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RESEARCH ARTICLE OPEN ACCESS

ASSESSMENT OF SYNERGISTIC ANTI-BACTERIAL AND ANTI-OXIDANT ACTIVITIES OF ESSENTIAL OIL EXTRACTED FROM PEELS OF CITRUS RETICULATA, CITRUS SINENSIS AND CITRUS LEMON

***Hifza Rashid and Misbah Ashravi**

Researcher, Bhimber AJK, Pakistan

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****Corresponding author: Hifza Rashid***

ABSTRACT

Nature provides a variety of drugs and medicinal agents derived from different parts of plants. Citrus plants have different medicinal properties. Essential oils extracted from peels of different citrus fruits are used to treat number of diseases like bacterial, fungal, insecticidal and diabetic etc. The present study was conducted to check the antibacterial and antioxidant properties of essential oils extracted from peels of *Citrus reticulata*, *Citrus sinensis* and *Citrus lemon* and their mixture. The composition of the essential oils was determined by GC/MS which showed that limonene, manitol, pinene and pyrazinoic acid were dominant among others. Their antibacterial activity was checked against *Staphylococcus aureus, E. coli* and *Salmonella enteritidis.* Well diffusion assay was performed to check the antibacterial activity. *Citrus lemon* was found active against *Staphylococcus aureus* while mixture showed highest antibacterial activity against all microbes. 5mg/ml of essential oils of mixture showed antibacterial activity against *Salmonella enteritidis* and *E. coli* while 10mg/ml of essential oil showed antimicrobial activity against *Staphylococcus aureus*. Their antioxidant activity was checked by DPPH assay. Different concentrations (20-100µg/ml) were used for evaluating antioxidant potential. 100µg/ml of sample showed highest antioxidant activity while using ascorbic acid as standard.

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INTRODUCTION

Essential oils are substances composed of lipids substances. They are obtained from plants by hydro distillation which involves heating in presence of water (Sadgrove and Jones 2015). As they cause aroma in plants, they are widely used in cosmetics and perfumes industry. They produced metabolites which help in protection against pathogens. These compounds are highly useful in agriculture field (Alonso-gato *et al.,* 2021). They are mixture of approximately 400 compounds containing both volatile and non-volatile components. Volatile composition is of 80-99 percent while 1-15 percent is of non-volatile components (Denkova-kostova *et al.,* 2021). Their composition differs greatly because of different factors such as environmental and genetic factors (Maggi 2021). Essential oils can be extracted from leaves, shoots, flowers and peels of aromatic plants. In recent years, they are used as herbal medicines. They are extracted from different parts of plants using steam distillation method. Their chemical composition is measured by GC/MS techniques. However, storage and handling process may affect its composition and properties (Irshad and Subhani). They have numerous applications such as in food, flavor and cosmetic industries. Most important application is aromatherapy. However, they have adverse effects such as hyperactivity and abnormalities in pregnancy which are actually mild (De Groot and Schmidt 2016).

Many properties of food diminish with time because of many factors such as light, oxygen and microorganisms. Essential oils have power to decrease growth of microorganisms which causes contamination (Viuda-Martos *et al.,* 2008). Use of essential oils as antioxidant is getting interest because synthetic antioxidants may cause harms to human health (Amorati *et al.,* 2013). Besides health hazards, there are other concerns regarding auto oxidation of fats which causes rancidity. So, they have ability to protect cells from free radical. This property has enabled their use in diet which resulted in low rate of cardiovascular diseases. Thus, they have been called as an effective way of promoting health (Anthony *et al.,* 2012). In recent years, extension of essential oils to food is mainly because of their antioxidant and antimicrobial activities. They can be degraded easily so they are unstable volatile compounds having low water solubility (Fern and Viuda-martos 2018). Contamination of microbial organisms may result in spoiling of food. The most popular way to increase food's shelf life is by adding one or more stabilizers. Synthetic chemical preservatives are widely employed today; while they work well, they can be hazardous to human health and the agricultural ecosystem (Guo *et al.,* 2018). Natural antibacterial treatments are becoming more and more popular as consumers demand foods with natural components and without artificial preservatives. Plants and their extracts have been tested for this aim to see how well they apply to food safety and preservation (Moosavy *et al.,* 2017). Synthetic drugs have side effects and most of microbes

have developed resistance to synthetic drugs as well. To overcome this dilemma there is dire need to focus on some antimicrobial compounds especially from plant materials because these have low side effects and environment friendly. So this study involved the assessment of synergistic potential of essential oils extracted from citrus peels of *Citrus sinensis*, *Citrus reticulata* and *Citrus lemon.* Comparison was also made between essential oil of mixture and their individual´s essential oil. Identification of compounds present in essential oils of samples were done by using GC/MS apparatus. Antimicrobial and antioxidant activities were carried out to evaluate essential oils potential against *Staphylococcus aureus*, *E. coli* and *Salmonella enteritidis.* Antioxidant activity was evaluated against DPPH assay.

MATERIALS AND METHODS

MATERIALS

Citrus reticulata, *Citrus sinensis* and *Citrus lemon.*

METHODS

Experimental work was conducted at post graduate Molecular Biochemistry laboratory at the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal sciences, Lahore.

Extraction of essential oil: Fruit peels of the selected plants were collected from the Corporation chowk and Madina market Raiwand, Lahore, Punjab, Pakistan. The scientific, common, and local names of the plant are listed in table 2.1. Essential oils were extracted from peels of selected citrus peel plants by using Clevenger apparatus. This apparatus uses hydro distillation principle. Sample was mixed with water and brought to boil until steam passed through sample. Two layers were formed. Distillate was then collected in Eppendorf tubes. Tubes were tightly closed by using parafilms. For mixture of samples, equal concentrations of sample were loaded in flask. Collected oil phase was centrifuged at 1500 rpm for 10 minutes to separate water from oil. Thus pure oil was extracted. Essential oils were preserved in amber bottles to avoid their evaporation.

Scientific Name	English Name	Local Name
Citrus reticulata	Sweet orange	Fruiter
Citrus sinensis	Raspberry orange	Red blood orange
Citrus lemon	Lemon	Lemon

Gas chromatography/mass spectrometry analysis : The composition of the essential oils was determined by GC/MS. The GC/MS was used for checking the detailed structure of compounds present in the samples.

Antibacterial Activity Assay: Gram-positive and gram-negative bacterial strains of *Staphylococcus aureus*, *E. coli* and *Salmonella enteritidis* were selected in the present study.

The bacterial strains were selected from the Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore. The antibacterial activity of the selected essential oils was checked by using the well diffusion method. The culture of microbial strains was maintained on a Nutrient agar medium. The microbial strains were cultured on the sterilized nutrient agar plates by using the sterilized loop in laminar air flow to avoid any type of contamination. The culture plates were placed in the incubator at 37ºC for 24 hours for bacterial growth. Petri plates were sterilized by using the autoclave at 121ºC under 15psi for 15 minutes and then placed into the hot air oven at 180ºC for one and half hour. Then 25ml sterilized nutrient agar medium was poured in plates in laminar air flow (EN 1822.1M laminar air flow, Singapore). Plates were placed for some time for the solidification of nutrient agar medium. 100 microliters of the bacterial suspension were taken with the pipette and transferred on to the surface of the plate and then spread by using the sterilized spreader. Essential oils were added to each well by using the sterilized micropipette and then placed undisturbed for 25 to 30 minutes in the laminar air flow for homogenous diffusion. These plates were incubated at 37ºC for 18 to 24 hours. The activity was assessed after 24 h by measuring the inhibitory zone in millimeters (mm) around each well.

Antioxidant Assay: The antioxidant activity of the selected essential oils was evaluated by performing DPPH radical scavenging assay. 4mg DPPH was dissolved in 50ml methanol and stored for 30 min to make the DPPH stock solution. The absorbance was measured at 517nm using the same amounts of ascorbic acid as a reference.

DPPH free radical scavenging: 50µl of various concentration of mixture of selected essential oils were mixed with 1ml of DDPH solution in each test tube and incubated at room temperature for 30 min. The absorbance at 517nm of the reaction mixture was measured using a UV-Visible spectrophotometer while keeping deionized water in the reference cell.

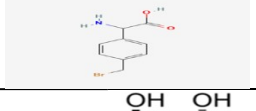
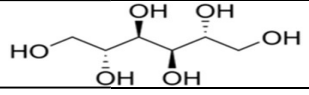
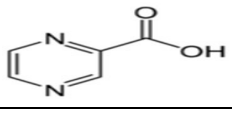
2% radical scavenging activity: Equation was used to calculate the % scavenging activity of mixture of selected essential oils at varied concentrations. (A° = absorbance of control; Ai = absorbance of test).

RESULTS AND DISCUSSION

Essential oils were extracted from peels of selected citrus peel plants by using Clevenger apparatus. Sample was mixed with water and brought to boil until steam passed through sample. Two layers were formed. Distillate was then collected in Eppendorf tubes. Tubes were tightly closed by using parafilms. For mixture of samples, equal concentrations of samples were loaded in flask.

GC/MS for identification of compounds: The chemical composition of essential oils is very complex, normally containing about 20–60 components. Essential oils have been widely used for antibacterial, antifungal, insecticidal and cosmetic applications GC/MS was done

Table 1. Main compounds identified in GC/MS of *Citrus sinensis*

Name of compound	Molecular formula	Molecular weight	Retention time	Structure
Benzene acetic acid	$C_9H10BrNO2$	244.08g/mol	17.332	
Manitol	$C_6H_{14}O_6$	182.172 g/mol	10.015	
Pyrazinoic acid	$C_5H_4N_2O_2$	124.10 g/mol	19.5	

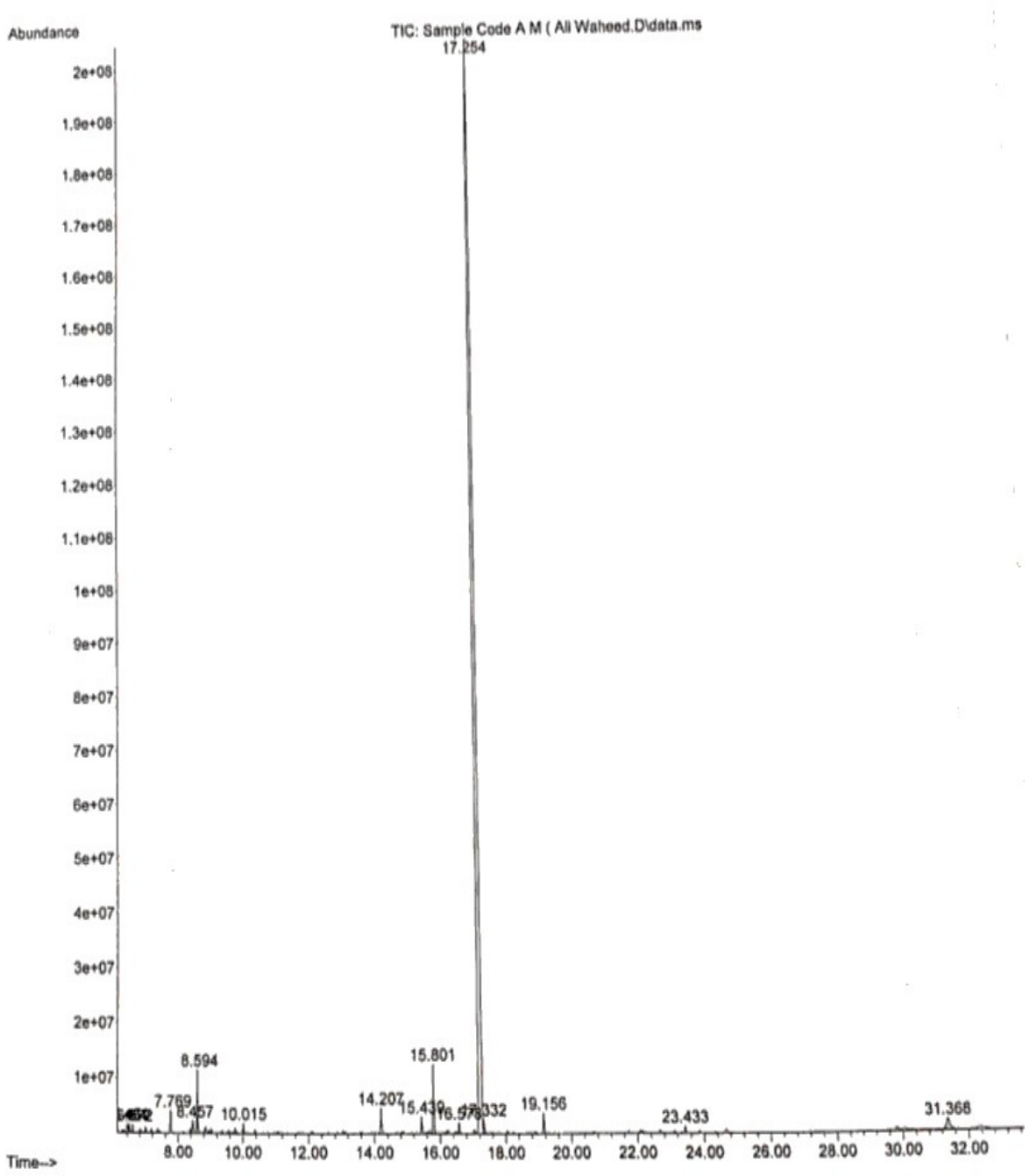
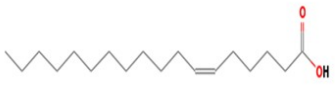


Figure 1. GC/MS analysis of *Citrus sinensis*

Name of compound	Molecular formula	Molecular weight	Retention time	Structure
1-pentanol	C_5H12O	88.15g/mol	29.51	
6-octadenoic acid	$C_{18}H_{34}O_2$	282.4614g/mol	31.604	
3-methyl-1,5-heptadiene	C_8H_{14}	110.19700g/mol	6.409	

for identification of compounds present in the samples of essential oil which shows that D-limonene, manitol, pinene and pyrazinoic acid were dominant among others. The results of determining the chemical composition of the essential oils samples.

Antibacterial Acitvity: The present study evaluated the antibacterial activity of essential oils from peels of *Citrus reticulata*, *Citrus sinensis* and *Citrus lemon*. All the oil extracts were checked for antimicrobial activity against Staphylococcus aureus, *E. coli*, and *Salmonella enteritidis*.

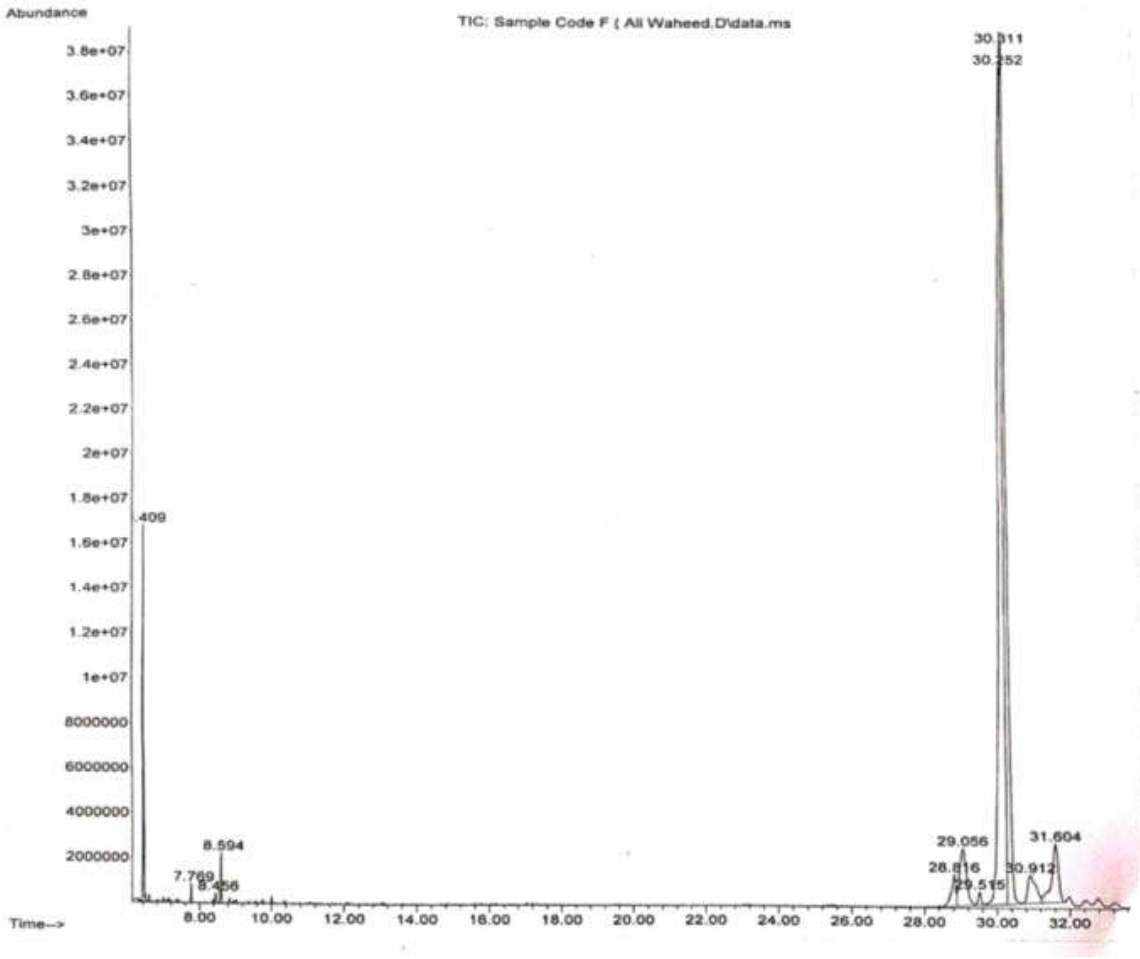
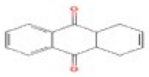
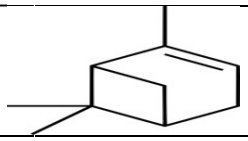


Figure 2.

Table 3. Compounds identified in GC/MS of *Citrus lemon*

Name of compound	Molecular formula	Molecular weight	Retention time	Structure
1-pentene	C_5H_{10}	70.135g/mol	6.4	
Anthracenedione	$C_{14}H_{12}O_2$	212.24g/mol	31.4	
Pinene	$C_{10}H_{16}$	136.238g/mol	19.8	

Antimicrobial activity was checked by using the well diffusion method. Oil extract from peels of *Citrus reticulata* showed more antimicrobial activity against *E. coli*, and *Salmonella enteritidis* as compared the oil extract from *Citrus sinensis* and *Citrus lemon*. According to our results, *Citrus lemon* showed the highest antimicrobial activity against gram-positive bacterial strains. According to our findings gram positive bacteria (*S. aureus*) showed more activity as compared to the gram negative bacteria (*E. coli,* and *Salmonella enteritidis*). The chemical makeup of each bacterial cell wall varies which is thought to be cause. In the cell wall of GPBs, there is thick coating of peptidoglycan that prevents oil from penetrating the cell. The outer layer of GNB, on the other hand, comprises a thin film of peptidoglycan that allows particles to penetrate the cell wall more easily. Another explanation of increased anti-GNB activity might be a strong interaction of oil with bacteria's strongly negative surface.

GPB surface is partly negative due to the presence of teichoic acid in its cell wall. As a result, bacteria have become more resistant to the examined material as a result of reduced contact (Prabuseenivasan *et al.,* 2006). Essential oils are potential sources of novel antimicrobial compounds especially those against bacterial pathogens. Essential oils showed antibacterial activity due to their hydrophobicity, which enables them to divide the lipids of bacterial cell membrane and mitochondrial membrane, thereby disturbing the cell structure and making it more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions would lead to bacterial death (Saeb *et al.,* 2016). Antimicrobial activity of the peel extract is directly concerned with the components that they contain. The studies showed that essential oils, protopine and alkaloids, lactones, polyacetylene, hypericin and pseudohypericin compounds are effective toward various bacteria (Denkova-kostova *et al.,* 2021).

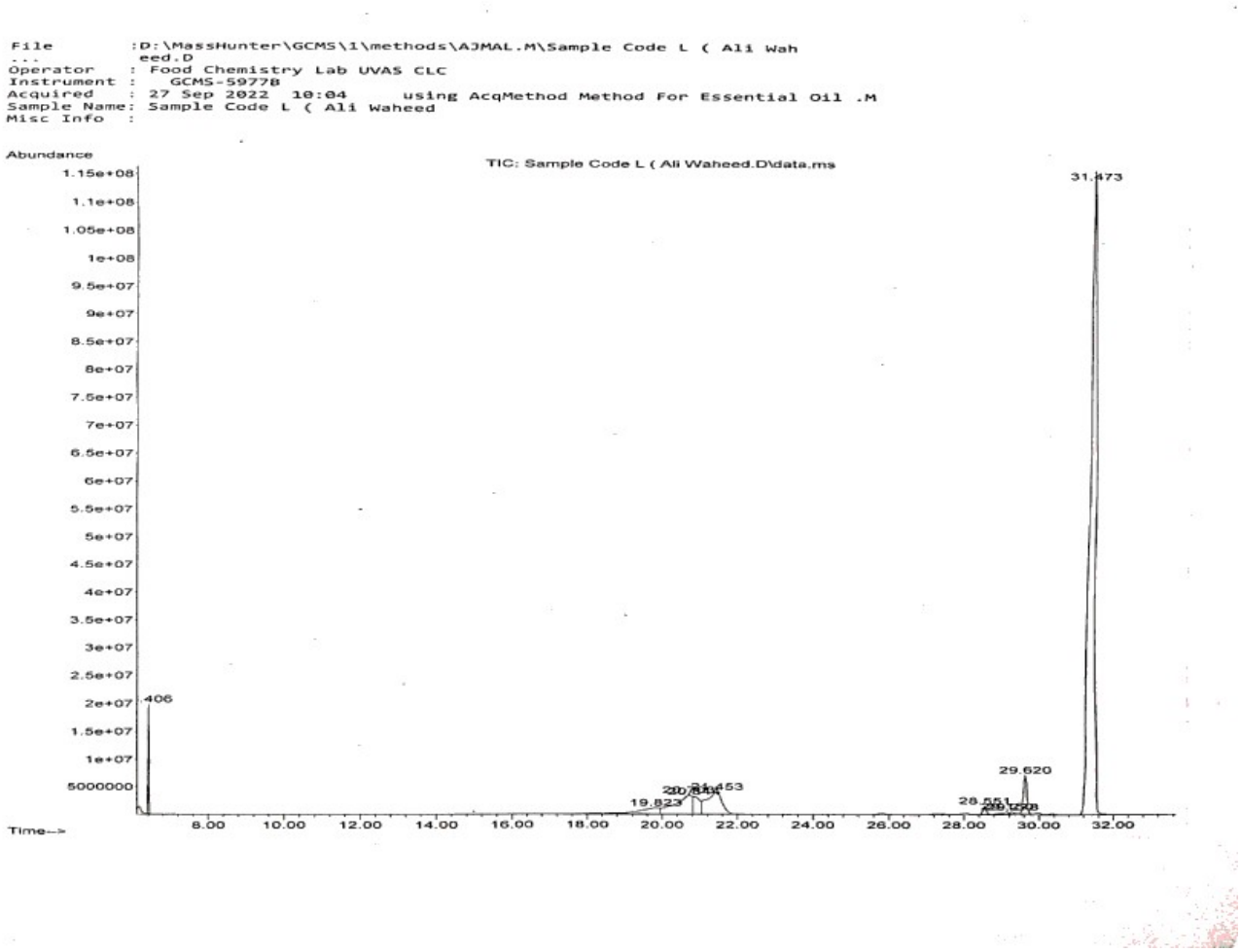


Figure 3. GC/MS analysis of *Citrus reticulate*

Table 4. Compounds identified in the GC/MS of mixture of essential oils

Name of compound	Molecular formula	Molecular weight	Retention time	Structure
Limonene	$C_{10}H16$	136.23g/mol	8.5	
Pyrazinoic acid	$C_5H_4N_2O_2$	124.10 g/mol	19.5	
Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4g/mol	21.21	

GC/MS analysis which showed presence of limonene present in higher concentration. The antimicrobial activity of the selected essential oil was compared with CLSI standards Ciprofloxacin. GC/MS analysis which showed presence of limonene present in higher concentration.

Antioxidant Activity: Free radicals are extremely erratic molecules that take electrons from other molecules to stabilize themselves, denaturing the intended target molecule in the process. Antioxidants have the power to stop other molecules from oxidizing. Using a DPPH free radical scavenging experiment, the antioxidant capacity of the mixture of oils at various doses was evaluated.

A stable radical is the DPPH radical. When it accepts electrons or free radical species, it loses the ability to absorb, which causes a visible discoloration. It has been widely utilized for antiradical activity screening. Synthetic antioxidants such as butylhydroxytoluene (BHT) being toxic to human nature has provided a large area for study of natural oxidants such as essential oils (Amorati *et al.,* 2013). According to our result, absorbance of the sample was found to be decreasing as the concentration was increased. The free radical scavenging activity of the sample continued to increase despite the decrease in absorbance. When the concentration of the sample was increased, the antioxidant activity of the sample increased after oxidation process. The scavenging activity checked was based on the capability of the antioxidants to retard color loss.

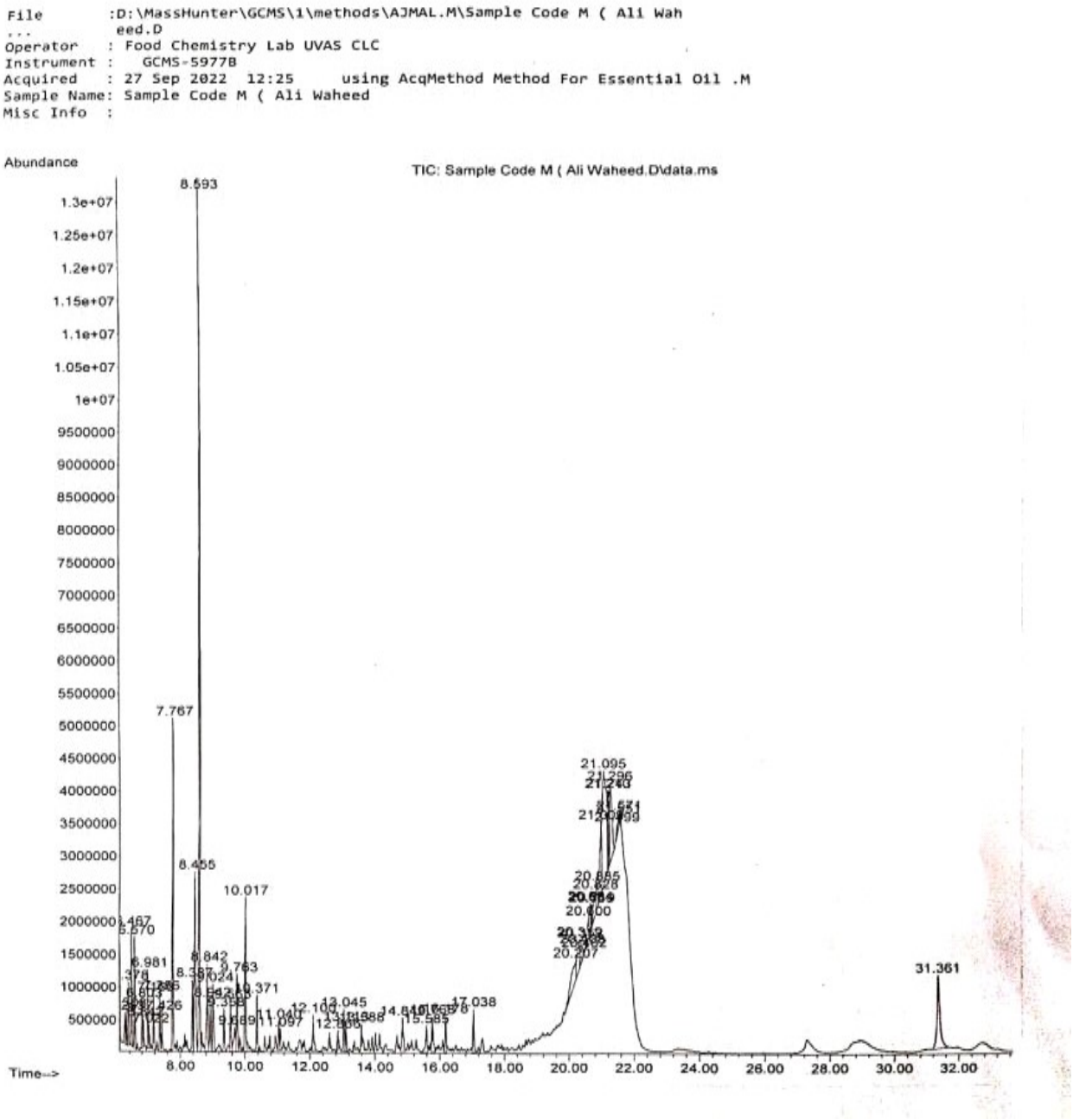


Figure 4. GC/MS analysis of mixture of esseontial oil

Table 5. Antibacterial activity of essential oils of *Citrus lemon, Citrus sinensis, Citrus reticulata* and their mixture in mm against gram-negative and positive bacterial strains

Bacterial strains	Standard (Ciproflaxin) ZOI (mm)	*Citrus lemon* ZOI (mm)	*Citrus reticulata* ZOI (mm)	*Citrus sinensis* ZOI (mm	Mixture ZOI (mm)
E.coli	25.3 ± 0.59	7 ± 0.1	11 ± 0.27	10 ± 0.27	14 ± 0.31
Salmonella enteritidis	25.3 ± 0.5	5 ± 0.08	10 ± 0.19	8 ± 0.09	11 ± 0.21
Staphylococcus aureus	25.3 ± 0.5	24 ± 0.5	15 ± 0.35	14 ± 0.31	17 ± 0.36

Table 5. %RSA and IC_{50} value of mixture of essential oils of *Citrus lemon, Citrus sinensis, Citrus* against DPPH radical scavenging assay

Sample	Concentraton (µg/ml)	%RSA	IC_{50}
Mixture (*Citrus reticulata* + *Citrus sinensis* + *Citrus lemon*	20	35.18	49.73
	40	43.51	
	60	53.70	
	80	62.03	
	100	67.59	
Ascorbic acid	20	12.06	46.71
	40	20.68	
	60	29.31	
	80	34.48	
	100	50.10	

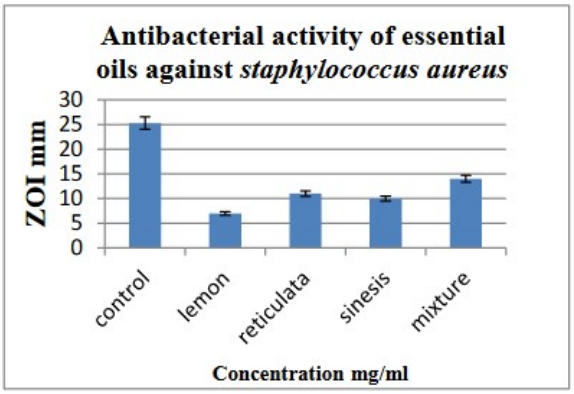


Figure 5. Mean value of zone of inhibition of Citrus reticulata, Citrus sinensis and Citrus lemon and their mixture against Staphylococcus aureus

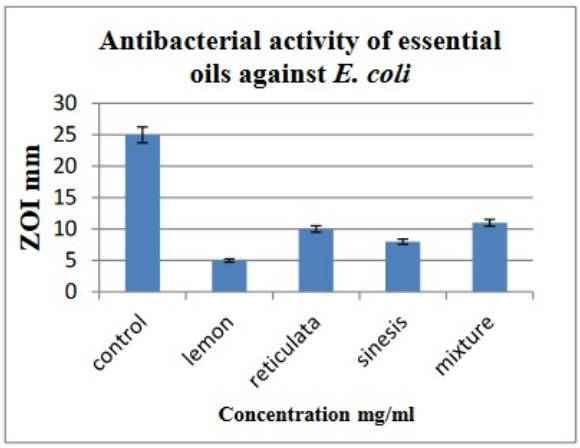


Figure 6. Mean value of zone of inhibition of *Citrus reticulata*, *Citrus Sinensis*, *Citrus lemon* and their mixture against *E.coli*

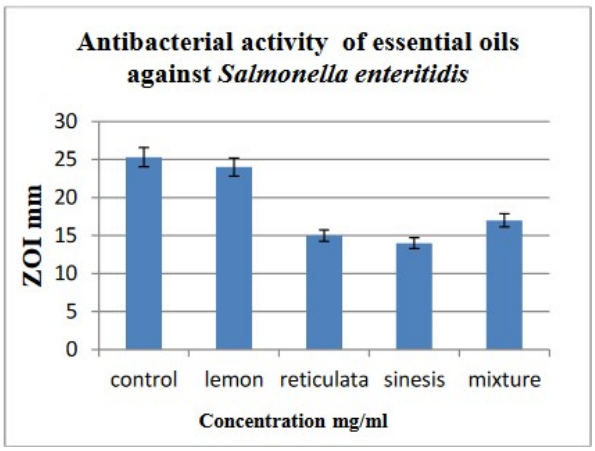


Figure 7 Mean value of zone of inhibition of Citrus reticulata, Citrus sinensis, Citrus lemon and their mixture against S. Enteritidis

The highest concentration of natural antioxidants, including flavonoids, phenolic acids, and carotenoids, as well as reducing sugar, is found in citrus peel. The free radicals are stabilized by the phenolic, flavonoid, and carotenoid content's reaction with the Citrus peel demonstrated antioxidant activity because it could donate hydrogen to free radicals, which helped to stabilize them. Their increased phenolic content is probably what gives them their ability to donate hydrogen (Nasır and Yabalak 2021). The IC_{50} value of a sample refers to the amount of that sample that is required to scavenge 50% of the radicals. The lower IC_{50} values had better antioxidant potential. The antioxidant of essential oil compared with standard ascorbic acid.

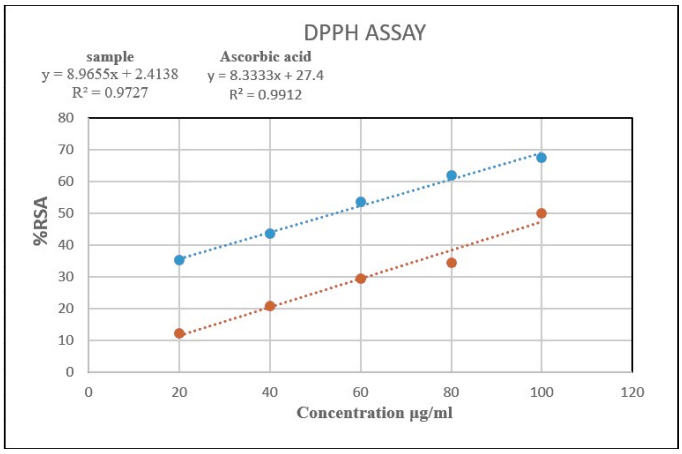


Figure 9. DPPH assay with concentration in µg/ml and %RSA

CONCLUSION

All the antibacterial agents that are in use show toxic side effects and are relatively expensive. In search of efficacious, less toxic, and more economical antibacterial agents this study was performed and it was found that the essential oils of *Citrus lemon, Citrus sinensis, Citrus reticulate* and their mixture showed significant antibacterial activity against *Staphylococcus aureus*, *E. coli*, and *Salmonella enteritidis*. Antioxidant activity of mixture was found higher than others. The essential oils extracted from mixture of peels of *Citrus sinensis*, *Citrus reticulata, Citrus lemon* and their mixture can be used for the treatment of several ailments. The essential oils provide bioactive compounds which can be used as an alternative to synthetic drugs. Due to the resistance to the drugs, they are used for the treatment of different kinds of diseases.

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