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Study the Effect of Some Citrus Peel Extracts Against Plant Pathogenic Fungi

Ahmed Mshari*, Alaa M. Alrudainy and Najwa M.J.A. Abu-Mejdad[1]

Department of Medical Laboratory Technology, College of Health and Medical Technology
Southern Technical University, Basrah, Iraq
[1]Department of Biology, College of Science, University of Basrah, Basrah, Iraq
✉ ahmed.mshari@stu.edu.iq

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Abstract: The safest and most effective and preventive method that inhibits the growth of phytotoxic fungi is the biological method using metabolic materials, which are environmentally friendly.

The current study focusses on extracting bioactive compounds from some citrus peels, and evaluating antimicrobial activity against some pathogenic plant fungi, also detecting the active compound using GC-MS. The five extracts of peels citrus included, *Citrus aurantifolia* (key lime), *Citrus singensis* (orange), *Citrus maxima* (pomelo), *Citrus limon* (lemon), and *Citrus reticulate* (mandarin orange). The extracts were evaluated against seven fungal species, including *Alternaria solani*, *Alternaria alternate*, *Rhizoctonia solani, Macrophomina phaseolina, Trichothecium roseum*, *Fusarium solani,* and *Fusarium equiseti*. The results showed that peels extract of pomelo (PPE) is the most effective anti-fungal, which was clear for all types of fungi used in the examination, and which recorded the highest inhibitory diameter against the fungus *F. equiseti*, followed by peels extract of the orange (OPE) and lemon peels extract (LPE), respectively, while lowest antifungal activities have been recorded in peels extracts of mandarin orange (MOPE) and key lime peels extract (KPE), respectively. The diameters of inhibition that represent the antifungal activity of the other extracts ranged between 0 and 25 mm, which constitute significant differences between them and (PPE). The most active extract (PPE) was analysed using GC-MS, and the result included the detection of 50 different compounds. According to the GC-MS results, furfural (4.91%), auraptenol (1.38%), 2-methoxy-4-vinylphenol (0.92%) and hexadecanoic acid-, methyl ester (0.80%) were the major components in the essential oil obtained from peels extract of pomelo (PPE), in addition of ethyl oleate (0.65%), alpha-terpineol (0.32%), osthole (0.31%), which were known as antifungal compounds.

Key words: Peels extract, antimicrobial activity, GC-MS, fungi.

Introduction

Fungi are considered the main cause of plant diseases that affect plant yield and quality, and causes global agricultural production losses (Moore et al., 2020). Pathogenic fungi are thought to be responsible for 70–80% of these diseases, which affect crop growth and yield (Li et al., 2017). The increasing use of fungicides has led to a number of ecological and environmental toxicity, also the development of resistance (Kottadiyil et al., 2021; Shuping and Eloff, 2017; Zamanova et al., 2020).

Many researchers looking for safe, cheap (Abed & Ibrahim, 2021) and effective antifungal agents (Ahmed, 2019; Brauer et al., 2019). Plant extracts have the ability, as antifungal, and proved a good alternative

*Corresponding Author

to chemical fungicidal (Rasheed et al., 2020; Saha et al., 2005). Regarding antifungal properties, distilled citrus essential oils (mainly containing terpenes) have been shown to inhibit *Aspergillus* sp., *Penicillium* sp. *Fusarium* sp., *Candida* sp., *Cladosporium* sp., *Eurotium* sp and *Rhizopus* sp. (Jing et al., 2014). Several plant extracts have shown different fungicidal activity against *Alternaria alternata*, *Fusarium solani* (lira-De León, et al., 2014), *Fusarium oxysporum*, *Alternaria solani* and *Helminthosporium oryzae* (Chaudhary and Vashistha, 2019).

Plant extracts have been used for different modes of action against fungi, such as the effect on cell walls or plasma membrane (Alyousef, 2021). Change of the metabolic pathway (Dhamgaye et al., 2014), inhibition of proteins or RNA/DNA synthesis and effect on mitochondria (Al Aboody and Mickymaray, 2020).

Citrus peels have been identified as one of the richest sources of bioactive compounds (Mondello et al., 2005). A large number of industrial by-products are produced from citrus fruits, where citrus peels which make up the majority of citrus "residue," have numerous potential uses, and contain several important compounds such as flavonoids, limonoids, alkaloids, essential oils, and pectin (Liu et al., 2021; Xiao et al., 2021).

GC-MS considers one of the more important techniques used for the detection of the compound, Several peels extract were analysed by GC-MS such as lime, lemon, valencia orange, navel, tangelo, and tangerine (Smith et al., 2001). Cholke et al. (2017) used GC-MS spectroscopy and detected 15 compounds in orange peel oil extract, 65% of the detected components were identified to be limonene, which is characterised by its anti-microbial activities. While Ahmed et al. (2019) analysed the grapefruit peels extract using GC-MS and found about 99% limonene, volatile fraction, benzopyran, and myrcene. The current study aimed to study the effect of some citrus peels against some plant pathogenic fungi in vitro, which also aims to detect the important active compound by using GC-mass.

Materials and Methods

Pathogenic Fungi

Seven isolates of plant pathogenic fungi were used in this study, which included *Alternaria alternate*, *Alternaria solani*, *Trichothecium roseum*, *F. equiseti*, *F. solani, Macrophomina phaseolina*, and *Rhizoctonia solani*. All isolates were taken from the department of Plant Protection/College of Agriculture at the University of Basra in Iraq.

Plant Extracts

Five citrus peels were used for extraction of the anti-fungal agents, including orange (*Citrus Singensis*), pomelo (*Citrus maxima*), lemon (*Citrus limon*), mandarin orange (*Citrus reticulate*), and key lime (*Citrus aurantifolia*).

Anti-fungal Agent's Extraction

The anti-fungal agents were extracted according to Harborne (1984) and mixed 20 g for each dry peel powder with 300 ml of HCl 36% (2 mole). The extraction was done by the reflex condenser in a water bath at 100ºC for 40 minutes. The extract was allowed to cool and filtered using Whatman No.1. The filtrate was transferred to a separation funnel and mixed well with 50 ml of ethyl acetate. Two layers were formed, and the ethyl acetate layer was isolated. The process was repeated three times, the ethyl acetate extract was collected and dried, and then 0.5 g of each extract was dissolved in 1 ml of distilled water and kept at a temperature of 20°C until use.

Antifungal Activity

The agar diffusion method (NCCLS, 1998) was used to evaluate the effect of extract against fungi. Fungi suspensions were prepared from active fungi isolates, 7 old on potato dextrose agar (PDA). About 0.2 ml of the fungi suspension with a concentration of 3×10^6 reproductive units/ml was spread on PDA in L-shape and let the plates to dry. Make wells by cork borer (6 mm). Transfer 100 µl from each extract (concentration 0.5 g/1 ml) to the wells. Ethyl acetate was dried, followed by adding sterile distilled water and the plates were used as a control.

Identification of Bio-active Compounds

The current study also focusses on the identification of the bio-active compounds that were extracted from citrus peels by GC–MS analysis. The extract was solved with ethanol and then injected into the GC-MS. Experiment conditions for the GC-MS system included using Agilent technologies-7890B-GC, a system coupled to an Agilent Technologies-5977A-MSD with signal detector (EI), HP-5ms –phenyl (l5%), methyl siloxane 95% (30 m*250 µm*0.25). The temperature of the oven was adjusted to 40°C and held for 4 min and then raised (10°C/min to 300°C for 20 min), purge flow of 3 ml/min, and the helium gas (carrier gas), adjusted at flow rate 1 ml/min. The sample was injected (0.5 µl) in split mode at 290°C (injection temperature).

Statistical Analysis

In this research, all experiments were conducted three times using CRD (completely randomized design), and analysis of variance (ANOVA) was used to compare means using the Duncan Test ($p< 0.05$) in SPSS Statistics version 23.

Results and Discussion

After extracting the active compounds from the citrus fruits used in the experiment, it was found that they differ in their ability to inhibit the pathogenic fungal species used in the sensitivity test. PPE extract recorded higher antifungal activity with differing significance against all tested fungi especially *F. equiseti*, which had an inhibition zone with a diameter of 35 mm.

Followed by OPE and LPE with diameters ranging from (11-25 mm for all fungal isolates, while MOPE and KPE showed the lowest activity and no difference significantly between their data (Table 1, Figure 1). Several of researches documented that peel extract has good antifungal activity (Cheng et al., 2022; Olakunleet al., 2019; Suklampoo et al., 2012). The antifungal activity may be due to one or more active compounds, PPE extract contains several compounds that have antifungal abilities such as furfural, alpha-terpineol, and osthole. There were differences in the composition of citrus polyphenols which may be caused by the genetic background and/or tissues of fruits, environmental factors, and extraction solutions and methods (Salas et al., 2011). This is in agreement with Kong et al. (2019), Jung et al. (2007) and Li et al. (2018). Also, PPE extract contains several components that have antimicrobial action, alone or in combination for instance 2-methoxy-4-vinyl phenol (Rubab et al., 2020), nootkatone (Yamaguchi, 2019), ethyl oleate (Akin-Osanaiye et al., 2011), hexadecanoic acid-, methyl ester (Shaaban et al, 2021) and auraptenol (Tan et al., 2017).

The active components affect fungi in one or more mechanisms. Furfural effect on some enzymes that are important in metabolism such as aldehyde dehydrogenase (Lewis et al., 2008). Also, cause damage to the mitochondrial membranes and accumulation of kinds of reactive oxygen, which led to the death of the cell (Almeida et al., 2007). Li et al. (2014) mention that alpha-terpineol have antimicrobial action during its effect on the cell wall and the plasma membrane making morphological changing. While 2-methoxy-4-vinylphenol has an antimicrobial effect during interaction with the DNA gyrase (Rubab et al., 2020). May one or more of these mechanisms be caused by the antifungal action and maybe the bio-action caused by one or more of the other components.

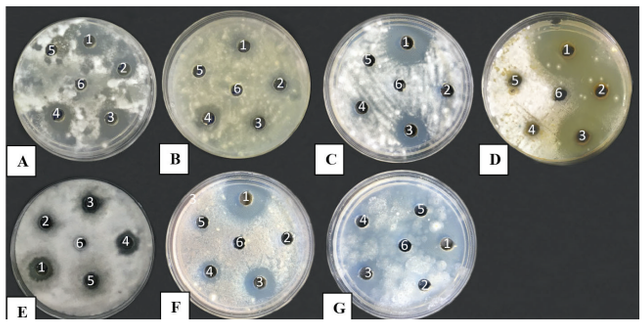

Figure 1: Inhibition zone of fungi by peels extract. A = *Alternaria alternate*, B = *Rhizoctonia solani*, C = *Fusarium solani,* D = *Fusarium equiseti*, E = *Macrophomina phaseolina,* F=*Trichothecium roseum*, G = *Alternaria solani.* 1 = PPE, 2 = KPE, 3 = OPE, 4 = LPE, 5 = MOPE and 6 = control. The concentration of each agent was 0.5 g/1 ml. p<0.05 by Duncan test.

Table 1: Antifungal activity of peels extract

Plant pathogenic fungi	*Inhibition zone (mm)*					
	OPE	*PPE*	*LPE*	*MOPE*	*KPE*	*Control*
Rhizoctonia solani	15	20	11	0	11	0
Trichothecium roseum	20	27	12	0	11	0
Fusarium solani	20	27	12	0	0	0
Fusarium equiseti	25	35	21	11	0	0
Macrophomina phaseolina	18	22	11	0	0	0
Alternaria alternate	20	28	15	11	15	0
Alternaria solani	18	25	11	11	0	0

OPE = Orange Peels extracts, PPE = Pomelo Peels extracts, LPE = Lemon Peels extracts, MOPE = Mandarin Orange Peels extracts, KPE = Key lime Peels extracts. Each treatment was applied in concentration of 0.5g /1ml. Duncan test analyzed ($p < 0.05$).

GC-MS Analysis

The analyses result of the PPE extract by GC-MS included the detection of 50 compounds (Figure 2 and Table 2). Several compounds with a different percentage in their peak area were mentioned in the current paper and were known to have an antimicrobial effect such as Furfural (4.91%), Auraptenol (1.38%), 2-Methoxy-4-vinylphenol (0.92%), Hexadecanoic acid-, methyl

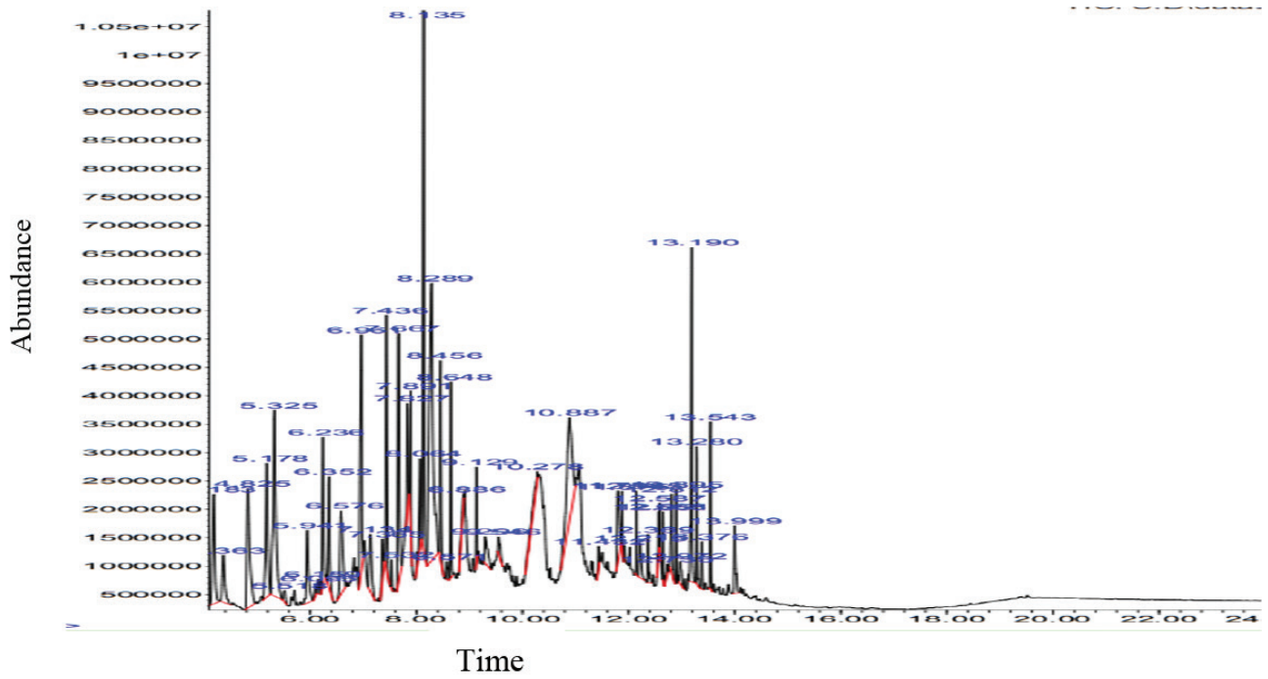

Figure 2: The chemical compounds PPE extract.

Table 2: Chemical constituents of PPE extract by GC-MS

Peak	*Library/ID*	*R.T.*	*Area*	*Area Pct*
1	Furfural	4.153	39389149	4 9124
2	Carbamic acid, methylnitroso-, ethyl ester	4.706	2528312	0.3153
3	Cyclopentane, 1,2-dimethyl-, cis-	4.957	3376080	0.421
4	2-Methyl-3-(methylthio)-1-propene	5.724	2572555	0.3208
5	2-Propanol, l-(2-ethoxypropoxy)-	6 204	20051744	2.5007
6	Ethoxyacetaldehyde dietliylacetal	6.358	6831735	0.852
7	Propane dioic acid, dimethyl ester	6.5 5	11011393	1.373 3
8	2-Furancafb oxaldehyde, 5-methyl-	7.339	50461508	6.293 3
9	Pentanoic acid. 4-oxo-, methyl ester	7 959	6SSO551	0.8581
10	4,4-Dimethyl-5-methylene 1,3 dioxolan-2-one	8 069	6420840	0.8008
11	Propanedioic acid, methyl, ethyl ester	8 224	59476761	7.4176
12	2-Hexanone. 5-methyl-	9.072	37710521	4.7031
13	Octadecanoic acid, 3-hydroxy-, methyl ester	9.235	3078327	0.3839
14	Pentanoic acid, 4-oxo-, ethyl ester	9.456	24286424	3.0289
15	Diethyl mal onate	9.633	24098198	3.0054
16	Furyl hydroxymethyl ketone	9.862	19233566	2.3987

(*Contd.*)

17	Levoglucosenone	10.371	25997435	3.2423
18	DehydromevaIonic lactone	10.481	2348941	0.2929
19	Pentanoic acid, 3-hydroxy-, ethyl ester	IO. 607	2453044	0.305 9
20	Butanedioic acid, hydroxy-, dimethyl ester	10.695	9119405	1.1373
21	1-(2-Thienyl)-! -propanone	11.108	60173467	7.5045
22	2-Furancarbox aldehyde, 5 -(clil oromethyl) -	11.455	30445903	3.7971
23	L-. alpha. -T erpineol	11.646	2604238	0.3248
24	2-Propoxy-succinic acid, dimethyl ester	11.772	17171820	2.1416
25	Hexanoic acid, 3-hydroxy-. ethyl ester	11.89	12486015	1.5572
26	2-Furancafb oxamide, 3-methyl-N-[2-[[(3-methyl-2-furanyl) carbonvl] amino] ethyl]-	12.296	80376291	10.0241
27	5-Hydroxymetliylfurfura 1	12.65	5827503	0.7268
28	Butanedioic acid hydroxy-. diethyl ester	12.886	26245409	3.2732
29	3-Acetoxy-3-hydroxy-2-methylpr opionic acid, methyl ester	13.232	15008528	1.8718
30	2-I\Ietlioxy-4-vinylplieiiol	13.512	7417280	0.925
31	2-t-Butyl-5-methyl-1,3 dioxolane-4-carboxylic acid	14.198	9029256	1.1261
32	D-Allose	17.215	3326844	0.4149
33	Pyrimeth anil	19.354	2516750	0.3139
34	Kootkatoiie	19.464	2402639	0.2996
35	Hexadecanoic acid, methyl ester	20.548	6488700	0 8092
36	Hexa decanoic acid, ethyl ester	21.212	6755835	0.8426
37	4-(Phenyl tlii pjpyridine 1-oxide	21.677	5867912	0.7318
38	9.12-Octadecadienoic acid (Z,Z.)-. methyl ester	22.156	2411675	0.3008
39	4-(Phenylthio)pyiidine 1-oxide	22.223	9385502	1.1705
40	Methyl stearate	22.444	8003533	0 9982
41	Osthole	22.65	2488585	0.3104
42	Ethyl Oleate	22.813	5284210	0.659
43	Octadecanoic acid, ethyl ester	23.041	8090802	1.009
44	Glaucyl alcohol	23.351	4202935	0.5242
45	Dihydrooros elol	23.491	7387822	0 9214
46	Iso auraptene	23.594	38720494	4.829
47	9-Octadecenoic acid, 12-hydroxy-, methyl ester-, [R-(Z)]-	23.904	14661212	1.8285
48	Auraptenol	23.978	11078358	1.3816
49	9-Octadecenoic acid, 12-hydroxy-, ethyl ester, [R-(2L]]-	24.45	23128114	2.8844
50	8-(2,3-Dihydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one	25.21	15515589	1.935

R.T. = retention time, Area = peak area, Area Pct = peak area percentage.

ester (0.80%), Ethyl Oleate (0.65%), alpha-Terpineol (0.32%), Osthole (0.31%), and Nootkatone (0.29%).

Many researchers mention that peels of citrus are rich in many compounds, Visakh et al. (2022) detection of different 25 chemical compounds in Pomelo. While Zulkaida et al. (2017) identify 14 peaks in white Pomelo and 20 peaks in red Pomelo. Shao et al. (2014) analysed four different varieties of pomelo under the same conditions, including Xiangyou, Yuhuan, Guanxi sweet, and Huangjin sweet, which recorded 16, 34, 20, and 9 volatile compounds, respectively.

Conclusion

The current study provided evidence that citrus peel extracts inhibit the growth of *Alternaria solani, Alternaria alternate, Rhizoctonia solani, Macrophomina phaseolina, Trichothecium roseum, Fusarium solani, and Fusarium equiseti*, and therefore are good candidates for further investigation as antifungal ingredients. Amongst the citrus extracts investigated, peels extract of Pomelo was the most effective. Furfural, auraptenol, 2-methoxy-4-vinylphenol, hexadecanoic acid-methyl ester were the major components in the essential oil obtained from peels extract of pomelo (PPE), in addition to ethyl oleate, alpha-terpineol and osthole. This study could obtain good information about the antifungal biological activity of citrus peel extracts prepared with solvents, which makes it possible to use citrus peel by-products to improve food safety and extend shelf life. These extracts may also be developed as antifungal agents for raw food storage, or considered natural antifungal ingredients added directly to foodstuffs or packaging materials.

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