Pak. J. Agri. Sci., Vol. 58(X), xxx-xxx; 2021
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/21.938
http://www.pakjas.com.pk

CHEMICAL PROFILING AND *IN-VITRO* ANTI-*FUNGAL* AND ANTIOXIDANT ACTIVITIES OF CITRUS PEEL ESSENTIAL OIL

Tehmina Sharif[1], Haq Nawaz Bhatti[1,*], Bushra Sultana[1], Muhammad Kashif Saleemi[2], and Muhammad Anjum Zia[3]

[1]Department of Chemistry, University of Agriculture, Faisalabad, Pakistan, 38000; [2]Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faislabad-38000, Pakistan; [3]Department of Biochemistry, University of Agriculture, Faislabad-38000, Pakistan
[*]Corresponding author's e-mail: hnbhatti2005@yahoo.com

The bioactive phytochemicals derived from citrus wastes are considered to have various physiological and biological activities that may be potentially helpful in nutraceutical, pharmaceutical, and processed food industries. This study assessed the anti-*Fusarium* and antioxidant potential and chemical constituents of essential oil extracted from the peels of *Citrus paradisi, Citrus sinensis,* and *Citrus aurantium*. Phytochemical screening by Gas chromatography identified 24, 26, and 39 constituents in *Citrus paradisi, Citrus sinensis,* and *Citrus aurantium,* respectively. Amongst which, limonene (84-87 %) was found to be present in major proportion followed by other major components like a-myrcene, geranial, (+)-valencene, a-pinene, and neral. The estimation of polyphenol contents (PPC) (1.041±0.04 - 7.705±0.26 GAE µg/5mg) and the free radical scavenging activity of these major chemical components was determined by 1,1-diphenyl-2-picrylhydrazyl free radical scavenging ability (IC_{50}: 0.71±0.02-2.99±0.1 µg, and reducing power (RP) (0.75±0.026-1.972±0.067).. Anti-*Fusarium* activity was evaluated against two major food spoilage *Fusarium* species namely, *Fusarium graminearum* and *Fusarium culmorum.* Dose-dependent activity of growth inhibition effect of essential oils was observed. *Citrus aurantium* peel essential oil proved to be most effective with 100% inhibition of *F. graminearum.*

Keywords: GC-MS, Citrus peels, essential oils, polyphenols, Reducing power.

INTRODUCTION

Among the 160 genera of family *Rutaceae* around the world, Citrus is an important genus due to its high yield production and refreshing flavor. Genus citrus includes many economically influential fruits like oranges, lemons, mandarins, grapefruits, and limes. Mostly, it is grown in the region having mild winters and temperate summers (Kamal *et al.,* 2013). Globally, the crop yield of various types of citrus in the fiscal year 2016-17 for grapefruit, oranges, mandarin/tangerine, and limes/lemon was reported as 28.5, 50, 186, and 934 million metric tons, respectively (Duke, 2017). Per annum production of citrus fruits of Pakistan makes it stand amongst the top ten citrus production countries of the world (Kamal *et al.,* 2013).

Fruit processing industries generate a large amount of agro-industrial waste (careful estimate of more than 15x160 tons worldwide) (Mahato *et al.,* 2018). The primary waste fractions of the citrus fruit industry are peels, seeds, and membrane residues which comprises approximately ∼55–60% of the wet/fresh fruit mass (Ahmad *et al.,* 2006). This agro-industrial waste could be a potential source of important secondary plant metabolites. The by-products obtained from this waste would have promising medicinal importance with a high economic impact in multiple fields (El Kamali *et al.,* 2015).

The volatile odoriferous oils found in citrus peels are produced by non woody segments of aromatic plants like leaves, flowers, fruits, buds, stems, seed, twigs, peels, and roots. These volatile components are stored in secretory, sometimes endo- and/or endodermis cells and/or cavities between the cells. Essential oils are complex compositions of more than 400 various kinds of molecules. The major portion i-e 85-99% of these are volatile whereas 1-15% of compounds are non-volatile in nature (Espina *et al.,* 2011). The volatile components of citrus peel essential oils belong to diverse classes of organic compounds including a large ratio of monoterpene hydrocarbons (70-95) along with a lower ratio of sesquiterpene hydrocarbons, which give the distinguish flavour to citrus oils. But makes little contribution to odor perception. Esters and aldehydes contribute a higher impact on the aroma identity of the oil (Tongnuanchan and Benjakul, 2014). The class of substance-related to aldehydes constituents the total content of oxygenated compounds. It is used as a critical indicator to authorize the prize of essential oil as it represents a reference to the quality of essential oil (Ahmad *et al.,* 2006; Javed *et al.,* 2014).

Essential oils of citrus peel exhibit a wide range of medicinal activities. These biological effects owe to the chemical

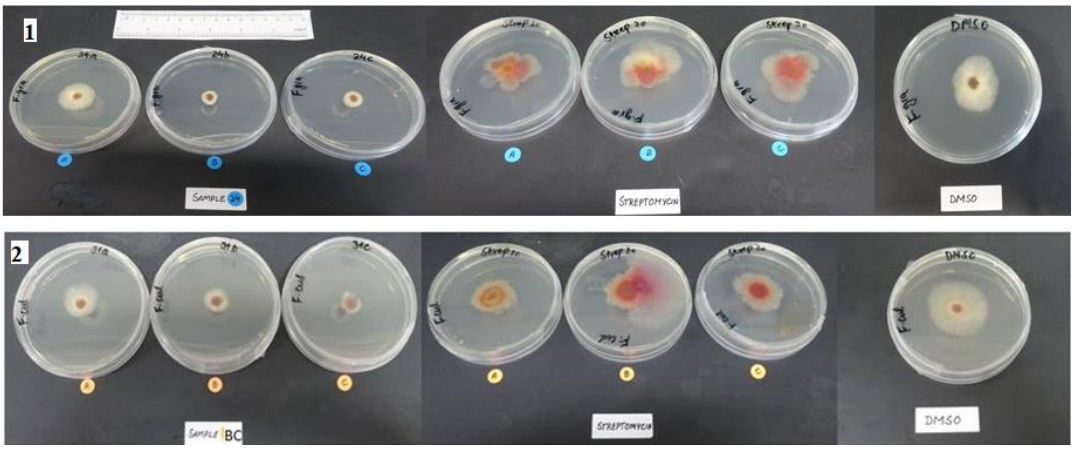

Figure 1. *In-vitro* effect of selected essential oils composition of *C. aurantium, C. paradisi, C. sinensis* against *Fusarium graminearum* (1) *and Fusarium culmorum* (2) at 4 days of incubation using poisoned food technique.

constituents of peel oils, like flavonoids (flavonol, flavone, and flavanone), phenolics, coumarines, carotenes and terpenes. Thus essential oils from citrus peels are being used in pharmaceutical industries as an antivirul, antimicrobial, antidiabetic, antioxidant, antimutagenic and antihepatotoxic agent (Javed *et al.*, 2014).

Botanicals extracts, both volatiles, and non-volatiles exhibit tremendous biological activities by inhibiting the growth of pathogenic bacteria, pests, insects, and fungi and the generation of free radicals (Holley and Patel, 2005; Friday *et al*., 2018). In recent times, exploration of botanicals has revealed that phytochemicals extracted from medicinal plants exhibited remarkable antifungal and free-radical scavenging abilities when applied as green-preservative on store food (Abate and Yayinie, 2018). An essential oil can be served as green-origin fungicides, environment friendly, and GRAS (generally regarded as safe) by EPA (Environmental Protection Agency) (Sivakumar and Bautista-Banos, 2014). Moreover, they have minimal residue on food items with a high rate of action as well as, are economically feasible in their application.

Despite high acreage production of citrus and being one of the largest citrus-producing countries, Pakistan still needs to import large amounts of citrus essential oils to fulfill its increasing industrial needs in the sector of processed food, flavor, scents, and pharmaceuticals. According to a careful estimate, the present import of citrus peel essential oil is worth 1.8 million US dollars (UN) that urges the need to explore the content and compositions, and yield of citrus essential oil from local resources. There is 1) limited data available on the profiling of the essential oils obtained from peels of citrus species of Pakistan. So, there is a dire need to 2) focus on the extraction of essential oils from citrus waste. And 3) phytochemical composition as well as the 4) free radical scavenging ability of essential oils extracted from the peels of three citrus cultivars are evaluated.

MATERIAL AND METHOD

***Collection of Plant materials*:** The peels of *Citrus paradisi*, *Citrus aurantium*, and *Citrus sinensis* were gathered from nearby markets of Faisalabad, Pakistan. The fruit peel samples were further being distinguished and verified from the Department of Botany, University of Agriculture, Faisalabad.

***Extraction of volatiles*:** The Hydrodistillation process was carried for the extraction of essential oil using a Clevenger-apparatus (Lin *et al*., 2009). 1Kg of fresh peels of each citrus species was placed into Clevenger apparatus and filled the jar with distilled water up to the mark (5 Litre). The plant material was boiled under 1.5torr pressure for 4 hours (Sharma *et al.*, 2017). A heterogeneous vapor mixture of essential oil and water was condensed afterward. The essential oils were collected using a separating funnel and were subsequently any moisture was removed with anhydrous sodium sulfate and stored at 4°C until further use. The extraction yield was calculated and estimated as a percentage (v/w) based as expressed below:

Yield of EO (%) = volume of EO obtained (mL) / mass of fresh plant materials (g) × 100

***Profiling of Essential oils*:** The identification of compounds in each pure sample was done by comparing their linear retention indices and mass spectral pattern in reference to homologous series of even (C8-C24) normal alkanes with NIST (National Institute of Standards and Technology) (version 1.1) mass spectra database. The quantification was done by calculation of each peak area obtained by FID detection.

***Quantitative analysis (GC-FID)*:** Quantitative analysis of EO was carried out on Hewlett-Packed 5890 II GC (Rai *et al*., 2021). The instrument was equipped with HP 5890 FID (flame ionization detector) and HP-1capillary column having dimensions; 50 m x 0.35 mm, film thickness 0.17 µm. Carrier

gas was Helium with the flow rate of 2 mL/min. For the initial 2 minutes the temperature was kept at 50 °C then ramped to 300 °C at 4°C. Injector temperature was set at 230 °C while FID transfer line temperature was kept at 300 °C. 1µL of 0.5% essential oil sample was injected manually at 1:1 split ratio. The compounds were identified by comparing their retention indices to that of homologous series of n-alkanes (C12 - C20) run under same operational conditions.

***Qualitative analysis (GC-MS*)**: Compounds were identified by comparing retention indices with homologous n-alkane series (C12-20), Xcalibur Data system, a mass spectral database of NIST (version 1.1), and with mass spectra of reported literature. Quantification was done by calculating component relative peaks area. GC-MS analysis was carried out on ISQ LT Trace 1300 GC-MS (Thermo Fisher Scientific, Hemel Hempstead, UK) system (Ibarra *et al*., 2019). The instrument was fitted with programmable temperature vaporizing GC injector at temperature 290 °C and incorporated single quadrupole mass-spectrometer. Helium was used as carrier gas. The GC temperature was programmed as 10 °C / minute increase from 50 to 100°C and ramped at 4 °C / minute to 350 °C, isotherm for 10 minutes. The stationary phase used was Dimethylpolysiloxane having column (HP 1) dimension; 50 m x 0.32mm with film thickness 0.17 µm. electron ionization mode (EI) was selected for mass spectrometer operation. The experimental conditions were set as; 70 eV electron energy, ion source temperature and transfer line temperature was kept at 350 °C and 400 °C, respectively, emission current of 350 mA and mass analyzer scanning the m/z range 50-600 at cycle time of 1.0/seconds.

***Polyphenol content determination*:** The polyphenol content (PPC) was determined using some modification in the previously reported method (Folin and Denis, 1915). Folin-Ciocalteu (FC) reagent was used for estimation of PP contents, gallic acid served as the standard. The essential oil emulsion was prepared in dimethyl sulfoxide (DMSO) having concentration of 20 mg/mL. 5µL of emulsion was mixed with 80 µL of FC reagent (1:10). After 5 minutes, 160 µL of $NaHCO_3$ (7.5%) was added leading to 30 minutes of shaking at intermediate speed. Serial dilutions of gallic acid (20-200 mg/L) were prepared to establish the standard curve. Absorbance was taken at 765nm. Polyphenol contents are expressed as gallic acid equivalent (GAE) in µg/mg of oil.

***DPPH free radical scavenging assay*:** The antioxidant activity of citrus peel essential oils was evaluated by spectrophotometric analysis using 1,1-diphenyl-2-picrylhydrazyl free radical scavenging protocol (Balogun *et al.*, 2014). Following the microassay, 25 µL of emulsified *Citrus paradisi*, *Citrus sinensis,* and *Citrus aurantium* essential oil in DMSO (10 µL/mL) was introduced into 96 micro-well plate. Four serial dilutions (10, 1.00, 0.1, 0.01 µL/mL) were assed to find out IC_{50} value for each of the three citrus oils. 125 µL of 0.5 tris-HCl buffer (pH 7.4) and 125 µL of 0.3 mM of DPPH, dissolve in methanol, was added in each well. In negative control, DMSO was added instead of sample emulsion while positive control contained butylated hydroxyanisole (BHA). The reaction mixture was then incubated in dark for 30 min. the absorbance was taken at 517 nm in an ELISA reader. The percentage inhibition of DPPH free radical ability of essential oils was assessed using the equation:

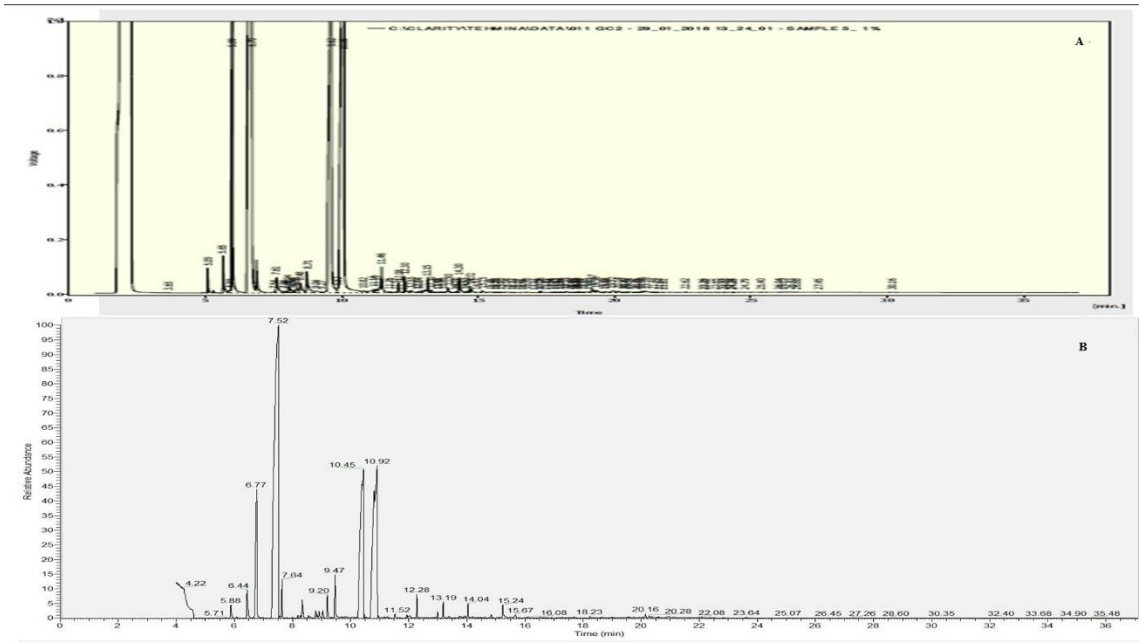

Figure 2. Typical GC-FID (A) and GC-MS (B) chromatogram of *C. aurantium* essential oil.

Scavenging effect % = (Absorbance of negative control- Absorbance of EO emulsion) / Absorbance of negative control * 100

Inhibition concentration emulsified essential oils to scavenge 50 percent (IC_{50}) of the DPPH free radical, is calculated through linear regression analysis.

***Reducing Power (RP) Assay*:** The free radical quenching capacity of emulsified essential oils was determined by using another protocol, named reducing power assay. Some modifications were made into a previously reported method (Wang *et al.*, 2017). Four dilutions 10, 7.5, 5, 2.5 µL/mL of essential oils were assessed. 500 µL of each dilution was mixed with 250 µL of phosphate buffer (pH 6.6, 2mM) and 250 µL of 1% w/v potassium ferricyanide. The mixture was incubated at 50°C for 30 minutes. After incubation, 250 µL of 10% w/v TCA was added in the reaction mixture. Took 100 µL from the upper layer of the solution and mixed with 250 µL of distilled water. 750 µL of $FeCl_3$ (0.1% w/v) was added to the reaction mixture and centrifuged at 3000 rpm for 10 minutes. The absorbance was taken at 700nm on an ELISA reader. BHA and DMSO were used as a positive and negative control, respectively.

***Antifungal activity*:** The fungal growth inhibition effect of all the three essential oils was assessed using the poisoned food technique (Rosello *et al*., 2015; Anzlovar *et al*., 2017). In the autoclaved PDA media, (500µL/20mL) essential emulsion was mixed at ~45 °C. The concentration used were 0.03%, 0.015% and 0.01%. 7- day old stock cultures were used to inoculate the prepared test media (90 mm, diameter) with fungal mycelia disks (7 mm, diameter). Negative and positive control plates were prepared using DMSO and Streptomysin. The test plates were incubated for 4 days at 25 °C in the dark. The growth of the fungal colony was measured in a perpendicular fashion to evaluate the efficacy of essential oil. Percentage inhibition was calculated as follows

% Inhibition= (diameter of control- diameter of treatment)/ diameter of control × 100

***Statistical analysis*:** Analysis were carried out in triplicate. Results were analyzed for standard deviation and analysis of variance using STATISTICA 10.0, Statsoft, 2010.

RESULTS AND DISCUSSION

***Profiling of essential oils by GC-MS*:** The phytochemical constituents of essential oils, extracted from the peels of *Citrus paradisi, Citrus sinensis,* and *Citrus aurantium* is present in the Table 1, 2 and 3 respectively.

There are significant variations in the type and number of compounds as evident from the GC-FID and GC-MS analysis of all the three citrus peel oils. Limonene is the major compound in all the three oils, constituting 87.40 % composition of *Citrus paradisi* peel oil, 84.548% in *Citrus sinensis* and 32.104% in *Citrus aurantium*. a-pinene (1.35 %), a-myrcene (5.19 %), Sabinene (0.48 %), b-thujene (0.27 %), a-trans-Ocimene (0.41 %), Linalool oxide (0.13%), a-Linalool (0.63 %), Farnesol (0.08 %), trans-Limonene oxide (0.24 %), (R)-citronellol (0.25 %), 1-Terpinen-4-ol (0.07 %), a-Terpineol (0.11 %), Decanal (0.46 %), Octyl acetate (0.16 %), (-)-Borneol (0.36%), Neral (0.46 %), Nerol acetate (0.06 %), Copaene (0.39 %), β-cis-Caryophyllene (0.51 %), cis-a-Bisabolene (0.08 %), Germacrene D (0.16 %), Humulen-(v1) (0.47 %) and Cubenene (0.27 %) forms the remaining ≈ 11% composition of *C. paradisi* essential oil. Table 1 summarizes the retention indexes, relative concentration and retention time for corresponding peak of each compound.

Table 1. Chemical composition of essential oil of peels of *Citrus paradisi*

	Citrus paradisi				
No.	**RT (min)**	**SI**	**RSI**	**Name of compound**	**Area %**
1	5.88	941	941	α-pinene	1.35
2	6.42	905	910	Sabinene	0.48
3	6.73	904	912	α-myrcene	5.19
4	6.94	842	923	b-thujene	0.27
5	7.61	903	903	Limonene	87.4
6	7.67	897	898	α-trans-Ocimene	0.41
7	7.94	879	914	Linalool oxide	0.13
8	8.34	905	906	α-Linalool	0.63
9	8.70	799	835	Farnesol	0.08
10	8.87	946	961	trans-Limonene oxide	0.24
11	9.07	924	939	(R)-citronellol	0.25
12	9.49	867	868	1-Terpinen-4-ol	0.07
13	9.67	884	892	α-Terpineol	0.11
14	9.85	886	888	Decanal	0.46
15	10.00	783	852	Octyl acetate	0.16
16	10.31	738	748	(-)-Borneol	0.36
17	10.72	895	899	Neral	0.46
18	12.31	891	893	Nerol acetate	0.06
19	12.45	901	905	Copaene	0.39
20	12.98	937	938	β-cis-Caryophyllene	0.51
21	13.38	859	877	cis-a-Bisabolene	0.08
22	13.71	900	939	Germacrene D	0.16
23	13.87	897	901	Humulen-(v1)	0.47
24	14.20	871	881	Cubenene	0.27

RT(min): retention time, SI: A direct matching factor for the unknown and the library spectrum, RSI: A reverse search matching factor ignoring any peaks in the unknown that are not in the library spectrum

The profiling of *Citrus sinensis* (sweet orange) peel oil displayed a slight lower amount of limonene (84.548 %) as compared to *C. paradisi*. Other chemical constituents identified were (-)-α-Pinene (0.007 %), Sabinene (0.957 %), a-myrcene (4.806 %), 3-Carene (0.969 %), (+)-Camphene (0.254 %), Ocimene (0.254 %), β-Linalool (1.39 %), (+)-trans-Limonene oxide (0.464 %), a-Citronellal (0.465 %), Terpinene-4-ol (0.181 %), α-Terpinol (0.248 %), Decanal

(0.351 %), β-Citral (Neral) (0.77 %), α-Citral (Geranial) (1.475 %), Pentadecyl hexanoate (0.067 %), β-Elemene (0.054 %), cis-Caryophyllene (0.076 %), β-Farnesene (0.056 %), Aromadendrene (0.077 %), (+)-Valencene (1.959 %), a-Farnesene (0.013 %), (-)-a-Panasinsen (0.091 %), (+)-Viridiflorol (0.124 %), Nootkatone (0.179 %), methoxyacetic acid, 3-tridecyl ester (0.134 %).

Table 2. Chemical composition of essential oil of peels of *Citrus sinensis*

Citrus sinensis					
No.	**RT (min)**	**SI**	**RSI**	**Name of compound**	**Area %**
1	5.87	950	955	(-)-α-Pinene	0.007
2	6.45	907	907	Sabinene	0.957
3	6.72	905	914	a-myrcene	4.806
4	7.07	930	944	3-Carene	0.969
5	7.57	899	899	(-)-Limonene	84.548
6	7.61	859	868	(+)-Camphene	0.287
7	7.67	893	906	Ocimene	0.254
8	8.35	910	910	β-Linalool	1.390
9	8.87	939	953	(+)-trans-Limonene oxide	0.464
10	9.06	917	926	α-Citronellal	0.465
11	9.49	891	891	Terpinene-4-ol	0.181
12	9.65	877	883	α-Terpinol	0.248
13	9.85	887	892	Decanal	0.351
14	10.29	802	806	β-Citral (Neral)	0.770
15	10.69	908	908	α-Citral (Geranial)	1.475
16	12.38	679	722	pentadecyl hexanoate	0.067
17	12.61	886	899	β-Elemene	0.054
18	12.99	910	914	cis-Caryophyllene	0.076
19	13.40	886	903	β-Farnesene	0.056
20	13.72	850	885	Aromadendrene	0.077
21	13.88	926	926	(+)-Valencene	1.959
22	14.02	876	913	a-Farnesene	0.013
23	14.17	870	881	(-)-a-Panasinsen	0.091
24	15.69	872	880	(+)-Viridiflorol	0.124
25	17.23	813	854	Nootkatone	0.179
26	18.41	775	853	methoxyacetic acid, 3-tridecyl ester	0.134

The largest numbers of compounds were identified in the essential oil of *Citrus aurantium.* Table 3 represents the relation abundance and retention time of all the 39 identified volatile constitutions of the essential oil. Among these Limonene shares the largest ratio (32.104 %) of relative concentration followed by Geranial (28.789 %). The remaining composition of *C. aurantium* essential oil was contributed by tricyclene (0.015 %), a-pinene (0.5 %), Sabinene (0.765 %), b-pinene (0.12 %), a-myrcene (8.247 %), E-a- Ocimene (0.91 %), α-Terpinolene (0.065 %), humulene epoxide (0.703 %), a-linalool (0.095 %), Perilla Alcohol (0.197 %), neral (20.908 %), terpineol (0.143 %), methyl geranate (0.158 %), citronellol acetate (0.304 %), cis-geraniol (0.146 %), Nerol acetate (0.911 %), Viridiflorol (0.23 %), a-franesene (0.38 %), z(a) franesene (0.141 %), a-selinene (0.03 %), cis-a-Bisabolene (0.09 %), beta bisabolene (0.528 %), globulol (0.101 %), c- elemene (0.056 %), Caryophyllene oxide (0.202 %), beta selinene (0.058 %), Germacrene D-4-ol (0.489 %), tau-Muurolol (0.085 %), α-cadinol (0.212 %) and Nerolidyl acetate (0.092).

Table 3. Chemical composition of essential oil of peels of *Citrus aurantium*

Citrus aurantium					
No.	**RT (min)**	**SI**	**RSI**	**Name of compound**	**Area %**
1	5.71	874	898	Tricyclene	0.015
2	5.87	948	954	α-pinene	0.500
3	6.44	849	870	Sabinene	0.765
4	6.69	905	910	β-pinene	0.120
5	6.77	913	914	α-myrcene	8.247
6	7.50	905	905	Limonene	32.104
7	7.65	916	917	E-α- Ocimene	0.910
8	8.23	872	924	α-Terpinolene	0.065
9	8.29	761	763	humulene epoxide	0.703
10	8.34	910	910	α-linalool	0.095
11	8.54	743	757	Farnesol	0.047
12	8.79	947	959	cis limonene oxide	0.200
13	8.86	922	945	trans limonene oxide	0.178
14	8.92	823	826	Myrtanal	0.102
15	9.03	913	927	α-citronellal	0.237
16	9.19	852	853	cis-verbenol	0.516
17	9.45	808	817	artemiseole	0.950
18	9.78	803	813	Perilla Alcohol	0.197
19	10.45	892	894	Neral	20.908
20	10.53	851	859	Terpineol	0.143
21	10.92	934	936	Geranial	28.789
22	11.51	810	832	methyl geranate	0.158
23	11.94	845	855	citronellol acetate	0.304
24	12.05	848	854	cis-geraniol	0.146
25	12.28	920	920	Nerol acetate	0.911
26	12.96	805	829	Viridiflorol	0.230
27	13.19	932	937	α-franesene	0.380
28	13.37	888	905	z(a) franesene	0.141
29	13.73	816	864	α-selinene	0.030
30	13.94	773	842	cis-a-Bisabolene	0.090
31	14.03	888	902	beta bisabolene	0.528
32	14.16	794	817	Globulol	0.101
33	14.62	838	879	γ- elemene	0.056
34	14.84	912	917	Caryophyllene oxide	0.202
35	15.11	810	841	beta selinene	0.058
36	15.22	832	846	Germacrene D-4-ol	0.489
37	15.50	868	895	tau-Muurolol	0.085
38	15.64	855	872	α-cadinol	0.212
39	16.02	803	806	Nerolidyl acetate	0.092

Our results are in agreement with the former work on the evaluation of essential oil obtained from the five citrus species, reposted by Javed *et al.* (2014). The GC-MS profiling of volatiles of Malta, Mandarin, Grape fruit, Mousami, and Tangerine showed that limonene was found in the highest ratio in all the five EO's. The relative abundance of limonene was 89.84, 88.57, 87.84, 87.45, and 58.50% in grapefruit, malta, mousami, mandarin, and tangerine, respectively. Followed by limonene, α-terpineol was present in higher amounts in all the essential oils. Other than these, carvone, spathulenol, limonene oxide, carveol, and eugenol were also identified in considerable amounts. There are many reports available in the literature about the composition of various species of citrus (Feger *et al.,* 2006). In the evaluation of citrus volatiles, Limonene was present in the highest amounts. Same results were reported by Espina *et al.* (2011) where limonene (85.50%) and α-terpineol (0.36%) was found as a major component in citrus essential oil. Ayoola *et al.* (2008) found that the majority of compounds present in citrus peel volatile oils are hydrocarbon in nature. The results of the present study are in conformity with the studies done by (Gancel *et al.,* 2002; Mahmud *et al.,* 2009). The GC-MS evaluation of the composition of volatiles from sweet orange, grapefruit peel, tangerine, mousami, bergamote and vietnames pummelo revealed the presence of limonene, β-pinene, valencene, γ-terpinene, α-pinene, linalool, myrcene, butyle butyrate, decanal and octanal (Choi *et al.,* 2000). Profiling data of Hyuganatsu oil showed that limonene (80.35-82.39%) as relatively higher in abundance. Other than this, α-terpinene, linalool, myrcene, and α-pinene were identified in the ranges of 7.71-9.03%, 1.37-2.01%, 2.11-2.28% and 1.17-1.43% respectively. Similar results were reported by profiling of *Citrus limon, C. paradisi* and *C. sinensis* carried (Ahmad *et al.,* 2006). They extracted the citrus oils by cold pressing method and the maximum yield observed was 1.21% for *C. sinensis* peel. The composition of *C. sinensis* was the mixture of limonene, citronellol, citral, borneol, α-terinolene and linalool in relative abundance of 61.08%, 4.18%, 7.74%, 7.63%, 2.06% and 1.28% respectively. The constituents found in *C. limon* were limonene, α-pinene, β-pinene, myrcene and terpinene in the relative abundance of 53.61%, 11.80%, 2.63%, 11.16% and 18.53% respectively. The variations among chemical composition of citrus essential oils may attribute to the variations among genetic makeup, geological and environmental factors.

***Total phenolics*:** Phenolics being ubiquitous in plants constitute an indispensable portion in human nutrition. These secondary metabolites perform important morphological and physiological activities in plants. Essential oils contain various compounds which structurally resembles with plant phenolics. These plant phenolics exhibit a broad-ranging chemical and physiological activities prominently free radical scavenging ability. As antioxidants, essential oils show multi-pathway mode of action like acting as reducing agents, prevents chain initiation, free-radical scavenging, termination of peroxide and singlet oxygen generation and deactivation of transition metal ion catalyst (Yildirim *et al.,* 2000). Due to these activities, essential oils can play an important role as green antioxidants, which could be useful in food industries for their lipid peroxidation ability. The antioxidant capacity of phenolics are usually linked to the hydroxyl group (-OH) present in the aromatic ring of these organic molecules. The hydrogen atom of this hydroxyl group plays vital role in stabilizing the free radicals and hence prevent them from oxidation (Hamid *et al.,* 2010).

The total phenolic contents found in *Citrus paradisi*, *C. sinensis* and *C. aurantium* were 1.041, 2.604 and 7.705 µg GAE/5mg respectively. Table 4. depicts that peels of *C. aurantium* are found to have highest phenolic contents in their essential oil among the three citrus species.

Our results are in line with the studies reported (Lin *et al.,* 2009). Their study investigated the antioxidant activity of 42 essential oils by measuring total phenolic content and DPPH free radical scavenging activity. The phenolics found in *Citrus paradisi*, *Citrus aurantifolia* and *Citrus aurantium bigarade* were 5.78 ± 0.354, 7.89 ± 0.331 and 7.87 ± 0.228 µg GAE/5 mg essential oil/mL EtOH, respectively. In another studies (Sajid *et al.,* 2016), the essential oils from the peels of Citrus *grandis* and *Citrus pseudolimon* were evaluated for the chemical profiling and free radical scavenging ability. Their studies find out the highest total phenolic contents in Citrus *grandis* 59 µg GAE/10mg and *Citrus pseudolimon* peel oil contains 40 µg GAE/10mg.

***Antioxidant activity*:** The results for the DPPH assay are presented in Table 4. To elude the free radical scavenging capacity of three citrus essential oils, IC_{50} value, that indicates

Table 4. Yield, Polyphenol contents, and IC_{50} of essential oils of fresh peels of *Citrus sinensis, Citrus paradisi,* and *Citrus aurantium.*

Sr. No.	Sample	Yield (%)	Polyphenols (GAE)[A] µg/5mL	DPPH[B] IC_{50} (µg)
1	*Citrus sinensis*	0.27	2.604±0.09b	2.99±0.12e
2	*Citrus paradisi*	0.31	1.041±0.04[a]	1.19±0.04b
3	*Citrus aurantium*	0.28	7.705±0.26[d]	0.71±0.02ab
4	Control	-	0.983±0.11a	-

A: Gallic Acid Equivalent, B: 1,1-diphenyl-2-picrylhydrazyl free radical scavenging (DFRS) ability. Different letters in the same column indicate a significant difference ($p < 0.05$).

the minimum amount of antioxidant which can neutralize 50% of free radical, was evaluated. It has inverse relation i-e the lower the IC_{50}, the higher the antiradical efficacy of the tested sample. For the determination of IC_{50}, the linear regression equation was used. The IC_{50} value ranged from 0.71±0.02 to 2.99±0.1 µg/g. The lower IC_{50} value for an essential oil means it has a higher radical scavenging capacity. The essential oils extracted from fresh peels of *Citrus aurantium* exhibited the lowest IC_{50} values among the three citrus oils as apparent from Table 4.

Various studies are reported about the co-relation of biological activates of essential oils and their chemical compositions. The lower antioxidant capacity of citrus essential oils could be linked to the lower p-cymene, c-terpinene, and (-)-camphor contents which exhibit higher radical scavenging activity and higher relative abundance of mono-sesquiterpene hydrocarbons. These terpenes possess lower free radical scavenging potential as they are incompetent of donating their hydrogen required for reducing the DPPH free radical as well as essential oils have low solubility in the methanolic medium for the DPPH test (Mata *et al*., 2007).

These results were comparable with the study reported (Sajid *et al.*, 2016). They studied the antioxidant potential of essential oil of peels of *Citrus grandis* and *Citrus pseudolimon*. *C. pseudolimon* showed the stronger DPPH radical scavenging potential with IC_{50} value 37.23µg/mL while for *C. grandis* the IC_{50} was 332.64 µg/mL. Another study about the antioxidant activity of essential oils obtained from peel of *Citrus paradisi*, *Citrus reticulata* and *Citrus sinensis* is carried out (Kamal *et al.*, 2013). The results of in vitro DPPH assay revealed that *Citrus sinensis* held the lowest present radical scavenging ability (14.05 ± 0.28) while maximum percentage inhibition was seen for *Citrus reticulate* (24.08 ± 0.48). These results are in close resemblance with the present study. Also these results are in agreement with the poor performance given by other oils with similar patterns and by single monoterpenic hydrocarbons (Ruberto and Baratta, 2000).

Reducing power (RP) of citrus peel EOs was determined by ferrous ion chelating assay. As represented in Table 5, it can be seen that there is a direct relationship between the concentration of essential oils and the ferric reducing ability of EO's. This assay is primarily employed to assess the electron/hydrogen giving the ability of natural antioxidants (Bouchekrit *et al.*, 2016). According to the reported literature, the reducing power of essential oils could be correlated to the antioxidant ability of bioactive molecules (Ray *et al*, 2018). Thus, the highest absorbance at 700nm represents the greater reducing power of the sample. *Citrus aurantium* showed the highest reducing power (1.972±0.067) at the concentration of 10 (µg/g) followed by *Citrus sinensis* (0.922±0.031). The lowest ferric reducing power was exhibited by *Citrus paradisi* (0.711±0.024). The increase in the reducing capacity of essential oils with concentration-dependent manner could be linked to the presence of certain compounds with possible synergistic and antagonistic impact. Our findings are in agreement with the study of Ray *et al.* (2018). They evaluated the essential oil of *H. coronarium* collected from different geological locations for ferric reducing ability. They reported an increase in absorbance with an increase in concentration and the EC_{50} (0.39 ± 0.01 to1.66 ± 0.04 mg/ml). According to writer's best knowledge, this is the first report on the reducing power ability of essential oils of *Citrus aurantium*, *Citrus paradisi* and *Citrus sinensis* (citrus species of Pakistan).

***Fungal growth inhibition assay*:** Poisoned food technique was used to evaluate the antifungal potential of citrus peel oils. In this technique the efficacy and dosage of potential

Table 5. Reducing power of essential oils of fresh peels of *Citrus sinensis, Citrus paradisi* and *Citrus aurantium.*

Sr. No.	Sample	Reducing power			
		10 (µg/mL)	7.5 (µg/mL)	5 (µg/mL)	2.5 (µg/mL)
1	*Citrus sinensis*	0.922±0.031^{b}	0.868±0.03^{b}	0.746±0.025^{b}	0.75±0.026^{ab}
2	*Citrus paradisi*	0.711±0.024^{ab}	0.692±0.024^{ab}	0.654±0.022^{a}	0.632±0.021^{ab}
3	*Citrus aurantium*	1.972±0.067^{e}	1.795±0.061^{de}	1.722±0.059^{de}	1.602±0.054^{d}
4	Control	0.699±0.028^{a}	0.67±0.026^{a}	0.599±0.041^{a}	0.533±0.037^{a}

Different letters in the same column indicate a significant difference ($p < 0.05$).

Table 6. Percentage growth inhibition of *F. culmorum* and *F. graminearum* by essential oils of fresh peels of *Citrus sinensis, Citrus paradisi* and *Citrus aurantium.*

Sr. No.	Essential oil	Code	10µl/ml		15µl/ml		30µl/ml	
			F. culmorum	*F. graminearum*	*F. culmorum*	*F. graminearum*	*F. culmorum*	*F. graminearum*
1	*Citrus sinensis*	A	05.44±0.141^{a}	31.82±0.212^{a}	18.29±0.353^{a}	13.63±0.212^{a}	47.56±0.212^{cd}	12.12±0.104^{a}
2	*Citrus paradisi*	B	19.51±0.000^{ab}	25.46±0.071^{a}	13.41±0.354^{a}	15.15±0.566^{a}	03.66±0.071^{a}	19.69±0.231^{a}
3	*Citrus aurantium*	C	87.80±0.141^{f}	72.73±0.283^{e}	71.95±0.071^{f}	89.40±0.212^{f}	92.68±0.283^{f}	100.00±0.000^{f}
4	33% Each	ABC	29.27±0.848^{b}	80.30±0.071^{f}	58.54±0.141^{e}	89.39±0.071^{f}	74.39±0.071^{e}	66.67±0.419^{d}
5	50% Each	AB	42.68±0.071^{c}	42.42±0.000^{bc}	56.09±0.000^{e}	84.85±0.141^{f}	04.76±0.283^{a}	84.85±0.100^{ef}
6	-	AC	28.05±0.071^{b}	86.36±0.071^{f}	34.15±0.283^{bc}	87.88±0.141^{f}	76.83±0.071^{ef}	66.67±0.551^{d}
7	-	BC	56.09±0.000^{d}	71.21±0.071^{e}	70.73±0.141^{f}	84.85±0.141^{f}	93.90±0.071^{f}	89.39±0.100^{f}

fungicide is examined by mixing the suspected fungicide with nutrient media (usually ager, PDY or PDA). The fungus under study is grown on same nutrient media then fungal disk are obtained using sterile borer. These fungal disks are placed in the center of poisoned plate. The effect of fungicidal activity is measured by the growth inhibition zone created by the sample. (Balamurugan, 2014; Qadoos *et al*., 2016) Essential oils of citrus peels exhibited an inhibitory effect on the growth of *Fusarium culmorum* and *Fusarium graminearum* in a dose-dependent manner (Table 6). *Fusarium culmorum* was more suppressed as its growth was mostly reduced by all tested doses. *Citrus aurantium* exhibited a strong inhibitory effect against fungus *Fusarium graminearum* (100%) at 30µl/ml concentration while on *Fusarium culmorum* 50% (v/v) mixture of *Citrus aurantium* and *Citrus paradisi* at 30µl/ml concentration exhibited 93.90% inhibition. It is quite evident from the Table 6 that all the compositions containing *Citrus aurantium* exhibited higher growth inhibition of *Fusarium graminearum.*

The present study indicated that essential oils extracted from peels of *Citrus aurantium* and all the compositions have proved most effective and significant fungicidal activity in comparison of their inhibition potential against tested fungal strains. The fungitoxicity of *Citrus aurantium* and *Citrus paradisi* can be attributed to their compositions having mono and sesquiterpenes/ terpenoids as major and many minor components. Fungicidal effects of essential oils have been widely reported for a broad range of fungal strains (Souza *et al*., 2009; Amer *et al*., 2021). Limonene, a-myrcene, Neral and Geranial are found in major quantities in all the citrus EO's. However, they differ in relative abundance in each sample (Table 1,2 and 3). The difference in the antifungal efficacy of these EO's against *F. graminearum* and *F. culmorum* can be linked to the variation in the concentration of these compounds in these EO's. Henry *et al*. (2009) have reported the fungicidal effects of neral and geranial. Phenolic and flavonoids can also exert synergistic antimicrobial/antifungal potential (Orhan *et al*., 2010). The polarity of each compound in the EO's might also play an important role in the fungal growth inhibition. From the results, it can be concluded that *Citrus aurantium* and *Citrus paradisi* could be considered as green fungicide in alteration of synthetic fungicides. However, clinical studies can further explore their possible practical implementation.

***Conclusion*:** In light of the results of the present studies, it could be suggested that like other essential oils extracted from medicinal plants, peels of local Pakistani citrus species, *Citrus aurantium, Citrus paradisi*, and *Citrus sinensis* could be a great source of medicinally active volatile compounds. The antioxidant and antifungal potential of the bioactive compounds in citrus peel essential oils could be useful as natural food preservatives and for medicinal purposes.

***Acknowledgment*:** The authors are grateful to the Higher Education Commission (HEC) Pakistan for the financial support (PIN: IRSIP 37 PSc 11) under the program International Research Support Initiative Program (IRSIP).

REFERENCES

Abate, L. and M. Yayinie. 2018. Effect of solvent on antioxidant activity of crude extracts of Otostegia integrifolia leave. Chem. Int. 4:183-188.

Ahmad, M. M., Z. Iqbal, F. M. Anjum and J. I. Sultan. 2006. Genetic variability to essential oil composition in four citrus fruit species. Pak. J. Bot. 38:319-324.

Amer, M.W. and A.M. Awwad. 2021. Green synthesis of copper nanoparticles by Citrus limon fruits extract, characterization and antibacterial activity. Chem Int. 7:1-8.

Ayoola, G.A., F. M. Lawore, T. Adelowotan, I. E. Aibinu, E. Adenipekun, H. A. B. Coker and T. O. Odugbemi. 2008. Chemical analysis and antimicrobial activity of the essential oil of *Syzigium aromaticum* (clove). Afr. J. Microbiol Res. 2:162-166.

Balamurugan, S. 2014. In vitro antifungal activity of Citrus aurantifolia Linn plant extracts against phytopathogenic fungi Macrophomina phaseolina. Int. Lett. Nat. Sci. 8:70-74.

Balogun, A. O., O. A. Lasode and A. G. McDonald. 2014. Thermo-analytical and physico-chemical characterization of woody and non-woody biomass from an agro-ecological zone in Nigeria. BioResources. 9:5099-5113.

Bouchekrit, M., H. Laouer, M. Hajji, M. Nasri, S. A. Haroutounian and S. Akkal. (2016). Essential oils from Elaeoselinum asclepium: Chemical composition, antimicrobial and antioxidant properties. Asian Pac. J. Trop. Biomed. 6:851-857.

Brewer, M. S. 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Compr. Rev. Food Sci. F. 10:221-247.

Choi, H. S., H. S. Song, H. Ukeda and M. Sawamura. 2000. Radical-scavenging activities of citrus essential oils and their components: detection using 1, 1-diphenyl-2-picrylhydrazyl. J. Agr. Food Chem. 48:4156-4161.

Chu, Y. H., C. L. Chang and H. F. Hsu. 2000. Flavonoid content of several vegetables and their antioxidant activity. J. Sci. Food Agr. 80:561-566.

Duke, J. A. 2017. Handbook of Phytochemical Constituent Grass, Herbs and Other Economic Plants: Herbal Reference Library. Routledge.

El Kamali, H. H., B. O. Burham and A. A. El-Egami. 2015. Essential oil composition of internal fruit peel of *Citrus paradisi* from Sudan. American Research Thoughts, 1:2079-2085.

Espina, L., M. Somolinos, S. Lorán, P. Conchello, D. García and R. Pagán. 2011. Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. Food Control, 22:896-902.

Feger, W., H. Brandauer, P. Gabris and H. Ziegler. 2006. Nonvolatiles of commercial lime and grapefruit oils separated by high-speed countercurrent chromatography. J. Agr. Food Chem. 54:2242-2252.

Folin, O. and W. Denis. 1915. A colorimetric method for the determination of phenols (and phenol derivatives) in urine. J. Biol. Chem, 22:305-308.

Friday, C., U. Akwada and O.U. Igwe. 2018. Phytochemical screening and antimicrobial studies of Afzelia africana and Detarium microcarpum seeds. Chem. Int. 4:170-176.

Gancel, A. L., D. Olle, P. Ollitrault, F. Luro and J. M. Brillouet. 2002. Leaf and peel volatile compounds of an interspecific citrus somatic hybrid [*Citrus aurantifolia* (Christm.) Swing.+ *Citrus paradisi* Macfayden]. Flavour Frag. J. 17:416-424.

Guo, J. J., Z. P. Gao, J. L. Xia, M. A. Ritenour, G. Y. Li and Y. Shan. 2018. Comparative analysis of chemical composition, antimicrobial and antioxidant activity of citrus essential oils from the main cultivated varieties in China. LWT-Food Sci. Technol. 97:825-839.

Hamid, A. A., O. O. Aiyelaagbe, L. A. Usman, O. M. Ameen and A. Lawal. 2010. Antioxidants: Its medicinal and pharmacological applications. Afr. J. Pure Appl. Chem. 4:142-151.

Javed, S., A. Javaid, S. Nawaz, M. K. Saeed, Z. Mahmood, S. Z. Siddiqui and R. Ahmad. 2014. Phytochemistry, GC-MS analysis, antioxidant and antimicrobial potential of essential oil from five citrus species. J. Agr. Sci. 6:201-208.

Kamal, G. M., M. Y. Ashraf, A. I. Hussain, A. Shahzadi and M. I. Chughtai. 2013. Antioxidant potential of peel essential oils of three Pakistani citrus species: Citrus reticulata, Citrus sinensis and Citrus paradisii. Pak. J. Bot. 45:1449-1454.

Lin, C. W., C. W. Yu, S. C. Wu and K. H. Yih. 2009. DPPH Free-Radical Scavenging Activity, Total Phenolic Contents and Chemical Composition Analysis of Forty-Two Kinds of Essential Oils. J. Food Drug Anal. 17:386-395.

Mahato, N., K. Sharma, M. Sinha and M. H. Cho. 2018. Citrus waste derived nutra-/pharmaceuticals for health benefits: Current trends and future perspectives. J. Funct. Foods. 40:307-316.

Mahmud, S., M. Saleem, S. Siddique, R. Ahmed, R. Khanum and Z. Perveen. 2009. Volatile components, antioxidant and antimicrobial activity of Citrus acida var. sour lime peel oil. J. Saudi Chem. Soc. 13:195-198.

Mata, A. T., C. Proença, A. R. Ferreira, M. L. M. Serralheiro, J. M. F. Nogueira and M. E. M. Araújo. 2007. Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. Food Chem. 103:778-786.

Qadoos, M., M.I. Khan, M. Suleman, H. Khan, M. Aqeel and M. Rafiq. 2016. Comparison of poison food technique and drench method for In Vitro control of Alternaria Sp, the cause of leaf spot of bitter gourd. MRJASSS. 4:126-130.

Ray, A., S. Jena, B. Dash, B. Kar, T. Halder, T. Chatterjee, B. Ghosh, P. C. Panda, S. Nayak and N. Mahapatra. 2018. Chemical diversity, antioxidant and antimicrobial activities of the essential oils from Indian populations of Hedychium coronarium Koen. Ind. Crop Prod. 112:353-362.

Rai, R., R.K. Singh and S. Suthar. 2021. Production of compost with biopesticide property from toxic weed Lantana: quantification of alkaloids in compost and bacterial pathogen suppression. J. Hazard. Mater. 401:123332-123344.

Ruberto, G, and M. T. Baratta. 2000. Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem. 69:167-174.

Sajid, A., R. A. Sarfraz, M. A. Hanif and M. Shahid. 2016. Evaluation of Chemical Composition and Biological Activities of Citrus pseudolimon and Citrus grandis Peel Essential Oils. J. Chem. Soc. Pakistan. 38:266-273.

Sharma, K., N. Mahato, M. H. Cho and Y. R. Lee. 2017. Converting citrus wastes into value-added products: Economic and environmentally friendly approaches. Nutrition 34:29-46.

Tongnuanchan, P. and S. Benjakul. 2014. Essential oils: extraction, bioactivities, and their uses for food preservation. J. Food Sci. 79:1231-1249.

Wang, H. F., K. H. Yih, C. H. Yang and K. F. Huang. 2017. Anti-oxidant activity and major chemical component analyses of twenty-six commercially available essential oils. J. Food Drug Anal. 25:881-889.

Yıldırım, A., A. Mavi, M. Oktay, A. A. Kara, O. F. Algur and V. Bilaloglu. 2000. Comparison of antioxidant and antimicrobial activities of Tilia (*Tilia argentea* Desf ex DC), sage (*Salvia triloba* L.), and Black tea (*Camellia sinensis*) extracts. J. Agr. Food Chem. 48:5030-5034.