Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 3038-3043
Received: 28-05-2019
Accepted: 30-06-2019

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Phytopharmacognostical profile of *Manilkara zapota* (L.) P. Royen seeds

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Abstract
The objective of the present study is to develop the pharmacognostic and phytochemical profiles of *Manilkara zapota* seeds. The present study considers the detail investigation of macroscopy, microscopy, preliminary qualititative phytochemical analysis, physicochemical evaluations, and chromatography & infra red spectroscopy profiling. Microscopical investigation was adopted to identify the cellular powder characters of seed including profuse oil globules, sclerenchymatous cells and numerous sclereids in variable shape and sizes. Physicochemical parameters reveal the data of quality, purity while phytochemical screening reflects the presence of various secondary metabolites. The chromatographic and FTIR fingerprint data represent the authentic plant sample. Data obtained from botanical and chemical screening in combination may be considered as standard for identification and authentication of seeds and may be helpful in developing pharmacopoeial standards.

Keywords: *Manilkara zapota*, microscopy, physicochemical, phytochemical, chromatography, infra red

1. Introduction
Manilkara zapota (L.) P.Royen (family: Sapotaceae) commonly known as sapodilla is an ornamental evergreen tree with a dense, widely spreading crown that can grow 9 - 30 metres tall. The straight, cylindrical bole can range in diameter up to 50-150 cm [1, 2]. A tree with a wide range of local uses as a food and medicine, it is also very important commercially as the source of an edible fruit, latex and a timber. The edible fruit is greatly enjoyed and very widely eaten in the tropics. The tree is widely cultivated commercially and in gardens in the tropics for this fruit and also for the latex contained in the sap. This latex is coagulated and used commercially to make chewing gum. The pulverized roots are used to treat thrush in babies [3]. The tree yields a timber traded internationally noted for its strength and durability, it is also very hard, tough, dense, and resistant to insects [1]. The bark is astringent, febrifuge and tonic. Tannin from the bark is used to cure diarrhoea and fever [3]. A leaf decoction is taken for fever, haemorrhage, wounds and ulcers. For neuralgia, leaf with tallow is applied as a compress on the temples. Matured and old leaves contain poisonous alkaloids [4]. The flowers are used as one of the ingredients of a powder that is rubbed on the body of a woman after childbirth [4]. The fruit is eaten as a remedy for indigestion and diarrhea [4]. Seeds are antipyretic, and when ground with water they act as a diuretic. They are used to expel urinary and gall bladder stones. The seeds contain hydrcyanic acid. The pulverized roots are used to treat thrush in babies. The plant is a source of sapotin, a glucoside used in medicine as a febrifuge [3]. The present work, focused on pharmacognostic, phytochemical, physicochemical characterization and chromatographic fingerprint profiling of *Manilkara zapota* seeds.

2. Material and methods
2.1 Material and reagents
The work has been carried out by using the chemicals, reagents and solvents of Emplura grade of Merck and aluminum supported Thin Layer Chromatography plates were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

2.2 Plant materials collection and authentication
The ripe fruits of *Manilkara zapota* were collected from fruit market of Salt Lake area, Kolkata (22°31′31.8″ N: 88°21′59.4″ E), West Bengal in the month of May, 2019 and authenticated in Department of Pharmacognosy, Central Ayurveda Research Institute for Drug Development, Kolkata. The seeds were separated from fruit pulp (two seeds per fruit) for the experiment and deposited in the Department, available for reference.

2.3 Plant sample processing
The seeds were thoroughly washed with distilled water, shed dried at 30-35 °C. A small portion of the fresh and shed dried seed was used for macroscopic or morphological study. Seeds were pulverized with a grinder (National SM 2000) to obtain coarse and fine powder. Fine seed powder (sieved in 60 #) used for powder microscopy, analysis of physicochemical and phytochemical features. Coarse seed powder (sieved in 25 #) of plant material was used for chromatographic analysis. The whole and powdered plant samples were stored at room temperature in airtight, light-resistant containers as per standard guidelines [5].

2.4 Macroscopy of plant material
The morphological and organoleptic parameters *viz.* texture, shape, size, colour, odour etc. of the whole plant material were noted mainly by naked eye observation [5, 6] with the help of simple microscope Olympus OIC DM.

2.5 Powder microscopy of cytomorphological features
Fine dried powdered seed sample (~ 2 g) was separately treated with different solutions *i.e.* 50% glycerin, aqueous saturated chloral hydrate (for maceration), phloroglucinol in conc. HCl (for staining lignified tissues) and 0.02 N iodine reagent (for starch grains), mounted on slides with 50% glycerin following a standard protocol and observed under the binocular compound microscope (Olympus OIC-07964) at 10× and 40× magnifications [5, 6]. The photomicrographs of different cellular structures and inclusions were taken using Magcam DC14 camera attached to an Olympus CX21i trinocular compound microscope.

2.6 Fluorescence analysis
The coarse seed powder (~ 0.5 g each) was treated with eighteen (18) different reagents (5 ml each) such as, acids and alkaline solutions along with other solvents (including distilled water) inside clean test tubes, which were shaken well and allowed to stand for about 24 hours. The individual solutions were observed under normal daylight and UV (254 nm and 365 nm) light for their characteristic colors and compared with the standard colour chart [7].

2.7 Physicochemical evaluation
The physicochemical constant like ash values, loss on drying, extractive values and pH value of the plant material were determined by using finely divided powder as per standard guidelines [5]. Extractability was studied with different solvents like hexane, acetone, chloroform, ethyl acetate, methanol, ethanol, water and with equivolume aqueous ethanol. Extractions were performed by conventional cold and hot extraction method [5, 6] and microwave assisted extraction was also done.

2.8 Phytochemical
The finely powdered plant materials were subjected to soxhlet extraction for 1 h, separately with petroleum ether, chloroform, ethyl acetate, acetone, methanol, ethanol, water and with equivolume aqueous ethanol. The individual extracts were evaporated to dryness and used for screening the presence of secondary metabolites [8].

2.9 Fingerprint analysis
Given that the methanol extract of the plant materials gave the maximum extractive value, the same was used for the fingerprinting analysis. For this, the coarsely powdered plant material (1 g) was extracted with methanol (25 ml) using a Soxhlet apparatus. The extract was filtered and final volume made up to 20 ml using methanol and used for the fingerprinting analysis by High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC) and Fourier Transmission Infra Red (FTIR) spectroscopy (FTIR).

2.9.1 High Performance Thin Layer Chromatography (HPTLC)
The extract (2 µL) was applied in the form of 8 mm band, 15 mm from the bottom of a 5 × 10 cm preactivated aluminium supported precoated silica gel $60F_{254}$ plate, with the help of ATS-4 applicator attached to a CAMAG HPTLC system. The plate was developed in a pre-saturated twin trough chamber using the mobile phase as hexane: ethyl acetate: acetone: 1,4-dioxan: formic acid (4:3:2:1:0.5, v/v) to a distance of 8 cm, dried for 5 min in ambient air. Images of the developed plate were captured under 254 nm and 366 nm UV light. Densitometric scanning [9] of the developed plate at 254 nm and 366 nm were performed. An image was also captured using visible light after derivatising the plate with aqueous 20% sulphuric acid [10].

2.9.2 High Performance Liquid Chromatography (HPLC)
This was carried out with a HPLC equipment (Agilent model Infinity 1260), equipped with quaternary LC-2010 AHT VP pumps, a variable wavelength programmable UV/VIS detector, SPD-10AVP column oven and Class-VP software for analysis. The chromatographic separation was performed using a Phenomenex C_{18} (250 mm × 4.6 mm, 5 µm particle sizes) column at 25 ºC. The optimized mobile phase was found to be methanol: water (0.1% aqueous orthophosphoric acid) 75:25 (v/v) at a flow rate of 0.5 ml/min. An auto sampler with injection volume 20 µl was used for sample loading and the peaks were detected at 254 nm UV.

2.9.3 Fourier Transmission Infra Red (FTIR) spectroscopy
A drop of the methanolic extract was placed between two potassium bromide pellets to obtain a thin layer, which was analysed with a FTIR spectrophotometer (Agilent Cary 630).

3. Results & discussion
3.1 Morphological characters
The seeds are hard, flattened, more or less ovo-elongated with one sided apical beak like bent notch, length 1.9 to 2 cm, width 0.8 to 1.1 cm, surface of the external shell is black, shining, glossy and embedded in pale brownish, juicy, sweet, fragrant fleshy fruit pulp of globular berries. Inside the hard shell, seed kernel is soft, nutty, oily, white mass, powder, coarse and oily. Pictorial representation are given in fig. 1(1a, 1b, 1c and 1d).

Fig 1a: Fresh ripe fruits of *Manilkara zapota* (dorsal & lateral view)

Fig 1b: Longitudinal sections of fruit showing elongated seeds embedded in pulp

Fig 1c: Isolated seeds

Fig 1d: Seed powder

3.2. Powder microscopy

Fine powder is oily, sticky granular mass with mixed black and cream coloured particles with stale oily taste and no characteristic odour, shows the presence of numerous sclereids of different shape and sizes i.e. oval to elongated, beaker shaped, polygonal, rectangular etc. single or in groups, few xylem vessels with spiral thickenings on wall, few prismatic crystal of calcium oxalate, profuse oil globules throughout the sample, aseptate fibres and sclerenchymatopus cells containing oil globules. Starch grains more or less absent in seed sample represented in fig. 2.

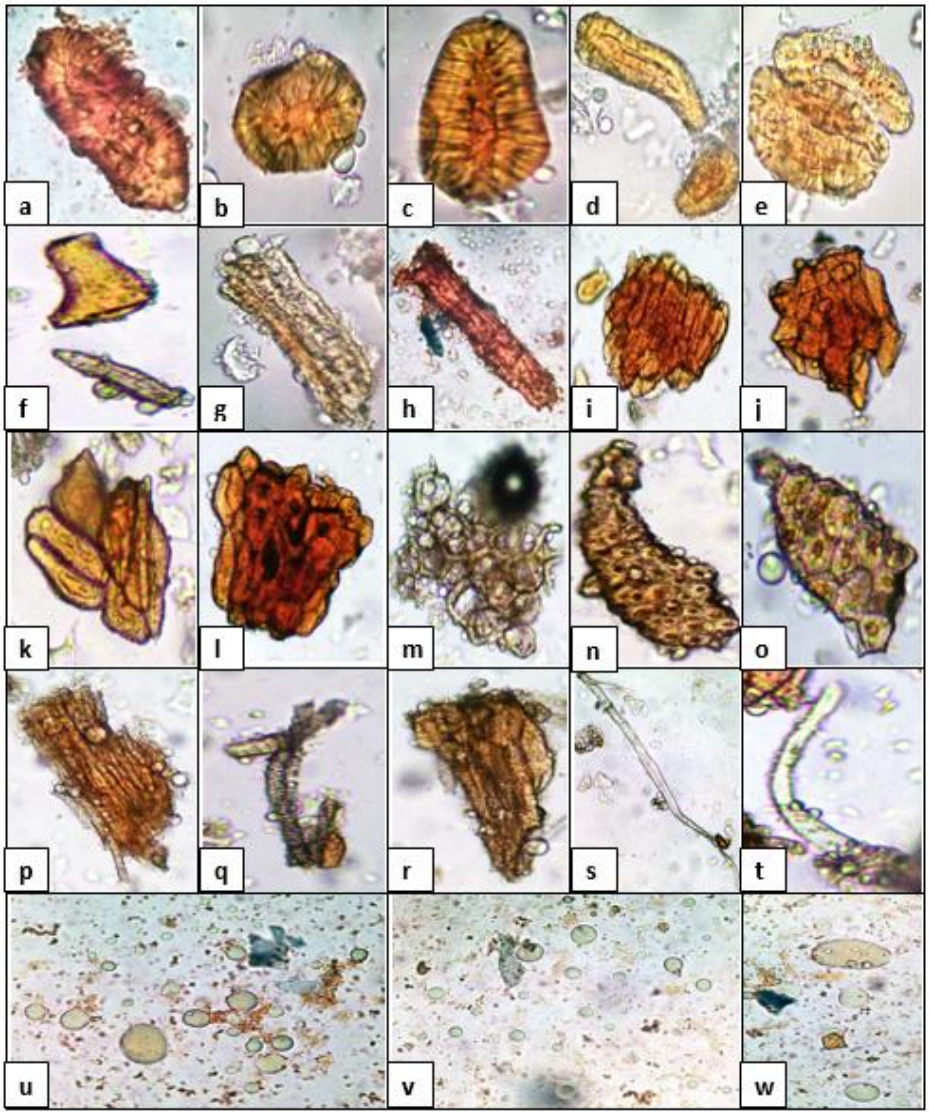


Fig 2: Photomicrographs of *Manilkara zapota* seed powder

a, b, c, d, e: Oval to spindle shaped lignified sclereids; **f, g, h:** Beaker shaped and elongated rectangular sclereids; **i, j, k, l:** Groups of elongated to polygonal sclereids; **m:** Groups of polygonal sclerenchymatous cells with oil globules; **n,o:** Groups of isodiametric hexagonal thick walled stone cells (may be of testa); **p, q:** Spiral xylem vessels in group; **r:** Groups of sclereids overlapped by xylem vessels; **s, t:** Aseptate thick walled fibre; **u, v, w**: Profuse oil globules.

3.3. Fluorescence analysis

Dried seed powders treated with different reagents reveals the presence of chromophoric compounds in them. Different shades of pink fluorescence with concentrated and 50% HNO_3 and pale bluish fluorescence with acetone under 366 nm UV light. No fluorescence was observed under normal daylight and short UV (254 nm) light, indicating very small amount of chromophores in the sample (Table 1).

Table 1: Florescence analysis of *Manilkara zapota* seed powder

Sr. No.	Fluorescence Analysis Reagents	Visible/Day Light	Short UV (254 nm)	Long UV (366 nm)
1.	1N Hcl	Slight turbid	No colour	No colour
2.	1N NaOH	Pale yellowish brown	Light pale brownish tinge	Bluish tinge
3.	1N NaOH + Methanol	Clear colourless	No colour	No colour
4.	50% KOH	Turbid orange	Pale brownish tinge	Bluish tinge
5.	50% H_2SO_4	Light blackish brown	Brownish tinge	Pale brownish tinge
6.	Conc. H_2SO_4	Turbid black	Black	Turbid black
7.	Conc. HNO_3	Turbid with greenish tinge+	Brownish with greenish tinge	Light pinkish tinge '+ ve'
8.	Acetic acid	Clear colourless	Light pinkish tinge	No colour
9.	50% HNO_3	Turbid++ with greenish tinge	Brownish tinge	Light pinkish tinge '+ ve'
10.	Iodin solution	Clear wine red	Brownish black	Blackish grey
11.	Distilled water	Turbid white	No colour	Fade bluish tinge
12.	Chloroform	Turbid grey	Fade pinkish tinge	No colour
13.	Acetone	Clear white	Fade pinkish tinge	Pale bluish tinge '+ ve'
14.	Ammonia	Turbid pale orange	Pinkish tinge	Bluish tinge
15.	Ethanol	Clear	Fade bluish tinge	No colour
16.	Toluene	Clear	Fade bluish tinge	No colour
17.	$K_2Cr_2O_7$	Clear bright orange	Yellowish green	Black
18.	$FeCl_3$	Clear greenish yellow	Brownish with greenish tinge	Pale blackish grey

3.4. Physicochemical

Investigation of the physiochemical parameters of the plant samples showed (Table Y) its total ash value as 5.33%, water soluble and acid insoluble ash contents as 2.53% and 2.34% respectively. Moderately high value (16.34%) of loss on drying suggests that the plant material bears significant amounts of water and\or volatile contents. The extractive values of different solvents for the plant samples revealed maximum and least extraction by methanol and hexane respectively. The extraction yields of the respective solvents were not significantly different under conventional and microwave assisted extraction. Based on the best extraction yield in methanol, the same was used for the subsequent finger printing analyses.

Table 2: Physico-chemical evaluation of *M. zapota* seeds.[a]

Physicochemical Parameters	Percentage		
Loss on drying (LOD)	16.34±0.17		
Ash values			
Total ash value	5.33±0.15		
Water soluble ash value	2.53±0.13		
Acid insoluble ash value	2.34±0.28		
Sulphated Ash	1.65±0.22		
pH value (10% aq. suspension)	6.72±0.09		
Extractive values	**Conventional extraction**		**Microwave assisted extraction**
	Cold extraction	**Hot extraction**	
Hexane	6.12±0.13	6.23±0.34	7.32±0.27
Acetone	16.38±0.11	16.65±0.22	12.32±0.15
Chloroform	9.35±0.16	9.92±0.23	6.35±0.02
Ethyl acetate	11.27±0.09	9.16±0.21	8.30±0.17
Methanol	19.31±0.31	21.07±0.23	19.12±0.12
Alcohol	11.19±0.33	10.98±0.21	6.86±0.05
Water	7.23±0.21	6.54±0.19	6.77±0.05
Hydroalcoholic (1:1)	8.37±0.43	8.87±0.43	8.63±0.03

[a]Values are expressed as Mean ± S.D.

3.5 Phytochemical

Phytoconstituents are basically divided into two groups like primary and secondary metabolites, according to their functions in plant metabolism. Primary metabolites consist of common sugars, amino acids, proteins and chlorophyll while alkaloids, terpenoids, flavonoids, tannins etc. contribute as the secondary metabolites. In present study, different qualitative tests were carried out with the *M. zapota* seeds samples after extraction with various solvents. The results of the phytochemical screening (Table X) revealed the presence of alkaloids, steroids, phenolics, glycosides, but less terpenoids, flavonoids. The phenolics and glycosides were primarily present in the protic, polar solvents.

Table 3: Phytochemical screening of *M. zapota* seeds

Phytochemical class	Hexane	Chloroform	Actone	Ethyl Acetate	Methanol	Ethanol
Alkaloids	-	-	+	-	+	+
Steroids	-	+	+	+	+	+
Triterpenoids	-	-	-	-	-	-
Flavonoids	-	-	+	-	-	-
Tannins	-	-	+	-	-	-
Glycoside	-	+	+	+	+	+
Phenolic	-	-	+	-	+	+
Oils	+	-	+	-	-	-
Saponins	+	+	+	+	+	+
Phlobatannins	-	-	+	-	-	-
Anthraquinones	-	+	+	+	+	+
Fatty ester	+	-	-	-	-	-

3.6 High Performance Thin Layer Chromatography (HPTLC)

The HPTLC experimental condition was optimized by using pre-activated and precoated TLC silica gel 60 F_{254} plates and different combinations of polar and apolar solvents as the mobile phases (data not shown). Best result was obtained with hexane: ethyl acetate: formic acid (6:4:0.5, v/v) as the mobile phase, which showed six bands at R_f values of 0.06, 0.21, 0.27, 0.40, 0.48 and 0.63, when visualized under UV at 254 nm and at 366 nm, five bands at R_f values 0.03, 0.17, 0.21, 0.34 and 0.59, were seen, while bands at R_f 0.12, 0.33, 0.37, 0.49 and 0.63, were seen after derivatiztaion followed by exposure to visible light. Pictorial representations of the bands at different visualization are represented in Fig. 3.

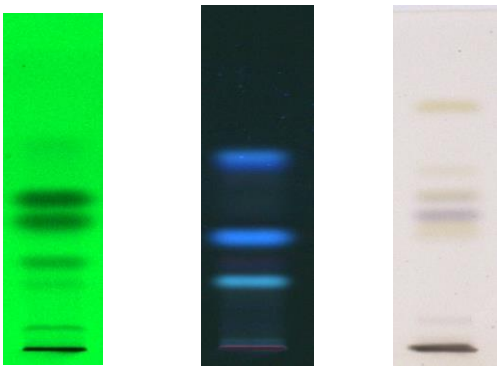


Fig 3: HPTLC profiles of *M. zapota* seed methanol extract.

3.7 High Performance Liquid Chromatography (HPLC)

HPLC method was developed for best separation of the chemical constituents of the *M. zapota* seeds methanol extract. Separated peaks were detected under UV (254 nm). The HPLC fingerprint analysis showed (Fig. 4a) five peaks and retention times were 2.678, 3.434, 8.801, 11.627, 12.847 min respectively. The relative ratios of the peaks of the HPLC chromatograms, determined from the areas under the curves are represented in Table 4.

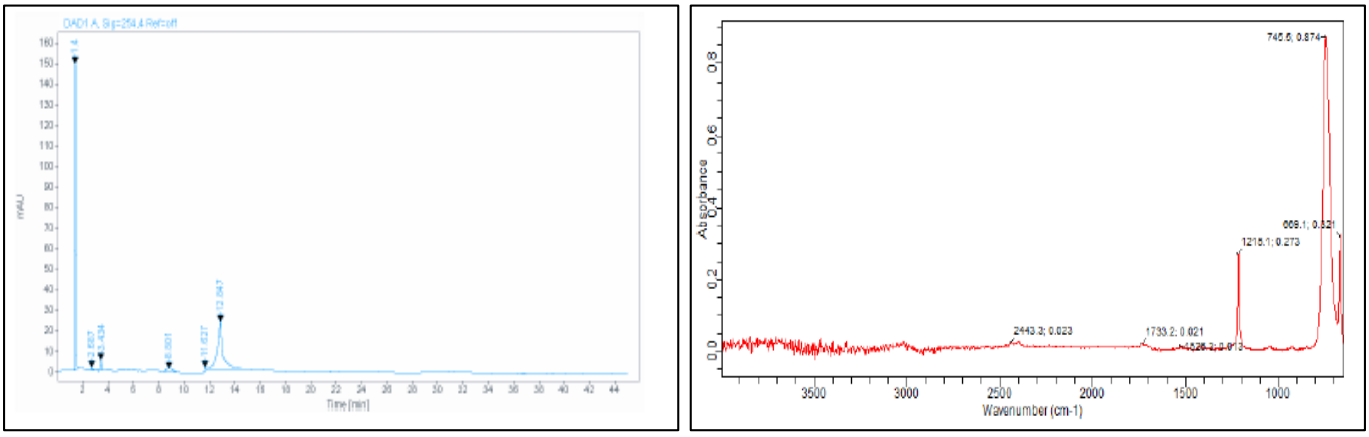
Table 4: Relative ratios of the HPLC peaks[a]

Sl. No.	Peak Retention time (Minute)	Realtive ratio (%)
1.	2.678	1.577
2.	3.434	5.523
3.	8.801	9.468
4.	11.627	1.529
5.	12.847	81.79

[a]The peaks were recorded by detecting the chromatogram at 254 nm.

3.8. Fourier Transmission Infra Red (FTIR) spectroscopy

The FTIR spectrum of the methanol extract using methanol is shown in (Fig. 4b). The absorption spectrum revealed distinct peaks at 669.1, 745.5 and 1215.1 cm^{-1}. The intense peaks in the region of 745 cm^{-1} indicated presence of disubstituted C-H bonds. The peak at 745.5 cm^{-1} may account for the C-N stretch of some amine containing group.



(a) (b)

Fig 4: HPLC chromatogram (a) and FTIR spectrum (b) of *M. zapota* seed, methanol extract.

4. Conclusion

The present investigations furnished a set of qualitative and quantitative phyto-pharmacognostic parameters along with HPTLC, HPLC and FTIR fingerprinting profile of *M. zapota* seeds. These data can serve as diagnostic tools for establishment of quality standards, authentication and identification of the medicinally important plant and help in compiling of a suitable monograph of this.

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