

**CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF ESSENTIAL OIL
FROM CITRUS SINENSIS PEEL**M. Vinodhini¹, M. Kalaiselvi^{1*}, R. Amsaveni² and V. Bhuvaneshwari²¹Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore-29, Tamil Nadu, India.²Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore-29, Tamil Nadu, India.***Corresponding Author: Dr. M. Kalaiselvi**

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ABSTRACT

The present study was aimed to evaluate the *in vitro* thrombolytic, anti-haemolytic and anti-inflammatory activities from essential oil of *C. sinensis* peel. *C. sinensis* collected and was subjected to hydrodistillation by using Clevenger apparatus. The identification of compounds present in the oil of *C. sinensis* was carried out by gas chromatography and mass spectrometry (GC-MS). *In vitro* thrombolytic, hemolytic and anti-inflammatory activities were studied by standard protocols. From the GC-MS report it has been estimated that 10 major compounds present in the essential oil. The essential oil of *C. sinensis* shows potent thrombolytic activity and the percentage of inhibition were found to be $90 \pm 1.65\%$ at 100 μ L. In hemolytic activity, the percentage of inhibition of *C. sinensis* is about 68%. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation assay. The results showed that essential oil of *C. sinensis* peel at a concentration range of 100 μ l significantly protects the heat induced protein denaturation at 69%. The results obtained in the present study indicate that essential oil of *C. sinensis* peel can be a potential source of thrombolytic, anti-haemolytic and anti-inflammatory agents.

KEYWORDS: *Citrus sinensis*; GC-MS; Thrombolytic activity; Hemolytic activity; Anti-inflammatory.**INTRODUCTION**

Traditional Indian medicines have been used for pharmaceutical and dietary therapy for several decades. During several periods, 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. Phytochemicals from medicinal plants showing antimicrobial, antioxidant and anti-inflammatory activities have a potential of filling this need because their no side effects than synthetic drugs.^[1] In this growing interest, many of the phytochemical bioactive compounds from medicinal plants have shown many pharmacological activities. The rapid emergence of multiple drug resistance strains of pathogens to current antimicrobial agents has generated an urgent intensive for new antibiotics for medicinal plants.^[2]

C. sinensis belonging to the family Rutaceae. Its fruits and leaves are used for decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorders, and so forth as well as blood pressure modulator.^[3] Its peel and inner part of the fruit contains hesperidin and naringin.^[4] The essential oil of citrus family contains α -pinene, β -pinene, sabinene, β -myrcene,

p-cymene, limonene, γ -terpinene, neryl acetate, β -bisabolene, and α -bergamotene.^[5]

Limonene, γ -terpinene was isolated from the peel, and their minimum inhibitory concentration (MIC) and maximal tolerated concentration (MTC) were estimated against *Pseudomonas putida*.^[6] The leaf extract of this family was evaluated for its antagonistic activity on the hypertensive action of angiotensin II.^[7] The fruit has shown anti-inflammatory and antithrombotic actions.^[8]

No scientific studies have been carried out regarding *in vitro* thrombolytic, anti-haemolytic and anti-inflammatory activities from essential oil of *C. sinensis* peel. Hence the study was undertaken to evaluate the above mentioned activities from essential oil of *C. sinensis* peel.

MATERIALS AND METHODS**Collection of Plant Material**

The peel of *C. sinensis* was collected from Coimbatore District. The plant was authenticated by Botanical Survey of India, TNAU, Coimbatore.

Extraction of Essential Oil

The fresh peels of *C. sinensis* were subjected for 3 hrs to water distillation using a Clevenger type apparatus. The

obtained essential oil was dried over anhydrous sodium sulphate (Na_2SO_4) and preserved in a sealed vial at 4°C until further analysis.

GC-MS Analysis

The analysis of the essential oil from *C. sinensis* 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 $3^\circ\text{C}/\text{min}$ rate, held for 10 min and finally increased to 250°C at $10^\circ\text{C}/\text{min}$. Diluted samples (1/100 in petroleum ether) of 1.0 μl 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 and standards of the main components. 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.^[9]

In vitro thrombolytic activity

About 3 mL of fresh blood was drawn from volunteers (n=4) 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 μL concentration of oil was added to the clot tube, 100 μL of distilled water in a clot tube serves as negative 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.^[10]

% of clot lysis = (Weight of the clot before lysis - Weight of the clot after lysis)/ Weight of the clot before lysis $\times 100$

In vitro Antihaemolytic Activity

The human venous blood samples were collected in EDTA tubes from well nourished healthy adults (25-30 years of age) and 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 of *C. sinensis* (20-100 μL) were added to 2.0 mL of RBC suspension 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 spectrophotometrically at 540 nm.^[11] The percentage of haemolysis inhibition was calculated using the formula:

$$\text{Inhibition percentage} = \left[\frac{\text{A control} - \text{A sample}}{\text{A control}} \right] \times 100.$$

Assessment of in vitro anti-inflammatory activity from C. sinensis oil

Inhibition of albumin denaturation

The reaction mixture consisted of essential oil at different concentrations (20-100 μL) 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 spectrophotometrically at 660 nm. The experiment was performed in triplicates.^[12] Percent inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = \frac{(\text{Abs}^{\text{control}} - \text{Abs}^{\text{sample}})}{\text{Abs}^{\text{control}}} \times 100$$

RESULTS AND DISCUSSION

Percentage Yield of Essential Oils by Clevenger Apparatus

Percentage yield of *C. sinensis* essential oils by Clevenger apparatus unit were shown in Table 1. Percentage of citrus oil yielded in the range of 1%.

Table 1: Percentage yield of citrus essential oil by Clevenger apparatus unit.

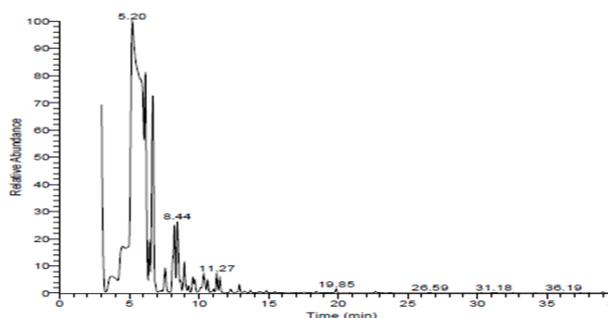
Essential oil (EO)	Raw Material Input (g)	Time extract (minutes)	Oil volume (ml)	Productivity (ml/1000g) %
<i>C. sinensis</i>	1000 g	90	10	1

GC-MS analysis from essential oil of C. sinensis Peel

The Peak destionogram of GC-MS analysis was indicated in Figure 1. The GC-MS analysis of the essential oil from *C. sinensis* resulted in the identification of 10 constituents eluted from 5 min to 30 min. The major compounds present in essential oil are terpenes, followed by essential fatty acids (Table 2). Usually terpenes have some biological activity such as anti-tumor, nematocidal, analgesic, antibacterial, anti inflammatory, sedative, fungicide, pesticide, insecticide nematocidal chemopreventive effect.^[13]

Table 2: Chemical composition of essential oil from *C. sinensis*.

S. No	Constituents
1	Ocimene-X
2	Pinene
3	Delta 3-Carene
4	DL-Limonene

Figure 1: Peak destimogram of GC-MS analysis from essential oil of *C. sinensis***Thrombolytic activity of essential oil from citrus *sinensis***

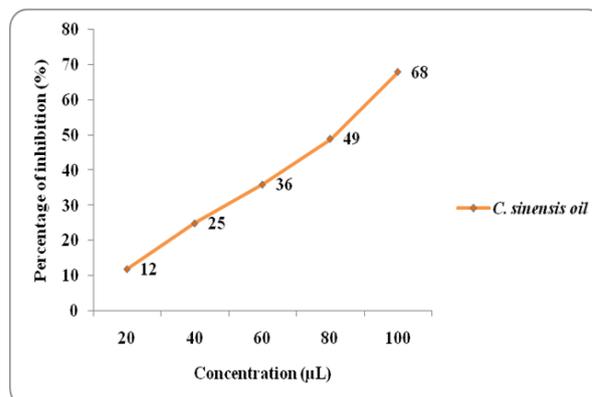
The result showed that the increasing concentration of the oil with increased percentage of clot lysis activity in a dose dependent manner. Essential oil of *C. sinensis* at maximum concentration of 100 μL showed clot lysis of $90 \pm 1.65\%$. From the results it is evident that the essential oil of *C. sinensis* showed the highest percentage of clot lysis (Table 3).

Sherwani *et al.*^[14] investigated the thrombolytic activity of *Bougainvillea spectabilis* using a simple and rapid *in vitro* clot lysis model. Their results indicated maximum 84.24% clot lysis at 800 $\mu\text{g/mL}$ concentration in 72 h of incubation as mentioned. The results indicated clearly that concentrations plant enhanced the percentage of clot lysis.

The present study was carried out to investigate the thrombolytic activity of the *C. sinensis* peel oil extract. An *in vitro* thrombolytic method was used to investigate the thrombolytic activity of plant extracts in blood sample from healthy human volunteers, along with streptokinase as a positive control and water as a negative control.^[15]

Table 3: Percentage inhibition of thrombolytic activity of essential oil.

S. No	Concentration of <i>C. sinensis</i> (μL)	Percentage of Inhibition (%)
1	20	47 ± 1.19
2	40	67 ± 1.22
3	60	76 ± 1.09
4	80	86 ± 1.39
5	100	90 ± 1.65

In vitro* Antihaemolytic Activity*Figure 2: *In vitro* Antihaemolytic Activity of *C. sinensis* oil**

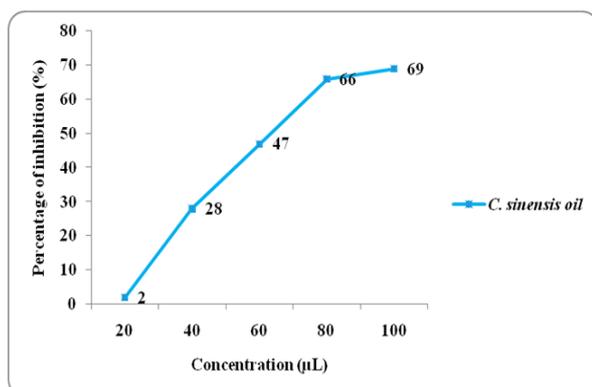
At 100 μL concentration of *C. sinensis* oil showed that percentage of inhibition is about 68% of antihaemolytic activity. The IC_{50} value for *C. sinensis* oil was 82 μL , (Figure 2). Hence the above result clearly indicates that *C. sinensis* peel oil possesses higher antihaemolytic activity towards RBCs. When red blood cells were treated with H_2O_2 (toxicant) alone, the percentage of haemolysis was found to be increased. This may be because of the oxidizing nature of H_2O_2 with respect to cell membrane degradation and release of haemoglobin from the cell. H_2O_2 also cause mobilization of Fe^{2+} by Ca^{2+} via Fenton reduction stimulating the production of OH radicals.^[16] All these factors caused destabilization of cell membrane, which is probably the key event of the lysis of the cell. Lipid peroxidation is regarded as one of the primary rationale in cellular damage.^[17]

The erythrocyte membranes are susceptible to peroxidation because they are rich in polyunsaturated fatty acids. They contain haemoglobin, which may catalyze the oxidation as they are continuously exposed to high concentration of oxygen. The oxidation of erythrocytes serves as good models for the oxidative damage of biological membranes.^[18] It has been found that certain chemicals, having ability to generate radicals that attack the erythrocyte membrane, inducing the chain oxidations of lipids and proteins and eventually causing membrane damage leading to haemolysis.^[19]

In the present investigation, when red blood cells were treated with extracts along with H_2O_2 a remarkable reduction in haemolysis was found. This may be because of the activity of the bioactive components present in the extracts showing potent antihaemolytic nature of the extracts.

In vitro Anti-Inflammatory Activity Inhibition of albumin denaturation

Figure 3: *In vitro* Anti-Inflammatory Activity of *C. sinensis*



To investigate on the mechanism of anti-inflammatory activity, the ability of oil to inhibit protein denaturation was studied. It was found that the essential oil from *C. sinensis* peel was effective in inhibiting heat induced albumin denaturation at different concentrations. The percentage of inhibition increased with increasing concentration of the extracts. At 100 µL concentration about 69% of inhibition of albumin denaturation was shown by *C. sinensis* peel oil, (Figure 3). The IC₅₀ value for *C. sinensis* oil is 64 µL. From the results it was evident that the albumin denaturation inhibition was exhibited by *C. sinensis* essential oil. Sakat *et al.*^[12] reported that the *Oxalis corniculata* L. maximum inhibition, 85.92±1.48% was observed at 800µg/mL. IC₅₀ value was found to be 288.04±2.78 µg/mL at correlation coefficient value (r) of 0.946.

CONCLUSION

In conclusion, it can be claimed that essential oil of *C. sinensis* peel possesses potent thrombolytic, anti-haemolytic and anti-inflammatory activity. This may be due to the presence of terpenoid constituents from the peel oil.

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