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Characterization of pharmacognostical and preliminary phytochemical features of seeds of a Folk Plant - *Gnetum ula* Brongn

Ramya Krishna KV[1], T Shridhara Bairy[2], Sunil Kumar KN[3], Sudhakar[4]*

1 *P.G. Scholar, Department of Dravyaguna, SDM College of Ayurveda, Kuthpady, Udupi- 574118 India*

2 *Professor and Head, Department of PG Studies in Dravyaguna, SDM College of Ayurveda, Kuthpady, Udupi- 574118 India*

3 *Senior Research Officer, Department of Pharmacognosy and Phytochemistry, SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi- 574118 India*

4 *Research Officer, Department of Pharmacognosy and Phytochemistry, SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi- 574118 India*

ABSTRACT

Ayurveda opines that every plant has its own medicinal values, but there are many less explored plants which are not popular though beneficial either as food or as medicine. But this knowledge is passed from tradition to tradition in folk lore practices but within a few groups of a society. One such plant from gymnosperm group is *Gnetum ula* Brongn. (Gnetaceae) found commonly in and around Udupi. Locally known as *kumti beeja*, the plant is dioecious, branched woody climber. Seeds are roasted or boiled and consumed as food and the seed oil is used in rheumatism by folk practitioners. On account of these utilities of this less explored plant material, a detailed pharmacognostical study including macro and microscopy. Seed is enclosed by 3 layer envelope, outer fleshy and fibrous sarcotesta, hard sclerotesta and inner endosperm with two cotyledons having an embryo. Inner cotyledon contained of large parenchyma cells containing aleurone grains and starch. Preliminary phytochemical study of the seed revealed the presence of alkaloids, saponins, tannins, resin etc. HPTLC photo documentation showed a single spot under short UV, 4 spots under long UV and 3 spots under white light after derivatisation with vanillin sulphuric acid reagent. Results of this study can be utilized for identification of the drug as well as systematic document on purity standards of this extra pharmacopoeial drug.

Keywords: Folk lore, Gnetaceae, *Kumti*, Pharmacopoeia, Standardization.

INTRODUCTION

Human's passion for herbal medicine dates back to Vedic period. The usage of these plants either for food or medicine was a common practice since ages. Some of them were documented in Ayurveda classics, but there are some local health practitioners who use locally available and readily accessible source plants for curing different diseases.

In the family Gnetaceae, the sole genus *Gnetum* includes many species of woody trees, shrubs or even climbers; in India, *Gnetum* is represented by 5 species. [1] *G. ula* with synonym *G. scandens* Brandis Hook. f. (non Roxb.) in part, and *G. funiculare* B. Smith ex Wight[2] is a large dioecious, branched woody climber with thick scaly bark, swollen joints and compressed trunk of about 1 to 1.5 m in girth (Figure 1.1 to 1.4). The plant is distributed in Assam, Sikkim, Terrain Himalayas, Evergreen forests of the Eastern and Western Ghats up to 1800 m, Andaman Islands and Malaysia.[3] In Karnataka it occurs in Udupi, Chikmangaluru, Hassan, North Canara and Shimoga.[4,5] Flowering season of the plant is from March to April and fruits are sets April onwards.[3] Seeds, locally known as *Kumti Beeja* in Udupi, are used for edible purpose either roasted or boiled and the seed oil is used in rheumatism by folklore practitioners.[2] Stem and leaf extracts are useful in jaundice and liver enlargement,[6] while leaf paste is applied externally cures arthritis.[7] *G. ula* is one of the common climbers seen conserved in the sacred groves.[8] Stem is reported to contain stilbene gnetol, gnetin and butanedione. Seed kernel contains fixed oil with fatty acids, sterculic and malavalic acids[4] Keeping these utilities of the seed in mind, a pharmacognostical study by macro and microscopical and preliminary phytochemical study has been carried out in the present investigation.

***Corresponding author:**
Prof. T Shridhara Bairy
Professor and Head, Department of PG Studies in Dravyaguna, SDM College of Ayurveda, Kuthpady, Udupi- 574118 India
Email: drtsbairy[at]yahoo.com

Fig. 1.1 Climber

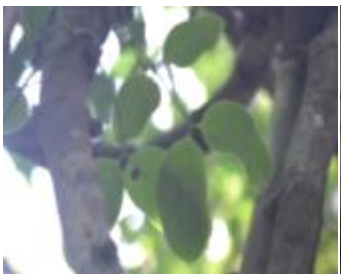


Fig. 1.2 Leaves

Fig. 1.3 Fruiting Spikes

Fig. 1.4 Fruits

Fig. 1.5 Layers of fruit

Fig. 1.6 Seeds

Figure 1: Macroscopy of *Gnetum ula*

MATERIALS AND METHODS

Collection of sample

Seeds of *G. ula* were collected from Barkur, Tantradi and Hebri of Udupi district during September and October 2014. The authenticity of seeds was confirmed by experts at SDM Centre for Research in Ayurveda and Allied Sciences, Udupi with the help of Pharmacognosist. Botanical characters were also compared with various floras and herbarium samples for further confirmation.[3]

Preservation of sample

The collected seeds are dried and were stored in air tight containers at SDM Centre for Research in Ayurveda and Allied Sciences, Udupi for pharmacognostical & phytochemical studies. For microscopic examination sample was preserved in fixative solution FAA (Formalin 5 ml + Acetic acid – 5 ml + 70% Ethyl alcohol – 90 ml) for more than 48 h.

Macroscopy

Air dried samples of were keenly observed under naked eyes to record the specific botanical characters and it was also recorded using Canon Ixus digital camera with size indicating rulers.[9]

Microscopy

The histology of seeds including stalk, seed coat, testa and cotyledon was recorded following standard microscopy procedures.[10] The preserved specimens were cut into thin transverse section using a sharp blade and the sections were stained with saffranine. Transverse sections were photographed using Zeiss AXIO trinocular microscope attached with Zeiss AxioCam camera under bright field light. Magnifications of the figures are indicated by the scale-bars.[11]

Physico-chemical analysis

G.ula seed powder was tested for pharmacopoeial constants like loss on drying at 105^{o}C, total ash, acid insoluble ash, alcohol soluble extractive, water soluble extractive as per standard protocol.[12]

Preliminary phytochemical analysis

Preliminary phytochemical investigation was done to detect the presence of alkaloids, steroids, carbohydrates, tannin, flavanoids, saponins, triterpenoids, coumarins and phenols in aqueous extracts of raw and roasted *G. ula* seeds.[13, 14]

HPTLC finger printing

One gram of seed powder of *G.ula* was extracted with 10 ml of ethanol by cold percolation. 4, 8 and 12 µl of the above extract was applied on a pre-coated silica gel F_{254} on aluminium plates to a band width of 7 mm, using Linomat 5 TLC applicator. The plate was developed in toluene: ethyl acetate (8.0:2.0) using CAMAG (Muttenz, Switzerland) twin trough chamber. The developed plates were visualized under long and short UV and then scanned under 254 and 366 nm using CAMAG Scanner 4. The plate was derivatised with vanillin-sulphuric acid reagent and R_f, colour of the spots and densitometric scan were recorded.[15, 16]

RESULTS

Macroscopical study

Fruiting spikes 7.5 to 25 cm long. Pseudo fruit oblong, olive shaped, solitary, ellipsoid, 2.5 to 3.7 cm long, stalked, green in colour, reddish orange on ripening. Seeds terete, top rounded, rugose, and hard enclosed by outer fleshy and fibrous sarcotesta, the layer next to sclerotesta is had bristles like and pricking. There is an inner thin papery layer which encloses endosperm with two cotyledons having an embryo (Figure 1.5 and 1.6).

Microscopical study

Stalk of strobilus

TS of stalk is circular with wavy irregular outline, it shows outermost thick-walled epidermis with few simple trichomes; about 20 layers of thin walled parenchyma forms the cortex embedding few solitary fibres with thick wall and narrow lumen; pericycle is formed by cap-like patches of porous tiny stone cells; vascular bundles are oval shaped patches with outer phloem tissue and inner xylem having protoxylem towards inner side; thick-walled fibres are found distributed in phloem as well as xylem; the central pith region shows simple and pitted

parenchyma cells which are lignified and there are few solitary thick walled fibres and branched sclereids in the pith (Figure 2).

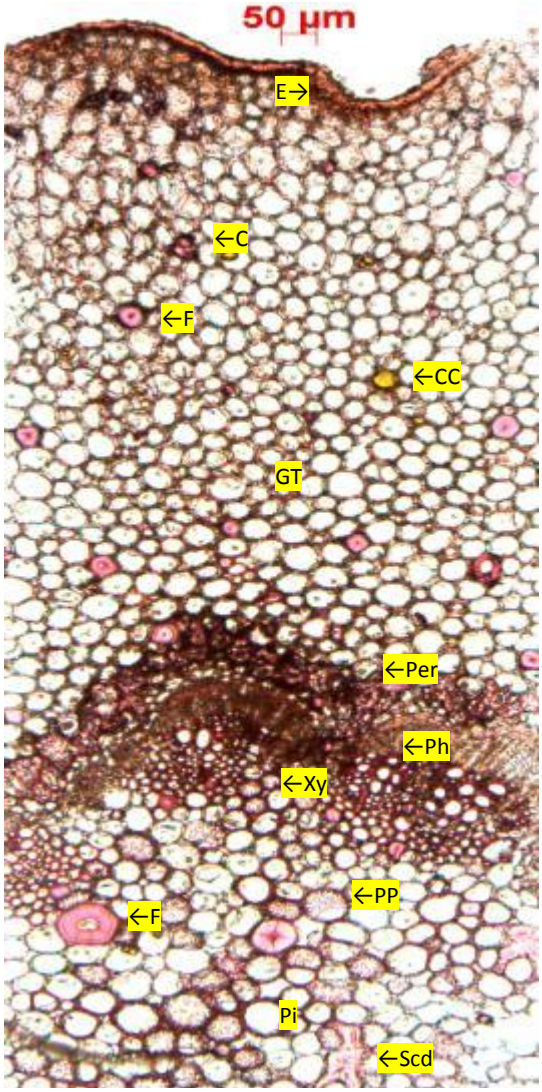


Fig. 2.1 TS of stalk

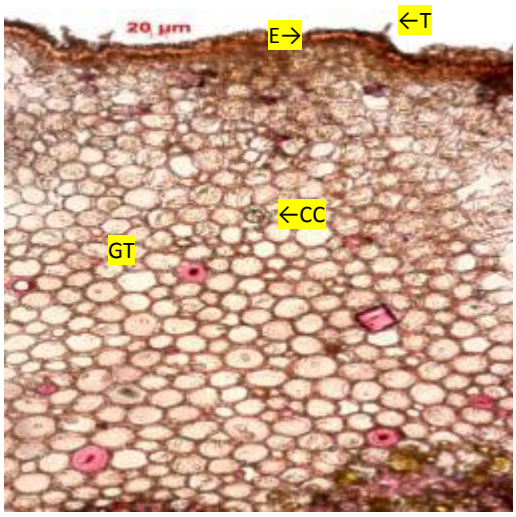


Fig. 2.2 Outer region enlarged

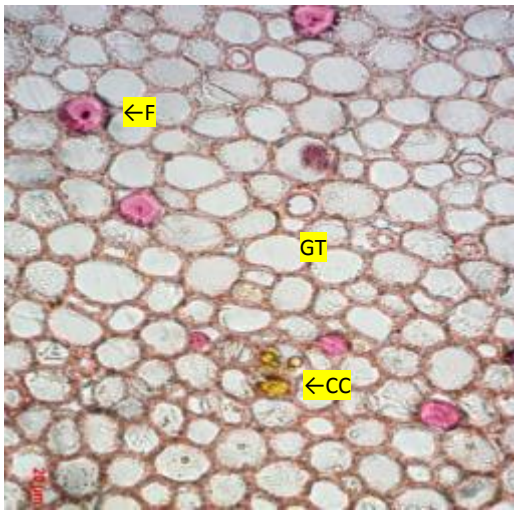


Fig. 2.3 Cortex

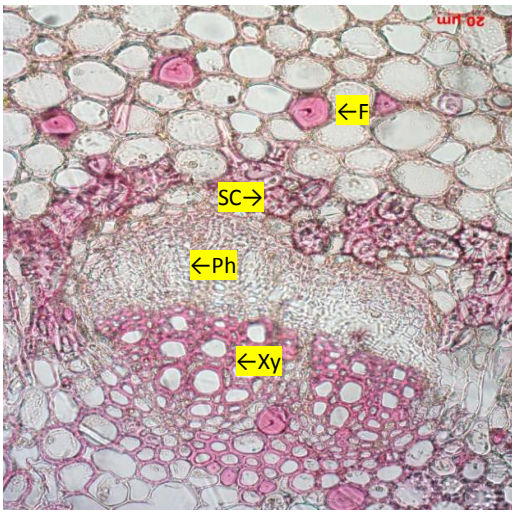


Fig. 2.4 Vascular bundle

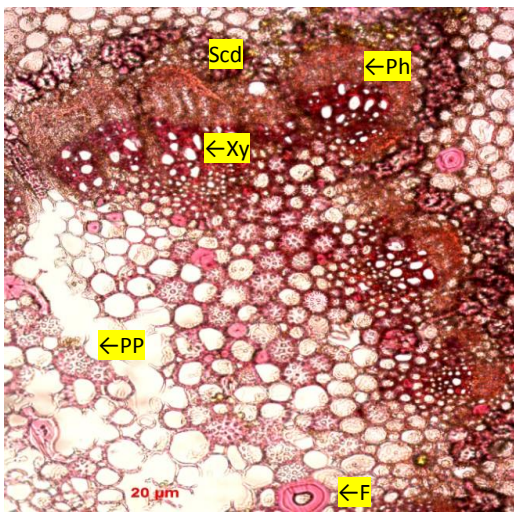


Fig. 2.5 Xylem and pith

E – epidermis; F – fibre; CC – cell content ; Ck – cork; Ct – cortex; GT – ground tissue; Per – pericycle; Ph – phloem; Pi – pith; PP – pitted parenchyma; Scd – sclereid; Xy – xylem; T – trichome.

Figure 2: Microscopy of stalk of strobilus of *Gnetum ula*

Strobilus

TS through stobilus (naked seed) shows four distinct layers which in turn envelopes the embryo and the endosperm. The outmost layer is thick-walled epidermis which has groups of thick-walled porous stone cells beneath it in discontinuous patches; majority of the tissue is formed by thick-walled parenchyma which has lot of simple starch granules and encloses some thick-walled narrow to broad lumened fibres; the third regions is hard and made up of compactly arranged porous stone cells; followed by stone cells region there are elongated palisade like columnar sclereids which forms the innermost region of the naked seed which is similar to cells found in the testa of other seeds; the embryo has prominently cotyledon, the TS through that shows large polygonal cells filled with lot of aleurone and starch grains (Figure 3).

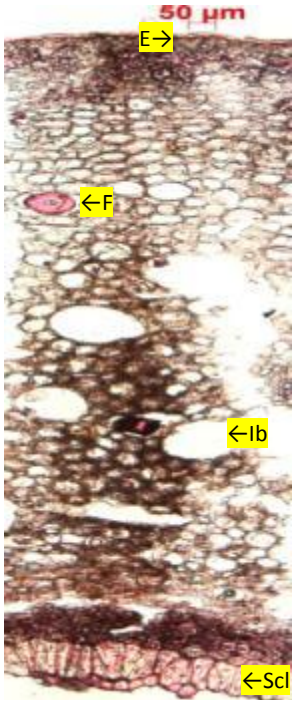


Fig. 3.1 TS of false seed coat

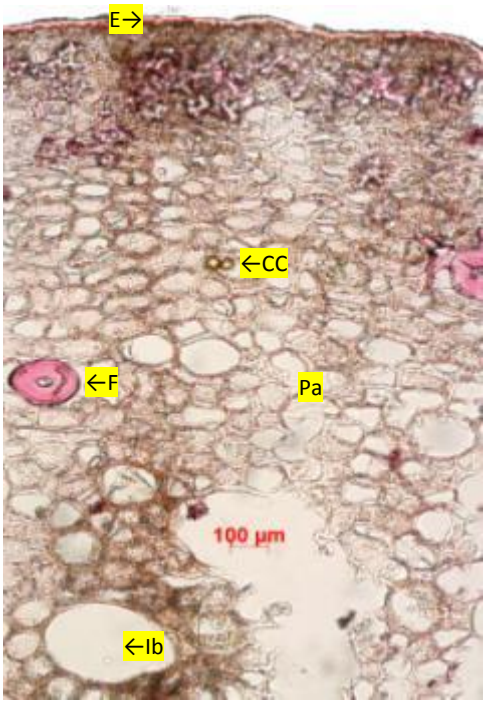


Fig. 3.2 Outer portion of false seed coat

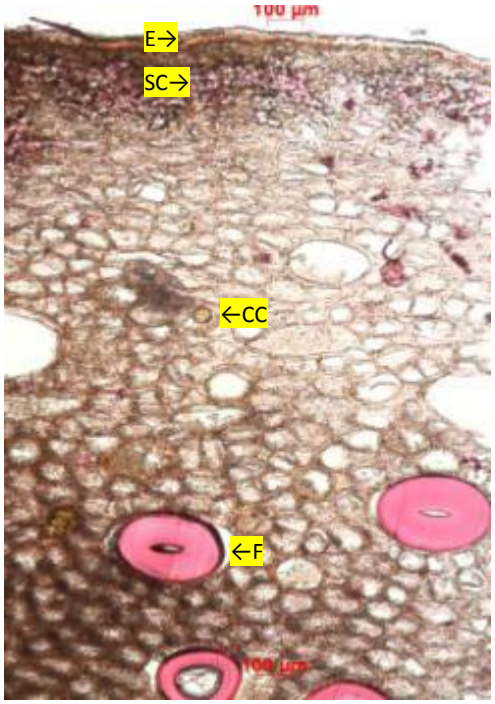


Fig. 3.3 Outer portion of false seed coat enlarged

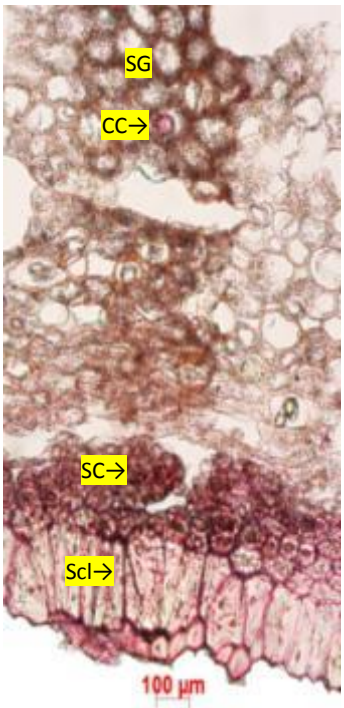


Fig. 3.4 Inner portion of false seed coat enlarged

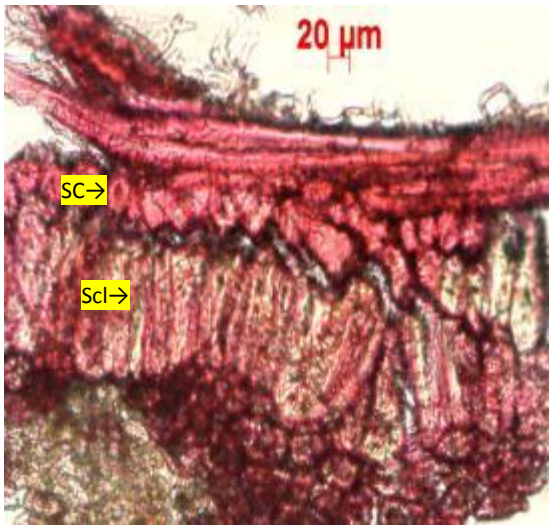


Fig. 3.5 Inner testa

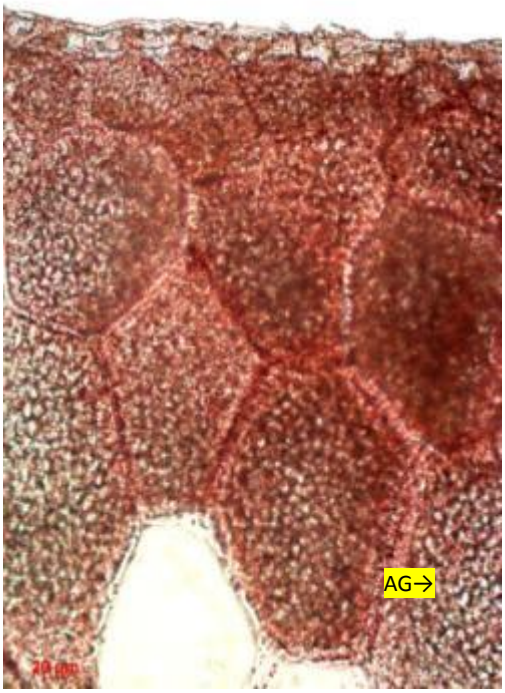


Fig. 3.6 TS of cotyledon

CC – content cell; E – epidermis; F – fibre; Ib – idioblast; Pa – parenchyma; SC – stone cell; Scl – sclereid; SG – starch grains; AG – aleurone grains

Figure 3: Microscopy of strobilus of *Gnetum ula*

Physico-chemical analysis

G. ula roasted seed powder was tested for loss on drying at 105°C, total ash, acid insoluble ash, ethanol and water soluble extractive as per standard protocol. Total ash was 1.29% w/w, loss on drying was 17.51% w/w, acid insoluble ash nil, water soluble ash 0.99% w/w, ethanol soluble extractive value 2.88 % w/w and water soluble extractive value 4.70% w/w. These parameters would help to determine the quality and purity of the drug (Table 1).

Phytochemical study

The preliminary phytochemical studies are essential to know the basic constituents present in the drug. Action of any drug depends upon these basic components. Preliminary phytochemical test were conducted for *G. ula* seed water extract (GU) and roasted *G. ula* seed water extract (GUR). Test for alkaloids (Dragendrof's test, Wagners's test, Mayer's test and Hager's test), carbohydrates (Molisch's test, Fehling's test and Benedict's), steroids (Libermann-Burchard and Salkowski), saponins, phenol, coumarin, triterpenoids, quinone, resin and tannins showed positive in both GU and GUR (Table 2 and 3).

Table 1: Physico-chemical parameters of seeds of *Gnetum ula*

Parameter	Results n = 3 %w/w
Loss on drying	17.51
Total Ash	1.29
Acid Insoluble Ash	0.0
Water soluble Ash	0.99
Alcohol soluble extractive value	2.88
Water soluble extractive value	4.70

Table 2: Preliminary phytochemical tests of aqueous extracts of seed of *Gnetum ula*

Tests	Colour if positive	*G.ula*	*G.ula* (Roasted)
Alkaloids			
Dragendrof's test	Orange precipitate	Orange precipitate	Orange precipitate
Wagners test	Red precipitate	Red precipitate	Red precipitate
Mayers test	Dull white precipitate	Dull white precipitate	Dull white precipitate
Hagers test	Yellow precipitate	Yellow precipitate	Yellow precipitate
Steroids			
Liebermann- buchard test	Bluish green	Brownish ring	Brownish ring
Salkowski test	Bluish red to cherry red color in chloroform layer and green fluorescence in acid layer	No bluish red to cherry red color in chloroform layer and green fluorescence in acid layer	No bluish red to cherry red color in chloroform layer and green fluorescence in acid layer
Carbohydrate			
Molish test	Violet ring	Violet ring	Violet ring
Fehlings test	Brick red precipitate	Brick red precipitate	Brick red precipitate
Benedicts test	Red precipitate	Red precipitate	Red precipitate
Tannin			
With $FeCl_3$	Dark blue or green or brown	Dark green	Dark green
Flavanoids			
Shinoda's test	Red or pink	Yellow color	Yellow color
Saponins			
With $NaHCO_3$	Stable froth	Stable froth	Stable froth
Triterpenoids			
Tin and thionyl chloride test	Pink	Pink	Pink
Coumarins			
With 2 N NaOH	Yellow	Yellow	Yellow
Phenols			
With alcoholic ferric chloride	Blue to blue black, brown	Brown color	Brown color
Carboxylic acid			
With water and $NaHCO_3$	Brisk effervescence	No effervescence	No effervescence
Resin			
With aqueous acetone	Turbidity	Turbidity	Turbidity
Quinone			
5% NaOH	Pink/purple/red	Red	Red color

HPTLC

HPTLC finger print profile of ethanol extract of *G.ula* has been obtained with suitable solvent system. The developed plates were visualized under UV light and white and then under light after derivatisation with vanillin sulphuric acid reagent. R_f, colour of the spots and densitometric scan at 254 and 366 nm were recorded. On photo documentation there was a single spot under short UV, 4 spots under long UV and 3 spots under white light post derivatisation. Densitometric scan at 254 nm showed 4 peaks at R_f 0.03 (74.45%), 0.10 (13.40%), 0.52 (1.77%) and 0.74 (10.37%). There were 3 peak at 366 nm with R_f 0.04 (96.74%), 0.19 (1.43%) and 0.26 (1.82%) (Table 4 and Figure 4).

Table 3: Inference of preliminary phytochemical screening of aqueous extracts of seed of *Gnetum ula*

Test	Water extract	
	G. ula	***G. ula* (Roasted)**
Alkaloid	+	+
Steroid	-	-
Carbohydrate	+	+
Tannin	+	+
Flavanoids	-	-
Saponins	+	+
Terpenoid	+	+
Coumarins	+	+
Phenol	+	+
Carboxylic acid	-	-
Resins	+	+
Quinone	+	+

Table 4: Rf values of ethanolic extracts of seed of *Gnetum ula*

At 254nm	At 366nm	Post derivatisation
-	0.32 (F. blue)	0.32 (D. purple)
-	-	0.35 (D. purple)
-	0.45 (FL. blue)	-
-	-	0.47 (D. purple)
-	0.63 (F aqua. blue)	-
0.68 (L. green)	-	-
-	0.89 (FL. blue)	-

L- light; D – dark; F- fluorescent

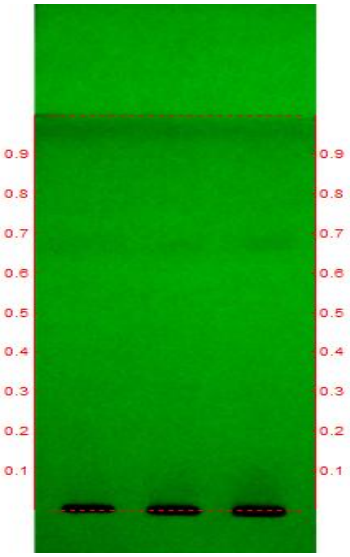


Fig. 4.1 Under short UV

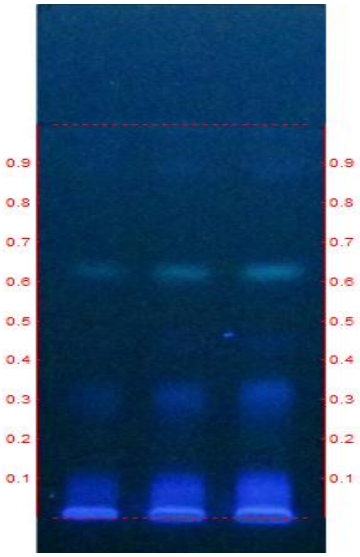


Fig. 4.2 Under long UV

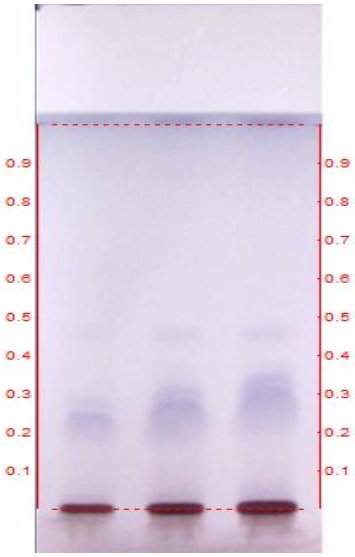
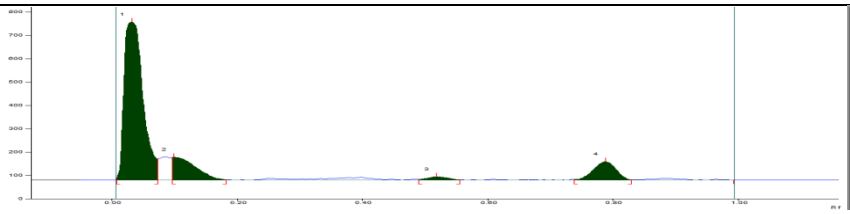


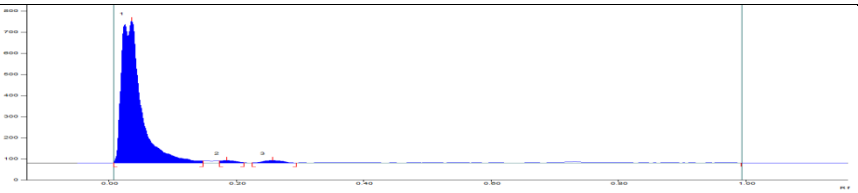
Fig. 4.3 Under white light post derivatisation

Track 1: *G. ula*-4µl, Track 2: *G. ula*-8µl, Track 3: *G. ula*-12µl



Peak	Max Position	Area %
1	0.03 Rf	74.45 %
2	0.10 Rf	13.40 %
3	0.52 Rf	1.77 %
4	0.79 Rf	10.37 %

Fig. 4.4 Densitometric scan of the sample at 254nm (12 µl)



Peak	Max Position	Area %
1	0.04 Rf	96.74 %
2	0.19 Rf	1.43 %
3	0.26 Rf	1.82 %

Fig. 4.5 Densitometric scan of the sample at 366nm (12 µl)

Solvent system- Toluene: Ethyl actetate (8.0:2.0)

Figure 4: HPTLC of ethanolic extract of seed of *Gnetum ula*

DISCUSSION

The macroscopic features recorded can be used for preliminary identification of the particular plant. In many of studies reported earlier, the macro-microscopic studies have been proved to be effective in establishing the authenticity and detection of adulterants/substitutes for herbal raw drugs.[17,18] *G.ula* is a Gymnosperm, the false fruit or strobilus contained four layers. Microscopic features revealed outer layer of epidermis containing fibres groups cells and many layers of parenchyma, the innermost layers contained continuous layers of small sized stone cells, most of the parenchyma contains starch grains. Middle sclerotesta which contained continuous rows of thick walled stone cells pith pits arranged as palisade cells. And inner cotyledon contained of large parenchyma cells containing aleurone grains and starch. The characters were comparable to microscopic structures of dicotyledonous seeds, particularly the sclerotesta part.

Any matter other than the described parts of the drug is to be considered as foreign matter, any raw drug must be made free from foreign matter before any physico- chemical analysis is done. Total ash indicative of the total inorganic composition of the drug was found to be 1.29% w/w, acid insoluble ash indicating the silicious matters was found to be nil for the test sample which is rare phenomena in the ash composition of plants, water soluble ash indicating the ash which is readily soluble in water was found to be 0.99% w/w. Loss on drying indicates the moisture and volatile matter content in sample and it was 17.51% w/w. The solvent used for the extraction is in a position to dissolve appreciable quantities of substances likewise various solvents are used to extract these chemical constituents. The extract obtained by percolating coarse powder is indicative of approximate quantity of their chemical constituents. Ethanol soluble extractive value of the test sample was found to be 2.88 % w/w and the water solubility was 4.70% w/w. All these pharmacopoeial parameter helps to determine the quality and purity of herbal drugs. Preliminary phytochemical tests were conducted by using the water extracts of raw and roasted seeds of *G.ula* for comparison of any phytochemical changes on rasting. Both the samples were found to be positive for alkaloids, carbohydrates, saponins, tannins, phenol, coumarins, resin, quinone and triterpenoids in aqueous extract. These preliminary analyses of chemical composition are one of the preliminary methods to analyze chemistry of herbs. HPTLC photo documentation revealed presence of phyto constituents with different R_f values. Densitometric scan of the plates showed diagnostic bands under 254 nm, 366 nm and post derivatisation. HPTLC fingerprinting is an effective technique of screening herbal raw drugs for authenticity and quality.[19, 20]

CONCLUSION

Ayurveda *Acharyas* have opined to make use of the drug found in the vicinity but after thorough examination before incorporating in medicine. Folklore medicine has tremendous source of information regarding the utility of locally available plants for use as food or medicines. Such plants have to be properly explored and scientifically documented before putting it in use. Use of Gymnosperm in Ayurveda is very rare. *Gnetum ula* Brongn locally called as *Kumti beeja* of family Gnetaceae is not considered as a source for any classical Ayurvedic drug. There are many numbers of valuable plants which has to be explored to include in Ayurvedic Pharmacopieia. These less explored plants need a systematic and scientific documentation. The current study has evolved standards for one of extra pharmacopoeial drug which is in verge of extinction.

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Conflicts of Interest: Nil

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