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Table: 5, Figs : 2

Chemical composition and antimicrobial activities of persimmon (*Diospyros kaki*) leaf extracts

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ABSTRACT

The chemical composition of Persimmon leaves was determined by gas chromatography-mass spectrometry (GC-MS) and the antimicrobial activities of different polar extracts (distilled water, ethanol, ethyl acetate, petroleum ether and n-hexane) were studied. In total, 72 compounds (alkanes, alcohols, aldehydes, esters, acids, phenols, flavonoids, ethers, amides, coumarins, phytosterols and ketones) were identified in five extracts [water, ethanol (95%), ethyl acetate, petroleum ether, or n-hexane]. The various extracts differed in their inhibitory acivity against 5 bacteria and 14 fungi and the effects were concentration dependant.

Keywords: Antimicrobial activity, chemical composition, *Diospyros kaki*; extract; GC-MS; MIC; MBC; persimmon leaf, polar extracts.

INTRODUCTION

Plants have evolved several strategies to interact with other organisms for self defence, sexual attraction, symbiosis and other developmental processes (9). Trees especially multipurpose trees, are an integral part of agroforestry programmes (24). Allelochemicals are found in all the plant parts (17, 20) and they participate in chemical interactions in the environment. In plant-plant interactions, these compounds released by the donor plant, inhibit or stimulate the growth and/or development of a neighbouring plants (23). Plant secondary metabolites found in plant extracts may also have antibacterial, insecticidal, antifungal, acaricidal and cytotoxic activities (3,19).

Persimmon (*Diospyros kaki*) is an important fruit tree and widely distributed in China. Its fruit, leaves, flower, peel, root and pedicel are used in Chinese medicine. Its leaves are used to wrap the foods to maintain quality, as ingredients in health-promoting tea and traditional herbal medicines in China, Korea and Japan (7,28). Recently, its leaf extracts have been found effective as antimicrobials, radical scavenger, neuroprotection, lowers the blood pressure and inhibits the thrombosis (4,22). Persimmon leaves have attracted attention due to their high polyphenol content (15) and several bioactive constituents viz., flavonoids, triterpenes, phenolic compounds, alkaloids and organic acids have been isolated and identified (11). This study, aimed to determine the chemical composition of different polar solvent extracts of persimmon leaves and evaluate their antimicrobial activities.

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MATERIALS AND METHODS

Persimmon leaf extracts

Fresh leaves of 6-years old Persimmon trees were collected in May 2016, from the Nursery garden, of the Weifang Engineering Vocational College, Qingzhou, Shandong, China. They were washed with distilled water, cut into 2-3 cm pieces and dried in shade for 120 h at room temperature (25 ºC).The leaves were then powdered using a mortar and pestle and passed through 0. 84 mm mesh sieve.

One hundred g dried leaf powder was mixed with 1.0 L of either distilled water, ethanol (95%), ethyl acetate, petroleum ether, or n-hexane in sterile conical flasks which were then sealed and incubated on a oscillating shaker (100 rpm) for 2-3 h at room temperature (25 ºC). The mixture was then filtered first through one-layer of filter paper, then through 3-layers of filter paper and diluted with distilled water or the respective solvents to give 12.5 mg, 25 mg, 50 mg, 100 mg/ml concentrations. The extracts were then centrifuged at 3000 rpm for 10 min, filtered through filter paper and stored at 4ºC. Five ml of each extracts of 100 mg/ml concentration was membrane-filtered (0.45 µm) for the GC-MS analysis.

GCMS analysis: Analysis of the Persimmon leaf extracts (100 mg/ml) was done using an Agilent 6890N- Agilent 5975 GCMS system with a 30 m fused silica capillary column (HP Part No. 19091Z-433) and high purity helium as the carrier gas (0.8 ml min^{-1}). The injector temperature was 280°C and the detector temperature was 325 ^{o}C. The temperature programme was as under: initial temperature 50 ^{o}C for 3 min, increasing to 100 ^{o}C at 5 ^{o}C min^{-1}; increasing to 280 ^{o}C at 15 ^{o}C/min^{-1}. Sample injected was 1 µl. Fractions were scanned with a scanning range of m/z 20-700 amu manually. The compounds were identified by matching their recorded mass spectra with the GC-MS data system. The HP Chem Station software operated the sampling, analysis and integration for each GC-MS sample. Each sample test was repeated twice. The analysis was conducted using the software package Sherlock Version 6.0 (MIDI Inc.)(8).

Organisms and inoculation conditions:

There were 14 Fungi and 5 Barceria used in this study (Table 1).

Table 1. Test fungi and Bacteria used in this study.

S. No.	Fungi	S. No.	Fungi	S. No.	Bacteria
1	*Fusarium oxysporium*	8	*Alternaria brassicae*	1	*Staphylococcus aureus*
2	*Rhizoctonia cerealis*	9	*Bipolaris sorokininan*	2	*Streptococcus pneumoniae*
3	*Sclerotinia sclerotiorum*	10	*Phacidiopycnis washingtonensis*	3	*Bacillus subtilis*
4	*Colletotrichum lagenarium*	11	*Alternaria bressicae*	4	*Escherichia coli*
5	*Colletotrichum orbicular*	12	*Marssonina zanthoxyli*	5	*Salmonella typhimurium*
6	*Colletotrichum trifolii*	13	*Piricularia oryzae*		
7	*Thanatephorus cucumeris*	14	*Cladosporium fulvum*		

All these fungi and bacteria were provided by Microbial Laboratory of our University. The bacterial strains were first grown in 1 L of Muller Hinton broth medium at 37 ^{o}C for 24 h prior to seeding on the nutrient agar plates (30). Suspensions were adjusted

to 0.5 Mc Farland standard turbidity equivalent to 1.5×10^8 CFU/ml (16). Potato sucrose agar (potato extract from 200 g potatoes, sucrose 20 g, agar 20 g and distilled water1L) was used for growing fungi.

Preparation of sample:

Put 8-10 ml sterile distilled water into agar media tubes on which either the bacteria or fungi were growing and scrap the surfaces (bacteria 1-2 rings and fungi 3-4 rings) to get a suspension which was then homogenized.

Antimicrobial assay

The antibacterial activity was determined by the disk diffusion test (12,26) and micro dilution methods (21). Ten to 15 ml agar medium were put in Petri dishes at room temperature and after the media solidified, 0.2 ml bacterial suspension was applied and spread with aseptic spatula. After the plates dried, filter paper discs impregnated with the extracts were kept (10).

Each sample was replicated thrice. The plates were then incubated for 24 h at 37°C (for bacteria) and for 72 h at 26-28°C (for fungi) . The extent of bacteriostasis was determined by measuring the diameter of inhibition zone (13,27). The antifungal activity was determined using the hyphal growth method (31). First, 1 mL of each different polar persimmon leaf extract was mixed with 9 ml of sterile cooled PDA medium and it was poured into 9-cm dia Petri plates, with three replications for each concentration. Then fungal discs of 4 mm dia obtained by punching hole in the centre of these plates and incubated for 72 h at 26-28°C, after which the diameter of the inhibition zone of fungal growth zone was determined. The percentage of hypha growth inhibition was determined (31).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) Test:

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the growth of a microorganism (14) and the minimum bactericidal concentrations (MBC) is the lowest concentration of antimicrobial that will inactivate the organism as determined after subculture on fresh growth medium (1,22). Micro plates with 96 wells were prepared by adding 100 µl of each different persimmon leaf extract into a separate well. Then 100 µl of MH broth (18) and 20 µl of each bacterial suspension were added to the wells (2) (standardized at 1.5×10^6CFU/ml) by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV 120 01 spectrophotometer). The final volume in each well was 220 µL. The plates were covered with sterile plate sealer, shaken for 30s and then incubated at 37°C for 24 h. After incubation, the wells were examined to assess the microbial growth. A concentration gradient (0.156 mg/ml, 0.312 mg/ml, 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml and 40 mg/ml) was prepared for each sample by multiplying according to the liquid times than dilution method (29). Each test was performed in triplicate and sterile water was used as control.

From the MIC assay, the concentrations showing complete absence of growth of bacteria were identified and 50 µL of each culture from that well was transferred to MH

agar plates and incubated at 37 ^{o}C for 24 h. The absence of growth on the agar surface indicated the lowest concentration of sample was defined as MBC.

RESULTS AND DISCUSSION

Chemical composition of Persimmon leaf extracts

A total of 72 compounds were identified in different polar extracts of persimmon leaves. Thirty seven in distilled water extract, 39 in the ethanol extract, 50 in the ethyl acetate extract 14 in the ether extract and 23 in the n-hexane extract. Only three compounds (namely heneicosane, dibutyl phthalate and 2,6-di-tert-butyl-kinone) were common in all the five extracts and among these extracts the ethylacetate extract was the most complex.

Table 2. The organic compounds extracted from the Persimmon leaves by 5-solvents.

Component	Various solvents chemical components total Peak Area (%)				
	Distilled water	Ethanol	Ethyl acetate	Petroleum ether	N-Hexane
Hepta decane	1.92	----	----	0.08	----
Octa decane	0.06	0.63	1.89	----	0.02
Nonadecane	0.55	0.97	----	----	0.28
Heneicosane	2.41	1.94	3.75	0.36	1.04
5-Propyl-tridecane	----	----	1.07	----	----
5-eicosylene	----	2.17	1.58	----	----
Neophytadiene	0.34	----	3.59	----	----
4- (2,6,6- trimethyl -2- cyclohexenyl) 3-butylene -2- ethanol	1.29	2.95	----	----	----
3,7- two methyl -1,6- methyl -3- alcohol	----	----	2.11	----	----
4- methyl -1- (1- methyl ethyl) -3-cyclohexenylene	----	1.25	3.18	----	----
2-(3- tolyl) aminoethanol	----	0.87	----	----	----
3,7,11,15-tetramethyl-1- hexadecenol	0.68	----	1.49	1.21	----
1-Eicosanol	0.02	0.29	1.87	----	----
3,7,11,15-tetramethyl-2-hexadecenol-1-ethanol	----	----	2.04	----	----
Octadecanol	----	----	1.18	0.56	1.54
3,7-dimethyl-1,6- octadiene-3-ethanol	0.28	----	3.39	----	----
2,5-Dimethyl Benzaldehyde	----	0.31	----	----	0.03
(E)-2-hexenal	0.91	5.72	3.05	----	0.22
Octadecanal	----	----	----	----	1.12
2-methyl-2-hexenal	0.84	----	----	----	2.34
5-methyl furfural	0.17	0.79	----	----	0.07
2,4-Dimethyl Benzaldehyde	0.83	0.95	----	----	0.02
Lauric aldehyde	----	1.53	----	----	0.09
z-7-hexadecenal	----	2.39	----	----	1.68
Indoleacetic acid	0.15	1.93	2.54	----	----
Tetradecanoic acid	----	----	1.73	0.38	----
Octadecanoic acid	0.55	----	1.04	0.06	----

9-Hexadecenoic acid	----	1.81	----	----	----
1,2-Benzenedicarboxylic acid	0.24	1.76	0.31	----	----
Protocatechuic acid	0.93	----	2.79	----	----
Syringic acid	----	----	0.39	----	----
P-hydroxybenzoic acid	0.89	----	1.03	----	0.43
Linolenic acid	----	----	0.81	0.15	----
Octadecanoic acid	0.03	0.09	0.78	----	----
Oleic acid	0.84	----	----	----	----
n-Hexadecanoic acid	0.18	2.24	----	----	1.08
Tetradecanoic acid, methyl ester	0.69	0.78	1.07	----	0.34
Hexadecanoic acid, ethyl ester	0.46	----	3.61	----	----
Stearic acid, 3-(octadecyloxy)propyl ester	----	----	0.68	----	----
Hexadecanoic acid, methyl ester	----	----	1.67	----	----
9-Octadecenoic acid, methyl ester	1.79	----	1.01	----	----
Dibutyl phthalate	1.42	3.73	5.86	0.25	2.14
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	----	2.46	1.97	----	----
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-alpha	0.54	0.92	1.38	----	----
(Z) -butyrate-2-hexenyl acetate	----	0.51	1.16	----	----
2,4-diethyl phthalate	0.85	0.32	1.99	----	----
Caproic acid - (Z) -3- hexene ester	0.84	----	1.72	0.23	----
2-methoxy-4-(1- propenyl) phenol	----	0.74	1.35	----	----
4- methyl -2,6-Ditert-butylphenol	----	----	1.27	----	----
Phenol, 2,4-bis(1,1-dimethylethyl)	1.71	4.51	5.37	----	----
2-methoxy-4-(1-propenyl) phenol	----	1.18	2.64	----	----
Kaempferol	----	----	0.93	----	----
2-methoxy-3-propenyl phenol	----	0.54	1.85	----	----
2,3,6-Trimethylanisole	0.49	1.03	1.67	----	0.31
3-tert-Butyl-4-hydroxyl-anisole	0.26	----	3.59	----	0.05
1- propylene -1- (2- propylene oxide) ether	----	----	1.43	----	----
2,5-dimethyl-2- propyl - cyclohexanone	----	----	----	0.37	----
Trimethyl-tetrahydrogen benzofuranone	----	0.83	2.07	----	----
1- (2- hydroxy -5- methyl phenyl) ethyl ketone	----	3.45	----	0.01	----
2-Cyclohexyl cyclohexanone ylidene	----	----	1.75	----	----
2,2,4-trimethyl-3-cyclohexene-1-ketone	----	0.54	----	----	----
4-ethyl-phenylketone	0.35	2.96	----	----	----
Jiao ketone-2	----	----	0.48	----	----
Ethyl ketone	----	----	0.59	----	----
9-Octadecenamide	0.75	6.85	----	0.26	----
Octadecanamide	1.32	----	----	----	----
Stearamide	----	----	3.07	----	----
2,6-di-tert-butyl-kinone	1.02	1.43	3.25	0.88	1.06
Vitamin C	1.95	1.17	----	----	0.04
Isoquercitrin	0.57	0.42	1.49	----	0.01
(Z)-6-tridecylene-4- alkyne	----	----	----	----	0.23
6-Hydroxy-7-methoxycoumarin	----	2.78	1.91	0.01	0.12

The analysis was conducted using the software package Sherlock Version 6.0 (MIDI Inc.)

Distilled water extract : GC-MS analysis of the aqueous extract indicated the presence of alkanes, aldehydes, esters, acids, phenols, flavonoids, ethers, amides, phytosterols and ketones. Esters were the most abundant (6.59%) with 7-compounds (9-octadecenoic acid, methyl ester 1.79% each, dibutyl phthalate 1.42%), alkanes 4.94% with four compounds (heneicosane 2.41%, heptadecane1.92%) and acids 3.81% with nine compounds (e.g. protocatechuic acid 0.93% and P-hydroxybenzoic acid 0.89%).

Ethanol extract : This extract contained alkanes, alkenes, alcohols, aldehydes, esters, acids, phenols, flavonoids, amides and coumarins. Aldehydes were the most abundant (11.69%) with 6-compounds [(E)-2-hexenal 5.72% and z-7-hexadecenal 2.39%), followed by esters 8.72% (with 6-compounds, dibutyl phthalate 3.73% and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl)ester 2.46%)] and acids 7.83% with 5-compounds (n-hexadecanoic acid 2.24% and indoleacetic acid 1.93%).

Ethyl acetate extract : The extract contained alkanes, alkenes, alcohols, aldehydes, esters, acids, phenols, flavonoids, amides, phytosterols and coumarins. Esters were the most abundant (22.12%) with **11** compounds (dibutyl phthalate 5.86%, hexadecanoic acid, ethyl ester 3.61%), followed by phenols (13.41%) with 6 compounds (phenol, 2,4-bis(1,1-dimethylethyl 5.37% and 2-methoxy-4-(1-propenyl) phenol 2.64%) and acids 11.42% with nine components (protocatechuic acid 2.79% and indoleacetic acid 2.54 %).

Petroleum ether extract : This extract contained alkanes, alcohols, esters, acids, ketones, amides and coumarins. Alcohols were the most abundant (1.77%) with two compounds (3,7,11,15-tetramethyl-1- hexadecenol1.21% and oetadeeanol 0.56%), followed by acids 0.59% with three compounds (tetradecanoic acid 0.38% and linolenic acid 0.15%).

n-Hexane extract : This extract contained alkanes, alcohols, aldehydes, esters, acids, ethers and amides. Aldehydes were the most abundant (5.57%) with 8-compounds (2-methyl-2-hexenal 2.34% and z-7-hexadecenal 1.68%), followed by esters 2.48% with two compounds (dibutyl phthalate 2.14% and tetradecanoic acid, methyl ester 0.34%) and alcohols (1.54%) with only one compound (Octadecanol).

Inhibitory effects of persimmon leaf extracts on fungi

In the preliminary testing, 5-fungi out of 14 were strongly inhibited by the distilled water extract of 100 mg/ml concentration, Therefore only these 5-fungi (*Fusarium oxysporium, Rhizoctonia cerealis, Sclerotinia sclerotiorum, Colletotrichum trifolii* and *Thanatephorus cucumeris*) were selected to test the effect of all the extracts. The distilled water extracts strongly inhibited the *F oxysporium, R cerealis, S sclerotiorum, C trifolii and T cucumeris* (89.03%, 86.14%, 80.59%, 77.92% and 73.25% respectively) (Table 3).

The inhibitory effects of extracts were concentration-dependent. At all concentrations, the distilled water extract strongly inhibited all 5-fungi (>30%) and the sequence of inhibition was : *Fusarium oxysporium >Rhizoctonia cerealis >Sclerotinia sclerotiorum >Colletotrichum trifolii >Thanatephorus cucumeris.* The ethanol extract at 25-100 mg/ml concentrations, inhibited all 5-fungi (>30%). The sequence of inhibition was : *Rhizoctonia cerealis > Sclerotinia sclerotiorum > Colletotrichum trifolii > Fusarium oxysporium > Thanatephorus cucumeris.* The ethyl acetate extract at 25-100 mg/ml concentrations, inhibited all the five plant pathogenic fungi (> 30%). The sequence

of inhibition was *Thanatephorus cucumeris* > *Rhizoctonia cerealis* > *Sclerotinia sclerotiorum* > *Colletotrichum trifolii* > *Fusarium oxysporium*. The petroleum ether extract also inhibited the 5-fungi slightly at all test concentrations and the n-hexane extract inhibited all 5-plant pathogenic fungi (> 30%) at 100 mg/ml concentrations. The order of inhibitory effects of n-hexane extract was : *Fusarium oxysporium* > *Sclerotinia sclerotiorum* > *Thanatephorus cucumeris* > *Rhizoctonia cerealis* > *Colletotrichum trifolii.* These results showed that the order of inhibition varied with the extracts.

Table 3. The inhibitory effects of different solvents extracts on selected pathogenic fungi.

Extract	Plant pathogenic fungus	Fungi inhibition (%)				
		mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Distilled water	*Sclerotinia sclerotiorum*	0	39.66±0.15a	54.71±0.03a	74.35±0.24a	80.59±0.61a
	Rhizoctonia cerealis	0	42.19±0.52ab	63.39±0.06b	77.08±0.17ab	86.14±0.28ab
	Colletotrichum trifolii	0	35.78±0.46ab	52.16±0.35a	61.99±0.11ab	77.92±0.98ab
	Fusarium oxysporium	0	50.27±0.09ab	64.35±0.26a	78.21±0.43a	89.03±0.59ab
	Thanatephorus cucumeris	0	34.23±0.41a	51.87±0.22a	60.84±0.09b	73.25±0.87b
Ethanol	*Sclerotinia sclerotiorum*	0	35.04±0.23a	49.61±0.46a	65.93±0.07b	74.89±0.24a
	Rhizoctonia cerealis	0	39.41±0.04a	54.1±0.09a	69.59±0.11ab	80.62±0.06a
	Colletotrichum trifolii	0	32.16±0.17b	46.24±0.15ab	63.75±0.18ab	73.27±0.59ab
	Fusarium oxysporium	0	19.01±0.34ab	39.65±0.01ab	56.07±0.25a	71.77±0.14ab
	Thanatephorus cucumeris	0	11.73±0.08b	36.59±0.39ab	51.19±0.33ab	69.63±0.02ab
Ethyl acetate	*Sclerotinia sclerotiorum*	0	31.17±0.24ab	45.46±0.23a	59.34±0.22ab	73.21±0.41a
	Rhizoctonia cerealis	0	34.73±0.15ab	46.27±0.54a	63.78±0.09ab	80.48±0.12a
	Colletotrichum trifolii	0	29.22±0.08ab	38.97±0.04b	51.83±0.63ab	69.60±0.02ab
	Fusarium oxysporium	0	25.49±0.37ab	35.21±0.44ab	50.81±0.14a	66.72±0.17b
	Thanatephorus cucumeris	0	35.62±0.16a	53.92±0.06ab	66.27±0.05a	83.79±0.34ab
Petroleum ether	*Sclerotinia sclerotiorum*	0	3.72±0.05ab	9.85±0.05a	14.17±0.02a	15.35±0.06a
	Rhizoctonia cerealis	0	4.45±0.23ab	9.91±0.03a	26.97±0.16ab	29.74±0.09a
	Colletotrichum trifolii	0	2.64±0.02a	5.59±0.24ab	10.76±0.21ab	12.57±0.15ab
	Fusarium oxysporium	0	1.79±0.01ab	4.93±0.19ab	5.88±0.03b	9.67±0.04ab
	Thanatephorus cucumeris	0	2.58±0.11a	5.22±0.07a	8.43±0.01b	10.61±0.18a
N-Hexane	*Sclerotinia sclerotiorum*	0	13.24±0.04a	21.14±0.17ab	32.46±0.03a	41.23±0.02a
	Rhizoctonia cerealis	0	10.58±0.07ab	17.53±0.03b	24.95±0.06ab	37.91±0.03ab
	Colletotrichum trifolii	0	9.37±0.16ab	14.25±0.31a	21.62±0.29b	32.11±0.17ab
	Fusarium oxysporium	0	16.57±0.02ab	26.76±0.06b	35.48±0.01ab	47.54±0.04b
	Thanatephorus cucumeris	0	11.69±0.19ab	20.66±0.09a	29.08±0.07a	39.5±0.02b

Note: Each value is mean of three replicates. Means within each column followed by the same letter are not significantly different at 5% level as determined by Duncan's multiple range tests. The Data in parenthesis inficatr % Inhibition over control.

Disk diffusion test

The inhibition zones obtained using the 4-concentrations of persimmon extract in different polarities against the bacteria are shown in Table 4. The leaf extracts inhibited the bacteria in a concentration-dependent manner with higher concentrations showing greater inhibition.

Table 4. The inhibitory effects of different solvent extracts on bacteria.

Extract	Bacteria	Bacteria inhibition zone (mm)				
		0 mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml	100mg/ml
Distilled-water	*S. aureus*	5.00±0.00	5.00±0.00	5.09±0.02	5.48±0.12	6.45±0.05
	B. subtilis	5.00±0.00	5.00±0.00	5.18±0.00	5.96±0.09	6.71±0.10
	E. coli	5.00±0.00	5.00±0.00	5.94±0.02	6.76±0.06	7.35±0.15
	S. pneumoniae	5.00±0.00	5.00±0.00	5.59±0.05	6.23±0.01	6.79±0.09
	S. typhimurium	5.00±0.00	5.03±0.01	6.26±0.07	6.79±0.03	7.95±0.01
Ethanol	*S. aureus*	5.00±0.00	9.31±0.01	10.10±0.03	11.01±0.15	12.41±0.08
	B. subtilis	5.00±0.00	10.37±0.03	13.76±0.15	15.28±0.45	18.32±0.12
	E. coli	5.00±0.00	9.79±0.27	10.50±0.02	11.84±0.33	13.35±0.14
	S. pneumoniae	5.00±0.00	9.73±0.12	11.90±0.31	14.77±0.18	15.67±0.02
	S. typhimurium	5.00±0.00	10.26±0.22	10.91±0.04	12.97±0.21	13.73±0.07
Ethyl acetate	*S. aureus*	5.00±0.00	8.54±0.01	9.74±0.12	13.28±0.19	14.63±0.14
	B. subtilis	5.00±0.00	9.01±0.05	10.43±0.08	13.72±0.22	15.08±0.06
	E. coli	5.00±0.00	5.03±0.01	5.91±0.02	8.79±0.17	11.22±0.05
	S. pneumoniae	5.00±0.00	8.20±0.03	8.99±0.06	12.46±0.15	13.95±0.11
	S. typhimurium	5.00±0.00	7.12±0.12	8.83±0.02	10.11±0.03	12.57±0.01
Petroleum ether	*S. aureus*	5.00±0.00	5.00±0.00	5.25±0.02	6.02±0.01	6.99±0.05
	B. subtilis	5.00±0.00	5.00±0.00	5.14±0.00	5.67±0.12	6.67±0.10
	E. coli	5.00±0.00	5.05±0.01	5.75±0.05	6.59±0.06	7.04±0.15
	S. pneumoniae	5.00±0.00	5.00±0.00	5.00±0.00	5.39±0.09	6.46±0.09
	S. typhimurium	5.00±0.00	5.21±0.03	6.12±0.07	6.77±0.03	7.29±0.01
N-Hexane	*S. aureus*	5.00±0.00	6.73±0.03	7.68±0.27	9.58±0.07	10.49±0.28
	B. subtilis	5.00±0.00	5.71±0.04	6.37±0.03	7.93±0.42	8.28±0.15
	E. coli	5.00±0.00	5.99±0.08	6.53±0.02	8.15±0.04	9.26±0.03
	S. pneumoniae	5.00±0.00	6.18±0.29	7.34±0.01	8.79±0.06	9.87±0.06
	S. typhimurium	5.00±0.00	6.07±0.03	7.21±0.12	8.54±0.03	9.44±0.12

Note: Values in table are average of three replicates.

The distilled water extracts of all concentrations, weakly inhibited the bacteria, whereas the ethanol extracts inhibited all 5-bacteria at all concentrations. The sequence of inhibition by the ethanol extract was: *B. subtilis*>*S. pneumonia* > *S. typhimurium* > *E. coli* > *S.aureus*. Ethyl extract strongly inhibited (> 8.00 mm) all the bacteria at 50 and 100 mg/ml concentrations. The sequence of inhibition was : *B. subtilis* > *S. aureus* > *S. pneumonia* > *S. typhimurium* > *E. coli*.

The petroleum ether extract at all test concentrations, weakly inhibited the 5-test bacteria (< 8.00 mm). At 5- and 100 mg/ml and 50 mg/ml, the n-hexane extract inhibited all five bacteria (> 8.00 mm). The order of inhibition was : *S. aureus* > *S. pneumonia* > *S. typhimurium* > *E. coli* > *B. subtilis*. These results showed that the order of bacterial inhibition was extract dependant.

MIC and MBC of leaf extracts

MIC : The MIC values (Table 5) of persimmon leaf extracts in the ranges of 1.25-20 mg/ml for *S. aureus*, 0.312-20 mg/ml for *B. subtilis*, 2.5-20 mg/ml for *E. coli*, 1.25-40 mg/ml for *S. pneumoniae and* 2.5-20 mg/ml for *S. typhimurium*). The MIC value of ethanol extracts was 0.312 mg/ml for *B. Subtilis.* The MIC value of ethyl acetate extracts was 1.25 mg/ml for *S. aureus and B. Subtilis*, respectively. The MIC value of petroleum ether extracts was 10 mg/ml for *E. Coli* and *S. typhimurium*, respectively. The MIC value of n-hexane extracts was 2.5 mg/ml for *S. aureus and S. pneumonia*, respectively. The ethanol and ethyl acetate extracts were strong inhibitory to all the bacteria,

Table 5. MIC and MBC of Persimmon leaf extracts against bacteria.

Extraction reagent		*S. aureus*	*B. subtilis*	*E. coli*	*S. pneumoniae*	*S.typhimurium*
MIC (mg/ml)	Distilled-water	20	20	20	20	20
	Ethanol	1.25	0.312	2.5	1.25	2.5
	Ethyl acetate	1.25	1.25	2.5	2.5	2.5
	Petroleum ether	20	40	10	20	10
	N-Hexane	2.5	6.25	6.25	2.5	6.25
MBC (mg/ml)	Water	40	40	20	40	20
	Ethanol	2.5	0.625	2.5	2.5	5
	Ethyl acetate	2.5	1.25	5	5	5
	Petroleum ether	40	40	20	40	20
	N-Hexane	5	10	10	5	10

Values in table are average of three replicates.

MBC: The MBC values (Table 5) of extracts varied among bacterial strains, with 2.5-40 mg/ml for *S. aureus*, 0.625-40 mg/ml for *B. subtilis*, 2.5-20 mg/ml for *E. coli*, 2.5-40 mg/ml for *S. pneumonia* and 5-20 mg/ml for *S. typhimurium. B. subtilis* was the most sensitive bacteria**.** The average MBC value ranged between 0.625-40 mg/ml**.** The MBC values of distilled water extracts was 20 mg/ml in *E. coli and S. typhimurium*, respectively. The MBC values of ethanol extracts was 0.625 mg/ml in *B. subtilis.* The MBC values of ethyl acetate extract was 1.25 mg/ml in *B. subtilis*. The MBC values of petroleum ether extracts was 20 mg/ml for *E. coli* and *S. typhimurium,* respectively. The MBC values of n-hexane extracts was 5 mg/ml for *S. aureus* and *S. pneumoniae,* respectively.

In this study, in 5-extracts a total of 72 compounds were identified (Figs 1, 2), including alkanes, alcohols, aldehydes, esters, acids, phenols, flavonoids, ethers, amides, coumarins, phytosterols and ketones. Among these, distilled water extract had 37 chemical compounds, 39 in the ethanol extract had 50 in the ethyl acetate extract (the most complex), 14 in the ether extract and 23 in the n-hexane extract. This indicated that there were significant differences in extraction of leaf components by various solvents of different polarities. Thus, extractions using different polarity solvents were necessary for the comprehensive evaluation of active allelopathic compounds in persimmon leaf extracts.

Ethyl acetate is medium polarity organic solvent and substances were most abundant in this extract, suggesting that many of the organic compounds present in the persimmon leaves had medium to weak polarity. Thus, extraction using a medium polarity solvent may be the best to extract most of the leaf components.

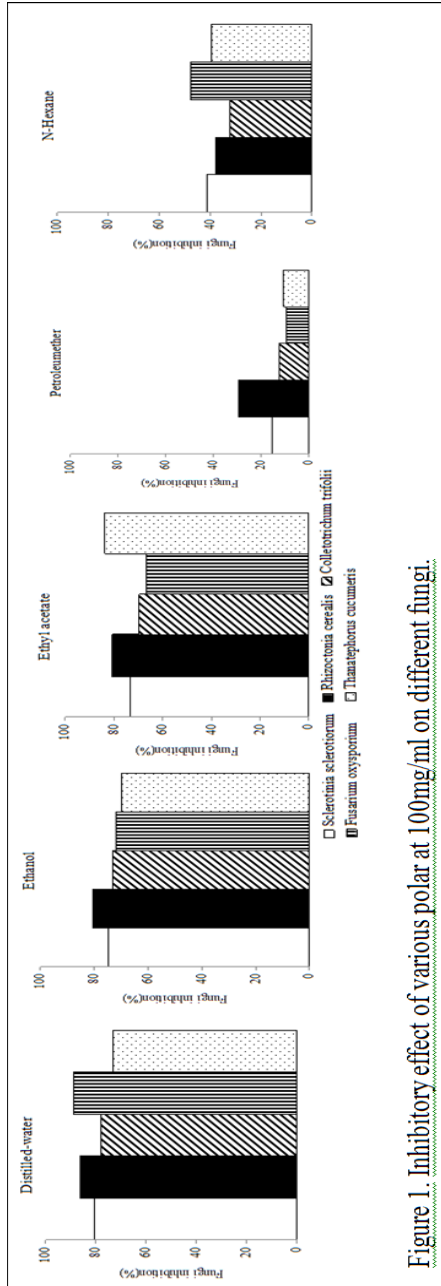

Figure 1. Inhibitory effect of various polar at 100mg/ml on different fungi.

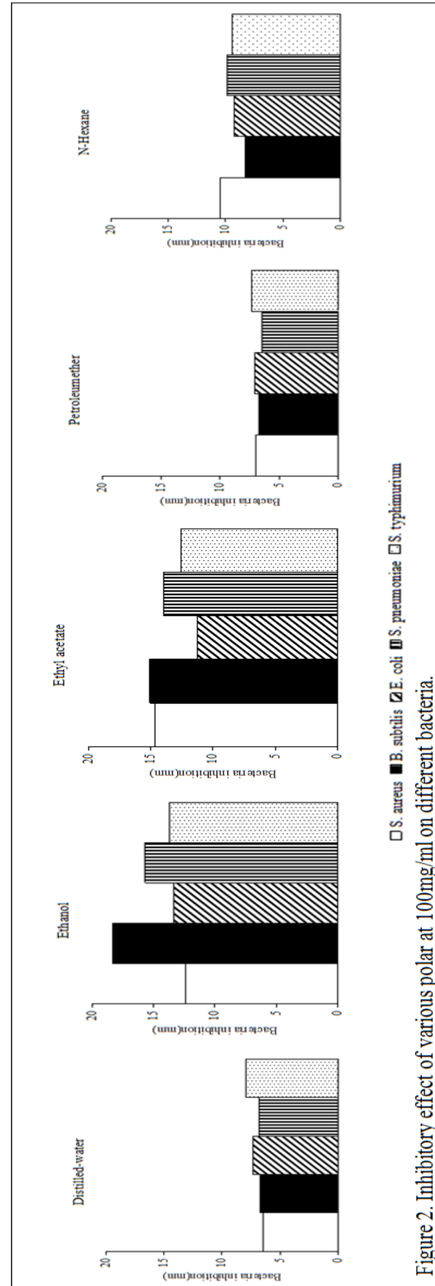

Figure 2. Inhibitory effect of various polar at 100mg/ml on different bacteria.

The antimicrobial activities of extracts from plant leaves, stems and peels of plants have been well recognized. In the present study, the leaf extracts with different polarities were effective against some pathogenic bacteria and plant pathogenic fungi. The Gram-positive bacteria were more susceptible to the ethanol and ethyl acetate extracts than the Gram-negative bacteria. This is probably due to the differences in the cell wall structure of these bacteria. These results are in agreement with Ji *et al*. (13). Many studies have highlighted the potential importance of persimmon extracts as source of polyphenolics, which have strong antimicrobial activity (6,25). The lipid rich nature of the cell walls of Gram-negative bacteria, however, may present a barrier to most polyphenols and thus only slight inhibitory effects were found.

Antibacterial and antifungal activities of the extracts showed that the distilled water extract was strongly inhibitory to five fungi, but weakly inhibited the bacteria. The ethanol and ethyl acetate extracts had strong inhibitory effects against five bacteria, but less inhibitory to five fungi. The petroleum ether extract had weak inhibitory effects on both bacteria and fungi. The n-hexane extract had variable inhibitory effects on bacteria and only slight inhibitory to fungi. Several mechanisms may be responsible for the bactericidal effects such as damage to cell membrane resulting in increased permeability, changes in the intracellular pH and membrane potential, dissipation of cellular components and decreases in the cytoplasmic ATP concentration, which may all lead to bacterial death (5). Secondary effects may also be involved including the inhibition of enzymes, loss of turgor pressure, changes in macromolecular synthesis and other cellular processes (5). Further studies are needed to establish these observations.

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