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Bioactive properties of the extracts of peels, pomace, seeds, and essential oils of *Citrus limon* and *Citrus aurantifolia*

Folasade Oluwatobi[1], Olakunle Afolabi[2], Pius Okiki[1], Funmilayo Adeniyi[3], Oghenerobor Akpor[1]*

[1]Department of Biological Sciences, Afe Babalola University, Ado-Ekiti, Nigeria.
[2]Biochemistry Program, Department of Chemical Sciences, Afe Babalola University, Ado-Ekiti, Nigeria.
[3]Department of Pharmacology and Toxicology, College of Pharmacy, Afe Babalola University, Ado-Ekiti, Nigeria.

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ABSTRACT

Citrus fruit wastes contain a high concentration of plant secondary bioactive compounds with multifunctional properties. The purpose of this study was to investigate into the antioxidant and antimicrobial properties of lemon and lime essential oils and extracts. Antimicrobial properties of the extracts and essential oils were determined using the agar-well diffusion method, while antioxidant potentials were determined using the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity assay. The samples were screened for their antibacterial activities, and all tested bacterial isolates were susceptible to all lemon seed extracts. The most sensitive bacterial isolate was *Klebsiella pneumoniae* (28 mm) to ethanol extract of lime peel, while the majority of the bacterial isolates were susceptible to lime pomace. None of the bacterial isolates tested positive for sensitivity to lime seed ethanol extract. *Pseudomonas aeruginosa* was found to be resistant to all lime extracts. Furthermore, all of the extracts demonstrated a significant ($p < 0.05$) increase in their inhibitory activities against DPPH free radical in a concentration-dependent manner. The gas chromatography-mass spectroscopy analysis on the extracts and essential oils revealed the presence of tetradecanoic acid, 1,4-butanediol, 2-(1-ethoxyethoxy)-3-methyl-, n-hexadecanoic acid, 1,2-benzenedicarboxylic acid, diisooctyl ester, stigmasterol, and spathulenol. Therefore, the findings of this study revealed that extracts and essential oils of lemon and lime could be explored for possible bioactivities and health benefits.

1. INTRODUCTION

Fruits and vegetables are regarded as medicinal plants, which play important roles in human nutrition and community health because they are rich in secondary metabolites and dietary fibers. [1,2]. Many chemical compounds are found in these plants, including alkaloids, flavonoids, glycosides, saponins, resins, oleoresins, sesquiterpene, phenolic compounds, fats, and oils [3,4]. Citrus fruits are one of the most abundant and widely available fruit crops worldwide, due to their high productivity, low cost, nutritive values, sweet taste, flavor, and healthy dietary properties [4-6]. Citrus fruits belong to *Rutaceae* family which composes about 1300 species and the commonly cultivated citrus fruits include Lemon (*Citrus limon*), Orange (*Citrus sinensis*), Grapefruit (*Citrus paradisi*), Lime (*Citrus latifolia*), and Mandarin/ Tangerine (*Citrus reticulate*) [4,7]. Oil extract of lemon leaf has been reported to inhibit the growth of bacteria due to monoterpenes and alkaloids in it. Furthermore, lemon juice plus hot water has been described as the best antibacterial gargle [8].

**Corresponding Author:*
Oghenerobor Akpor,
Department of Biological Sciences,
Afe Babalola University, Ado-Ekiti, Nigeria.
E-mail: akporob @ abuad.edu.ng

Several studies have documented global production of citrus fruit to be of about 160 million tons per annum, with 75% being freshly consumed while the remaining are processed into other products. About 50–60% of waste can be generated from the fresh fruit mass which are used as livestock feeds or discarded on an open land thereby causing environmental pollution, underutilization of its high-value application potentials, and reduction in the nation's economy [7-10].

Citrus fruit wastes especially the peels, pomace, and seeds are part of the important agro-wastes generated from crops [11,12]. Documented evidence shows that bio-based polymers were first generated from plant-based biopolymers especially polymers obtained from agriculture wastes [13]. Moreover, agro-wastes like citrus fruit wastes possess a significant number of compounds such as fibers (cellulose, hemicelluloses, lignin, and pectin), lipids, carbohydrates, peptides, carotenes, essential oils, phenols, some bioactive components such terpenoids, carotenoids, coumarins, limonoids and vitamins, and so on, that are bioactive and of multi-functional values [4,14,15]. They have a lot of high-value application potentials and are therefore used after conversion or recycling as ingredients incorporated into some food products to aid their nutritional and antimicrobial properties, shelf lives, and other qualities [2].

Several studies have reported citrus fruit wastes as an important source of bioactive compounds and antioxidants such as ascorbic acid, flavonoids phenolic compounds, and pectins, which are essential in human nutrition. *Eriocitrin*, *Narirutin*, *Naringin,* and *Hesperidine*, are main flavonoids found in citrus species and its by-products or wastes. For instance, the Citrus wastes possess naturally occurring flavonoids especially the peel, which has the highest concentration of flavonoids such as flavanones and polymethoxylated flavones, compared to other plant where they are scarcely found [4,16]. Despite the development of various antibiotics by the pharmaceutical industry, bacterial populations are rapidly developing antibacterial resistance, posing a serious threat to the success of infectious disease treatment. On the other hand, commercial antibiotic-related side effects are frequently mentioned, therefore, new antimicrobial compounds need to be discovered and researched frequently. Plant-based bioactive components are important source for the discovery of novel and natural antimicrobial and antioxidant potentials and they have demonstrated ability to prevent enzymatic activity, interact with proteins, boost immune system, and hinder pathogenesis of metabolic-related human disease condition by harming microbial cells [17,18].

The essential oils obtained from citrus fruit peels comprise many volatile compounds, mainly aldehydes, ketones, esters, alcohols, and terpenes, which give it characteristic aromas and flavors. Citrus essential oils are greatly utilized as flavoring agent in the food and beverage industries, and as fragrance materials in the perfumery, toiletries, fine chemicals, and cosmetic products. They can also be used to some extent as a traditional medicine [19]. Attention has been drawn to the application of agricultural and food processing wastes as one of the major sources of bioactive compounds for their natural antibacterial, antifungal, anticancer, antidiabetic, and antiviral activities which will consequently enhance the nation's health systems and economy, the prevent environmental pollution and sustains the ecosystem [20]. However, a few information has been reported on the bioactive components of citrus fruit waste extracts and essential oils to establish the facts about their biological significance and potential health effects. Hence, this study aimed to investigate the bioactive properties of essential oils and ethanol extracts of two citrus fruits (lemon and lime) wastes.

2. MATERIALS AND METHODS

2.1. Collection of Plant Materials

Lemon (*Citrus limon*) and lime (*Citrus aurantifolia*) fruits were obtained from a local market in Ado-Ekiti, Ekiti State, Nigeria. A voucher sample was deposited and authenticated at the Department of Biotechnology and Plant Science, University of Ado-Ekiti, Ekiti State Nigeria, Herbarium numbers: UHAE2023003 (Lemon) and UHAE2023002 (Lime) were thereafter provided. A total of six bacterial and five fungal pathogens were used for antibacterial and antifungal assay, respectively. The bacterial species were *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus subtilis,* and *S. aureus* while the fungal cultures were, *Aspergillus niger, Alternaria* sp.*, Corynespora* sp.*, Fusarium* sp.*,* and *Rhizopus* sp. All the bacterial and fungal pathogens were obtained from the Microbiology Laboratory in the Department of Biological Sciences, Afe Babalola University, Ado-Ekiti, Nigeria. Before use, the bacterial and fungal pathogens were sub cultured on nutrient agar and sabouraud dextrose agar plates, respectively, to ascertain their purity.

2.2. Preparation of Plant Extracts

This study employed two citrus fruits: Lemon (*Citrus limon*) and lime (*Citrus aurantifolia*). Peels, pomace, seeds, and essential oils were used for each of the fruits. The fruit samples were washed several times with distilled water before use to remove sand and other debris or dirt. To obtain the pomace and seeds, the samples were peeled, cut, and the juice was squeezed out. Each fruit part was then air-dried for 14 days before being pulverized with a sterile electric blender and weighed. 200 g of each of the pulverized peels, pomace, and seed were soaked in 400 mL of absolute ethanol for 24 h for extraction. The solution was then filtered using Whatman No. 1 filter paper and transferred to a rotatory evaporator for concentration of the extracts.

2.3. Preparation of the Essential Oils

The essential oils were prepared using the method described by the Association of Official Analytical Chemist (AOAC) 920.85 with Soxhlet apparatus [21]. 100 g of the air-dried respective sample was wrapped with a filter paper and placed into the thimble of the Soxhlet extractor. The material sample was continuously extracted for 6 h using n-hexane as the solvent. At the end of the extraction, the sample was removed from the thimble and the solvent distilled off. The oil was poured into a sample bottle and kept for further analysis.

2.4. Antimicrobial Assay

In vitro, antibacterial and antifungal activity was evaluated by the agar-well diffusion methods. The respective peel, pomace and seed extracts, and essential oils were tested against all the bacterial and fungal species. For stock extract solution, 1 g of the respective extracts were dissolved in 20 mL of dimethylsulfoxide. Antibacterial assay of the extracts and essential oils was carried out as described elsewhere [22]. For antifungal assay, mycelial discs of young actively growing respective fungal cultures were cut separately with a sterile cork borer and inoculated at the center of already prepared plates containing the different extracts and the control plates (without extracts) and incubated at 28 ± 2°C for 3 d.

The mycelial growth diameter (cm) of each pathogen was measured and the percentage of growth inhibition was calculated according to [23] as follows:

$$\text{\% Mycelial growth inhibition} = \frac{D_o - D_t}{D_o} \times 100$$

Where Do = Diameter of mycelial growth of fungal pathogen in the control plates; D_t = Diameter of mycelial growth of fungal pathogen in the treatment plates.

2.5. Qualitative Phytochemical and *In Vitro* Antioxidant Assays

The qualitative phytochemical screening of the respective extracts was determined as described [24]. The DPPH (Sigma–Aldrich, Sternheim, Germany) free radical scavenging activity assay of the extracts was carried out according to the method described [25]. A properly diluted portion (1 mL) was combined with an equal volume of a methanol-based 0.4 mM DPPH solution. The mixture was measured at 516 nm for absorbance after 30 min of dark incubation, using Quercetin as a standard. The DPPH free radical scavenging activity was reported as a percentage (%) control inhibition.

2.6. Gas Chromatography-Mass Spectrophotometric (GS-MS) Analysis

GC-MS analysis of the respective samples was carried using a Varian 3800/4000 gas chromatograph mass spectrometer equipped with an Agilent equipped with a BP5 (30 m × 0.25 mm × 0.25 microns) capillary column.

2.6.1. Identification of compounds

Organic compounds in the samples were identified in Wiley's NIST 08 Mass Spectral Library to obtained comparison scores that were higher than 95%. Otherwise, fragmentation peaks of the compounds were evaluated, and the compounds were identified using the memory background for the identification of the compounds that appeared in GCMS chromatograms.

3. RESULTS

3.1. Antibacterial Properties of the Extracts and Essential Oil

Antibacterial assay of the lemon extracts and essential oil revealed sensitivity of all the bacterial species to the seed extracts. However, *B. subtilis, P. aeruginosa,* and *S. aureus* were observed to be resistant to the pomace, peel extracts, and essential oil, respectively. In general, highest zone of inhibition (16 mm) was recorded against *Klebsiella pneumonia,* for the peel extract. None of the lime extracts and essential oil inhibited the growth of *P. aeruginosa. B. subtilis* and *Salmonella typhi* were observed to be resistant to the pomace extract while all the seed extract did not inhibit the growth of any of the bacterial species [Table 1].

3.2. Fungal Inhibitory Potential of the Extracts and Essential Oil

In general, all the extracts and essential oils showed mycelia inhibition against the test fungal pathogens. The highest mycelial inhibition of 84, 71, 78, and 67% was observed against *Corynespora* sp. when treated with the lemon peel, pomace and seed extracts and essential oil, respectively. In the lime extracts and essential oil, the highest mycelial inhibition of 62% (peel and pomace), 73% (seed), and 67 (essential oil) was observed against *Corynespora* sp. [Table 2].

3.3. Phytochemical Components of the Extracts

Phytochemical screening of the extracts revealed the presence of alkaloids, saponins, carbohydrates, reducing sugars, flavonoids, terpenoids, phenols, and tannins in the lemon peel, and seed extracts. Reducing sugars were however absent in the lemon pomace. In the case of the lime peel and pomace, terpenoids were observed to be absent, while flavonoids, phenols, and tannins were absent in the lime seed extract [Table 3].

3.4. DPPH Scavenging Activity of the Extracts

The antioxidant potential of the extracts revealed DPPH free radical inhibiting ability in a concentration-dependent manner. At the concentrations studied, all the lemon and lime extracts (peel, pomace, and seed) showed higher DPPH scavenging ability than Quercetin [Figure 1].

3.5. Compounds Detected in the Lemon Extracts and Essential Oils

A total of 15 compounds were detected from each of the extracts and essential oils. The major compounds identified in

Table 1: Antibacterial potential of the extracts and essential oil.

Extracts	Zone of inhibition (mm)					*Salmonella typhi*
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Bacillus subtilis*	
Lemon extracts						
Peel	5	8	14	16	Resistant	13
Pomace	10	Resistant	10	11	8	10
Seed	8	8	10	13	8	10
Oil	7	5	Resistant	15	9	8
Lime extracts						
Peel	9	Resistant	8	28	Resistant	Resistant
Pomace	11	Resistant	11	28	8	13
Seed	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
Oil	5	R	11	9	15	5

Table 2: Fungal inhibitory potential of the extracts and essential oil.

Extracts	Percentage mycelial inhibition				
	Aspergillus niger	*Alternaria sp.*	*Corynespora sp.*	*Fusarium sp.*	*Rhizopus sp.*
Lemon extracts					
Peel	35	44	84	25	25
Pomace	31	29	71	22	10
Seed	38	49	78	13	65
Oil	22	60	67	37	65
Lime extracts					
Peel	38	40	62	50	5
Pomace	18	56	62	25	10
Seed	38	40	73	50	65
Oil	18	60	67	37	60

Table 3: Phytochemical composition of the extracts.

Extracts	Alkaloids	Saponins	Carbohydrates	Reducing sugars	Flavonoids	Terpenoids	Phenols	Tannins
Lemon								
Peel	+	+	+	+	+	+	+	+
Pomace	+	+	+	−	+	+	+	+
Seed	+	+	+	+	+	+	+	+
Lime								
Peel	+	+	+	+	+	−	+	+
Pomace	+	+	+	+	+	−	+	+
Seed	+	+	+	+	−	+	−	−

"+" and "−" represent present and absent, respectively,

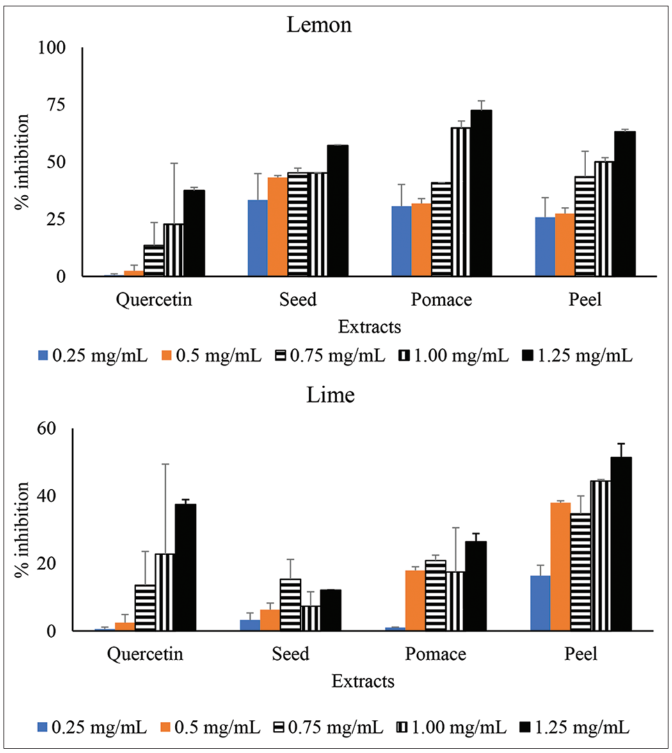

Figure 1: 2,2-diphenylpicrylhydrazyl scavenging activity of the extracts at different concentrations.

the lemon extracts and essential oil were tetradecanoic acid, 1,4-butanediol, 2-(1-ethoxyethoxy)-3-methyl-, n-hexadecanoic acid, 1,2-benzenedicarboxylic acid, diisooctyl ester, stigmasterol, spathulenol, octadecane and diethyl phthalate, and 3,7,11,15-tetramethyl-2-hexadecen-1-ol. The highest detection of tetradecanoic acid (20.36%) was detected in the peel extract [Figure 2]. In the lime extracts and essential oil, the dominant compounds detected were n-hexadecanoic acid, 1,2-benzenedicarboxylic acid, diisooctyl ester, tetradecanoic acid, 9,12,15-octadecatrienoic acid, methyl ester, stigmasterol, spathulenol, benzoic acid, 4-hydroxy-3,5-dimethoxy-, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, and 9,12-octadecadienoic acid (Z, Z)-. The highest detection of n-hexadecenoic acid (18.90%) was observed in the pomace extract [Figure 3].

4. DISCUSSION

The results in this study revealed the susceptibility of all the microorganisms tested against the lemon seed extracts. This is consistent with the findings [22] and [24] that reported a remarkable bioactivity of lemon extract against most of the tested bacterial species in this study. Furthermore, *S. typhi*, among the bacterial isolates tested, was most vulnerable to the essential oil of citrus species. This antibacterial activity of lemon extracts and essential oil could be due to the synergistic effect of the available bioactive constituents. These compounds could also be responsible for the inhibitory action against the tested species by the interaction of the phytochemicals with the microbes' intracellular sites through the reported formation of hydrogen bond which alters their cellular protein structures and consequently results to microbial death [18,22,27].

Similarly, all the extracts and essential oils showed mycelia inhibition of the tested fungal pathogens and the highest mycelial inhibition was demonstrated against *Corynespora* sp. when treated with the lemon peel, pomace, seed extracts, and essential oils. This observation corroborates several other studies that reported inhibitory effect of citrus extracts against the growth of different fungal species [23,28,29].

The presence of alkaloids, saponins, carbohydrates, reducing sugars, flavonoids, terpenoids, phenols, and tannins was confirmed by phytochemical screening of the lemon peel and seed extracts. However, reports of previous studies have linked the antimicrobial activity of some of these metabolites to the presence of oxygenated terpenes and phenolic groups in particular, as well as the synergetic effects of all the chemical constituents in the extracts and essential oils [22,30]. Moreover, therapeutic properties (antidiabetic, anticarcinogenic, antithrombotic, stimulatory, antibacterial, antifungal, and antiviral) of citrus extracts and essential oils have been related to the presence of some bioactive components such as flavonoids, carotenoids, and alkaloids. [15,18,19,31].

In addition, at the various concentrations investigated, all the lemon and lime extracts (peel, pomace, and seed) showed higher antioxidant capacity through their DPPH free radical scavenging ability in a favorable competitive manner with quercetin. This effect could possibly be linked to the ability of lemon and lime extracts to release hydrogen atoms (H^+) and consequently neutralize the effects of unstable electrophiles/free radicals [18]. The antioxidant activity of lemon and lime extracts could also be due to the presence of detected bioactive compounds as revealed in the result of GC-MS analysis

A total of 15 compounds were detected from each of the extracts and essential oils which are in consistent with past report [32]. Moreover, the highest level or concentration of tetradecanoic acid was confirmed in the peel extract while n-hexadecenoic acid in the pomace extract.

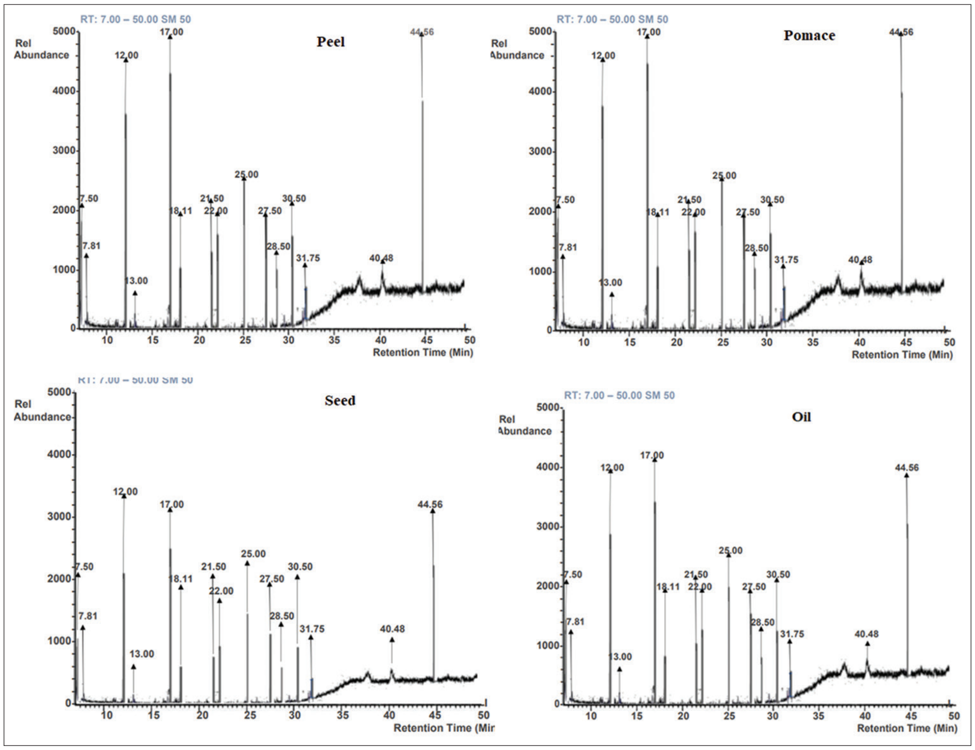

Figure 2: Gas chromatograms of the lemon extracts and essential oil.

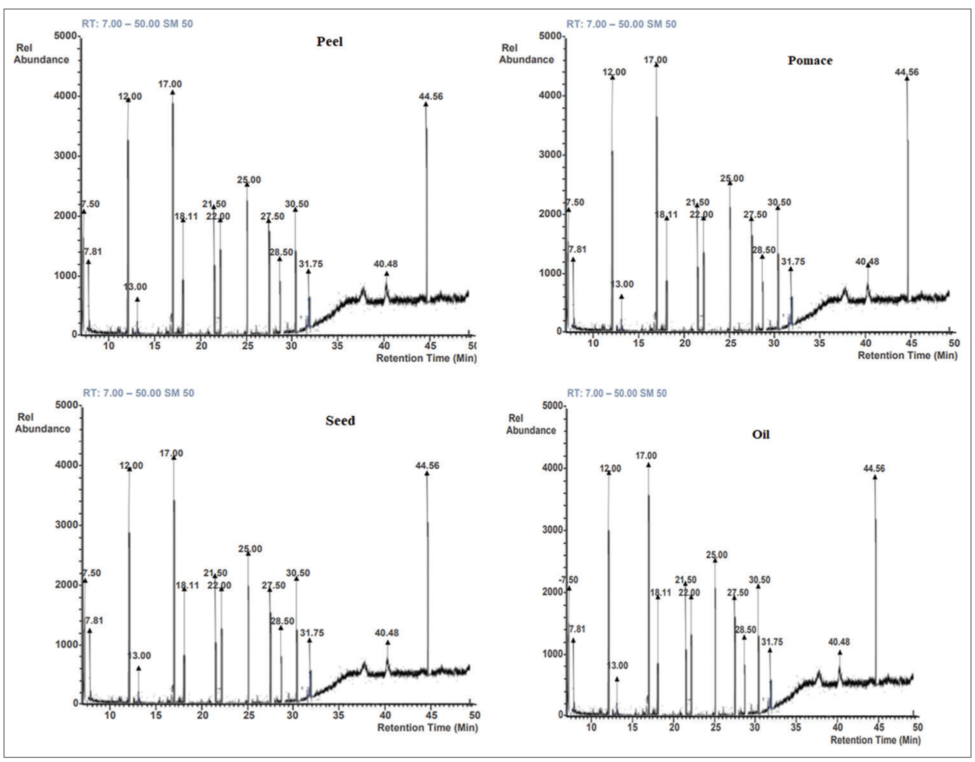

Figure 3: Gas chromatograms of the lime extracts and essential oil.

Several studies have reported citrus extracts especially tetradecanoic acid detected in lemon peel and n-Hexadecanoic acid in lime pomace to possess or exhibit l antibacterial, anti-inflammatory, antioxidant, and antifungal effects when used alone or combined [33]. The differences in the n-hexadecanoic acid (lemon, 16.33%; lime, 13.12%), 1,2-benzenedicarboxylic acid, diisooctyl ester (lemon, 15.33%; lime, 10.33%) and octadecane (lemon, 10.75%; lime, 5.08%) could also be another factor which made lemon extracts to be more effective on all the bacterial and fungal isolates compared to lime extracts.

5. CONCLUSION

The findings of this work revealed that all lemon extracts and essential oil possessed higher antimicrobial activity against many of the bacterial and fungal isolates tested, most especially *K. pneumoniae* and *salmonella typhi*. Whereas, lemon seed and peel demonstrated a higher antioxidant activity and percentage composition of tetradecanoic acid. Therefore, it could be suggested that lemon extracts and its essential oil possess antimicrobial activity than lime extract and essential oil, an observation that could possibly be linked to the activities of available phytochemicals (secondary metabolites). The findings of this study revealed that extracts and essential oils of lemon and lime could be explored for their possible bioactivities and potential health benefits.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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