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Research Article

ISOLATION AND IDENTIFICATION OF PHYTOSTEROLS FROM *MANILKARAZ APOTA* L. AND *MADHUCA LONGIFOLIA* L.

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ABSTRACT

In the present investigation selected plants were *Manilkara zapota* L. and *Madhuca longifolia* L. Both plant belongs to family Sapotaceae and have many medicinal values. In the present study, phytosterols from *Manilkara zapota* L. and *Madhuca longifolia* L. were identified and quantified *in vivo*. Phytosterols were identified using chromatographic and spectral studies. ß-sitosterol, campesterol and stigmasterol were identified by IR and GC-MS. GC-MS profiling showed various compounds. In *Manilkara zapota* L. 1,3-Butanedione, 1-Phenyl found in highest amount (31.49%), while 5H,10H-Dipyrrolo[1,2-A:1',2'-D]Pyrazine-5,10-Dione, Octahydro-, (5AS-CIS) and Pyrimidine, 2-phenyl-4-hydroxy-5-dimethylamino found in minimum amount (0.16 %). In *Madhuca longifolia* L. Ethyl Oleate found in highest amount (22.72%), while 2-Ethylhexyl salicylate found in lowest amount (area of % 0.16). It is the first report on phytosterols from the experimental plants.

INTRODUCTION

A medicinal plant can be described as any plant in which one or more of its organs contain substances that can be used therapeutic purposes or which are precursors for the synthesis of useful drugs [1].The use of plant-based remedies is also widespread in many developed countries and pharmaceuticals are based or devised from plants or plant products[2].Plant sterols are triterpenes that are important structural components of plant membranes, and free phytosterols serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell membranes[3]. Plant sterols structurally similar to cholesterol that act in the intestine to lower cholesterol absorption. Because they have very low systemic absorption and are already present in healthy diets, increasing the intake of phytosterols may be a practical way to reduce coronary heart disease with minimum risk[4]. Studies now show that phytosterols consumption, of about 2 grams/day, results in an approximately 9% reduction in LDL-cholesterol, which is the "bad" cholesterol known to contribute to heart disease. Based on this tremendous benefit many food manufacturers and supplement developers are putting phytosterols into their product to both benefit consumers and promote a healthy heart diet[5].

Sapodilla (*Manilkara zapota* L.) is one of the edible fruits cultivated all over India. In India it ranks fifth position in production and consumption next to mango, banana, citrus and grapes. It is also commercially important because it is a source of chicle, the principle ingredient in chewing gum. It is a rich source of sugar, protein, phenol, carotenoids, amino acids, pectin, vitamin C and mineral like Phosphorus, Calcium, Iron and Magnesium [6]. This plant has antioxidative property and its fruit is used for preventing biliousness and attacks of fever whereas seeds are diuretic [7].

Madhuca longifolia L. commonly known as the Butter nut tree is a medium to large sized deciduous tree distributed in Nepal, India and Sri Lanka. Mahua seeds are of economic importance as they are good source of edible fats [8]. The plant belongs to the family of Sapotaceae. It is both wild and cultivated [9]. Ethnomedical uses say to possess significant antipyretic, hepatoprotective, anti-inflammatory, analgesic, antitumour, antiprogestational, antiestrogenic and wound healing activity. Traditionally *Madhuca longifolia* L. bark is used in rheumatism, ulcers, bleedings and tonsillitis [10].

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MATERIALS AND METHODS

Collection of plant Material

Plant parts of *Manilkara zapota* L. (leaves and bark) and *Madhuca longifolia* L. (leaves and seeds) were collected from the fields at Jaipur and authenticated. The voucher (RUBL* No. 211568 for *Manilkara zapota* and RUBL* No. 211564 for *Madhuca longifolia* of experimental plant was deposited in the Herbarium of Department of Botany, University of Rajasthan, Jaipur. The plant parts were separated and washed thoroughly 2-3 times with running tap water and then air dried under shade after complete shade drying the plant material was powdered and used for phytochemical analysis.

Extraction

Dried and powdered plant material and was defatted in petroleum ether (60-80^0 C) for 24 h on a water bath. Defatted material was air dried and hydrolyzed in 30% HCl (v/v) for 4 h. Each hydrolyzed sample was washed with distilled water till pH 7 was achieved and was dried later. The dried preparation was again extracted with benzene for 24 h. The extract was filtered and dried *in vacuo*. The crude extract was dissolved in benzene before chromatographic examination [7].

Thin layer chromatography (TLC)

Glass plates coated with silica gels G were used. Each of the extract was co-chromatographed separately with authentic sterols as marker. These plates were developed in an airtight chromatographic chamber, saturated with solvent mixture (Hexane: Acetone:: 8:2),[8]. Other solvents such as benzene and ethyl acetate (85:15),[9] benzene: ethyl acetate (3:1), was also used but hexane: acetone (8:2) gave better separation. These plates were air dried and visualized under UV light and fluorescent spots corresponding to that of standards marker were marked. These developed plates were sprayed with 50% sulphuric acid [10] and anisaldehyde reagent, separately and heated at 110^0 C for 10 min.

Preparative thin layer chromatography

PTLC was performed using silica gel G coated plates (0.4-0.5mμ) along with the reference markers. These plates were developed in hexane: acetone (8:2), air dried and examined under UV light. Each spot coinciding with that of standard marker was marked, scraped from 50 plates, and eluted with chloroform. The eluted reactions were subjected to crystallization, separately and their melting point, mixed melting point were determined. The isolated compounds were also subjected to UV and IR spectral studies.

Identification

Melting point and IR spectra of each of the isolated compounds was taken and a comparison of the TLC colour reaction was made, which was found to be in accordance with those of studied authentic compounds.

RESULT AND DISCUSSION

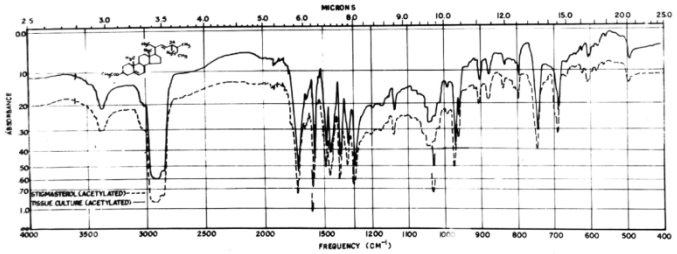

Fig 1 Infra-red Spectra of Isolated and Standard Stigmasterol

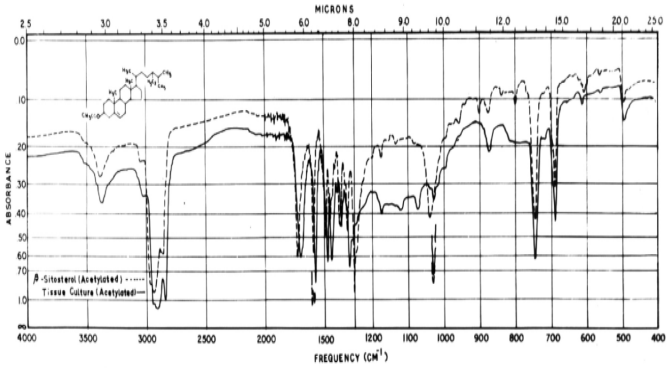

Fig 2 Infra-red Spectra of Isolated and Standard β-sitosterol

Table 1 Chromatographic Behavior and Physico-chemical Characteristics of Isolated Phyto-sterols

Isolated Compounds	R_fValue			Color After Spray		M.P. (ºC)	IR Spectral Peaks (rept.) ν (KBr) cm^{-1}
	S_1	S_2	S_3	R_1	R_2		
β-sitosterol	0.89	0.90	0.71	PU-BN	PK	136-137	3350 (O-H), 2830, 1665 (C=C), 1470, 1300, 1052 (C-O) and 880
Stigmasterol	0.83	0.64	0.65	GY	PU	167-69	3400 (O-H). 2950, 1750, 1640 (C=O), 1035 (C-O), 991, 957, 935, 810 and 715

Abbreviations: S_1 - Hexane : acetone (8 : 2), S_2 - Benzene : acetone (2 : 1), S_3 – Benzene : ethyl acetate (3 : 2),R_1 - 50% H_2SO_4, R_2 - Anisaldehyde reagent, BN - Brown, PK- Pink, PU – Purple, BL – Blue, GY – Gray

Gas Chromatography-Mass Spectrometry (Gc-Ms) Analysis

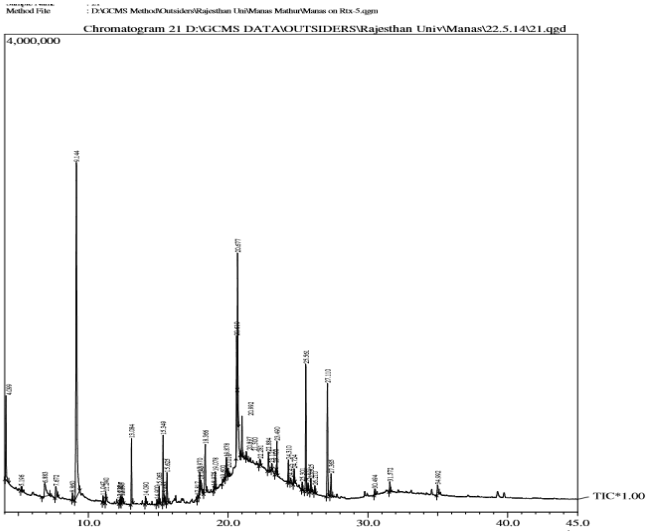

Fig 3 showing GC-MS of phytosterol isolated from leaves of *Manilkara zapota* L.

Table 2 GC-MS profiling of phytosterols isolated from leaves of *Manilkara zapota*L.

S.NO.	R.Time	Area	Area %	Compound Name
1.	4.099	2180041	4.87	Benzoic acid, methyl ester
2.	5.196	97181	0.22	Benzoic acid, ethyl ester
3.	6.883	989777	2.21	Hydroquinone
4.	7.672	470616	1.05	2-METHOXY-4-VINYLPHENOL
5.	8.860	145137	0.32	1-Tridecene
6.	9.144	14083307	31.49	1,3-BUTANEDIONE, 1-PHENYL-
7.	11.047	123198	0.28	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-
8.	11.240	187616	0.42	Diphenylamine
9.	12.225	78299	0.18	BENZENAMINE, N-PHENYL-
10.	12.307	108813	0.24	1-TRIDECENE
11.	12.395	278137	0.62	1,3,6,9B-TETRAAZAPHENALENE, 2-METHYL-
12.	13.084	1291863	2.89	2,5-Dimethyl-4-phenylpyridine
13.	14.090	167153	0.37	2-CYCLOHEXEN-1-ONE, 3-PHENYL-
14.	14.900	91040	0.20	2(4H)-BENZOFURANONE, 5,6,7,7A-TETRAHYDRO-6-HYDROXY-4,4,7A-TRIMETHYL-, (6S-CIS)-
15.	15.063	317462	0.71	Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-
16.	15.349	1479353	3.31	5,6,7,7A-TETRAHYDRO-6-HYDROXY-4,4,7A-TRIMETHYL-, (6S-CIS)-
17.	15.463	84097	0.19	1-Hexadecanol
18.	15.625	801877	1.79	PLUCHIDIOL
19.	17.817	73713	0.16	5H,10H-DIPYRROLO[1,2-A:1',2'-D]PYRAZINE-5,10-DIONE, OCTAHYDRO-, (5AS-CIS)-
20.	17.970	421580	0.94	n-Hexadecanoic acid
21.	18.043	329627	0.74	Phthalic acid, bis-(10-hydroxy-decyl ester
22.	18.366	1798149	4.02	HEXADECANOIC ACID, ETHYL ESTER
23.	18.975	70869	0.16	Pyrimidine, 2-phenyl-4-hydroxy-5-dimethylamino-
24.	19.078	464953	1.04	Hexadecanoicacid, trimethylsilyl ester
25.	19.600	129163	0.29	n-Heptadecanol-1
26.	19.878	693816	1.55	2-Buten-1-one, 3-amino-1-phenyl-4,4,4-trifluoro-
27.	20.010	153647	0.34	Phytol
28.	20.610	1685255	3.77	n-Propyl 9,12-octadecadienoate
29.	20.677	3158411	7.06	Ethyl Oleate
30.	20.992	939307	2.10	Octadecanoic acid, ethyl ester
31.	21.300	228659	0.51	trans-9-Octadecenoic acid, trimethylsilyl ester
32.	22.884	522082	1.17	Pyridine, 2-methyl-4,6-diphenyl-
33.	23.121	111213	0.25	9-OCTADECENAMIDE
34.	23.400	85350	0.19	Tetradecanamide
35.	23.490	532052	1.19	Hexanedioic acid, bis(2-ethylhexyl) ester
36.	24.310	474565	1.06	1,2-Propanediol, 3-benzyloxy-1,2-diacetyl-
37.	24.475	174770	0.39	OCTADECANOIC ACID, 3-OXO-, METHYL ESTER
38.	24.724	425025	0.95	GAILLARDIN
39.	25.301	185763	0.42	Cyclohex-2-enone, 2-benzoyl-3-(2-hydroxyethylamino)-5,5-dimethyl-
40.	25.561	3197446	7.15	Bis(2-ethylhexyl) phthalate
41.	25.722	173435	0.39	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
42.	25.925	355118	0.79	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
43.	26.210	200960	0.45	BENZYL MYRISTATE
44.	27.110	3387192	7.57	2,4-DIBENZOYL-2,4-DIAMINO-NITROBENZOL
45.	27.365	702505	1.57	Methanone, [4-(2-hydroxy-5-methylbenzylidenamino)phenyl](phenyl)-
46.	30.494	155672	0.35	Squalene
47.	31.572	251191	0.56	Cholesta-3,5-diene
48.	34.992	390830	0.87	CHOLEST-5-EN-3-OL (3.BETA.)-

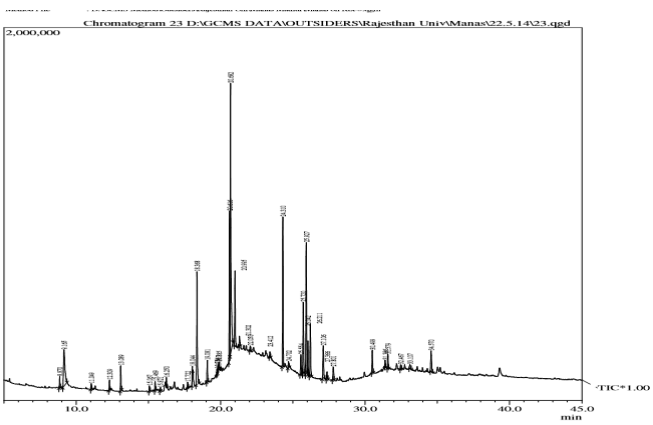

Fig 4 showing GC-MS of phytosterols isolated from seed of *Madhuca longifolia* L.

Two sterols were spotted which were common in plant parts on thin layer chromatography. The R_f values of the spots matched with authentic standards and were identified as β-sitosterol, stigmasterol. . Among the various solvent systems tested best results were obtained in the solvent system Hexane: Acetone (8:2) with R_f values viz., β-sitosterol, 0.89; stigmasterol, 0.83. The characteristic colours were also developed when TLC plates were sprayed with anisaldehyde reagent (β-sitosterol - pink; stigmasterol - Purple) and with 50% sulphuric acid (β-sitosterol-Purple brown; stigmasterol- Gray) corresponding to their authentic samples. The isolated sterols were also identified and characterized with their mp, which also corresponded with those of their respective standards separately (β-sitosterol 136-137^{o}C; stigmasterol; 167-169^{o}C).
The characteristic peaks of IR spectra of isolates (β-sitosterol, stigmasterol) were also found to be superimposable with the IR spectra of reference compounds.

Table 3 GC-MS profiling of phytosterols isolated from seed of *Madhuca longifolia* L.

S.NO.	R.Time	Area	Area %	Compound Name
1.	8.870	125591	0.54	1-Tridecene
2.	9.165	1398181	5.96	3-Buten-2-one, 4-hydroxy-4-phenyl-
3.	11.049	61807	0.26	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-
4.	12.309	124861	0.53	PHOSPHONIC ACID, DIOCTADECYL ESTER
5.	13.089	330972	1.41	2,4-Dimethyl-6-phenylpyridine
6.	15.067	78146	0.33	BENZENE, 1,1'-(1,2-CYCLOBUTANEDIYL)BIS-, CIS-
7.	15.469	132600	0.56	PHOSPHONIC ACID, DIOCTADECYL ESTER
8.	15.821	38607	0.16	2-Ethylhexyl salicylate
9.	16.250	81079	0.35	FARNESYL ACETATE 3
10.	17.722	93379	0.40	Cyclododecene, (Z)-
11.	18.044	161396	0.69	Dibutyl phthalate
12.	18.368	1801733	7.68	HEXADECANOIC ACID, ETHYL ESTER
13.	19.081	309090	1.32	Hexadecanoic acid, trimethylsilyl ester
14.	19.754	53865	0.23	6,11-HEXADECADIEN-1-OL
15.	19.817	54249	0.23	9-Octadecenoic acid (Z)-, methyl ester
16.	19.885	126386	0.54	2-Buten-1-one, 3-amino-1-phenyl-4,4,4-trifluoro-
17.	20.616	2061150	8.78	Linoleic acid ethyl ester
18.	20.682	5333270	22.72	Ethyl Oleate
19.	20.995	1088620	4.64	Octadecanoic acid, ethyl ester
20.	21.302	165942	0.71	OELSAEURE, TRIMETHYLSILYLESTER
21.	22.050	62180	0.26	ETHYL 9-HEXADECANOATE
22.	23.412	84159	0.36	ETHYL NONADECANOATE
23.	24.310	2076274	8.85	Benzonitrile, m-phenethyl-
24.	24.702	98330	0.42	Oxalic acid, decyl 3,5-difluorophenyl ester
25.	25.554	345981	1.47	Bis(2-ethylhexyl) phthalate
26.	25.720	1188041	5.06	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
27.	25.927	2172771	9.26	(2,3 Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
28.	26.042	511084	2.18	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
29.	26.211	913935	3.89	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
30.	27.106	688488	2.93	2,4-DIBENZOYL-2,4-DIAMINO-NITROBENZOL
31.	27.366	141042	0.60	2,4-DIBENZOYL-2,4-DIAMINO-NITROBENZOL
32.	27.801	257908	1.10	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-
33.	30.499	366920	1.56	Squalene
34.	31.386	94569	0.40	Hentriacontane
35.	31.579	229362	0.98	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate
36.	32.467	74828	0.32	DOCOSAHEXAENOIC ACID, 1,2,3-PROPANETRIYL ESTER
37.	34.570	461805	1.97	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate

The main compounds of the phytosterols in *Manilkara zapota*L.and *Madhuca longifolia* L. were identified by using IR and GC-MS. β-sitosterol,stigmasterol, were reported by TLC shown in Table-1.In *Manilkara zapota*L.48 compounds were identified in GC-MS analysis. From which one compound 1,3-Butanedione, 1-Phenyl found in highest amount (31.49%), while two compounds 5H,10H-Dipyrrolo[1,2-A:1',2'-D]Pyrazine-5,10-Dione, Octahydro-, (5AS-CIS) and Pyrimidine, 2-phenyl-4-hydroxy-5-dimethylamino found in lowest amount (0.16%). In *Madhuca longifolia* L. 37 compounds were identified in GC-MS analysis. From which one compound Ethyl Oleate found in highest amount (22.72%), while one compound 2-Ethylhexyl salicylate found in lowest amount (0.16%). It is the first report on phytosterols from the experimental plants.

Among these 7 peaks, 2 peaks were identified by mass spectroscopy (MS) shown in Table -2. Studies have found that consuming 1-2 grams each day of phytosterols can lower low-density lipoprotein (LDL) cholesterol by 6 to 10 percent, which may reduce risk for coronary heart disease .They are also effective when combined with cholesterol-lowering medication; adding phytosterols to statin medications can lower LDL more than doubling the statin dose . β-sitosterol promotes apoptosis in various cancer cells and inhibits their growth[11]. Penicillamine is known to be effective therapy for wilson'sdisease[12].

This investigation has given preliminary information to determine the chemical composition of sterols found in *Manilkara zapota* L.and *Madhuca longifolia* L.using IR and GC-MS. The presence of these bioactive compounds in *Manilkara zapota* L. and *Madhuca longifolia* L. lends credence to its use by the human community. It also holds for the production of novel drugs with isolation of specific compounds with reduced toxicity and more potency.

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