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Phytochemical investigation and assessment of antimicrobial, anti-inflammatory and antioxidant activities of Sudanese *Citrus paradisi* peel extract

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The peel of grape fruit is used in traditional medicine for the treatment of several diseases. The objective of this study was to evaluate the antimicrobial, anti-inflammatory and antioxidant activities of peel extract from *Citrus paradisi.* Qualitative phytochemical screening of peel indicates the presence of alkaloids, flavonoids, sterols, triterpenes, tannins, saponins, coumarins, glycosides, reducing sugars, anthraquinones, lignin and carbohydrates. Extract was assessed for their effectiveness against four bacterial strains including both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria as well as fungal species (*Candida albicans* and *Aspergillus niger*) using disc diffusion method. Antibacterial effects of peel extract showed different degrees of inhibition profiles against tested bacteria with inhibition zone that ranged from 13 to 17 mm. Peel extract showed high antifungal activity against *A. niger* (24 mm) and *C. albicans* (22 mm). The *C. paradisi* peel showed high anti-inflammatory activity with inhibition percentage 77.57%. The antioxidant potential of extract was determined on the basis of their scavenging activity of the 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical stability. The peel extract showed DPPH scavenging activity (55%) and vitamin C content was 23.08 mg/kg by HPLC. The quantitative analysis of chemical composition of the extract was determined by Gas Chromatography–Mass Spectrometry (GC-MS). The results showed high amounts of Naringenin (28.09%). The peel extract of *C. paradisi* is a natural source of chemical constituents which have medicinal uses in treating many disease.

Key words: *Citrus paradisi* peel, antimicrobial activity, phytochemical screening, vitamin C, Naringenin.

INTRODUCTION

Citrus is one of the most consumed fruits in the world and contain a high amount of useful by-products which

include essential oils. It is mostly consumed fresh or used as raw materials for juice and wine. The second largest world produced citrus species is grape fruit, with an average of more than 60 million annual production. Grapefruit (*Citrus paradisi*) belongs to the family Rutaceae. The yield of grapefruit and oranges juice is about half of the fruit weight thereby generating a very high amount of waste annually (Okunowo et al., 2013). It has been used as a folk medicine in many countries as antibacterial, anti-fungal, anti- inflammatory, antimicrobial, antioxidant, antiviral, astringent, and preservative. It has also been used for cancer prevention, cellular regeneration, lowering of cholesterol, cleansing, detoxification, heart health maintenance, Lupus nephritis, rheumatoid arthritis and weight loss.

In Sudan, *C .paradisi* fruit peel is used for treatment of malaria, gastro protective and antiulcer and this action is attributed to the antioxidant activity of citrus flavonoids found in grapefruit such as naringenin. The major flavonoid exhibited the potent antibacterial and anti *helicobacter pylori* activity *in vitro* and was also recently implicated in cytoprotection against injury induced by algal toxins in isolated hepatocytes. Moreover naringenin showed gastro protective activity due to increased expression of prostaglandins biosynthesis. Furthermore, it was shown to exhibit anticancer activity against human breast cancers. Therapeutic efficacy of citrus fruits such as red grapes and grapefruits is emphasized by the fact that they contain different classes of polyphenolic flavonoids, which were shown to inhibit platelet aggregation thus decreasing the risk of coronary thrombosis and myocardial infarction (Gupta et al., 2011).

An important component of *C. paradisi* is vitamin C. It is an essential micronutrient for humans, with pleiotropic functions related to its ability to donate electrons and a potent antioxidant and a cofactor for a family of biosynthetic and gene regulatory enzymes. Vitamin C contributes to immune defense by supporting various cellular functions of both the innate and adaptive immune system (Traber and Stevens, 2011). It supports epithelial barrier function against pathogens and promotes the oxidant scavenging activity of the skin, thereby potentially protecting against environmental oxidative stress. Vitamin C deficiency results in impaired immunity and higher susceptibility to infections. Furthermore, supplementation with vitamin C appears to be able to both prevent and treat respiratory and systemic infections. Prophylactic prevention of infection requires dietary vitamin C intakes that provide at least adequate, if not saturating plasma levels (that is 100 to 200 mg/day), which optimize cell and tissue levels (Carr and Maggini, 2017).

In the present paper, results on phytochemical screening of the 96% ethanolic extract of *C. paradisi* fruits peel and assessment of it's antimicrobial, anti-inflammatory and antioxidant activities in addition to determination of vitamin C and naringenin content by HPLC and GC-MS analysis was reported.

MATERIAL AND METHODS

Preparation of peel extract

The peel of fresh fruit of *C. paradisi* was air dried and ground to powder using a pestle and mortar. A hundred grams of powder was extracted with 96% ethanol at room temperature for 72 h. The extract was first filtered through Whatman number 4 filter paper. After filtration, the extract was vacuum concentrated.

Phytochemical analysis

Qualitative preliminary phytochemical analysis was performed initially with different chemical reagents to detect the nature of phytoconstituents and their presence in peel. The presence of sterols/terpenes, flavonoids, tannins, alkaloids, lignins, saponins and coumarins were evaluated by standard qualitative methods of Trease and Evans (Trease and Evans, 2002).

Antimicrobial activity

Test microorganisms

Six microorganisms were used in this study, consisting of four bacterial strains and two fungal strains. Two were Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*), while the other two were Gram negative (*Escherichia coli*, and *Salmonella typhi*). The two fungal strains used were *Candida albicans*, *Aspergillus niger*. Standard strains of microorganism used in this study were obtained from Medicinal and Aromatic Institute of Research, National Research Center, Khartoum.

Culture media

Mueller Hinton agar

Thirty eight grams of the powder of Mueller Hinton agar were weighed, dissolved in 1 liter of distilled water and allowed to soak for 10 min. The medium was placed in water bath to dissolve, swirled to mix and sterilized by autoclaving for 15 min at 121°C, cooled to 47°C mixed well then poured into sterile Petri dishes.

Sabouraud Dextrose agar

Sixty two grams of the powdered Sabouraud dextrose agar, was weighed, dispersed in 1 L water and allowed to soak for 10 min, swirled to mix then sterilized by autoclaving for 15 min at 121°C, cooled to 47°C, mixed well and then poured in to sterile Petri dishes.

Antibacterial assay

The disc-diffusion assay (Kil et al., 2009) with some modifications

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was employed to investigate the inhibition of bacterial growth by peel extract. Extract solution (20 mg/ml) was prepared by diluting with dimethyl sulfoxide (DMSO) 30%. Base plates were prepared by pouring 10 ml Mueller-Hinton (MH) agar into sterile Petri dishes. About 0.1 ml of the standardized bacterial stock suspension 10^8 to 10^9 C.F.U/ ml were streaked on Mueller Hinton agar medium plates using sterile cotton swab. Sterilized filter paper disc (6 mm diameter) were soaked in the prepared extracts, and then were placed on surface of the test bacteria plates. The plates were incubated for 24 h and the diameters of the inhibition zones were measured.

Antifungal assay

The same method described for bacteria was employed to antifungal activity, Sabouraud Dextrose Agar was used. The inoculated medium was incubated at 25˚C for two days for the *Candida albicans* and three days for *Aspergillus niger*.

Anti-inflammatory activity

Inhibition of albumin denaturation

Inhibition of protein denaturation was evaluated by the method of (Sakat et al (2010)) with slight modification: 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract with different concentrations. This mixture was kept at room temperature for 10 min, followed by heating at 51°C for 20 min. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Standard (Aspirin) was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using: % Inhibition = (A0 – A1) /A0 *100
Where, A0 is the absorbance of the control and A1 is the absorbance of the sample.

Antioxidant activity

The DPPH radical scavenging was determined according to the method of Shimada et al (1992), with some modification. In 96-wells plate, the test samples were allowed to react with 2,2, Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour .The concentration of DPPH was kept as (300 µl). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using Shimadzu UV spectrophotometer double beam. Percentage radical scavenging activity by samples was determined in comparison with DMSO treated control group. Ascorbic acid was used as standard. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = (A0 - A1)/A0 \times 100$$

Where, A0 is the absorbance of the control and A1 is the absorbance of the sample.

Quantitative estimation by HPLC to determine ascorbic acid (vitamin C) in peel extract

The HPLC analysis system was Waters 2996 Photodiode array detector and Waters 2695 Separation Module HPLC pump (Waters, Milford, USA). The chromatographic assay was performed on a Intersil ODS-3 column (4.6 mm x 250) reversed phase matrix (5 µm) (Waters) and elution was carried out in a gradient system with acetic acid 0.1% (w/v) methanol (95:5%). UV detector was set at 254 nm and the volume of injection was 20 µl.

GC-MS analysis

The qualitative and quantitative analysis of the sample was carried out by using GC-MS technique model (GC-MS-QP2010-Ultra) from japans 'Simadzu Company, with capillary column (Rtx-5ms-30 m×0.25 mm×0.25 µm).The sample was injected by using split mode, Helium as the carrier gas passed with flow rate 1.61 ml/min. The temperature program was started from 60°C with rate 10°C /min to 300°C as final temperature degree with 2 minhold time: the injection port temperature was 300°C. The ion source temperature was 200°C and the interface temperature was 250°C. The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 26 min .Identification of components for the sample was achieved by comparing their retention times and mass fragmentation patterns with those available in the library from the National Institute of Standards and Technology (NIST).

RESULTS AND DISCUSSION

Qualitative preliminary phytochemical analysis

Qualitative preliminary phytochemical analysis was performed initially with different chemical reagents to detect the nature of phytoconstituents and their presence in peel extract of *C. paradisi*. Phytochemical screening showed that the peel extract was rich in chemical constituents, results are presented in Table 1. Preliminary phytochemical analysis of peel extract of *C. paradisi* revealed presence of flavonoids, sterols, triterpenoids, coumarins, glycosides, reducing sugars and carbohydrates, but alkaloids, tannins, saponins, anthraquinones and lignin were not detected, and might be present in trace undetectable amounts by qualitative methods. These principles have been known for many years to exhibit biological activity, such as effects on the central nervous system, antibacterial, antitumor and anthehelmintic activity (Harborne, 1973). Compared with previous studies, Mathew et al. (2012) reported the presence of flavonoids, alkaloids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars.

Generally, phytochemicals are known to confer certain health benefits such as anti-inflammatory, antimicrobial, antihypertensive, and antidiabetic effects (Oikeh et al., 2016; Oikeh et al., 2013).

Antimicrobial activity

The antibacterial activity of the ethanolic extract from peel of *C. paradisi* was determined against the Gram positive *B. subtilis* and *S. typhi* and the Gram negative *E. coli* and *S. aureus* and two fungi; *C. albicans* and *A. niger* using the disc diffusion method. The results are presented in Table 2. Different extracts showed variable activity

Table 1. Preliminary phytochemical screening of peel 96% ethanolic extract of *C. paradisi* fruit.

Test	Specific test	Grape fruit peel
Alkaloids	Wagner's	-ve
	Mayer's	-ve
	Dragendroff's	-ve
Flavonoids	$FeCl_3$	+ve
	Lead acetate	-ve
Sterols	Salkowski	+ve
	Lebermann	+ve
Triterpenes	Salkowski	+ve
	Liebermann	+ve
Tannins	$FecCl_3$	-ve
	Gelatin	-ve
	HNO_3	-ve
	lead acetate	-ve
Saponins	Foam test	-ve
Coumarins	UV lamp	+ve
Glycosides	Keller kiliani	+ve
	Kedd's	-ve
Reducing sugars	Fehling's	+ve
Anthraquinones	Ammonia test 25%	-ve
Lignins	Labat test	-ve
Carbohydrates	Molisch	+ve

against the tested bacteria. Generally, the Gram-positive strains showed higher susceptibility values than the Gram negative strains. The highest antibacterial activity was showed by *C. paradisi* against *B. subtilis* (17 mm) followed with inhibition zone against *S. aureus* (15 mm) , and against *S. typhi* (14 mm), while the lower zones of inhibition was observed in the Gram negative organisms *E. coli* (13 mm). *C. paradisi* extract exhibited high antifungal activity against *C. albicans* and *A. niger* with inhibition zone (22 and 24 mm) respectively (Figure 1).

In vitro anti-inflammatory activity

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation (Chandra et al., 2012). Results showed in Table 3. The *Citrus paradisi* peel showed high anti-inflammatory activity with inhibition percentage 77.57%. Aspirin a standard anti-inflammation drug showed the maximum inhibition of 88.59%.

Antioxidant activity

The *in vitro* antioxidant activity of the ethanolic extract from peel of *C. paradisi* fruit was assessed by DPPH

Table 2. Antimicrobial activity of *Citrus paradisi* peel extract.

Extract (20 mg/ml)	MDIZ (Mean diameter of growth inhibition zone, mm)					
	Bacteria strain				Fungi strain	
Peel extract	***B. subtilis***	***S. aureus***	***E. coli***	***S. typhi***	***C. albicans***	***A. niger***
	17±1.41	15±0.00	13 ±0.70	14±0.00	22±1.41	24 ±0.0

Interpretation of results: MDIZ* (mm):< 9 mm inactive; 9-12 mm partially active; 13-18 mm active;>18 mm: Very active.

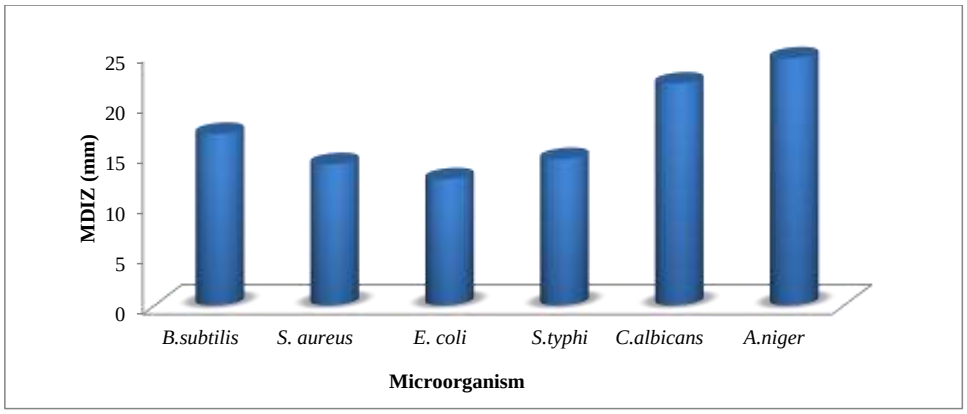

Figure 1. Antimicrobial activity of peel extract against bacteria and fungi microorganisms.

Table 3. Effect of peel ethanolic extract on protein denaturation.

Sample	Inhibition (%)
Grape fruit peel	77.57
Aspirin (Control +)	89.59

Table 4. Antioxidant activity by DPPH assay of *C. paradisi* peel extract.

Sample	DPPH %
Grape fruit peel	55
Ascorbic acid	93.5

assays. Results are shown in Table 4. The extract showed moderate antioxidant activity (55.8 %) compared with ascorbic acid (93.5%). The supplementation of natural antioxidants through a balanced diet containing enough fruits could be much more effective and economical than the use of individual antioxidants, such as vitamin C or vitamin E for protecting of the body against various oxidative stresses (Pisoschi and Pop, 2015).

Barros et al. (2012) stated that antioxidant capacity of all peels was higher than those of pulps, both in terms of the DPPH radical scavenging capacity and the FRAP assay and the antioxidant capacity of citrus does not seem to be a property of a single phytochemical compound, but is correlated both to vitamin C and phenolic constituents.

Determination of vitamin C in *C. paradisi* peel extract by HPLC

C. paradisi peel contains about 23.08 mg/kg of vitamin C (Figure 2 and 3). Previous studies have shown that grape fruit has high vitamin C content and is therefore valuable to the immune system. It helps protect against colds and flu; has a positive effect on obesity and also has diuretic properties. It is used with great success to combat muscle fatigue and stiffness while stimulating the lymphatic system

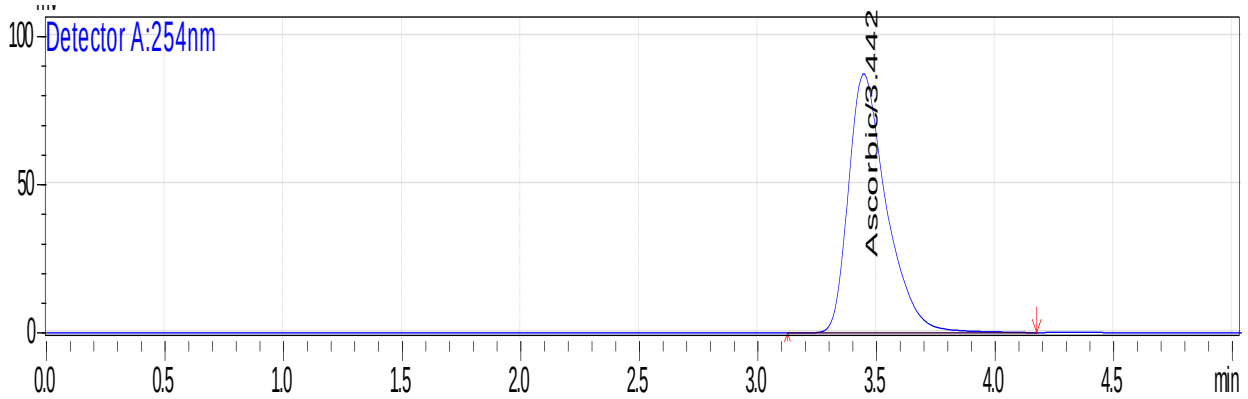

Figure 2. Diagram of vitamin C standard by HPLC.

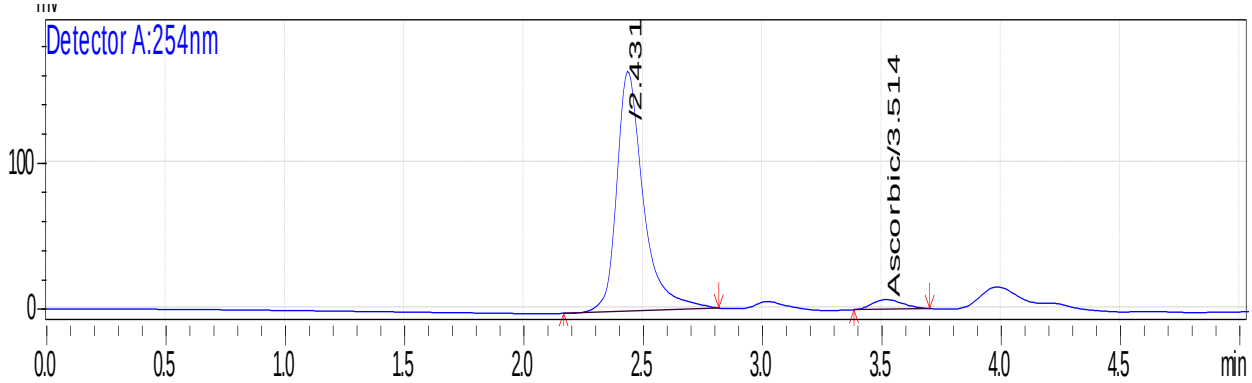

Figure 3. Diagram of vitamin C in *C. paradisi* peel extract by HPLC.

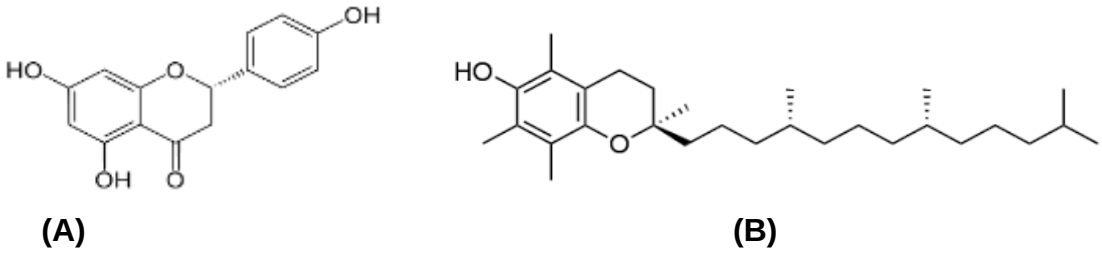

Figure 4. Chemical structure of Naringenin (A) and vitamin E (B).

and therapy clearing the body of toxins (Faleye et al., 2012).

GC-MS analysis

The results of GC-MS analysis of peel ethanolic extract showed different types of chemical constituents (Table 5). The main component in grapefruit peel was found to be Naringenin (28.09%). Citrus flavonoids constitute an important series of flavonoids. Naringenin is a flavanone aglycone of naringin (Figure 4) which has been reported to have numerous bioactive effects on human health such as being an antioxidant, an anti-inflammatory, anti-diabetic and anti-neurodegenerative (Moran et al., 2016).

The results were in agreement with those obtained by Gupta et al. (2011), who reported that, Citrus peel was rich in flavanone glycosides and poly methoxy flavones. Grapefruit peel is candied and is an important source of chemical constituents. Several pharmacological activities

Table 5. Chemical composition of ethanol extract of *C.paradisi* peel using GC-MS.

S/N	R.Time	Name	Formula	Area%
1	4.131	Beta - Myrcene	$C_{10}H_{16}$	0.12
2	4.667	D-Limonene	$C_{10}H_{16}$	0.75
3	5.257	Alpha -Methyl- alpha- [4-mthyl-3-penten]oxiranemethanol	$C_{10}H_{18}O_2$	1.90
4	5.471	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)	$C_{13}H_{22}O_4$	1.64
5	5.590	1,6-Octadien -3-ol,3,7-dimethyl	$C_{10}H_{18}O$	0.46
6	6.373	Ethoxycitronellal	$C_{12}H_{22}O_2$	0.29
7	6.942	L.alpha-Terpineol	$C_{10}H_{18}O$	0.33
8	7.689	(-) Carvone	$C_{10}H_{14}O$	0.06
9	8.012	2-Furanmethanol,5-ethenyltetrahydro-alpha,alpha,5-trimethyl,cis	$C_{10}H_{18}O_2$	0.62
10	8.390	Artemiseole	$C_{10}H_{16}O$	0.17
11	9.446	Geranyl acetate	$C_{12}H_{20}O_2$	0.31
12	9.487	Alpha -Copaene	$C_{15}H_{24}$	0.30
13	10.095	Caryophyllene	$C_{15}H_{24}$	1.30
14	10.538	1,3-Propanediol,2-(hydroxymethyl)-2-nitro methane	$C_4H_9NO_5$	17.94
15	11.068	D-Allose	$C_6H_{12}O_6$	4.82
16	11.343	Naphthalene ,1,2,3,5,6,8a-hexahydro-4,7-dimethyl -1(1-methylethyl)	$C_{15}H_{24}$	0.87
17	11.674	Cyclohexanemethanol ,4-ethenyl-alpha.,4-trimethyl-3-(1-methylethyl)	$C_{15}H_{26}O$	0.59
18	11.724	1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl	$C_{15}H_{26}O$	0.15
19	11.858	3-Oxabicyclo(4.3.0)nonan-2-one,8-isopropylidene	$C_{11}H_{16}O_2$	0.63
20	12.727	1,2,3,5-Cyclohexanetetrol	$C_6H_{12}O_4$	2.23
21	15.647	Benzenemethanol ,alpha-(1-(ethylmethylamino)ethyl	$C_{12}H_{19}NO$	0.44
22	16.003	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	1.14
23	16.031	Isopropyl palmitate	$C_{19}H_{38}O_2$	0.40
24	17.285	7H-Furo(3,2)-(1)benzopyran-7-one,4-methoxy	$C_{12}H_8O_4$	0.56
25	17.722	1(2H)-Naphthalenone,3,4-dihydro-5-methoxy-8-methyl	$C_{12}H_{14}O_2$	2.21
26	17.886	Osthole	$C_{15}H_{16}O_3$	1.92
27	18.375	2,3,5,6-Tetramethylterphthalaldehyde	$C_{12}H_{14}O_2$	0.32
28	18.649	(5,6-Dihydro-2H-(1,4)oxazin-3-yl)-p-tolyl-amine	$C_{11}H_{14}N_2O$	0.54
29	18.806	2H-1-Benzopyran-2-one,7-methoxy-6-(3-methyl-2-oxobutyl)	$C_{15}H_{16}O_4$	3.36
30	18.995	Cholest -5-en-3-ol(3.beta),carbonochloridate	$C_{28}H_{45}CLO_2$	2.82
31	19.227	7-Methoxy-1-methyl-8(1H)-cycloheptapyrazolone	$C_{10}H_{10}N_2O_2$	1.11
32	19.522	Phenacetic amide ,2-methoxy-6-nitrose-alpha,alpha.,dimethyl	$C_{11}H_{14}N_2O_3$	0.51
33	20.443	2,3-Dihydroxydihydrosuberosin	$C_{15}H_{18}O_5$	4.29
34	20.653	Octaethylene glycol monomethyl ether	$C_{19}H_{38}O_{10}$	2.63
35	21.065	Methyl 6-O-(1-methylpropyl).beta-d-galactopyranoside	$C_{11}H_{22}O_6$	1.84
36	21.890	2H-1-Benzopyran-2-one,7(3,7-dimethyl-	$C_{19}H_{22}O_3$	5.34
37	23.158	Isocyclocitral	$C_{10}H_{16}O$	6.41
38	23.997	Naringenin	$C_{15}H_{12}O_5$	28.09
39	25.524	Vitamin E	$C_{29}H_{50}O_2$	0.95

of the peel were reported; anti HIV, anti-inflammatory effect, anti atherogenic, antibacterial, apoptotic activity, anxiolytic, antidepressant and antioxidant.

Conclusion

This study demonstrated support for the claimed uses of the plants in the traditional medicine probably due to the phytochemicals present. The peel of grapefruit is a very important part, as rich source of chemical constituents which is for prevention and cure of diseases. The peel (96% ethanolic extract of *C. paradise*) showed various degree of inhibitory activity against tested microorganism species of bacteria and fungi. Analysis of the peel extract showed high amount of vitamin C and naringenin which might be the cause of the effectiveness against inflammation and antioxidant activity. The results of the present study gave solid grounds that the *C. paradisi* peel extract passes a medicinal potential to develop new phyto-

pharmaceutical drugs and cosmeceuticals.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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