration of heavy metals such as Pb, Cu, Zn, Mn, Cr and Fe was also estimated found to be in permissible limits (Table 2.). The essential heavy metals such as Cu, Zn, Fe and Mn play many biochemical and physiological functions in plants [12].

Table 2: Concenteration of various elements in the fruit powder of *S. anacardium*

Elements	Concentration (mg/Kg)
Na	74.0
K	8890
Mn	44.3
Cr	2.2
Pb	1.4
Cu	5.9
Fe	178
Zn	10.4

Preliminary Phytochemical Screening

The methanol extract of *S. anacardium* revealed the presence of sugars, flavonoids, steroids, saponins and tannins (Table. 4). Sugars were also seen in petroleum ether, chloroform and ethyl acetate extracts while steroids in ethyl acetate and chloroform extracts. The petroleum ether extract also contains quinones. The yield of extract was maximum in petroleum and lower in both ethyl acetate and methanol (Table. 3).

Table 3: Yield of extract from *S. anacardium* using different solvents

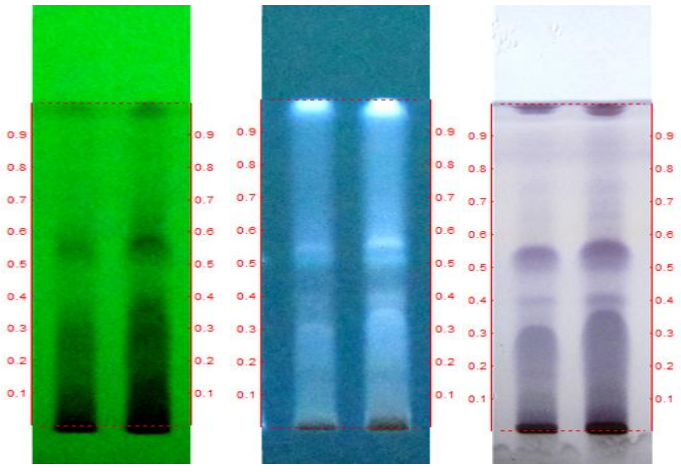
Sample (g)	Solvents	Volume (ml)	Yield (g)	% w/w
40	Petroleum Ether	200	3.572	8.93
	Chloroform	200	1.585	3.962
	Ethyl acetate	200	0.26	0.65
	Methanol	200	0.324	0.81

Table 4: Preliminary phytochemical tests of *S. anacardium* successive extracts

Test	Pet ether	Chloroform	Ethyl Acetate	Methanol
Alkaloid	–	–	–	–
Carbohydrate	+	+	+	+
Carboxylic acid	–	–	–	–
Coumarins	–	–	–	–
Flavanoids	–	–	–	+
Phenol	–	–	–	–
Quinone	+	–	–	–
Resins	–	–	–	–
Steroid	–	+	+	+
Saponins	–	–	–	+
Tannin	–	–	+	+
Terpenoid	–	–	–	–

HPTLC Fingerprinting

HPTLC fingerprinting of ethanol extract of *S. anacardium* yield the following results. Photodocumentation under 254 nm gives 7 spots (Fig. 3a), 11 spots under 366 nm (Fig 3b), and 13 spots under 620 nm post-derivatisation with vanillin sulphuric acid spray reagent (Fig 3c; Table. 5). Densitometric scan at 254 nm (Table. 6) revealed 5 peaks corresponding to 5 different compounds in the ethanol extract with R_f 0.66 (59.91%), 0.35 (19.51%) and 0.42 (17.80%) being the major peaks (Fig. 4). Densitometric scan at 366 nm (Table. 7) showed 7 peaks, and peaks with R_f 0.29 (26.65%), 0.36 (24.31%), 0.65 (16.59%), 0.59 (11.56%), 0.46 (9.55%) and 0.07(8.95%) were the major peaks detected (Fig. 5). Densitometric scan at 620 nm (Table. 8) showed 9 peaks, peaks with R_f 0.63 (42.51%), 0.23 (14.94%), 0.46 (9.17%), 0.42(7.23%), 0.96(6.86%) and 0.76 (6.41%) being the major ones (Fig. 6). HPTLC is still increasingly finding its way in pharmaceutical analysis and with the advancements in the stationary phases and the introduction of densitometers as detection equipment, the technique achieves for given applications, a precision and trueness when compared to High Performance Liquid Chromatography [13].



At 254 nm **(a)** | At 366 nm **(b)** | After derivatisation **(c)**

Track 1- S. anacardium– 5 µl: Track 2- S. anacardium– 10 µl
Solvent system: Toluene: Ethyl Acetate (8.0:1.0)

Figure 3: HPTLC photo documentation of ethanol extract of fruits of *S. anacardium*

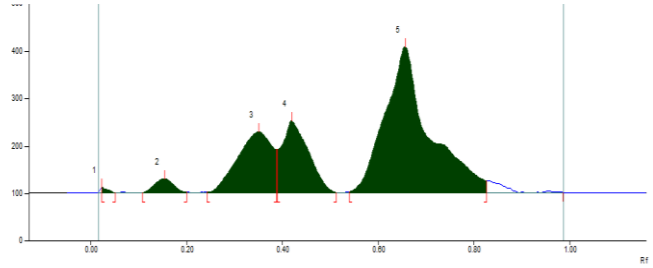


Figure 4: Densitometric scan of ethanol extract of fruits of *S. anacardium* at 254 nm

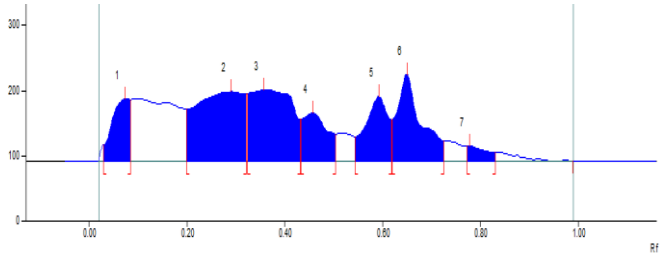


Figure 5: Densitometric scan of ethanol extract of fruits of *S. anacardium* at 366 nm

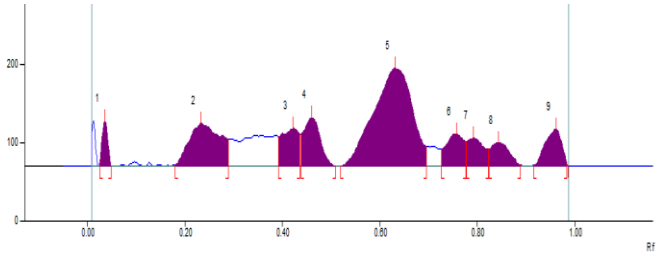


Figure 6: Densitometric scan of ethanol extract of fruits of *S. anacardium* at 620 nm

Table 5: R_f values of ethanol extract of nuts of *S. anacardium* (10µl)

At 254 nm	At 366 nm	After derivatisation
0.06(D Green)	0.06(F L Violet)	-
-	-	0.08(Violet)
-	-	0.12(Violet)
0.14(Green)	0.14(F L Violet)	-
-	0.19(F L Green)	0.19(Violet)
-	0.26(F L Violet)	-
0.32(Green)	-	-
-	0.35(F Violet)	-
0.37(Green)	-	0.37(Violet)
-	0.41(F Violet)	0.41(Violet)
-	0.52(F Violet)	0.52(Violet)
0.58(Green)	0.58(F Violet)	0.58(Violet)
-	-	0.62(L Violet)
0.65(L Green)	0.65(F L Violet)	-
-	0.70(F L Violet)	-
0.70(L Green)	-	0.70(L Violet)
-	-	0.76(L Violet)
-	0.80(F L Violet)	0.80(L Violet)
-	-	0.85(L Violet)
-	-	0.88(L Violet)

*L-Light, D-Dark, F-Fluorescence

Table 6: Details of bands obtained by densitometric scan of ethanol extract of nuts of *S. anacardium* at at 254 nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.02	11.3	0.02	11.3	1.80	0.05	0.1	96.6	0.28
2	0.11	0.1	0.15	29.8	4.73	0.20	1.5	851.2	2.50
3	0.24	1.2	0.35	128.6	20.43	0.39	90.5	6654.8	19.51
4	0.39	90.7	0.42	152.2	24.17	0.51	0.7	6071.8	17.80
5	0.54	2.5	0.66	307.8	48.88	0.83	25.6	20437.0	59.91

Table 7: Details of bands obtained by densitometric scan of ethanol extract of nuts of *S. anacardium* at 366 nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.03	25.2	0.07	96.2	15.03	0.09	94.2	2520.0	8.95
2	0.20	79.7	0.29	106.2	16.59	0.32	3.5	7501.8	26.65
3	0.32	103.5	0.36	109.2	17.06	0.43	64.4	6843.4	24.31
4	0.43	64.5	0.46	73.5	11.48	0.50	41.3	2689.4	9.55
5	0.55	37.4	0.59	98.3	15.36	0.62	62.9	3253.4	11.56
6	0.62	63.3	0.65	133.2	20.81	0.73	31.3	4671.3	16.59
7	0.77	23.3	0.78	23.4	3.66	0.83	14.0	670.3	2.38

Table 8: Details of bands obtained by densitometric scan of ethanol extract of nuts of *S. anacardium* at 620nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.03	4.3	0.04	57.5	11.53	0.05	0.7	492.9	2.95
2	0.18	2.0	0.23	54.1	10.85	0.29	33.9	2492.9	14.94
3	0.39	37.7	0.42	47.7	9.55	0.44	40.8	1206.6	7.23
4	0.44	40.9	0.46	61.7	12.36	0.51	0.1	1530.1	9.17
5	0.52	0.0	0.63	124.4	24.95	0.70	25.5	7094.8	42.51
6	0.73	21.9	0.76	41.1	8.25	0.78	31.4	1070.6	6.41
7	0.78	31.5	0.79	35.3	7.07	0.82	21.2	860.0	5.15
8	0.83	21.6	0.85	29.7	5.95	0.89	1.2	797.8	4.78
9	0.92	1.1	0.96	47.3	9.48	0.99	3.4	1144.7	6.86

GC-MS Analysis

Gas liquid chromatogram of the diethyl ether extract of the nuts of *S. anacardium* showed 11 peaks (Fig. 7) indicating the presence of as much as compounds (Table. 9). Among these n-Nonadecanol-1, 1-Octadecene, 9- Octadecanoic acid, cis-Vaccenic acid and p-Thiocresol were identified. The results revealed that 9- Octadecanoic acid (27.84%) was the major component followed by n-Nonadecanol-1 (3.67%). However two compounds with the highest composition at peaks, Peak 8 (35.43%) and Peak 9 (18.87%) have not been matched with the library. Eicosane, Glucobrassicin, Octadecane, Hepatadecane, Tetracosane, Icosane and Phenol 2, 4-bis (1, 1-dimethylethyl) were reported in the ethyl acetate extract of *S. anacardium*[14].

Table 9: List of phytochemicals identified by GC-MS of diethyl ether extract of nuts of *S. anacardium*

Peak	RT	% Area	Name	Match
1	14.664	0.45	-	-
2	14.720	3.67	n-Nonadecanol-1	##
3	15.302	0.29	1-Octadecene	##
4	15.552	27.84	9- Octadecanoic acid	##
5	15.627	6.24	cis-Vaccenic acid	##
6	16.622	0.33	-	-
7	17.360	0.96	-	-
8	18.623	35.43	-	-
9	18.667	18.87	-	-
10	18.736	4.75	p-Thiocresol	##
11	21.57	1.18	-	-

Identified
- Unidentified

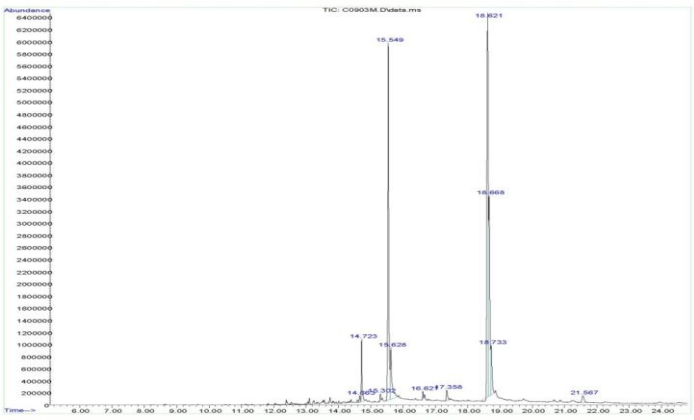


Figure 7: GLC of the diethyl ether extract of fruits of S. *anacardium*

CONCLUSION

In the present study the fruits of Bhallataka have been standardized as per pharmacopoeial testing protocol. The results of powder microscopy, physicochemical and preliminary phytochemical analyses have been reported. The ethanol extract of drug was subjected to HPTLC fingerprinting and photo documentation, R_f values and densitometric scan at 254 nm, 366 nm and after derivatisation has been developed. The GC-MS analysis of the diethyl ether fraction showed the presence of 11 compounds of which five were identified. Results obtained from the study can be used for analytical standardization of the drug *S. anacardium* Linn.

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CONFLICTS OF INTEREST

No conflicts of interest.

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Nil.

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