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RESEARCH ARTICLE

Antioxidant and Wound Healing Potential of Essential Oil from *Citrus reticulata* Peel and Its Chemical Characterization. Medicinal Values of Peels Essential Oil

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Abstract: ***Background*:** Fruit peels are considered as waste and contribute to a major proportion of the biomass. They can be a good source of various therapeutic benefits. Peels biomass of citrus fruits is usually considered as garbage. Such peels may have many important and valuable medicinal components with pharmacological activities. *Citrus reticulata,* (Rutaceae family, local name tangerine) is a local seasonal fruit in Pakistan, a very good example of wastage of its peels.

***Objective*:** The study is based on the exploration of a citrus fruit peel derived essential oil, its chemical characterization, identification of various bioactive components and the exploration of pharmacological potentials (antibacterial and wound healing activity).

***Method*:** Essential oil was recovered by hydro-distillation of freshly collected peels. Chemical constituents of oil were determined by gas chromatography-mass spectroscopy (GC-MS) analysis. Antioxidant activities were evaluated by total phenolic contents, total flavonoid content, DPPH scavenging activity and reducing power assay. Antibacterial activity was determined using disc diffusion assay. *In vivo* wound healing potential was determined in rabbits after topical administration of oil. Wound scoring was calculated followed by histological study.

***Results*:** GC-MS analysis showed the presence of various components with the greatest proportion of D-Limonene (89.31%). Total flavonoid and phenolic contents were found to be 14.63 ± 0.95 mg CE/g and 17.03 ± 3.24 mg GAE/g respectively, while DPPH activity was found to be 73.32%. A better antibacterial activity was shown against *E. coli*. *In vivo* studies showed significant reduction in wound diameter in essential oil treatment groups. Further, the essential oil was found non-irritant in draize scoring.

***Conclusion*:** The study concluded that essential oil of this fruit peel might be used for antibacterial and wound healing purposes.

Keywords: Peels, essential oil, anti-bacterial, wound healing, rabbits, Draize scoring.

1. INTRODUCTION

Large quantity of waste is generated each day and contributes to serious management and environmental issues. Half of the total citric fruit mass corresponds to peels, membrane segments, and seeds [1]. Highly valuable citrus peels are usually considered as a waste product and are thrown away. Medicinal plants have great therapeutic potential [2]. The benefits of using peels are easy available and economic improvement for industrial and health use [3].

Currently, food processing waste is a major environmental issue. Peels of fruits and vegetables are considered as the chief by-product obtained through food processing. Peels contain bioactive compounds that have favorable effects on public health [4, 5].

Citrus peels essential oils are medically important, show diverse biological activities due to the presence of flavo-

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noids, terpenes and carotenes. Citrus essential oils are widely used in pharmaceuticals as an antioxidant, carminative, antimicrobial, insect repellent, anti-diabetic and larvicidal [6]. *Citrus reticulata* (tangerine) belongs to genus citrus and family Rutaceae. The fruit has various secondary metabolites *e.g*., coumarins, alkaloids, phenol acids, carotenoids, flavonoids and D-Limonene. D-Limonene exhibit various biological activities including anti-inflammatory, anti-cancer, antioxidant, cardio and neuro-protective effects, which are vital to human health [7, 8].

Medicinal plants are used globally as classical treatments for wound healing. The plant-derived components from medicinal plants have proven to have wound healing properties and many of these provide the new inputs for the pharmaceutical industry. These compounds belong to the families of phenolics, essential oil, terpenoids, tannins, saponins, flavonoids and alkaloids [9].

There is little information about the antibacterial and wound healing activity of *Citrus reticulata* peels essential oil. The current study was designed to determine various chemical components of *Citrus reticulata* peel essential oil. Pharmacological potentials (antimicrobial & antioxidant) were determined followed by *in vivo* wound healing potential using rabbits as experimental animals.

2. MATERIALS AND METHODS

2.1. Sample Collection and Recovery of Essential Oil

Mature fruits of *Citrus reticulata* were collected from the market of Faisalabad. It was identified from University of Agriculture, Faisalabad under the voucher number "IPPP/206". After washing the fruits, peels were removed carefully. Essential oil was recovered by hydro distillation method using 10 Kg of fresh peels and water (20 L) as solvent for 3-4 hours. Pure essential oil was recovered by separating the mixture of aqueous and oil layer using a separating funnel [10] and stored in a sealed vial at 4^{o}C.

2.2. GC-MS Analysis of *Citrus reticulata* Peel Essential Oil

Characterization of essential oil for its chemical constituents was done by the GC-MS analysis. Agilent 7980A gas chromatograph was used. For carrier gas nitrogen was used. Split ratio was 1:50, 70 eV electrical energy, with ionization temperature 250°C. The temperature of the column was maintained from 70°C for 2 minutes to 250°C for 15 minutes at a 10°C/min rate. The injector and detector temperature were 200°C and 250°C, respectively. Chemical constituents were separated on the basis of retention time and mass to charge ratio.[11] Constituents were identified after comparing to NIST library as in our previous study [12].

2.3. *In Vitro* Determination of Antioxidant Potential

2.3.1. Determination of Total Phenolic Content

The total phenolic content of essential oil was determined by Folin-Ciocalteu method [13]. Mixed essential oil (1 ml) with Folin-Ciocalteu (5ml of 10%), and sodium carbonate (4 ml of 20%) were incubated for 1 hour. Absorbance of resulting blue color solution was taken at 765 nm. Quantifications were done w.r.t. gallic acid and standard curve was drawn by different gallic acid concentrations. One ml aliquots of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 & 0.10 mg/ml solutions of gallic acid were mixed with Folin-Ciocalteu (5 ml) reagent that was diluted 10 times with sodium carbonate (4 ml of 20%). After an hour, absorbance was determined at 765 nm. Total contents of phenolic composites in essential oil in gallic acid equivalents (GAE) were measured by the given method.

$$T = \frac{C \times V}{M}$$

Where

T = Total contents of phenolic compounds in mg GAE/g essential oil

C = Gallic acid concentrations (mg/ml) determined using calibration curve

V = Volume (ml) of essential oil

M = Weight (grams) of the essential oil

2.3.2. Total Flavonoids Contents

Total flavonoid contents of the essential oil were evaluated by the method of [14]. Distilled water (2ml) was mixed with essential oil (0.5 ml) and sodium nitrite (0.5 ml of 5%) solution and incubated for 6 min. Aluminum chloride (0.5 ml of 10%) and sodium hydroxide (4%) were added to the mixture and incubated for 6 min. Volume was made up to (5 ml) using methanol, incubated again for 15 minutes and absorbance was taken at 510 nm. By using linear regression curve, total flavonoid contents were represented as catechin equivalents from linear regression curve.

2.3.3. DPPH Scavenging Activity

DPPH radical scavenging activity was peformed by [15] with little alterations. 0.004% DPPH (1 ml) in the methanol solution (freshly prepared) was added to 3 ml of essential oil with different concentrations. The solutions were kept in dark for 30 minutes and absorbance was recorded at 517 nm. Antioxidant potential of ascorbic acid, taken as standard, was also evaluated. Solution without essential oil was taken as control. Experiment was executed in triplicate. Percentage inhibition of the DPPH radical specimens was determined by the following formula:

$$DPPH\ inhibition\ (\%) = \frac{Absorbance\ of\ Blank - Absorbance\ of\ Sample}{Absorbance\ of\ Blank} \times 100$$

2.4. Determination of Antibacterial Activity

Antibacterial activity was determined by disc diffusion method against *Escherichia coli* and *Staphylococcus aureus*, received from Institute of Microbiology, University of Agriculture, Faisalabad. Nutrient agar media (2%) was sterilized and transferred (15 ml) to petri plates. Inoculum (0.01 ml) was added into petri plates. Gentamicin was used as standard which is known to possess antibacterial activity against these bacterial strains [16]. The discs (1 into 3 mm diameter) soaked with peel essential oil were placed on the medium. The plates were kept for 1-2 hours at 40^{o}C and then incubat-

ed for 24 hours at 37°C. The diameter of zones of inhibition was determined by vernier calliper [17].

2.5. *In Vivo* Wound Healing Potential

2.5.1. Test Animals

Rabbits of either sex weighing 1300-1500 g were selected and kept in animal house of Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad. All *in vivo* experiments were conducted after taking approval (letter vide number 2356/ORIC) of Institutional Biosafety and Bioethical Committee. Rabbits were provided routine diet and water.

2.5.2. Animals Grouping and Experiment Protocol

The rabbits were categorized into 4 groups with 5 rabbits per group. The following treatment plan was used for 8 days.

Group 1: Negative control, received no treatment.

Group 2: Positive control, treated with pyodine.

Group 3: Received 0.5 mL essential oil treatment with occlusive dressing.

Group 4: Received 0.5 mL essential oil treatment without dressing.

2.5.3. Wound Induction and Treatment Application

Hairs of dorsal side were fully shaved with clipper. Animals were anesthetized with intramuscular lignocaine injection. Then incisions of 1.5 cm were created on each rabbit with the help of sharp and sterilized blade [18]. Wound size was measured with help of vernier calliper. Wounded area was traced manually and photographed.

2.6. Draize's Scoring for Dermal Irritation Testing

Draize's scoring was recorded in order to assess the dermal toxicity tendency of the essential oil. For this purpose animals were divided into two groups (n=4 in each group). Hairs were clipped dorsally. Both groups received essential oil application with and without occlusive dressing, respectively. Erythema score was calculated following 2 h, 4 h & 6 h post application of essential oil in both groups. The following criteria were used; 0= non-significant changes, 1=Erythema (very slight), 2= Minor red in defined areas, 3= Red in defined areas, 4= Beet red [19].

2.7. Histopathological Studies

Histopathological studies were performed on the 8th day of treatment. Skin samples were collected and fixed in 10 % formalin solution. Then, thick sections of about 5 µm were cut and stained by haemtoxylin and eosin. These sections were observed under light microscope [18, 20].

2.8. Statistical Analysis

The results were expressed in terms of average values ± standard deviation. All data were analyzed statistically with two way ANOVA followed by Tukey's multiple comparison test using Graph pad prism.

3. RESULTS AND DISCUSSION

3.1. GC-MS Analysis of *Citrus reticulata* Peel Essential Oil

GC-MS analysis resulted in the identification of eight compounds with the D-limonene as the major compound. The peaks were identified as Beta-myrcene, D-limonene, Caryophylene, Isopropyl myrstate, Hexadecanoic acid, *etc*. The components have been shown in Table **1**. The results are comparable with another study, which also utilized GC-MS analysis to detect components of essential oil of *Citrus reticulata* [21]. These components are limonene derivatives, the main component of *Citrus reticulata*. D-Limonene has been shown to have antimicrobial characteristics and the oil extract had antimicrobial potential against a wide range of organisms [22]. Various studies reported different percentages of D-Limonene *i.e*. 58.5% and 80.83% [21].

3.2. Antioxidant Activities

Total phenolic content was determined as 17.03 ± 3.24 mg GAE/g by using standard curve. The value of R^2 from the regression curve and equation was 0.9968 and Y= 0.0055x + 0.0987. Studies show that oils from citrus peel with high total phenolic contents have more antioxidant activity. Phenolic compounds have the ability to scavenge radicals. It may be described by their potential to donate hydrogen atom from phenolic hydrogen groups [23].

Table 1. Components identified by GC-MS analysis.

Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area (%)	Base m/z
Beta-Myrcene	$C_{10}H_{16}$	136	6.794	0.88	93.00
D-limonene	$C_{10}H_{16}$	136	7.525	89.31	68.05
Caryophyllene	$C_{15}H_{24}$	204	13.385	0.97	93.05
Iso-propyl Myristate	$C_{17}H_{34}O_2$	270	17.893	1.25	60.00
Hexadecanoic acid	$C_{16}H_{32}O_2$	256	18.915	0.92	74.00
9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	294	20.501	0.79	67.05
9-Octadecenoic acid	$C_{19}H_{36}O_2$	296	20.553	2.66	55.10
1,2-Benzenedicarboxylic acid	$C_{24}H_{38}O_4$	390	24.109	2.45	149.00

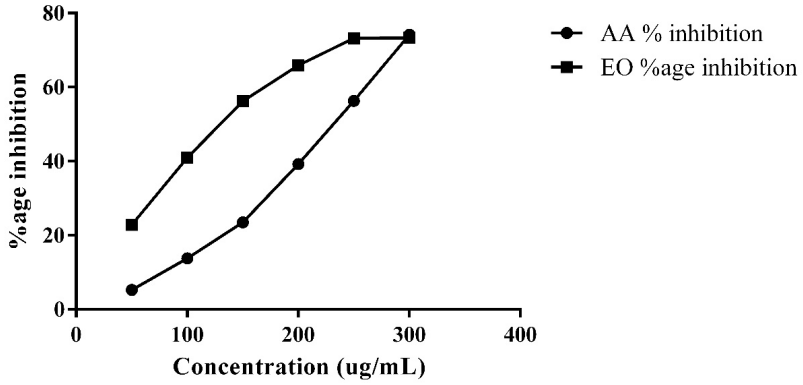

Fig. (1). Percentage inhibition versus concentration of *Citrus reticulata* essential oil in DPPH activity. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 2. Antibacterial activity of *Citrus reticulata* peel essential oil against *E. coli* and *S. aureus.*

Drug	Zone of Inhibition Diameter (cm) for *Staphylococcus aureus*	Zone of Inhibition Diameter (cm) for *Escherichia coli*
Gentamicin	1.3 ±0.42	1.0 ±0.31
100μl E.O.	0.8 ± 0.25	0.6±0.50
75μl E.O.	0.65 ± 0.51	0.3 ± 0.22
50μl E.O.	0.5 ± 0.64	0.2 ± 0.83
25μl E.O.	N/A	N/A

Total flavonoid content was found to be 14.63 ± 0.95 mg CE/g by using standard curve. The value of R^2 and equation from linear regression curve was $R^2 = 0.9835$ and $Y = 0.0038 + 0.0285$. It has natural antioxidant activity due to flavonoids being important phenolic compounds found in essential oil of citrus peels [21, 24]. Comparable results of TPC and TFC have also been reported by other researchers [25].

Percentage inhibition of radical scavenging activity of tangerine peel essential oil was 73.32 % ± 0.01 (Fig. **1**). The secondary metabolites in plants are phenols and known to have many therapeutic uses like antioxidant, anti-carcinogenic, anti-mutagenic, free radical scavenging actions and in many cardiovascular complications as well. Many flavonoids are found to be strong antioxidants, having the ability to scavenge reactive oxygen species due to their phenolic hydroxyl groups [26, 27].

3.3. Antibacterial Activity

Essential oil showed more activity at a concentration of 100 µl against *S. aureus* (gram positive) and *E. coli* (gram negative) with inhibition zone value of 0.6 cm and 0.8 cm respectively, as shown in Table **2**. Oil showed better antibacterial potential against gram positive bacteria. This may be due to periplasmic spaces containing peptidoglycan and the external membrane of gram negative bacteria. The results are comparable with another research which studied the antimicrobial activities of oil obtained from leaves of *C. reticulata* and *C. limon*. Minimum inhibitory concentration of oil of *C. reticulata* leaves against *E. coli* and *B. subtilis* were 900 and 1800 µg/mL respectively [28]. Antibacterial activity is due to the presence of monoterpenes in the oil [29]. Comparable results have also been reported in another study [17].

3.4. Wound Healing Activity

The wound diameter was determined daily throughout the study. The wound area measurement showed that there is significant decrease in all treated groups when compared with negative and standard drug treatment groups (Fig. **2**). Results have been expressed in terms of wound diameter ± SD. A significant decrease was observed in wounds treated with essential oil. Essential oil without occlusive dressing showed better results than with occlusive dressing. There was significantly improved wound contraction and healing in group of essential oil treated animals without occlusive dressing, as shown in Fig. (**3**). Wound healing potential is related to the presence of various flavonoids which has antioxidant activity.

The results are comparable with another study aimed to observe the effect of oral treatment with different citrus peel extracts on wound repair of skin in diabetic patients [30]. In another study, wound healing effect of *Citrus sinensis* in rats was evaluated. They conclude that topical administration of *Citrus sinensis* methanol extract quickens scar development and stimulates wound healing such as wound contraction, fibroplasias, collagen synthesis and epithelization [31]. Another study of topical administration of *Citrus tamurana* extract reported comparable results regarding wound closure and quick formation of scar [32].

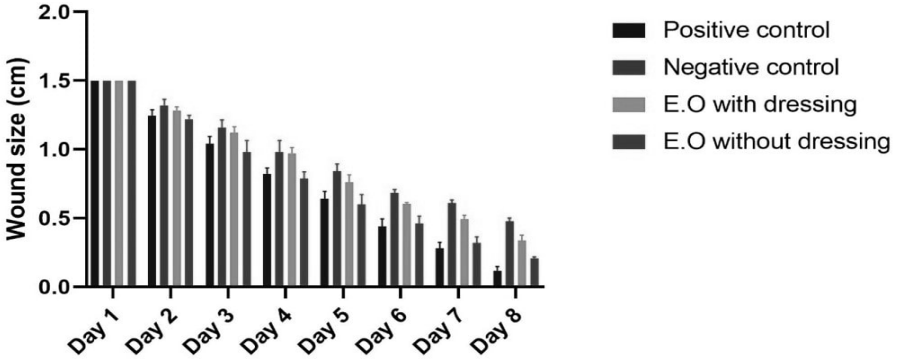

Fig. (2). Effect of various treatments on wound size at different days of application. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

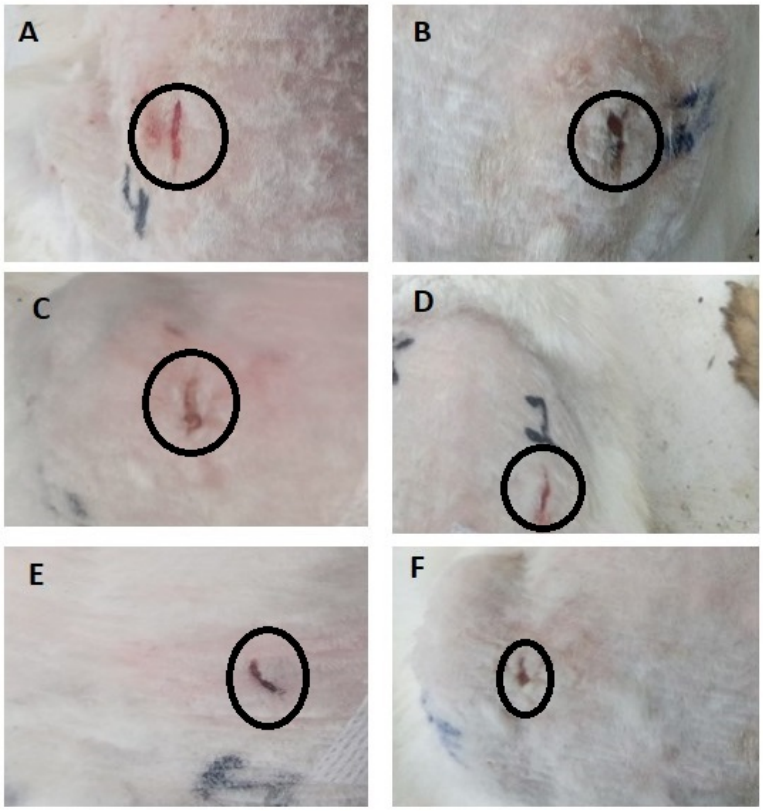

Fig. (3). Healing process of wounds during treatment period by *Citrus reticulata* peel essential oil without occlusive dressing: (**A**): wound at 3rd day of treatment, (**B**): wound at 4th day of treatment, (**C**): wound at 5th day of treatment, (**D**): wound at 6th day of treatment, (**E**): wound at 7th day of treatment, (**F**): wound at 8th day of treatment. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

3.5. Dermal Irritation Testing

Skin is a very important site for protection of other body organs as well as for drug absorption [33].

Draize scoring for essential oil was determined as shown in Fig. (**4**). The values fall in range of 0.17-0.25 at time intervals of 2 h, 4 h and 6 h. Both the treatment showed almost negligible signs of erythema and yielded non-significant results "($p > 0.05$)". The results are comparable to our previous study [34].

3.6. Histological Examination

Histopathological studies showed that healing process was swift in essential oil treated animals as shown in Fig. (**5**). On 8th day of treatment, smooth epidermis layer was present in pyodine and essential oil treatment groups, while in group one epidermis layer was broken (red arrows) and more inflammatory cells were present (see yellow arrow). The results are comparable with our previous study [35].

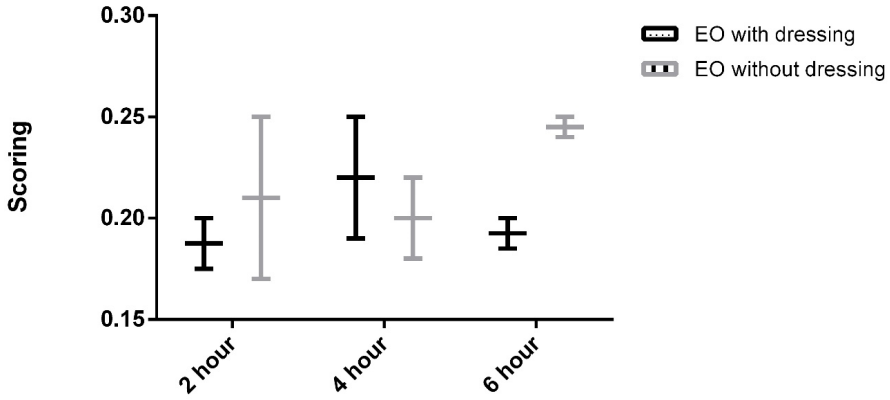

Fig. (4). Dermal toxicity studies of *Citrus reticulata* peel essential oil at 2, 4 and 6 hours following treatment application. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

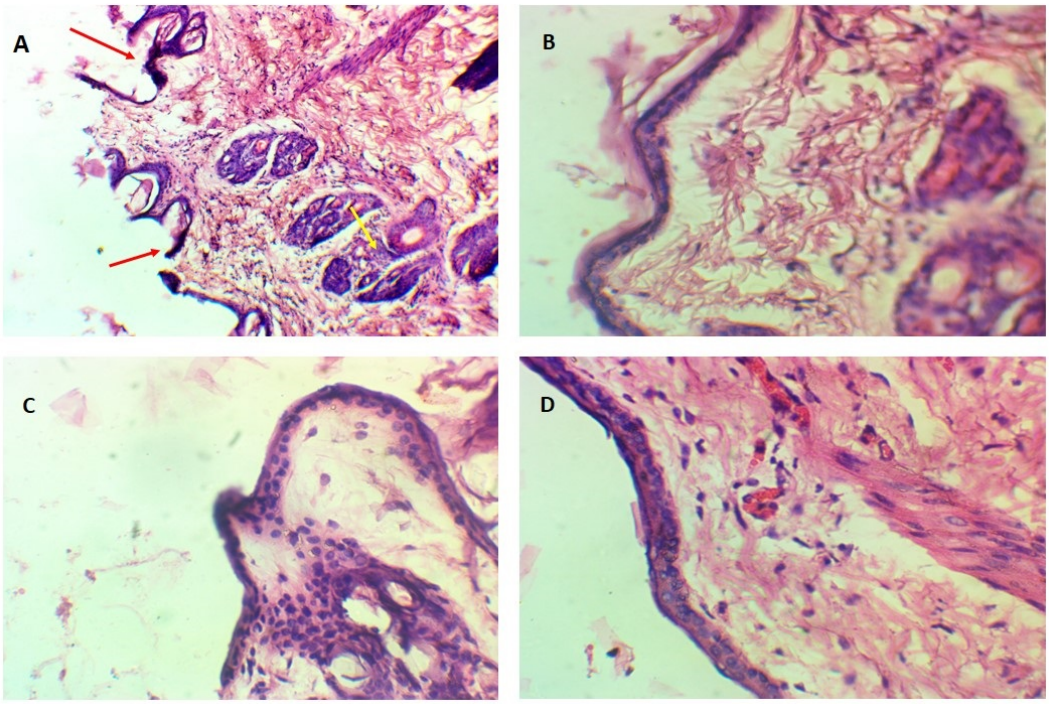

Fig. (5). Histological studies of skin biopsy from incision wound area (**A**) Photomicrograph of no treatment group (**B**) Standard drug treatment group (**C**) Essential oil treatment with dressing (**D**) Essential oil treatment without dressing. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

CONCLUSION

The present study indicated that tangerine peel essential oil shows antibacterial and antioxidant potentials. Further, tangerine peel oil improved the wound healing process in experimental model. That is why it can be utilized in the management of wound healing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All *in vivo* experiments were conducted after taking approval for animal handling (letter vide number 2356/ORIC) from Institutional Biosafety and Bioethical Committee, University of Agriculture, Faisalabad, Pakistan.

HUMAN AND ANIMAL RIGHTS

The study was not on human subjects. All animal experiments were carried out after approval of Institutional Bioethics Committee. The *in vivo* experiments were carried out following the guidelines of National Biosafety Committee 2005 & Punjab Biosafety rules 2014.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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