Microwave Assisted Extraction of Citrus limetta Peel and assessment of its Bio-Actives Using HRLC-MS/MS

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Abstract

*Citrus limetta*, often known as sweet lime, is one of India's most important crops due to its high demand in the agro-processing industry. Peel of *Citrus limetta* has significant levels of polyphenolic chemicals that have pharmacological effects. In current study, the peel is valued for the bioactive compounds it contains. The total phenolic content (TPC) was 16.66 mg GAE/g with an extraction yield of 12.91%. Radical scavenging activity (RSA) was 78.05%, total flavanoid content (TFC) was 9.59 mg QE/g, and total phenolic content (TPC) was 9.59 mg QE/g. Sweet lime peel (SLP) extract was obtained by microwave assisted extraction (MAE) in methanol. LC-MS/MS results showed 18 flavonoid compounds along with polyphenols like rutin, curcumin diglucoside, carotenoid, coumeroic acid, coumaric acids, flavonoid-7-o-glycosides, and Gardenin B that embrace anticancer, antimicrobial, anti-inflammatory, and antitumor activity. This method uses Q-TOF LC/MS with Agilent MassHunter Profiler software to investigate bioactive components in sweet lime peel.

Introduction

In the pharmacy sector, "extraction" refers to the process of separating the medicinally active components of plant or animal tissues from the inert or inactive ones using specified solvents and established extraction processes. Microwaves are electromagnetic fields that range in frequency from 300 MHz to 300 GHz. The temperature, mass of food, water content, density, physical shape, thermal characteristics, electrical conductivity, and dielectric properties of molecules are all affected by the frequency, power, and speed of microwave heating. There is no heating when the frequency is less than 2450 MHz and the electrical component changes at a much slower rate. MAE is widely used in the extraction of organic and organometallic compounds; it uses water or alcohol as a solvent at elevated temperatures and pressures.[1]. Sweet lime (*Citrus limetta*) is also known as *Mosambi* which contains a wealth of different classes of bioactive secondary metabolites in addition to vitamins (principally vitamin C), minerals, and dietary fibers, it promotes good health, as presence of many bioactive components such as flavonoids, volatile oils, limonoids, coumarins, alkaloids, sterols, and carotenoids[2]. By scavenging free radicals, citrus flavonoids demonstrated anti-oxidant properties[3]. Hesperetin had quite considerable antimicrobial effects against *Salmonella typhi* and *S. typhimurium*[4]. Apparently anticancer effects of limonin are delivered through selective cytotoxicity, antiproliferative activities, and apoptosis,[5]. Liquid chromatography/Mass Spectroscopy (LC/MS) is technique that combines high performance liquid chromatography (HPLC), a powerful analytical separation technique, with mass spectroscopy, a powerful analysis and detection tool. The Current Research work mainly focuses on the extraction of sweet lime peel by using novel MAE methodology and the quantification of different Bio-actives present in the extract by using high resolution liquid chromatography with mass spectroscopy.

Materials And Methods

Chemicals
Acetonitrile, formic acid, FolinCiocalteu reagent, 2, 2-diphenyl-1-picrylhydrazil were procured from Sigma-Aldrich, 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) is a waste product that was collected from various regions of Aurangabad City and cleansed with a Na$_2$CO$_3$ (5%) solution before being air-dried at room temperature and ground into powder for extraction. The powder (100 g) was extracted in methanol (1:10 Solid-Solvent ratio), at 60 °C, for 20 minutes using a microwave extractor (Make Microsynth). The mixture was filtered using Whatman No. 1 filter paper, and the filtrate was then concentrated using a rotary evaporator under decreased pressure to yield an orange-yellow semi-solid extract. The extract was then stored at 4 °C for additional analysis. The% yield was computed as follows:

\[
\text{%Yield of extract (g/100 g) = (weight of the extract residue after solvent removalx 100) / weight of dried powder}^{[6]}
\]

**Preliminary phytochemical analysis of SLP Extract**

Phytochemical analysis of the SLP extract was performed to detect the presence of different classes of secondary compounds, including alkaloids, phenolic, flavonoids, tannins, saponins, terpenes and glycosides \[^7\].

**Antioxidant Activity of SLP Extract**

The total antioxidant activity of MESLP was measured in terms of the percentage of radical scavenging activity using 2,2-diphenyl-1-picrylhydrazil solution (1 mg/mL) was made by dissolving DPPH in methanol. The DPPH solution was diluted to 5 mL and the absorbance was calculated at 517 nm in a UV-Spectrophotometer \[^8\].

The following formula was used to determine the antioxidant activity.

\[
\text{% Free Radical scavenging} = \frac{\text{(Absorbance of Control } - \text{ Absorbance of sample)}}{\text{Absorbance of Control}}
\]

**Determination of Total Phenolic Content**

The Folin Ciocalteu reagent (FC Reagent) was used to calculate the total phenolic content. The standard for the calibration curve was gallic acid. The total phenolic content was expressed as gallic acid equivalents (mg/g) \[^9\].
Determination of Total Flavonoids Content

The total flavonoid concentration was assessed using a colorimetric assay. The standard for the calibration curve was Quercetin. The total flavonoids in the extract were estimated as mg/g of Quercetin equivalents $^{[10]}$.

HRLC-MS/MS Characterization of Phenolic Compounds

G6550A MS Q-TOF was applied in a positive and negative mode with Dual AJS ESI Ion source. *Phenomenex Synergy* 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 (13L/min) at 300°C and nebulization was set as 35 psig. Agilent MassHunter Workstation 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 $^{[11]}$. The compounds obtained were listed and transferred automatically to MS/MS analysis in a further Q-TOF LC/MS analysis.

Statistical analysis

All experimental results were obtained in triplicate and the average values of the results were mentioned as mean ±SD.

Results And Discussion

Phytochemical screening of methanolic extract of SLP

The preliminary phytochemical screening of methanolic extract of SLP revealed the presence of alkaloids, flavonoids, Phenolic compounds and tannins, steroids, glycosides and terpenoids which were shown in Table 1.

Table 1: Phytoconstituents of methanolic extract of SLP
<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Phytoconstituents</th>
<th>Test</th>
<th>Observations</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Dragendorff's test</td>
<td>Orange red precipitate</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayer's test</td>
<td>White precipitate</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hager's test</td>
<td>Yellow precipitate</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>Alkaline reagent test</td>
<td>Deep yellow turn</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shinod's test</td>
<td>colorless</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phenolic compounds and</td>
<td>Ferric chloride test</td>
<td>Deep blue color</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>tannins</td>
<td>Lead tetra acetic acid test</td>
<td>Precipitate</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Glycosides</td>
<td>Keller Killiani test</td>
<td>Deep blue color</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Saponins</td>
<td>Foam Test</td>
<td>Foam appears</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Terpenoids</td>
<td>Horizon test</td>
<td>Red precipitate</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Steroids</td>
<td>Salkowski test.</td>
<td>Red color</td>
<td>+</td>
</tr>
</tbody>
</table>

**Extraction yield**

The extraction yield of crude methanolic extract of SLP was found to be 12.91 %, high yield of extraction was due to migration of targeted compounds from the matrices of the peel cells to the surroundings at a more rapid rate due to microwave energy and solvent selected, MAE generates direct heat which results in faster diffusion rate of compounds into solvent [12].

**TPC and TFC of MESLP**

The results for TPC and TFC suggested that MESLP contains fair amount of phenolic and flavonoid substances. The proportion of TPC and TFC was 16.66 ±0.05 mg GAE/g and 9.59 ±0.08 mg QE/g of extract respectively as shown in Table 2.

**DPPH scavenging assay**

Based on MESLP’s capacity to neutralize stable free radicals by donating an electron or hydrogen, its potential antioxidant activity was assessed [13] as shown in Table 2.

**Table 2:** TPC, TFC and free radical scavenging activity of methanolic extract of SLP
% Extraction Yield  | TPC mg GAE/g  | TFC mg QE/g  | % Free radical scavenging activity  
--- | --- | --- | ---  
12.91±0.04  | 16.66± 0.05  | 9.59± 0.08  | 78.05±0.07  

(Results are mean ± SD of 3 determinations)

**HRLC–MS/MS analysis of MESLP**

The results of the phytochemical study were based on qualitative analysis, which revealed that MESLP has significant biological activities. As a result, the next procedure was to follow the right methods to identify the chemical components of the fraction that were present. For understanding the chemical content of extracts, LC-MS/MS analysis is recognized as a powerful analytical method. Identification was based on retention time and standard comparison. The LC-MS/MS analysis of the MESLP data provided in Table 3 revealed the presence of 18 flavones and flavonols as well as phenolic acids and hydroxycinnamic acid.

The chemical profile of MESLP was determined by HRLC-MS/MS analysis is shown in fig 1 and 2 founded on the polarity of compounds. Based on their high-resolution mass, UV spectra, retention times, and MS fragmentation patterns, 18 of the chemicals found in the MESLP extract were identified as flavones and flavonols along with phenolic acids, and hydroxycinnamic acid. The Pharmacological action of identified compound from SLP is given in Table 4.

Table 4. Pharmacological action of identified compound from SLP
<table>
<thead>
<tr>
<th>Sr No</th>
<th>Compound</th>
<th>Pharmacological action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>4',5,7-Trihydroxy-3-methoxyflavanone</td>
<td>Anticarcinogenic</td>
<td>[16]</td>
</tr>
<tr>
<td>3.</td>
<td>4-Feruloyl-1,5-quinolactone</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>4.</td>
<td>Gardenin B</td>
<td>Treating hypertension</td>
<td>[17]</td>
</tr>
<tr>
<td>5.</td>
<td>Sinensetin</td>
<td>Anti-inflammatory, Suppresses the Progression of pulmonary fibrosis.</td>
<td>[18]</td>
</tr>
<tr>
<td>6.</td>
<td>2'-Hydroxy-3,4',5',7,8-pentamethoxyflavone</td>
<td>Antioxidant Activity</td>
<td>[19]</td>
</tr>
<tr>
<td>7.</td>
<td>7-Hydroxyflavanone beta-D-glucopyranoside</td>
<td>Anticarcinogenic Activity</td>
<td>[20]</td>
</tr>
<tr>
<td>8.</td>
<td>2-(2,5-Dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-1-benzopyran-4-one</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>9.</td>
<td>5,6,7,8,3',4',5'-Heptamethoxyflavone</td>
<td>Anticarcinogenic Activity</td>
<td>[21]</td>
</tr>
<tr>
<td>10.</td>
<td>Kaempferol 3-rhamnoside 7-xylsode</td>
<td>Anti-Inflammatory, Anticancer</td>
<td>[22]</td>
</tr>
<tr>
<td>11.</td>
<td>Rutin</td>
<td>Antioxidant, Cytoprotective, Vasoprotective, Anticarcinogenic Activity, Neuroprotective and Cardio protective Activity</td>
<td>[23] [24] [25]</td>
</tr>
<tr>
<td>12.</td>
<td>Scoparin 2''-glucoside</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>13.</td>
<td>Curcumin diglucoside</td>
<td>Antibacterial</td>
<td>[26]</td>
</tr>
<tr>
<td>14.</td>
<td>Poncirin</td>
<td>Anti-Inflammatory</td>
<td>[27]</td>
</tr>
<tr>
<td>15.</td>
<td>(S)-Naringenin 8-C-(2''-rhamnosylglucoside)</td>
<td>Treatments of Osteoporosis, Effective against cardiovascular, gastrointestinal, rheumatologic disorders</td>
<td>[28]</td>
</tr>
<tr>
<td>16.</td>
<td>b-D-fructosyl-a-D-(6-O-(E))-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>No.</td>
<td>Compound</td>
<td>Activity</td>
<td>Reference</td>
</tr>
<tr>
<td>-----</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>17.</td>
<td>Isorhamnetin 3-O-[β-D-glucopyranosyl-(1-&gt;2)-α-L-rhamnopyranoside]</td>
<td>Anti-diarrheal activity</td>
<td>[29]</td>
</tr>
<tr>
<td>19.</td>
<td>Allivicin</td>
<td>Anti-inflammatory, Anticancer</td>
<td>[31]</td>
</tr>
<tr>
<td>20.</td>
<td>3-Hydroxy-b,e-caroten-3′-one</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>22.</td>
<td>Kaempferol 3-rhamnoside 7-xylloside</td>
<td>Antimalarial, Anti-Inflammatory, Antiparasitic, Anticancer</td>
<td>[21]</td>
</tr>
<tr>
<td>23.</td>
<td>Coumeroic acid</td>
<td>Antimicrobial Activity, Antioxidant, Anticancer Activity</td>
<td>[33]</td>
</tr>
<tr>
<td>24.</td>
<td>m-Coumaric acid</td>
<td>Antioxidant, Anti-Thrombosis</td>
<td>[34]</td>
</tr>
</tbody>
</table>

**Pharmacological Activity not reported.**

**Conclusion**

An Agilent 6550 iFunnel Q-TOF LC/MS and a collection of Agilent MassHunter statistical and qualitative analytical software tools were used to evaluate MESLP. An untargeted screening method produced the desired results, identifying 18 flavone and flavonol compounds, 3 phenolic moieties, and 2 hydroxycinnamic acids with bioactive components that have antioxidant, anticancer, anti-inflammatory, and neuroprotective properties that may be valorize in future functional foods.

**Abbreviations**

FTIR: Fourier-transform infrared spectroscopy

GAE: Gallic Acid Equivalent

HRLC-MS: High performance liquid chromatography and Mass Spectroscopy

MAE: Microwave Assisted Extraction

MESLP: Methanolic Extract of Sweet lime peel

QE: Quercitin Equivalent
Declarations

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Consent for publication: All authors give consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text (“Material”) to be published in the above Journal and Article

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' contributions: Deo S. K. has made a substantial contribution to the concept or design of the article, Sakhale B. K. has revised it critically and approved the version to be published;

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Data Availability: Data analyzed in this study were a re-analysis of existing data, which are openly available at locations cited in the reference section

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### Table

Table 3 is available in the Supplementary Files section

### Figures

**Figure 1**

*LC-MS analysis of MESLP at positive polarity*
Figure 2

**LC-MS analysis of MESLP at negative polarity**

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Table3.docx