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Original Article

Steroid and Fatty Acid Contents from the Leaves of *Carica Papaya*

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Abstract

Aim: To extract and identify the non-polar entities from the leaves of *Carica papaya*, a plant used for medicinal purpose as folk medicine.

Materials and methods: Petroleum ether extract of the *Carica papaya* leaves was used for this study. Saponification process and methylation process was performed to separate fatty acids and unsaponifiable matters. Phytochemical constituents were separated using chemical process and separated fractions were analyzed by thin layer chromatography (TLC) and gas chromatography coupled with mass spectroscopy (GC-MS).

Results: The chemical composition of the steroids, triterpenoids and fatty acid methyl esters (FAMEs) in leaves of *Carica papaya,* which were analyzed by gas chromatography coupled with mass spectroscopy (GC-MS). A total of 15 fatty acid components were identified in saponifiable matter, from unsaponifiable portion 2 steroids (campesterol, β- or γ-sitosterol), 1 triterpene (squalene), and 1 diterpene (phytol) were identified.

Conclusions: The results indicate that the extract is rich in non-polar compounds. In this study, GC-MS method is at the central focus for identification of these phytoconstituents. The current method can be used for direct analysis of non-polar entities of plant material.

Keywords

campesterol, *Carica papaya*, fatty acid methyl ester, GC-MS, steroid, squalene, phytol, β- or γ-sitosterol

INTRODUCTION

Carica papaya is a plant of the Caricaceae family widely distributed in India. *Carica papaya* is known for its biological activity of ripe fruits, leaves, and seeds. It is used as a herbal medicine in diseases such as dengue, malaria, colon cancer, it relieves nausea, treats chronic skin ulcer, asthma, colic, and eases menstrual pain.[1-5] *Carica papaya* plant has many biological activities such as antibacterial, antifungal, antioxidant, anti-inflammatory, anti-cancer, hepatoprotective, and antidiabetic activity.[6-8] The therapeutic potential of *Carica papaya* plant is due to the presence of varied bioactive compounds such as a lycopene, beta-carotene, beta-cryptoxanthin, glycyl-endopeptidase, caricain, quercetin, kaempferol, benzyl glucosinolate, benzyl iso-

thiocynate, carpaine, pseudocarpaine, dehydrocarpaine I and II, p-coumaric acid, ferulic acid, caffeic acid, papain, chymopapain, protease omega.[9-14] The reported composition of *Carica papaya* fruit shows the presence of vitamins (vitamin A, C, B1, and B2, thiamine, folate, riboflavin, niacin), minerals (potassium in high amount, along with sodium, calcium, phosphorus, zinc, iron, copper, manganese, and magnesium are present in considerable amounts) and fiber contents.[15]

Preliminary phytochemical analysis of *Carica papaya* leaves indicated the presence of more than 50 ingredients; some of these are alkaloids (carpaine, pseudocarpaine, dehydrocarpaine I and II, choline, and methyl derivative of carpaine), glucosinolates (3-indoylmethyl glucosinolate, benzyl glucosinolate), cyanogenic glycosides (R-prunasin, sambunigrin, tetraphyllin B), vitamins (vitamin A, B most especially B12, tocopherols), steroids, saponins, tannins, enzyme (papain, chymopapain, myrosin), terpenoids (linalool, beta-carotene), flavonoids, protein (cystatin), some fermenting agents, fatty acids, and glycosides (rutins, carposide).[16-18] No accurate determination of steroid and fatty acids content in *Carica papaya* has been reported so far. Hence, the present investigation was undertaken to determine the easy, rapid extraction and detection method for steroids and terpenoids from unsaponifiable matter and methyl ester of fatty acids from saponifiable matter in *Carica papaya* leaves.

AIM

For identification of the non-polar component, first, it is necessary to separate lipids from the plant material. Many classical methods and references are readily available for the extraction and identification of steroids and terpenoids from other medicinal plants[19-23]; yet it is necessary to determine a specific method to isolate these molecules or fractions which ought to be standardized.

MATERIALS AND METHODS[24-27]

Chemicals and reagents

Petroleum ether (60°-80°C), methanol (MeOH), potassium hydroxide (KOH), conc. sulphuric acid (H_2SO_4), anhydrous sodium sulphate (Na_2SO_4), acetic anhydride, n-hexane, diethyl ether, glacial acetic acid, chloroform, and benzene were purchased from Merck (India).

Plant materials

Fresh leaves of *Carica papaya* were collected from Rajkot (Gujarat), India in winter, 2019 and were authenticated by the Department of Botany, Shri M. & N. Virani Science College (Autonomous), Rajkot, Gujarat, India. The leaves were prewashed with distilled water to remove dust particles and shed dried for three days at room temperature and grinded to obtain powder (144 gm) for extraction purposes.

Preparation of extract

The leaves powder (20 gm) was extracted with petroleum ether (60°-80°C) by continuous Soxhlet's extraction method for 12 hours (52 cycles). The extract was concentrated and dried under reduced pressure, which yielded a brownish mass (2.3 gm). This crude extract was utilized for further experiment.

Saponification of petroleum ether extract

The crude extract of petroleum ether (2.3 gm) was taken in RBF and 7.5% methanolic KOH (50 ml) was added to it. The resultant mixture was refluxed for 5-6 hours, upon cooling at room temperature; distilled water (20 ml) was added. The mixture was then extracted with diethyl ether, until diethyl ether remains transparent after partition process (liquid-liquid extraction).The combined diethyl ether extract was dried over anhydrous sodium sulphate (Na_2SO_4) and evaporated to obtained unsaponifiable fraction (108 mg) (fraction A).

The aqueous portion left after ether extraction was labeled as saponifiable portion, which was acidified with 5N H_2SO_4 and the aqueous layer was further extracted 3-4 times with diethyl ether. Ether layer was partitioned with distilled water and extractive was dried over anhydrous Na_2SO_4, evaporated to obtain fatty acid portion (144 mg) (fraction B).

Investigation of unsaponifiable fraction (fraction A)

The unsaponifiable fraction (fraction A) was tested with Liebermann-Burchard reagent for the confirmation of steroid and triterpenoids present by thin layer chromatography (TLC F_{254}) with solvent system chloroform: methanol (9.5:0.5) which gave a strong positive test, and then it was analyzed by GC-MS.

Investigation of saponifiable fraction (fraction B)

Methylation of fatty acid

An accurately weighed portion of (fraction B) fatty acid (140 mg) was taken into RBF and refluxed in water bath for 5 hours with solution of methanol: benzene: conc. H_2SO_4 (43:5:2). Cooled the same and diluted with water (40 ml) and extracted with n-hexane until the green color stop extracted. The combined extract was dried over anhydrous sodium sulphate and studied further for GC-MS analysis.

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Instruments

GC-MS analysis was carried out on a Shimadzu GC-MS (model no. TQ8040) sampler and gas chromatograph interfaced with mass-spectrometer (GC-MS) instrument employing the following conditions.

GC-MS protocol for unsaponifiable fraction (fraction A)

SH-Rxi-5Sil MS capillary column (30 m×0.25 mm ID×0.25 µM df), operating in an electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 µl was employed (split ratio of 5:1); injector temperature 250°C; ion-source temperature 290°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 15°C/min, to 180°C, then 5°C/min to 300°C, ending with an isothermal at 300°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da.

GC-MS protocol saponifiable fraction (fraction B)

SH-Rxi-5Sil MS capillary column (30 m×0.25 mm ID×0.25 µM df), operating in an electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 µl was employed (split ratio of 5:1) injector temperature 100°C; ion-source temperature 250°C. The oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of 10°C/min, to 100°C, then 15°C/min to 280°C, ending with an isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da.

RESULTS

Interpretation of GC-MS was conducted using the similarity search from the database of National Institute Standard and Technology (NIST). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST 17 Library having >260,000 patterns.

Analysis of unsaponifiable fraction (fraction A)

The R_f value of the track at 254 nm, 366 nm and post chromatic derivatization with Lieberman-Burchard spray reagent are presented in **Table 1**. It revealed that from sample, 10 components were observed at 254 nm and 5 components were observed on 366 nm at different R_f values. After sprayed with Lieberman-Burchard reagent it was confirmed as a steroid or triterpenoids compounds (**Fig. 1**). An unsaponifiable fraction was injected into GC-MS and two steroids, one triterpene, and one diterpene compound were identified and confirmed the result by GC-MS **(Fig. 2)**. The comparison of obtained mass spectrums with the NIST library database gave us a confirmatory structure of compounds.

GC-MS fragment

The peak at 17.00 minutes had a mass [M+] 296. The daughter ion spectra of this compound (inserts) revealed the characteristic fragments *m/z*: 29, 43, 57, 71, 81, 85, 111, 123, 137, and 196. **(Fig. 3)**

Compound-1: Phytol, M.F.: $C_{20}H_{40}O$, M.W.: 296, RT: 17.00 minutes

GC-MS fragment

The peak at 28.085 minutes had a mass [M+] 410. The daughter ion spectra of this compound (inserts) revealed the characteristic fragments *m/z:* 41, 43, 69, 70, 81, 95, 105, 137, 161, and 189. **(Fig. 4)**

Compound-2: Squalene, M.F.: $C_{30}H_{50}$, M.W.: 410, RT: 28.085 minutes

GC-MS fragment

The peak at 34.230 minutes had a mass [M+] 400. The daughter ion spectra of this compound (inserts) revealed the characteristic fragments *m/z:* 41, 43, 57, 81, 95, 121, 145, 159, 173, 199, 213, 231, 255, 273, 289, 315, 367, and

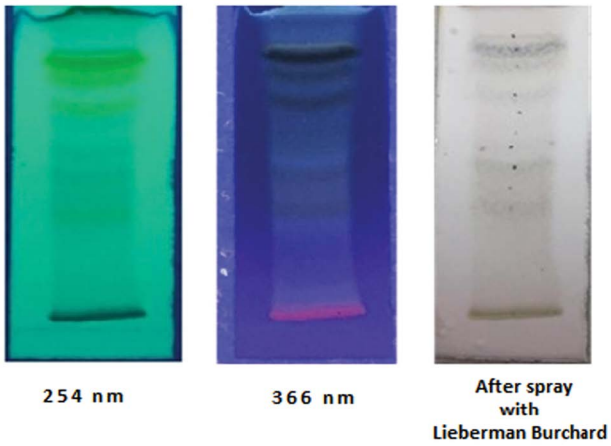

Figure 1. TLC profile of unsaponifiable fraction.

Table 1. TLC analysis of unsaponifiable fraction

	UV light		After spraying of Liebermann–Burchard reagent
	254 nm	365 nm	
R_f value	0.32, 0.37, 0.43, 0.50, 0.56, 0.62, 0.66, 0.75, 0.81, 0.84.	0.32, 0.47, 0.67, 0.77, 0.92.	0.32, 0.37, 0.43, 0.62, 0.66, 0.75, 0.81, 0.84.

382. **(Fig. 5)**

Compound-3: Campesterol, M.F.: $C_{28}H_{48}O$, M.W.: 400, RT: 34.230 minutes

GC-MS fragment

The peak at 35.855 minutes had a mass [M+] 414. The daughter ion spectra of this compound (inserts) revealed the characteristic fragments *m/z:* 41, 43, 57, 81, 95, 107, 119, 145, 159, 173, 199, 213, 231, 255, 303, 329, 381, 396, and 414. **(Fig. 6)**

Compound-4: β- or γ-Sitosterol, M.F.: $C_{29}H_{50}O$, M.W.: 414, RT: 35.855 minutes

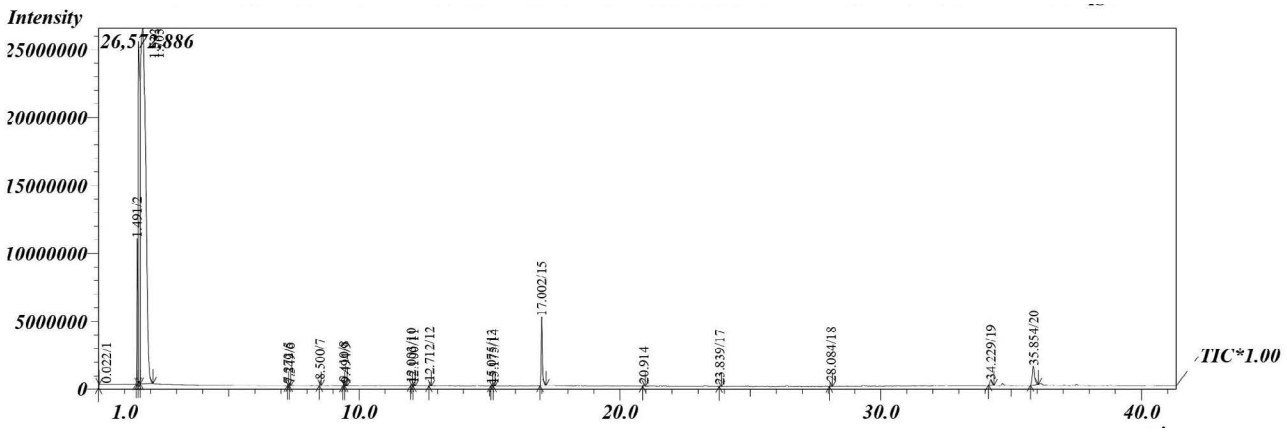

Figure 2. GC chromatogram of unsaponifiable fraction.

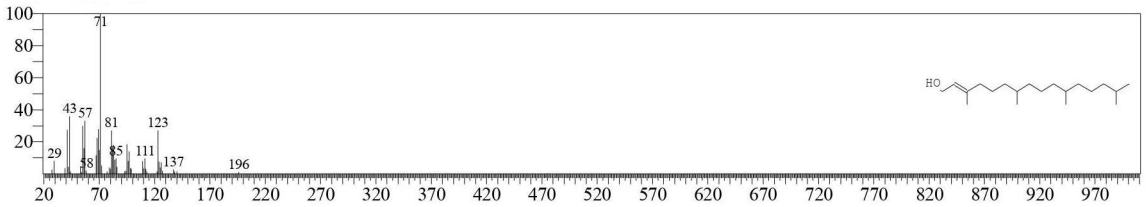

Figure 3. Mass spectra of Phytol.

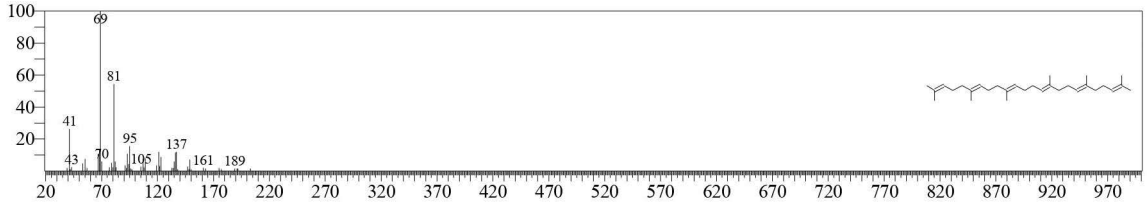

Figure 4. Mass spectra of squalene.

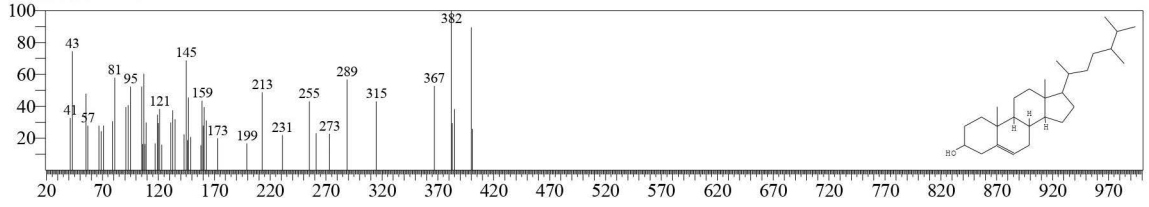

Figure 5. Mass spectra of campesterol.

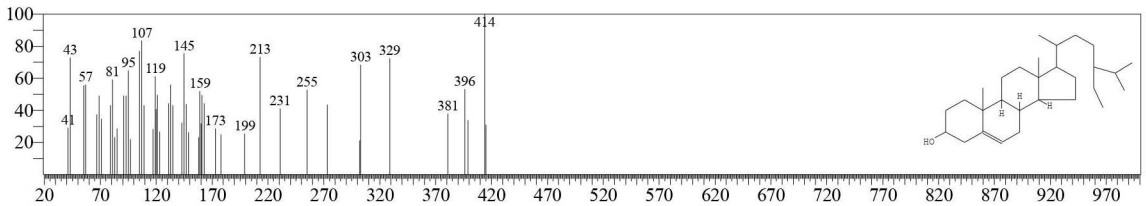

Figure 6. Mass spectra of β- or γ-sitosterol.

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Analysis of saponifiable fraction (fraction B)

The major fatty acid components were identified by noting their retention time and comparing it with the retention time of an authentic sample of methyl ester of fatty acid. GC analysis of the ester residue showed several peaks (**Fig. 7**). **Table 2** shows that 20 compounds were identified in *Carica papaya* leaves. Major FAMEs fraction was identified as 2-methylpentane (11.99%), 3-methylpentane (11.72%), acetyl valeryl (37.49%), methyl-cyclopentane (19.55%), and other identified compounds.

DISCUSSION

Qualitative test of extract gave positive test with Lieberman-Burchard reagent, which suggests the presence of steroid or triterpenoids. TLC results revealed that unsaponifiable load in *Carica papaya* was higher. The unsaponifiable matter was separated through extraction with non-polar solvents like diethyl ether. From the unsaponifiable fraction, one triterpene, one diterpene and two steroid compounds were identified in saponifiable fraction; the mixed fatty acids were liberated by addition of 5N sulphuric acid. It was difficult to separate higher fatty acids through simple chemical methods; hence, they were converted into their methyl esters which were amenable to separation through various chromatographic techniques like adsorption TLC, reversed phase TLC and GC-MS. In the present study, GC-MS technique was employed to study the unsaponifiable and saponifiable matter in the test plant. The comparison of the mass spectrums with the database gave more than 95% match as well as confirmatory compound structure match.

CONCLUSIONS

In the present study, we have extracted and identified fifteen fatty acid components in saponifiable fraction and two steroids (campesterol, β- or γ-sitosterol), one triterpe-

Table 2. Fatty acid methyl ester identified in *Carica papaya* leaves

Peak#	RT	Name	MF	MW	Area %
1	1.710	2-methylpentane	C_6H_{14}	86	11.99
2	1.760	3-methylpentane	C_6H_{14}	86	11.72
3	1.904	Acetyl valeryl	$C_7H_{12}O_2$	128	37.49
4	2.025	Methyl-cyclopentane	C_6H_{12}	84	19.55
5	2.218	Cyclohexane	C_6H_{12}	84	15.40
6	2.357	3-methylene-tridecane	$C_{14}H_{18}$	196	0.06
7	2.405	1,2-dimethyl-cyclopentane,	C_7H_{14}	98	0.10
8	2.462	Heptane	C_7H_{16}	100	1.02
9	2.744	Methyl-cyclohexane	C_7H_{14}	98	0.57
10	2.844	Ethyl-cyclopentane	C_7H_{14}	98	0.05
11	3.002	1,2,3-trimethyl-, (1.alpha., 2.alpha., 3.beta.)-, cyclopentane	C_8H_{16}	112	0.04
12	3.186	2-methyl-heptane	C_8H_{18}	114	0.30
13	3.226	Toluene	C_7H_8	92	0.45
14	3.285	3-methyl-heptane	C_8H_{18}	114	0.23
15	3.422	*cis*-1,3-dimethyl-cyclohexane,	C_8H_{16}	112	0.04
16	3.666	Octane	C_8H_{18}	114	0.50
17	4.835	p-xylene	C_8H_{10}	106	0.11
18	5.248	2-propenoic acid, butyl ester	$C_7H_{12}O_2$	128	0.08
19	21.802	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.06
20	23.610	Methyl ester hexadecanoic acid	$C_{17}H_{34}O_2$	270	0.22
					100.00

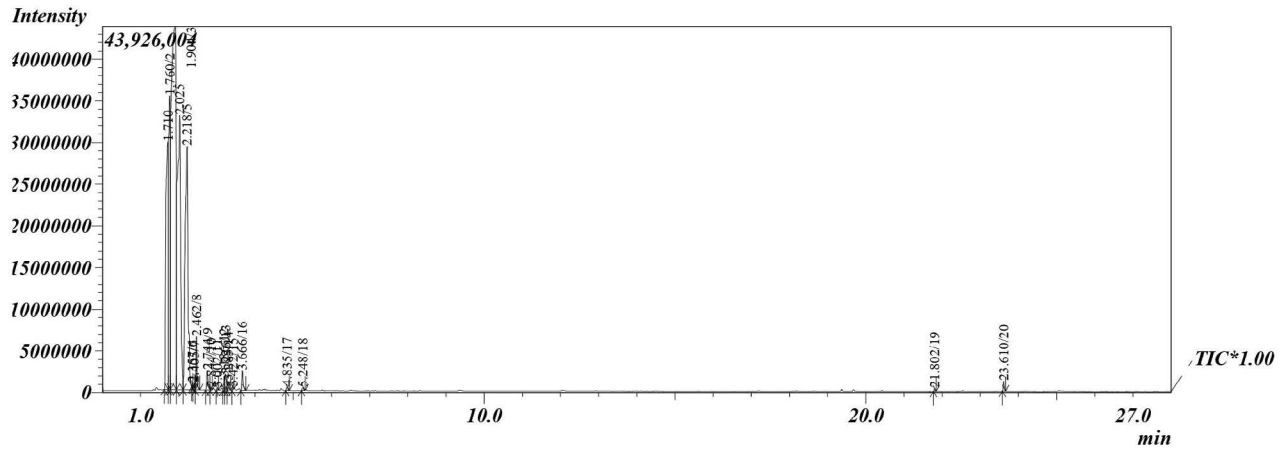

Figure 7. GC chromatogram of saponifiable fraction.

ne (squalene), one diterpene (phytol) from the unsaponifiable fraction of *Carica papaya* leaves; it is the first time reporting in this plant. These results indicate that the extract is rich in non-polar compounds. In this study, GC-MS method is at central focus for identification of these phytoconstituents. This method is a direct and fast analytical approach for identification of non-polar entities of plant material, and also this method can be applied in the analysis of any other low concentration minor component fatty acids in complex sample matrices.

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Conflict of Interest

We declare that we have no conflict of interest.

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Содержание стероидов и жирных кислот в листьях *Carica papaya*

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Резюме

Цель: Извлечь и идентифицировать неполярные вещества из листьев *Carica papaya*, растения, используемого в лечебных целях в народной медицине.

Материалы и методы: В исследовании использовали экстракт петролейного эфира из листьев *Carica papaya*. Процесс омыления и метилирования был проведён для высвобождения жирных кислот и неомыляемого вещества. Фитохимические соединения были разделены с помощью химического процесса, и разделённые фракции были проанализированы с помощью тонкослойной хроматографии (ТСХ) и газовой хроматографии в сочетании с масс-спектроскопией (ГХ-МС).

Результаты: Химический состав стероидов, тритерпеноидов и метиловых эфиров жирных кислот (МЭЖК) в листьях *Carica papaya*, который был проанализирован с помощью газовой хроматографии в сочетании с масс-спектроскопией (ГХ-МС). В омыляемом материале неомыляемой части 2 стероидов (кампестерин, β- или γ-ситостерин), 1 тритерпен (сквален) и 1 дитерпен (фитол) были идентифицированы в общей сложности 15 компонентов жирных кислот.

Заключение: Результаты показывают, что экстракт богат неполярными ингредиентами. В этом исследовании метод ГХ-МС является ключом к определению этих фитонутриентов. Настоящий метод может использоваться для прямого анализа неполярных компонентов растительного материала.

Ключевые слова

кампестерин, *Carica papaya*, метиловый эфир жирных кислот, GC-MS, стероид, сквален, фитол, β- или γ-ситостерин