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Qualitative and Quantitative Evaluation of Flavonoids from *Manilkara zapota* L.

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A B S T R A C T

Selected plant for study was *Manilkara zapota* L. belongs to family Sapotaceae and have many medicinal values. Flavonoids from *Manilkara zapota* L. were identified and quantified. Flavonoids were identified using chromatographic and spectral studies. Kaempferol, luteolin and quercetin were identified by IR and GC-MS. GC-MS profiling showed various compounds. In *Manilkara zapota* L. one compound 1,3-Butanedione, 1-Phenyl- found in highest amount with area of 24.79%, at the retention time of 9.155 while one compound 1-Heptacosanol found in lowest amount with area of 0.16% at the retention time of 23.383.

***Key words*:** Flavonoids, GC-MS, IR, *Manilkara zapota* L.

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]. Flavonoids are one of the most promising metabolites which have drawn attention by several workers. More than 5000 flavonoids have been identified [3]. They play a major role in the successful medical treatments in ancient times and their use has persevered till date [4]. Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, isoflavanoids, anthocyanidins, and catechins [5-6].

MATERIALS AND METHODS

Collection and identification of plant materials

Fresh and disease-free plant parts (leaves and stem bark) of *Manilkara zapota* L. were collected from the Jhunjhunu district of Rajasthan. The collected plants were identified and authenticated by the Department of Botany, University of Rajasthan. The voucher RUBL* No. 211568 for *Manilkara zapota* L. of experimental plants were deposited in the Herbarium of Department of Botany, University of Rajasthan, Jaipur.

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Extraction

Various plants parts were air dried and powdered, separately. Each of these extracted separately with 80% methanol on water bath [7] for 24 hrs. The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fr1), diethyl ether (FrII) and ethyl acetate (FrIII) separately. Each step was repeated thrice for complete extraction, fraction 1 was discarded in each case because it contained fatty substance, whereas fraction II and fraction III were concentrated and used for determining flavonoids. Fraction III was further hydrolyzed by refluxing with 7% sulphuric acid (10mLg^{-1} plant material for 2 hrs.), filtered and filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo.* Both fraction II and III were taken up in small volume of ethanol (2-5mL) before chromatographic examination.

Qualitative estimation

Thin layer chromatography (TLC): Thin glass plates (20x20 cm) were coated with Silica gel G (250mμ thick). The freshly prepared plates were air dried at room temperature; thereafter these were kept at 100°C for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis. Each of the extract was co- chromatographed with authentic flavonoid as a marker (kaempferol, luteolin and quercetin). These plates were developed in an air tight chromatographic chamber saturated with solvent mixture

(Benzene: Acetic Acid: Water: 125:72:3). The developed plates were air dried and visualized under UV light by exposure to ammonia fumes. The mouth of a 100 mL containing concentrated NH_4OH was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The developed plates were also sprayed with 5% $FeCl_3$, 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber separately. The coloured spots thus developed were noted and the R_f value of each spot was calculated. Several others solvent systems such as n- butanol, acetic acid, water (4:1:5), tertiary butanol, acetic acid, water (3:1:1) were also tested, but the solvent system containing benzene, acetic acid, water (125:72:3) gave better results.

Identification: The identity of the isolated flavonoids were confirmed by mp, mmp performed in capillaries (Toshniwal Melting Point Apparatus), IR (Infra-red spectrophotometer; Perkin, Elmer 337, Grating Infra-red spectrophotometer), UV (Ultraviolet and visible spectrophotometer; Carl Zeiss, Jena, DDR, VSU-2P spectrophotometer) analysis along with their respective authentic samples.

Quantitative estimation

The isolated flavonoids were estimated by spectrophotometer following the method of Mabry [8]. Stock solution ($1mgL^{-1}$) of kaempferol, luteolin and quercetin were prepared separately by dissolving authentic compounds in methanol. Different concentrations ranging from 20µg to 160µg of each of the compounds spotted separately on silica gel G plates. For each concentration of reference authentic standards separate plates were used and developed in the same manner as described earlier. These developed plates were air dried and visualized under UV light. The fluorescent spots were marked and collected along with the absorbance in separate test tubes. Spectroscopy methanol grade (5mL) was added to each test tube, shaken vigorously, centrifuged and supernatants were collected separately. The volume of each of the eluate was made up to 10mL by adding methanol. To each of these samples, 3mL of 0.1 M $AlCl_3$ solution was added again shaken vigorously and kept at room temperature for 20 min. Five such replicates were run in each case and their optical densities were measured using spectrophotometer at 426nm for kaempferol and luteolin and at 440nm for quercetin against blank (10ml of spectroscopic grade methanol and 3mL of 0.1 M $AlCl_3$). The standard curves were plotted between concentration and their respective average optical density of each of the compound. The regression curve so achieved followed Beer's law.

Each of the plant extract sample (ether and ethyl acetate sample) was dissolved in 5 mL of spectroscopic grade methanol and 0.1mL was applied on silica gel G coated plates along with standard markers, separately. The plates were developed as above and the spots coinciding with that of standard markers were marked on each plate under UV. Each spot was collected along with the silica gel, eluted in methanol and test samples were prepared in the same way as described above. The optical density in each case was recorded and concentration of each sample was computed using the regression curve of authentic flavonoids samples. The concentrations were calculated on mg/g dry weight basis.

RESULTS AND DISCUSSION

Qualitative analysis

Three spots (which were yellowish brown in colour after keeping plates in iodine chamber) of flavonoids were observed in different plant parts of *Manilkara zapota* L. on thin layer chromatography plates developed and sprayed with 5% $FeCl_3$. The R_f values of these spots matched with their respective authentic standards and were identified as Kaempferol, luteolin and quercetin in *Manilkara zapota* L. Solvent system Benzene: Acetic Acid: Water (125:72:3) gave best results with R_f values viz., kaempferol, 0.84; luteolin, 0.58 and quercetin, 0.76. When other solvents viz n-Butanol: Acetic acid: Water (4:1:5) and conc. HCl: Acetic acid:Water (3:30:10), the R_f value of kaempferol was found to be 0.82 and 0.56 that of luteolin was found to be 0.84 and 0.78 and quercetin was 0.62 and 0.44 respectively. The isolated flavonoids viz., kaempferol, luteolin and quercetin were also identified and characterized by super imposable IR peaks (Fig. 1,2,3), the melting point of kaempferol, 276-278°C; luteolin 326-329°C; quercetin 315-320°C (Table 1).

Table 1 Chromatographic behavior and physico-chemical characteristics of isolated flavonoids in *Manilkara zapota* L.

Isolated compounds	R_f Value			Physical Appearance			Color After Spray				Melting point (°C)	IR Spectral Peaks $V(KB_r)cm^{-1}$
							R_1		R_2			
	S^1	S^2	S^3	Day light	UV ammonia	Iodine vapor	Visible	UV	Visible	UV		
Kaempferol	.84	.82	.56	GN-YW	BT-YW	YW-BN	BN	BK	YW	YW-GN	276-278	(O-H) (3410CM^{-1} (270,295,344,1690)
Luteolin	.58	.84	.78	GN-YW	YW	YW-BN	TN	BK	DL-YW	YW-GN	326-329	3421,2965,1736(LACTONE),1510(FURAN),1461, 1388,1360,1274,1242,1187,1136,1028,93,850 CM^{-1}
Quercetin	.76	.62	.44	GN-YW	YW	YW-BN	BT-GY	BK	DL-YW	YW-GN	315-320	3423,1739,1655(OH)160, 1508,1305,1203, (C=C),1088.

Abbreviations: S1- Benzene: acetic acid: water (125: 72: 3), S2- n-butanol: acetic acid: water (4: 1: 5), S3- Conc. Hydrochloric acid: acetic acid : water (3: 30: 10), R1- 5% FeCl3 solution, R2- %% alc. AlCl3 solution, YW- Yellow, BK- Black, BN, Brown, BT-Bright, DL- Dull, GN- Green, GY- Gray

Functional screening of flavonoids

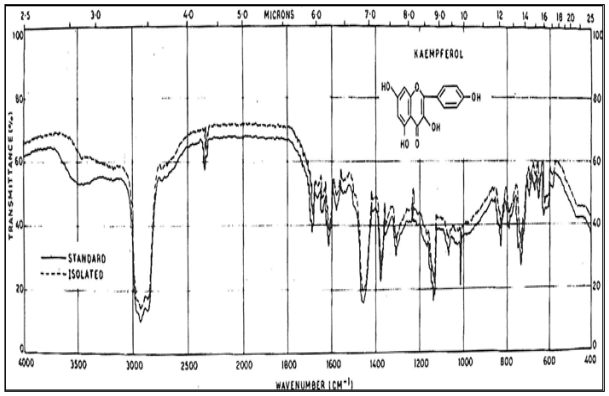

Fig 1 Infrared spectra of standard and isolated Kaempferol

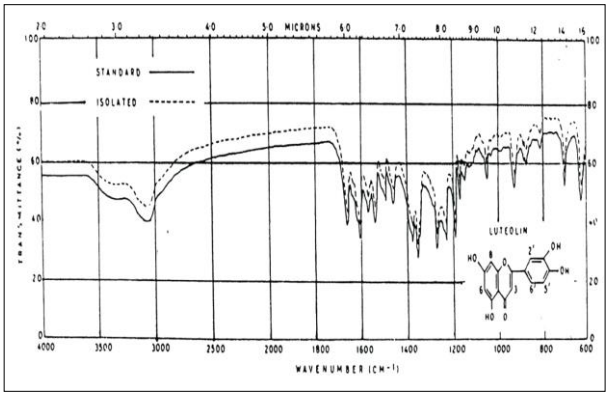

Fig 2 Infrared spectra of standard and isolated Luteolin

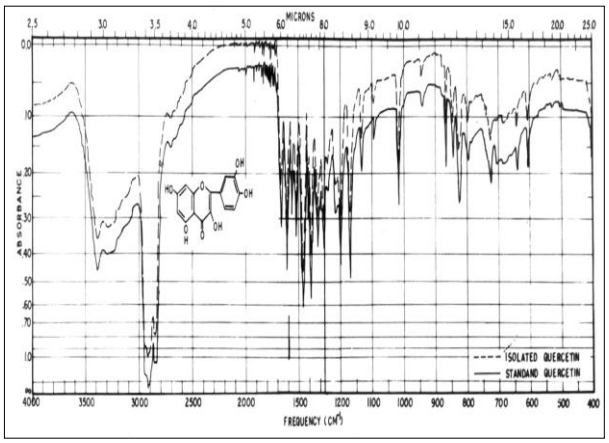

Fig 3 Infrared spectra of standard and isolated Quercetin

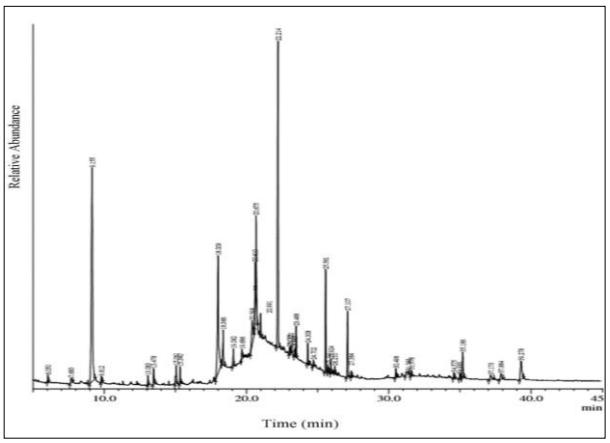

Fig 4 Showing GC-MS of flavonoids isolated from leaves of *Manilkara zapota* L.

Table 2 Flavonoids content (mg/gdw) in different plant parts of *Manilkara zapota* L.

Plant parts	Free flavonoids (mg/gdw)				Bound flavonoids (mg/gdw)				Total flavonoids (Free + bound) (mg/gdw)
	Kaemferol	Quercetin	Luteolin	Total	Kaemferol	Quercetin	Luteolin	Total	
Leaves	.84	1.07	.32	2.23	.74	1.28	.49	2.51	4.74
Stem bark	.12	.19	.07	.38	.62	1.12	.37	2.11	2.49

Quantitative analysis

In the case of *Manilkara zapota* L., total flavonoid content (free and bound) was found to be more in leaves (4.74±0.046 mg/gdw) than in stem bark (2.49±0.022 mg/gdw). Flavonoid content in its bound form was more as compared to the free form in plant parts. Individually, all the isolated flavonoids were more in stem with the highest level of quercetin (1.28±0.017 mg/gdw) followed by kaempferol (0.84±0.007 mg/gdw). Here, maximum flavonoid content was observed in leaves (4.74±0.046 mg/gdw) with a maximum level of quercetin (1.28±0.017mg/gdw) (Table 2).

Gas chromatography-mass spectrometry (GC-MS) analysis of flavonoids

In *Manilkara zapota* L. total thirty-seven compounds were identified in GC-MS analysis of flavonoids from leaves. From which one compound 1,3-Butanedione, 1-Phenyl- found in highest amount with area of percentage 24.79%, at the retention time of 9.155 while one compound 1-Heptacosanol found in lowest amount with area of percentage 0.16% at the retention time of 23.383 (Table 3, Fig 4).

In the present investigation, all the experimental plant parts show the presence of these flavonoids: Kaempferol, luteolin and quercetin. In *Manilkara zapota* L., maximum content of flavonoids was observed in leaves (4.74±0.046 mg/gdw) with a maximum level of quercetin (1.28±0.017 mg/gdw). In GC-MS analysis of flavonoids, total thirty-seven compounds were found from leaves of *Manilkara zapota* L. Maximum area was observed in 1,3-Butanedione, 1-Phenyl- of 24.79%, at retention time of 9.155.

CONCLUSION

From the above investigation, it can be concluded, that *Manilkara zapota* L. are good source of flavonoids. *Manilkara zapota* L. are easily available in rural areas thus they can serve as low-cost supplements for food and medicine as compared to synthetic drugs.

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Table 3 GC-MS profiling of flavonoids isolated from leaves of *Manilkara zapota* L.

S. No.	R. Time	Area	Area%	Compound Name
1.	6.050	237142	0.30	4-(2-Methoxyethyl) phenol
2.	7.683	402688	0.52	2-METHOXY-4-VINYLPHENOL
3.	9.155	19283003	24.79	1,3-BUTANEDIONE, 1-PHENYL-
4.	9.812	272585	0.35	2,5-Pyrrolidinedione, 1-(benzoyloxy)-
5.	13.083	309500	0.40	2,4-Dimethyl-6-phenylpyridine
6.	13.478	814289	1.05	2-BUTEN-1-ONE, 3-AMINO-1-PHENYL-
7.	15.061	956762	1.23	(2-PHENYLCYCLOBUTYL) BENZENE
8.	15.340	681204	0.88	2(4H)-BENZOFURANONE,5,6,7,7A-TETRAHYDRO-6-HYDROXY
9.	18.009	9802608	12.60	n-Hexadecanoic acid
10.	18.366	1925828	2.48	HEXADECANOIC ACID, ETHYL ESTER
11.	19.082	759077	0.98	Hexadecanoic acid, trimethylsilyl ester
12.	19.686	624112	0.80	Dibenzoylmethane
13.	20.398	2166123	2.78	Oleic Acid
14.	20.610	1387096	1.78	n-Propyl 9,12-octadecadienoate
15.	20.675	3086219	3.97	Ethyl Oleate
16.	20.991	969311	1.25	Octadecanoic acid, ethyl ester
17.	22.214	15810287	20.32	Stibine, triphenyl-
18.	23.033	267528	0.34	9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-
19.	23.122	154233	0.20	Bis(dodecanamido)methane
20.	23.383	238019	0.16	1-Heptacosanol
21.	23.488	861415	1.11	Hexanedioic acid, bis(2-ethylhexyl) ester
22.	24.308	808854	1.04	Benzonitrile, m-phenethyl-
23.	24.702	278386	0.36	2-Ethylbutyric acid, eicosyl ester
24.	25.561	4348274	5.59	Bis(2-ethylhexyl) phthalate
25.	25.721	304702	0.39	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
26.	25.924	631456	0.81	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
27.	26.210	296749	0.38	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-
28.	27.107	3381974	4.35	2,4-DIBENZOYL-2,4-DIAMINO NITROBENZOL
29.	27.364	283403	0.36	2,4-DIBENZOYL-2,4-DIAMINO NITROBENZOL
30.	30.498	391078	0.50	Squalene
31.	31.383	517206	0.66	Hentriacontane
32.	31.576	247876	0.32	Cholesta-3,5-diene
33.	34.575	338473	0.44	Stigmast-5-en-3-ol, oleate
34.	34.983	287645	0.37	Cholesterol
35.	35.186	1625845	2.09	Vitamin E
36.	37.884	527975	0.68	Stigmasterol
37.	39.278	2044021	2.63	STIGMAST-5-EN-3-OL, (3. BETA.)-

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