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Phytochemical constituents and antibacterial activity of *Citrus lemon* leaves

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Abstract

Background: The study investigated the phytochemical constituents and antibacterial activity of *Citrus lemon* volatile oil extracted from pruning leaves collected from a private farm at Nobariya district against Gram-positive "*Staphylococcus aureus* NRRL B-313 and *Bacillus cereus* NRC" and Gram-negative "*Pseudomonas aeruginosa* NRC B-32 and *Escherichia coli* NRC B-3703".

Results: The oil obtained from powdered dried *C. lemon* leaves was analyzed by GC–MS to identify their constituents. The analysis revealed the presence of sabinene, carene, limonene, and β-ocimene. The leaves volatile oil showed a remarkable inhibition against *S. aureus* (32 ± 0.01 mm) and *P. aeruginosa* (49 ± 0.01 mm) and it had a strong effect on the DNA, RNA, lipids, and protein biosynthesis in cells of *S. aureus* and had a strong effect on the lipids biosynthesis in cells of *P. aeruginosa*.

Conclusion: The results in this study suggested that *C. lemon* leaves could be beneficial in developing a novel antibiotic and studied its mode of action on the pathogenic microorganism's cells.

Keywords: *Citrus lemon*, GC–MS, Antibacterial, MIC, Mode of action

Background

Bacteria are responsible for increasing the mortality rates in many developing countries; about 50,000 people died every day as a result of infections (Sapkota et al. 2012). Disease caused by microbes that had become resistant to drug therapy was an increasing problem of public health. Many researchers have been interested in developing modern antimicrobial reagents with the emergence of antibiotic-resistant microbes, which increases the cost of healthcare (Maiti et al. 2014). Agriculture crops (fruits) produce large amount of wastes or by-products every year. These wastes included pruning materials and juice production wastes of different industrial nutritional companies (El-gengaihi et al. 2020).*Citrus lemon* contains about 5% citric acid that gives lemons pH 2–3, and it is used as antibacterial due to the low pH (Sapkota et al. 2012). The same finding was revealed by Mathew et al. (2012) on their study on *Citrus lemon*, and they reported that the extract of pulp revealed the presence of carbohydrates, alkaloids, fixed oils, tannins, proteins, cardiac glycosides, sterols, phenols, and flavonoids. Also, Oikeh et al. (2016) reported that juice of *Citrus lemon,* contained: flavonoids, alkaloids, steroids, saponins, terpenoids, reducing sugars, and cardiac glycosides. This investigation suggested that this juice had beneficial antimicrobial roles which could be controlled by the unwanted microbial growth. To determine antimicrobial susceptibility *in vitro*, various methods were commercially available, and microbiology clinical laboratories choose an instrument or manual-based method for performing routine antimicrobial activity testing (Chiang et al. 2009). The commonly used methods include disk diffusion (Hubert et al. 1998) broth micro-dilution and rapid automated instrument-based methods (Wayne 2006). In many countries, the disk diffusion method was the commonly used method in clinical laboratories. This test provided the greatest flexibility and cost-effectiveness

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in which the test took 24 h (Liao et al. 2008). Therefore, this work aimed to estimate the *C. lemon* leaves oil constituents and its potential as a novel antibiotic against pathogenic bacteria, and the effect on the DNA, RNA, lipids, and protein biosynthesis in *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells.

Methods

Plant material

Citrus lemon as pruning materials were collected on February 2017 from a private farm situated in Nubaria district. The pruning wastes composed of leaves were freshly weighed, then oven-dried at 50 °C and again weighed. Water distilled using a Clevenger apparatus was used to collect higher quantity to determine volatile oils percentage, and then GC–MS analyses of the volatile oils were adopted.

GC–MS analyses of the volatile oils obtained from *Citrus lemon* leaves

The GC–MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, NRC Cairo, Egypt. Samples were diluted with hexane (1:19, v/v). The GC was equipped with HP-5MS column (30 m × 0.25 mm internal diameter and 0.25 μm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1 mL/min at a split ratio of 1:10, injection volume of 1 µl and the following temperature program: 40 °C for 1 min; rising at 4 °C/min to 150 °C and held for 6 min; rising at 4 °C/min to 210 °C and held for 1 min. The injector and detector were held at 280 °C and 220 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 50–550 and solvent delay 5 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Antibacterial activity

Bacterial stains and culture media

Four bacterial strains of importance were used to trial the antibacterial properties of the volatile oil obtained. Two of the bacterial strains were Gram-positive *Staphylococcus aureus* NRRL B-313 and *Bacillus cereus* NRC, while the others were Gram-negative *Escherichia coli* NRC B-3703 and *Pseudomonas aeruginosa* NRC B-32. Nutrient agar media was used in this study for bacterial strains growth. The media was sterilized at 121 °C by autoclaving after that used for sub-culturing, and agar media was utilized for agar well diffusion assay.

Agar-well diffusion assay

About 20 mL nutrients of agar media were sited into Petri dishes (10 mL). Tween 20 (0.5% v/v) was added to the agar after autoclaving to improve oil solubility, and 100 µL of the fresh cultures was spread over the plate using a sterile swap spreader to get a uniform microbial growth for all plates. A well was done as 9 mm diameter in the agar plate. The wells loaded with 50 µL of the *Citrus lemon* leaves volatile oil. The plates were left for 1 h at refrigerator to allow the diffusion of oil, and then plates were incubated at 37 °C for 24 h. The inhibition zone appeared was measured with a ruler.

Minimal inhibitory concentration (MIC) determination

Determination of the MIC for *Pseudomonas aeruginosa* and *Staphylococcus aureus* was measured by optical density assay for the different microbial strains *P. aeruginosa* and *S. aureus* by added different volumes of volatile oil 1, 2, 3, 4, and 5 µL. *C. limon* leaves volatile oil at 5 mL nutrient broth culture was then inoculated with 50 µL of the *S. aureus* and *P. aeruginosa* fresh cultures and incubated in shaking incubator at 150 rpm at 37 °C for 24 h. Microbial growth was measured at 620 nm, and the results were expressed as growth inhibition percentage.

Mode of action

The effects of different concentrations of lemon leaves volatile oil on several biochemical activities were studied according to Ramadan et al. (2012). After inoculating flasks with *S. aureus* and *P. aeruginosa* strains, during the middle logarithmic growth phase, volatile oil was applied to lemon leaves at concentrations of 1/8, 1/4, 1/2 MIC. Then, the flasks were shaken at 120 rpm at 37 °C. Samples were withdrawn at the onset of the experiment and after incubation periods of 20, 40, 60, 80, 100, and 120 min in the case of *P. aeruginosa*, and 20, 40, 60, 80, 100, 120, and 180 min in the case of *S. aureus.* The bacterial cells were subjected to determination of acid-soluble phosphorus, total lipids, soluble protein, DNA, and RNA.

Acid-soluble phosphorous was determined according to Hogeboom and Schneider (1950) and Toribara et al. (1956). Bacterial cells were collected, washed two times with ice-cold saline and extracted two times with 5% ice-cold trichloroacetic acid. The suspensions were finally centrifuged at 5000 rpm. 1 mL of extract was added to 4 mL reagent (40 mL of 6 N H_2SO_4, 80 mL distilled water, 40 mL ascorbic acid, and 40 mL from a solution of ammonium molybdate), mixed and incubated for 2 h at 37 °C, and then cooled to room temperature. The absorbance was measured at 680 nm. Total lipid was determined according to Bligh and Dyer (1959) and Kinght et al. (1972). The residue after removing of

the acid soluble phosphorous was extracted three times with chloroform: methanol mixture (2:1, v/v). 0.1 mL of volatile oil was added to 5 mL of concentrated H_2SO_4. The mixture was heated in a water bath for 10 min and then cooled, and 0.4 mL aliquot was placed in a dry test tube. 6 mL of phosphor-vanillin reagent (0.6 gm vanillin dissolved in 10 mL ethanol before diluting to 100 mL with distilled water was mixed with 400 mL of concentrated orthophosphoric acid) was then added to each test tube. The mixture was set in the dark for 45 min, and the absorbance was measured at 525 nm. Soluble protein was determined according to Bradford (1976). The de-lipidated cells were solubilized in 1 N KOH for 20 h at 37 °C. The soluble proteins were determined at 595 nm. RNA was extracted according to Burton (1957) and Malik and Singh (1980). The residue of the sample after hydrolysis by 1 N KOH was subjected to extraction of RNA. To each sample, HCl (6 N) was added, and then the solution was completed with the same volume of 10% TCA. After adjusting the concentration, then the residue was washed with 5% TCA. 1 mL of RNA was added to 3 mL of reagent (0.2 gm orcinol was dissolved in 15 mL distilled water, and 135 mg of ferric ammonium sulfate, then 85 mL of concentrated HCl was added), mixed, and heated in a water bath for 20 min. The tubes were cooled and measured at 670 nm. DNA was extracted according to Burton (1957 and Malik and Singh (1980). Remaining portions after extraction of RNA were hydrolyzed by 5% TCA, and the supernatants were heated for 30 min at 90 °C, cooled, and centrifuged at 5,000 rpm. The residue was washed one time with 5% TCA. 1 mL of DNA extract was added to 2.5 mL of the diphenylamine reagent (1 gm

Table 1 GC–MS volatile oil of lemon leaves

Retention time	Concentration (%)	Compound’s name
7.94	0.10	Biocyclo (3.1.0) hex-2coc,2 methyl-5-(1-methyl ethyl)
8.14	3.52	α-Pinene
9.49	29.50	Sabinene
9.70	1.90	β-Pinene
10.16	3.96	β-Myrcene
10.56	0.81	α-Phyllandrene
10.85	7.18	3-Carene
11.00	1.61	α-Terpinene
11.33	0.35	O-cymene
11.43	7.86	D-limonene
12.19	8.37	β-Ocimene
12.52	2.48	γ-Terpinolene
12.54	2.64	γ-Terpinene
12.80	0.62	Trans-sabinene hydrate
13.51	0.32	Cyclohexane, 3-methyl, 6-1 methyl diene
14.03	1.38	Linalool
14.70	1.19	Cyclohexane 1-Ol-1-methyl
15.90	4.45	Citronellal
16.70	1.39	Terpinen-4-ol
17.23	0.37	α-Terpineol
18.58	0.56	Citronellol
19.02	0.61	z-Citral
19.60	0.65	Geraniol
20.05	0.37	E-citral
22.81	3.71	Citronellyl acetate
24.05	5.18	β-Elemene
24.90	0.72	Transcaryophyllene
25.90	1.40	Humulene
26.45	1.98	Methyl anthranilate
27.18	0.37	Valencene
34.91	2.62	2,6,11-dodecatriene
37.40	0.98	2,6,9,11-dodecatriene

Table 2 Antibacterial activity of *Citrus lemon* leaves oil against some pathogenic bacteria by well diffusion method (mm)

Samples	Gram + ve		Gram − ve	
	B. cereus	*St. aureus*	*P. aeruginosa*	*E. coli*
Citrus limon leaves volatile oil	0.00	32.0 ± 0.01	49.0 ± 0.01	0.00

of diphenylamine was dissolved in 98 mL of acetic acid, and then 2 mL of H_2SO_4 was added), and the mixture was heated for 5 min in a water bath. The samples were cooled, and absorbance was measured at 540 nm.

Results

Volatile oil determination

Leaves emerged from the pruning process of the *Citrus lemon* included in hydro-distillation by Clevenger device for 3 h. The obtained oil was measured as ml, and the percentage was calculated as pointed *Citrus lemon* leaves volatile oil percentage; it had oil percentages amounted to 0.56%.

GC–MS analyses of the volatile oils obtained from *Citrus lemon* leaves

Citrus lemon leaves oil sample was subjected to GC–MS analysis to characterize its components and its percentage. Table 1 describes the components, their signals, and their area percentages. This table includes the chemical analyses of *Citrus lemon* leaves volatile oils. The oil contained sabinene 29.5%, 3-carene, 7.18%, limonene 7.86%, and β-ocimene 8.27%.

Antibacterial activity

The results in Table 2 show that crude oil showed a remarkable inhibition against *P. aeruginosa* and *S. aureus* 49 ± 0.01 and 32 ± 0.01 mm, respectively, while it didn't affect *B. cereus* and *E. coli*. The minimum inhibitory concentration (MIC) by optical density assay for *P. aeruginosa* and *S. aureus* was 1 and 2 μL/5 mL, respectively.

Mode of action

The different concentrations of *C. lemon* leaves oil impact on the total lipids, acid soluble phosphorus, protein, RNA, and DNA biosynthesis in the *S. aureus* and *P. aeruginosa* cells were studied, and the data are presented in Figs. 1, 2, 3, 4 and 5. It was found that *Citrus lemon* leaves oil had a strong effect on the total lipids in cells of *P. aeruginosa* indicated in Fig. 2a, whereas it had a trivial effect on acid-soluble phosphorus, protein, RNA, and DNA biosynthesis indicated in Figs. 1a, 3a, 4a and 5a and this is the best state. In the case of *S. aureus* cells, the *C. lemon* leaves oil had a strong effect on acid-soluble phosphorus, lipid, protein, DNA, and RNA biosynthesis (Figs. 1b, 2b, 3b, 4b and 5b). This impact increased with the increasing of incubation period and concentration (1/8–1/2 MIC).

Discussion

Essential oils and plant extracts had been used for many years (Jones 1996), in pharmaceuticals, food preservation, natural therapies, and alternative medicine

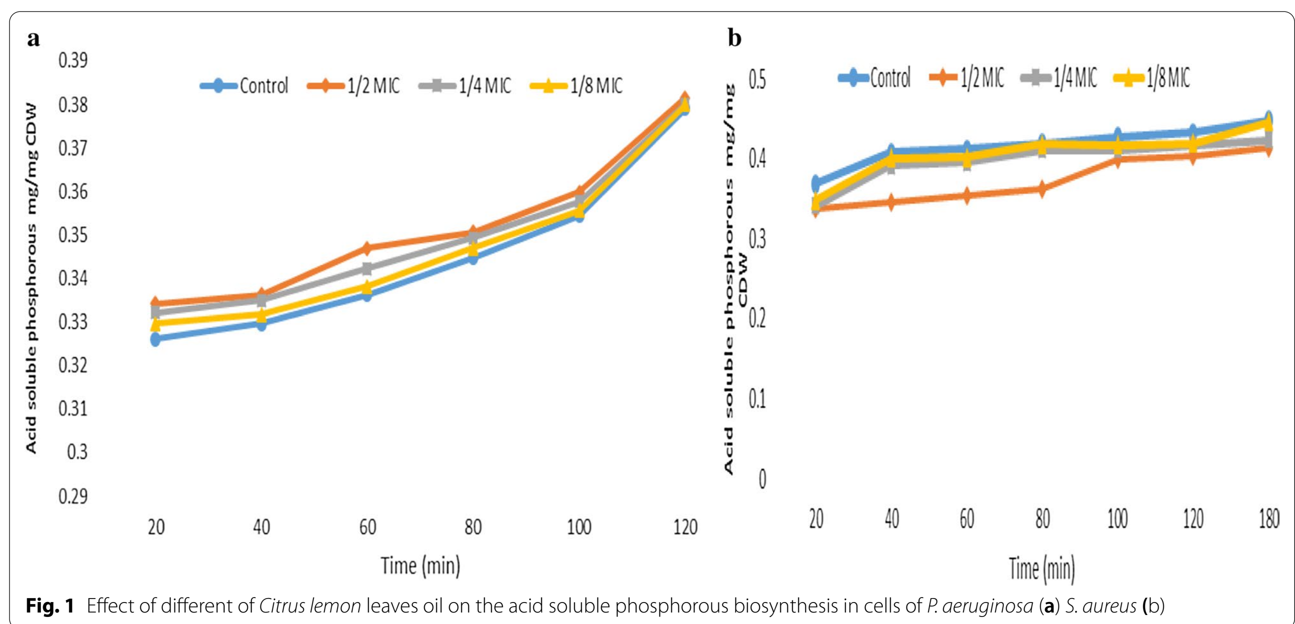

Fig. 1 Effect of different of *Citrus lemon* leaves oil on the acid soluble phosphorous biosynthesis in cells of *P. aeruginosa* (**a**) *S. aureus* (b)

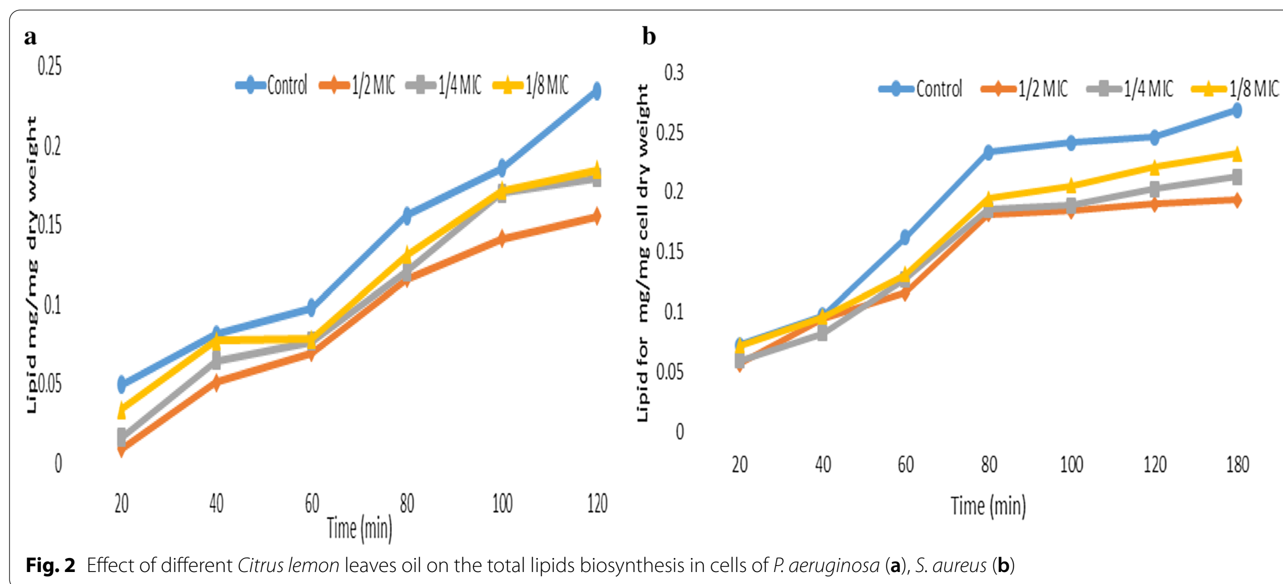

Fig. 2 Effect of different *Citrus lemon* leaves oil on the total lipids biosynthesis in cells of *P. aeruginosa* (**a**), *S. aureus* (**b**)

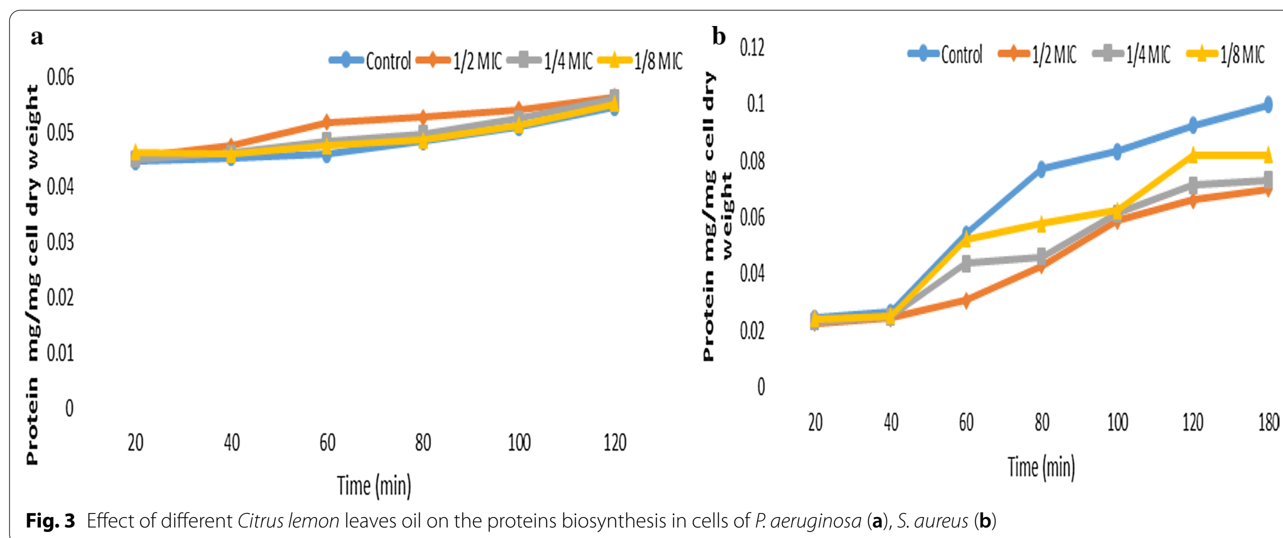

Fig. 3 Effect of different *Citrus lemon* leaves oil on the proteins biosynthesis in cells of *P. aeruginosa* (**a**), *S. aureus* (**b**)

(Reynolds 1996; Lis-Balchin and Deans 1997). It was necessary to examine those plants scientifically which had been used in medicine to improve the quality of healthcare. *C. lemon* leaves oil sample was subjected to GC–MS analysis to characterize its components and its percentage went parallel with those stated by Martos et al. (2008) to some extent and was parallel with the finding obtained by Rouseff and Perez-Cacho (2007). Essential oils are possible sources of modern antimicrobial compounds (Mitscher et al. 1987; El-gengaihi et al. 2020), especially against pathogenic bacteria. This work showed that *C. lemon* leaves essential oil inhibited bacterial growth. The antimicrobial activity of many essential oils had been previously classified as weak, medium, or strong (Zaika 1988). In this work, *Citrus lemon* leaves oil was very strong essential oil as an anti-bacterial activity against Gram-negative (*E. coli and P. aeruginosa)* and Gram-positive (*B. cereus and S. aureus*). *C. sinensis* essential oil inhibited the *Aspergillus niger* growth*;* in addition, the lemon essential oil antibacterial activity was reported against various bacteria Gram-negative and Gram-positive (Baratta et al. 1998). Usually, the essential oils antimicrobial activity at the cellular level had many mode action sites. The primary mechanism is making an irreversible damage of bacterial membrane resulting in cytoplasmic losses, energy substrate (glucose and ATP) loss causing bacterial lysis, ions leakage, and death. Another possible action mode is the protease inhibition and therefore cell

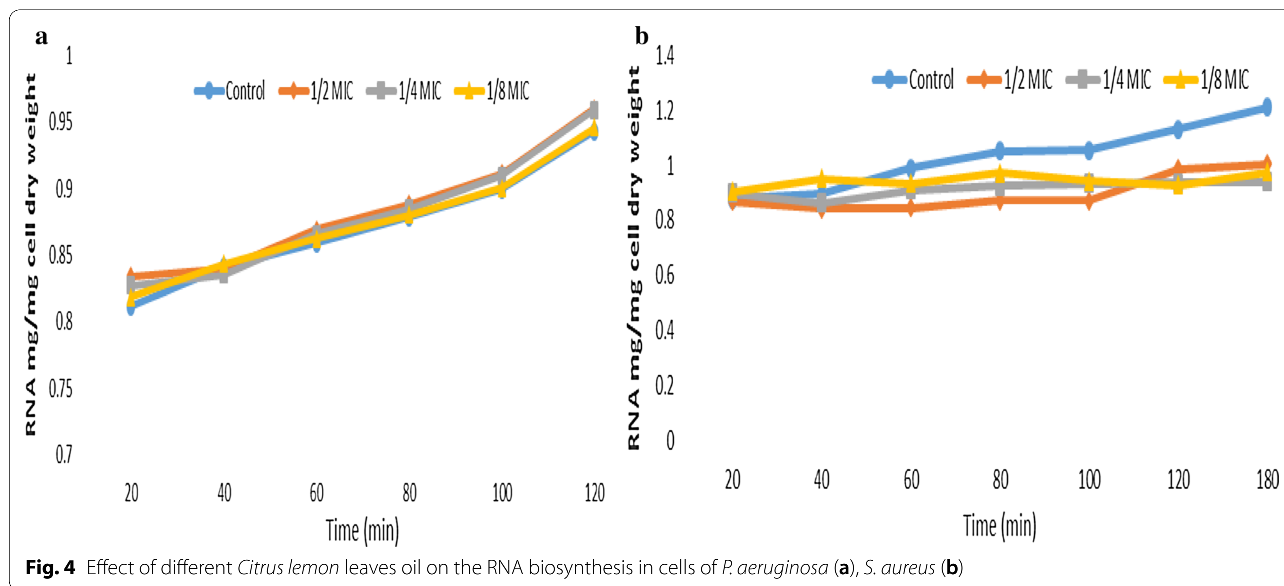

Fig. 4 Effect of different *Citrus lemon* leaves oil on the RNA biosynthesis in cells of *P. aeruginosa* (**a**), *S. aureus* (**b**)

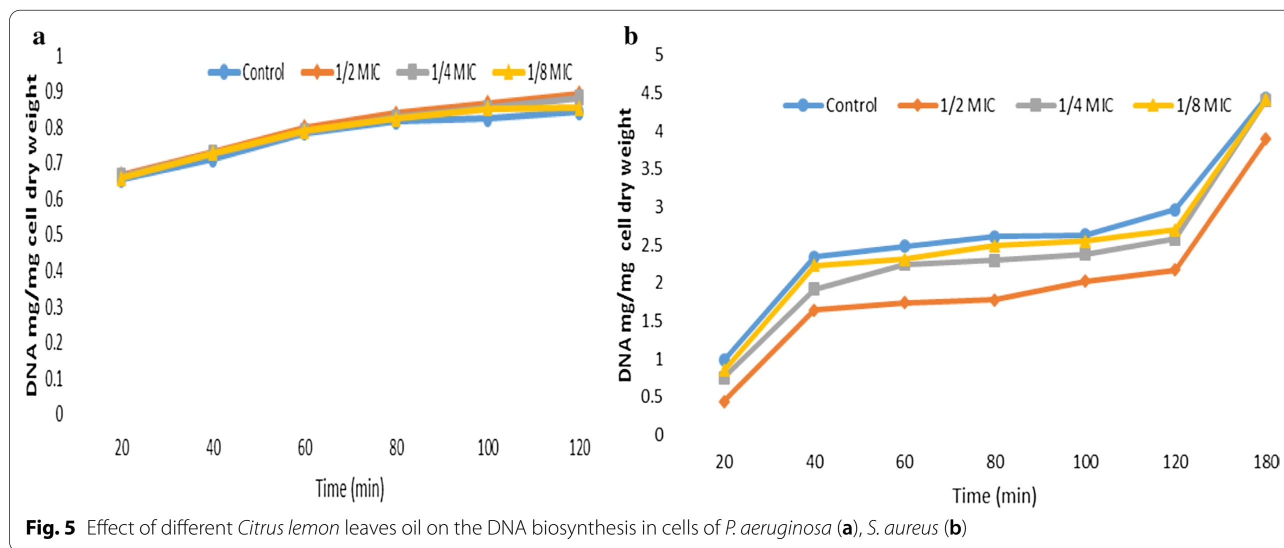

Fig. 5 Effect of different *Citrus lemon* leaves oil on the DNA biosynthesis in cells of *P. aeruginosa* (**a**), *S. aureus* (**b**)

content coagulation (Burt 2004; Di Pasqua et al. 2007; Bakkali et al. 2008). These results revealed that *C. lemon* leaves oil greatly impacts the proteins biosynthesis via inhibiting some steps in the complex translation process. The same action and the most important antibiotic was tetracycline. Otherwise, some chemotherapeutic agents impact the RNA or/and DNA synthesis or could bind to RNA or/and DNA, so their messages could not be read. A lot of these drugs were un-selective and affect animal and bacterial cells, so that these drugs don't have applications in therapeutic (Shuichi et al. 2000). These data suggested that *C. lemon* leaves oil have potential as natural food preservatives applications able to inhibit bacterial growth against *P. aeruginosa*.

Conclusions

Food rich in antimicrobial activity had become a significant approach for a lot of consumers, to obtain their requirements to decrease the health problem risk or a specific disease and to treat minor disease. The antimicrobial development and description of in agricultural products and novel food were demanded to provide scientific proof for improving of the human diet nutritional value and quality. This was also important for improved the agricultural and food products using. Knowledge of the composition, functional properties and analysis of lemon leaf oil will aid in identifying the medicinal, industrial and nutritional applications of it. It can be concluded that the *C. lemon* leaves oil seems to be a well source of antibiotic agent. *C. lemon*

leaves oil essential could be nutritionally considered as a non-conventional supply for edible purposes, pharmaceutical industries, and provide health benefits to the consumers specially when these essential oils were extracted from a waste product like pruning process. The *C. lemon* leaves oil had potential applications as natural food preservatives inhibiting bacterial growth against *P. aeruginosa*.

Abbreviations

TCA: Trichloroacetic acid; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; MIC: Minimal inhibitory concentration; GC–MS: Gas chromatography–mass spectrometry.

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Authors' contributions

All authors certify that they have participated sufficiently in contributing to the intellectual content, concept, design of this work, and writing the manuscript. MA (corresponding author) confirms that all listed authors have approved the manuscript before submission, including the names and order of authors, and that all authors receive the submission and all substantive correspondence with editors, as well as the full reviews. EMH: Supervisor on the work and design the experiments. MM: Collection of plant materials. SAA: Antibacterial Activity and mode of action. SE: PI of the whole project and supervisor on the work. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available.

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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