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Phytochemical investigation and *In-Vitro* evaluation of antibacterial and antioxidant activities of grapefruit (*Citrus paradisi*) peel essential oils

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Abstract

Peels are usually considered as waste and thrown away. Many fruits and vegetable peels have therapeutic potential. Medicinal plants are now a days used largely in the treatment and prevention of different diseases as these drugs are cheaper and have very less side effects than the synthetic drugs. The objective of this study was to investigate the grapefruit peel's essential oil for its qualitative phytochemical analysis, antioxidant activity, antibacterial activity against different strains, *in-vivo* wound healing activity, acute skin irritation studies and GC/MS analysis of the essential oil for chemical composition. Phytochemical investigation revealed the presence of alkaloids, flavonoids and saponins qualitatively. Antioxidant activity was evaluated through total phenolic contents, along with total flavonoid contents, DPPH scavenging assay and reducing power assay. Essential oil shows highest total phenolic contents and lowest reducing power activity. Antibacterial activity was evaluated through disc diffusion method and its shows minimal activity against tested strains. *In-vivo* wound healing activity was recorded in rabbits after giving surgical incision on dorsal side of the animals. Grapefruit essential oil shows good wound healing activities but it shows less activity than synthetic drug pyodine. Acute skin irritation was observed 24h prior to the application of essential oil which shows non-significant results. GC/MS analysis was conducted on the grapefruit peel essential oil to find active constituents.

Keywords

Antibacterial activity
Antioxidant activity
Essential oil
Grapefruit
Wound healing

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Introduction

Fruits and vegetables are important part of human diet due to their delicious taste and flavor. Besides this, fruits and vegetables contain phytochemicals which are considered good against several chronic diseases. Fruits are used as such for their nutritive value and bioactive compounds. Fruits and vegetable by products which are formed in great quantities on industrial level are mostly thrown in waste. Different types of compounds with varying activities like antimicrobial, anti-proliferative, antioxidant, anti-inflammatory, etc. have been isolated from different peels (Parashar et al., 2014). The main advantage of using fruit peels for these compounds include cheaper, easy availability, and better absorption capacities (Pathak et al., 2015). With increasing world population, problems like antimicrobial resistance, food security, and environmental pollution are in rising (Edet, 2017). In developing countries infectious diseases are major cause of mortality. Use of regular antibiotics leads to drug resistance and have more side effects than natural antibiotics and these are much costly than natural antibiotics. (Ayepola & Adeniyi, 2008).

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Grape fruit (*Citrus paradisi)* belongs to Rutaceae family, genus Citrus, and taxa of flowering plants and cultivated in tropical and subtropical regions worldwide (Okunowo et al., 2013). World citrus production reached 135 million tons cultivated over 9.6 million hectares, out of which share of grapefruit is 8.4 million tons as per recent studies in 2013. Large quantities of citrus peel were generated during processing and juice making at industrial levels. Juice produced from grapefruit is around half of the total weight of the fruit, generating high amounts of waste. A total of 36 million tons citrus waste is produced annually. This is one of the major agro waste produced and may contribute towards health and environmental issues (Okunowo et al., 2013).

Essential oil from peel of different fruits and vegetables shows very important medicinal activities and shows a variety of biological effects due to presence of flavonoids, carotenes, terpenes, and coumarins. Pharmacologically essential oils from different sources are used as antimicrobials, antioxidant, anti-diabetic, insect repellent, larvicidal, carminative, and antiviral (Javed et al., 2014). *Citrus paradisi* peel essential oil shows a variety of antibacterial effect against different strains of bacteria. It inhibited the growth of, *Staphylococcus aureus*, *Escherichia coli*, *Lactococcus lactis* subsp. *diacetylactis*, *Lactococcus lactis, Leuconostoc mesenteroides* subsp. *dextranicum* and *Lactobacillus plantarum. Lactococcus lactis* subsp. *Lactis* is most sensitive out of these strains according to the minimum inhibitory concentration (Vasek et al., 2015).

Essential oil from grapefruit peel is fundamental product of this fruit mainly extracted by distillation or solvent extraction methods. This is complex mixture of volatile and non-volatile compounds. It shows natural antioxidant activity because of flavonoids, glycosides which are important phenolic compounds found in essential oils of citrus peel (Javed et al., 2014). Chemical characterization of different citrus peel essential oils by GC/MS shows that highest amount of limonene is present in grapefruit essential oil 89.84% then followed by other species, Malta 88.57%, Mausami 87.84%, Mandarin 87.45%. Other chemical compounds identified include limonene oxide, α-terpineol, carveol, carvone, spathulenol, eugenol, and caryophyllene oxide. Second major component identified is α-terpineol with highest amount present in mandarin (Javed et al., 2014).

The phytochemical content, antimicrobial activity, antioxidant activity, characterization of essential oils from peel of grapefruit were evaluated in different regions of the world but there is little information available about grapefruit Essential Oils cultivated in Pakistan.

Materials and Methods

Collection of sample and Recovery of Oil: Mature fruits of *Citrus paradisi* was collected from local market of Faisalabad, and was identified by experts from institute of horticulture, University of Agriculture, Faisalabad. After washing, grapefruits were peeled and stored at ambient temperature for further processing. Essential oil from grapefruit peels was extracted by hydro distillation method and two layers was separated. Pure essential oil obtained was stored at 4˚C in dark brown sealed vials (Javed et al., 2014). Essential oil was analyzed for the presence of different phytochemical contents (saponins, alkaloids, steroids, anthraquinones, proteins, coumarins, anthocyanins, phenols, tannins, amino acids, terpenoids, cardiac glycosides, resins and fixed oils) with methods as described below (Kumari, 2014).

Tests for coumarins: A few drops of ammonia was added to a filter paper and added a drop of essential oil to this and checkde for fluorescence.

Mayer's test for alkaloids: Essential oil was treated with Mayer's reagent and formation of yellow precipitate indicated presence of alkaloids.

Ninhydrin test for amino acids: Essential oil and 0.25% ninhydrin reagent was boiled and checked for blue color which was indicative of amino acids.

Biuret test for proteins: Essential oil was treated with 1ml of 10% NaOH and added a drop of 0.7% $CuSO_4$ and observed for purple-violet color.

Tests for phenols: Ferric chloride test was used to test the phenols in essential oil.

Ferric chloride test for flavonoids: Essential oil was treated with $FeCl_3$ for blackish color. Other tests included stain test for fixed oils and fats, acetone-H_2O for resins, salkowski test for terpenoids, foam test for saponins, Keller-Killani test for cardiac glycosides, and Borntrager's test for anthroquinones.

Characterization of essential oil: Citrus essential oil was characterized for their chemical composition by using GC/MS with helium as carrier gas, split ratio 1:1000, the chemical constituents was identified by their retention time and was compared with standards (Javed et al., 2014).

Antioxidant activity: Essential oil antioxidant activity from grapefruit was tested *in-vitro* by, total phenolic contents (TPC), total flavonoids contents (TFC), DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity and by evaluating reducing powers.

Determination of TPC: Total phenolic content of grapefruit peel essential oil was determined by Folin-Ciocalteu method (Sultana et al., 2009).

Determination of TFC: Total flavonoids content was determined by previously described spectrophotometric method (Sultana et al., 2009).

DPPH scavenging activity: Freshly prepared solution of DPPH was added to grapefruit essential oil and kept in dark for half hour and checked for absorbance. Low absorbance indicated higher scavenging activity (Sultana et al., 2009).

Reducing power determination: Determination of reducing power was done by previously described method (Sultana et al., 2009).

***In vitro* Evaluation**

Antibacterial activity: *In vitro* micro dilution method was used to determine antibacterial activity. Antibacterial activity will be tested on two G +ve and two G –ve strains.

***In vivo* Evaluations**

Wound Healing Activity: Wound healing activity was evaluated by topical application of grapefruit essential oil on albino rabbits to check whether it is effective or not effective (Fahimi et al., 2015). Rabbits was used of either sex and purchased from local market of Faisalabad. Rabbits was separated from each other and kept in separate cages and provided normal rabbit diet. Six albino rabbits were selected, and three wounds of uniform size (2 cm) was created on each rabbit. One for control and was unattended, second wound was treated with grapefruit essential oil and applied by occlusive dressing and third one was treated with standard healing cream (Fahimi et al., 2015).

Skin Irritation Testing: Skin irritation was analyzed by applying essential oil from grapefruit peel on skin of rabbits by dividing them into two groups as showed in Table 1. Dermal toxicity and other signs of irritation was assessed by draize's scoring method (Raza et al., 2016).

Results

Phytochemical Screening: Phytochemical screening was conducted to examine the presence of phytochemical constituents. The grapefruit peel was analyzed for alkaloids, tannins, saponins, phenols and flavonoids. The phytochemical parameters of grape fruit peel essential oil analyzed has been shown in Table 2.

Antioxidant activity: TPC, TFC, DPPH and reducing power assay were recorded to access the antioxidant activity. Results of these activities were shown in Table 3.

Figure 1 shows the values of four antioxidant parameters total phenolic contents, total flavonoid contents, DPPH scavenging assay, and reducing power assay. According to this Fig. grapefruit essential oil shows highest total phenolic contents which are 120.78 mgGAE/g, followed by DPPH scavenging assay of 82.37% and total flavonoid contents of 75.57 mgCE/g. While it shows lowest reducing power assay of 0.81%. Significant difference was observed between these four parameters.

Antibacterial Activity: The objective of this study was to check the antibacterial activity of grapefruit peel essential oil 50 mg against Gram negative and Gram positive strains. Results of this activity showed in the Figure 2. Grapefruit essential oil shows very low antibacterial activity against the tested bacterial strains. No significant difference was observed between the results of all the bacterial strains against grapefruit essential oil. Disc diffusion method was used in this study to examine the antibacterial activity of four bacterial strains: two Gram +ve and two Gram –ve against essential oil. It shows very low antibacterial activity that could not be measured through scale.

Table 1: Groups for skin irritation analysis by applying essential oil from grapefruit peel on skin of rabbits

Group -1	Group -2
Control 3 Rabbits	Treated group with essential oil 3 Rabbits

Table 2: Phytochemical characters present in grapefruit peel essential oil

Serial No.	Constituents	Grape fruit Essential Oil
1	Alkaloids	+
2	Tannins	-
3	Saponins	+
4	Phenols	-
5	Flavonoids	+

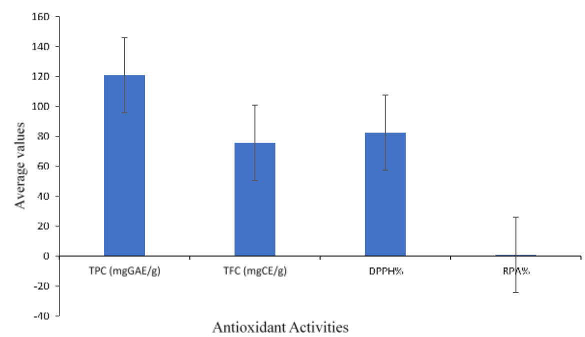


Figure 1: Antioxidant Activities of Grapefruit peel Essential Oil.

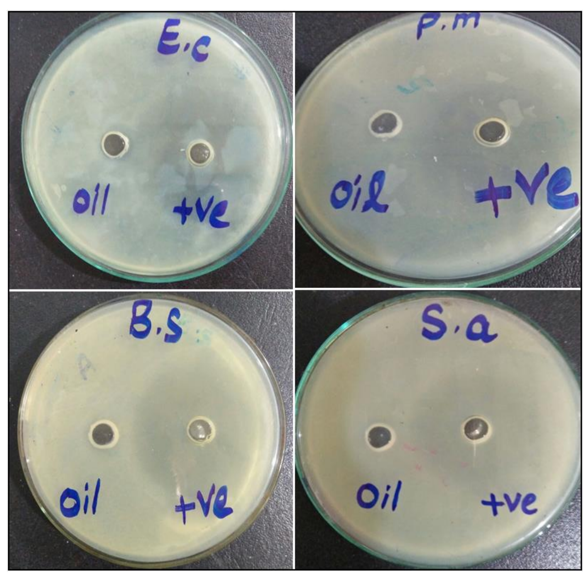
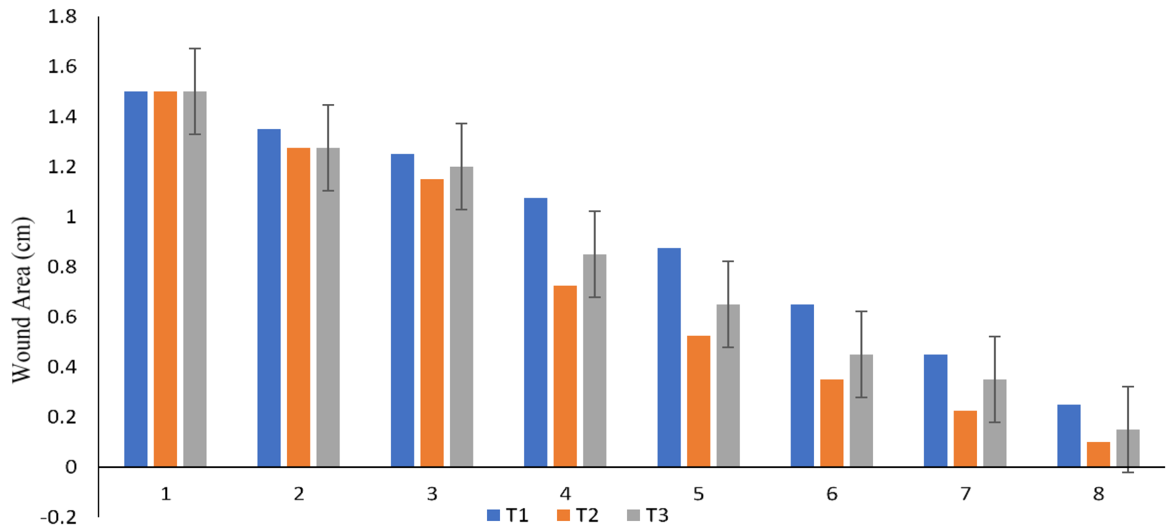


Figure 2: Showing zone of inhibitions of grapefruit peel essential oil against two G +ve and two G –ve bacterial strains.



T1=Control, T2=Pyodine, T3=Grapefruit peel Essential Oil

Figure 3: ***In Vivo*** **Wound Healing Activity.**

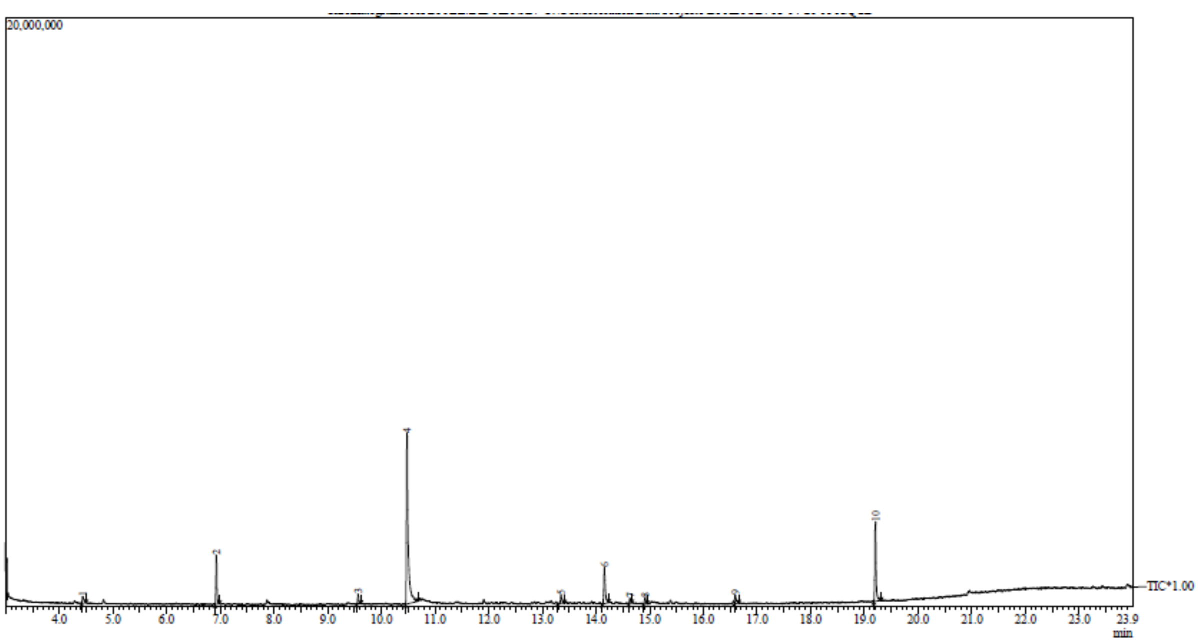


Figure 4: The GC/MS chromatogram of grapefruit essential oil. Showing various components.

Table 3: Antioxidant activities of grapefruit peel Essential Oil (mean ± SEM)

Solvent	Total Phenolic content (mgGAE/g)	Total Flavonoid content (mgCE/g)	DPPH radical scavenging activity (%)	Reducing power assay (RPA %)
Grape fruit peel Essential oil	120.78 ± 1.29	75.57 ± 0.83	82.37 ± 0.78	0.81 ± 0.02

Table 4: Effect of grapefruit peel essential oil on rabbit's skin

Treatments	Mean ± S.E
(T_1, Control)	0.00 ± 0.00 A
(T_2, Grape fruit peel essential oil)	0.75 ± 0.35 A

Means in column with the same letters are not significantly different at 5% level of DMR's Test.

Table 5: Components identified in grapefruit essential oil according to their Retention time, Area%, height% and Base m/z

Peak #	Compound	R. Time mints	Area %	Height %	Base m/z Ratio
1	unidentified	4.438	2.09	1.74	90.95
2	D-Limonene	6.928	9.25	2.92	68.00
3	1,6-Octadien-3-ol	9.568	1.79	2.57	42.95
4	Triacetin	10.479	53.49	44.86	43.00
5	Cyclopentane acetic acid	13.350	1.91	2.28	82.95
6	Octanal	14.157	9.21	9.43	129.00
7	Isopropyl-Myristate	14.635	1.05	1.36	43.05
8	Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	14.917	1.60	1.77	243.00
9	9-Octadecenoic- acid	16.595	2.30	2.22	55.00
10	Di-n-octyl -phthalate	19.206	17.31	20.83	148.95
Total			100	100	

***In Vivo* Wound healing Activity:** Figure 3 shows the effect of essential oil and pyodine on wound healing activity in rabbits with incision wound. The rabbits shows significant increase in wound healing which were treated with pyodine and essential oil as compared to control. Pyodine shows better results than essential oil while essential oil shows good wound healing activity than control animal.

Acute Skin Irritation Studies: The effect of grapefruit peel essential oil on skin of rabbits was recorded after 24 hours. Data revealed very less irritation with essential oil as described in Table 4.

GC/MS Analysis: GC/MS analysis was conducted on the grapefruit peel essential oil. The peaks formed in the chromatogram were identified and compared with the database of spectra of known components (NIST-147) stored in the GC-MS library. Details of components found in grapefruit essential oil was given in the Table 5 of grapefruit peel essential oil. Phytochemical by GC-MS analysis of the studied grapefruit peel essential oil revealed the presence of different compounds like limonene, triacetin compounds etc. The grapefruit peel essential oil examination ten peaks were observed indicating presence of ten compounds (Figure 4).

Discussion

Pure essential oil was used to analyze the presence of different phytochemical constituents. Phytochemical tests for Saponins, flavonoids, alkaloids, phenols and tannins were performed according to the standard procedures. Phytochemical investigation shows positive results for flavonoids Saponins and alkaloids while negative for phenols and tannins. Phytochemicals present in plants contribute towards their flavor and color and are non-nutritional, secondary metabolites of plants. They produce antioxidants which gives protection against different diseases, like garlic and onions have alkyl sulfide, and carotenoids can be obtained from fruits vegetables and carrots. Phytochemicals present in plants can save plant itself and humans from ailments by working with nutrients and nutritional fibers as they are biologically active compounds. Medicinal plants are plants which have some kind of substances in their parts which can be used as therapeutic agent or as beneficial purpose or used as precursor in drug production. These plant parts are used in crude or in purified form. Garlic plant shows different biologically active phytochemicals like alkaloids, flavonoids, tannins, Saponins, and cardiac glycosides on phytochemical investigation (Huzaifa et al., 2014).

Antioxidant activity is highest in grapefruit peel essential oil due higher TPC than other antioxidant values. Grapefruit peel essential oil have various explore able secondary metabolites which are pharmacologically active. These results shows that grapefruit peel can serve as source of natural antioxidant and essential oil can be used in fruit industry as preservative agent. For the production of energy for various biological activities oxidation is an important process. On the other hand free radicals and ROS which are continuously produced in human body, may cause harmful effects to tissues and even death of cells. The free radical role has been documented in various diseases including cancer, ageing, cardiovascular diseases etc (Shukla et al., 2009).

Grapefruit essential oil shows highest ability of extraction of total phenolic content. TPC mean value was 120.78 mg GAE/g observed in grapefruit essential oil. In one of the similar studies conducted on different citrus fruits lemon, grapefruit, and mandarin shows the presence of TPC in comparison. This study shows TPC value of grapefruit peel extract was 59.68 ug GAE/g while TPC for lemon is 142.37 ug GAE/g and mandarin shows lowest TPC of 52.35 ugGAE/g (Diab, 2016). Grapefruit peel essential oil shows good TFC mean value of 75.57 mg CE/g. Flavonoids produce good antioxidant activity and produce a noticeable effect. Rogerio et al. (2003) reported that flavonoids have different beneficial actions including anti proliferative action on T-cells which can interfere with lymphocyte activation and IL-5 production during *Toxocara cannis* infection.

Grapefruit peel essential oil shows DPPH scavenging assay mean percentage of 82.37%. DPPH is a stable organic and free radical with deep violet color and gives absorption at 515 to 528 nm. With increase in concentration of phenolic compounds and degree of hydroxylation it loses its chromophores and eventually became yellowish in color. Ayoola et al. (2008) investigated the antioxidant activity of different plants like *Magnifera indica*, *Carica papaya*, *Psidium guajva* and *Vermonia amygdalina*. They used the DPPH radical inhibition activity. At different concentrations (0.05, 0.1 and 0.5 mg/ml) *Psidium gujava* shows the good DPPH radical activity. At 1, 2 and 5mg/ml concentration maximum activity was shown by *Magnifera indica*.

Grapefruit essential oil shows lowest reducing power assay than TPC, TFC, and DPPH scavenging assay. Its mean value was 0.81% in grapefruit essential oil. This assay was used to determine the antioxidant potential of different plant materials and extracts and nutritional supplement. In this procedure reduction of ferric ions into ferrous ions occurred as well as its color changed from yellow to bluish green color. The intensity of color change determine the reducing power of the compounds (Zou et al., 2004).

Secondary metabolites of plant origin shows varying degree of antibacterial activity. This activity is useful for different contagious ailments management with no or very less side effects. This antibacterial activity is due to the presence of different compounds like alkaloids, phenolics, flavonoids, fatty acids, resins, and tannins etc. that can cause physiological action against bacteria (Joshi et al., 2013). In the present study grapefruit essential oil was used to study the antibacterial activity against four bacterial strains two gram positive strains *Staph. Aureus* and *Bacillus subtillus* and two gram negative strains *E. coli* and *Pasterulla multocida*. Essential oil was tested by using Disc Diffusion method. Our preliminary research shows that the essential oil was not effective against the tested strains. Results shown by grapefruit essential oil were non-significant. Although grapefruit essential oil has good antibacterial activity as described in literature. In this case the strains used were probably resistant enough that they show almost no effect against grapefruit essential oil.

Grapefruit essential oil shows good wound healing activity. In this present study we checked the wound healing activity of grapefruit peel essential oil on rabbits by giving surgical incision. The best results are shown by commercially available pyodine while grapefruit shows wound healing better than the control wounds. Fahimi et al. (2015) studied the burn wound healing activity of traditionally used poly herbal products in rat model. They found a significant improvement in wound healing percentage PHC (poly herbal cream) treated rats then the other tested medicines. PHC shows 87% while 32.0 % for control 57% for cream base, 70.8% for SS group. Ramasamy & Bhaskar (2016) studied the wound healing activity of *Citrus sinensis* extracts in albino rats model. They checked the efficacy of methanolic extracts of *Citrus sinensis* in 5% in 10% w/w. The biochemical and histological studies reveled that collagen synthesis was increased at wound site. Increased protein and DNA amount at site suggesting more cellular growth. Extract treated wounds heal much faster than the control groups and wound contraction was significantly better than the control group. Wet and dry granulation tissue also increased at wound site of tested animals. Raza et al. (2016) studied the *in vitro* and *in vivo* toxicity effects of different compounds: methy *p*-coumarate, methyl ferulate and pulgenone 1, 2-epoxide. According to this study they performed dermal and ocular toxicological studies on healthy albino rabbits. Dermal toxicity for 96 h and ocular testing for 12 h post application. Results showed their non-irritant behavior. Okunowo et al. (2013) studied the antimicrobial activities and chemical constituents of grapefruit essential oil through GC/MS analysis. The components of oil shown by GC/MS are higher in D-limonene 75.05% then followed by β-myrene, α-pinene, caryophylline, octanal and some minor compounds. These findings are related to our study as D-limonene and octanal is identified in grapefruit peel essential oil in the present study with some other compounds. Javed et al. (2014) investigated the phytoconstituents, antioxidant activity and antimicrobial activity of five citrus plants including *C. paradisi*, *C. sinensis*, *C. reticulate*, *C. sinensis* var mousami, and *C. reticulate*. GC/MS analysis revealed that limonene was the key element found in citrus peel essential oil. Grapefruit shows highest amount of limonene which was 89.9% and followed by Malta, Mousami, Mandarin and tangerine. Other chemical constituents identified were limonene oxide, carvone, α-terpineol, carveol, spathulenol, eugenol, and caryophyllene oxide. The second major component was the *α*-Terpineol (12.55%) from all five *Citrus* essential oils with Mandarin peel oil containing highest of it.

Conclusion: Phytochemical analysis of essential oil revealed the presence of compounds like Saponins, alkaloids, and phenols are important phytochemicals found qualitatively in grapefruit essential oil. Grapefruit essential oil shows good wound healing activity and can be explored for its clinical potential. It inhibits the growth of gram positive and gram negative bacterial strains. It needs to be investigated in composition with other potent compounds for wound healing. Grapefruit essential oil shows no toxic effect on skin. So it should be investigated for its use in cosmetics and other skin applications.

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