GC-MS Analysis and *In Vitro* Thrombolytic, Anti-hemolytic, Anti-Inflammatory Activities of Essential Oil of *Citrus limetta* Peel

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Abstract: Essential oils are oily liquids which contain many chemical constituents that act against various degenerative disorders. They are responsible for the fragrance and having different biological activities. In the present study, the fresh peel of *Citrus limetta* Risso were collected and subjected to hydrodistillation by using Clevenger apparatus. The identification of compounds present in the oil was carried out by GC-MS. From the GC-MS analysis it has been identified as 43 compounds were present in the essential oil. Out of these constituents, α– pinene, limonene, β-myrcene and hexadecane are some of the important terpenoids present in the oil sample. Even though they are presented in a small amount they are having many biological properties. Based on the literature survey the essential oil of *C. limetta* was assessed for its *in vitro* thrombolytic, anti-haemolytic and anti-inflammatory activities. In thrombolytic activity using clot lysis method, the essential oil was found to have significant thrombolytic effect showed a maximum effect of 72±1.17% at 100 µg/mL concentration. Concordantly, in anti-hemolytic activity the percentage of inhibition of *C. limetta* was found to be 76%. Anti-inflammatory activity was evaluated using albumin denaturation. The essential oil showed mean inhibition of protein denaturation as 70%. The present exploration revealed that the essential oil of *C. limetta* possesses significant thrombolytic properties as well as anti-haemolytic and anti-inflammatory effects.

Keywords
Essential oil; GC-MS; α– Pinene, Limonene, Thrombolytic activity; Hemolytic activity; Anti-inflammatory

Introduction

Inflammation is characterized by redness, warmth, swelling and pain [1,2]. The mechanism of inflammation is attributed to release of reactive oxygen species (ROS) from activated neutrophil and macrophages. This over production leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes [3]. Thrombosis reflects a pathological progression of atherosclerotic lesions and arterial vascular thrombosis. Uncontrolled platelet aggregation is critical in arterial thrombosis and may cause life threatening disorders. Thromboembolic anarchies such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of mortality in developed countries [4]. Antithrombotic agents are considered as a key tool in the treatment and prevention of cardiovascular thrombotic diseases [5]. Oxidative stress is among the major causative factor for producing free radicals and reactive oxygen species (ROS). It is linked to inflammation, playing together an important role in the pathogenesis of cancer [6], cardiovascular diseases [7], type diabetes and obesity [8]. Traditional Indian medicines have been used to treat several degenerative disorders for several years. During the last decades, there is increasing interest to unlock the secrets of ancient herbal remedies. The increase in prevalence of multiple drug resistance has shown the

development of new synthetic antibacterial, antioxidative and anti-inflammatory drugs.

C. limetta Risso belongs to the family Rutaceae. Its fruits and leaves are used as common cold decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorders, and so forth as well as blood pressure modulator [9]. Its peel and inner part of the fruit contains hespiridin and naringin [10]. The essential oil extracted from citrus species contains α-pinene, β-pinene, sabinene, β-myrcene, p-cymene, limonene, γ-terpinene, neryl acetate, β-bisabolene, and α-bergamotene [11]. No scientific studies have been carried out regarding *in vitro* thrombolytic, anti-haemolytic and anti-inflammatory activities from essential oil of *C. limetta* and peel. Hence the study was undertaken to evaluate the thrombolytic, anti-hemolytic and anti-inflammatory activities of essential oil of *C. limetta* peel.

Materials and Methods

Collection of Plant Material

The fresh peels of *C. limetta* (100 g) were collected from local market of Coimbatore. The plant was authentified by Botanical Survey of India (No.BSI/SRC/5/23/2017/Tech/3406), TNAU, Coimbatore.

Isolation of Essential Oil

The fresh peels of *C. limetta* (250 g) were subjected for hydrodistillation for 3 hrs using a Clevenger type apparatus. The obtained essential oil was dried over anhydrous sodium sulphate and preserved in a sealed vial at 4°C until further analysis.

GC-MS Analysis of Essential Oil

The analysis of the essential oil from *C. limetta* was performed using a Hewlett Packard 5890 II GC equipped with a FID detector and HP-5 ms capillary column (30m x 0.25m, film thickness 0.25 µm). For GC-MS detection, an electron ionization system was used with ionization energy of 70eV. Helium was the carrier gas, at a flow rate of 1ml/min. Injector and MS transfer line temperature were set at 220 and 290°C respectively. Column temperature was initially at 50°C, and then gradually increased to 150°C at a 3°C/min rate, held for 10 min and finally increased to 250Vc at 10Vc/min. Diluted samples (1/100 in acetone) of 1.0µl were injected manually and split less. The components were identified based on the comparison of their relative retention time and mass spectra with those of Wiley 7N library data. The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature [12].

Animals and diets

Female albino rats of Sprague-Dawley strain weighing 180±10 g were used for this study. The animals were purchased from Kerala Veterinary and Animal Sciences University, Kerala. The animals were under standard conditions and were housed four per cage with a wire mesh top and a hygienic bed of husk in a specific pathogen free animal room for 12 h light and 12 h dark cycle, with temperature of 24±2ºC. They were provided standard rat feed and water *ad libitum*. All the animal experiments were done according to the ethical guidelines given by Institutional Animal Ethical committee (IAEC-CPCSEA NO: 04/2016/IAEC/KASC).

In vitro thrombolytic activity

About 3 mL of the blood sample from rats were collected and immediately distributed in the 5 different pre-weighed sterile microfuge tubes (0.5 mL/tube). The tubes were incubated at 37°C for 45 min. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube-weight of tube alone). About 20-100 µg/mL concentration of oil was added to the clot tube, 100

µL of distilled water in a clot tube serves as negative control and standard streptokinase has been acted as a positive control. All the tubes were incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption [13].
% of clot lysis = (Weight of the clot before lysis - Weight of the clot after lysis)/ Weight of the clot before lysis ×100

***In vitro* Antihaemolytic Activity**

The blood samples from rats were collected in EDTA tubes the tubes were centrifuged at 1,500 rpm for 10 min. Further the plasma was discarded from the tubes and the settled RBCs were washed three times with saline (0.9% NaCl). Then the RBCs were diluted with saline phosphate buffer to give 4% (v/v) suspension. Different concentrations of essential oil from *C. limetta* peel (20-100µg/mL) were added to 2.0 mL of RBC suspension (standard vitamin C has been acted as positive control) and the volume was made up to 5.0 mL with saline. This mixture was pre-incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in buffered saline was added to induce oxidative degradation of the membrane lipids. The tubes were incubated for one hour and further the reaction mixture was centrifuged at 1500 rpm for 10 min. Then the extent of haemolysis was measured at 540 nm [14]. The percentage of haemolysis inhibition was calculated using the formula:
Inhibition percentage = [A control-A sample/A control] × 100.

Assessment of *in vitro* anti-inflammatory activity from essential oil of *C. limetta* peel
Inhibition of albumin denaturation

The reaction mixture consisted of essential oil (Diclofenac sodium has been used as positive control) at different concentrations (20-100µg/mL) and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using small amount of 1N HCl. The samples were incubated at 37 °C for 20 min and then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured at 660 nm. The experiment was performed in triplicates [15]. Percent inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = \frac{(Abs^{control} - Abs^{sample})}{Abs^{control}} X100$$

Results and Discussion

Percentage Yield of *C. limetta* Essential Oil by Clevenger Apparatus

The volume of essential oil which is extracted from the peels of *C. limetta* was increases when increasing the time. It was examined that 4 ml of essential oil was extracted from 250 g of the peels of *C. limetta*.

GC-MS analysis from essential oil of *C. limetta* peel

The gas chromatogram was indicated in **figure 1**. The GC-MS analysis of the essential oil from *C. limetta* resulted in the identification of 43 constituents eluted from 5 min to 30 min. The major compounds present in essential oils are terpenes **(Table 1).**

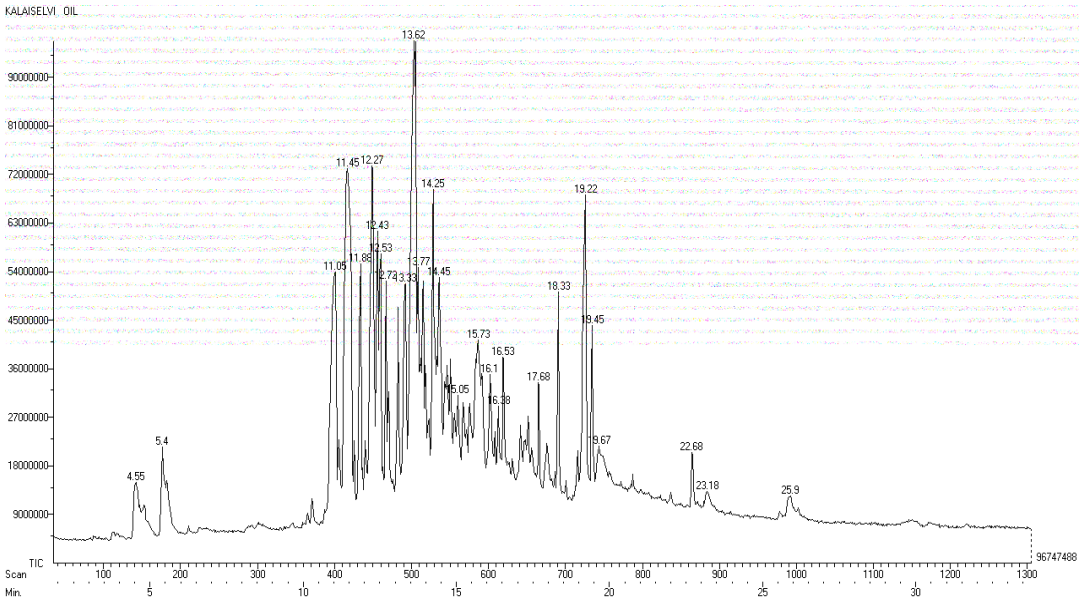

Figure 1: Chromatogram of essential oil of *C. Limetta*

Terpinoids are considered as one of the important secondary metabolite which possesses various biological activities such as anti-tumor, nematicide, analgesic, antibacterial, anti-inflammatory, dative, fungicide, pesticide, insecticide and nematicide chemopreventive effect [16].

Table 1: Chemical composition of essential oil from *C. limetta*

S. No	Constituents	Retention Time	Oil (%)
1	α –Pinene	6.894	0.41
2	α –Pinene	6.980	0.74
3	1,6-Octadiene	7.988	1.44
4	Limonene	5.45	45.23
5	Bicyclo[3.1.1]Hept-2-Ene	8.250	1.37
6	2,6-Octadien-1-Ol, 3,7-Dimethy	8.308	1.06
7	1,6-Octadiene	8.547	6.33
8	Cyclohexene	9.575	5.78
9	Cyclohexene	9.613	6.35
10	1-Octanol	9.723	1.24
11	4-Thujanol	9.782	0.24
12	Cyclohexene	10.004	0.72
13	1,6-Octadien-3-ol	10.188	4.29
14	trans-p-Mentha-2,8-dienol	10.469	0.26
15	Trans-Limonene Oxide	10.707	0.50
16	6-Octenal, 3,7-dimethyl-	10.868	0.38
17	1-Nonanol	11.292	0.25
18	3-Cyclohexen-1-Ol, 4-Methyl-	11.324	0.75
19	3-Cyclohexene-1-methanol	11.537	1.18
20	6-Octen-1-ol, 3,7-dimethyl-	12.008	1.06
21	2,6-Octadienal, 3,7-dimethyl-, (Z)-	12.222	0.73
22	2-Cyclohexen-1-One, 2-Methyl	12.307	0.22
23	α-Cadinol	12.287	0.34
24	2,6-OctadienaL, 3,7-Dimethyl-	12.643	1.11
25	p-Mentha-1(7),8(10)-dien-9-ol	12.959	0.18
26	Undecanal	13.104	0.26

27	6-Octen-1-ol, 3,7-dimethyl-, acetate	13.778	0.05
28	1,6-Cyclodecadiene, 1-Methyl	14.602	0.34
29	Dodecanal	14.770	0.27
30	Lupeol	15.672	0.21
31	1,6-Cyclodecadiene, 1-Methyl	16.613	0.17
32	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro	16.915	0.13
33	α-Farnesene	17.053	0.29
34	E-11(12-Cyclopropyl)dodecen-1-ol	18.082	0.19
35	Caryophyllene oxide	19.483	0.05
36	Hexadecane	21.892	0.03
37	β-Myrcene	21.922	0.02
38	Hexadecane	23.790	0.06
39	Nonadecane	25.409	0.08
40	Hentriacontane	26.842	0.09
41	Heneicosane	28.150	0.07
42	Trans- Limonene oxide	28.343	0.05
43	Pentacosane	29.360	0.05

Thrombolytic activity of essential oil from peel of *C. limetta*

Pathological enlargement of blood clots requires clinical intervention with fibrinolytic agents such as urokinase, tissue plasminogen activator and streptokinase. Naturally occurring plant sources and their supplements having antithrombotic effect and there is indication that consuming such sources leads to anticipation of coronary events and stroke [17].

In vitro thrombolytic activity was studied using clot lysis method. The percentage yields of clot lysis of C. limetta as well as the standard are shown in **Table 2**. It was clearly noted that the percentage of clot lysis of *C. limetta* peel oil was found to be increased in a dose dependent manner. The essential oil of *C. limetta* at maximum concentration of 100 µg/mL showed clot lysis of 72±1.17% which was compared with the standard streptokinase showed clot lysis of 83±0.33 %. From this result it is evident that the essential oil of *C. limetta* showed the highest percentage of clot lysis. These results were concordance with the previous report of Fathima, *et al*., [18]. The positive control, streptokinase is one of the important enzyme secreted by streptococci species which can able to unite and activate the inactive precursor of the enzyme plasmin (plasminogen). Plasmin is an important proteolytic enzyme that causes degradation of fibrin clots. From the above mentioned results, it is clearly indicated that essential oil of *C. limetta* peel having receptor for plasminogen, so that plasminogen can able to bind and activate the receptor, which may leads to clot lysis. Our findings were coincidence with the previous report of Arfan, *et al*., [19].

Table 2: Thrombolytic activity of essential oil from peel of *C. limetta*

S. No	Concentration (µg/mL)	Thrombolytic activity of *C. limetta* Percentage of Inhibition (%)	Standard Streptokinase Percentage of Inhibition (%)
1	20	15 ± 1.14^{a}	25 ± 0.64^{a}
2	40	23 ± 1.19^{b}	49 ± 0.51^{b}
3	60	47 ± 1.11^{c}	65 ± 0.45^{c}
4	80	59 ± 1.41^{d}	74 ± 0.76^{d}
5	100	72 ± 1.17^{e}	83 ± 0.33^{e}

Values are expressed as Mean±SD for triplicates. Values not sharing common superscript letters (a-e) differ significantly at $p< 0.05$ (DMRT)

***In vitro* anti-hemolytic activity**

Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes. Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. This haemolysis relates to concentration and potency of extract. Furthermore the haemolytic activity of each extract is related to their chemical composition [20]. The haemolysis level was found to be much greater, while treating with hydrogen peroxide with red blood cells which leads to the destruction of cell membrane and subsequent liberation of haemoglobin from the cells. Mobilization of Fe^{2+} by Ca^{2+} via fenton reaction is also caused due to hydrogen peroxide which further leads to the production of OH radicals [21]. In the present study, *C. limetta* peel oil showed the best result with IC_{50} value of 38 µg/ml, which infers that this extract possesses higher antihaemolytic activity towards RBC. The antihaemolytic activity of the essential oil was tested in the presence of toxicant H_2O_2, where distilled water was taken as a negative control. At 100 µg/ml concentration of *C. limetta* oil and standard vitamin C showed that percentage of inhibition is about 76% and 84%. The IC_{50} value for *C. limetta* oil was 38 and 35 µg/ml (**Figure 2**). Hence the above result was clearly indicates that *C. limetta* peel oil possesses higher antihaemolytic activity towards RBCs against. Our studies support previous reports by James and Alewo, [22] who worked *In vitro* antihemolytic activity of *Gymnema sylvestre* extracts against hydrogen peroxide (H_2O_2) induced haemolysis in human erythrocytes.

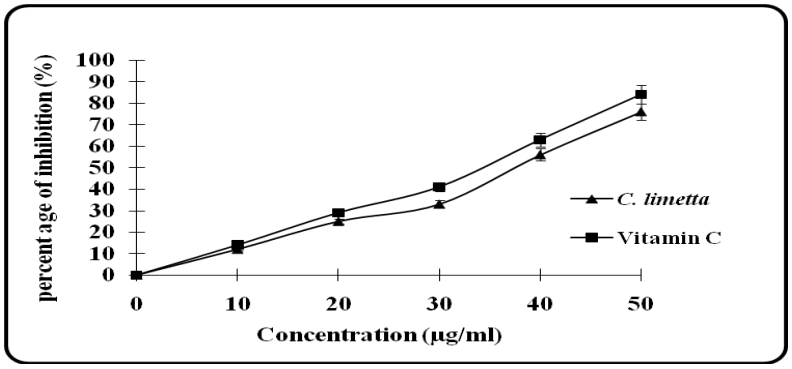

Figure 2: Antihaemolytic activity of *C. limetta* oil

***In Vitro* Anti-Inflammatory Activity**

Inhibition of albumin denaturation

Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair [23]. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [24]. So, by controlling the production of auto antigen and inhibiting denaturation of protein and membrane lysis in rheumatic disease leads to anti- inflammatory activity [25]. To investigate on the mechanism of anti-inflammatory activity, the ability of oil to inhibit protein denaturation was studied. When increasing the concentration of essential oil extracted from *C. limetta* peel the inhibition of heat induced albumin denaturation is also increased at different concentrations. The percentage of inhibition of albumin denaturation was increased with increasing concentration of the extracts. At 100 µg/ml concentration about 70% and 79% of inhibition of albumin denaturation was shown by *C. limetta* peel oil and standard diclofenac. The IC_{50} value for *C. limetta* oil and standard showed 38, 34 µg/mL respectively **(Figure 3).**

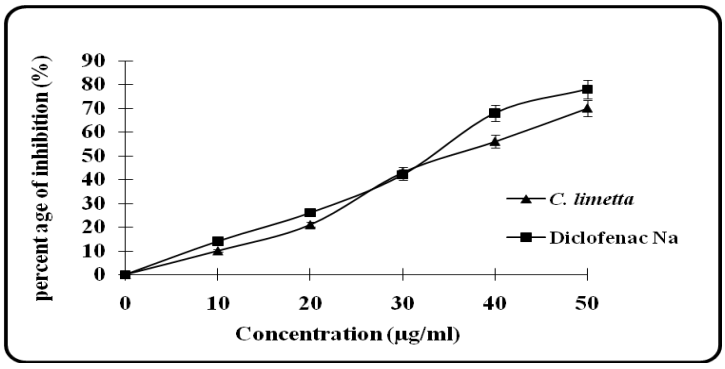

Figure 3: Anti-Inflammatory Activity of *C. limetta* oil

Several authors suggest that anti-inflammatory drugs have shown dose dependent ability to inhibit heat induced protein denaturation. The oil may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. The extracellular release of neutrophils lysosomal constituents like bactericidal enzymes and proteinases cause further tissue inflammation and damage [26]. The essential oil extracted from the peels of *C. limetta* contains more number of terpinoid compounds. These compounds may be involved in membrane stabilization and cause expansion of membrane or the shrinkage of cells and an interaction with membrane protein. The above result was accordance with the previous report of Sangeetha and Vidhya, [27] who were studied in *Pedalium murex*.

Conclusion

The result indicates the efficacy of *C. limetta* oil as an effective therapeutic agent. The result of present study authentifies the theombolytic, anti-hemolytic and anti-inflammatory property of the essential oil of *C. limetta*. This may be due to the presence of phytoconstituents in the *C. limetta* peel oil. Hence it is justifies the traditional use of essential oil may be used in the treatment of various types of pains and inflammation in future.

Conflict of Interest

We, the authors declare that they have no conflict of interests.

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