

ORIGINAL ARTICLE

## Antibacterial and wound healing potential of kinnow (*Citrus Mandarin*) peel's essential oil

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### Abstract

*Citrus mandarin* commonly named as kinnow is most widely produced and well cultivated in the Pakistan. The peel of *Citrus* fruit is much more effective as that of the fruit because it is composed of limonene which plays an important role therapeutically. Now a day in Pakistan even throughout the country the *Citrus* fruits are used for medicinal purpose because it has great efficacy and less adverse effect. The *Citrus mandarin* belongs to the family *Rutaceae* that has a great antioxidant activity, anti-inflammatory activity, anti-diabetic activity, antimicrobial activity and cytotoxic activity. The essential oil was extracted through the process of hydro-distillation at boiling temperature. The objective of this study is to check the wound healing potential, antimicrobial activity, GC-MS analysis, and skin irritation of the essential oil of kinnow peel. The wound healing activity of kinnow peel essential oil was within 8 days, whereas antimicrobial activity was checked against gram positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*). GC-MS analysis showed that essential oil composed of limonene, hexanal, decanal, octanal, hexa-decanal and other constituents. Limonene is greater and chief constituent of essential oil of kinnow. The skin irritation showed that it is nontoxic and nonirritant to the skin of rabbit.

### Keywords

Anti-microbial activity  
*In vitro In vivo* studies and GC-MS Analysis  
Phyto-chemicals  
Wound healing

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### Introduction

Wound healing is a solemn issue now a days because many infectious microbes contaminate wound surface if not treated well. So, there is a need of time to use antibiotics and wound healing agents in order to treat that wound. In market there are large number of wound healing agents and antimicrobials are available. The antibiotic resistance is getting stronger day by day and even the early use of penicillin shifted to macrolides. So, it is a need of time to use an alternative of these antibiotics, as there are number of causalities in the world. The essential oil obtained from citrus species also have antimicrobial activity so there should be some measurements to shift from synthetic antibiotics to natural antibiotics to prevent the resistance in human being.

The essential oil of *Citrus* is present in flavedo part. The essential oil of *Citrus* is volatile in nature due to presence of monoterpenes. The essential oil of *Citrus* fruit is divided into three fractions: terpenes hydrocarbons, oxygenated compounds and nonvolatile compounds. The quantity of terpenes hydrocarbons is about more than 95% having little contribution of flavor and fragrance. The industries who deal in fruits and vegetables produce large amount by products and peels, which are harmful for living organisms as well as they produce environmental pollution as well. So, there is a great need to deal with these by product which are harmful and peels of the fruits in order to prevent the future complications related to environmental pollution as well as organism's fatalities. The essential oils that are obtained from plants and vegetables have medicinal

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activity as well as biological effect due to the presence of flavonoids, flavones, carotenes, terpenes and coumarins. Due to antioxidant, antidiabetic, antimicrobial and antiviral effects the essential oils are also well known pharmacological agent (Javed et al., 2014). The essential oils of *Citrus mandarin* show a great antimicrobial activity against both gram positive and gram-negative bacteria. They inhibited the growth of *staphylococcus aureus*, *Lactococcus lactis* species as well as *E-coli* bacterial strains on higher concentration in minute region (Vasek et al., 2015). The 0-10% is volatile in nature which contains esters, waxes and other related substances, whereas the non-volatile portion is about 90% which contains terpenes, monoterpenes, sesquiterpenes and oxygenated compounds which is enclosed in glandular sac like structure of citrus peel (Ahmad et al., 2006). In our studies we use the waste material to treat that wound on the skin surface. Normally the peels of citrus fruits are thrown away in garbage or use as a food of animals rarely. But in our studies we collect the peel of kinnow (*Citrus mandarin*) and extracted the essential oil from those peels through the process of hydro distillation in a hydrodistillator chamber. The recovery of essential oils takes place in a separating funnel and stored at 4°C.

## Materials and Methods

The research plan was proposed to study and evaluate the antimicrobial activity, wound healing potential, GC-MS (gas chromatography-mass spectroscopy), skin irritation of *Citrus Mandarin* peels essential oils.

**Sample collection:** Fresh and healthy sample of kinnow peel was collected from the University of Agriculture Faisalabad market. The proper storage, preservation and purification of these peels were performed in the institute of Horticulture sciences of University of Agriculture Faisalabad.

### Phase-I (*In-vitro* studies)

#### Antibacterial activity

**Preparation of disc:** The Whatman's filter paper no 1 was used to prepare the disc. The paper was cut into 3 mm of diameter and then sterilized and autoclaved.

**Media preparation:** The nutrient agar media was used as a medium in order to study the antimicrobial activities. The method of growth of bacterial species was introduced by the scientist named Cruickshank in 1975.

**Preparation:** First of all washed, cleaned and dry all the glass apparatus. Then the peptone and the agar were added in the flask and added some water in order to mix the peptone and agar. Then took NaCl and beef extract in the separate flask and mix them. Finally mixed the both solution of peptone-agar and NaCl-beef and maintained the PH 4.5 by mixing HCl in them. Then the

cotton wool cloth was used to plug the solution and added in the autoclave for sterilization. The autoclave process was performed at 121 degree for 15 minutes at pressure of 15 PSI. At last, the content was allowed to dry and they removed from autoclave.

**Poured plates sterility testing:** In poured plate method the plates were incubated in the autoclave for 15 minutes at 121°C as a result there was no any growth of bacterial species. If there was any contamination on the plates, those plates were discarded, only the plates were kept who have no contamination.

**Disc diffusion method:** The prepared media was first sterilized and then cooled down at the temperature of 45°C. Then with the help of pipette the prepared media was placed on the petri plates and then mixed homogeneously. Then the petri plates were inoculated and the drops of essential oil of kinnow peel were placed on the petri plates. Then these plates were placed at 4°C and then incubated for 34 hours at 37°C. An antibiotic named as chloramphenicol was used as standard.

### Phase-II (*In-vivo* studies)

**Animals:** The 4 healthy rabbits were purchased from the local market of the Faisalabad having weight about 1000 gm to 1600 gm. All the rabbits were kept at animal house of the University of Agriculture Faisalabad and provided the healthy diet during experiment. The rabbits were kept at proper temperature and dark and night periods for one week before experiment.

**Feed:** All the rabbits were fed with fresh green fodder named as alfalfa. They were with free access to feed.

**Incision of wounds:** The hairs of all four rabbits were clipped with the help of clipper. The four groups were made with help of the permanent marker. And then the anesthesia named as lignocaine was applied on the skin of the rabbit with the help of the syringe. Then the incisions were made on the skin of the rabbits in all four groups. About 1.5 cm of size, wounds was produced on the skin of the rabbits and then applied the essential oils and compared with pyodine.

**Application of essential oil:** On each rabbit 4 wounds were placed with the help of surgical blade and number 1, 2, 3 and 4. The first wound left untreated, the second was treated with the standard which was pyodine and the third wound was treated with essential oil in patch form while forth was treated with essential oil in naked form. The trial period was for 10 days and the wounds were monitored on daily basis.

**Acute skin irritation studies:** In acute skin irritation studies the activity of skin against essential oil was checked on each animal. In this case the hair was clipped from the skin of the rabbits and a little mark was placed on their skin on the specific region. After that an essential oil was applied in order to check the skin irritation against essential oil and left the animal for 24 hours.

**GC-MS analysis of kinnow essential oil:** The GC-MS analysis of essential oils was performed on the gas chromatography having number of HP 5890 and the mass chromatography of serial number about 5972 with electron impact ionization of 70 eV. A capillary column of 5 HP having thickness of 0.25  $\mu\text{m}$  and about 30 mm-0.25 mm of radius. The temperature of the column was from 50°C to 240°C and rises about 5°C/min. Neon was used as carrier gas having flow rate of about 1.2 ml/min and the split ratio was 60:1. The time ratio was 1 sec and the mass ratio was about 40-300 m/z. The chemical ratio of kinnow peel essential oil was obtained through retention time and compared with standard.

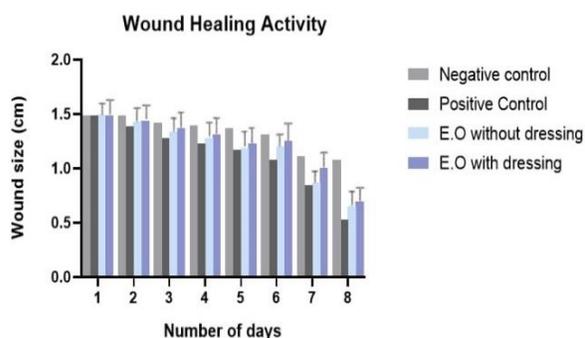
## Results

**Antibacterial activity:** The purpose of this study was to check the activity of essential oil of kinnow against specific gram positive and gram-negative bacteria. The disc diffusion method was used in order to check the activity of organism. There were four different concentrations used of 25, 50, 75 and 100  $\mu\text{l}$ . The different gram positive and gram-negative bacterial strains were used. There was lesser activity noted that cannot be measure through any mean. The lesser activity was seen at 100  $\mu\text{l}$  concentration of essential oil.

**Acute skin irritation:** The effect of skin irritation was also checked against the essential oil. The essential was applied on the skin of rabbit and check the level of irritation after 24 hours. After 8 days of complete trail the readings were observed that there was less irritation and swelling as shown in the Table 1.

**In Vivo Wound Healing Activity:** The effect of pyodine as well as the effect of kinnow essential oil was checked against the rabbit skin. The 8 days clinical in vivo trails were conducted. The effect of pyodine was greater than the essential oil. But the essential oils that were applied in naked form show greater activity as well as the essential oil applied in the patch form. The effect of pyodine and kinnow essential oil was seen that was  $P < 0.001$  which is more significant. The above-mentioned treatment showed that the rabbits treated with the pyodine show greater healing activity, whereas the essential oil of kinnow applied in naked form show good wound healing activity as compared to essential oil applied in the patch form as shown in the Figure 2.

**GC/MS Analysis of Kinnow Essential Oil:** GC/MS analysis was conducted in order to analyze the essential oil of kinnow. The chromatogram was developing during the analytical studies with known component of database spectra (NIST-147) stored at GC/MS library. Detail of constituents found in analysis of kinnow essential oil presented in Table 2. About 5 peaks were identified on the chromatogram during the studies as shown in the Figure 3.



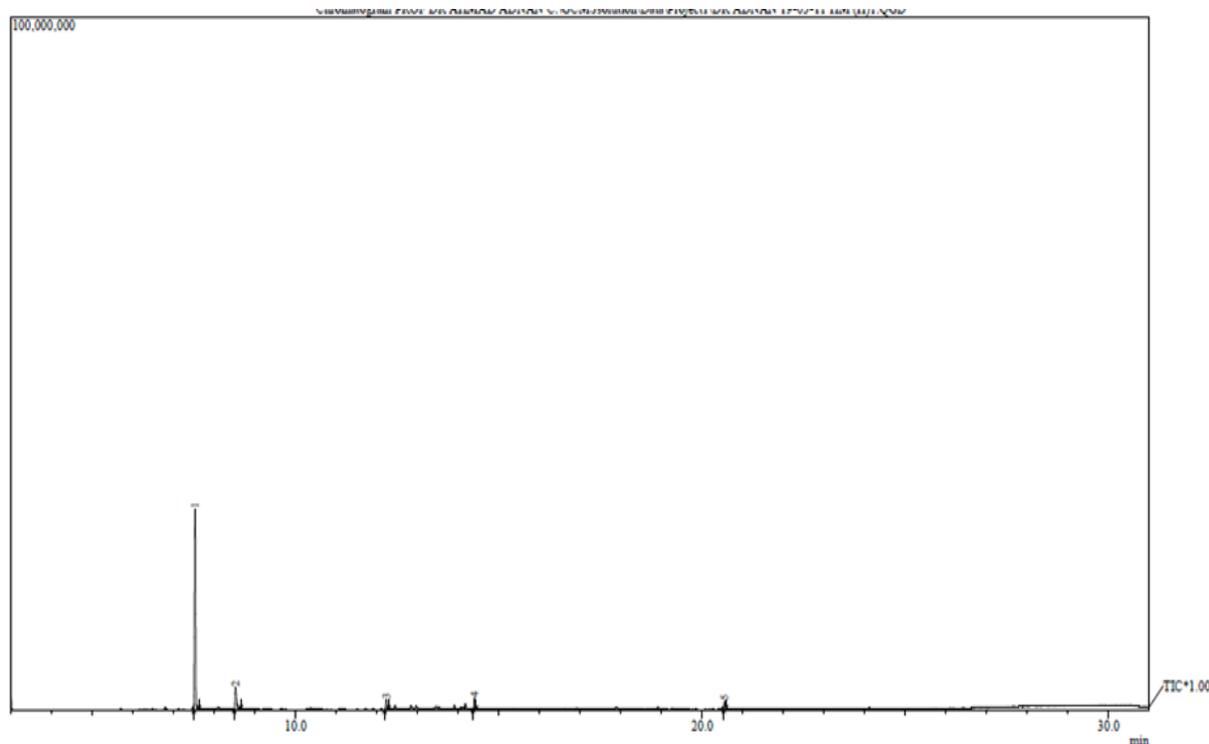
**Figure 2:** *In vivo* wound healing activity wound area v/s number of days.

## Discussion

The components obtained from the essential oil plays an important role in the antibacterial activity as they are tested in both gram positive and gram-negative bacteria. The limonene is the major constituent that plays an important role in the treatment of bacterial infection due to its antibacterial activity. The essential oil of *Citrus* fruits are volatile in nature and plays an important role in the antibacterial activity in order to treat against bacterial infections inside the body, though this power is not too much as that of antimicrobial medicinal agents. The medicinal plants are also present that plays a major role in various disease like garlic plants have a great activity against cardiac disease (Huzaifa et al., 2014).

The presences of phenolic content in the essential oil that has been already discussed they are volatile in nature and plays an important role as anti-inflammatory, anti-diabetic, anti-allergic, and cardio-protective agents. The major activity of included is the anti-inflammatory and anti-carminative activity (Han et al., 2017). There are aldehydes, ketones, tannins and flavonoids are also present in the essential oils of the citrus fruit. The presence of tannins shows the greater activity against any microbial infection as well as they also play an important role in the treatment of wound. The tannins also have anti-diuretic, anti-inflammatory and wound healing potential (Khanbabaee & Van Ree, 2001).

The antibiotics are major concern for our society and throughout of the world because in the world there is a great overuse and misuse of antibiotics that leads towards the resistance. The antibiotic resistance is much more sudden and dangerous for the human consumption because the overuse and even more than normal use of antibiotics there are great chances for the organs like liver, stomach and pancreas to be affected. In recent survey of international journal in the world there are more than 10 million people died due to misuse and overuse and antibiotic resistance. The Asian region is much more effective than the remaining world because of the poly-pharmacy as well as the dispensing of



**Figure 3: The GC/MS chromatogram of kinnow fruit essential oil; showing various constituents.**

**Table 1: Draize's Score results.**

Day 1			Day 4			Day 8		
Redness	Swelling	Pus	Redness	Swelling	Pus	Redness	Swelling	Pus
-	-	-	+	-	-	++	+	-
-	-	-	+	-	-	+	++	-

**Table 2: Components identified according to their Retention time, Area%, Height% and Base m/z.**

Peak	Compound	Time (m)	Area%	Height%	Base m/z ratio
1	D-limonene	7.523	74.27	79.93	68.05
2	Decanal	8.521	13.82	8.66	57.05
3	Copaene	12.226	4.63	4.09	119.05
4	Naphthalene, 1,2 4,5,8, hexahydro- 4,7 dimethyl-1-(1-methylethyl)	14.393	4.07	4.25	161.11
5	Octadecenoic acid	20.551	3.21	3.07	55.10
Total			100	100	

antibiotics without prescription. In the GC-MS analysis of the citrus peels essential oil the different constituents are reported. The constituents are D-limonene, cyclohexene, cyclohexanol, 1 methyl 4 acetate, decanal, deca-aldehyde, capraldehyde, capric acid, dodecanal, hexadecanal, palmitaldehyde, copaene, cubebene, naphthalene, octadecenoic acid, and various methyl esters. The different studies are conducted in case of different oils in order to check their safety, efficacy and other major factors. In this case the lavender oil was used in order to check its efficacy. The study was performed in-vitro basis and the result was not too much abrupt because the lavender oil is toxic and irritant for the hepatic and liver cells of the skin. The tested amount of the oil was 0.25% v/v (Prashar et al., 2004).

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It used the two basic constituents of the citrus essential oil that was obtained from the peels. The *In-vitro* and *In-vivo* clinical studies were performed in order to check the efficacy or toxicity of methyl p-coumarate, methyl ferulate and pulgenone 1, 2 epoxide. The study was conducted on albino rabbits and the dermal and ocular region was used for the examination of the oil efficacy or toxicity. The results indicated that the 96 hours were taken in order to produce the dermal infection whereas the more than 12 hours were taken in order to produce the ocular toxicity testing. The results showed that these major components of the essential oil have no irritancy and toxicity. The other component of essential oil like limonene which is the chief constituent have a great anti-inflammatory activity and meanwhile the components like decanal, hexanal, deodecanal have also a great anti-inflammation activity. The various clinical studies was conducted on the essential oil but there was no evidence for the production of any type of infection.

The different bacterial strains are used to check the antibacterial activity of different therapeutic activity acquiring plants. The bacterial strains included *Staphylococcus aureus*, *klebshiella pneumonia*, *E-coli* and *bacillus cereus*. The studies showed that the *bacillus cereus* is the most species of gram-positive bacteria (Parekh and Chanda). The antibacterial activity of citrus essential oil of different ethanolic, methanolic and tween 80 extract was checked against different bacterial strains which included *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella typhmurium* etc. the different zone of inhibition showed different values at 80ug/ml concentration. The values are 32.67, 12.00, 12.67, and 24.67 respectively (Okunowo et al., 2013). The different concentration of essential oil of citrus fruit is required in order to act against microbial species. The lowest concentration that acts against the microbial species is about 4.29 ppm in order to require acting against *Lactococcus lactis* bacterial strain of minimum inhibitory concentration (Vasek et al., 2015). According to our research, the antibacterial activity of essential oil of citrus mandarin is not too good and significant. But although the essential oils are very significant in order to act against bacterial strain and have antibacterial activity. The strain used in our research may be resistant against essential oil that why it may not show any kind of result.

There are many secondary metabolites present in the citrus essential oil that are very useful in order to act against various bacterial infections. These secondary metabolites included phenolics, flavonoids, alkaloids, fatty acids, tannins and resins. These essential oils play an important role in order to treat bacterial infection that

are contagious to health and also considered harmful for human being (Joshi et al., 2010). The methanolic extract and alcoholic extract used to study the reducing power of the essential oil. The study demonstrated that the ethanolic extract of propolis showed a greater reducing activity as compared to methanolic activity of citrus essential oil (Geckil et al., 2005). The antioxidant activity of *Aloe barbendesis*, *Acacia nilotica*, *Eugenia jambolana* and *Moringa olifera* was checked. The studies revealed that the antioxidant activity of methanolic extract of *Moringa olifera* showed 80% of antioxidant activity whereas the *Moringa olifera* bark has the less reducing power. The change of color of ferric ion into ferrous ions takes place and changed from yellow to bluish green. The reducing power activity of compound is determined by the mean of change in the color of the compound (Zou et al., 2004).

The volatile hydrocarbon of various citrus essential oil was studied. The essential oil was extracted through the process of the hydro-distillation and majorly and most commonly process in order to extract the oil from the peels of the citrus mandarin. The GC-MS analysis used to analyze the oil in order to check the various constituents' proportion in the essential oil. The 78, 90, 67, and 42 % percent of various constituents found in the oil. The limonene is the basic constituent (Njoroge et al., 2006).

The more than 21 species of medicinal plants were studied in order to check the antimicrobial activity. From 21 the 19 species showed the greater medicinal and antimicrobial activity which included fennel, clove, oranges, mandarins, rosemary oil etc. these species are most widely and commonly used throughout the region (Prabuseenivasan et al., 2006). The extraction of essential oil from the peel of the citrus fruit is obtained from the process of cold pressing. The oil is very economical and most widely used throughout the region as it is used in perfume industry, food industry as well as various antimicrobial agents. The study of essential oil is most commonly done by the various processes like GC-MS, flame spectroscopy, flam ionization and many other detection processes. The most common process and most widely used process is the GC-MS which shows accurate values of the constituents of the essential oil obtained from citrus species (Di Vaio et al., 2010). The essential oil of the citrus species is mostly volatile in nature. The antifungal activity of various citrus essential oils was reported. The many researchers are still on the way in order to check the antifungal activity of various essential oils obtained from the citrus species that are volatile in their nature. The antifungal activity is basically used to check and inhibits or kills the growth of specific fungal species that are harmful and injurious to human health (Jing et al., 2014). The extraction process of essential oil from the citrus peels included various methods like hydro-distillation, effleurage, hydro-diffusion, and

steam distillation, cold pressing, microwave assisted process and solvent extraction. The most widely and most commonly used process in order to extract the essential oil is the hydro-distillation method. It extracts the exact and accurate amount of oil with lesser water content. But the major concern of this process is that it is time taking process. The oil extracted from this method is used in aromatherapy, Phyto-therapy, antibacterial and anti-fungal use (Hamid et al., 2011).

The analytical characterization of mandarin essential oil takes place in order to check the new constituents. The mandarin peels obtained in the three different productive regions and for the non-volatile portion the GC-MS process takes place whereas the oxygen containing part of essential oil is observed against GC/MS analysis. The various process of analyzing the essential oil of mandarin caused the identification of three different constituents in the oil. These constituents included Dimethyl-nobiletin, isosinesitin, and dimethyl-tangaritin. The studies and the analysis of essential oil reveals that these three newly discovered constituents also have a greater and major contribution in the oil for various activities like antimicrobial as well as wound healing and anti-fungal (Angelini et al., 2009).

**Conclusion:** In conclusion, GC-MS analysis of essential oil obtained from the peel of citrus mandarin, shows that it composed of various constituents like limonene, decanal, hexanal and others but the limonene is the chief constituent of essential oil of citrus fruit. Kinnow essential oil has a great affinity in order to treat the wound, so it can be use clinically. Meanwhile the essential oil also has minute activity against gram positive and negative bacteria, but it need some investigations. The essential oil of Kinnow is totally non-toxic and non-irritant for the skin. So, there should some measurements in order to use this essential oil in cosmetics.

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