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CHEMICAL CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT OF THE LEAVES OF *CITRUS LIMON* (L.) BURM. f.

Shahnaz Sultana[1]*, Andleeb Khan[1], Farah Islam[1], Fakhrul Islam[1], Ibrahim A. Khardali[2], Mohsen M. Fageeh[2], Hassan A. Alhazmi[1] and Mohd. M. Safhi[1]

[1]College of Pharmacy, Jazan University, Jazan, Saudi Arabia.
[2]Poison Control & Medical Forensic Chemistry Centre, Jazan, Saudi Arabia.

*** Corresponding Author: Shahnaz Sultana**
College of Pharmacy, Jazan University, Jazan, Saudi Arabia.

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ABSTRACT
Citrus limon (L.) Burm.f. (Rutaceae) is a small evergreen tree found in Asia, Mexico and South Africa. Its leaves are sedative, antispasmodic and antiseptic; used to treat insomnia, nervousness, palpitation, migraine headache and asthma. GC-MS analysis of an ethanolic extract of the leaves indicated that the predominent constitutents of the extract were *n*-tetracontane (9.07) followed by *n*- hexacosane (5.88%), 9-octadecenamide (4.60%), 5,7-dimethoxy-2H-1-benzopyran-2-one (4.06%), *n*-hexatriacontane (3.11%), methoxsalen (2.61%), *n*-dotriacontane (2.37%), *n*-hexadecanoic acid (2.36%), tetradecanamide (2.14%) and stigmast-5-en-3β-ol (2.04%). Fifty five compounds were present in traces. The leaf ethanolic extract showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus pyogenes.*

KEYWORDS: *Citrus limon,* leaves, ethanolic extract, chemical composition, antimicrobial activity.

INTRODUCTION
Citrus limon (L.) Burm.f. (Rutaceae) is a small evergreen, straggling, bushy plant with thorny branches. Its leaves are ovate with alternate arrangement and lustrous; petiole margined or winged. Oil glands are present in the leaves and other aerial parts like branches and fruit peels. It is a native to Asia and distributed all over India, Mexico, Argentina, China, Brazil, United States, Turkey, Iran, Spain, Italy and some parts of Middle East.[1,2,3]

Lemon leaf is sedative, antispasmodic and antiseptic. It is used to treat insomnia, nervousness, palpitation, migraine, headache and asthma. The plant parts are useful to relieve acne, loss of appetite, melasma, asthma, conjunctivitis, constipation, cholera, cough, diarrhoea, dysentery, dyspepsia, fever, headache, influenza, jaundice, leprosy, night blindness, palpitation, pain, rheumatic gout, scabies, scurvy, sciatica, sore-throat and vomiting [4]. Infusions of the aerial (leaves) parts of *C. limon* are taken orally in folk medicine for the treatment of obesity, diabetes, blood lipid lowering, cardiovascular diseases, brain disorders, and certain types of cancer.[5,6,7] A leaf extract showed an antioxidant activity. A monoterpene hydrocarbon fraction of the leaves exhibited a positive Pearson's correlation coefficient with all antioxidant assays.[8] The composition of lemon leaf oil has been studied many times without specification of the varieties.[9]

The essential oil of the leaves contained neral, geranial and limonene as major components; riboflavin and thiamine were detected in the leaves.[10] Most varieties of lemon leaf oil exhibited the limonene/β-pinene/geranial/neral chemotype or similar composition[9] whereas humulene epoxide (0.1) was the minor constituents present in leaf oil.[11]

The main objective of our study was to analyze chemical constituents of the leaves cultivated in the southern region of Saudi Arabia in Jazan and to investigate the antibacterial activity of the same.

MATERIAL AND METHODS
Plant material
The leaves of *C. limon* were collected from Jazan, Saudi Arabia and identified by Dr. Yahiya Masruhi, Department of Botany, Faculty of Science, Jazan University. A voucher specimen of the sample is preserved in the herbarium of the Department.

Preparation of the leaf extract
The air-dried coarsely powder of the leaves (500 g) was exhaustively extracted with ethanol in a Soxhlet apparatus for 30 h. The extract was concentrated on a steam-bath and dried under reduced pressure to get 53 g of dark brown mass. The residue was stored at 4°C in the dark for subsequent experiments.

GC-MS analysis of the extract

GC-MS analysis were carried out on a Shimadzu Gas Chromatograph instrument fitted with a capillary column TR-5MS (30 m x 0.25 mm), film thickness 0.25 µm. The carrier gas He, flow rate 1.2 ml/min. The initial temperature was 70°C and then heated at a rate of 15°C per minute to 290°C and held for 16 minutes. The chromatograph was coupled to Shimadzu QP2010 Ultra MS detector 70eV.

Identification of constituents: The most constituents were identified by GC-MS by comparing their retention indices with those of authentic standard available in the laboratory or with the retention indices, which were in close agreement with reference. Further identification was achieved by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer data base using the NIST08 and Wiley 9 built libraries.

Antimicrobial activity

Microbial strains

Pure cultures of pathogenic bacterial species *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Streptococcus pyogenes* were obtained from Microbiology Department, College of Pharmacy, Jazan University, KSA. All the bacterial cultures were maintained on nutrient agar medium at 4^0C.

Standard antimicrobial substance

Antibiotic tetracycline (50 µg/ml) was prepared in dimethyl sulphoxide.

Preparation and Sterilization of Media

Distilled deionized water was used to prepare nutrient agar media. The media was composed of peptone (5.1 g), sodium chloride (5.0 g), beef extract (1.5 g), yeast extract (1.5 g) and agar (1.5 g). Dehydrated nutrient agar medium (28 g) was accurately weighed and suspended in 1000 ml of distilled water in a conical flask and heat to dissolve completely. Finally the nutrient agar medium was sterilized by autoclaving at 15-lbs/in^2 pressure for 20 minutes.[12]

Methods of preparation of test organisms

The test organisms were maintained on slants of nutrient agar medium and transferred to a fresh slant once in a month. The slants were incubated at 37^0C for 24 hours. Using 10 ml of sterilized normal saline solution, the cells/ mycelium were washed from the slants. A dilution factor was determined which gave optical density of 1.5 at 600 nm. The amount of suspension to be added to each 100 ml agar or nutrient broth was determined by use of test plates or test broth. The test organisms were stored under refrigeration.

Anti-microbial assay

In vitro antimicrobial assay was carried out by Agar well diffusion.[13] A Previously liquefied and sterilized nutrient agar was poured into petri-plates of 100 mm size (to make uniform thickness) and kept for solidifying. Microbial suspensions were spread over the solidified media. Holes were made in each plate with a stainless steel borer having 6 mm ID. Different dilutions (75 µl) of the ethanolic extract of the Citrus leaves were made having concentration of 3 µLml-1, 5 µLml-1, 7 µLml-1 and 9 µLml-1 of solution. Tetracycline solution was used as a standard. The plates were labeled as Co (control), S (standard), A *(E. coli)*, B *(Staph. aureus)*, C *(B. subtilis)* and D *(Strep. pyogenes)* with four different holes, labeled as 4, 7, 10 and 13 for different concentrations. All dilutions were made in DMSO solvent. The plates were then left for standing for 3 hours at 4^0C for proper diffusion of the drug/test solutions. After diffusion process all the Petri plate were incubated for 24 h at 37^0C. After 24 h the plates were examined and the diameter of each zone of inhibition was accurately measured.[14]

RESULTS AND DISCUSSION

The chemical composition of the ethanolic extract of the leaves of *C. limon* is tabulated in Table 1. The ethanolic extract was consisted mainly four each of aliphatic alcohols (1.75%), aliphatic esters (1.93%) and heterocyclic compounds (5.8%), two each of aromatic compounds (1.52%), diterpenes (2.23%) and fatty acids (1.58%), ten aliphatic alkanes (24.4%), one each of aliphatic acid (2.36%) and fatty ester (1.31%), three aliphatic amide (7.22%) and five sterols (7.77%). The predominant constituents were *n*-tetracontane (9.07) followed by *n*- hexacosane (5.88%), 9-octadecenamide (4.60%), 5,7-dimethoxy-2H-1-benzopyran-2-one (4.06%), *n*-hexatriacontane (3.11%), methoxsalen (2.61%), *n*-dotriacontane (2.37%), *n*-hexadecanoic acid (2.36%), tetradecanamide (2.14%) and stigmast-5-en-3β-ol (2.04%). Thirty seven compounds were present in traces.

Maximum percentage of compounds present in the leaf extract was of aliphatic alkanes (24.4%) including cyclotetradecane, *n*-hexadecane, 3-methyl-heptadecane, *n*-pentacosane, *n*-heneicosane, *n*- hexacosane, *n*-tetracontane, *n*-dotriacontane, *n*-pentatriacontane and 1-cyclopentyl-4-(3-cyclopentylpropyl)-dodecane.

Dodecanamide, tetradecanamide and 9-octadecenamide were aliphatic amides (7.22%). 7-Methoxy-2H-1-benzopyran-2-one, 5,7-dimethoxy-2H-1-benzopyran-2-one, 1,1-dimethyl-2,4-bis-(1-methylethyl)-cis-cyclohexane and psoralen were the heterocyclic compounds (5.8%). The phytosterols (4.66 %) detected in the leaf extract were cholesterol, (24R)-ergost-5-en-3β-ol, sigmasterol, stigmast-5-en-3β-ol and 27,28-dihydrolanosterol. Methoxsalen and isopimpinelin were coumarins (4.44%). The diterpenes (2.23%) determined in the leaf extract were 2,6,10,14-tetramethyl-hexadecane and 3,7,11,15-tetramethyl-2-hexadecen-1-ol. Methyl tetradecanoate, 2-propenoic acid 2-[hydroxymethyl]-2-[(1-oxo-2-propenyl)oxy]methyl]-1,3-propanediyl ester, methyl hexadecanoate and 3-(4-methoxyphenyl)-,2-ethylhexyl-2-propenoate were

aliphatic esters (1.93%). The aliphatic alcohols (1.75%) present in the leaf extract were 1-heptacosanol, 5,9-dimethyl- 4,8-decadiene-3-ol, 1-octadecanol and *n*-pentadecanol. (Z,Z)-9,12-Octadecadienoic acid and octadecanoic acid were fatty acids (1.58%). 2,4-Bis-(1,1-dimethylethyl) phenol and 4,5,7-tris(1,1-dimethylethyl-3,4-dihydro)- 1,4-epoxynaphthalene-1(2H)-methanol were the aromatic compounds (1.52%), Ethyl linoleolate was a fatty ester (1.31%).

The leaf extract of Jazan region was devoid of alkaloids and flavonoids. *C. limon* is cultivated mainly for its alkaloids which exhibited anticancer and antibacterial potentials in crude extracts of different parts.[15] The Citrus flavonoids have large spectrum of biological activities including antibacterial, antiseptic, antidiabetic, anticancer, antioxidant and antiviral effects.[16,17] The flavonoids have the capacity to modulate enzymatic properties and inhibit cell proliferation.[18] In plant, they appear to play a defensive role against invading bacteria, fungi and viruses. The flavonoids are generally present in glycosylated form in plants and sugar moiety is a major factor for establishing their bioavailability.[19] The immature fruits from cultivars Lisbon and Fino-49 are ideal for obtaining the flavanone hesperidin, while the mature fruits of cultivar Fino-49 and the leaves of cultivar Eureka are the most interesting for obtaining the flavone diosmin and the flavanone eriocitrin.[20] In lemons, eriocitrin and hesperidin glycosides predominated.[21] Quercetin, myricitin, rutin, tangeritin, naringin and hesperidin are found amongst the common flavonoids in citrus fruits.[22] Limonflavonyl lactones A and B were isolated as flavanoids from the fruit peels.[23] l-Synephrine was detected in several samples of citrus leaves.[24]

The volatile oil was examined for antibacterial activity against *E coli*, *S aureus*, *B subtilis* and *S pyogenes*. The alcoholic extract showed significant antimicrobial activity against clinically isolated pathogenic microbial strains in comparison to standard, tetracycline. The observations were recorded in the Table 2. The antimicrobial activity is mainly due to the presence of monoterpene hydrocarbons in volatile oil.[25,26] The potential antimicrobial activity of present leaves extract of *C. limon* can be useful for treatment of skin disorder and/or in aroma therapy, it can be incorporated into cosmetic formulations. The presence of coumarin and tetrazene present in fruit peel showed good antimicrobials with broad spectrum activity.[27]

Table 1. Chemical constituents of the ethanolic extract of the leaves of *C. limon*.

S.No	Retention time	Component	% Area
1	4.3	(E,Z)-2,6 Dimethyl-2,4,6-Octatriene	0.14
2	5.4	Geraniol	0.12
3	5.6	Citral	0.27
4	7.7	2,4-Bis-(1,1-dimethylethyl) Phenol	0.2
5	8.2	*n*- Pentadecanol	0.19
6	8.7	Decahydro-1,1,7 trimethyl-4methylene-[1ar-(1aα,4aα, 7β,7aβ, 7bα)]- 1H-Cycloprop(e) azulen-7-ol	0.24
7	8.9	Cyclotetradecane	0.17
8	9.0	7-(5-Hexynyl)]-tricyclo[4.2.2.0-(2,5)-dec-7-ene	0.14
9	9.2	Methyl tetradecanoate	0.12
10	9.5	7-Methoxy-2H-1-Benzopyran-2-one	1.31
11	9.7	*n*-Hexadecane	0.14
12	10.0	2-Propenoic acid,2-[hydroxymethyl]-2-[(1-oxo-2-propenyl)oxy]methyl]-1,3-propanediyl ester	0.14
13	10.1	Neophytadiene	0.31
14	10.1	4-(2,4-Dimethylcyclohexylbutan-2-one)	0.17
15	10.2	1,1-Dimethyl-2,4-bis-(1-methylethyl)-,cis, cyclohexane	0.1
17	10.3	Psoralene	0.33
18	10.4	1-Octadecanol	0.74
20	10.5	5,9-Dimethyl- 4,8-decadiene-3-ol	0.24
21	10.6	Methyl hexadecanoate	0.72
22	10.7	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	0.36
23	10.8	3,5-bis-(1,1-Dimethylethyl)-4-hrdroxy-methyl benzenepropanoate	0.34
24	10.9	n-Hexadecanoic acid	2.36
25	11.0	Dodecanamide	0.48
26	11.1	1-Nonadecene	0.79
27	11.1	2,6,10,14-Tetramethyl- hexadecane	0.55
28	11.2	3-Methyl-Heptadecane	0.2
29	11.3	5,7-Dimethoxy-2H-1-Benzopyran-2-one	4.06
30	11.7	Methoxsalen	2.61
31	11.8	*n*-Pentacosane	0.74

32	11.9	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.68
33	12.0	4,6-bis-(4'-Methyl-3'pentenyl)-6-methyl-1-formyl-1,3-Cyclohexadiene	0.72
34	12.1	(Z,Z)-9,12-Octadecadienoic acid	0.62
35	12.1	Ethyl linoleolate	1.31
36	12.2	Octadecanoic acid	0.96
37	12.3	Tetradecanamide	2.14
38	12.4	*n*-Heneicosane	1.30
39	12.5	N-[(3α.,5β)-24-0xo-3-[(trimethylsilyl)oxy]cholan-24-yl]-methyl ester Glycine	1.18
40	12.8	Hexadecamethyl-heptasiloxane	1.87
41	12.9	Isopimpinelin	1.83
42	13.0	*n*- Hexacosane	5.88
43	13.2	3-(4-Methoxyphenyl)-,2-ethylhexyl-2-propenoate	0.95
44	13.4	9-Octadecenamide	4.60
45	13.5	*n*-Tetracontane	9.07
47	13.7	(Z)-7-hexadecenal	0.76
49	14.1	Eicosamethylcyclodecasiloxane	0.61
50	14.3	Octadecamethyl-cyclononasiloxane	1.74
51	14.4	*n*-Dotriacontane	2.37
53	15.0	*n*-Hexatriacontane	3.11
54	16.3	*n*-Pentatriacontane	0.80
55	16.3	1-Heptacosanol	0.58
56	16.5	1-Cyclopentyl-4-(3-cyclopentylpropyl)- dodecane	0.62
58	17.1	Nanodecyl-cyclohexane	0.54
59	18.6	α -Tocophenol-β-D-mannoside	0.71
60	18.8	Cholesterol	0.56
61	20.0	(24R)-Ergost-5-en-3β-ol	0.65
62	20.4	Sigmasterol	1.90
63	21.3	Stigmast-5-en-3β-ol	2.04
64	21.6	27,28-Dihydrolanosterol	1.30
65	26.7	4,5,7 Tris(1,1-dimethylethyl-3,4-dihydro)- 1,4-epoxynaphthalene-1(2H)-methanol	1.32

Table 2. Antimicrobial activity of the leaf ethanolic extract of *C. limon* on tested pathogenic microbes

Sample conc. (µl/ml)	Zone of inhibition (mm) *E. coli*	Zone of inhibition (mm) *S. aureus*	Zone of inhibition (mm) *B. subtilis*	Zone of inhibition (mm) *S. pyogenes*
4(A)	00	7	16	00
7(B)	15	13	22	12
10(C)	15	18	28	16
13(D)	21	25	31	16
50 (Co)	---	---	---	---
50 (S)	20	16	34	24

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