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GC-MS Profiling of Bioactive Compounds Antibacterial and Cytotoxic Potential of *Citrus limo* Extracts and their Toxicity Against *Salmonella enterica*: A Food Borne Pathogen

Aniqa Tahir[1], Shagufta Saeed[1]*, Muhammad Tayyab[1], Aftab Ahmad Anjum[2], Rida Waseem[1] and Tahir Mehmood[1,3]*

[1]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan.*
[2]*Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.*
[3]*Centre for Applied Molecular Biology, University of the Punjab, Lahore, 53700 Punjab, Pakistan*

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Antimicrobial, Potential, Minimum inhibitory concentration, Cytotoxicity, GCMS analysis, Food pathogen, *Salmonella enterica*

ABSTRACT

Salmonellosis is a major food-borne disease in humans worldwide caused by *Salmonella enterica*. The potential of the peel and leaves of lemons was tested at the minimum inhibitory concentration (MIC) by using the broth micro-dilution method, whereas antibacterial activity was evaluated using the disc diffusion method. The MTT assay was used to measure cytotoxicity, and GC-MS was used to look into the contents of the extract. Certain phytochemicals are becoming more and more well-liked as potent therapeutic alternatives for the management and control of microbial medication resistance. The five components in the ethanolic lemon peel extract that were identified using GC-MS are: Heptanedioic acid, 3-methyl-dimethyl ester, 3,3,5-trimethylcyclohexan-1-ol, trimethylsilyl ether, 2,2,6,6-Tetramethylthiabicyclohexane, 9,12-Octadecadien-1-ol, (Z, Z), - and Nonadecanoic acid. The ethanolic extract of lemon peels at 100 mg/mL, with a mean zone of inhibition (MZI) of 14.6 ± 0.5 mm, displayed the highest antibacterial activity, followed by the ethanolic extract of leaves at 8 ± 0.9 mm. The least microbial-resistant extracts were aqueous extracts, which had MZIs of 3.9 ± 0.8 mm and 2 ± 0.7 mm for peels and leaves, respectively. The MIC of the ethanolic peel extract was found to be 25 µg/mL. 50% of the cells survived according to the MTT assay at a concentration of 495.4 µg/mL. The findings suggested that inexpensive and widely accessible byproducts of *Citrus limon* demonstrated strong antibacterial effects against enteric illnesses, and this study might provide the basis for the formulation of improved pharmaceuticals from plant extracts.

INTRODUCTION

Salmonellosis or Salmonella infections are a major global health concern, costing billions of dollars on account of disease management in industrialized countries. Poultry products, the leading cause of disease, are inexpensive and the main source of animal protein in staple food in all ethnic groups of Pakistan. Salmonella infections implicate a spectrum of diseases including gastroenteritis, bacteremia and enteric fever. Inappropriate cooking, inadequate packing, cross-contamination and consumption of raw constituents in food preparation are factors contributing to the outbreak of Salmonellosis (Javed, 2016). The genus Salmonella comprises approximately 2600 known serovars. Salmonellae are rod-shaped, gram-negative, facultative anaerobes belonging to the Enterobacteriaceae family that play a dynamic role in food microbiology. Infections caused by bacterial agents remain a challenge to antibiotic therapies resulting from rise in antibiotic resistance among various strains of Salmonella (Rahimifard and Moghni, 2016). Furthermore, lack of innovation in antibacterial drug development has been observed and for more than 45 years, no novel

* Corresponding author: tahir.camb@pu.edu.pk, shagufta.saeed@uvas.edu.pk
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class of antibiotics has been introduced into the market for the treatment of gram-negative infections. Limited antibacterial discovery and development over the last few decades demands alternative approaches for the treatment or prevention of infections (Tse *et al*., 2017). In this regard, herbal medications provide a approachable foundation for elemental health maintenance and equivalent health care association (Rahimifard and Moghni, 2016).

Citrus fruits have been exploited since ancient eras, as Asian medicine due to the emerging frequency of innumerable subordinate metabolites possessing an extensive range of biological activities. Extensive research on consuming plant parts (leaves, seeds, peels, stem and bark) having multitude of applications is being carried out globally. Citrus cultivation is an imperative, economic and industrial farming accomplishment throughout the world. It belongs to Rutaceae family with around 140 genera and 1300 species but most important species are 6% *Citrus paradise* (Grapefruit), 11% *Citrus limon* (lemon), 17% *Citrus aurantifolia* (Key lime) and 56% *Citrussinensis* (Orange) (Al-Jabri and Hossain, 2014). Lemon is a pale yellow elliptically shaped fruit. It is cultivated in Southeast Asia primarily for its dietary fibers, alkaloids, ascorbic acid, essential oils and minerals that have led to their use in new fields such as pharmacology and food technology. Its peels are of marvelous origin of various flavanones and poly-methoxylated flavones with antimicrobial potential which are scarce in other plants.[5] Lemon is a rich source of calcium, potassium and Vitamin C. Its flavonoids possess large spectrum biological activities including antibacterial, antiviral, antitumor and antidiabetic. In plants, they seem to play a protective role against invading pathogens (Dhanavade *et al*., 2011). Lemon juice has been reported to exhibit antimicrobial activity against *Klebisella pneumonia* (Makni *et al*., 2018)

The current study's objectives include the identifying of bioactive substances in C*itrus limon* ethanolic extract as well as evaluating its antibacterial effects on *Salmonella enterica*, a pathogen that can be found in food. A safe cytotoxicity profile of the effectiveness of lemon peel and leaf extracts against enteric foodborne pathogens has been added into this study.

MATERIALS AND METHODS

All the experimental work was carried out at the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore. The fresh peels and leaves of *Citrus limon* were collected from household gardens of Shahdara, Lahore. It was authenticated by the Department of Botany, University of the Punjab, Lahore. The identified plant sample was deposited as a voucher specimen with herbarium number LAH # 141118A and submitted in University Herbarium for future reference.

Sample pre-treatment

Collected samples were rinsed thoroughly with distilled water and shade dried for about fifteen days. The dried samples were then crushed properly using an electric grinder and powder obtained was dried in the oven at 40 °C for 24 h. It was then stored in a secured container for further use (Suja *et al*., 2017).

Extraction of bioactives

The extraction of *Citrus limon* peels and leaves was done using ethanol and distilled water in the ratio (1:5) plant powder to solvent. For preparation of ethanolic extract, the powdered sample 50 g was added in 250 ml of the 95 % ethanol in Soxhlet apparatus and extracted for 6 h at 78.37 °C. The extract obtained was sieved using Whatman filter paper no. 1 and concentrated at 40 °C on a rotary evaporator and then stored at 4 °C, Anbari and Hasan (2015). To obtain aqueous extract, the powdered sample 50 g was mixed in 250 mL of distilled water in a conical flask and kept on a mechanical shaker at 130 rpm for 48 h. The obtained substance was sieved through Whatman filter paper no. 1 in a Buchner flask and concentrated in the oven at about 40 °C and later kept at 4 °C for further use (Ali *et al*., 2016).

Qualitative phytochemical analysis

The qualitative phytochemical investigation (alkaloids, saponins, flavonoids, steroids, tannins, phenol) of extracts (aqueous and ethanolic) of *Citrus limon* (peels and leaves) was carried out according to Mathew *et al*. (2012).

Antimicrobial assay

The characterized gram-negative strain of *Salmonella enterica* (NRRL B-4212) was procured from the ARS culture collection database and grown on the nutrient agar medium for 48 h at 37 °C (Mattick *et al*., 2001). The antimicrobial susceptibility assay of aqueous and ethanolic extracts of *Citrus limon* was evaluated by agar well diffusion assay. Bacterial suspension of 0.1 mL (0.5 McFarland standard having 1.5×10^8 cells/mL) grown for about 48 h on nutrient agar medium was swabbed uniformly on agar plates. It was kept at room temperature in order to solidify the agar medium. The wells of six millimeter were made with help of sterile corn borer. The plant extract (25 µL) was added in each well. Streptomycin (20 µg/mL) and DMSO was utilized as positive and negative controls respectively. The plates were kept for incubation at about 37 °C for 48 h. After two days the zone of inhibition in mm

were calculated with the help of scale (Ali *et al*., 2016).

Minimum inhibitor concentration (MIC)

The MIC of *Citrus limon* extract showing maximum anti-Salmonella activity was evaluated by broth micro-dilution method. In 96 well microtiter plate, nutrient broth (100 µL) was added into wells. Two-fold successive dilutions (100 to 0.048 µg/mL) of extracts were prepared and added in wells. The standard inoculum (100 µL) was mixed into wells except control. It was then incubated for 48 h at 37 °C. Microbial suspension was used as a growth control whereas nutrient broth used as sterility control. The absorbance was taken at 630 nm by means of an ELISA reader (Multiskan ™ Microplate photometer). The of extract minimum concentration which shows less growth was termed as MIC (Adham, 2015).

GCMS profiling

To characterize the bioactive components, the extract with the highest antibacterial activity was analysed by GC-MS. The carrier gas such as helium was used to isolate components at a persistent flow proportion of 1mL/min. The sample of about 1µL was introduced into apparatus. The oven temperature was maintained at 100 °C for 1 min followed by 100-280 °C by the side of 5 °C min^{-1} and 280 °C for 25 min. Relative fraction of every component was demonstrated as a ratio obtained by the peak zone standardization. Identification of constituents was done by comparing sample mass spectra with the Wiley Registry of Mass Spectral Data edition 7th and National Institute of Standards and Technology (NIST) 05 MS reference library data (Ben-Hsouna *et al*., 2017).

Cytotoxic analysis

The cytotoxic evaluation was performed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. A monolayer of Vero cell lines (ATCC CCL81.4) was grown on 96 wells microtiter plates. Two-fold serial dilutions (5000 to 9.77 µg) of *Citrus limon* extract were made. In the unicellular layer, 100µL of plant extract from each dilution was added. It was then incubated for 24 h at 37 °C. Following incubation, 10 µL of dye was added. It was incubated for next 3 h at 37 °C. Following addition of dimethyl sulfoxide (DMSO) in order to retain the color of dye the optical density (O.D) value was recorded at 570 nm (Mattick *et al*., 2001).

Statistical analysis

All experimental tests were performed in triplicate. The outcomes were expressed in terms of Mean ± S.D (Makni *et al*., 2018).

RESULTS AND DISCUSSION

Table I shows the phytochemical analysis of lemon peels and leaves in aqueous and ethanolic extracts. Aqueous extracts indicated various phytocomponents except for presence of saponins and tannins in aqueous leaves. The results of ethanolic peels and leaves extracts specified the occurrence of alkaloids, saponins, flavonoids, steroids, tannins and phenols. It could be inferred that during Soxhlet extraction more metabolites are extracted with ethanol as compared to water due to polarity difference between solvents.

Table II shows the antimicrobial activity of *Citrus limon* extracts against *Salmonella enterica*.

Table I. Phytochemical analysis of *Citrus limon* peels and leaves.

S.	Phyto-chemicals	Test performed	Aqueous extracts		Ethanolic extracts	
			Peels	Leaves	Peels	Leaves
1	Alkaloids	Mayer's test	+	+	+	+
2	Saponins	Froth test	-	+	+	+
3	Flavo-noids	Alkaline reagent test	+	+	+	+
4	Steroids	Chloroform test	+	+	+	+
5	Tannins	Lead acetate test	+	-	+	+
6	Phenols	Ferric chloride test	+	+	+	+

+, Present; -, Absent.

Table II. Antimicrobial activity of different *Citrus limon* extracts against *Salmonella enteritidis.*

S. No	Extracts	Zone of growth inhibition (mm)
1	Ethanolic peels	14.6 ± 0.5
2	Ethanolic leaves	8 ± 0.9
3	Aqueous peels	3.9 ± 0.8
4	Aqueous leaves	2 ± 0.7
5	Positive control*	20 ± 0.8
6	Negative control**	0 ± 0

*Streptomycin = 20 µg/mL, ** DMSO.

According to present results, at 100 mg/mL concentration of extract, *Citrus limon* ethanolic peels showed the highest mean zone of inhibition (MZI) of 14.6 mm whereas ethanolic extract of leaves showed 8 mm MZI against test microbe. In case of aqueous extracts, 3.9 mm MZI was shown by aqueous extract of peels and 2 mm least MZI was shown by aqueous extract of leaves as

compared to the control antibiotic having 20 mm MZI on nutrient agar medium plate after 48 h of incubation.

The MIC of extract by the broth micro-dilution technique exhibiting significant activity is shown in Table III. The results of MIC revealed that ethanolic extract of *Citrus limon* at 25 µg/mL against *Salmonella enterica* showed best antimicrobial effect.

Table III. Minimum Inhibitory concentration of ethanolic extract of *Citrus limon* peels against *Salmonella enteritidis.*

Sr. No.	Concentration (µg/mL)	O.D at 630 nm
1	100	0.223 ± 0.01
2	50	0.162 ± 0.05
3	25	0.000 ± 0.04
4	12.5	0.432 ± 0.05
5	6.25	0.768 ± 0.04
6	3.12	0.862 ± 0.08
7	1.56	1.007 ± 0.05
8	0.78	1.103 ± 0.02
9	0.39	1.120 ± 0.01
10	0.19	1.127 ± 0.02
11	0.09	1.134 ± 0.00
12	0.04	1.163 ± 0.09

Figure 1 shows a gas chromatogram of ethanolic *Citrus limon* peels extract. Five components were identified: Heptanedioic acid, 3-methyl-dimethyl ester, 3, 3, 5-trimethylcyclohexan-1-ol, trimethylsilyl ether, 2, 2, 6, 6-Tetramethylthiabicyclohexane, 9, 12-Octadecadien-1-ol, (Z, Z)- and Nonadecanoic acid as shown in Table IV along with molecular formula, molecular weight and peak area.

However, analysis of *Citrus limon* ethanolic peels' cytotoxic effects was done. The plant extract concentration used in the MTT experiment had an IC50 value of 495.4 µg/mL (Fig. 2). This concentration was greater than the lemon plant extract's MIC value, which was 25 µg/mL, as shown in Table V, showing that the antimicrobial concentration was found to be safe for Vero cell lines.

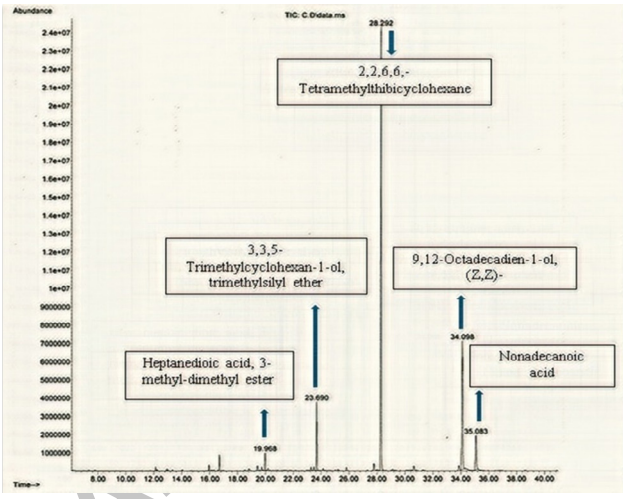

Fig. 1. Gas chromatogram of ethanolic *Citrus limon* peel extract (The five components in the extract were identified).

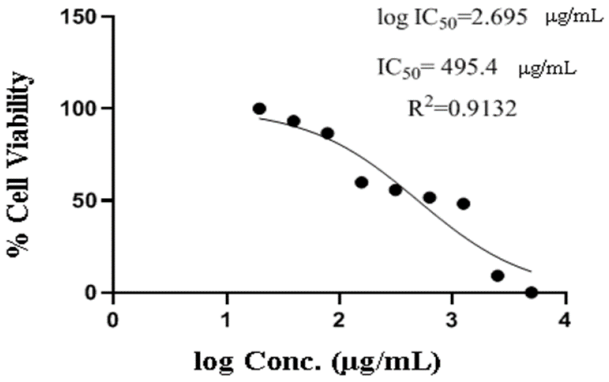

Fig. 2. Cytotoxic study of *Citrus limon* peels extract.

Table IV. Phytocomponents detected in ethanolic lemon peel extract by GCMS.

S. No	RT (min)	Phytocomponents	MF	MW (g/mol)	% PA
1	19.968	Heptanedioic acid, 3-methyl-dimethyl ester	$C_{10}H_{18}O_4$	202.247	2.04
2	23.690	3,3,5-trimethylcyclohexan-1-ol, trimethylsilyl ether	$C_{12}H_{26}OSi$	214.424	7.64
3	28.292	2,2,6,6, -Tetramethylthiasbicyclohexane	$C_{11}H_{24}$	156.313	58.18
4	34.098	9,12-Octadecadien-1-ol, (Z, Z)	$C_{18}H_{34}O$	266.462	24.67
5	35.083	Nonadecanoic acid	$C_{19}H_{38}O_2$	298.511	7.48

*RT, retention time; MF, molecular formula; MW, molecular weight; PA, peak area.

Table V. % Cell survival of ethanolic extract of *Citrus limon* peel.

Plant Name	Conc. µg/mL	O.D $_{590}$	% Cell survival	IC_{50}
Postive control	10	0.342	0.08	(µg/mL)
Citrus limon	5000	0.101	2.03	495.4±0.21
	2500	0.123	10.97	
	1250	0.217	49.18	
	625	0.225	52.43	
	312.5	0.235	56.50	
	156.5	0.245	60.56	
	78.13	0.309	86.58	
	39.06	0.325	93.08	
	19.53	0.341	99.59	

After the modern era of pharmacological industries underwent a revolution, the indiscriminate use of antibiotics led to the emergence of resistance, which prompted the consumption of natural plant extracts along with their subordinate metabolites as an impending antimicrobial alternative to synthetic antibiotics. Our research aims to assess the in vitro anti-Salmonella activity of several *Citrus limon* medicinal plant components. In line with our findings, prior research examined the presence of key phytochemicals such as alkaloids, saponins, glycosides, tannins, and flavonoids in lemon peel extract (John *et al*., 2017). These secondary metabolites might interpret antibacterial activity but it could be difficult to associate their mode of action to a particular phytochemical. Researchers while studying phytochemical analysis of Citrus plants showed that phenols could function as antioxidants, anti-inflammatory and antimicrobial. Flavonoids act as chemical messengers, regulators and the inhibitors of bacterial cell cycle (Ezeabara *et al*., 2013).

Antimicrobial activity assessed in a previous study reported that lemon peel extract has broad spectrum antimicrobial activity attributed to presence of essential oils: limonene and γ-terpinene that exert their lethal influence through the interference of the microbial membrane permeability and obstruction of respiration and ion- transport procedures (Moosavy *et al*., 2017). It is perceived that alteration in antimicrobial action could account for the distribution of altered phytocomponents in several parts of the plant or the extraction procedure and solvent used. The slight activity of aqueous extracts is due to the fact that water is a poor extracting solvent of all bioactive components that are liable for antimicrobial action (Kumar *et al*., 2011). Therefore, it could be inferred that activity of extract depends on availability of active constituents.

The antibacterial potential of lemon zest and juice against various microbial strains corresponding to gram positive and gram-negative types has been evaluated with a range of diameter 16 to 32 mm zone of inhibition (Makni *et al*., 2018). Our findings are in agreement with the results of ethanolic extracts of *Citrus limon* but contradict with those of aqueous extracts. The sensitivity of bacteria was linked to polyphenols, chief components of *Citrus limon* having the potential to inhibit enzymes responsible for energy production in bacterial cells and exterminate microbial growth.

In an analogous study the MIC of methanolic Citrus fruit juice concentrates was recorded against gram negative bacterial strains and was found to be 12.5 µg/mL for the *Pseudomonas aeruginosa* that is contradictory to present study; however, an MIC of 25 µg/mL was recorded for *Salmonella* spp. in the same study which corresponds with our results carried against test organism (Oikeh *et al*., 2016).

The GC-MS analysis revealed that for instance, 9, 12-Octadecadien-1-ol, (Z, Z)- compound was indicated in ethanolic extract of *Entada pursaetha* seed, an African herb demonstrated by scientists (Kalpana *et al*., 2012). It was proved to be a linoleyl alcohol, fatty alcohol, formed by the reduction of a linoleic acid owning antibacterial effect that correlates with present results of this study. Earlier studies reviewed various reports on antibacterial potential of long chain fatty alcohol and documented an emerging increase in activity with length of carbon chain (Hughes *et al*., 2008). Nonadecanoic acid, a conjugate acid of nonadecanoate acts as anticancer, antifungal and antibacterial. It was also identified in the chloroform extracts of *Ophiorrhizashendurunii* from South India. It had also found application in the field of metal lubrication and used as pheromones by insects (Rajan *et al*., 2016). The crude hexane extract of the *Heliotropium indicum*, a Thai folk medicine has earlier been reported for the presence of Heptanedioic acid, 3-methyl-dimethyl ester containing antibacterial activity that was in accordance with present findings (Machan *et al*., 2007). Hence, maximum antimicrobial activity of the ethanolic extract of *Citrus limon* peels is due to the presence of these components.

Researchers mainly focus on cytotoxicity of drugs before clinical utilization. IC_{50} value of 70.52 µg/ml after 80% confluency demonstrated that lemon leaves extract displayed substantial dose dependent inhibition of Sh-Sy5y cell lines that confirms its safety (Krishna *et al*., 2017) like lemon peel extract. Therefore, it may be concluded from the results above that lemon peel extract also had a

significant impact on mammalian Vero cell lines and could be used in future for various therapeutic purposes.

CONCLUSION

The current study utilizes the locally accessible by-products of *Citrus limon* that are inexpensive and possessed the strong antimicrobial potential against enteric pathogens. However, supplementary evaluation conducted with the pure components is mandatory for definite conclusion of the bioactive compounds contributing to antimicrobial activity. After proper pharmacological evaluation and clinical testing in an animal model, this discovery might serve as the foundation for the formulation of improved pharmaceuticals from plant extracts.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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