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Pharmacognosy and quality characterization of *Balanites aegyptiaca* (L.) Delile fruits

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Fruits of *Balanites aegyptiaca* (L.) Delile syn. *B. roxburghii* Planch (Family Balanitaceae) is considered as source of *Ingudi* of Ayurveda. It is a thorny tree with ovoid pulpy drupe type of fruit. In Ayurveda, *Ingudi* bark, leaf and fruits are said to be beneficial as blood purifier, diuretic, in leprosy, poisoning, ulcers, worm infestation, leucoderma, anorexia and constipation. Seed extract is hypotensive and the seed oil is used for burns and freckles. The unripe fruits are cathartic and the ripe ones are used in whooping cough and skin troubles. The fruits are also reported to possess antihperglycemic activity. In the present study, systematic pharmacognostical evaluation of ripe fruits has been carried out as per standard methodologies used for drug standardisation. Macroscopical, microscopical and physico-chemical features of the fruit have been documented. Preliminary phytochemical investigations indicated presence of carbohydrates, coumarins, triterpenoids and saponins. HPTLC fingerprint profile has been developed for methanol extract of pulp using diosgenin as standard. The result obtained from standardization of fruit established set diagnostic tests to authenticate genuine fruits. These parameters can be utilized for rapid identification of the drug as whole and as powder.

Keywords: *Balanites aegyptiaca* (L.) Delile, Diosgenin, Fruit pulp, HPTLC, Macroscopy, Microscopy, Physico-chemical, Quality standards.

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Introduction

Balanites aegyptiaca (L.) Delile is a small spiny evergreen tree native to much of Africa and parts of the Middle East with glabrous or puberulous branches bearing very strong sharp ascending spines and bifoliate leaves of two elliptical or obovate, coracious leaflets. It is distributed in drier parts of Western Rajasthan and peninsular India from South East Punjab to West Bengal and Sikkim. The fruits are used as drastic purgative[1], for wound abscesses[2], in pneumonia[3], skin diseases[4], as antidiabetic[5,6] and anthelmintic as well as purgative[7]. The seeds are used in cough[1,7] and in colic[1,8]. The seed oil is used in skin diseases[9]; for healing of burns, ulcers and as wormicidal[1,7]. The plant is used as an alternative source of diosgenin, which is generally obtained from various *Dioscorea* sp. and is an important raw material used in the synthesis of steroidal drugs. Most of the diosgenin in *B. aegyptiaca* is present as saponin which on acidic hydrolysis yields diosgenin[10]. A saponin with glucose, xylose and rhamnose as sugars was obtained from the seed kernel and named balanitisin[11]. A pregnane glycoside and furostanol saponin was also reported from the fruits of *B. aegyptiaca*[12,13]. Different yields of diosgenin have been obtained by acid hydrolysis of saponins isolated from pericarp[14,15]; epicarp (0.15 %), fruit pulp (0.32 %), seed kernel (0.6 %)[8]; seed oil (0.45 %) and defatted seeds (0.4 %)[15]. A mixture of sapogenins, diosgenin and yamogenin was isolated in varying yields and ratio from different plant parts[16]. The *n*-hexane extract of kernel yielded an oil rich fraction and fatty acids present were identified as palmitic, stearic, oleic and linoleic acids[17]. The seed oil showed amino acids and *β*-sitosterol[18].

Aqueous extract of the fruit exhibited spermicidal activity[19]. The aqueous solution of the ethanolic extract of fruit pulp (pericarp) produced triphasic response on blood pressure of anaesthetized dogs and cats[20]. The aqueous alcoholic extract of seed kernel produced non specific cardiac depression, increased tone and motility of rabbit intestine and increased oesophageal ciliary movements in frogs[21]. Saponin from fruit exhibited no cardiovascular activity in dogs or haemolytic activity in human blood cells[22]. The oil

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and unsaponifiable matter showed antibacterial activity against *Escherichia coli*, *Bacillus anthracis* and many other micro-organisms[23]. Daya *et al*[24] reviewed phytochemical constituents, traditional uses, and pharmacological activity of *B. aegyptiaca*. Pharmacognostical and phytochemical studies of stem bark[25], leaf[26] and nutritional value of fruit[27] pulp have been studied earlier but there is no report on pharmacognostical features of fruit yet, though this medicinal drug is an important commodity in herbal therapeutics. To authenticate and standardize the crude drug material and to avoid possible substitution and adulteration of *Ingudi*, a set of quality specifications are proposed in the present study.

Materials and Methods

Plant material

Fruit of *B. aegyptiaca* was collected from local crude drug market. It was identified and authenticated by comparison with the botanical description mentioned in Floras[28,29]. A voucher specimen (No. 74.12020201) was deposited in the Pharmacognosy Department of SDM Centre for Research in Ayurveda and Allied Sceinces, Udupi. The whole fruit was used for the study of macroscopic and microscopic characters. Pulp from shade-dried fruits was used for physico-chemical analysis, extraction, phytochemical study and HPTLC fingerprinting.

Macro and microscopical study

The fruits were examined for macroscopy as per standard procedures. Transverse section of fruit pericarp was taken using blade, stained as per standard methodology[30] and then examined microscopically. Photomicrographs of the microscopical sections were captured with the help of Zeiss Axio Lab.A1 microscope fitted with Ziess Axio CamERc5s provided with Zeiss Axio Vision software.

Physico-chemical and preliminary phytochemical analysis

Percentage of ash (total, acid-insoluble and water soluble), extractive values (alcohol and water soluble), loss on drying and foaming index were estimated as per standard procedure[31,32]. Preliminary phytochemical tests were performed as per standard methods using methanol extracts to detect different phytoconstituents[33].

For determination of foaming index, 1 g of extract taken in a 150 mL conical flask was dissolved in 100 mL distilled water by shaking and gentle warming. It was then filtered in 100 mL volumetric flask and volume was made up to the mark with distilled water. In 10 stoppered test tubes (height 16 cm, diameter 16 mm), the solution was taken in successive portion of 1, 2, 3, up to 10 mL. The volume of the solution in each test tube was adjusted with distilled water to 10 ml. The tubes were corked and shaken in a lengthwise motion for 15 seconds, two shakes per second was maintained in each tube. The test tubes were allowed to stand for 15 minutes and height of the foam was measured.

Foaming index of a solution = 1000/a

where a = volume (in mL) of the solution used for preparation of dilution in the tube where foaming to a height of 1 cm is observed[34].

High performance thin layer chromatography

Pulp (1 g) was extracted with 10 mL methanol 2 times by cold percolation for 48 h. Methanolic extracts (20 µL) were applied on a pre-coated silica gel F_{254} on aluminum plates to a band width of 8 mm at speed of 5 sec/µL using Linomat 5 TLC applicator CAMAG Muttenz Switzerland. The plate was developed in chloroform : methanol (5 : 1) in CAMAG twin trough chamber to a height of 8 cm and the developed plates were visualized under short UV, long UV and white light. The plates were scanned under 254 nm using deuterium lamp, 366 nm using mercury vapour lamp and 620 nm using tungsten lamp after derivatisation. The derivatisation was done by dipping the plate vanillin-sulphuric acid reagent followed by heating in air circulated oven at 105 °C till the development of colour of the spots. R_f, colour of the spots and densitometric scan were recorded.

Pulp (10 g) was refluxed with 25 mL of 30 % HCl for 2 h. The hydrolysed product was cooled, filtered and the residue was extracted by refluxing with chloroform (15 mL x 3) on a water bath. Chloroform extracts were combined and dried under vacuum. Residue (10 mg) was dissolved in 10 mL of chloroform. HPTLC of the extract was carried out using toluene : acetone : formic acid (7 : 1 : 1) as mobile phase and the R_f values were recorded. Both chloroform and total methanol extract was fingerprinted using silica gel G 60 F_{254} (Merck) of 0.2 mm thickness as absorbent and the R_f values were determined and scanned under 254, 366, 540 and 620 nm[35,36].

Results and Discussion

Macroscopic study

Dried drupes are woody, ovoid, oblong to globular in shape, 5 to 6 cm long and 3 to 4 cm wide, surface smooth, granular to faintly transversely wrinkled, show 5 to 10 longitudinally running grooves from base to apex, a rim of calyx and stout pedicel is attached at the base or a scar left by its removal; apex flat or slightly depressed, outermost pericarp highly brittle, easily fragmented, on slight shaking the inner loosely placed hard ball of endocarp containing seed, moves easily inside the cavity of the pericarp making noise. The exposed inner surface of the pericarp devoid of stony endocarp is dark yellow in colour, granular and exhibits converging longitudinally running ridges (Plate 1a). Fracture hard, stony irregular. Odour is unpleasant, nauseating and bitter in taste. Macroscopic characters are the quickest means of identifying a plant drug under naked eye though an experienced eye can do it exactly. In a previous study, it has been shown that there is a significant difference in fruit morphology based on source of the plant which in turn is related to type of soil. Among geographical sources, there were no significant differences in the weight of seed kernel, but epicarp and endocarp did[37].

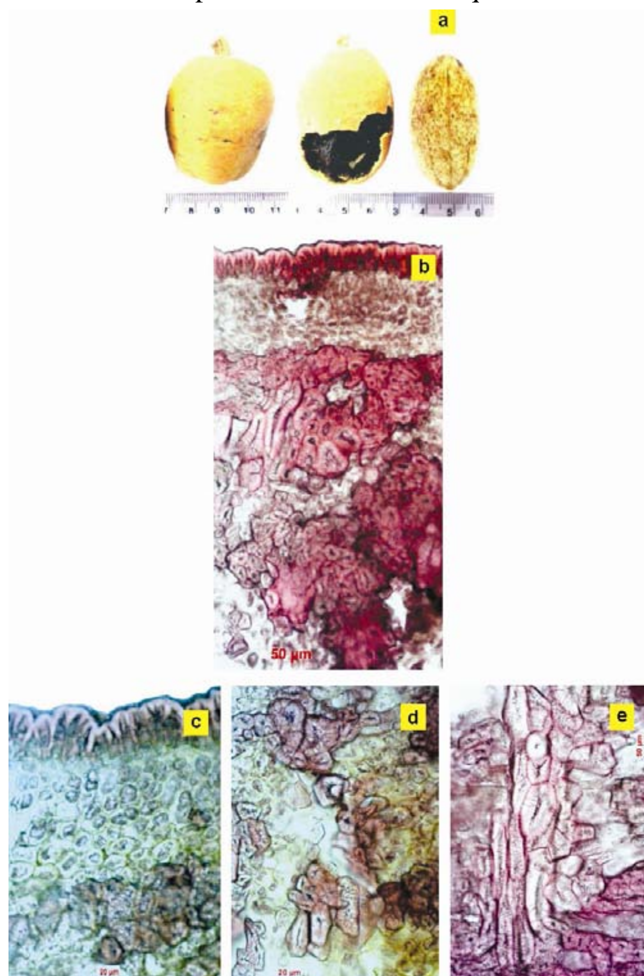


Plate 1—Macro- and micro-scopic characters of *Balanites aegyptiaca* fruit. a) Macroscopy of fruit, pulp and seed, b) Microscopy of fruit pericarp, c) Pericarp – Outer portion enlarged, d) Pericarp - Inner portion enlarged and e) Stone cells

Microscopic study

Longitudinal section of fruit is elliptical in outline showing outermost yellowish colored stony pericarp followed by brown colored gummy mesocarp (pulp), inner to it there is a wide stony multilayered endocarp enclosing thin papery white seed coat and large yellowish white cotyledons.

Enlarged portion of transverse section of fruit shows a layer of epicarp with thickened radial walled cells, having thin cuticle, embedding yellowish brown contents. The pulp is wide zone consisting of multiple rows of tangentially elongated thin walled parenchymatous cells traversed with few vascular bundles. Endocarp is wide, stony, traversed with groups of stone cells and sclereids of various sizes and thickness, the outermost 15 to 20 rows are occupied by thick and thin walled pitted squarish to triangular to rectangular stone cells. Underneath this lies radially elongated thin walled pitted sclereids embedded with groups of highly thickened, narrow lumened, small sized stone cells and fibers. The innermost zone of sclereids running tangentially and radially, embedded with small groups of stone cells, is very wide. Testa is stony, multilayered consisting of various types of stone cells and sclereids, the outermost 2 to 4 layers being occupied by tangentially elongated, spindle- shaped sclereids followed by few rows of small sized, thick walled stone cells and the innermost cells filled with pigments (Plate 1b-e). Isolated elements showed epicarp cells in surface view showing thick-walled elongated, narrow, radially arranged rectangular cells; parenchyma of different sizes and shapes containing colored contents; thin walled simple trichome; abundant sclereids, stone cells and fibers of various sizes, shapes and thickness; longitudinally cut fragments of small reticulate vessels and polygonal parenchymatous cells of cotyledon containing aleurone grains and oil globules (Plate 2a-p). Microscopic identification of drugs is performed for further confirmation of its botanical source under microscope. These features

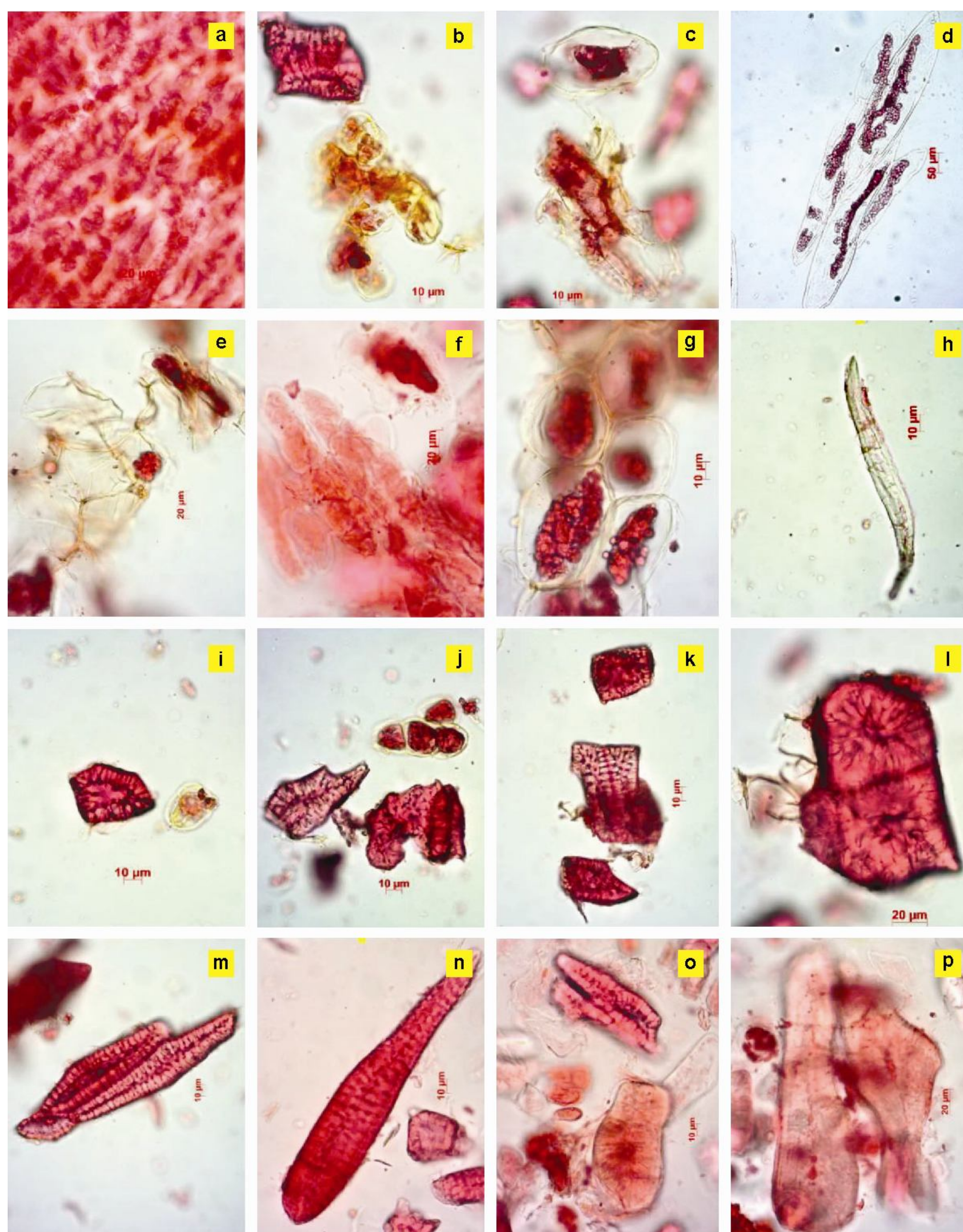


Plate 2—Microscopic characters of elements isolated from *Balanites aegyptiaca* fruit. a) Epicarp cells in surface view, b-g) Parenchyma of mesocarp with contents, h) Simple trichome, i-l) Stone cells and m-p) Sclereids of different size and shape.

are fingerprints of the botanical source for an experienced microscopist. Most of herbs have been standardized for their microscopic features; these results would aid in authentication of this botanical drug.

Physico-chemical parameters

The physico-chemical parameters such as ash value, acid insoluble ash, water soluble ash, loss on drying, total alcohol, water soluble extractive values and foaming index of *B. aegyptiaca* pulp are

presented in Table 1. This is an important observation towards quality affirmation of many botanical drugs and is part of every herbal monograph derivation. The parameters included under the physico-chemical characterization directly or indirectly project the standards of a drug. When compared to an earlier study[26] on leaves of *B. aegyptiaca*, total ash is found to be higher (12.174 %) while acid insoluble ash is found to be lesser (0.65 %); leaf is found to contain more of saponin (foaming index value of 500). The preliminary phytochemical investigation of the alcohol extract showed presence of carbohydrates, phytosterols, saponins, triterpenoids, amino acids and coumarins (Table 2). The medicinal activity of an herbal drug is due to the presence of these metabolites specifically related to the phylogenetic relationship of the plant. Preliminary examinations indicating presence of certain phytochemical moieties are helpful in proposing probable action of the plant drug.

High performance thin layer chromatography (HPTLC)

HPTLC fingerprinting revealed presence of various phytoconstituents with their respective R_f values and the photo documentation of the plates showed numerous bands under long UV and after derivatisation. Vanillin-sulphuric acid reagent was used as visualizing agent to effect visualization of the resolved spots. Total methanolic extract was finger printed using chloroform : methanol (5 : 1) as solvent system. Under short and long UV there were 7 and 11 spots, respectively (Plate 3 a-b); and after derivatisation with vanillin : sulphuric acid, 9 spots (Plate 3c) were observed (Table 3).

HPTLC fingerprinting of chloroform soluble fraction (T1), diosgenin (marker compound) (T2) and methanol extract (T3) was performed (Table 4). On photo documentation under long UV (Plate 4a), 1 spot was observed at R_f 0.27 (Fluorescent blue) in chloroform extract, no spots were observed in diosgenin track and in methanolic extract 4 spots were observed at R_f of 0.08 (fluorescent blue), 0.15 (fluorescent blue), 0.59 and 0.67 (fluorescent red). After derivatisation with vanillin/sulphuric acid (Plate 4b) chloroform extract showed 10 spots, diosgenin track showed a single spot at R_f 0.53 (green) and methanolic extract showed 10 spots. Densitometric scan of chloroform soluble fraction at 366 nm (Plate 4c) showed 2 peaks with R_f of 0.04 (67.20 %), 0.29 (32.80 %); methanolic extract (Plate 4d) showed 7 peaks with R_f 0.05 (76.92 %), 0.13 (2.73 %), 0.18 (2.63 %), 0.56 (1.61 %), 0.61 (10.68 %), 0.69 (4.09 %), 0.85 (1.33 %) and no peaks were detected in diosgenin track. Densitometric scan at 620 nm showed 11 peaks in chloroform soluble fraction (Plate 5a) at R_f of 0.04 (6.34 %), 0.08 (11.53 %), 0.12 (4.02 %), 0.16 (21.61 %), 0.24 (5.32 %), 0.28 (6.92 %), 0.40 (2.12 %),

Table 1—Physico-chemical parameters of *Balanites aegyptiaca* fruit

Parameter	% w/w (Mean±SEM)
Total ash	9.94±0.06
Acid insoluble ash	2.21±0.15
Alcohol soluble extractive	30.5±0.30
Water soluble extractive	88.6±0.05
Foaming index	333.33

Table 2—Preliminary phytochemical analysis of methanolic extract of *Balanites aegyptiaca* fruit

Tests	Alcohol extract	Inference
Alkaloid		
Dragendorff's test	Yellow turbidity	
Wagner's test	Reddish brown, no precipitate	-
Mayer's test	Reddish brown, no precipitate	
Hager's test	Reddish brown, no precipitate	
Amino acid		
Ninhydrine test	Purple color	+
Carbohydrate		
Molisch's test	Violet ring	
Fehling's test	Brick red precipitate	
Benedict's test	Red precipitate	+
Anthrone-sulphuric acid test	Red	
Steroids		
Liebermann-Buchard test	Bluish green	+
Salkowski test	Bluish red to cherry red	
Saponins		
On shaking with water	Stable froth	+
Tannins		
with $FeCl_3$	Yellowish brown color	-
Flavonoids		
Shinoda's test	Brown color	-
Phenol (ferric chloride test)	Brown color	-
Coumarins (with 2N NaOH)	Dark yellow	+
Triterpenoids		
Tin and thionyl chloride test	Pink	
Liebermann-Burchard test	Brown color	+

Table 3—R_f value of TLC of methanolic extract of *Balanites aegyptiaca* fruit

Short UV	Long UV	Post – derivatisation with VS
0.04 (L Green)	-	-
0.08 (L Green)	0.08F (M Blue)	-
-	-	0.11 (L Brown)
0.14 (L Green)	0.14 (F L Blue)	-
-	-	0.20 (L Brown)
0.24 (L Green)		-
-	0.27 (F L Blue)	-
-	0.32 (F L Blue)	0.32 (L Brown)
0.36 (Green)	-	-
-	0.42 (F L Blue)	0.42 (L Blue)
0.52 (D Green)	-	0.52 (L Blue)
-	-	0.59 (L Blue)
-	0.62 (F Blue)	-
-	0.70 (F Blue)	0.70 (L Blue)
-	0.78 (F Blue)	-
0.80 (L Green)	-	0.80 (Violet)
-	0.83 (F Blue)	-
-	0.87 (F Blue)	-
-	0.95 (F Pink)	0.95 (Blue)

F-Flourescent, L-Light, D-Dark, M-Medium, VS Vanillin/ sulphuric acid spray reagent

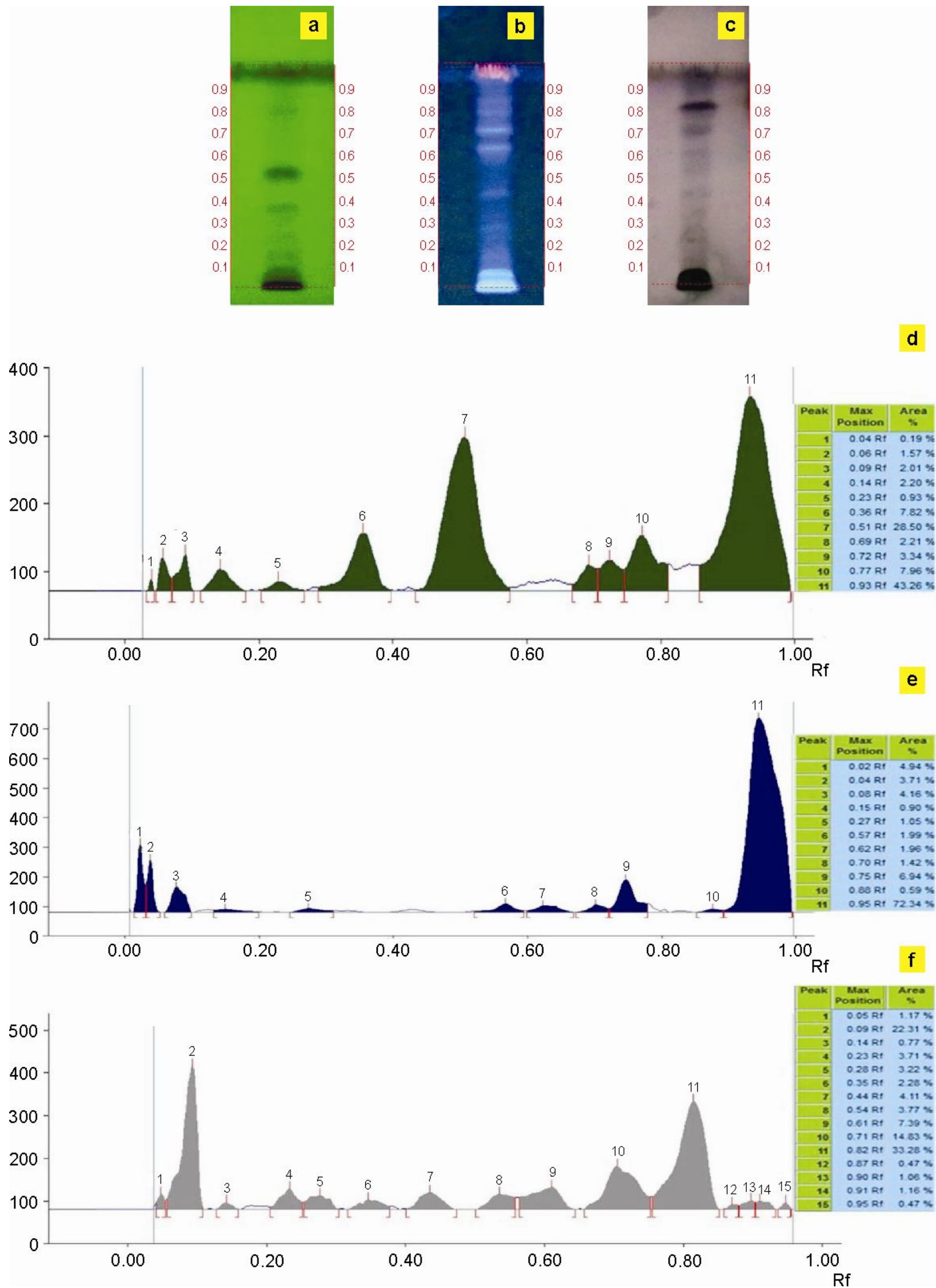
0.49 (24.64 %), 0.57 (2.26 %), 0.62 (7.85 %), 0.86 (7.39 %) with diosgenin peak evident at R_f of 0.48 (100.00 %) (Plate 5b); in methanolic extract (Plate 5c) there was no corresponding peak observed at R_f of 0.48, 10 other peaks found were at R_f of 0.04 (14.22 %), 0.11 (1.03 %), 0.14 (4.56 %), 0.23 (0.97 %), 0.29 (4.49 %), 0.41 (5.21 %), 0.55 (5.36 %), 0.61 (49.96 %) and 0.87(13.24 %). Three dimensional superimposition of densitograms of chloroform soluble fraction (T1), diosgenin (T2) and methanolic extract (T3) at 620 nm after derivatisation with vanillin/sulphuric acid spray reagent (Plate 5d) showed presence of diosgenin in soluble fraction. HPTLC with marker compound is an identity test proposed in most of standardization documents for herbal drugs. As using HPTLC is simple, cost effective and useful in rapid detection of herbal drugs, this chromatographic technique is more in use as identity test unlike other chromatographies. Standardisation of fingerprint for *Ingudi* has been performed in this study for quick identity and quality control.

The pharmacognostical studies are the first and foremost study required for every herbal drug before further studies are taken. This primary analysis, though appears simple but play an important role in herbal drug research[38,39].

Table 4—TLC (R_f value) of chloroform soluble fraction and methanolic extract of *Balanites aegyptiaca* fruit

Long UV			Post – derivatisation with VS		
T1	T2	T3	T1	T2	T3
-	-	-	0.03 (Green)	-	-
-	-	0.08 (F L Blue)	0.08 (Green)	-	-
-	-	-	0.10 (Green)	-	0.10 (Green)
-	-	-	0.12 (Green)	-	-
-	-	0.15 (F L Blue)	-	-	0.15 L (Green)
-	-		0.17 (Green)	-	0.17 L (Green)
-	-	-	-	-	0.20 L (Green)
-	-	-	-	-	0.22 L (Green)
-	-	-	0.24 (Green)	-	-
0.27 (F M Blue)	-	-	0.27 (F M Blue)	-	-
-	-	-	-	-	0.29 L (Green)
-	-	-	-	-	0.43 L (Green)
-	-	-	0.53 (Green)	0.53 (Green)	-
-	-	0.59 (F Red)	-	-	-
-	-	-	-	-	0.60 L (Green)
	-	-	0.65 (L Green)		0.65 (Green)
-	-	0.67 (F Red)	-	-	-
-	-	-	0.95 (Violet)	-	0.95 (Violet)

F-Flourescent, L-Light, D-Dark, M-Medium, T1-Chloroform soluble fraction, T2-Diosgenin (marker), T3-Methanolic extract, VS-Vanillin/sulphuric acid spray reagent



Peak	Max Position	Area %
1	0.04 Rf	0.19 %
2	0.06 Rf	1.57 %
3	0.09 Rf	2.01 %
4	0.14 Rf	2.20 %
5	0.23 Rf	0.93 %
6	0.36 Rf	7.82 %
7	0.51 Rf	28.50 %
8	0.69 Rf	2.21 %
9	0.72 Rf	3.34 %
10	0.77 Rf	7.96 %
11	0.93 Rf	43.26 %

Peak	Max Position	Area %
1	0.02 Rf	4.94 %
2	0.04 Rf	3.71 %
3	0.08 Rf	4.16 %
4	0.15 Rf	0.90 %
5	0.27 Rf	1.05 %
6	0.57 Rf	1.99 %
7	0.62 Rf	1.96 %
8	0.70 Rf	1.42 %
9	0.75 Rf	6.94 %
10	0.88 Rf	0.59 %
11	0.95 Rf	72.34 %

Peak	Max Position	Area %
1	0.05 Rf	1.17 %
2	0.09 Rf	22.31 %
3	0.14 Rf	0.77 %
4	0.23 Rf	3.71 %
5	0.28 Rf	3.22 %
6	0.35 Rf	2.28 %
7	0.44 Rf	4.11 %
8	0.54 Rf	3.77 %
9	0.61 Rf	7.39 %
10	0.71 Rf	14.83 %
11	0.82 Rf	33.28 %
12	0.87 Rf	0.47 %
13	0.90 Rf	1.06 %
14	0.91 Rf	1.16 %
15	0.95 Rf	0.47 %

Plate 3—HPTLC fingerprinting of total methanol extract of *Balanites aegyptiaca* fruit. a) Photo-documentation under short UV, b) Photo-documentation under long UV, c) Photo-documentation post derivatisation with vanillin/sulphuric acid, d) densitometric scan at 254 nm, e) Densitometric scan at 366 nm and f) Densitometric scan at 620 nm post derivatisation with vanillin/sulphuric acid; solvent system - chloroform : methanol (5: 1).

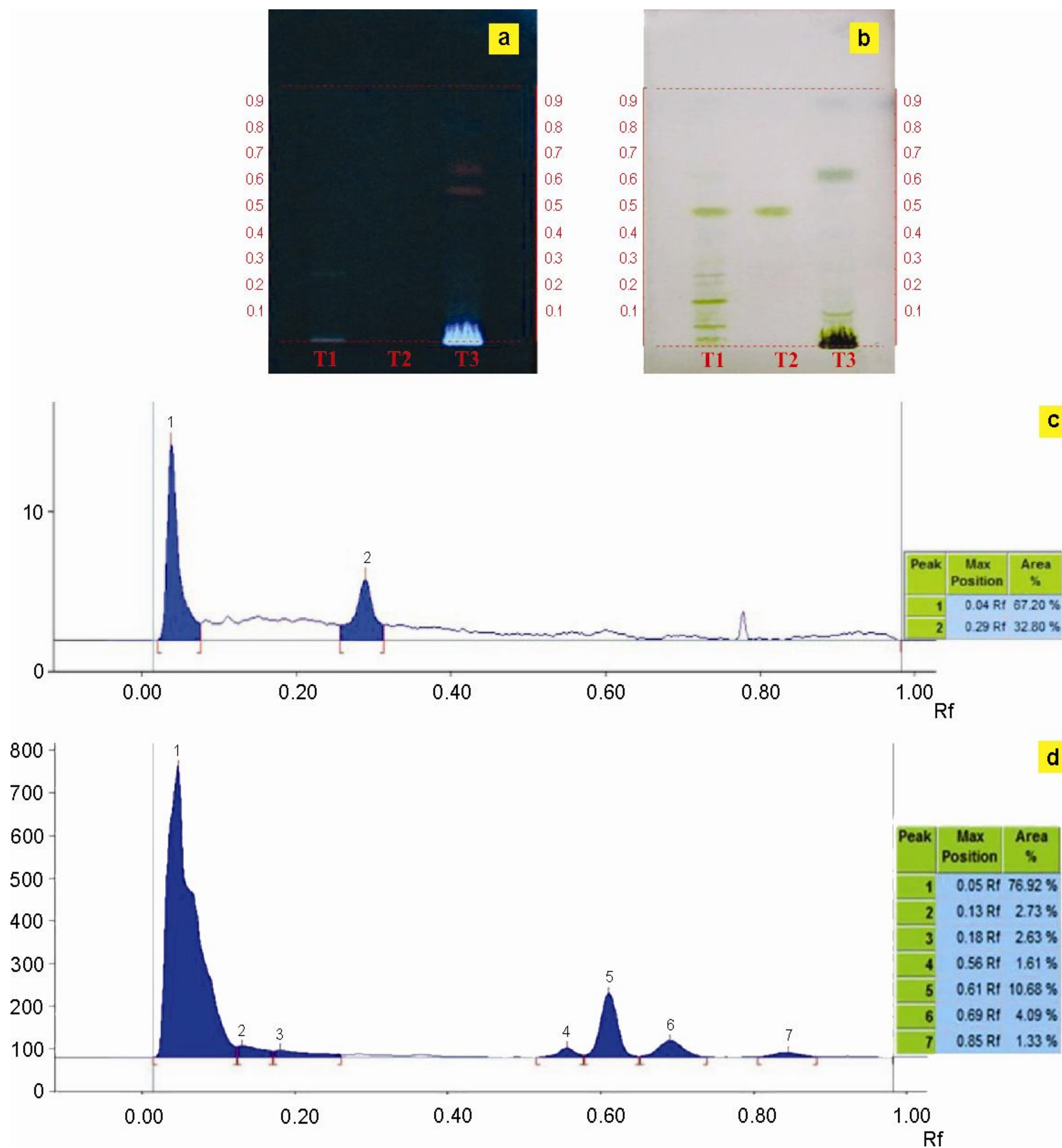


Plate 4—Comparative HPTLC fingerprint of chloroform soluble fraction and methanol extract of *Balanites aegyptiaca* fruit using Diosgenin. a) Photo-documentation of chloroform soluble fraction (T1), Diosgenin (T2), Methanolic extract (T3) under long UV, b) At 620 nm after derivatisation with vaniliin-sulphuric aci spray reagent, c) Densitometric scan of chloroform soluble fraction (A) at 366 nm and d) Densitometric scan of methanolic extract (C) at 366 nm, solvent system – toluene : acetone : formic acid (7 : 1: 1), (Diosgenin not detected).

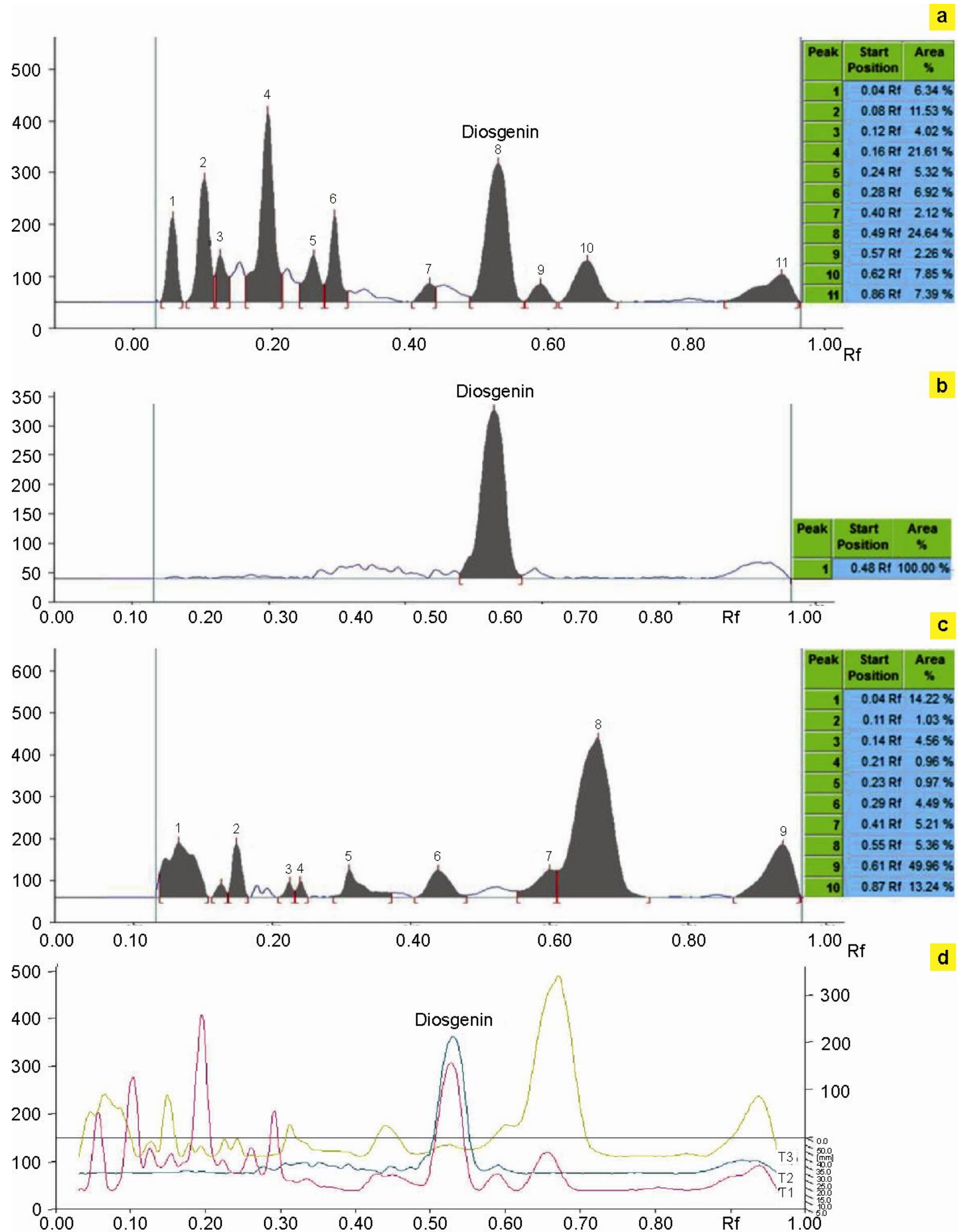


Plate 5—HPTLC detection of diosgenin in chloroform soluble fraction and methanolic extract of *Balanites aegyptiaca* fruit at 620 nm after derivatisation with vanillin-sulphuric acid spray reagent. a) Densitometogram of chloroform soluble fraction, b) Densitometogram of Diosgenin in chloroform, c) Densitometogram of methanolic extract and d) 3-D densitometric scan of chloroform soluble fraction (T1), diosgenin (T2) and methanolic extract (T3), solvent system – toluene : acetone : formic acid (7 : 1 : 1).

Conclusion

Macro- and micro- scopical and physico-chemical pharmacopeial standards for the fruit of *B. aegyptiaca* have been derived as per standard methods. Phytochemical entities such as carbohydrate, triterpene, phytosterol, saponins, amino acids and coumarins were detected by colour tests. HPTLC fingerprint profile has also been recorded for identification of different extracts of *B. aegyptiaca*. The set of data obtained in the present investigation can serve as standard for identification and quality control of this traditional medicament. The result of the study can serve as quality control document for *Ingudi*.

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