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RESEARCH ARTICLE

Citrus for wellness: Exploring the bioactive properties of *Citrus medica* fruit peel with emphasis on its anticancer, antioxidant, antimicrobial and anthelmintic properties

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Abstract

Citrus medica (Citron) is an underutilised plant consisting of various bioactive elements with numerous medicinal benefits. The present study aimed to evaluate the bioactive properties, including anthelmintic, antimicrobial, antioxidant and anticancer activities, of chloroform extract obtained from the of fruit peel of *C. medica*. The different types of phytochemicals present in the chloroform extract were analysed using GC-MS. The major components detected included n-hexadecanoic acid, octadecanoic acid, t-tetradecenal, 1-nonadecene etc. Anthelmintic study was conducted using *Eisenia fetida* as a test organism, revealing a significant anthelmintic effect in the *C. medica* fruit peel extract compared to the standard drug. Antimicrobial properties were assessed against five test bacterial and fungal strains. Antibacterial tests showed zones of inhibition ranging from 8 to 11 mm, while no prominent zones of inhibition were observed in antifungal tests. The DPPH assay demonstrated significant antioxidant properties of Citron fruit peel extract compared to the standard ascorbic acid. The Chloroform extract of citron fruit peel exhibited significant cytotoxic properties against FaDu (human hypopharyngeal tumour) cell line. The present study indicates the potential of the chloroform extract of *C. medica* fruit peel to be employed as an anthelmintic, antibacterial, antioxidant and anticancer agent. Hence, it emphasises the prominence that can be given to the dietary consumption of citrus fruit peel in various forms, such as dried peel, powder etc.

Keywords

Anthelmintic; antibacterial; antioxidant; anticancer; FaDu cell line; hypopharyngeal tumour; *Citrus medica*

Introduction

Herbal drugs have been used since ancient times for the treatment of parasitic infections in humans and could be valuable in preventing the advancement of resistance (1). In today's modern epoch of global industrialization and hectic intellectual practices, many Ayurvedic or traditional medicines have been replaced by allopathic medicines. Citron/ kimb, also known scientifically as *C. medica*, belongs to the Rutaceae family, comprising about 140 genera and 1300 species (2). It is a short tree with yellowish-orange round or oblong fruit, ranging in size from 8 cm to 12 cm in length. *C. medica* L. popularly known as 'Bara nimbu' in India, is also referred to as 'bijapura' in Ayurvedic literature (3). Since ancient time, herbal medicines

have been utilised to treat various human parasitic diseases. Essential oils from *C. medica* have been reported to show antifungal activity against some fungi. Additionally, *C. medica* have been found to be applicable for the treatment of diabetes and Alzheimer's disease (4).

Several parasitic worms and microorganisms are pathogenic or infectious to human beings. For instance, juvenile parasites attack individuals by infesting the gastrointestinal tract (GIT) or skin, advancing into other tissues where adult worms exhibit characteristic traits of tissue distribution. Drugs used for expel worms from the GIT are called anthelmintics. Many currently existing anthelmintic drugs are known to produce various side effects such as loss of appetite, nausea, abdominal pain, vomiting and diarrhoea (1, 5). Chemotherapy remains the effective tool to treat, cure, and control helminthic infections is, as effective vaccines against helminths have not yet been identified. The indiscriminate use of synthetic anthelmintics may result in resistance to parasites. Pathogenic bacteria persistently undergo mutations, gaining resistance to various drugs. Hence, there is a dire need to develop new potent drugs (4). Earthworms have been used for their anthelmintic activity due to their anatomical and physiological resemblance to the human intestinal roundworm parasite *Ascaris lumbricoides* and their easy accessibility (5-7).

One of the applications of natural products from medicinal plants is to utilise their antibacterial properties in treating various microbial diseases. Microbial infections are prominent causes of medical issues, physical inabilities and mortalities worldwide. Numerous species and hybrids belonging to the genus *Citrus* have been explored and are now well-established for their economic and pharmaceutical importance. People, in general, are becoming progressively aware of issues but are also being misguided due to the misuse of traditional antibiotics. Scientists have realised that the effective lifespan of antibiotics is limited owing to microbial resistance. Hence, this has necessitated the need for novel substances with antimicrobial properties to be extracted from various sources, including plants. These substances should have the least or no toxicity towards human cells but can either inhibit or kill microorganisms, making them potential candidates for developing new antimicrobial drugs (4, 5). Traditionally, *C. medica* L. has been claimed to possess anthelmintic properties, but this has not yet been scientifically demonstrated. Thus, the present study was designed to evaluate the *in-vitro* anthelmintic, antimicrobial, antioxidant and anticancer activities of the chloroform extract of *C. medica* L. fruit peel.

Citrus fruits were highlighted as the second most highly produced fruits in the world in 2021, accounting for about 161.8 million tons of Citrus fruits produced on more than 10.2 million hectares (8). According to this report, China, Brazil and India were identified as the highest producers of Citrus fruits, with India also being the world's largest producer of lemons and limes. The native lineage of Citron is in India (9) and different wild and semi-wild populations of *C. medica* predominantly occur in the forests at the foothills of the Himalayas. Additionally, the distribution of this fruit is spread across Indian states such as Assam, Bihar, Meghalaya, Odisha, Gujarat, Karnataka and Maharashtra (10).

The global production of Citron has been reported to be on a large scale. There are reports of mass-scale production of 'Ethrog' or 'Etrog' Citron in Israel due to its religious importance in Jewish communities. Another similar acidic variety of Citron called 'Diamante', resembling 'Ethrog' or 'Etrog', is largely cultivated in Italy. 'Corsican', a sweet variety of Citron, has been reported to be extensively produced by the French (11). Apart from the fruit, Citron peel also holds commercial significance in the global market. Constituting about 70% of the entire fruit, the Citron peel is thick and very rough in nature. The coloration of this peel varies based on the ripening stages of the fruit, from green when unripe to golden yellow when fully ripened Citron (12). Some of the commercially important products obtained from this fruit include liquors and candies. Additionally, oils are obtained on a minor scale, which are then used as flavouring agents in beverages and sweets (13).

Materials and Methods

Collection of sample

C. medica fruits were collected from their natural habitat, Mysore, Karnataka and authenticated by the Regional Ayurveda Research Institute for Metabolic Disorders, Bangalore. The average weight of these fruits was noted, and subsequently, they were washed and the peel was separated.

Extraction and analysis of phytochemicals

Fresh fruits of *C. medica* were transported to laboratory conditions and subsequently washed using distilled water. The fruit peel was removed and kept for drying for one week at 50 °C. Following this, 30 g of dried fruit peel was weighed, and then extracted successively using Soxhlet extraction method with 300 mL of chloroform. The extract was concentrated using a rotary evaporator at 50 °C and 153 rpm. The phytochemicals were analysed using GC-MS.

Anthelmintic activity

Earthworms (*Eisenia fetida*) were taken and carefully washed using normal saline to remove all faecal matter. Different concentrations (50 and 100 mg/mL) of *C. medica* L. chloroform extract were prepared using DMSO as the suspending agent and the final volume was adjusted to 10 mL. Albendazole (10 and 30 mg/mL) was used as the positive control. Normal saline served as the negative control and DMSO was used as the vehicle control. Two groups of roughly equal-sized earthworms (each consisting of two earthworms) were released into 10 mL of the desired concentrations of *C. medica* L fruit peel extract. Observations on the time taken for paralysis and death were noted. When the worms failed to be revived in normal saline, they were considered paralysed. The time of death for worms was recorded when their body colours began to fade and they stopped moving when shaken vigorously or submerged in warm water (50 ^{0}C) (1- 4, 14-15).

Antibacterial activity

The antibacterial activity of the chloroform extract of *C. medica* fruit peel against 5 bacteria (*Vibrio harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *Pseudomonas aeruginosa* and *Streptococcus* spp.) was evaluated using the agar well diffusion method. Petri dishes plated with Muller Hinton Agar (MHA) growth media were inoculated with 200 µL of an overnight culture of test bacteria and were spread plated using sterilised cotton swabs. Subsequently, a sterile borer was used to create wells with a 6 mm diameter in these MHA plates inoculated with the bacterial culture. Fifty µL of different concentrations (50 mg/mL and 100 mg/mL) of peel extract was poured into each well of the inoculated plates. A commercially available standard antibiotic (ampicillin 20 mg/mL) was used for the comparative study as the positive control. The plates were incubation at 37 °C for 24 h and subsequently observed for the diameters of the zone of inhibition (ZOI), which were measured and expressed in millimetres. The mean values of the ZOI were calculated to ascertain the antimicrobial activity of citron fruit peel extract (16, 17).

Antifungal activity

The antifungal activity of the chloroform extract of *C. medica* fruit peel against 5 different fungi (*Aspergillus* spp, *Penicillium* spp., *Trichoderma* spp., *Alternaria* spp. and *Fusarium* spp.) was evaluated through the agar well diffusion method. Fungal spores from each of these pure cultures were suspended in sterile water and inoculated into Martin's Rose Bengal Agar (MRBA) plates. These inocula were subsequently spread-plated using sterilised cotton swabs, and a sterile borer was used to create agar plate wells with a diameter of 6 mm in MRBA plates containing the fungal inoculum. Fifty µL of different concentrations (50 mg/mL and 100 mg/mL) of fruit peel extract was poured into each well of the inoculated plates. The plates were incubation at 27 °C for 48 h, after which they were observed for the diameters of the zone of inhibition (ZOI), which were measured and expressed in mm. The mean values of the ZOI were calculated to ascertain the antifungal activity of citron fruit peel extract (18, 19).

Antioxidant activity (DPPH assay)

This assay was performed to analyse the antioxidant properties of the chloroform extract of the fruit peel of *C. medica* according to the protocol by Blois 1958 (with modifications) (20). For this assay, different concentrations (30-900 µg/mL) of fruit peel extract were tested for their free radical scavenging activity (RSA) against the standard antioxidant, ascorbic acid. Various concentrations of the extract and standard were prepared in methanol. 1 mL of each of these test concentrations was mixed with 1 mL of 1 mM of DPPH reagent (prepared in methanol) and kept for incubation at room temperature for 30 min under dark conditions. Following this, the absorbance was noted at a wavelength of 517 nm and RSA was calculated based on the following formula:

$$\text{Radical Scavenging Activity (\%)} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Anticancer activity (MTT assay)

Citron fruit peel extract was tested to probe for its anticancer properties using the FaDu (human hypopharyngeal tumour) cell line. For this assay, DMEM growth media containing 10% FBS (Foetal Bovine Serum) was used to adjust the cell count to 5 x 10^5 cells/mL. Subsequently, 100 µL of the diluted cell suspension (50,000 cells/well) was added to each well of the 96 well microtiter plate. A monolayer was obtained upon incubation for 24 h, after which the supernatant was removed. This monolayer was washed once with DMEM medium and the cells in microtiter plates were treated with 100 µL of different test concentrations of Citron fruit peel extract, and drugs. These plates were incubation at 37 °C for 24 h with a 5% CO_2 atmosphere. After the incubation period, the test solutions were discarded, and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added per well. The plates were incubation at 37 °C for 24 h with a 5% CO_2 atmosphere. Subsequently, the supernatant from these plates was removed, and 100 µL of DMSO was added to solubilize formazan. The absorbance was then recorded at a wavelength of 590 nm using a microplate reader. The % of growth inhibition was calculated using the following formula and the dose-response curve of the cell line was used to generate the IC50 values and perform a nonlinear regression analysis:

$$\text{\% Inhibition} = \frac{\text{(OD of Control} - \text{OD of sample)}}{\text{(OD of Control)}} \times 100$$

Nonlinear regression analysis (curve fit) based on a variable sigmoid dose-response curve was performed to derive the IC50 values, and subsequently, these values were computed using Graph Pad Prism 6 (Graph pad, SanDiego, CA, USA).

Statistical analysis

All experiments were conducted in triplicates, and the data obtained was statistically analysed, expressed as mean values ±SD.

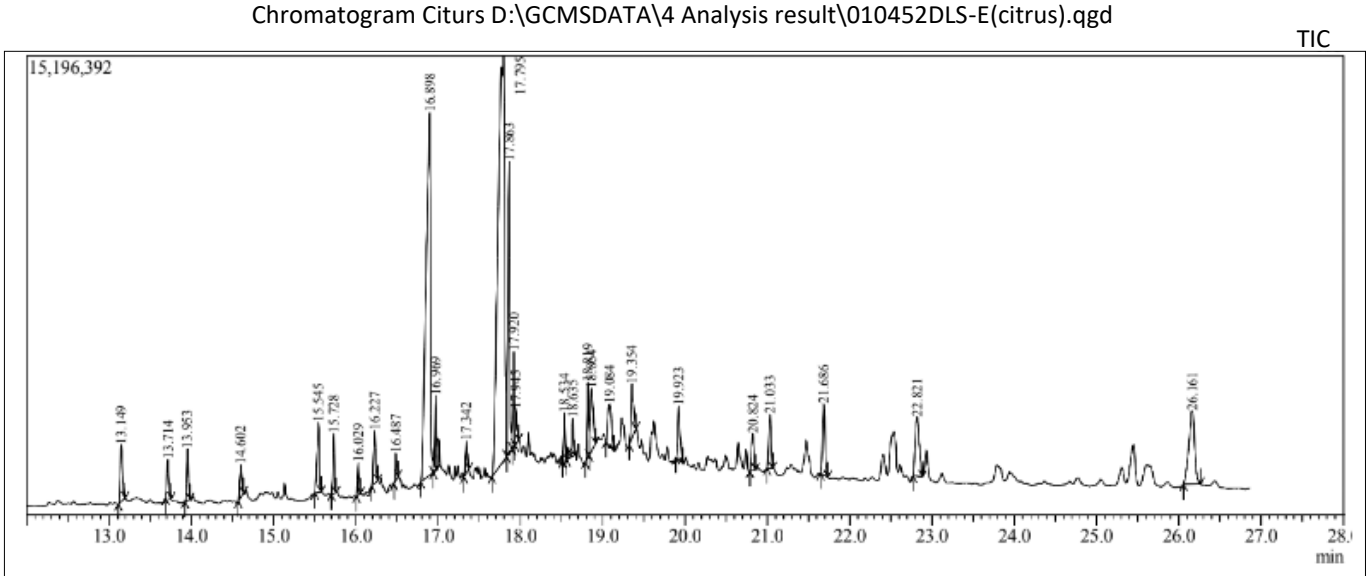
Results

Extraction of phytochemicals

C. medica fruit peel is known to be rich in phytochemicals. As depicted in Fig. 1, the present study also confirmed the presence of major phytochemicals through GC-MS analysis of the chloroform extract of Citron fruit peel. Key phytochemicals identified include n-hexadecanoic acid, octadecanoic acid, t-tetradecenal, 1-nonadecene etc.

Anthelmintic activities

The chloroform extract of *C. medica* fruit peel exhibited varying degrees of anthelmintic activity against worms, causing paralysis followed by death at all tested concentrations. As shown in Table 1 and Fig. 2, the extract of *C. medica* L. fruit peel demonstrated robust anthelmintic activity compared to the standard drug. Paralysis of earthworms occurred within 20 and 45 min when exposed to concentrations of 30 mg/mL and 15 mg/mL of *C. medica* L. fruit peel extracts respectively. The vehicle control DMSO



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	13.149	13.115	13.195	3345482	1.52	1883938	2.38	1.78		2,4-Di-tert-butylphenol
2	13.714	13.685	13.755	2084434	0.95	1291478	1.63	1.61		5-Chloro-2-(3,4-dimethoxybenzamido)benz
3	13.953	13.925	13.985	2350074	1.07	1716813	2.17	1.37		1-Heptadecene
4	14.602	14.570	14.635	1872329	0.85	1070308	1.35	1.75		6-Methoxychroman-2-one
5	15.545	15.500	15.580	4107627	1.87	2300570	2.91	1.79		Tetradecanoic acid
6	15.728	15.700	15.760	2386473	1.08	1990363	2.52	1.20		1-Nonadecene
7	16.029	16.005	16.055	1212729	0.55	1073924	1.36	1.13		Neophytadiene
8	16.227	16.190	16.270	2888246	1.31	1736663	2.20	1.66		Pentadecanoic acid
9	16.487	16.465	16.510	1107950	0.50	895233	1.13	1.24	MI	Ethanedione, di-2-pyridinyl-
10	16.898	16.790	16.940	43371083	19.70	12000428	15.18	3.61		n-Hexadecanoic acid
11	16.969	16.940	16.985	3139668	1.43	2499285	3.16	1.26	V	1-Nonadecene
12	17.342	17.315	17.375	1632308	0.74	1080847	1.37	1.51		Heptadecanoic acid
13	17.795	17.655	17.835	77381961	35.15	13409203	16.96	5.77		7-Tetradecenal, (Z)-
14	17.863	17.835	17.900	15798567	7.18	9669369	12.23	1.63	V	Octadecanoic acid
15	17.920	17.900	17.935	3663675	1.66	3155170	3.99	1.16	V	1-Nonadecene
16	17.945	17.935	17.980	1412016	0.64	1147263	1.45	1.23	V	Non-7-ynamide
17	18.534	18.510	18.560	1941130	0.88	1589552	2.01	1.22		cis-9-Hexadecenal
18	18.635	18.560	18.655	1860705	0.85	1247860	1.58	1.49		Cyclohexane, tetradecyl-
19	18.819	18.790	18.835	2905715	1.32	2485073	3.14	1.17		1-Heptacosanol
20	18.864	18.835	18.915	5274013	2.40	2080813	2.63	2.53	V	Succinic acid, di(neryl) ester
21	19.084	19.040	19.135	4158539	1.89	1399096	1.77	2.97		Carbonic acid, methyl ester, [(E)-3,7-dimetl
22	19.354	19.320	19.405	3907620	1.78	1800351	2.28	2.17		1-Heptacosanol
23	19.923	19.890	19.975	3429558	1.56	1778864	2.25	1.93		Heneicosyl trifluoroacetate
24	20.824	20.790	20.855	2122732	0.96	1222404	1.55	1.74		(Z)-3,7-Dimethylocta-2,6-dien-1-yl palmitat
25	21.033	20.995	21.065	3337052	1.52	1776853	2.25	1.88		(Z)-3,7-Dimethylocta-2,6-dien-1-yl palmitat
26	21.686	21.645	21.735	4885784	2.22	2350052	2.97	2.08		Squalene
27	22.821	22.770	22.885	6622808	3.01	1959048	2.48	3.38		(9Z,12Z)-(E)-3,7-Dimethylocta-2,6-dien-1-
28	26.161	26.055	26.265	11941312	5.42	2432008	3.08	4.91	MI	Vitamin E
				220141590	100.00	79042829	100.00			

Fig. 1. GCMS Chromatogram depicting the major peaks obtained corresponding to the phytochemicals detected in Citron fruit peel extract.

Table 1. Anthelmintic activity of the chloroform extract of *Citrus medica* fruit peel extract

Samples	Time for paralysis (in minutes)	Time for death (in minutes)
Saline	180	-
Albendazole (10 mg/mL)	66	90
Albendazole (30 mg/mL)	18	57
DMSO	180	-
C. medica L. fruit peel extract (15 mg/mL)	45	60
C. medica L. fruit peel extract (30 mg/mL)	20	48

and the negative control normal saline did not have any negative impact on the worms. According to the results, the chloroform extract of *C. medica* L. fruit peel exhibited significant anthelmintic activity in a dose-dependent manner compared with standard anthelmintic drugs. It was thus inferred that the *C. medica* L. peel comprised active constituents responsible for the anthelmintic activity of its chloroform extract.

Antifungal activity

The antifungal activity of *C. medica* L. fruit peel against test pathogens (*A. niger*, *P*enicillium spp., *Trichoderma* spp., *Alternaria* spp. and *Fusarium* spp.) was studied at different concentrations. It was observed that there was no significant reduction in fungal growth, leading to the conclsion that the components present in the chloroform extract of Citron fruit peel were not potent enough to inhibit fungal growth.

Antibacterial activity

The chloroform extract of Citron fruit peel exhibited good antibacterial activities. The fruit peel extract demonstrated inhibitory effects against all tested bacteria, namely *P. aeruginosa*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and *Streptococcus* sp. While the zone of inhibition pro-

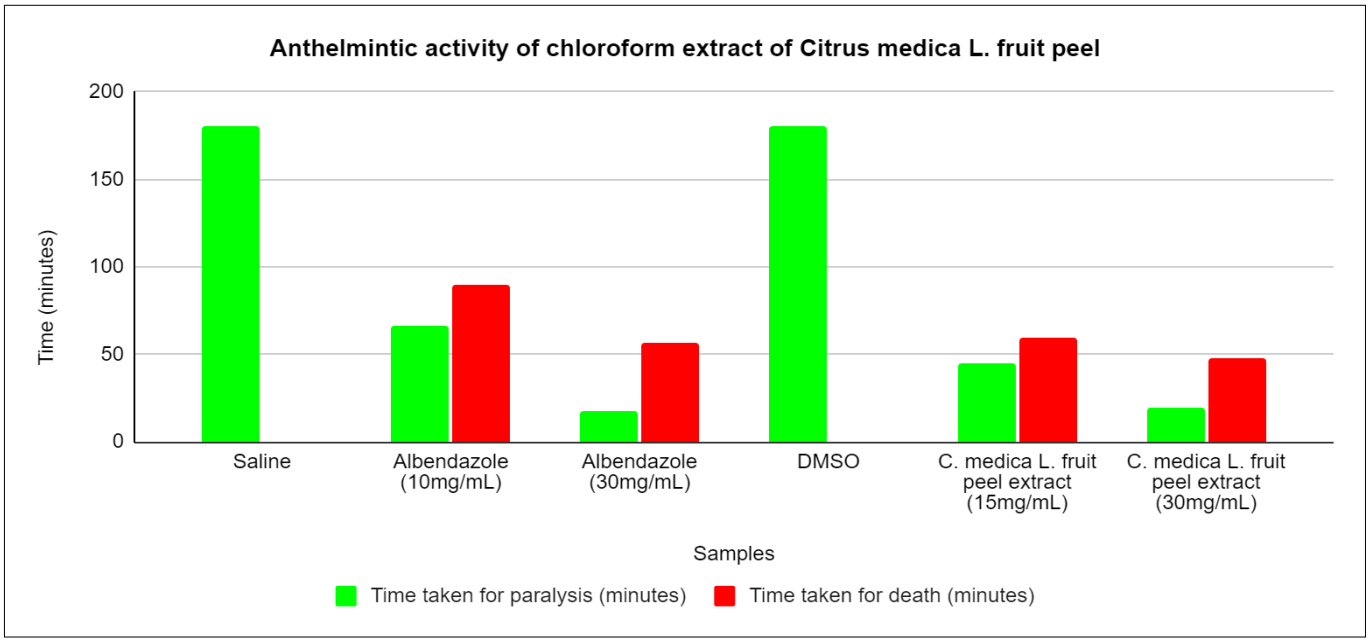


Fig. 2. Anthelmintic activity of chloroform extract of *Citrus medica* L. fruit peel.

duced by the fruit peel extract against these test organisms was lower than the positive control ampicillin, substantial antibacterial activity was observed in all these tested microorganisms (as depicted in Table 2). Among all organisms tested, the highest antibacterial activity was observed against *Vibrio* sp. and the least was observed against *Streptococcus* sp. Thus, it can be inferred that Citron fruit peel extract demonstrated higher antibacterial activity against the tested Gram- negative bacterial organisms compared to the Gram positive bacteria tested.

Table 2. Antibacterial activity of the chloroform extract of *Citrus medica* fruit peel extract

Bacterial species	Diameters of ZOI (mm)	
	Citron fruit peel extract	Ampicillin
Pseudomonas aeruginosa	10	19
Vibrio harveyi	11	20
Vibrio parahaemolyticus	11	20
Vibrio vulnificus	11	21
Streptococcus sp	9	18

Antioxidant activity (DPPH assay)

C. medica fruit peel extracts displayed notable antioxidant properties, comparable to the standard ascorbic acid (Fig. 3). The radical scavenging activity (RSA) increased with the rising concentrations of Citron fruit peel extract. It was observed that at the highest concentration of the fruit peel extract (900 µg/mL), a maximum RSA of 79% was achieved, while at its lowest concentration (30 µg/mL), the least RSA of 68% was observed.

Anticancer activity (MTT assay)

Citron fruit peel extract demonstarted significant antioxidant, antibacterial, antifungal and anthelmintic properties. Therefore, further investigation was conducted to assess its anticancer properties on the FaDu cell line (human hypopharyngeal tumour). The crude extract of Citron fruit peel exhibited anticancer properties against the FaDu cell line with an IC_{50} value of 124.4 µg/mL. As depicted in Fig. 4 and Table 3, increasing concentrations of this extract (10, 20, 40, 80, 160 and 320 µg/mL) corresponds to an increase in the percentage of cell growth inhibition in the FaDu cell line. Doxorubicin was used as the positive control for this assay.

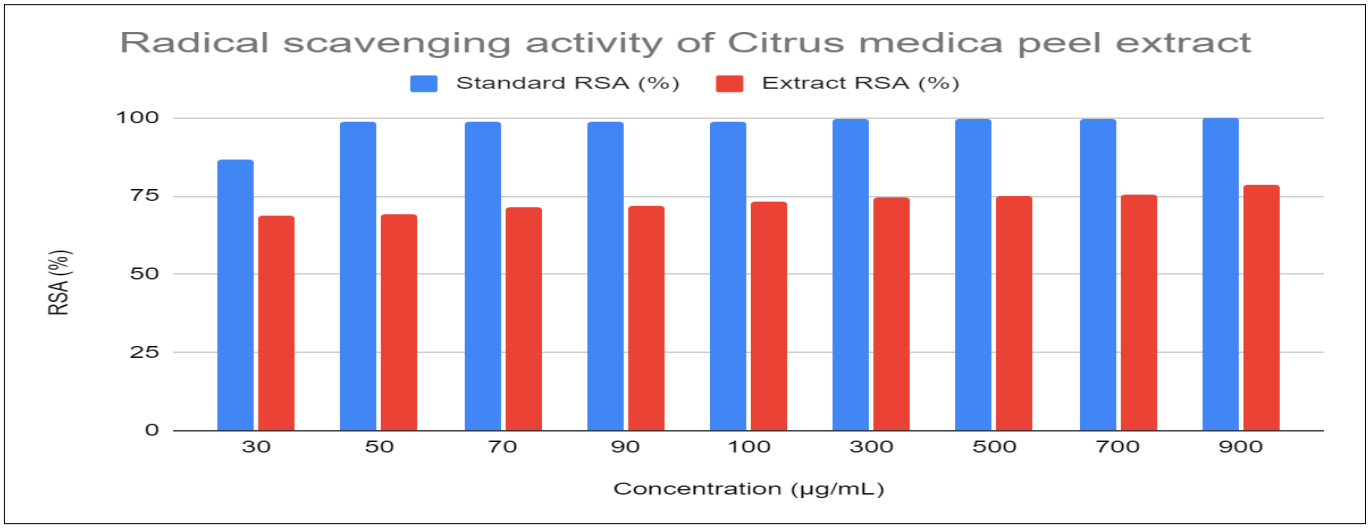


Fig. 3. Radical scavenging activity of *Citrus medica* L. peel extract.

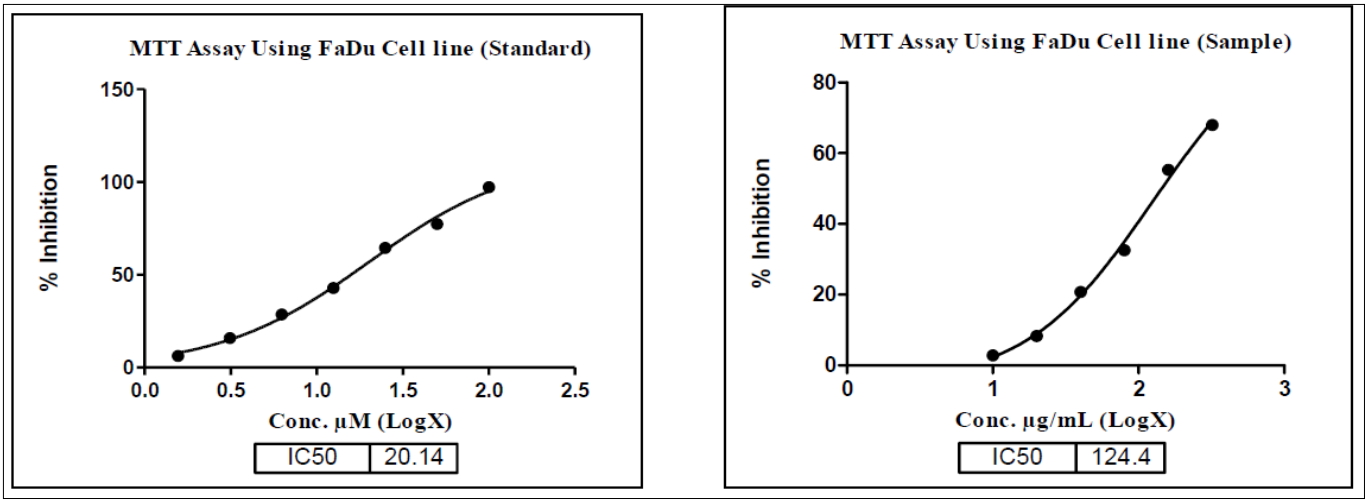


Fig. 4. MTT assay to test the anticancer properties of Citron fruit peel extract against FaDu cell line, inhibitory action by the standard drug Doxorubicin on the left and citron peel extract on the right.

Table 3. MTT assay to test the anticancer properties of Citron fruit peel extract against FaDu cell line

FaDu				
Compound name	**Conc. (µM)**	**OD at 590 nm**	**% Inhibition**	**IC_{50}**
Control	0	0.568	0.00	
	1.56	0.533	6.23	
	3.125	0.479	15.75	
	6.25	0.406	28.55	
Doxorubicin	12.5	0.325	42.81	**20.14**
	25	0.202	64.40	
	50	0.129	77.38	
	100	0.016	97.21	
Compound name	**Conc. (µg/mL)**	**OD at 590 nm**	**% Inhibition**	**IC_{50}**
	10	0.552	2.82	
	20	0.521	8.27	
	40	0.450	20.77	
Citron fruit peel extract	80	0.383	32.57	**124.4**
	160	0.254	55.28	
	320	0.182	67.96	

Discussion

The phytochemicals present in *C. medica* fruit peel, as investigated in our current study, exhibit various medicinal properties according to findings from different studies. N-hexadecanoic acid, is versatile and serves as a nematicide, antioxidant, pesticide, flavour enhancer, antifibrinolytic, haemolytic, lubricant and anti-alopecic agent. On other hand, octadecanoic acid demonstrates hypocholesterolemic, nematicidal, antiarthritic, hepatoprotective, antiandrogenic, antihistaminic, anti-coronary, insectifuge, anti-eczema and anti-acne properties (4-6). N-hexadecanoic acid, also known as palmitic acid, is an essential fatty acid vital in the dietary composition of birds, invertebrates and mammals. Despite its commercial importance, it occurs naturally and is found in various trees and plants (14, 21). Free fatty acids, including N-hexadecanoic acid, are known for their diverse bioactive properties, contributing to host defence against various potential pathogenic microorganisms.

Fatty acids functions as anionic surfactants and possess antibacterial and antifungal properties at low pH (22). Research has demonstrated that palmitic acid, at low concentrations, serves as an antiviral agent in animals. However, negative effects are observed at high concentrations, particularly in aquacultured fish (23). An alkylbenzene, 2,4-di-tert-butylphenol, is a member of the phenol class, featuring 2 tert-butyl substituents at positions 2 and 4. As a volatile compound, it plays various roles as a bacterial metabolite and an antioxidant marine metabolite. Octadecanoic acid, also known as stearic acid, stands as a potent anti-inflammatory lipid. This fatty acid exerts profound and diverse effects on liver metabolism, with previous studies confirming its antimicrobial activity (24, 25).

Succinic acid, cyclohexene etc., were among the other phytochemicals evidently found. The antiviral activity of these phytochemicals is well-established through numerous reports, with some studies indicating

their efficacy surpassing that of standardised drugs in certain cases. For instances, dehydro-andrographolide succinic acid monoester has been reported to inhibit viruses, including HIV (26). Cyclohexene has demonstrated higher potential against the Herpes simplex virus compared to acyclovir (27). Nonadecene, another identified compound, possesses antimicrobial compound with anticancer and antioxidant properties (28). While the medicinal properties of phytochemicals from plant extracts have been implicated in complex diseases such as Alzheimer's, hypertension etc. (29, 30), it is important to administer some plant-produced chemical with caution due to their potential inhibitory effects too (31).

The chloroform extract of *C. medica* fruit peel demonstrated significant anthelmintic activity, including paralysis and death of worms at all tested concentrations. Both *in vivo* and *in vitro* studies on anthelmintic activity of various citrus fruits have consistently revealed their notable effects when compared to standard drugs (14). Ethanol extracts from different citrus fruits, such as *Citrus aurantifolia*, *C. limon*, *C. reticulata* and *C. sinensis* have demonstrated substantial anthelmintic activities. Additionally, seed coats of *C. sinensis* fruit have been reported to exhibit significant anthelmintic activity (4, 19, 20).

Conversely, the antifungal activity of *C. medica* L. fruit peel was studied against various fungal pathogens, but no significant reduction in their growth was observed. Consequently, it was concluded that the components present in the chloroform extract were not potent enough to inhibit fungal growth. While antifungal activity has been proven for peel extract of different citrus fruits like *C. sinensis*, *C. aurantium*, *C. aurantifolia* etc., several studies have shown that *C. medica* fruit peel extract demonstrates the least antifungal activity (32-34).

Various studies have consistently reported fruit juice as the most effective antibacterial and antifungal agent, followed by juiceless pulp extract. The varying sensitivity of test organisms to antimicrobial compounds can be attributed to factors such as the nature and combination of phytocompounds present in the phytochemical crude extracts and the intrinsic tolerance of these test microorganisms. Various phytochemicals such as phenols, flavonoids, alkaloids, glycosides tannins and acids, are known to be present in some of these extracts, displaying antimicrobial activity (18, 35, 36). The chloroform extract of Citron fruit peel exhibited potent antibacterial activities, inhibiting all tested bacteria, including *P. aeruginosa, V. harveyi, V. parahaemolyticus, V. vulnificus* and *Streptococcus* sp. The highest activity was observed against *Vibrio* sp., while the least was observed against *Streptococcus* sp. Previous studies have also demonstrated the anti-Vibrio effect of plant extracts (37, 38).

Studies on *C. aurantifolia* Linn. have demonstrated that the hydro alcoholic extract of its leaves exhibits antibacterial activity against *Pseudomonas* spp, *Staphylococcus aureus* and *Klebsiella pneumonia* (30). Essential oils from *C. acida* have also shown antibacterial activity against a range of bacteria including *Bacillus cereus*, *Escherichia coli*, *S. aureus*, *Bacillus subtilis*, *Salmonella typhimurium* and *Enterobacter aerogenes* (39). Peels of *C. sinensis* and *C. aurantium* are other rich sources of antibacterial components.Notably, *C. sinensis* peel extract has exhibited superior antibacterial activity compared to *C. aurantium* peel extract (40). Additionally, fruit peel extracts have been reported to exert stronger activity against Gram negative than Gram positive bacteria(41, 42).

Antibacterial properties of fingered Citron (*C. medica*) have been reported, indicating that fingered essential oil (FEO) exhibits the strongest antibacterial activity against *S. aureus*, Sichuan citron essential oil shows high antibacterial activity against *E. coli* and Cantonese Citron essential oil displays potent antibacterial activity against *Enterococcus faecalis*. These reports suggest the potential of Citron essential oils for use in nutraceutical or pharmaceutical products, such as natural drug candidates, antibacterial agents, and food additives (33). A similar study by Li *et al*. reported the antibacterial properties of *C. medica* L. var. *sarcodactylis* derived FCEO (finger citron essential oil) against food borne bacteria. This study demonstrated antibacterial activity of FCEO towards *E. coli*, *S. aureus*, *B. subtilis* and *Micrococcus luteus*. FCEO exhibited higher antibacterial activity against Gram-positive bacteria than Gram-negative ones. The antibacterial mechanisms of FCEO were found to involve a significant reduction in bacterial growth rate, mediated by cell wall lysis, intracellular cell-ingredients leakage, and cell lysis (34). The rind of *Citrullus lanatus* var. *citroides* fruit showed good biological activity, with a significant decrease in biological activity observed at higher temperature. Noteworthy antibacterial properties were observed against different Gram-negative opportunistic pathogens in the flesh and seeds of this fruit (35). The present study aligns with these findings, reporting good antibacterial activity of Citron fruit peel extract, consistent with similar studies involving antibacterial activity of citrus fruits. Plant extract nanoparticles enhance antimicrobial effects, and the green synthesis of nanoparticles and nanocatalysts holds valuable industrial applications .

Citron fruit peel extract exhibited significant antioxidant activity with an RSA of 79% at 900 µg/mL fruit peel extract. A study by Florinda Fratianni *et al*. reported an RSA of 50% in citrus peel ethanolic extract after 60 minutes of incubation (43). The antioxidant activity of Citron fruit peel extract obtained in this study is comparable to findings in earlier reports. A notably high RSA of 94.07% was obtained in pectic oligosaccharide extract of finger citron pomace (*C. medica* L. var. *sarcodactylis* Swingle), which is comparatively higher than the results obtained in this experiment (39). In a study conducted by Chen Q. *et al*., the highest antioxidant activity of 80% was found in *Citrus reticulata* (Shiyueju) peel extract, very similar to the RSA values reported in this experiment (79%) (44).

The citron fruit peel extract demonstrated potential anticancer properties against the FaDu cell line, with an IC_{50} value of 124.4 µg/mL. The results indicated that as the concentration of the extract increased (10, 20, 40, 80, 160 and 320 µg/mL), there was a corresponding increases in

the percentage of cell growth inhibition in the FaDu cell line. In the ongoing search for natural compound-based remedies for various disorders and ailments, fruit extracts have been widely used for treating different diseases. Citrus plants, in particular, produce a diverse range of bioactive compounds such as coumarins and furanocoumarins, which aid the plants in defending against herbivorous insects and pathogens. Coumarins, among their various biological properties, are known to exhibit antibacterial, antioxidant, anti-platelet-aggregating and anti-tumour effects. The antiproliferative properties of 5-Geranyloxy-7-methoxycoumarin against SW480 human colon cancer cell, compared to citropten and isopimpinellin have also been reported. Furthermore, the unique furocoumarin, Oxypeucedanin, found in citron, has been reported to possess anticancer properties when tested against DU145 human prostate carcinoma cell (45).

Citrus flavonoids have been reported to have inhibitory effects against a range of cancer types, including breast, rectal, lung, liver, uterine, ovarian, prostrate and gastric cancer. These compounds have demonstrated the ability to prevent cancer cell proliferation, migration, angiogenesis and induction of apoptosis (46). The antimutagenicity of *C. medica* fruit juice against the human astrocytoma cell line has revealed high anticancer effects of this fruit juice, particularly in half-ripe *C. medica* when compared to its ripened fruit (47). Various compounds, such as 3,5,6,7,8,30,50-hepta methoxyflavone, identified from citrus fruit peels, have shown significant anticancer properties, with IC50 values of 57 µg/mL obtained from compounds extracted from *C. canaliculata*, 31 µg/mL from *C. tamurana* and 45 µg/mL from *C. kinokuni* and *C. tachibana* (48). Other reports have demonstrated the anticancer effects of *Citrus hystrix* (Kaffir lime) leaf extract against cervical and neuroblastoma cell lines, with IC50 values of 17.6, 18.9, 6.4 and 9.4 µg/mL against HeLa, UKF-NB3, IMR5 and SKNAS parental cells (49). Extracts of *C. aurantium* have also been reported to exhibit high anticancer properties against Chang liver, MCF7, MDA-MB231 and HT29 cell lines with IC50 of 30 µg/mL (50). The present study has demonstrated the anticancer properties of Citron fruit peel extract against FaDu cell line, a less explored type of human cancer cell line. Although this extract exhibited significant anticancer properties, further isolation of specific bioactive compounds from this crude extract will help elucidate its specific antiproliferative properties against various cancer types.

Conclusion

The current study investigated the biological properties of Citron fruit peel extract. As an underutilised plant rich in numerous phytochemicals with various advantageous properties, *C. medica* comprises bioactive compounds demonstrating antimicrobial, antioxidant, anthelmintic and anticancer activities. Naturally synthesised bioactive compounds, as seen in the study, offer better efficacy and bioavailability compared to their synthetic or allopathic counterparts, with the added benefits of showing minimal toxicity humans/ animals and the environment. The fruit peel extract of *C. medica*, as evidenced by the GCMS chromatogram, contains a variety of phytochemical compounds contributing to its antibacterial, anthelmintic, antioxidant and anticancer properties. Therefore, *C. medica* stands out as a rich source of phytocompounds, making it a potential candidate for further exploration of its cytotoxic and antioxidant properties in a sustainable manner.

Novelty Statement

The field of natural medicine has gained substantial attention, especially in light of recent global pandemics and the increasing prevalence of lifestyle-associated disorders. Regarded as a potent, less toxic, and safer alternative to allopathy, natural medicine extracts lead drug compounds from organic and naturally occurring sources. With advancing technologies, research in this field aims to characterize novel bioactive compounds from plant and microbial sources, demonstrating diverse biological properties. Against this backdrop, our study focuses on exploring the remarkable biological properties of *C. medica*, a globally prevalent citrus plant recognized for its therapeutic values. Despite being native to India, this plant remains underutilized and less explored for its extensive range of benefits. Preliminary characterization of phyto-compounds from Citron fruit peel extract through GC-MS analysis revealed various compounds like n-hexadecanoic acid, octadecanoic acid, t-tetradecenal, 1-nonadecene etc., known for their noteworthy properties documented in the literature. The chloroform extract of Citron fruit peel was subsequently analysed for its biological properties, unveiling significant anthelminthic, antimicrobial, antioxidant and anticancer activities. Naturally synthesized bioactive compounds, as observed in our study, offer enhanced efficacy, bioavailability and minimal toxicity towards humans, animals and the environment compared to their synthetic counterparts. This study underscores the importance of researching key plant species with myriad therapeutic applications. *C. medica*, a globally prevalent yet less explored plant native to India, emerges as a rich source of phytocompounds, positioning it as a promising candidate for further exploration of its cytotoxic and antioxidant properties in a sustainable manner.

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Authors contributions

SS conceptualised and supervised the research design and experimental planning. SA and AP carried out sample collection and chloroform extraction of Citron fruit peel extracts. SJ, SB, IP and AA did the assays. All authors were involved in the manuscript preparation and editing.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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