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Antimicrobial activity of bioactive components of essential oils from *Citrus sinensis* against important pathogens

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Abstract

Alternative strategies to treat multidrug resistant pathogens are indispensable due to the scarcity of new therapeutically effective antibiotics. The present work was conducted to investigate the antimicrobial effects of essential oils extracted from *Citrus sinensis*, locally known as "Mousami", against various important pathogens as well as their phytochemical characterisation. Essential oils were extracted from *Cit. sinensis* peels by the steam distillation method, and a 0.23% yield was obtained. Chemical composition of the extracted essential oil was analysed through gas chromatography/mass spectrometry (GC/MS). The analysis revealed that the *Cit. sinensis* essential oil was composed of a variety of chemical compounds; mostly are monoterpene hydrocarbon and 0.62% of limonene (dipentene), as well as oxygenated monoterpenes and 0.50% limonene oxide, also known as eucalyptol. Standard reference microorganisms, i.e., *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella* Typhi (ATCC 24682), *Bacillus subtilis* (ATCC 6633), *Aspergillus flavus* (ATCC 204304), and *Candida albicans* (ATCC 10231) were used, and the *in vitro* antimicrobial effect of *Cit. sinensis* essential oils was observed against these strains by disc diffusion method. Statistical analysis of the resulting data was done by using Least Significant Difference (LSD) method and Analysis of Variance (ANOVA) to assess the significant association between biological activities of essential oils at $p < 0.05$. Using microbroth dilution assay, maximum sensitivity was exhibited by *E. coli* and *Can. albicans* among the tested microbial strains. The zones of inhibition were significantly different, having diameters of 34.0 ± 1.5 and 55.0 ± 0.5 mm for the said bacterial and fungal strains, respectively; and their MIC values were 0.0007 ± 0.0001 and 0.0007 ± 0.0006 mg/ml, respectively. Thin layer chromatography-bioautography (TLC-bioautography) showed dipentene as biologically most active antimicrobial component. Hence, it was established that broad spectrum antimicrobial effect against important microorganisms was elucidated by essential oil extracts from *Cit. sinensis* that may be used as a natural antimicrobial to treat various infections caused by pathogens of public health interest.

Introduction

Infectious diseases have created havoc, and are the leading cause of untimely death (Khalid *et al*., 2017). For example, the causative agents *Salmonella* Typhi and *S. Paratyphi* have become resistant to the first line of available drugs (Siddique *et al.,* 2018). The loosely regulated and/or immoderate administration of drugs is mainly responsible for the increase in antibiotic resistance (Anwar *et al.,* 2018). Worldwide rise in antibiotic resistance warrants development of alternative options in order to cure microbial infections. In such scenario, phytogenic compounds like essential oils have received more attention as a good alternative to antimicrobial drugs. Fruits and vegetables are considered as potential herbal remedies, and may be used as a promising alternative to antibiotics (Kotzekidou *et al.,* 2008). In the past, the antibacterial effect of essential oils has been demonstrated (Dongmo *et al.,* 2009), and citrus fruit peel essential oil has been accepted as an antibiotic (Bhuiyan *et al.,* 2009). There is a dire need for new antimicrobial agents having appropriate chemical structures and unique mechanisms of action against pathogenic microorganisms.

Universally, citrus fruits are recognised as one of the most lucrative fruit crops (Tao *et al.,* 2008). A total of 16 species have been ascribed to the genus

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Citrus of Rutaceae family, and essential oils obtained from its peels are frequently produced worldwide (Fisher and Philips, 2008). Citrus peels are commonly discarded as waste. Therefore, it becomes extremely beneficial to extract the citrus peel into essential oils in replacement of medicines and food additives (Singh *et al.,* 2010). Approximately 0.5 to 3 kg of essential oil can be extracted from every ton of citrus fruits (Sattar *et al.,* 1986). Citrus peel essential oil is a complicated blend of volatile organic compounds with monoterpene hydrocarbons constituting the major portion (Chanthaphon *et al.,* 2008). A dipentene named limonene is the most substantial component present in essential oils of *Citrus* spp. (Bhuiyan *et al.,* 2009). Appreciable fungicidal as well as bactericidal activities have been exerted by these essential oils against a wide range of microorganisms (Burt, 2004; Fisher and Phillips, 2006).

Pakistan is one of the leading citrus-producing countries in the world, but has limited resources to manage the peel waste. Essential oils of the peels have antimicrobial potential, and may also be a good economic and natural antimicrobial alternative both for growth promoter in animal feed as well as therapeutic options for infectious diseases. The present work aimed to isolate and determine the chemical composition of locally available citrus fruit peels extracted essential oils by GC/MS, as well as the assessment of its antimicrobial potential through different methods against several important fungal and bacterial strains including *Aspergillus flavus, Candida albicans, Bacillus subtilis, Staphylococcus aureus, Salmonella* Typhi, and *Escherichia coli*.

Materials and methods

Microorganisms

The bacterial strains *E. coli* (ATCC 25922), *B. subtilis* (ATCC 6633), *Sta. aureus* (ATCC 25923), and *Sal*. Typhi (ATCC 24682) were subcultured on Nutrient Agar Medium (Oxoid, England) at 37°C overnight or for 24 h. The fungal strains of *Candida albicans* (ATCC 10231) and *Aspergillus flavus* (ATCC 204304) were subcultured on Sabouraud's Dextrose Agar (SDA) Medium (Oxoid, England) at room temperature (25 - 28°C) for 2 d. All bacterial and fungal strains used in the present work were generously gifted by the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.

Plant material

One kilogram (1000 g) of the *Cit. sinensis* peels were sampled from local marketplace of Faisalabad, Pakistan. The collected peel samples were compared with authentic samples by a taxonomist in the Department of Botany, University of Agriculture, Faisalabad, Pakistan. A voucher sample of *Cit. sinensis* peels (No. 6390/11.08.37) was submitted in the herbarium of the University. The peels were sliced, splashed with 70% alcohol, and rinsed with sterilised distilled water. The peels were air-dried, and refrigerated at 4°C until further analyses (Naveed *et al.,* 2013).

Hydro distillation

The air-dried peelings of *Cit. sinensis* were subjected to hydro distillation for 4 - 6 h to extract essential oil, and sodium sulphate (anhydrous) was added in order to remove the water molecules from the extracted essential oil. Then, filtration was performed, and the obtained pure oil was kept in amber glass vial at 4°C (Ezejiofor *et al.,* 2011) until further analyses. The quantity of oil gained from peels was calculated using Eq. 1:

$$\text{Essential oil (\% v/w)} = [\text{volume of oil (mL)} / \text{weight of sample (g)}] \times 100 \quad \text{(Eq. 1)}$$

All chemicals and organic solvents used were of HPLC (high performance liquid chromatography) and/or analytic grade, and purchased from different commercial sources like Sigma-Aldrich and Fluka.

Chemical composition analysis

GC/MS Thermo TRACE™ GC Ultra (Thermo Fischer Scientific, USA) on 30 m × 0.25 mm × 0.25 µm TR5-MS capillary column, together with linear ion trap (Polaris Q) was employed for chemical profiling of *Cit. sinensis* peel essential oil (Cheng *et al.,* 2006). At the start, the column temperature was maintained at 100°C for approximately 2 min, and then eventually elevated at the rate of 8°C per minute to 270°C for 5 min. Next, 230°C was maintained as injection temperature, mode of injection was split (1:50), and volume of the injection was 0.2 µL. For mass spectrometry detection, the temperature of transfer line of mass spectrometry was fixed at 260°C with ionisation mode at a 70 eV ionisation potential. The ion source temperature was 200°C, and range of mass analysis was adjusted between 50 - 300 *m/z*. The carrier gas was helium at a steady flow rate of 1 mL/min. Essential oil (300 µL) with concentration of 0.1 mg/mL *n*-nexane (Lab-scan, Thailand) was used (Naveed *et al.,* 2013). Identification of compounds was done by matching the fragmentation pattern of their mass spectrum and their retention time with standardised compounds, and then by making a comparison with the results of mass spectra with

National Institute of Standards and Technology (NIST) library kept in GC/MS database for validation.

Evaluation of antimicrobial activity

Cit. sinensis peel essential oil was studied to evaluate antibiotic effect on public health pathogens by using disc diffusion method, microbroth dilution method, as well as TLC-bioautography.

Disc diffusion assay

For disc diffusion assay, 0.1 mL (1.5×10^5 CFU/mL) inoculum suspension for each bacterial strain was inoculated on the Mueller-Hinton agar medium (MHA) (Liofilchem, Italy). Sabouraud's dextrose agar medium was used for *Can. albicans* (1×10^6 CFU/mL) and *A. flavus* spores (5×10^6 spores/mL). Various concentrations (1.25, 2.5, 5, and 10 mg/mL) of essential oil of *Cit. sinensis* were solubilised in sterilised DMSO (dimethyl sulfoxide; MP Biomedicals, France), from which 20 µL was instantly inoculated on separate sterilised filter paper discs (6 mm); Whatman's filter paper No. 1, amoxicillin (10 µg/mL), and fluconazole (15 mg/mL) were used as positive control for bacteria and fungi, respectively, whereas DMSO was used as a negative control. Bacterial culture was incubated at 37°C overnight, whereas fungal culture was incubated at 28°C for 48 h (Natta *et al.,* 2008). The diameters of zones of inhibition were measured in millimetre (mm).

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of essential oil refers to the minimum or least concentration repressing the growth of microorganism, and was determined by microbroth dilution method in the present work. In a sterile 96-well microtitration plate, 50 µL of Muller Hinton Broth (Liofilchem, Italy) was poured in each well. Two-fold serial dilution of 50 µL *Cit. sinensis* essential oil (500 mg/mL) was transferred into each well in the first column of microtitration plate. A volume of 50 µL (1.5×105 CFU/mL) inoculum suspension of every selected strain was then inoculated in each well. Potato dextrose broth was used for antifungal susceptibility tests. Incubation of microtitration plates was done for 24 h at 37°C for bacteria, and for 48 h at 25°C for fungi. The concentration of the first well showing no turbidity was considered as MIC, which was established by measurement of absorbance at 600 nm by the ELISA reader. Fluconazole and amoxicillin were used as controls for antifungal and antibacterial susceptibility tests (Anwar *et al.,* 2018). All the tests were performed in triplicate.

TLC-bioautography

For TLC-bioautography, 10 µL of *Cit. sinensis* essential oil along with its reference standard were solubilised per mL of ethyl acetate (Lab-scan, Thailand). Next, 8 µL of the aliquot was smeared by capillary pipettes to the two TLC strips, each of 20 × 20 cm and coated with silica gel (TLC silica gel 60 F254 aluminium sheet; Merck, Germany); one for the reference TLC chromatogram and the other for bioautography. On TLC chromatographic plate, each standard was employed beside the point of its essential oil. TLC plates were prepared in ethyl acetate and *n*-hexane (9:1, v/v) at room temperature. UV light (254 nm) was used for examining dried TLC plates, and in addition, one of the TLC strip was observed by dipping in alcoholic vanillin sulphuric acid reagent made by solubilising 99.8% ethanol (Merck, USA) at 95 mL, 99% vanillin (Merck, USA) at 6 g, 98% concentrated sulphuric acid (AnalaR, England) at 1.5 mL. Heat gun was used for 1 min to dry up, and the compounds were observed for those who were separated, which were later recognised visually by the colour spots of the different compounds, as well as by computing the Rf value as described elsewhere (El-Baroty *et al.,* 2010).

The other TLC strip was utilised for bioautography, and positioned with silica side down on the Mueller Hinton agar for overnight, having a volume of 2 µL of bacteria culture, and incubated at 37°C. Next, 300 µL aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.05 g/30 mL; Sigma, USA) was incubated at 37°C for 24 h. As previously determined, inhibition zones (mm) were observed as clear zones or spots on the dark background, and were linked with the spots viewed on the TLC plate under UV light which were utilized as reference chromatogram (Horvath, 2010).

Statistical analysis

The data obtained were expressed as mean ± SEM of triplicate. Data were then assessed through Analysis of Variance (ANOVA), and statistical significance among the different means was evaluated by Least Significant Difference (LSD) method by Minitab software program version 15. A probability level ($p < 0.05$) was seen as statistically significant in all experimental data.

Results

Extraction yields

In the present work, *C. sinensis* essential oil obtained from its peels by hydro-distillation method had a volume of 2.3 mL that was obtained from

1000 g peels, which provided a 0.23% (w/w) production yield.

Essential oil composition

Chemical profiling of *Cit. sinensis* essential oil evaluated by GC/MS is presented in Table 1. Different chemical compounds were identified in different percentageS. Limonene, which is a dipentene, was the major aromatic component (0.62%) present in the essential oil with retention time of 3.01. Other constituent identified was eucalyptol, also known as limonene oxide, that was present as oxygenated monoterpenes (0.5%), with retention time 3.13 (Figure 1A).

Table 1. Chemical composition, retention time, and concentration (%) of *Citrus sinensis* essential oil by GC/MS analysis method.

Essential oil	Compound	Molecular weight	RT 9 (Min)	Concentration (%)
Citrus sinensis	Dipentene	136.0	3.010	0.62
	Eucalyptol	154.2	3.013	0.50
	Unidentified	-	3.440	-
	Unidentified	-	3.810	-

Antimicrobial activity

Antimicrobial activity of the extracted essential oil was tested by different methods. By disc diffusion technique, antimicrobial potential of different concentrations of *Cit. sinensis* peel's essential oil was determined against specified strains of bacteria and fungi. Prepared concentrations of *Cit. sinensis* essential oil demonstrated strong antibacterial activity (Table 2). Of all the bacterial species examined, *E. coli* demonstrated maximum sensitivity with a significantly highest inhibition zone (34 ± 1.5 mm) at 10 mg/mL concentration of essential oil. Other tested bacterial strains also showed a reasonable sensitivity for the extracted essential oil, and they were ranked in the order of their sensitivity as *Sta. aureus* and *B. subtilis* (22.3 ± 4.8 mm) > *Sal*. Typhi (21 ± 0.5 mm) at same concentration. Antifungal effect of essential oil extracted from *Cit. sinensis* was also determined against *A. flavus* and *Can. albicans*. Both tested fungal species showed considerably ($p < 0.05$) higher susceptibility to different concentrations of essential oil. A larger zone of inhibition of the growth of *Can. albicans* (55 ± 0.5 mm) as compared to that of *A. flavus* (53.6 ± 0.8 mm) was observed at highest tested concentration of 10 mg/mL of essential oil. In the present work, we found a significantly more profound antifungal effect of *Cit. sinensis* essential oil ($p < 0.05$) than the antibacterial one. The growth of microorganisms was effectively inhibited by *Cit. sinensis* essential oil when compared with the respective positive controls used. *S. aureus* was most susceptible (9.33 ± 0.3 mm) to positive control amoxicillin, while other bacteria were found resistant. In antifungal susceptibility testing, *Can. albicans* (17 ± 0.5 mm) and *A. flavus* (19 ± 0.5 mm) were found susceptible to the used positive control drug, fluconazole.

MIC

Minimum inhibitory concentration (MIC) of essential oil was also determined by microbroth

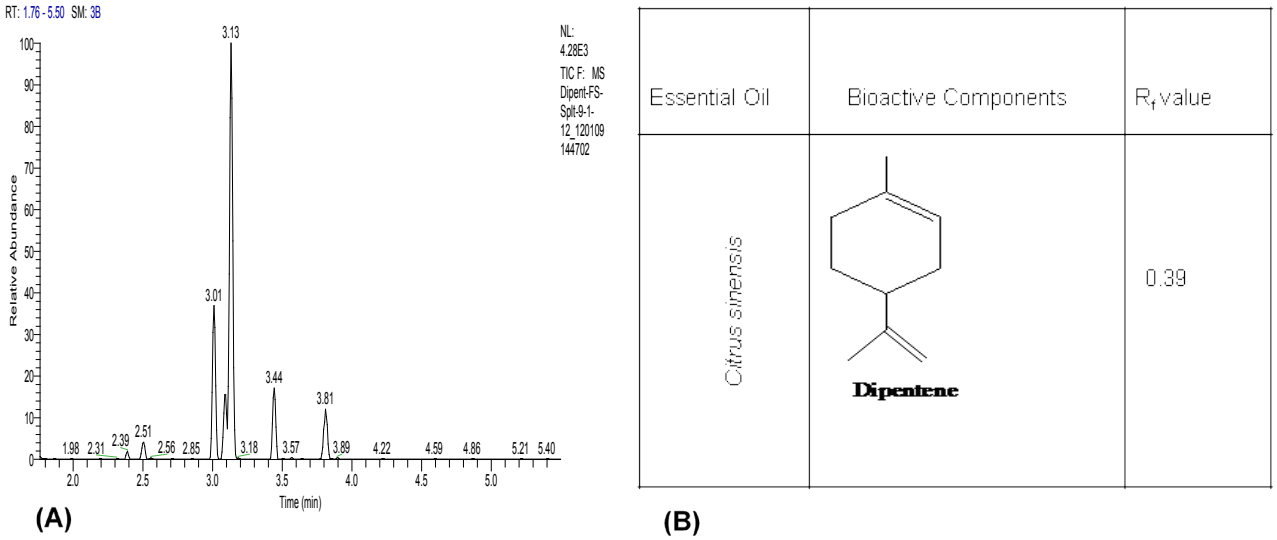

Figure 1. (A) A typical chromatogram of indigenous essential oil extracted from *Citrus sinensis* peels by GC/MS analysis showing dipentene at retention time, RT = 3.01, and eucalyptol at RT = 3.13. (B) Chemical structure of bioactive compound, dipentene, present in essential oil extracted from the peels of *Citrus sinensis*.

Table 2. Antimicrobial spectra of *Citrus sinensis* essential oil at various concentrations against some pathogens of public health interest, as tested by disc diffusion technique.

Microorganism	Diameters of zones of inhibition by *Citrus sinensis* essential oils (mm)[a]				Positive control
	10 mg/mL	5 mg/mL	2.5 mg/mL	1.25 mg/mL	
Bacterial strain					Amoxicillin 10 µg
Gram-positive					
Bacillus subtilis ATCC 6633	22.3 ± 4.8*	15.3 ± 4.3	12.0 ± 4.0	08.3 ± 0.8	R
Staphylococcus aureus ATCC 25923	22.3 ± 4.8*	15.3 ± 4.3	12.0 ± 4.0	08.3 ± 0.8	9.33 ± 0.3
Gram-negative					
Escherichia coli ATCC 25922	34.0 ± 1.5*	32.0 ± 0.5*	31.0 ± 0.5*	23.0 ± 4.3	R
Salmonella Typhi ATCC 24682	21.0 ± 0.5*	17.3 ± 2.6*	09.3 ± 1.2	08.6 ± 1.3	R
Fungal strains					Fluconazole 15 mg
Candida albicans ATCC10231	55.0 ± 0.5*	53.0 ± 0.5	32.0 ± 0.5	30.3 ± 0.3	17.0 ± 0.5
Aspergillus flavus ATCC204304	53.6 ± 0.8*	32.3 ± 0.3	28.0 ± 0.5	23.0 ± 0.5	19.0 ± 0.5

Value are mean ± SEM of three replicates (n = 3). a = diameter of zones of inhibition, includes 6 mm diameter of disc, * = illustrated a significant ($p < 0.05$) values, and R = resistant.

Table 3. Average minimum inhibitory concentrations (MICs) of *Citrus sinensis* essential oils against test microorganisms as assessed by microbroth dilution method.

Microorganism	Minimum inhibitory concentratio n (MIC) (mg/ mL)	Amoxicillin (positive control) (mg/ mL)
Bacterial strains		
Gram -positive		
Bacillus subtilis ATCC6633	0.008 0 ± 0.001 0	R
Staphylococcus aureus ATCC25923	0.81 00 ± 0.16 00	0.15 00 ± 0.02 00
Gram -negative		
Escherichia coli ATCC25922	0.0007 ± 0.0001	R
Salmonella Typhi ATCC24682	1.62 00 ± 0.32 00	R
Fungal strains		Fluconazole
Candida albicans ATCC10231	0.0007 ± 0.0006	0.005 0 ± 0.001 0
Aspergillus flavus ATCC204304	0.001 0 ± 0.0006	0.04 00 ± 0.001 0

Values are mean ± SEM of three replicates (n = 3). R = resistant.

dilution techniques. The peel of *Cit. sinensis* essential oil exhibited a strong inhibitory effect and significantly variable MIC values against tested microorganisms. Of all tested bacterial species, *E. coli* was established to be the most susceptible strain with least concentration, *i.e.,* 0.0007 ± 0.0001 mg/mL of *Cit. sinensis* essential oil necessary to inhibit its growth (Table 3), while other bacterial species also showed sensitivity to the essential oil preparations. *Cit. sinensis* essential oil showed MIC value 0.008 ± 0.001 mg/mL against *B. subtilis*, 0.81 ± 0.16 mg/mL against Sta. aureus, and 1.62 ± 0.32 mg/mL against *Sal.* Typhi. For fungi, *Can. albicans* was found to be more sensitive to *Cit. sinensis* essential oil at concentration of 0.0007 ± 0.0006 mg/mL as compared to *A. flavus*, which showed

clear inhibitory zones at concentration of 0.001 ± 0.0006 mg/mL.

TLC-bioautography

Detection of the biologically active components in essential oil extracted from *Cit. sinensis* peels was done by TLC which revealed the existence of dipentene as the principal bioactive component. Dark spots demonstrating the presence of main bioactive component dipentene were observed when extracted essential oil was run along with its reference standard on aluminium plates coated with silica, and the Rf value was found to be 0.39 (Figure 1B). Correlation of Rf values of these bands with accurate standard of the chemicals was established by running them parallel on TLC plates. At the time of TLC-bioautography assay, antibacterial effects of the essential oil along with their reference standard were shown as diameter (mm) of inhibition zone. Bioautography was employed for the evaluation of the dipentene antibacterial potential. When TLC strips inoculated with active compound were positioned on the agar, the active component diffused into the agar surface, inhibiting the growth of microbes, and hence clear inhibition zones were observed. As presented in Table 4, in TLC-bioautography assay, the tested *Cit. sinensis* essential oil, dipentene, with its reference standard revealed good antibacterial effect against *Sal*. Typhi, *B. subtilis*, and *Sta. aureus*, but unable to show any inhibitory activity against *E. coli*. The tested essential oil samples did not show more effective antibacterial activity against *B. subtilis* and *Sal*. Typhi with respect to their standard dipentene.

Table 4. Antibacterial activity, expressed as the diameters (mm) of the inhibition zones, of the main component identified in the essential oil extracted from the peels of *Citrus sinensis*, against selected bacteria detected by TLC-bioautography.

Bacterial strain	***Citrus sinensis* essential oil**	
	Dipentene R_f value (0.39)	**Dipentene standard**
Bacillus subtilis	10.8 ± 0.6	15.0 ± 0.5
Staphylococcus aureus	11.6 ± 0.3	11.0 ± 0.0
Escherichia coli	R	R
Salmonella Typhi	9.50 ± 0.2	10.0 ± 0.0

Values are mean ± SEM of three replicates (n = 3). R = resistant.

Discussion

Irrational use of antibiotics has rendered bacterial species to develop resistance against multiple therapeutic drugs. Consequently, the resistant microorganisms are unaffected by the first line antibiotics available for treatment around the world (Shanahan *et al.,* 2000). People in less developed countries are unable to bear the expenses of these costly therapeutic medications to which multidrug resistance pathogens are susceptible (Molloy *et al.,* 2010). Hence, clinically effective and affordable strategies are needed to combat such fatal bacterial infections. Essential oils are famous for vast range of biological actions, commonly as antimicrobial agent either alone or in combination. Citrus essential oils are famous for their several applications other than their use in cosmetics and food industry. They are already reported for their antimicrobial activities as well (Ambrosio *et al.,* 2019). When essential oils are combined particularly with other drugs, they possess synergistic effect of antibiotic potential against notorious drug resistant pathogens (Derakhshan *et al.,* 2010).

The antimicrobial activity of citrus essential oils evaluated in the present work on various bacterial and fungal strains showed that they had an overall selective antibacterial spectrum, having higher activity on certain pathogenic strains. This selectivity towards some bacterial strains has been reported previously (Ambrosio *et al.,* 2019).

Extraction yield

In the present work, essential oil of locally available *Cit. sinensis* peels was extracted through hydro distillation process. A total of 0.23% (w/w) essential oil was yielded from the fruit peels. The yield of essential oils is influenced by various factors including environmental in addition to intrinsic and extrinsic ones. This finding matches that reported by Kamal *et al.* (2011) who stated the production yield of fresh *Cit. sinensis* peels essential oil to be 0.2%. Essential oil yield from natural source depends on climate, geography, source, degree of freshness, period of harvest, and extraction method used among other several factors (Lawrence, 1986).

GC/MS

The component analysis of essential oil is important as the composition of natural source determines its characteristics and its antimicrobial potential. Biological activities of essential oil could be influenced by variation in chemical constituents of essential oil. In order to show a relationship with the

essential oil antimicrobial effects, it is mandatory to explore its chemical composition (Lin *et al.,* 2007). GC/MS technique was administered to chemically analyse the composition of extracted essential oil of *Cit. sinensis*. The chemical compounds in the essential oil were identified based on their retention time. Chemical analysis showed the presence of volatile compounds mainly monoterpene hydrocarbon, which are in accordance with Chanthaphon *et al.* (2008). Limonene, a dipentene, was the main monoterpene hydrocarbon present as a major aromatic component (Figure 1B). In several previous studies, it was found that aroma of oil was due to the presence of limonene that is also its chief component, which are similar to our results (Lota *et al.,* 2000; Baik *et al.,* 2008; Tao *et al.,* 2008; Chutia *et al.,* 2009; Singh *et al.,* 2010; Espina *et al.,* 2011). Limonene has been reported as a major compound in the composition of citrus essential oils, and responsible for most of its biological activity shown. Other minor compounds like oxygenated monoterpenes were also present in essential oil, *i.e.*, eucalyptol (limonene oxide).

Tao *et al.* (2008) reported that 6.33% of the entire quantity of essential oil were oxygenated monoterpenes, with 1.45% limonene oxide and 0.81% *trans*-limonene oxide being the major constituents, which are almost similar to the findings reported in the present work. Previous report by Dharmawan *et al.* (2007) demonstrated that terpenes also occurred in citrus juice in addition to limonene, but some terpenes did not exhibit aromatic character. The variation in the presence of active ingredient in essential oils as well as their percentage in the natural source relies on both geographical dissemination and environmental circumstances. Both the chemical and biological activities are always influenced by the type of active constituents present in essential oils (Al-Jabri and Hossain, 2014; Hossain *et al.,* 2014).

Disc diffusion test

Disc diffusion test was conducted to evaluate the antimicrobial potential of *Cit. sinensis* essential oil against selected strains of bacteria and fungi which may play a role in infections and global antimicrobial resistance (Ali *et al.,* 2008; Saeed *et al.,* 2009; Afzal *et al.,* 2012). This assay demonstrated that all the tested microbial strains were susceptible to all exposed concentrations of *Cit. sinensis* essential oil. The diameter of zone of inhibition increased in a dose-dependent manner in Gram-positive and Gram-negative bacterial strains. In both Gram-positive bacteria tested, the diameters of zones of inhibition were larger than the respective positive control amoxycillin, except lowest concentration of *Cit. sinensis* essential oil. It means that the growth inhibition by a range of 2.5 to 10 mg/mL concentration of *Cit. sinensis* essential oil was clearly evident. However, maximum antimicrobial activity was seen against a Gram-negative bacterium, *E. coli*, among the tested strains of bacteria and similarly against *Can. albicans* among tested fungi. As reported previously, *Cit. sinensis* essential oil showed a variable antimicrobial effect against microorganisms (Fisher and Phillips, 2006; Dubey *et al.,* 2011). Singh *et al.* (2010) described antimicrobial action of citrus species including *Cit. sinensis*, and the principal components were limonene, limelool, and citral.

The antifungal action of *Cit. sinensis* essential was also similar to that of the antibacterial one. There was a stronger inhibition of the growth of both tested strains of fungi in the presence of *Cit. sinensis* essential oil, where *Can. albicans* was found to be more sensitive.

Positive control against bacteria was amoxicillin which elucidated susceptibility against *Sta. aureus* and resistant to other tested bacteria, while positive control against fungi was fluconazole, which showed susceptibility against both *Can. albicans* and *A. flavus*.

Minimum inhibitory concentration

To investigate the MIC of essential oil, which is required to inhibit the growth of microorganism, MIC was performed by microbroth dilution assay. *Cit. sinensis* essential oil exhibited highly inhibitive response against the test microorganisms which is in agreement with previous literature (Fisher and Phillips, 2006: Chanthaphon *et al.,* 2008). It showed maximum inhibitory activity against *E. coli*, and all other tested strains were also found susceptible, which agrees with Dhiman *et al.* (2012). These MIC findings are in agreement with results determined by disc diffusion method. In both methods, *E. coli* was found highly sensitive to *Cit. sinensis* essential oil among the tested bacterial strains. All tested bacteria in MIC assay found to be resistant against amoxicillin except *Sta. aureus*.

The antifungal effects of extracted *Cit. sinensis* essential oil are presented in Table 3. The tested fungi also showed excellent susceptibility to *Cit. sinensis* essential oil where the growth of *Can. albicans* was repressed. The average MIC values were 0.0007 ± 0.0006 and 0.001 ± 0.0006 mg/mL for *Can. albicans* and *Aspergillus flavus*, respectively. Both presented lower values as compared to respective control fluconazole, meaning that both

were more sensitive to tested essential oil. The antifungal effect of *Cit. sinensis* essential oil was higher for *Can. albicans*, and was even significantly higher as compared to that of the positive control.

TLC-bioautography

For better understanding of antibacterial potential of the constituents of essential oil, TLC-bio autography was performed. TLC-bioautography is a sensitive and rapid screening technique for identification of antimicrobial agents. Analysis of *Cit. sinensis* essential oil was done using TLC plate coated with silica gel in duplicate method. Alcoholic-vanillin sulphuric acid reagent treatment was applied on TLC control plate to locate the components, whereas the other plate was utilised for bio autography assay. TLC analysis indicated that dipentene (limonene) was the principal component of Cit. sinensis essential oils, by means of tinted spot showing Rf value, whose comparison was made with its reference standard. In the extract, appearance of bands (spots) or inhibition zones having equivalent Rf value indicate antimicrobial effect against many microbes. Bioautography described the antimicrobial potential of the essential oil by showing inhibitory zones against tested bacterial species, except for *E. coli*. This might occur due to a little amount of bioactive compound applied on TLC, loss of compound by photo-oxidation, or evaporation (Suleimana *et al.,* 2009). To the best of our knowledge, none of the earlier studies used TLC-bioautography of particularly *Cit. sinensis* essential oil against the microorganisms.

Conclusion

It was concluded that the essential oil extract from peels of locally available *Cit. sinensis* contains several volatile and aromatic compounds including oxygenated monoterpenoids and monoterpene hydrocarbon, which showed great antimicrobial activity against different tested strains of bacteria and fungi. TLC-bioautography can be used to determine the biologically active components. The use of essential oils as alternative to synthetic antimicrobial agents against pathogenic microorganisms is growing. This is due to the fact that the intended main effect is achieved with minimum side effects. Based on the findings of the present work, further studies to clarify the possible mechanism of action of the bioactive compounds present in essential oil, particularly against public health microorganisms are warranted so these compounds can be further pursued for their therapeutic use and may possibly act as a natural substitute to synthetic drugs.

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