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Antimicrobial Properties of Orange (*Citrus reticulata* var. *Kinnow*) Peel Extracts against Pathogenic Bacteria

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A B S T R A C T

Citrus peels are known for the abundant amounts of polyphenols present, which have been proven to possess antimicrobial activity. The objective of this project was to determine the phenolic content and antibacterial capacity of orange (*Citrus reticulata* var. *Kinnow*) peel extracts against pathogenic strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae.* Peel powder of Orange was subjected to polyphenolic extraction using different solvents viz., petroleum ether, ethanol, acetone, and methanol. Pathogenic bacterial strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* were screened for the antibacterial activity of the extracts using disc diffusion technique. The total phenolic content of the extracts was determined by the method involving Folin-Ciocalteau reagent and gallic acid standards, and was expressed as mg GAE/ml extract. As compared to other solvent extracts, acetone extract possessed high phenolic content with 17.6 mg GAE/ml of extract. It was also noticed that acetone extract possessed comparatively higher antibacterial potential, and it was shown to inhibit all four pathogenic bacterial strains. The Minimum Inhibitory concentration (MIC) of 68.75 µg/ml of acetone extract was found to inhibit *Klebsiella pneumoniae* and *Escherichia coli*, with no significant difference. Maximum zone of inhibition at MIC of acetone was found to be 7.93±0.065 mm in case of *K. pneumoniae* and 7.75±0.12 mm in *E. coli*.

Introduction

In the recent years, there has been a profound shift in the preference for natural substances as antimicrobials. The prevalence of antibiotic resistance is a continual problem due to the evolution of a potent defense mechanism against antibiotics. Therefore, it is necessary to exploit and develop novel inhibitory agents against resistant microbial pathogens (Otang and Afolayan, 2015). Plants can produce antimicrobial compounds to protect themselves from biotic attack that could be essential for microbial infection resistance. Also, it has been proven that antimicrobials and antibiotics from plant sources work more efficiently with fewer side-effects and added beneficial effects (Khushwaha *et al.*, 2012).

Plant based extracts with potential antimicrobial activity are being researched and tested to replace antibiotic drugs used for

inhibiting pathogens. Natural antimicrobials, whether of animal, plant or microbial origin, which exhibit bacteriostatic or bactericidal effects lengthen the life of products they are incorporated into, and also reduce, if not completely avoid health-related issues (Viuda-Martos *et al.,* 2008).

Citrus species are known for an abundance of bioactive components, nutraceuticals, and functional compounds in the flavedo and albedo of the peels. In Citrus fruits, flavonoids are present as flavanones (neohesperidosides, rutinosides), flavanol glycosides, flavones (polymethoxyflavones, hydroxylated polymethoxyflavones) with predominant bioactive compounds like naringin and hesperidin (Escobedo-Avellaneda *et al.,* 2014; Ramful *et al.,* 2011). Phenolic compounds like flavonoids are known to exhibit antioxidant, antiatherogenic, anti-inflammatory, anti-carcinogenic, antiviral, antimicrobial and antiallergenic activities (Escobedo-Avellaneda *et al.,* 2014)

Certain *Citrus* species have the antibacterial potential against clinically significant bacterial strains. It was found that acid-hydrolyzed *Citrus unshiu* peel extract inhibited *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes* (Keun Young Min *et al.,* 2014). As an antimicrobial agent, these polyphenols can penetrate the semi permeable cell membrane where they react with the cytoplasm or cellular proteins (Sa *et al.,* 2015)

The objective of this study was to determine the antibacterial potential of extracts of methanol, ethanol, acetone and petroleum ether from *Citrus reticulata* var. Kinnow against pathogenic strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* and to determine the MIC and Zone of Inhibition of the bacteria.

Materials and Methods

Materials

Microorganisms

The extracts were screened for their antibacterial activities against various pathogenic bacterial strains, gram negative and gram positive, namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae,* provided by the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University.

Chemicals and apparatus

All chemicals and media were procured from Merck and Hi-media respectively. The plates used for experimentation were irradiated disposable Tarsonspetriplates and Eppendorf tubes for extracts.

Preparation of orange peel powder

Orange peels of *Citrus reticulata* var. *Kinnow* procured from the local fruit vendors was first washed thoroughly to remove any extraneous matter and to get rid of contaminants. It was then subjected to blanching operation and pressed to remove excess water. They were cut into 1x1 inch size, placed on a tray and dried in a tray drier at a constant temperature of 40°C. When peels were dried to a moisture content <5%, it was finely pulverized in a sterile grinder and sieved. It was then stored in air tight sealed PE-PA bags and placed at 4°C.

Preparation of extracts

Orange peel extracts were prepared according to the method by Yadav *et al.,* (2015) with slight modifications. 4 g of Orange Peel Powder, stored at 4°C, was taken in 4 different conical flasks. 20 ml of ethanol, methanol,

acetone, and petroleum ether was added respectively. The conical flasks were tightly stoppered with plugs of non-absorbent cotton and this was wrapped with aluminium foil as a precautionary measure. The conical flasks were placed in a shaker incubator pre-set at 30°C at 130 rpm for 36 hours for extraction to complete. After the extraction process was completed, the flasks were removed from the incubator and the contents were poured into centrifuge tubes (Tarson Tubes) that were tightly capped and were centrifuged at 4200 rpm at 10°C. The clear liquids were immediately transferred to clean, dry petri-plates and were placed in a tray drier at 35°C to concentrate it up to 80% and to ensure that the solvent used for extraction evaporated. The centrifuge tubes with pellets were discarded. When most of the solvents had evaporated, the extracts were carefully transferred into small Eppendorf tubes and stored at 10°C.

Total Phenolic Content (TPC) assay

The Total Phenolic Content of the extracts was determined by the method involving Folin-Ciocalteau reagent and Gallic acid standards (Hinneburg *et al.,* 2006). Gallic acid was used for generating the standard curve having concentrations ranging from 20 to 100 mg/ml. 2.5 ml of 10 times diluted FC reagent was added to each tube and mixed well for 1 min and 2 ml of 7.5 % Na_2CO_3 was added to it and allowed to incubate for 30 minutes at 37 °C and further the absorbance was measured at 760 nm in ultraviolet- 1800 spectro photometer (Shimadzu, Kyoto, Japan) and standard graph was plotted.

The reaction mixture was also incubated at 37 °C for 30 min and the absorbance was recorded at 760 nm. All procedures were performed with three replicates. The total phenolic content equivalent to Gallic acid was determined from standard graph. It was expressed as Gallic Acid Equivalents per gm of dry extract (mg GAE/g).

Antimicrobial testing

Preparation of inoculum

The bacterial isolates procured were inoculated in Mueller-Hinton Agar (MHA, Hi Media) and were incubated at 37°C for 3-7 hours until the culture attained turbidity to the Mc Farland Std no. 0.5 [~10^6 colony forming units CFU/ml]. (Singh *et al.,* 2014)

Determination of sensitivity of orange peel extract against pathogenic bacteria

The sensitivity of peel extract against four pathogens namely *Staphylococcus aureus, Salmonellatyphii, Klebsiella spp., and Escherichia coli*was performed according to protocol of Yadav *et al.,* (2015). Mueller Hinton Agar (MHA, Hi-Media) was prepared, autoclaved, and poured into sterile petriplates (Tarsons Irradiated Disposable). The Orange peel extracts, which were tray-dried at 35°C, were dissolved in respective solvents in the ratio of 2:1 and 10µl of extract solution was dropped onto petri plates (Tarsons-Irradiated Disposable Plates) swabbed with bacterial inoculum. The controls, which consisted of respective solvents for extracts, was set up next to the extract and 10µl of control was dropped adjacent to the spot of the extract. The plates were then incubated for 24 hours at 37℃. The clear zone around the drop of the extract was noticed.

Determination of minimum inhibitory concentration of extract against pathogenic bacteria

Solutions of the extracts were prepared for initial stock solution at a ratio of 1:1. From this stock solution, serial dilutions of the compound were prepared up to 10 dilutions to

determine the Minimum Inhibitory Concentration.0.5 ml of each of the diluted extract was dropped sequentially on to the prepared plates, after placing sterile Whatman no. 1 filter paper discs (5 mm diameter).Sterile distilled water was used as a negative control. The plates were inverted and incubated for 24 hours at 37°C. Antimicrobial activity was evaluated by measuring the diameter of inhibition zones with no bacterial growth in mm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration where no viability was observed after 24 h on the basis of zones of growth. All the determinations were conducted in triplicates (Singh *et al.,* 2014). The serial dilutions of the four different extracts have been tabulated in Table 1.

Statistical analysis

All data was expressed as mean ± standard errors of triplicate measurements. Statistical significance was tested by employing one- way analysis of variance and comparison between means was made with the help of Microsoft excel 2016.

Results and Discussion

Total phenolic content

The highest phenolic content was found in the acetone extract of Orange peel with 17.6 mg GAE/ml of extract, followed by methanol extract which contained 12.5 mg GAE/ml extract. This is in agreement with a study conducted by Yadav *et al.,* (2015), where it was found that acetone was a better solvent for the extraction polyphenols from different grape fractions. It was propounded by Alothman *et al.,* (2009) that the recovery of phenolic compounds was purely dependent on the solvent used and its polarity for the different plant materials it is used for. The recovery of polyphenolic compounds from plant materials is affected by their solubility in that specific solvent. Also, the solvent solubility plays a pivotal role in increasing the phenolic compounds solubility in it (Alothman *et al.,* 2009). The solvent which has the highest polyphenol content possesses maximum extractability of the compounds in comparison to the other solvents (Yadav *et al.,* 2015). The maximum predicted Total Phenolic Content comprising primarily of bioactive polyphenols from *Citrus sinensis* under the optimal Microwave Assisted Extraction (MAE) conditions with 51% acetone concentration in water (v/v), 122 s extraction time and 25 mL/g solvent to solid ratio) was 12.20 mg GAE/g dry weight, which was ideal (Nayak *et al.,* 2015). In a study conducted by Alothman *et al.,* (2009) where phenolic content was determined for different tropical fruits using different solvents for extraction, it was found that for pineapple extracts, 50% acetone and 70% ethanol gave the highest yield for total phenolics without significant differences between them.

In a study conducted for the determination of effects of different solvents extraction on concentration and antioxidant activity of black and black mate tea polyphenols, it was found that for black mate tea, 50% acetone showed the highest polyphenol content (Turkmen *et al.,* 2006).

The ethanolic extract contained 10.25 mg GAE/ml in the present study, which is a fairly good extraction potential. It has been stated that ethanolic mixtures and extracts have a higher acceptability for human consumption models (Alothman *et al.,* 2009). In a study conducted for the extraction of polyphenols from grapes marc, ethanol and methanol extracts of red and black currant contain twice more anthocyanins and polyphenols than water extracts, extracts made from grape marc had seven times higher values than water extracts (Lapornik *et al.,* 2005).

Table.1 Serial dilution of extract for the determination of MIC for pathogenic bacteria inhibition

Extract	Initial Concentration (mg GAE/ml extract)	Serial Dilutions used for determination of MIC (µg/ml)									
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
M	12.5	6250	3125	1562.5	781.25	390.63	195.32	97.65	48.82	24.41	12.21
E	10.25	5125	2562.5	1281.25	640.625	320.32	160.15	80.07	40.03	20.02	10.01
A	17.6	8800	4400	2200	1100	550	275	137.5	68.75	34.38	17.19
P	8.98	4490	2245	1122.5	561.25	280.63	140.32	70.16	35.08	17.54	8.77

Where M=Methanol extract, E=Ethanol extract, P=Petroleum ether extract, A=Acetone extract, D=Dilution

Table.2 Determination of Minimum Inhibitory Concentration (MIC) of different extracts against pathogenic bacteria strains

Extract	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*
Methanol	781.25 µg/ml^{c}	1562.5 µg/ml^{b}	3125 µg/ml^{a}	3125 µg/ml^{a}
Ethanol	320.32 µg/ml^{c}	640.625 µg/ml^{b}	1281.25 µg/ml^{a}	1281.25 µg/ml^{a}
Petroleum Ether	2245 µg/ml^{c}	ND	ND	4490 µg/ml^{c}
Acetone	275 µg/ml^{b}	68.75 µg/ml^{c}	550 µg/ml^{c}	68.75 µg/ml^{a}

Values are Mean±SEM of Triplicate Samples
Different superscripts in rows are significantly different ($p<0.05$)

Table.3 Diameter of zone of inhibition at MIC of extract

Diameter of ZI for:	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*
Methanol	5.02±0.956 mm^{b}	5.32±0.054 mm^{c}	4.12±0.026 mm^{a}	4.38±0.008 mm^{a}
Ethanol	6.91±0.087 mm^{b}	7.68±0.034 mm^{a}	4.03±0.023 mm^{d}	5.88±0.012 mm^{c}
Petroleum Ether	3.56±0.002 mm^{a}	ND	ND	2.76±0.092 mm^{b}
Acetone	7.21±0.029 mm^{d}	7.93±0.065 mm ^{a}	7.58±0.054 mm^{c}	7.75±0.12 mm^{b}

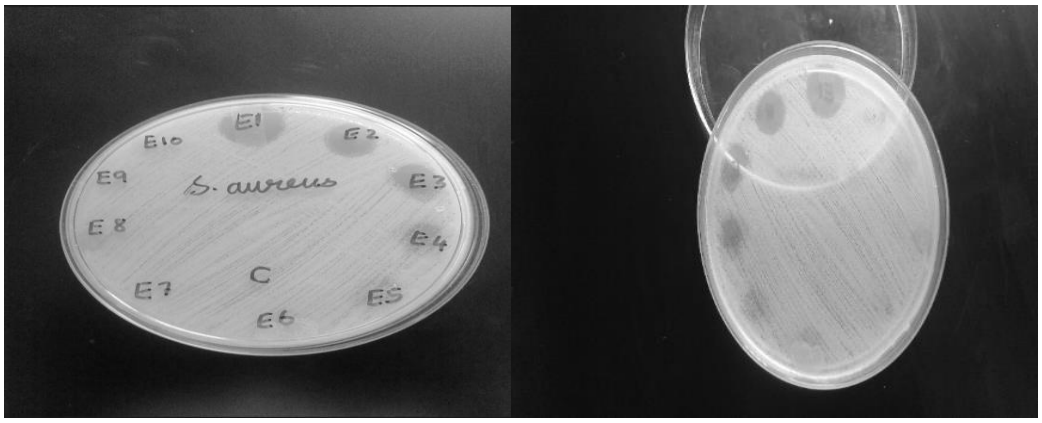
Where ZI = Zone of Inhibition
Values are Mean±SEM of Triplicate Samples
Different superscripts are significantly different ($p<0.05$)

Table.4 Diameter zone of inhibition at initial concentration

Diameter of ZI for:	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*
Methanol	18.52±0.12 mm^{c}	20.05±0.07 mm^{a}	17.54±0.05mm^{d}	19.97±0.09 mm^{a}
Ethanol	19.12±0.06 mm^{a}	19±0.08 mm^{a}	18.44±0.01 mm^{b}	17.03±0.07 mm^{c}
Petroleum Ether	5.02±0.05 mm^{a}	ND	ND	6.56±0.03 mm^{b}
Acetone	20±0.02 mm^{c}	22.96±0.08 mm^{b}	25.88±0.05 mm^{a}	22.14±0.97 mm^{b}

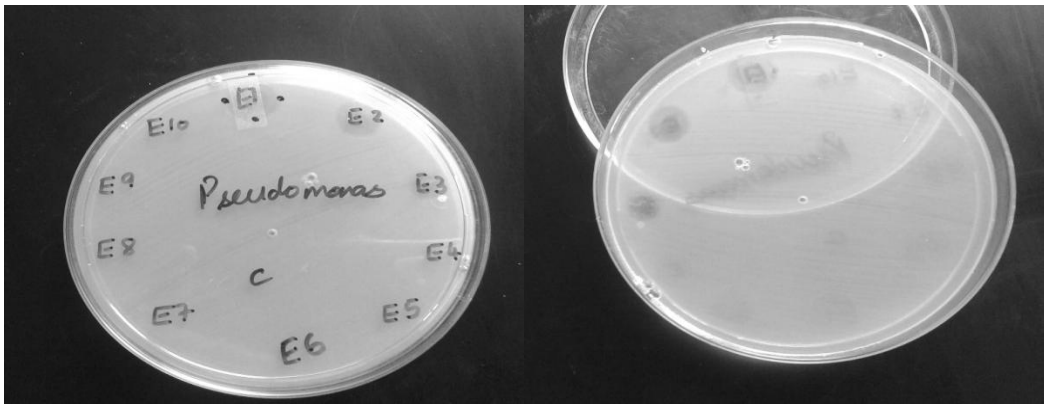
Where ZI = Zone of Inhibition; Values are Mean ± SEM of Triplicate Samples
Different superscripts are significantly different ($p<0.05$)

Fig.1 Zones of inhibition at different dilutions of ethanolic extract against *Staphylococcus aureus*



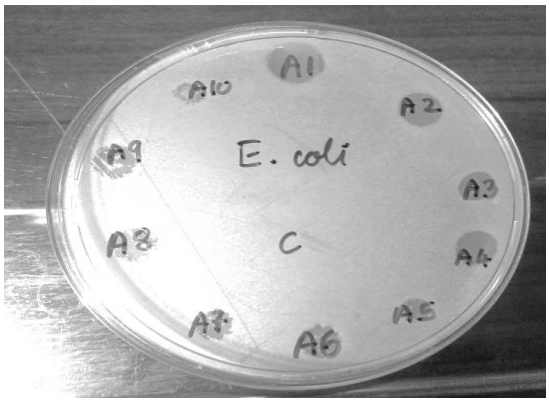
Where (E1>E2>..>E10), and C = control

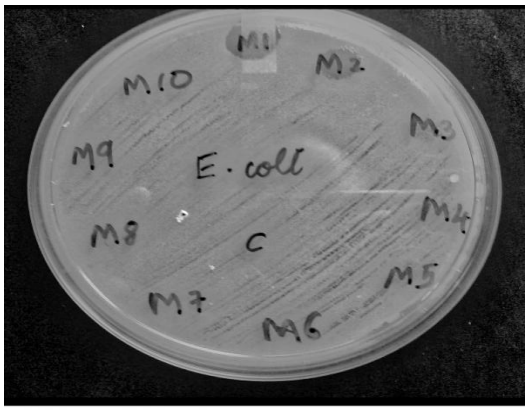
Fig.2 Zones of inhibition at different dilutions of Ethanolic Extract against *Pseudomonas aeruginosa*



Where (E1>E2>..>E10), and C = control

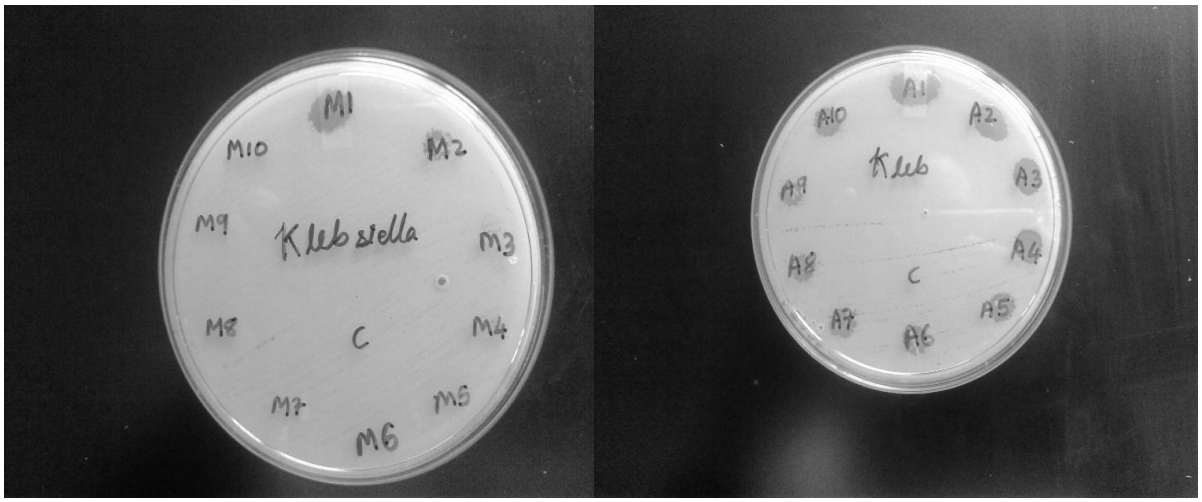
Fig.3 Zones of inhibition at different dilutions of Acetone and Methanol Extract against *Escherichia coli*





Where (E1>E2>..>E10), (M1>M2>..>M10) and C = control

Fig.4 Zones of inhibition at different dilutions of methanol and acetone extract against *Klebsiella pneumoniae*



Where (E1>E2>..>E10), (M1>M2>..>M10) and C = control

Antibacterial activity

The result of the disc diffusion assay, expressed as Zone of Inhibition of bacterial strains and the MICs of the extracts are summarized in Table 2, 3, and 4. Figure 1, 2, 3 and 4 represents the zone of inhibition in different extracts against pathogenic strains. The highest antibacterial activity was obtained with the acetone extract of *C. reticulata* var. *Kinnow* against *Klebsiella pneumoniae* and *Escherichia coli* with inhibition zone diameters of7.93±0.065 mm and 7.75±0.12 mmat MIC of 68.75 µg/ml, which were not significantly different (P <0.05). The methanol extract showed a zone of inhibition5.02±0.956 mm of at MIC of 781.25 µg/mlin the plate containing the strain of *Staphylococcus aureus*. The effectiveness of the extracts can be summarized as: Acetone> Methanol > Ethanol > Petroleum Ether. Sterile distilled water, used as negative control, did not show any inhibition against all tested microorganisms.

It has also been observed that Petroleum ether extracts were completely ineffective against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Antibacterial activity has been observed in Citrus peel by Dorman *et al.*, (2000) and Mandalari *et al.*, (2007). Espina *et al.*, (2011) previously demonstrated that mandarin peel had greater antimicrobial activity than lemon peel. It was reported that an acetone extract of sea buckthorn seed had higher antibacterial activities than an ethyl acetate extract, although it had the higher phenolic contents than the acetone one. (Turkmen *et al.*, 2007)

It should be taken into account that the area of inhibition of bacterial strain depends on the ability of the extract to diffuse uniformly through the agar (Samy and Ignacimuthu, 1998).

In the case of *Staphylococcus aureus*, the presence of a simple membrane structure presents little buffering capacity at the interface against localized protonation effects caused by phenolic compounds and polyphenols and can easily cause hyper acidification and therefore disrupt plasma membrane associated H^+-ATPase and affect the energy metabolism of the bacterial cell (Du *et al.*, 2011). While it has been noted in

several studies that Gram positive bacteria are more sensitive to plant extracts than gram negative bacteria, because of the presence of an additional lipopolysaccharide coat, nevertheless there are exceptions in which Gram-negative bacteria are more susceptible than Gram positive towards some natural extracts (Kalemba and Kunicka, 2003). The results obtained in this study are in agreement with this.

The presence of an additional lipopolysaccharide layer along with minor membrane components besides an intact plasma membrane around its cell can have potentially more buffering capacity and hydrophobicity and therefore could prevent the action of simple phenolic compounds and thereby reduce the sensitivity of these bacteria against polyphenols (Du *et al.*, 2011). In the present study, however, it was observed that Acetone extract was highly in inhibiting gram-negative bacteria *Klebsiella pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa*, to different degrees, at varying concentrations of applications. This phenomenon can be attributed to the fact that the acetone extract contained compounds other than simple phenolics, including terpenes limonene, linalool, monoterpenes, and sesquiterpenes.

In the gram negative bacterial cell, lipid constituents of cell membrane are pivotal for its normal functioning for they provide the membrane with its barrier function and play a role in a variety of processes in the bacterial cell. Toxic effects of these components on membrane structure and function have been generally used to explain the antimicrobial action of several essential oils and their monoterpenoid components. As a result of their lipophilic character, monoterpenes will preferentially partition from an aqueous phase into membrane structures (Sikkema *et al.*, 1994). This results in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes. Zengin *et al.*, (2014) have described the effects of selected essential oil components on outer membrane permeability in gram-negative bacteria, thereby proving that terpene and monoterpene uptake is also determined by the permeability of the outer envelope of the target microorganism.

The present study could determine the antibacterial activity exhibited by the extracts of methanol, ethanol, acetone and petroleum ether from *Citrus reticulata* var. Kinnow against pathogenic strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae.* The highest antibacterial activity was obtained with the acetone extract of *C. reticulata* var. *Kinnow* against *Klebsiella pneumoniae* and *Escherichia coli* and zone of inhibition at Minimum Inhibitory Concentrations were determined successfully. The effectiveness of the extracts was also determined subsequently from the results obtained.

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