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CHEMICAL CONSTITUENTS ISOLATED FROM *SEMECARPUS ANACARDIUM* LEAVES & ANALYSIS OF THEIR BIO-ACTIVE POTENTIAL

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ABSTRACT

Herbal drugs standardization is the need of the time as the use and practice of traditional herbal drugs and their formulations has increased vastly. In the present study, an attempt has been made to isolate the potential bioactives of *Semecarpus anacardium* as per pharmacognostic testing protocol which include powder microscopy, physico-chemical screening, HPTLC fingerprinting NMR & LC-MS analysis. Preliminary phytochemical tests indicate the presence of flavonoids, alkaloids, glycoside, phenols, steroids, protein, sugars and tannins. HPTLC profiling of the ethanol extract using Ethyl Acetate: Water: Formic Acid: Acetic acid (100:26:11:11) v/v/v/v) as mobile phase revealed the presence of phytochemicals with different Rf values. Upon further purification LC-MS & NMR analysis of the ethyl acetate fraction showed the presence of semecarpetin as bioflavonoids.

Keywords: *Semecarpus anacardium,* Phytochemicals, Flavonoids, Pharmacognostic, Fingerprinting.

1. INTRODUCTION

Standardization is the development of prescribing a set of standards or natural characteristics, definitive qualitative and quantitative values that transmit an assurance of efficacy, safety and reproducibility is important 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 bring about the process of prescribing a set of characteristics exhibited by the particular drug [2].

Semecarpus anacardium is a native of India, found in the outer Himalayas to coromandel Coast. It is closely related to the cashew. It is known as *Bhallaatak* in India and was called "marking nut" by Europeans, because it was used by washer men to mark cloth and clothing before washing, as it imparted a water insoluble mark to the cloth. It is a deciduous tree. The fruit is composed of two parts, a reddish orange accessory fruit and a black drupe that grows at the end. The nut is about 25 millimeters long, ovoid and smooth lustrous black. Leaves are alternate, simple, oblong-obviate, 25-60 cm long and 10-32 cm broad, rounded at the apex coriaceous glabrous above and more or less pubescent, beneath. The seed inside the black fruit, known as godambi is edible when properly prepared.

Semecarpus anacardium, 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 mentioned for various gastric and urinary disorders, 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, liver toxicity, menorrhagia ulcers, obesity, & pelvic inflammatory disease [3]. In this study, standardization of leavess of *Semecarpus anacardium* Linn. was carried out by performing physicochemical, preliminary phytochemical, HPTLC, IR NMR & LC-MS analysis.

A lot of phytopharmaceuticals from different parts of *S. anacardium* have been isolated and reported. Bhilwanols, phenolic compounds, biflavonoids, sterols and glycolsides 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].

2. MATERIAL AND METHODS

2.1. Sample Collection

Leaves of *S. anacardium* were collected from forest of Betul district and were authenticated by Dr. A.K Pathak, Professor, Department of Pharmacy, Barkatullah University, Bhopal & new voucher Specimen (Bot H-02/53/118) was deposited at the hebarium of Department of Pharmacy, Barkatullah University, Bhopal. The leaves were cleaned, shade dried, coarse powdered (Fig. 1) and stored at-20°C until further analyses.

Fig. 1: Dried and powdered leaves of *S. anacardium*

2.2. Powder microscopy

To study the microscopic characteristics, a pinch of powder was warmed with few drops of chloral hydrate on a microscopic slide and mounted in glycerine. Characters were observed under microscope and diagnostic characters were photographed using trino-cular microscope attached with camera under bright field light.

2.3. 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].

2.4. Elemental analysis

Two gram of finely powdered sample was taken into a pre weighed crucible and kept in muffle furnace overnight at 500°C. The ash obtained was kept in desiccators for few minutes & then again dried crude drug sample & calculated the % yield. Ash was then dissolved in 25 ml conc. HCL, filtered to a volumetric flask and made up to 100 ml with distilled water. The sample was boiled for 5 minutes & filtered through ash less filter paper. Ignited the filter paper in crucible, kept for few minutes in desiccators, weighed & calculated the percentage yield with reference to air dried drugs. Similar procedure was applied for water soluble ash instead of 25 ml HCl replaced by 25 ml of water.

2.5. 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 desiccators until use for further analyses/successive extraction.

2.6. Qualitative Phytochemical Tests

Hydro-alcoholic extract was prepared as per procedure, 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].

2.7. TLC (Thin Layer Chromatography)

2.7.1. Preparation of plates

A suspension of the Silica gel G in water was prepared using the spreading device designed for the purpose, a uniform layer (0.20 to 0.30 mm thick) of the suspension was spread on a 20 cm long flat glass plate. Allowed the coated plates to dry in air, heat at 100°C to 105°C for at least 1 hour and allowed to cool, protected from moisture. Stored the plates protected from moisture and used within 3 days of preparation. Pre coated plates of silica gel (Merck made) were also used.

2.7.2. Method

A tank was prepared by lining the walls with sheets of filter paper; and mobile phase was poured into the tank, saturating the filter paper in the process, sufficient of the mobile phase to form a layer of solvent 5 to 10 mm deep, closed the tank and allowed to stand for 1 hour at room temperature. About 5 mm wide narrow strip of the coating substance was removed from the vertical sides of the plate and solutions being examined was

applied the in the form of circular spots. Sides of the plate were marked for reference. Allowed the solvent to evaporate and placed the plate in the tank, ensuring that it is as nearly vertical as possible and that the spots or bands are above the level of the mobile phase. Remove the plate and dry and visualized in UV inspection cabinet.

2.7.3. Visualisation

The phrases *ultra-violet light (254 nm)* and *ultra-violet light (366 nm)* indicate that the plate should be examined under an ultra-violet light having a maximum output at about 254 or at about 365 nm, as the case may be.

2.7.4. Rf Value

Distance of each spot from the point of its application was measured, recorded and calculated the *Rf* value by dividing the distance travelled by the spots by the distance travelled by the front of the mobile phase.

2.7.5. Separation and Isolation of flavonoids

The concentrated residue was further extracted by following methodology to separate flavonoids. Specified amount of extract was weighed, dissolved in specified amount of 5M HCl and refluxed for six hours. The solution was filtered and filtrate was extracted with ethyl acetate with several volumes of ethyl acetate to completely extract the flavonoids. The ethyl acetate was evaporated and the residue was further extracted with amyl alcohol. The amyl alcohol residue was further fractionated by column chromatography.

2.8. Column Chromatography

The glass column of 250 ml capacity was used for further fractionation. Silica gel of column grade of about 230 mesh sizes with particle dimension of 200µm was used as adsorbent. The column was packed with activated silica slurry by wet packing method [9]. The sample was soaked on small portion of silica, dried and poured on column. The elution was carried out using hexane, ethyl acetate and methanol. The ethyl acetate fraction consisted of flavonoids.

2.9. HPTLC Fingerprinting

One gram of hydro-alcoholic extract was prepared and 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 isolated ethanolic extract was applied on a pre-coated silica gel F254 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 Ethyl Acetate: Water: Formic Acid: Acetic acid (100:26:11:11) v/v/v/v) 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].

2.10. FT-IR analysis

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried powder of different solvent extracts of each plant materials were used for FT-IR analysis.

FT-IR of ethyl alcohol extract sample was recorded in wave number range in the 4000-600cm^2 with Alpha-ECO-ATR spectrometer (Bruker Germany) including ATR unit. A resolution of 4cm^2 and a number of 32 scans per sample was used. Solid powder extract was analyzed by using IR apparatus shows 03 spectra of which in mean reference spectra was calculated OPUS-7 software.

2.11. LC-MS Analysis

One gram of hydro-alcoholic extract was prepared as per procedure and mixed with silica gel for column chromatography and extracted by maceration with diethyl ether extract which was made up to 10 ml in a volumetric flask and analyzed for composition by LC-MS. The study was carried out on a Waters Micromass Q-Tof Micro. The Mass Spectrometer is coupled with Waters 2795 HPLC having quaternary pumping configured for flow rates from 0.05- 5.0 ml/Min. The auto sampler is configured with a 100 micro-litre syringe 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.

3. RESULTS AND DISCUSSION

3.1. Powder Microscopy

Microscopic powder study of the leaves powder of *S. anacardium* shows diagnostic characters like unicellular ribbon shaped covering trichomes & shrunken stomata. It also contain lignified xylem cell, parenchyma with dark brown contents; many hyaline pitted parenchyma cells. Pericyclic fiber & cuticle was prominent in powder microscopy (Fig. 2).

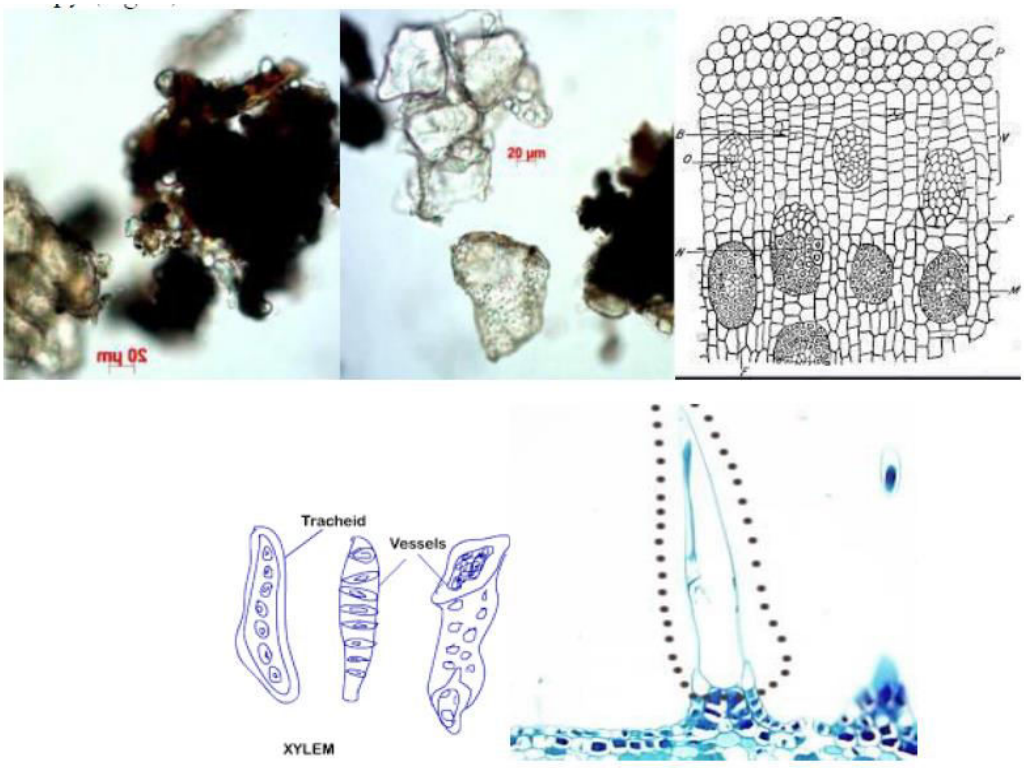

Fig. 2: Powder microscopy of *S. anacardium* leaves powder (a) Parenchyma with contents; (b) Pitted parenchyma (c) Pericyclic fiber (d) Lignified xylem Cell (e) Unicellular Trichomes

3.2. Physico-chemical Analysis

The total ash indicating total inorganic content was found to be 7.70 and acid insoluble part of total ash was found to be 1.38. 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 22.00 and 6.04 % 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 leaves of *S. anacardium*

Parameters	Results
Foreign matter	Nil
Total Ash	7.70
Acid insoluble Ash	1.38
Water soluble Ash	0.64
Alcohol soluble extractive	12.50
Water soluble extractive	2.30

3.3. Preliminary Phytochemical Screening

The ethnolic extract of *S. anacardium* revealed the presence of flavonoids, alkaloids, glycoside, phenols, steroids, protein, sugars 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 yield of extract was maximum in ethanol and lower in both ethyl acetate and chloroform (Table 3).

3.4. HPTLC Fingerprinting

HPTLC fingerprinting of ethanol extract of *S. anacardium* yield the following results. Photo documentation under 254, 366 nm gives 06 spots (Fig. 3a and 3 b), and 06 spots under 620 nm post-derivatisation with 10% methanolic sulphuric acid spray reagent (Fig 3c; Table 5). 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].

3.5. FT-IR interpretation

The identification study of isolated fraction in extracted sample was done by the FT-IR spectroscopy and predicts the corresponding functional groups in marker compound.

Table 2: Yield of extract from *S. anacardium* using different solvents

Sample (gm)	Solvent	Volume (ml)	Yield (gm)	% w/w
25	Petroleum Ether	200	1.36	5.45
	Chloroform	200	0.9	3.60
	Ethyl Acetate	200	1.625	6.50
	Ethanol	200	3.12	12.5

Table 3: Preliminary phytochemical tests of *S. anacardium* successive extracts

Test	Petroleum Ether	Ethyl Acetate	Chloroform	Ethanol	Aqueous Extract
Carbohydrate	-	-	-	+	-
Alkaloid	-	+	-	+	-
Flavanoids	-	+	+	+	-
Proteins	-	-	-	+	-
Glycoside	+	-	-	-	+
Phenols & Tannins	+	+	-	+	+
Steroid	+	+	+	+	-
Mucilage	-	+	-	+	+

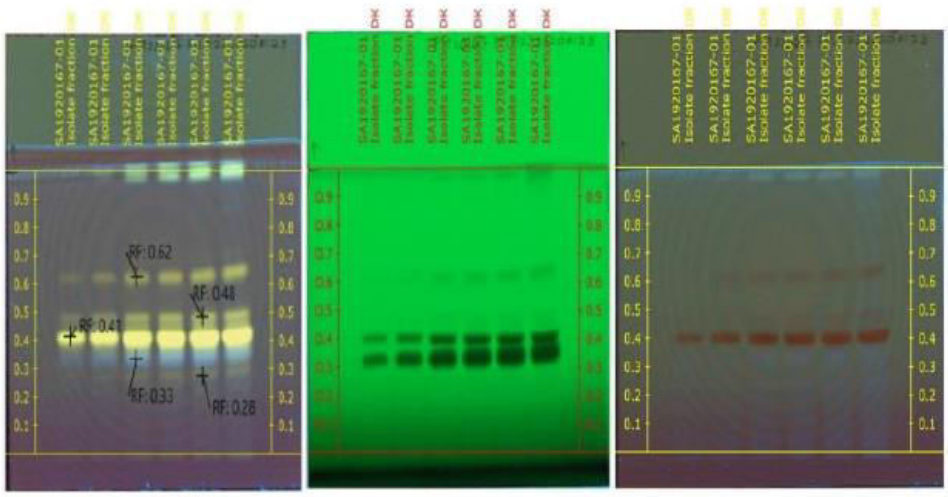

Fig. 3: HPTLC photo documentation of isolated ethyl acetate fraction of leaves of *S. anacardium* Track 1- *S. anacardium*-5 µl: Track 2- *S. anacardium*-10 µl Solvent system: Ethyl Acetate: Water: Formic Acid: Acetic acid (100:26:11:11) v/v/v/v

Table 4: Rf values of ethanol extract of nuts of *S. anacardium* (10µl)

Name	Track no.	No.	Colour	Intensity	Rf Value
DKT-SA-01	1	1	Yellow	High	0.41
	3	2	Blue	Low	0.33
			Yellow	Medium	0.62
	5	2	yellow	Medium	0.48
			yellow	Low	0.28

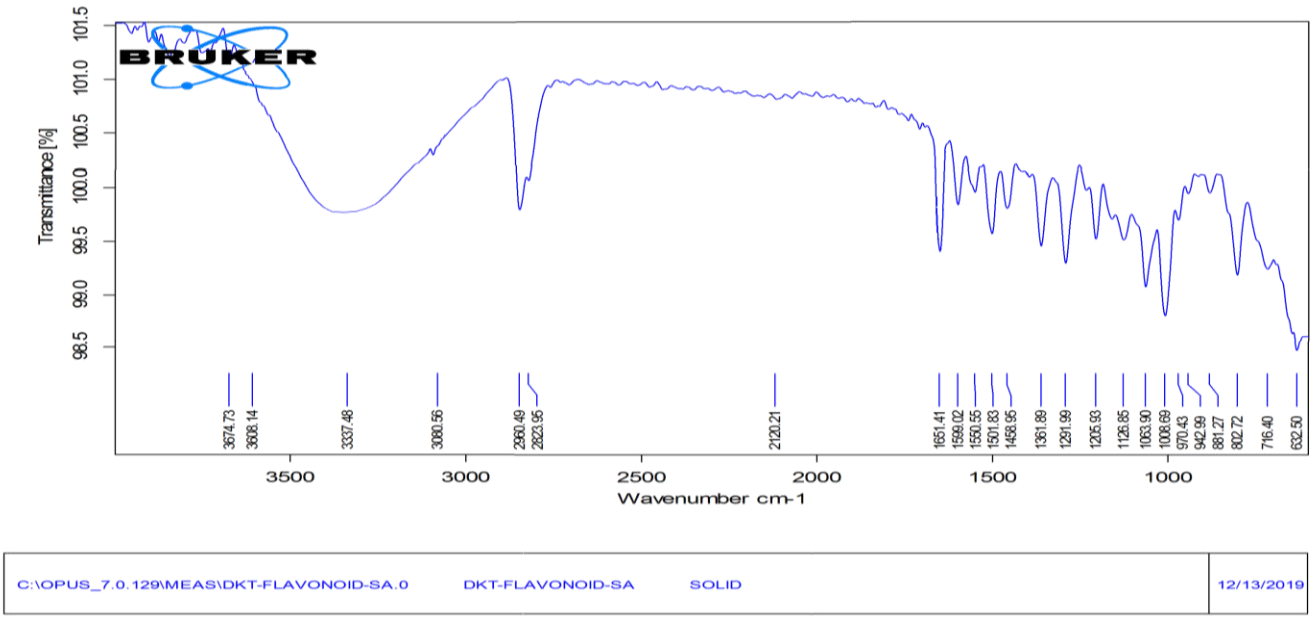

Fig 4: FT-IR Spectrum of isolated ethyl acetate fraction of leaves of *S. anacardium*

Table 5: IR interpretation of isolated ethyl acetate fraction of leaves of *S. anacardium*

S. No	Peak value (cm^{-1}) Observed	Frequency range	Functional groups
1	3337	3500-3200	O-H str.
2	3080	3100-3000	C-H str. Sp^2 ($-CH_2-$)
3	2960	3000-2840	C-H str. Sp^3 ($-CH_3$)
4	1651	1725-1650	C=O str. Aromatic ketone
5	1501	1550-1470	C-C str. Aromatic ring
6	1458	1465-1450	C-H bend methylene group
7	1361	1420-1330	O-H bend
8	1205	1275-1200	C-O alkyl aryl ether

3.6. LC-MS Analysis

Liquid chromatogram Mass spectrum of the ethyl alcohol extract of the leaves of *S. anacardium* showed single peaks (Fig. 5) indicating the presence of single compounds (Table 5) [14-16].

Table 5: LC-MS interpretation of isolated ethyl acetate fraction of leaves of *S. anacardium*

Peak	RT	% Area	Name	Match
01	7.82	99.56%	DKT SA	

LC-MS: (m/z): 609.63 (M+1): 283(100%), 234, 300, 338, 447 **^{1}H NMR (DMSO, 500 MHz):** δ, 7.56(d, 2H, *J* =7.5, CH), 6.87(d, 2H, *J* =9.00, CH), 6.40(s, 1H, CH), 6.21(s, 1H, CH), 5.36(d, 1H, *J* =3.5, CH), 5.10(s, 2H, OH), 4.77(d, 1H, *J* =8.5, CH), 4.04(q, 1H, CH), 3.83(s, 3H, O-CH_3), 3.75(t, 1H, *J* =5.0, 2.5, CH), 3.58(s, 6H, OH), 3.48-3.40(m, 4H, CH), 3.32-3.21(m, 2H, CH), 3.10(t, 2H, *J* =9.0, 9.0, CH), 1.01(s, 3H, CH_3)ppm.

^{13}C NMR (DMSO, 500 MHz): δ, 17.61, 61.88,

66.89, 68.13, 68.30, 69.89, 70.44, 71.74, 74.53, 75.77, 76.32, 93.49, 98.58, 100.62, 101.07, 103.87, 115.13, 116.19, 117.86, 121.49, 133.18, 152.88, 156.32, 161.10, 163.94, 170.58, 177.26 ppm.

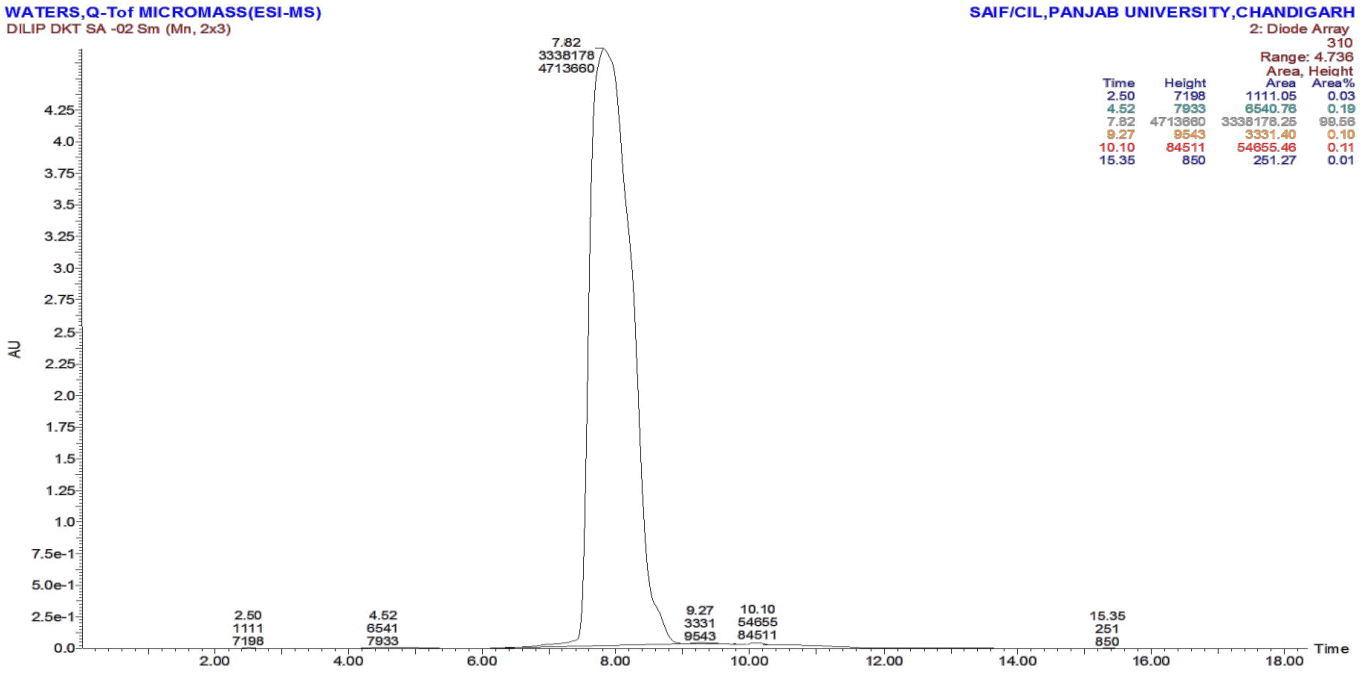

Fig. 5: LC-MS of the isolated ethyl acetate fraction of leaves of *S. anacardium*

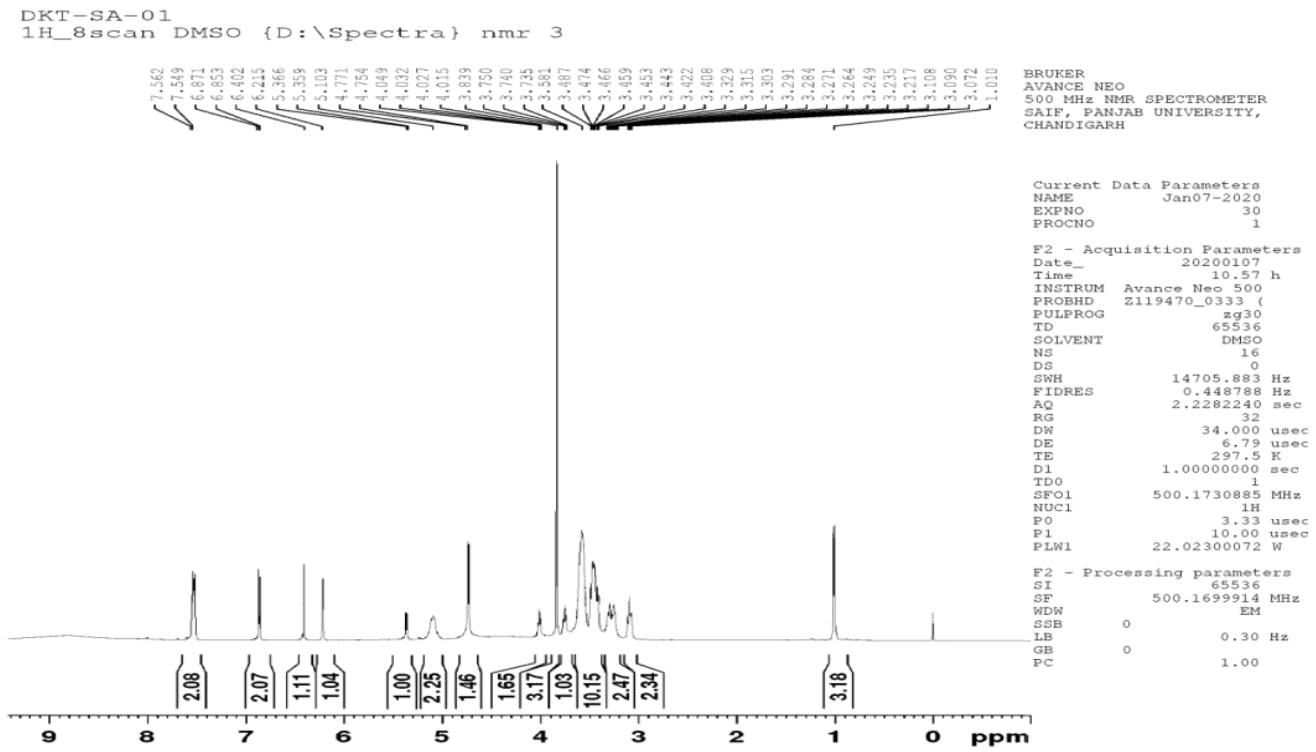

Fig. 6: ^{1}H NMR spectrum of pure compound of isolated ethyl acetate fraction of leaves of *S. anacardium*

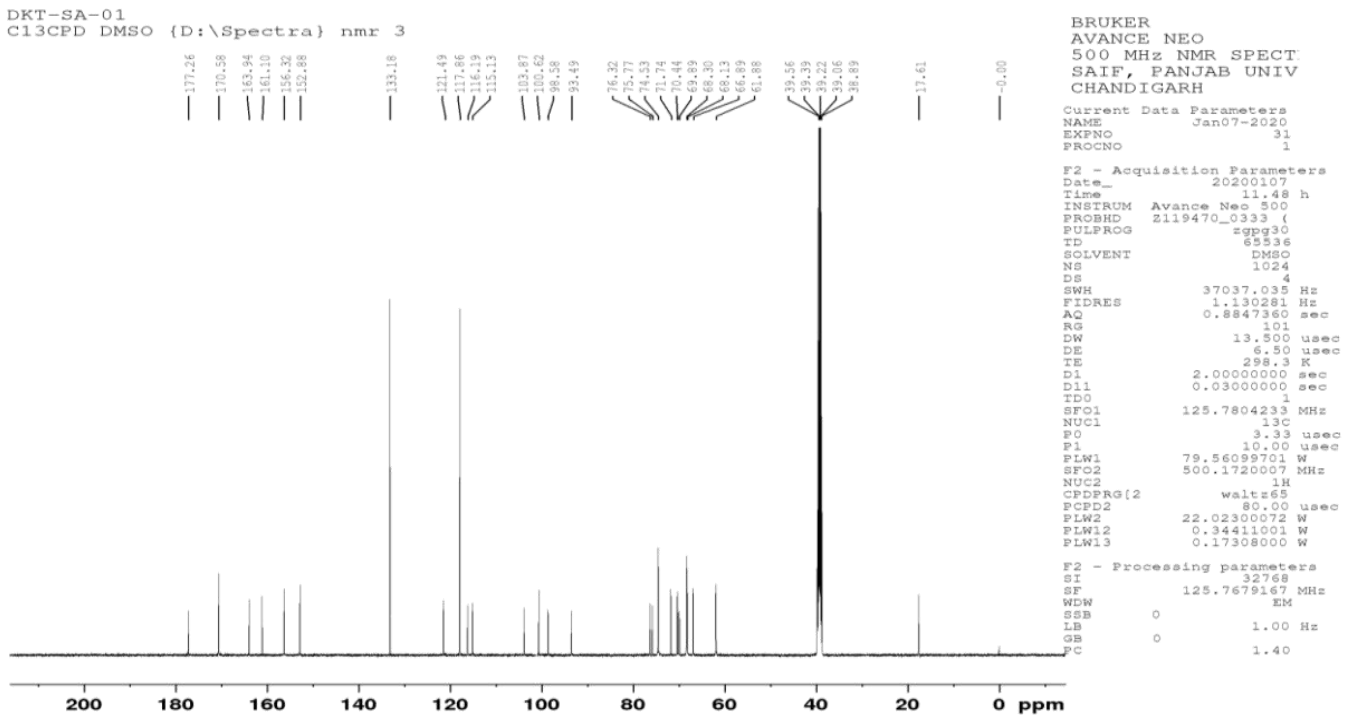

Fig. 7: ^{13}C NMR spectrum of pure compound of isolated ethyl acetate fraction of leaves of *S. anacardium*

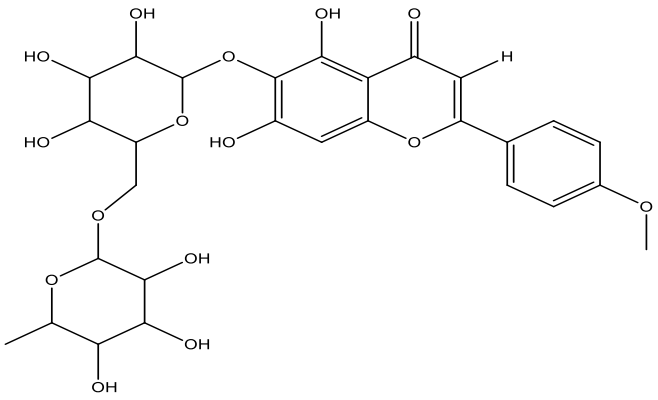

Fig. 8: Structure of flavonoid: 5,7-dihydroxy-2-(4-methoxyphenyl)-6-((3,4,5-trihydroxy-6-(((3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)-4H-chromen-4-one ($C_{28}H_{32}O_{15}$)

4. CONCLUSION

In the present study the leaves of Bhallantaka 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, Rf values and densitometric scan at 254 nm, 366 nm and after derivatisation has been developed. Further upon purification, The LC-MS analysis & NMR spectroscopy of the ethyl alcohol fraction showed the presence of bioflavonoid which was characterized as semecarpetin. 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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