Research Article

Quality Assessment of Bioactive Components in *Citrus Medica L.Var.Limetta* Leaves by Gas Chromatography: Mass Spectroscopy

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Abstract

Higher plants are rich source of novel natural substances that can be used to develop environmental safe insect control. Citrus leaves are excellent sources of antimicrobial and anti-fungicidal substances. Citrus leaves also consists main volatile components and these compounds, not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of applications in the agriculture industries**.** However, the compounds in leaves of *Citrus limetta* have biological value that cannot be underestimated. An essential part in this investigation is the identification of the biologically active compounds present in leaves of *Citrus limetta* which leads to further antimicrobial and insecticidal studies. This study aimed to investigate and characterize the chemical composition in the methanol extracts of *Citrus medica L*.var.*limetta* leaves by GC-MS technique. Gas chromatography–mass spectrometry revealed the presence of 32 different types of high and low molecular weight chemical entities with varying quantities in the methanol extract. Furthermore, it possess unique physicochemical characteristics which may be attributed to the compounds naturally present in significant quantities in the leaves of *C.limetta* that warrants further biological studies.

Keywords: *Citrus limetta*, gas chromatography, α-terpineol, eucalyptol, mass spectroscopy

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Introduction

Recently, natural plants are getting ample attention of researchers for sources of biologically active substances like antioxidants. A vast study is already being carried out on several plants, vegetables and fruits which are rich sources of antioxidants, vitamins, carotenoids, polyphenolic compounds and flavonoids which reported to prevent free radical damage, reducing the risk of chronic diseases [1]. During normal metabolic activities in a human cell, various physiological and biochemical reactions produce free radicals and other reactive oxygen species (ROS) as the by-products. Many these plants species are reported to synthesize certain bioactive compounds. These secondary metabolites are important medicinal properties which include antioxidant activity, free radical scavenging abilities, anti-carcinogenic, anti-inflammatory [2] and anti microbial and insecticide action.

Citrus medica L.var. *limetta* is a common variety of *Citrus medica* plant and is regarded as ethnopharmacological imperative plants in Asian countries. Its leaf and peel provide a unique aroma oil and flavor. In addition, it is believed to contain lots of bioactive compounds which are responsible for the medicinal effect on sore throat, cough and sputum. In contrast to its wide applications, it is not widely utilized due to limited availability which leads to few studies of *Citrus limetta*. Occurrence of new compounds in *Citrus limetta* exhibited the antioxidant activities during storage [3]. Experimental and pharmacological studies indicated that citrus plants have antifungal, anti-inflammatory, antitumor, hypoglycemic and acetyl cholinesterase inhibitory effects [4-7].

Citrus fruits and leaves have long been recognized for its secondary metabolites including antioxidant such as ascorbic acid, flavanones, phenolics and pectin that are important to human nutrition. Many researchers have found antioxidant in juice and edible parts of oranges of different origin and from different varieties [8, 9]. *Citrus limetta* plant (Kidarangai in Tamil) is widely used as food and medicine in ancient times and it was used mainly to combat sea sickness, pulmonary troubles, intestinal ailments, and other biological activities such as antimicrobial and anti-fungicidal. The leaves and peel of *Citrus* fruits are a rich source of flavanones and many polymethoxylated flavones which are very rare in other plants. Flavonoids are important due to their antioxidant, anticancer and anti thrombotic properties and lower blood sugar level [10, 11]. However, no studies have been carried out so far on analysis of active compounds of *Citrus medica L*.var.*limetta* leaves. This study is aimed to investigate and characterize the bioactive compounds in the methanol extracts of *Citrus limetta* leaves by gas chromatography–mass spectrometry (GC–MS) analysis.

Methods and materials

Collection and extraction of plant leaves

Fresh and healthy leaves of *Citrus limetta* were collected from July to January in Echangkottai village, Thanjavur, Tamilnadu. The collected plant leaves were washed with tap water to remove sand, dust and other contaminants, then air dried for a for weeks ensuring sufficient air flow to avoid damping. Dried plant leaves were crushed to powder by using an electric grinder at a speed of 6000 rpm for 60 s. 10 g powder of the plant leaves was extracted by using 100 ml of methanol. The standard soxhlet extraction method [12] was conducted using methanol. It was kept for 24 h at room temperature (25°C) and shaken by using an electric shaker to get a better extraction. Thereafter, the extract was filtered through Whatman filter paper No. 1. After filtering, methanol was removed at 50°C by using a rotary evaporator to obtain a solid extract, dried in vacuum desiccators at room temperature. Finally, dry material was stored in desiccators until required for further analysis.

Analysis of solvent extract

GC/MS Analysis of methanol extract

The methanol extracts of *Citrus limetta* leaves were analyzed by GC/MS and the analysis was performed on a Perkin elmer clarus SQ8C. Trace GC-MS equipped with DB-5 MS capilary standard non polar column (30Mts, ID: 0.25 mm, FILM: 0.25 IM).The injection temperature was 220ºC and the oven temperature was raised from 40ºC (3 min hold) to 250ºC at a rate of 5ºC/min, then held at 250ºC for 2 min; transfer line temperature was 250ºC. 1 µl of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned over the 40 to 500 m/z with an ionizing voltage of 70 eV and identification was based on standard mass library (NIST Version 2.0) that to detect the possibilities of essential components. Relative quantity of the chemical compounds are present in the methanol extracts of *Citrus limetta* leaves and was expressed as percentage based on peak area produced in the chromatogram.

Identification of chemical constituents

Bioactive compounds extracted from methanol extract of *Citrus limetta* were identified based on GC retention time on HP-5MS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems).

Results and Discussion

Physical properties

The methanol extract of *Citrus limetta* was green to dark green in color with sweetened agreeable odor; the pH was 4.07 in 10% aqueous solutions. Phytochemical studies of the *Citrus limetta* leaves revealed the presence of several bioactive secondary metabolites such as alkaloids, polyphenols, terpenoids, flavonoids, steroids, tannins and cardiac glycosides.

Bioactive compounds present in the extracts

Bioactive compounds present in methanol extract from *Citrus limetta* leaves are shown in **Table 1**. In this study, the leave samples of *Citrus limetta* were analyzed by GC/MS for determination of the components and results are given in **Figures 1-2** as a relative peak area of each constituent. The components were identified by matching mass spectra with MS libraries and the present research encompasses identification of chemical constituents in *Citrus limetta*. The identification and characterization were based on their elution order in a HP-5MS column. Constituents of leaf essential oils of *Citrus medica* that contain active compounds were also studied in several studies [13, 14].

There is abundance of active compounds present in the leaves of *Citrus limetta* and total of 32 compounds were exists in the methanol extract of leaves. The main compounds of them were the following: Propanal,2,3-ihydroxy,(S),calyptol(1.56%), linalool(2.87%), eucalptol(2.87%), terpineol(1.89%), Verbenol,2-Oxabicyclo[2.2.2] octan-6-ol(2.07%), 11,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-5-ol(2.146%), 2-Furanmethanol (1.16%), cyclo hexane(1.28%), Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene(1.68%), 1,3,3-Trimethyl-2-oxabicyclo [2.2.2]octane-6,7-endo,endo-diol(2.14%), Bicyclo[7.2.0]undec-4-ene,4,11,11-trimethyl-8-ethylene (1.6%), Caryophyllene(1.6%), 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane-6,7-endo,endo-diol(2.14%), 4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)butan-2-one(3.68%), -Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl-,acetate (3.10%), Cyclohexanemethanol,4-ethenyl-à,à,4-trimethyl-3-(1-methylethenyl) (2.91%), Caryophyllene oxide(2.99%)

Table 1 Biologically active chemical compounds of methanol extract from *Citrus limetta* leaves

S. No	Name of compounds	Peak Area (%)
1	Propanal, 2,3-dihydroxy	1.568
2	Eucalyptol	2.69
3	Bicyclo[3.1.0]hexan-2-ol,2-methyl-5-(1-methylethyl)	0.38
4	Linalool	2.86
5	2(3H)-Benzofuranone, hexahydro-3a,7a-dimethyl-, cis	0.63
6	3,7-Octadiene-2,6-diol, 2,6-dimethyl	0.85
7	Terpineol	1.89
8	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl	2.07
9	Bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl	0.38
10	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-5-ol	2.146
11	Verbenol	0.459
12	2 2-Octen-1-ol, 3,7-dimethyl-, isobutyrate, (Z)	0.651
13	Cholestan-3-ol, 2-methylene-, (3á,5à)	0.701
14	1,2-Cyclohexanediol,1-methyl-4-(1-methylethenyl)	1.30
15	(R)-lavandulyl acetate 1 685	0.40
16	2-Furanmethanol, 5-ethenyltetrahydro-à,à,5-trimethyl	1.166
17	cyclohexane	1.285
18	Propane-1,3-diyl bis((E)-2-methylbut-2-enoate)	0.95
19	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene	1.682
20	Caryophyllene	1.67
21	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane-6,7-endo,endo-diol	2.14
22	4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)butan-2-one	3.682
23	2,4-Heptanedione, 6-methyl	0.622
24	exo-2-Hydroxycineole	1.14
25	5-isopropyl-6,6-dimethylhept-3-yne-2,5-diol	1.193
26	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy	0.605
27	1,4-Dimethyl-7-(prop-1-en-2-yl)decahydroazulen-4-ol	0.95
28	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-, acetate	3.10
29	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl	1.08
30	Cyclohexanemethanol,4-ethenyl-à,à,4-trimethyl-3-(1-methylethenyl)	2.91
31	Caryophyllene oxide	2.919
32	Naphthalenemethanol	0.81

Eucalyptol is one of active compounds present in the leaves of *Citrus limetta* and a natural organic compound of monoterpenoid. Eucalyptol is used in flavorings, fragrances, and cosmetics, antiseptic [15]. Eucalyptol is used as an insecticide and insect repellent [16]. Linalool and linalyl acetate are also present in leaves and it is terpenes and terpenes are principal components of many essential oils known to possess several biological activities, attributable to these monoterpene compounds [17]. Essential oils containing (S)-cis-verbenol have been reported to have biological activity such as antimicrobial and anti-fungicidal [18-20]. Most of these 4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)butan-2-one are ionones used as fragrance and flavor ingredients.

In addition, one of the compounds in *C.limetta* is α-terpineol which attracts a great interest as it has a wide range of biological applications as an antioxidant, anticancer, anticonvulsant, antiulcer, antihypertensive, anti-nociceptive compound [21]. It is also used to enhance skin penetration, and also has insecticidal properties [22]. α- terpineol proved to possess a potent antioxidant activity against free radicals causing injury. cis-verbenol is a natural metabolite from alpha-pinene of host pine tree, has been suggested to have anti-ischemic activity [23]. *Citrus limetta* contain caryophyllene oxide and it is also isolated from *Annona squamosa* bark that has analgesic and anti-inflammatory activity oxide. Recently, (S)-cis-verbenol was reported to reduce ischemia/hypoxia-induced cell death in neuroblastoma SH-SY5Y cells [24].

Based on this study, some of the constituents present in *C.limetta* that was revealed by GC–MS are biologically active compounds. They were proven to possess synergic effect which may contribute to the active potential of the plant. These results showed that the *Citrus limetta* leaves could be used as a potent natural antiviral and antioxidant agents. However, studies *in vivo* are needed to assess the true antioxidant and antiviral activities of the leaves of *Citrus limetta* and to determine the metabolic pathways involved in their degradation.

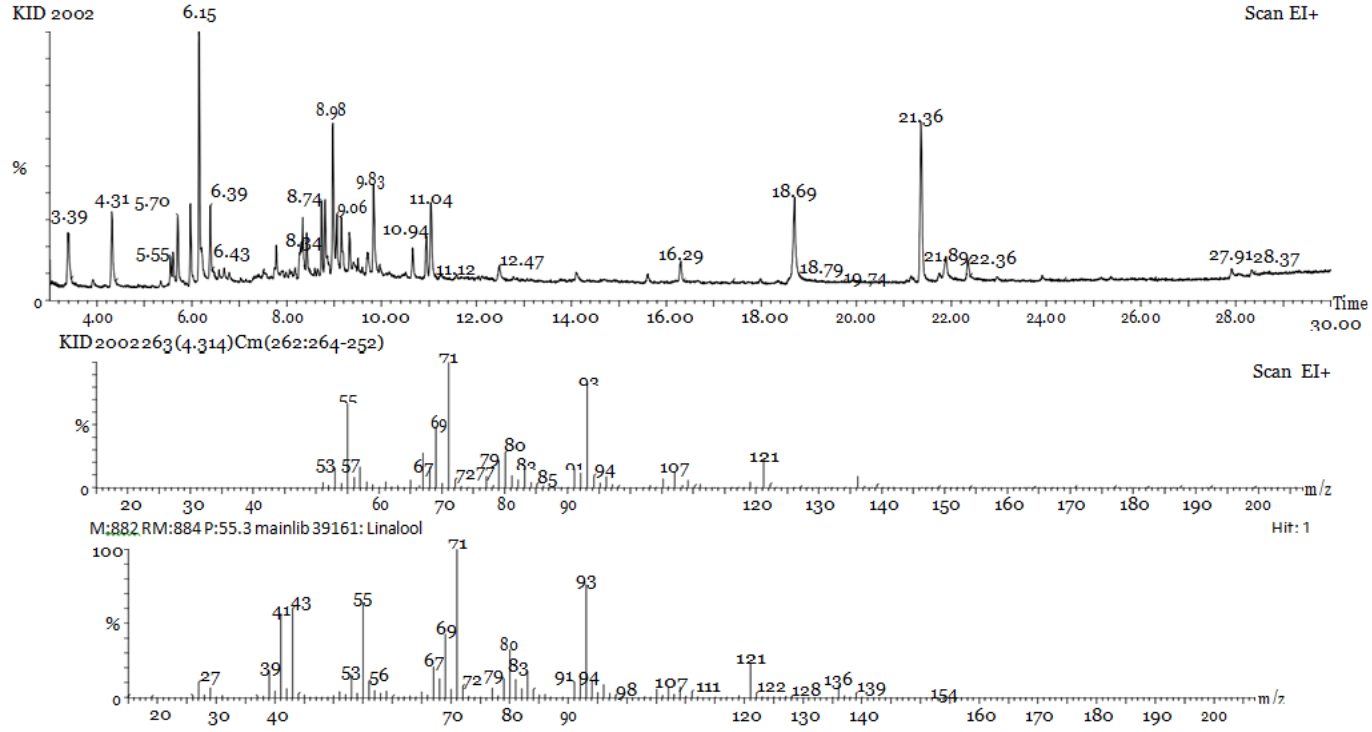

Figure 1 GC-MS spectrum of linalool

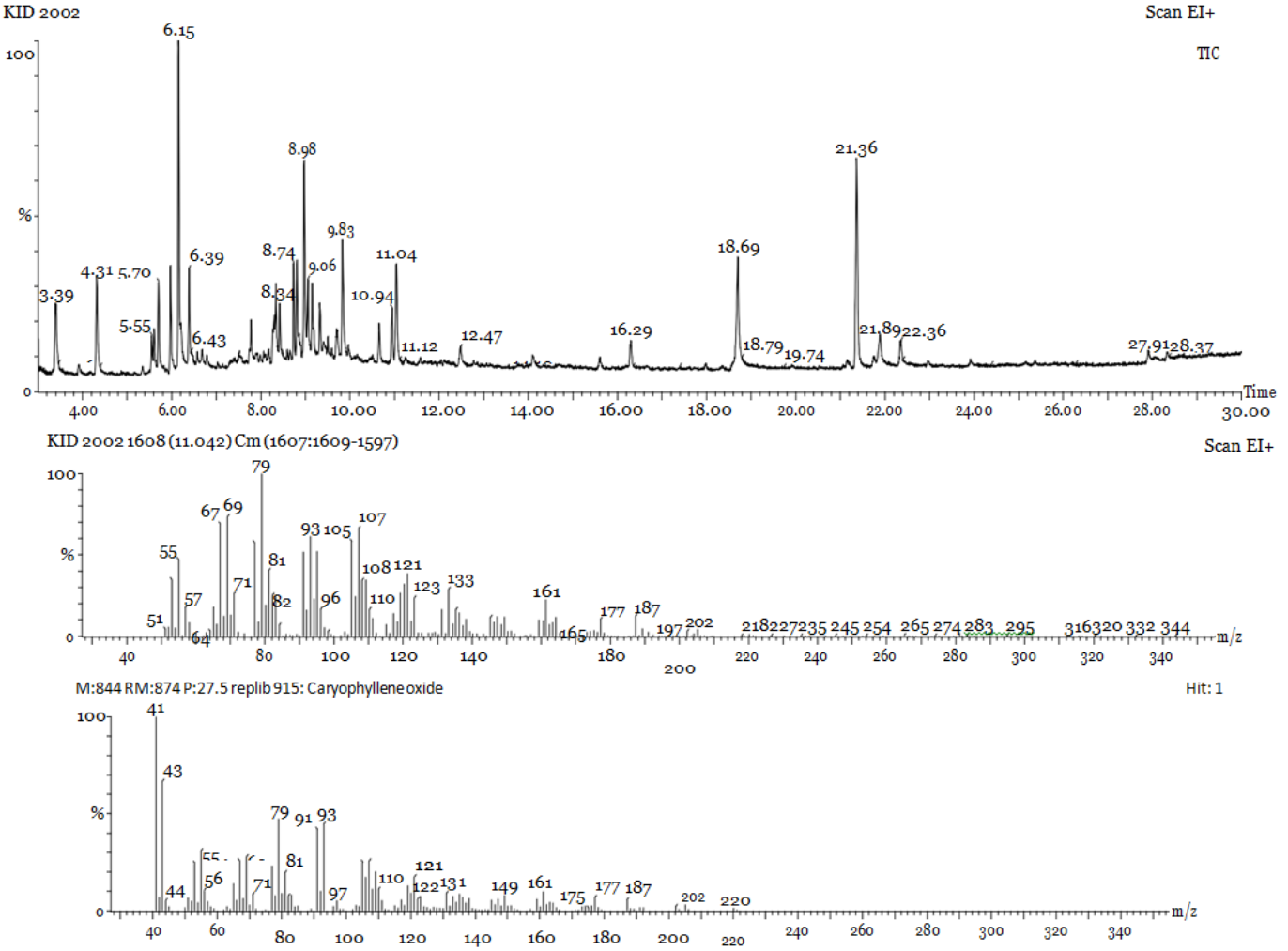

Figure 2 GC-MS spectrum of Caryophyllene oxide

Conclusion

It is concluded that there are 32 bioactive active compounds present in leaves of *Citrus limetta* which support the biological and medicinal application of the plant. The study revealed that major useful bioactive compounds present in methanol extracts. Leaves also serve as a potential source for bioactive compounds of insecticide. *Citrus limetta* leaves are a very rich source of important bioactive constituents to defensive, providing protection against insect, fungal, and viral attacks. Identification of these compounds in the leaves serves for the basis in determining the possible benefits of the plant leading to further biologic and pharmacologic studies. Researchers to work in various pharmacological activities and insecticidal studies may concentrate in it.

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