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Phytochemical analysis of *Citrus limetta* using High-Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS) and FTIR

Análisis fitoquímico de *Citrus limetta* mediante la cromatografía líquida con espectrometría de masa de alta resolución (HR-LCMS) y espectroscopía infrarroja por transformada de Fourier (FTIR)

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ABSTRACT

Sweetlime peel (*SLP*) is a rich source of bioactive chemicals. The nutraceutical sector claims that a large range of bioactive secondary metabolites produced by sweet lime peel can be exploited to develop novel pharmaceuticals and functional foods. It has been proven that it is critical to analyze complicated bioactive substances employing analytical methods such as HR-LCMS. The current project's purpose was to use HR-LCMS and FTIR to investigate the bioactive metabolites contained in *SLP* methanolic extract. Investigating the bioactive components of *SLP* using Q-TOF LC/MS and Agilent MassHunter Profiler software revealed 18 flavonoid compounds along with polyphenols like rutin, 4',5,7-Trihydroxy-3-methoxyflavanone, Curcumin diglucoside, carotenoid, Coumeroic acid, coumaric acids, flavonoid-7-o-glycosides, and Gardenin B that have anticancer, antimicrobial, anti-inflammatory, and antitumor activity. The results of the present study confirm the presence of important phytocompounds in *SLP* and are useful for further in-depth research to create functional foods from *SLP* to prevent a variety of ailments. Current research aims to discover all-natural therapies for a variety of illnesses and conditions.
Keywords: Sweet lime peel, HR-LCMS, FTIR, Bioactive compounds.

RESUMEN

La cáscara de lima dulce (CLD) es una rica fuente de compuestos bioactivos. El sector nutracéutico afirma que se puede explotar una amplia gama de metabolitos secundarios bioactivos, que son producidos por la piel de lima dulce, para desarrollar nuevos productos farmacéuticos y alimentos funcionales. Se ha demostrado que es fundamental analizar sustancias bioactivas complicadas empleando métodos analíticos como HR-LCMS. El propósito del presente proyecto es utilizar HR-LCMS y FTIR para investigar los metabolitos bioactivos contenidos en el extracto metanólico de CLD. La investigación de los componentes bioactivos de la CLD mediante LC/MS Q-TOF y el software *Agilent MassHunter Profiler* reveló 18 compuestos flavonoides junto con polifenoles que tienen actividad anticancerígena, antimicrobiana, antiinflamatoria y antitumoral como la rutina, la 4',5,7-trihidroxi-3-metoxiflavona,

el diglucósido de curcumina, los carotenoides, el ácido cumeroico, los ácidos cumáricos, los flavonoides-7-o-glucósidos y la Gardenina B. Los resultados del presente estudio confirman la presencia de importantes fitocompuestos en la CLD. Estos resultados son útiles para seguir investigando en profundidad con el fin de crear alimentos funcionales a partir de la CLD para prevenir diversas dolencias. La investigación actual tiene como objetivo descubrir terapias completamente naturales para diversas enfermedades y afecciones.
Palabras clave: cáscara de lima dulce, HR-LCMS, FTIR, componentes bioactivos.

INTRODUCTION

*Citrus limetta, a*lso known as sweet lime or mosambi, is a citrus fruit that also contains vitamins, minerals, dietary fibers, and a variety of bioactive secondary metabolites. These bioactive compounds include flavonoids, volatile oils, limonoids, coumarins, alkaloids, sterols, and carotenoids, among others (Favela 2016), Citrus flavonoids displayed antioxidant qualities by scavenging free radicals (Zou 2016). In comparison to *Salmonella typhi* and *S. typhimurium*, hesperetin demonstrated a sizable antibacterial impact (Kawaguchi 2004). Apoptosis, antiproliferative activity, and selective cytotoxicity appear to be the mechanisms by which limonin exerts its anticancer effects (Ke 2015).

The conventional methods for characterizing bioactive chemicals include extraction, analysis, chromatographic separation, and spectroscopic identification. To discover the functionally effective bio-actives and make it easier to comprehend their impact on the target, it is essential to decipher the intricate chemistry of bioactive crude extracts utilizing high throughput and high-resolution techniques (Marulasiddaswamy *et al*. 2021). Using HRLC-MS and FTIR analysis, the current study attempted to examine the phytoconstituents present in *SLP*.

MATERIALS AND METHODS

Chemicals: All the chemicals and reagents used for the research were of analytical grade and were procured from the reputed manufacturers.

Raw material collection and Preparation of extract: Sweet Lime Peel (*SLP*) were air-dried at room temperature and pulverized into powder for extraction. The powder (100 g) was extracted by Microwave (*Make Microsynth*) at 750 W in methanol (1:10 Solid-Solvent ratio) at 60 degree C and for 20 min. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator under reduced pressure to get an orange-yellow semi-solid extract, then the extract was stored at 4 degree C till further analysis.

HR-LCMS analysis of SLP: G6550A MS Q-TOF was applied in a positive and negative mode with Dual AJS ESI Ion source. *Phenomenex Synergi* Polar-RP, 150 × 3 mm, 4 µm at 35 °C was used for the separation of phenolic compounds, and the flow rate was set at 0.300 mL/min. An aliquot of 5 µL from the extract was injected. Mobile phase (A) taken was 0.1% formic acid in water and mobile phase (B) taken was 95% acetonitrile with 0.1% formic acid. A full scan mode was reached in the range of 100–1000 amu and the conditions maintained were as following; capillary voltage (3500 V), nozzle voltage (1000 V), nitrogen gas flow rate (13 L/min) at 300^{o}C and nebulization was

set as 35 psig. Agilent Mass Hunter Work station Software (LC/MS Data Acquisition for 6200 series TOF/6500 series Q-TOF) was used for extraction and identification of phenolic compounds present.

Identification of polyphenols by LC–MS/MS analysis: The Identification of the chemical nature of extract was done by HRLC–MS/MS analysis; it was based upon the method described by (Akdeniz *et al*. 2018). The compounds obtained were listed and transferred automatically to MS/MS analysis in a further Q-TOF LC/MS analysis.

FTIR analysis of SLP: Shimadzu spectrometers were used to study FTIR (IRAffinity 1S) , 2.0 mg of sample was combined with 200 mg of spectroscopic grade KBr before being compressed into a disc at 10 MPa for 3 minutes. The resulting spectra were collected with a resolution of 4 cm-1 in the 4000-400 cm-1 region. The obtained peaks and their associated functional groups were recognized. Peak values of the FTIR were recorded.

RESULT AND DISCUSSION

High Resolution-Liquid Chromatography-Mass spectrometry analysis (HR-LCMS) of SLP: Chromatogram displays in Fig. 1 and 2 the approximate concentrations of several compounds that are present in *SLP* and are eluted in accordance with the retention time and polarity. The height of the peak was used to calculate the relative concentration of the bioactive chemicals found in the plants. The molecules that were eluted at different times are examined by the mass spectrometer to ascertain the make-up and structure of the compounds. These mass spectra indicate the unique pattern of the molecule in the data base. The mobile phase was augmented with formic acid to optimize peak resolution. It was discovered that each resolved peak might represent many phytocompounds due to homology in polarity and chemical properties brought on by retaining the same retention time. Agilent iFunnel technology used electrospray to separate the contents, creating different ions fragments that were then concentrated using Agilent Jet Stream system for effective ion sampling and transmission. Table 1 lists the polarity, mass, and chemical formula for each molecule. Based on these values, 24 compounds were found in the methanolic extract of *SLP* having phytochemical properties.

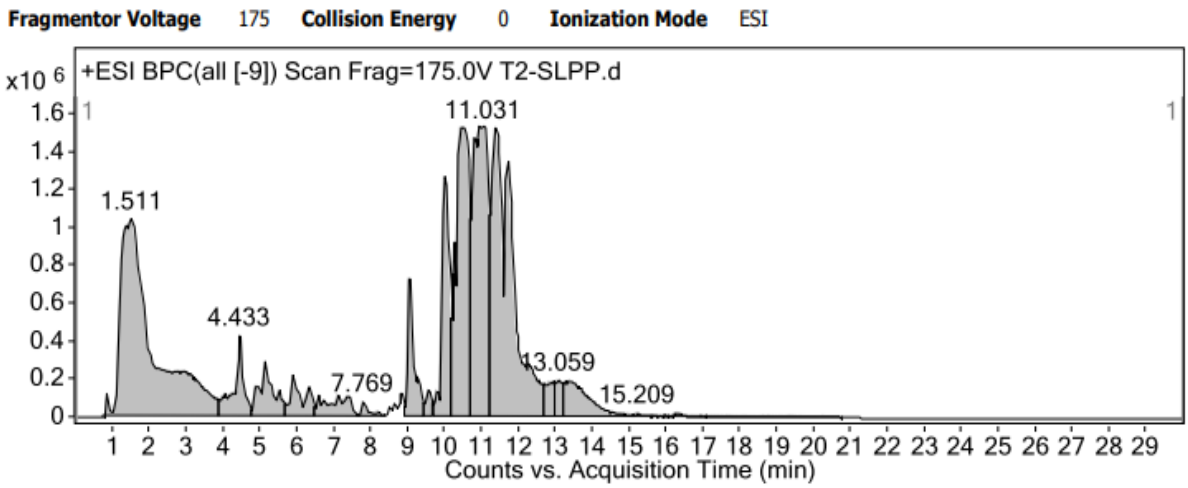

Figure 1 LC-MS analysis of MESLP at positive polarity

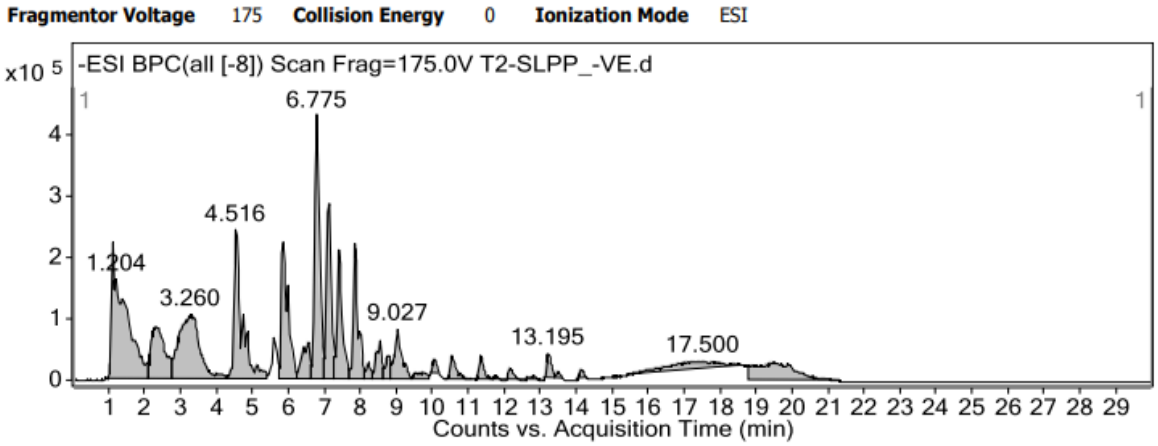

Figure 2 LC-MS analysis of MESLP at negative polarity

According to the HR-LCMS investigation, the primary compounds were predicted to belong to a variety of secondary metabolite categories, including alkaloids, steroid, fatty acid, ester, phenolic acids, triterpenoids, polypeptides, glycoside, coumarin, flavones, flavonols, diterpenoid, and hydroxycinnamic acid. The different mass spectra of the *SLP* bioactives that were identified are shown in Figure 3.

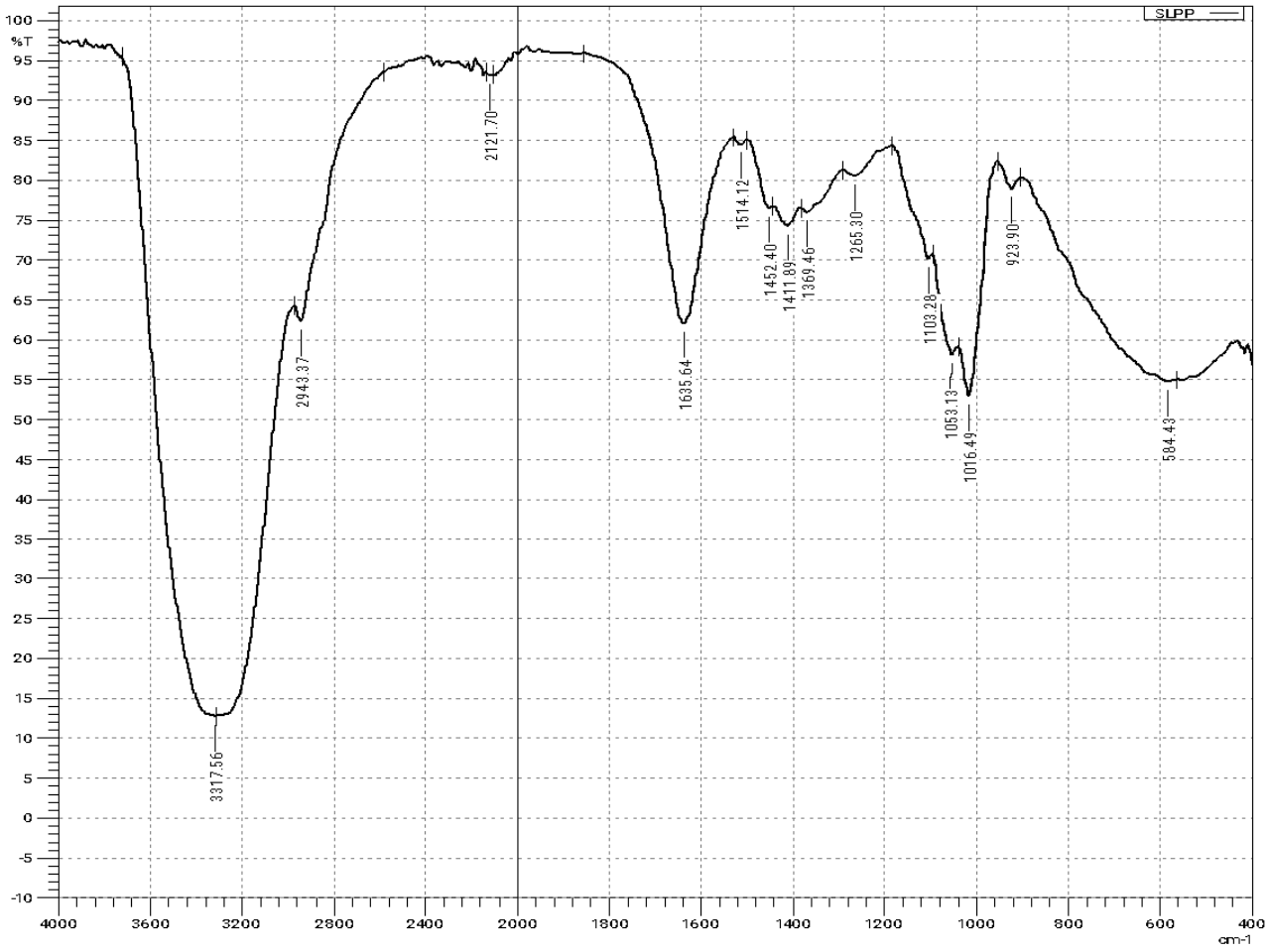

Figure 3. FTIR mass spectra of SLP

FT-IR analysis of SLP (Table 2): FTIR analysis confirmed the existence of bioactive compounds that were suggested by HR- LCMS investigations. The functional groups included in the methanol extract of *SLP* were identified using a range of peaks discovered by FTIR analysis, as illustrated in Figure 3. The functional group of the active components was measured using the FTIR spectrum based on the peak value in the infrared radiation band (Skoog *et al.,* 2017).

SLP methanolic extract's FTIR study shows distinct peaks at 3317.56 due to presence of O–H stretching of polyphenolic alcohols, peak at 2943.37 indicates C–H stretching of Alkanes, C≡C stretching at 2121.7 due to Alkyne. Presence of Imine and Oxime (C=N stretching) create the peak at 1635.64, peak at 1452.4 and 1514.12 due to presence of Nitro compound at , N-O stretching. peak at 1411.89 indicates O-H bending of Carboxylic acid .Peaks at 1016.49 ,1053.13, 1103.28,1265.3 C-O stretching of Alcohol, Ether, Ester, Carboxylic Acid, Anhydride, the peak at 584.43 (C-Br stretching) suggest the presence of Halo compounds.

Table 1. Chemical ingredients of Methanolic extract of SLP by HRLC-MS

n	Compound Name	Formula Ion	polarity	mass	m/z	Compound nature
1	Sakuranetin	$C_{16}H_{14}O_5$	Positive	286.084	287.091	Flavones and flavonols
2	4',5,7-Trihydroxy-3-methoxyflavanone	$C1_6H_{14}O_6$	Positive	302.078	303.086	Flavones and flavonols
3	4-Feruloyl-1,5-quinolactone	$C_{17}H_{18}O_8$	Positive	350.105	373.094	Phenolic acids
4	Gardenin B	$C_{19}H_{18}O_7$	Positive	358.104	359.111	Flavones and flavonols
5	Sinensetin	$C_{20}H_{20}O_7$	Positive	372.122	373.129	Flavones and flavonols
6	2'-Hydroxy-3,4',5',7,8-pentamethoxyflavone	$C_{20}H_{20}O_8$	Positive	388.115	389.122	Flavones and flavonols
7	7-Hydroxyflavanone beta-D-glucopyranoside	$C_{21}H_{22}O_8$	Positive	402.131	403.139	Flavones and flavonols
8	2-(2,5-Dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-1-benzopyran-4-one	$C_{21}H_{22}O_8$	Positive	402.135	403.143	Flavones and flavonols
9	5,6,7,8,3',4',5'-Heptamethoxyflavone	$C_{22}H_{24}O_9$	Positive	432.147	433.156	Flavones and flavonols
10	Kaempferol 3-rhamnoside 7-xyloside	$C_{26}H_{28}O_{14}$	Positive	564.146	565.153	Flavones and flavonols
11	Rutin	$C_{27}H_{30}O_{16}$	Positive	610.151	611.159	Flavones and flavonols
12	Scoparin 2''-glucoside	$C_{28}H_{32}O_{16}$	Positive	624.167	625.174	Flavones and flavonols
13	Curcumin diglucoside	$C_{33}H_{40}O_{16}$	Negative	692.243	737.241	Phenolic compound
14	Poncirin	$C_{28}H_{34}O_{14}$	Negative	594.204	593.197	Flavones and flavonols
15	(S)-Naringenin 8-C-(2''-rhamnosylglucoside)	$C_{27}H_{32}O_{14}$	Negative	580.187	579.180	Flavones and flavonols
16	b-D-fructosyl-a-D-(6-O-(E))-feruloylglucoside	$C_{21}H_{28}O_{12}$	Negative	472.164	471.157	Phenolic compound
17	Isorhamnetin 3-O-[b-D-glucopyranosyl-(1->2)-a-L-rhamnopyranoside]	$C_{28}H_{32}O_{16}$	Negative	624.176	623.169	Flavones and flavonols
18	Leucodelphinidin 3-[galactosyl-(1->4)-glucoside]	$C_{27}H_{34}O_{18}$	Negative	646.173	645.168	Flavones and flavonols
19	Allivicin	$C_{27}H_{30}O_{16}$	Negative	610.160	609.152	Flavones and flavonols
20	3-Hydroxy-b,e-caroten-3'-one	$C_{40}H_{54}O_2$	Negative	566.412	611.412	Tetraterpenoids
21	Nicotiflorin	$C_{27}H_{30}O15$	Negative	594.165	593.157	Flavones and flavonols
22	Kaempferol 3-rhamnoside 7-xyloside	$C_{26}H_{28}O_{14}$	Negative	564.154	563.147	Flavones and flavonols
23	Coumeroic acid	$C_{17}H_{14}N_2O_7$	Negative	358.076	417.090	Hydroxycinnamic acid,
24	m-Coumaric acid	$C_9H_8O_3$	Negative	164.046	223.060	Hydroxycinnamic acid,

Table 2. FTIR Absorption Frequencies for Functional Groups in SLP

Sr.No.	Peak	Area	Bond	Functional Group	Frequency in cm−1 (Intensity*)
1	584.43	12078.152	C-Br stretching	Halo compound	690-515 (s)
2	923.9	952.072	C=C bending	Alkenes	1000-650 (s)
3	1016.49	2980.001	C-O stretching	Alcohol, Ether, Ester, Carboxylic Acid, Anhydride	1300–1000 (s)
4	1053.13	2106.879			
5	1103.28	2055.975			
6	1265.3	1884.609			
7	1369.46	2027.386	S=O stretching	Sulfonamide	1370-1335 (s)
8	1411.89	1469.731	O-H bending	Carboxylic acid	1450 and 1375 (m)
9	1452.4	1102.912	N-O stretching	Nitro compound	1550 and 1350 (s)
10	1514.12	467.542			
11	1635.64	5642.94	C=N stretching	Imine and Oxime	1690–1640 (w-s)
12	2121.7	195.247	C≡C stretching	Alkyne	2140-2100 (w-m)
13	2943.37	7377.03	C–H stretching	Alkane	3000–2850 (s)
14	3317.56	22985.604	O-H stretching	PolyHydroxy compounds	3400–3200 (m-s)

(s = strong; m = medium; w = weak) Kennepohl et al., 2020.

As conclusion, the effective method to make functional food uses secondary metabolites made from natural sources. The phytochemical assessment of the current study has shown that *SLP* could be a source of beneficial substances. Using FTIR analysis and HR-LCMS high-resolution liquid chromatography, it was discovered that the methanolic extract of *SLP* contained functionally significant bioactive substances such as alkaloids, steroid, fatty acid, ester, phenol ethers, hydroxycinnamic acid, triterpenoids, polypeptides, glycoside, coumarin, flavonoid, diterpenoid, and flavons. The results of the experiments indicate that *SLP* are an excellent source of biogenic compounds with both structural and functional activity. The research is currently being done to segregate the components and utilize the phytochemical properties as functional diets with nutraceutical effects.

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CONFLICT OF INTEREST: There are no conflicts of interest from the authors.

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