Evaluation of Chemical Composition and Biological Activities of *Citrus pseudolimon* and *Citrus grandis* Peel Essential Oils

[1]Arfaa Sajid*, [1,2]Raja Adil Sarfraz, [1]Muhammad Asif Hanif and [1]Muhammad Shahid
[1]*Department of Chemistry and Biochemistry, University of Agriculture Faisalabad-38040, Pakistan.*
[2]*Central Hi-Tech Laboratory, University of Agriculture Faisalabad-38040, Pakistan.*
arfaasajid@yahoo.com*

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Summary: Essential oils and their volatile constituents are used extensively to prevent and treat human diseases. In the past decades, worldwide demand for citrus essential oils has greatly increased. Citrus essential oils containing 85–99% volatile and 1–15% non-volatile components. Essential oils from *Citrus pseudolimon* and *Citrus grandis* peels were extracted through steam distillation and characterized by GC-MS. *C. pseudolimon* has thirty six and *C. grandis* has thirty three total components; limonene 47.07% and 71.48% was the major component in both oils respectively. Antioxidant activity was checked by 2, 2-diphenyl-1-picrylhydrazyl radical assay and β-carotene/linoleic acid bleaching test. Both oils have modest activity. The antimicrobial potential was assessed against different bacterial and fungus strains. *C. pseudolimon* oil possessed strong activity against all tested strains while *C. grandis* has moderate activity. The antitumor activity was evaluated by potato disc assay, *C. pseudolimon* showed 81.25% inhibition. Hence the essential oils could have a great potential in pharmaceutical industry.

Keywords: *Citrus pseudolimon, Citrus grandis,* Antitumor activity, Antimicrobial, GC-MS.

Introduction

Essential oils (EOs) are natural, concentrated, hydrophobic substances having volatile aromatic components obtained through various methods mostly by hydro and steam distillation from different parts of plants [1]. Generally, essential oils are terpenoids which are responsible for aroma and flavor allied with herbs, spices and perfumes. Due to their diffusing nature they also called volatile oils [2]. Due to their healing properties many EOs widely used in folk medicine since ancient times and still today. The therapeutic potential of an EO is due to their compositions that represent a complex framework of many chemical components having various biological activities [3]. Phenolic components that present in essential oils have been renowned as bioactive compounds for the antimicrobial activity. Many plant phenolics are recognized as Generally Recognized As Safe (GRAS) substances, thus they could be used to inhibit growth of several food spoilage and food-borne microorganisms in the foods [2, 4]. EOs showed a broad variation in chemical composition and yield, which depend on the herbal source, chemo type of the plant species, and analytical methods used for extraction [5].

Currently, citrus has earned attention for providing massive amount of health benefits [6]. In genus citrus many species of mandarins, tangerines, lemons, grapefruit, limes and oranges are included. Recently, in citrus industry, fruits are marketed fresh and at the same time as processed juices that give multitude benefits for health, provide vitamin-C and some other constituents. Among them limonoids, flavonoids, dietary fibers, carotenoids (chiefly β-carotene), and folic acid are included [7]. Recently, *Citrus* peel essential oils showed a wide range of the biological activities *e.g.* antioxidant [8-9], antimicrobial [10], antiviral [11], anti-inflammatory [12] and anticancer [13] properties. It is well known that essential oils from *Citrus* spp. have pronounced antimicrobial effect against both bacteria and fungi [14]. Limonoids are distinctive secondary metabolites of the Citrus essential oils, derived from limonene compound and showed large number of pharmacological and biological activities [15-16]

The purpose of current research was evaluation and characterization of two citrus oils keeping in view their antioxidant, antimicrobial and antitumor activity. There are very few detailed reports available on antitumor and antimicrobial properties of *Citrus pseudolimon* and *Citrus grandis* essential oil.

Experimental

Plant Materials

The fruit samples of *Citrus pseudolimon* and *Citrus grandis* were purchased from Sargodha, Pakistan which is famous for Citrus fruit production and sample was identified and authenticated using

*To whom all correspondence should be addressed.

standard herbarium techniques from Department of Botany, University of Agriculture, Faisalabad, Pakistan.

Extraction of the Essential Oils

The fresh samples (peels) were treated in Clevenger type apparatus for 3h steam-distillation to attain an oil of musammi 0.45% (v/w) and galgal 0.70% (v/w). Both oils were dried over anhydrous sodium sulphate (10ml on 3g) and then stored at 4˚C for further testing. The %age yield of oils extracted from each plant was calculated as [17]:

% of oil (v/w) = {volume of oil (mL) / weight of sample (g)} × 100

GC-MS Analysis

Volatile components in the essential oils were determined by Hewlett-Packard GC-MS system on GC 5890 series II, equipped with the mass selective detector (MSD 5972) and HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The injector and detector temperature were set at 250˚C and 275°C, correspondingly. The oven temperature was put for 1 min at 40°C, then progressively raised to 240°C at 8°C/min, then stayed here for 2 min and at last reached up to 300°C at 10°C/min, for 1 min. Helium was used as a carrier gas, with flow rate of 0.7 mL/min. 10 µL of oil sample was diluted in 1mL of n-hexane and injected in split mode. In MS For detection, an electron ionization system was used which have 70 eV ionization energy. The compounds were analyzed by comparing the mass spectra with NIST 98 NIST/EPA/NIH mass spectral library and comparing their relative retention times by literature data [18].

Potato Disc Bioassay

The antitumor potential of essential oils was evaluated according to [19] method with slight changes. *Agrobacterium tumefaciens* was grown on nutrient broth for 48 hrs at 28˚C. Potatoes were obtained from local market washed them under cold running water then immerse in 40% bleaching solution (sodium hypochlorite) for 20 minutes. Trimmed the outer section of potato and again dip it in bleaching solution for 10 minutes, then disks of 0.5cm thickness were cut using cork borer (10mm). These potato disks were placed in Petri-plates and poured 50µl of suspension containing water, sample and *A. tumefaciens,* whereby Vincristine was used as positive control. After that petri-plates were incubated for 21 days at 28˚C. On 21st day potato disks were stained by Lugol's reagent (5% I_2 and 10% KI solution in distilled water). The stained disks were examined using dissecting microscope and count the total number of tumors. The following formula was used for calculating percentage inhibition:

%age inhibition = 100 × {1- (No. of tumors in sample /No. of tumors in negative control)}

Spectrophotometric DPPH Assay

DPPH$^{\bullet}$ (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging potential of *Citrus* essential oils were assessed according to the reported method [20] with minor modifications. For making essential oil dilutions (1 to 10 mg/mL) methanol used as solvent. 0.004% DPPH$^{\bullet}$ was prepared in methanol. These solution mixtures were kept in the dark for 30min and the absorbance was noted at 517nm using a double beam spectrophotometer (Lambda 25, Perkin Elmer, USA) against methanol. The methanol (1 mL) having DPPH radical solution (1 mL) was used as blank. Scavenging (%) of free radicals were calculated by using this equation:

$$\text{Scavenging (\%)} = 100 \times [(A_0 - A_1)/A_0]$$

Here, A_0 is the absorbance of control (having all reagents excluding essential oil) and A_1 is absorbance of the tested sample. IC_{50} values, that showed the concentration of the essential oil which is responsible for 50% scavenging, it was computed by plotting a graph of percentage inhibition versus concentration.

β-Carotene /linoleic Acid Bleaching Assay

Antioxidant activity was assessed by calculating the inhibition of the volatile organic compounds and conjugated diene hydroperoxides produced as a result of the linoleic acid oxidation. A stock solution of the linoleic acid and the β-carotene was prepared by mixing 0.5 mg of the β-carotene in 1 mL of the chloroform, and after that 25 µL of linoleic acid and 200 mg of Tween 40 were added in the β-carotene solution. The chloroform was evaporated and then added 100 mL of distilled water in residue. The samples solution (2 g/L) was prepared in the DMSO. After that 2.5 mLof the above mixture and 350 µL of oil was added in a test tube. Then put the test tubes in the hot water bath for 2 h at the 50˚ C. Two blanks were used, one as a positive control which have antioxidant BHT in place of oil while second as negative control that have the equal volume of DMSO in place of oil. The test tube with

the BHT retained yellow color throughout the incubation time. At 470 nm the absorbance was measured using an ultraviolet double beam spectrophotometer (Lambda 25, Perkin Elmer, USA). The percentage Inhibition (I %) of oil samples was calculated as:

$$I\% = 100 \times (A_{\beta\text{-carotein after 2h assay}}/A_{\text{initial }\beta\text{-carotein}})$$

Here, $A_{\beta\text{-carotein after 2h assay}}$ is the absorbance after 2 h of the β-carotene that left behind in the samples and $A_{\text{initial }\beta\text{-carotein}}$ is the absorbance of the β-carotene in the start of the experiments. All experiments were performed in triplicate and results were recorded as means ± SD and [21].

Assay for Total Phenolics

The total phenolics were calculated by Folin-Ciocalteu reagent as reported by [22]. Briefly, 200µL of tested sample were added in 2.5mL of the Folin-Ciocalteu reagent (10 times diluted in distilled water). The samples were incubated for 5 min at 25˚C and shake it at least 2 times. After that, 2mL of Na_2CO_3 (7.5 %) was added and glass tubes were again incubated for 90 min in dark with constant shaking. The absorbance of samples was noted at 765 nm by a double beam spectrophotometer (Lambda 25, Perkin Elmer, USA). Distilled water was used as blank. For standard curve different concentrations of gallic acid (0-1000µg/ml) were used. The amount of the total phenolic contents were determined as microgram of gallic acid equivalent per gram of oil sample (µg GAE/ g oil) using the standard curve.

Antimicrobial Activity Test

Disc Diffusion Assay

The essential oils were tested against different microorganisms. The antimicrobial ability of *Citrus* oils were checked through disc diffusion test by mixing 100µL suspension of bacteria and 200µL of fungus in 100mL of nutrient agar (NA) and sabouraud dextrose liquid medium respectively. Sterile 6mm diameter filter paper discs and loaded with 10µL of essential oil and then put on agar plates. Rifampicin and terbinafine were used as the positive reference standards for antibacterial and antifungal activity, correspondingly. The plates were incubated for 1 day for bacteria and four days for fungus at 28 ˚C. The diameter of clear zone around the discs were calculated and expressed in millimeters for its antimicrobial activity. Seven discs per put on each plate and experiment was run in triplicate [23].

Determination of Minimum Inhibitory Concentration Assays

Modified resazurin microtitre-plate assay was also used for determination of MIC of some essential oils as reported by [24]. 100 µL of 20mg/mL (w/v) essential oil solutions in 10% DMSO (v/v) was pipetted in first column of the 96 well plates. 50 µL of the nutrient broth was added in remaining all wells. Serial dilutions were done taking 50µL from each well using micropipette and at the end 50 µL was discarded to maintain the same volume in each well in the serially descending concentrations. After that 10 µL of the resazurin solution (prepared by dissolving 270 mg tablet in 40 mL of distilled water) were added in each well. In the end, 10µL of the bacterial suspension was added in each well to get a concentration of approx 5×10^5 CFU m/L. Each plate had two set of controls: one row with a refampicine which is used as positive control, and second with 10% DMSO (v/v) as negative control. Plates were enfolded loosely with paraffin film and incubated for 24 h at 28 °C. The change in color from purple to pink was noted visually. MIC value was the lowest concentration at which color was changed.

Results and Discussion

Essential Oil Composition by GC-MS

The steam-distillation of *C. pseudolimon and C.grandis* peels (petitgrain mandarin) yielded 0.70% (v/w) and 0.45 % (v/w) respectively. The GC-MS analysis of *C. pseudolimon and C.grandis* (peels) are shown in Table-1. Thirty six components were detected constituting 97.42% of *C. pseudolimon* essential oil whereby the main constituents were limonene (47.07%), cuminal (11.41%), eugenol (10.17%), β-pinene (5.23%), styrene glycol (3.49%), γ-terpinene (3.67%), 3-isobutyl-1-cyclohexene (2.57%), caryophyllene (2.04%), menthene (1.53%), α-pinene (1.01%), p-cumic aldehyde (1.47%), α-isopropylbenzyl alcohol (1.56%), caryophyllene Oxide (1.94%) and Eugenyl acetate (1.29%). All other components were present in amount lower than 1%.

Thirty three compounds constituting 98.74% of *C. grandis* essential oil have been identified. The major components were limonene (71.48%), β-Pinene (7.71%), 3-carene (3.99%), β-citronellal (2.90%), α-Pinene (2.29%), 6-octenal, 3, 7-dimethyl (2.90%), caryophyllene (1.63%), cuminal (1.13%), anethole (1.29%), eugenol (1.01%), 3-p-menthene (1.37%) and gemacrene D (2.05%). All other components found to be less than 1%. Oxygenated

compounds and hydrocarbons are the main group in Citrus essential oils. Major components were monoterpenes hydrocarbons and some amounts of sesquiterpenes, alcohols and esters were also present. These results were correlated with previous findings [25, 26] who reported that chemical compositions of the essential oils were different amongst citrus species. Usually, limonene was the major chemical component among citrus essential oils, ranging from 32 to 98% while in *C. grandis* and *C.pseudolimon* oil, limonene accounted for 71.48% and 40.07% in respectively.

Antitumor Assay

The results revealed that both oils inhibited the tumor formation on potato discs. Results are summarized in Fig. 1. For positive control Vincristeine injection was used. *C. pseudolimon* oil has greater inhibition (81.25%) than Vincristine (78.69%) and *C. grandis* (61.53%). The cytotoxic, antitumor and anti-inflammatory effects observed in the *Citrus* essential oils might be attributed to flavonoids and limonoids present in citrus plants. Flavonoids have a chemo preventive potential against cancer because they inhibit cell proliferation, signal transduction and the angiogenesis [27]. Therefore it can be concluded from this study that *C. pseudolimon* possessed potent anticancer activity which might serve as a stepping stone for the discovery of a new anticancer agents.

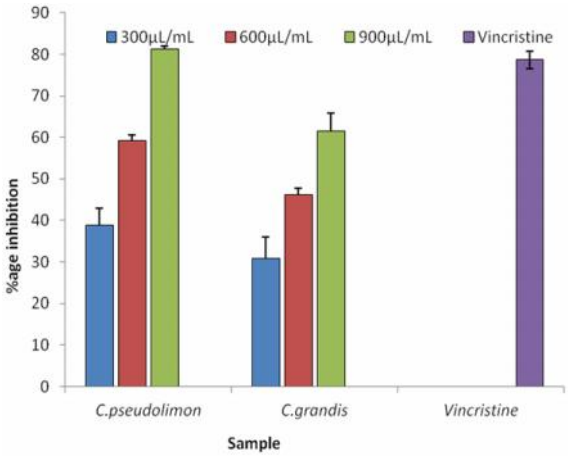

Fig. 1: Antitumor activity of *Citrus* essential oils.

Table-1: Volatile components of *Citrus* Peel essential oils.

Sr. No.	*C. pseudolimon*			*C. grandis*		
	Compounds	Rt	%age	Compounds	Rt	%age
1	α-thujene	9.1	0.43	α -phellandrene	9.12	0.22
2	α-pinene	9.27	1.01	α-pinene	9.33	2.29
3	Menthene	10.06	1.53	β-pinene	10.26	7.71
4	Pinocarveol	10.19	5.23	β –myrcene	10.33	0.56
5	β-myrcene	10.29	0.47	Menthene	10.81	1.36
6	3-methyl-4-methylidenebicyclo[3.2.1]oct-2-ene	10.64	0.68	Limonene	11.51	71.48
7	Sabinene	10.77	0.60	α-terpinene	11.87	3.99
8	Limonene	11.25	47.06	α-fenchene	11.97	0.10
9	γ-terpinen	11.72	3.66	α-terpinolene	12.31	0.04
10	Linalyl alcohol	12.41	0.67	2-Norbornanone	12.39	0.38
11	Carvomenthenol	12.90	0.98	Linalyl alcohol	12.44	0.12
12	α-Pinene oxide	13.13	0.85	Rose oxide	12.66	0.32
13	Bicyclo[3.3.1] non-2-en-9-ol, 9-methyl	13.19	2.57	β-cis-ocimene	12.94	0.20
14	3-Isobutyl-1-cyclohexene	13.46	0.34	Citronella	13.49	2.90
15	Bicyclo[3.3.1] heptan-3-one, 6,6-dimethyl-2-methylene	13.74	0.41	Isopregol	13.67	0.14
16	Norbornane	13.85	0.64	terpinen-4-ol	13.99	0.11
17	Isopregol	13.96	0.31	Camphene	14.21	0.14
18	Thujone	14.20	0.61	Anethole	15.89	1.29
19	Trans-p-Mentha-2,8-dien-1-ol	14.78	1.90	4-(1-Hydroxyethyl) benzaldehyde	16.02	0.49
20	p-cumic aldehyde	15.22	1.47	α-terpineol acetate	16.84	0.12
21	Citral	15.50	1.34	Eugenol	17.08	1.01
22	7-Propyl-cis-bicyclo[3.2.0]hept-6-en-2-one	15.71	0.87	Nerol acetate	17.27	0.11
23	α-Isopropylbenzyl alcohol	15.93	1.56	β-gurjunene	17.58	0.18
24	Styrene glycol	16.01	3.49	Caryophyllene	18.17	1.63
25	4-(1-Hydroxyethyl) benzaldehyde	16.20	0.71	α-humulen	18.66	0.15
26	Terpinyl butyrate	16.63	0.56	(−)-Isocaryophyllen	19.05	0.087
27	8-Hydroxylinaloo	16.88	0.71	Spathulenol	19.29	0.16
28	Eugenol	17.15	10.16	Eugenyl acetate	19.55	0.45
29	Caryophyllene	18.16	0.31	δ-cadinene	19.62	0.42
30	α-farnesene	18.23	0.52	(E,E)-alpha-farnesene	20.01	0.21
31	Isocaryophyllene	18.40	0.33	Cis,trans-α-Farnesene	20.62	0.03
32	α-humulen	18.65	0.45	1-Phenyl-5-(propyl amino)(1H)tetrazole	23.7	0.27
33	β-bisabolene	19.30	0.99	α-humulen	18.66	0.15
34	Eugenyl acetate	19.59	1.29			
35	Caryophyllene oxide	20.65	1.94			
36	Humulene epoxide II	20.99	0.65			

Antioxidant Activity

Antioxidant potential was determined by DPPH radical scavenging assay and β-carotein/linoleic acid bleaching assay. Result of the DPPH radical scavenging assay is shown in Fig. 2 and also presented as IC_{50} values (Table-2). *C. pseudolimon* has shown greater antioxidant activity (IC_{50}=37.23µg/mL) as compared to the *C.grandis* (IC_{50} 332.64 µg/mL).These results were comparable to those reported by [28] which exhibited some citrus species having DPPH scavenging potential up to 77.76%. The radical scavenging behavior of the essential oil was due to its phenolic components. In phenolic compounds, hydroxyl group attached to aromatic ring might be responsible for its antioxidant behavior, that has capacity to give hydrogen atoms with electrons and it stabilized the free radicals [20].

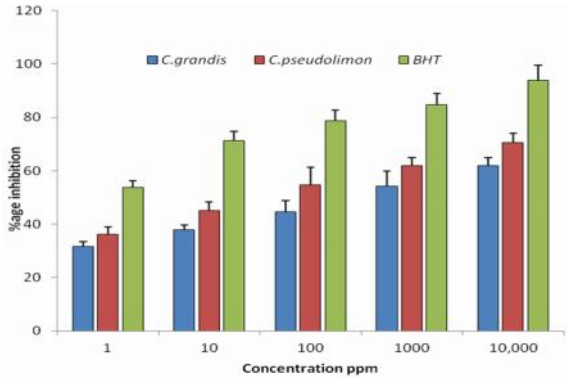

Fig. 2: DPPH radical scavenging activity of *Citrus* peel essential oils.

The lipid peroxidation inhibition was assessed with β-carotene/linoleic acid bleaching assay. This test is a measure of the plant's ability to inhibiting the conjugated diene hydroperoxides, formed during oxidation of the linoleic acid (21). The results of the Citrus peel essential oils and positive standard BHT were shown in Table-2 and in Fig. 3 at different time intervals. As more antioxidant components are present, the color will be depleted slowly. The slight decrease in the absorbance of the β-carotene associated with slower rate of linoleic acid oxidation and greater antioxidant activity of citrus essential oils. Based on these results the order of antioxidant activity was BHT > C. pseudolimon > C.grandis. These results were comparable to other's findings, who studied the antioxidant activity of some Pakistani citrus peel oils. Peroxidation inhibition via donating hydrogen atoms is the fundamental basis of the β-carotene/linoleic acid bleaching assay. The compounds having hydrogen atoms in the benzylic or allylic sites will have good potential in this assay due to comparatively simple abstraction of the atomic hydrogen from the functional groups of peroxy radicals which were formed in the test (29).

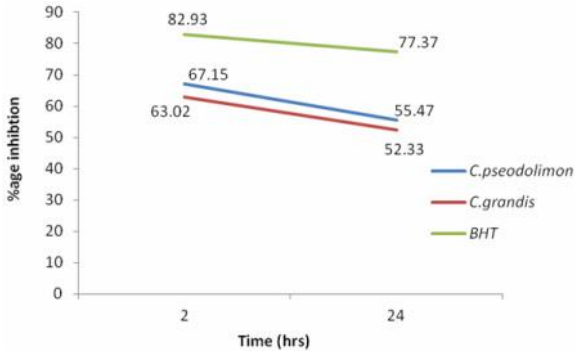

Fig. 3: β-Carotein linoleic acid bleaching assay of *Citrus* peel essential oils.

Table-2: Antioxidant activities (±S.D) of *Citrus* peel essential oils.

No.	Sample	DPPH IC_{50} (µg/mL)	β-Carotene/linoleic acid(%age inhb)
1	*C. Pseudolimon*	37.23±1.86	67.15±3.36
2	*C. grandis*	332.64±16.63	63.02±3.15
3	BHT	1.03±0.05	82.93±4.15

Total Phenolic Contents

Total phenolic contents of the *Citrus* oils were calculated with Folin-Ciocalteu reagent and expressed as µg GAE/10mg of oil. *C. grandis* had highest phenolic contents 59 µg GAE/10mg of oil while *C.pseudolimon* had 40 µgGAE/10mg .These results were comparable to previous findings (30) revealing that different essential oils have phenolic contents in range of 5.11 to 1107.20 µg GAE/5 mg of the essential oil. The results were presented in Fig. 4.

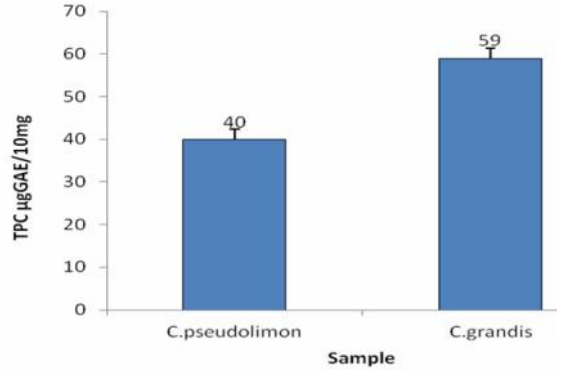

Fig. 4: Total Phenolic contents (TPC) in *Citrus* peel essential oils.

Antimicrobial Activity of Citrus Peel Essential Oils

The results showed that Citrus oils have varying levels of antibacterial activity against four tested bacteria viz, *Escherichia coli, Bacillus subtilis Staphylococcus aureus* and *Pasteurella multocida*. The antibacterial inhibition potential was checked by

inhibition zone diameters and MIC values qualitatively and quantitatively respectively (Fig. 5 a, b). *C. pseudolimon* oil has shown highest inhibition (18.2±1.3) against *Escherichia coli.* Minimum zone of inhibition was shown by *Citrus grandis* against *Escherichia coli.*

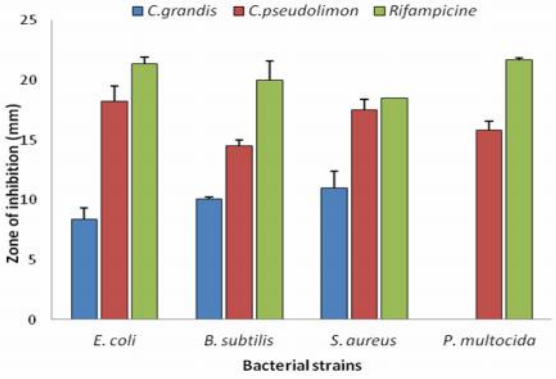

Fig. 5: (a). Antibacterial activity of *Citrus* peel essential oils.

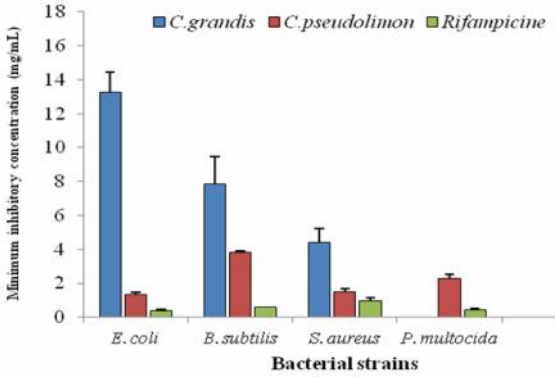

Fig. 5: (b). Minimum inhibitory concentration of *Citrus* peel essential oil.

Some researchers reported that there is a correlation in chemical composition of essential oils and their antimicrobial activity. A distinctive feature of the essential oils and its components is the hydrophobicity that plays a role in separating the lipid layer of the bacterial cell membrane and the mitochondria, destroying the structures of cells and makes the cell surface more permeable. Due to extensive leakage of some vital molecules and ions form the bacterial surface will cause their death [31]. In this study, both oils have been found effective against gram-positive as well as gram negative bacteria.

The antifungal behavior of Citrus essential oils was checked against four fungal strains by disc diffusion method. The essential oils showed antifungal activity at different extent against all the tested pathogens in (Fig. 6). *C. pseudolimon* showed highest inhibition (25.7±0.75mm) against *Penicillium notatum* while *C. grandis* showed highest inhibition (13±0.5 and 13±0.29 mm) against *Aspergillus niger and Penicillium notatum* respectively.

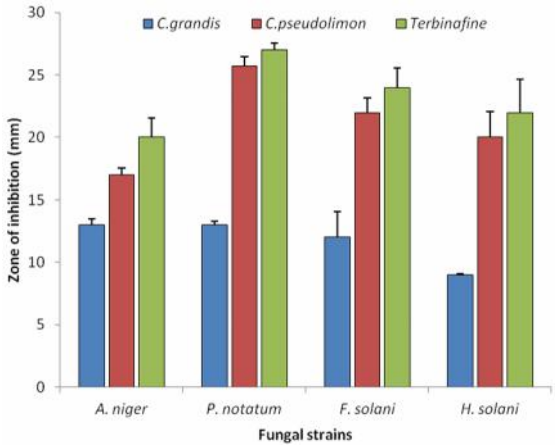

Fig. 6: Antifungal activity of Citrus peel essential oils.

The chemical compounds like α-terpineol, linalool and α-pinene, having antifungal and antibacterial potential, and these are present in significant amounts in the *C. pseudolimon* and C. *grandis* essential oil. Citral; one of the components of *Citrus limettioides* also found in *C. pseudolimon* essential oil used as fungicidal agent due to its aptitude to form a transferrable charged complex that donate electron to fungal cells and results in the fungal death [14, 32].

Conclusion

Antimicrobial properties of essential oils are of great interest in food, cosmetic and pharmaceutical industries since their possible use as natural additives emerged from the tendency to replace synthetic preservatives with natural ones. From this study it can be concluded that essential oils possess antibacterial and antitumor activity. Both oils have moderate antioxidant potential. GC-MS concluded that *Citrus pseudolimon* oil has thirty-seven components while *Citrus grandis* has thirty-three. It can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agents. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of these oils as an antibacterial agent. However a further study is

needed for isolation of active constituents from these oils.

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References

1. F. Bakkli, S. Averbeck, D. Averbeck and M. Idaomar, Biological Effects of Essential Oils- A Review, Food Chem Toxicol, **46**, 446 (2008).
2. N. Tabassum and G. M. Vidyasagar, Antifungal Investigation on Plant Essential Oils, A Review, *Int. J. Pharm Pharm Sci*, **5**, 19, (2013).
3. L. Pistelli, F. Mancianti, A. Bertoli, P. L. Cioni, M. Leonardi, F. Pisseri, L. Mugnaini and S. Nardoni, Antimycotic Activity of Some Aromatic Plants Essential Oils Against Canine Isolates of Malassezia pachydermatis: An in vitro Assay, *Open Mycol J.*, **6, 17** (2012).
4. S, Chanthaphon, S. Chanthachum, and T. Hongpattarakere, Antimicrobial Activities of Essential Oils and Crude Extracts from Tropical Citrus spp. Against Food-Related Microorganisms, Songklanakarin *J. Sci. Technol*, **30,** 125 (2008).
5. A. El Asbahani, K. Miladi, W. Badri, M. Sala, E. H. Ait Addi, H. Casabianca, A. El Mousadik, D. Hartmann, A. Jilale, F. N. R. Renaud, A. Elaissari. Essential Oils: From Extraction to Encapsulation. Int. J. Pharm, **483,** 1, 220-243, (2015).
6. J. R. Patil, G. K. Jayaprakasha, K. N. C. Murth, S. E. Tichy, M. B. Chetti and B. S. Patil, Apoptosis-Mediated Proliferation Inhibition of Human Colon Cancer Cells by Volatile Principles of Citrus aurantifolia, *Food Chem*, **114,** 1351 (2009).
7. M. Z. El-Readi, D. Hamdan, N. Farrag, A. El-Shazly and M. Wink, Inhibition of P-glycoprotein Activity by Limonin and other Secondary Metabolites from Citrus Species in Human Colon and Leukaemia Cell Lines, *Eur. J. Pharmacol*, **626,** 139 (2010).
8. D. I. Hamdan, R. H. Abdulla, M. E. Mohamed and A. M. El-Shazly, Chemical Composition and Biological Activity of Essential Oils of *Cleopatra mandarin* (Citrus reshni) Cultivated in Egypt, J. Pharmacognosy Phytother, **5**, 5,83-90, (2013).
9. G. Bertuzzi, B. Tirillini, P. Angelini and R. Venanzoni, Antioxidative Action of Citrus limonum Essential Oil on Skin, European J. Med Plants, **3**, 1, 1-9, (2013).
10. B. J. Seok, S. S. Kim, J. A. Lee, T. H. OH, J. Y. Kim, N. H. Lee, and C. G Hyun, Chemical Composition and Biological Activities of Essential Oils Extracted from Korean Endemic Citrus Species, J. Microbiol Biotechnol, **18**, 1,74-79, (2008).
11. F. Q. S. Guerra, J. M. Mendes, W. A. D. Oliveira, F. S. D. Souza, V. N. Trajano, H. D. M. Coutinho and E. D. O. Lima, Antibacterial Activity of the Essential Oil of *Citrus limon* Against Multidrug Resistant Acinetobacter Strains, Rev. Bras. Farm. **94**, 2, 142-147, (2013).
12. D. S. Janoti, M. Rana, A.K.S. Rawat, Comparative Antioxidant Activity of Essential Oil of Leaves of *Citrus limettioides* and *Citrus pseudolimon* of Nainital District, J. Pharmacogn Phytochem, **2**, 5, 24-26, (2014).
13. F. Spadaro, C. Circosta, R. Costa, F. Pizzimenti, D. R. Palumbo and F. Occhiuto, Volatile Fraction Composition and Biological Activity of Lemon Oil (Citrus limon L. Burm.): Comparative Study of Oils Extracted from Conventionally Grown and Biological Fruits, J. essent oil res, **24**, 2, 187-193, (2014).
14. N. Vasudeva and T. Sharma, Chemical Composition and Antimicrobial Activity of Essential Oil of Citrus limettioides Tanaka, J. Pharm Technol Drug Res,1-7, (2012).
15. S. Frassinetti, L. Caltavuturo, M. Cini, C. M. D. Croce and B. E. Maserti, Antibacterial and Antioxidant Activity of Essential Oils from Citrus spp, *J. Essent Oil Res*, **23,** 27 (2014).
16. A. Mohamed, G. A. El-Emary, and H. F. Ali, Influence of Some Citrus Essential Oils on Cell Viability, Glutathione-S-Transferase and Lipid Peroxidation in Ehrlich ascites Carcinoma Cells, *J. Am Sci*, **6,** 820 (2010).
17. R. M. Romeilah, S. A. Fayed and G. I. Mahmoud, Chemical Compositions, Antiviral and Antioxidant Activities of Seven Essential Oils, J. appl. sci. res., **6, 50-62,** (2010).
18. R. P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Carol Stream, Alluret, IL, USA, 1322-1326, (1995).
19. P. S. Coker, J. Radecke, C. Guy and N. D. Camper, Potato Disc Tumor Induction Assay: A Multiple Mode of Drug Induction Assay, *Phytomedicine*, **10,** 133 (2003).
20. A. Senthilkumar and V. Venkatesalu, Chemical Constituents, In Vitro Antioxidant and Antimicrobial Activities of Essential Oil from

the Fruit Pulp of Wood Apple, *Ind Crop Prod*, **46,** 66 (2013).

21. J. S. Ghomi, A. H. Ebrahimabadi, Z. D. Bidgoli and H. Batool, GC/MS Analysis and In Vitro Antioxidant Activity of Essential Oil and Methanol Extracts of Thymus Caramanicus Jalas and its Main Constituent Carvacrol, *Food Chem*, **115,** 1524 (2009).
22. A. Alizadeh, O. Alizadeh, M. Amari and M. Zare, Essential Oil Composition, Total Phenolic Content, Antioxidant Activity and Antifungal Properties of Iranian Thymus daenensis subsp. Daenensis celak. as in Influenced by Ontogenetical Variation, *J Essent Oil Bear Pl*, **16,** 59 (2013).
23. N. Djabou, V. Lorenzi, E. Guinoiseau, S. Andreani, M. C. Giuliani, J. M. Desjobert, J. M. Bolla, J. Costa, L. Berti, A. Luciani and A. Muselli, Phytochemical Composition of Corsican Teucrium Essential Oils and Antibacterial Activity against Foodborne or Toxi-Infectious Pathogens, *Food Control,* **30,** 354 (2013).
24. A. Gahlaut and A. K. Chhillar, Evaluation of Antibacterial Potential of Plant Extracts Using Resazurin based Microtiter Dilution Assay, *Int J. Pharm Pharm Sci*, **5**, 372 (2013).
25. N. G. Tao and Y. J. Liu, Chemical Composition and Antimicrobial Activity of the Essential Oil from the Peel of Shatian Pummelo (Citrus Grandis Osbeck), *Int J. Food Prop*, **15,** 709 (2014).
26. H. S. Choi, H. S. Song, H. Ukeda and M. Sawamura, Radical-Scavenging Activities of Citrus Essential Oils and Their Components: Detection Using 1,1-Diphenyl-2-picrylhydrazyl, *J. Agr food chem*, **48,** 4156 (2000).
27. S. Kundusen, M. Gupta and A. Bala, Antitumor Activity of Citrus maxima (Burm.) Merr. Leaves in Ehrlich's Ascites Carcinoma Cell-Treated Mice, ISRN Pharmacology, **5,** (2011).
28. K. Shahzad, S. Nawaz, R. Ahmad, N. Akram, and Z. Iqbal, Evaluation of Antibacterial, Antifungal and Antioxidant Activity of Essential Oil of Citrus Reticulate Fruit (Tangerine fruit peel), Pharmacologyonline, **3,** 614 (2009).
29. G. M. Kamal, M. Y. Ashraf, A. I. Hussain, A. Shahzadi and M. I. Chughtai, Antioxidant Potential of Peel Essential Oils of Three Pakistani Citrus species: Citrus reticulate, Citrus sinensis and Citrus paradish, *Pak. J. Bot.,* **45,** 4, 1449 (2013).
30. C. W. Lin, C. W. Yu, S. C. Wu and K. H. Yih, DPPH Free-Radical Scavenging Activity, Total Phenolic Contents and Chemical Composition Analysis of Forty-Two Kinds of Essential Oils, *J. Food Drug Anal*, **17, 386** (2009).
31. S. Prabuseenivasan, M. Jayakumar and S. Ignacimuthu, In Vitro Antibacterial Activity of Some Plant Essential Oils, *BMC Complement Altern Med*, **6** (2006).
32. A. El Asbahani, E. H. Ait Addi, K. Miladi, A. Bitar, H. Casabianca, A. El Mousadik, D. J. Hartmann, A. Jilale, F. N. R. Renaud and A. Elaissari. Preparation of Medical Cotton Textile Activated by Thymus leptobotrys Essential Oil Colloidal Particles: Evaluation of Antifungal Properties, *J. Coll Sci Biotechn*, **3** (2014).