



Phytochemical, Antimicrobial and Acute Toxicity Studies on Methanolic Extracts of *Citrus medica* L. and *Citrus hystrix* D. C. Fruits

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Authors' contributions

This work was carried out in collaboration among all authors. Author DN designed the study, performed the experiment, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author HD help in performing acute toxicity experiment. Author BD managed the analyses of the study and corrected the paper. Author MDD finalized the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study aims to investigate the phytochemical, antimicrobial and acute toxicity assay of methanol extract of *Citrus medica* L. fruit (CMF) and *Citrus hystrix* D.C. fruit (CHF).

Place and Duration of Study: Fruit samples were collected between February to August 2018, at the Department of Life Sciences, Manipur University.

Methodology: Phytochemical studies were conducted using Gas Chromatography-Mass Spectrometry (GC-MS), HR-LC-MS (High Resolution-Liquid Chromatography-Mass Spectrometry), Graphite Furnace-Atomic Absorption Spectrometry (GF-AAS), and Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) respectively. The standard filtered disc-diffusion method was used for antimicrobial assay. Acute toxicity was performed using 423-OECD guidelines.

Results: GC-MS and HR-LC-MS analysis showed presence of Ranitidine, 4-Methylesculetin, Diosmin and Avobenzone in CMF whereas 9-Octadecenamamide, Gamma-Sitosterol, n-Hexadecanoic acid, 2-Methoxy-4-Vinylphenol, Rhoifolin, Diosmin and Phytosphingosine in CHF.

GF-AAS and ICP-OES study prominently showed Pb content in both the samples. Highest element in CMF was Pb (4.26 ± 0.120 ppm) while in CHF was Cr (4.35 ± 0.70 ppm). Antimicrobial study exhibited highest inhibitory effect of CMF against *Staphylococcus aureus* and *Klebsiella pneumonia* while CHF against *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus* than Gentamicin ($p < 0.05$). No toxicity behaviour and mortality in mice were observed during acute toxicity study period even at a dose of 5000 mg/kg body weight. Changes in serum constituent level were observed however, no genotoxicity was recorded.

Conclusion: We concluded that CMF and CHF cultivation site selection should be the first step to avoid Pb content. The CMF and CHF have many health beneficial constituents. From this study also concluded that CMF and CHF may be a potential source of antiulcer, antimicrobial, antiarthritic, diuretic, antiinflammatory and anticancer effects. However, further study to understand whether changes in serum constituent level for prolonged period usages as medicine or nutraceuticals is highly recommended.

Keywords: *Citrus medica* fruit; *Citrus hystrix* fruit; antimicrobial and acute toxicity.

1. INTRODUCTION

Manipur is heavily endowed with abundant natural resources and is home to millions of medicinal plants. Various plants, including those used by the traditional medical practitioner, grow luxuriantly in Manipur, North-East India, within the Indo-Burmese mega-biodiversity hot-spot. Since the early civilization, the living population of this region has been widely using numerous medicinal plants to treat many diseases. The numerous medicinal plants used in Manipur include a variety of *Citrus* species. *Citrus medica* L. fruit is a rich source of numerous essential bioactive constituents however it is underused among the *Citrus* genus [1]. Similarly, *Citrus hystrix* D.C. fruit is also an underutilized fruit. The juice of *Citrus* fruits had roles in controlling inflammation and oxidative stress, and also in supporting innate as well as acquired immune responses [2]. A large amount of flavonoids had isolated and systematically studied for numerous biological activities, including anticancer, antiviral, antibacterial, antioxidant, analgesic, antiinflammatory, antidiabetic, antiulcer and protective effect of dyslipidemia, atherosclerosis and endothelial dysfunction [3-4]. *Citrus* species are richly available in India; thus, they stood in a great position as the "Citrus belt of the world" [5]. 17 species of *Citrus*, 52 cultivars, 7 hybrids, and a total of 33 species of *Citrus* had reported from Manipur ($23^{\circ}50'$ to $25^{\circ}42'$ N latitudes and $92^{\circ}58'$ to $94^{\circ}45'$ E longitudes) [5-7]. The taste of the *Citrus* fruits found in Manipur ranges from sweet to sour (acidic); some are generally consumed as edible fruit, while some are well known for their use in traditional medicine [5]. Among the varieties of *Citrus*, the fruit of *Citrus medica* L., also known as Citron, is widely used to treat urolithiasis, especially calcium oxalate stone [8].

However, the fresh juice of *Citrus medica* L. fruit increased the antiurolithiatic inhibitors while decreasing antiurolithiatic promoters though it does not directly affect the calcium oxalate kidney stone [8]. *Citrus medica* L. is also utilized as a digester to treat stomach problems [9]. The evaluations of phyto-chemical constituents of plants have been of use in investigating and assessing the possible therapeutic potentials of such plants. Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography with tandem mass spectrometry (LC-MS) have proven to be of significant advantage in plant phytochemical studies as it reveals in details of individual compounds present and also enables researchers to pin down to pharmacological properties to individual constituents [10-11]. So far, the phytochemicals identified from *Citrus medica* L. fruit are alkaloids, carbohydrates, flavonoids, tannins, steroids, phenols, amino acids and cardioactive glycosides [12]. The flavonoids isolated from *Citrus* fruits are acacetin 3,6-di-C-glucoside, apigenin 8-C-neohesperidoside, dihydrokaempferol 3-O-rhamnoside (engeletin), 8-prenylnaringenin, cosmosiin, diosmin, quercetin, didymin, dihydrokaempferol, excavaside A and B, glychalcone, glyflavanone, hesperidin, kaempferol, lemairone, marmesin, myricetin 3-O- β -D-rutinoside, naringin, nobiletin, rhoifolin, rhamnetin, rutin, tangeretin, and others [3] [13]. Besides, *Citrus hystrix* D.C. also known as *Citrus macroptera*, is one of the endangered *Citrus* species; they exist in their natural habitat in Northeast India [14]. In Manipur, it has been used to prevent kidney stone formation [15]. The CHF has pharmaceutical effects, such as antioxidant, antitumor, antiinflammation, antihemolytic, acetylcholinesterase, β -glucuronidase, peroxidation, tyrosinase, α -

amylase, and α -glucosidase inhibitory potentials [16]. This fruit is rich in phenolic compounds, flavonoids, glyceroglycolipids, α -tocopherol, limonoids, furanocoumarins, benzenoid derivatives, quinolinone alkaloids, β -pinene, geranial, d-limonene, terpinen-4-ol, sabinene, citronellal, bergamottin, 5-[(6', 7;-dihydroxy-3',7'-dimethyl-2-octenyl)terpinenepsoralen and oxypeucedanin [17, 18].

The use of medicinal plants without knowing its detailed phytochemical and toxic effects is unsafe. Thus, this study analyses phytochemical, antimicrobial and acute toxicity assay (using mice as a model organism) of indigenous *Citrus medica* L. fruit (CMF) and *Citrus hystrix* D.C. fruit (CHF) found in Manipur.

2. MATERIALS AND METHODS

2.1 Collection and Identification

The plant samples were collected from different locations of Manipur during their growing seasons. Herbariums were prepared for each plant sample, and identified at the Botanical Survey of India, Shillong. The part of the samples, month of collection and BSI identification letter number are provided at Table 1.

2.2 Sample Preparation

Fresh pulp of 100 gm of *Citrus medica* L. and *Citrus hystrix* D.C. fruits were measured separately. 500 mL of methanol was used as the solvent of extraction. Soxhlet extraction was performed separately for each pulp sample, and the process took 3 h at 30°C - 40°C. The dried residue of CMF and CHF from Soxhlet extraction was obtained using Rotatory Vacuum Evaporator for 4 h. The water bath temperature of the Rotatory Vacuum Evaporator was maintained at 30°C. The dried residue was collected separately in a sterilized Eppendorf tube and stored at -20°C. The extract yield was calculated as yield (g / 100 g) = $(W_1 \times 100) / W_2$, where W_1 is the weight of the residue of the extract after removal of the Solvent and W_2 is the total weight of the pulp sample.

2.3 Phytochemical Analysis

2.3.1 GC-MS analysis

GC-MS analysis was carried out using GC-MS 5975 C Agilent. CHF and CMF extracts of 50 mg

dissolved in methanol were taken and injected with the volume of 1 μ l into the Column (J & W 122-5532, DM-5MS of 30 X 250 μ m X 0.25 μ m) by using a hot needle fixed in 10 μ l Hamilton syringe. Injection temperatures were set at 70°C for 3 min followed by 10°C/min to 300°C for 9 min and hold for 35 min by maintaining at a maximum temperature of 325°C. Helium was used as the carrier gas and operated at a constant flow of 1 mL/min. The split ratio was maintained at 10:1 with a flow of 14.464 mL/min. The chromatography was subjected to MS by a solvent delay of 4.00 min. MS results were matched with the National Institute of Standards and Technology Library source (NIST), and compounds were identified.

2.3.2 LC-MS analysis

HR-LC-MS was performed using a 1290 Infinity UHPLC system, 1260 infinity Nano HPLC with Chip cube, and TOF/Q-TOF Mass Spectrometer. 3 μ L methanol extract of CMF and CHF were infused in Column Luna (r) 5 μ m C18150 X 2 mm by injection with needle wash at a flow rate of 0.300 mL/min. Two mobile phase solvents were prepared; Solvent A was made up of 0.1 % formic acid, and solvent B was 90% acetonitrile acidified and 0.1 % formic acid. The flow rate of the solvents was maintained at 0.2 mL/min keeping the maximum pressure limit of 1200.00 bar. Running time for Solvent A was from 95% to 5% in 15 min, followed by 5% to 95% in 10 min until 15 min. At the same time, Solvent B was from 5% to 95% in 15 min and flowed with 95% to 5% in 10 min until 15 min. Positive ionization mode was used for MS analysis. MS was maintained at a range of 250 m/z to 1000 m/z at the scan rate of 1.00 spectra/sec.

2.3.3 GF-AAS and ICP-OES

Powdered *Citrus medica* L. and *Citrus hystrix* D.C. fruit samples (0.5 g) were separately digested using a Teflon digestion vessel taking 10 mL concentrated nitric acid. Digested samples were kept for 30 min to cool down. Further, digested sample solutions were diluted to 12 mL with concentrated nitric acid. The final volume of 50 mL was obtained by adding double-distilled water, and the solutions were filtered. The filtered solution was ready for elemental analysis using GF-AAS (Model: Analytik Jena Vario-6) and ICP-OES (Thermo Scientific™ iCAP™ 7600).

Table 1. Identification of the plant samples

Name of the sample	Part of the sample	Month of collection	BSI identification letter no.
<i>Citrus medica</i> L.	Flower	March	BSI /ERC / Tech / Identification / 2018 / 20
	Leave	June	
	Fruit	August	
<i>Citrus hystrix</i> D.C.	Flower	February	BSI /ERC / Tech / Identification / 2018 / 398
	Leave	June	
	Fruit	August	

2.4 Antimicrobial Assay

Plant extracts were dissolved in DMSO solution (stock solution) and 5% DMSO in water used for antimicrobial activity, MIC was identified using dilution series. The antimicrobial activities of the extracts were determined using the standard filtered disc-diffusion methods [19]. *Enterococcus faecalis* (Ef), *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sa), *Escherichia coli* (Ec), *Salmonella typhimurium* (St) and *Klebsiella Pneumonia* (Kp) were selected for the study. Gentamicin was used as a standard antibiotic.

2.5 Acute Toxicity Assay

Acute toxicity was performed using 423-OECD guidelines. The CMF and CHF extract was dissolved in 70% ethanol for studying acute toxicity assay. A sequential administration to single mice was performed to identify the appropriate dose of CHF and CMF extracts. 21 Male mice weighing 25-30 g were taken for the acute toxicity assay. The weight and urine pH of all the mice before and after the experiment were recorded. Four groups were arranged where each group consists of three mice for each of the CHF and CMF extracts. One group served as control. Thus, a total of 7 groups were made, and all the mice were given *ad libitum* of standard food and water. Extract dosages of 1000, 2500, and 5000 mg/kg body weight were given separately for both the extracts. CHF and CMF extracts were orally administered once and were observed for death, behavior changes, and other signs of toxicity within 24 h.

2.5.1 Genotoxicity

Colchicine of 2 mg/kg body weight was injected into the mice, and bone marrow cells were collected by flushing in KCl from both femurs. Cells were incubated for 18-20 min at 37°C, then fixed in the 1:3 (acetone: methanol) for 30 min in cold condition and centrifuged twice at 1500

RPM for 5 min. Chromosomal aberration was evaluated under a microscope by analyzing 100 well spread metaphase cells per animal after staining with 3% Giemsa in PBS.

2.5.2 Analysis of blood serum

Collected blood was transferred into a sterilized Eppendorf tube and centrifuge at 3000 RPM, and the supernatant was taken for calcium, sodium, urea, uric acid, creatinine, phosphorous and albumin level analysis. Serum analyses were performed using kits from Beacon Diagnostics Pvt. Ltd. (albumin and calcium), Peerless Biotech Pvt. Ltd. (phosphorous and sodium), Medsource Ozone Biomedicals Pvt. Ltd. (creatinine, urea, and uric acid).

2.6 Statistics

Results are expressed as Mean±Standard deviation. Antimicrobial results were analyzed by one-way analysis of variance (ANOVA). Serum calcium, sodium, urea, uric acid, creatinine, phosphorous and albumin level of mice were analyzed using a t-test. The P-Value that is less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Collection and Identification

Identification of the two samples revealed that both the samples belong to the same family, "Rutaceae", and genus "*Citrus*", and the name of the two samples are *Citrus medica* L. and *Citrus hystrix* D.C..

3.2 Sample Preparation

The yield of the plant samples obtained from 100 g of the CMF after removing solvent from soxhlet extract is 3.24±0.17 g while for CHF is 4.29±0.19 g (Fig. 1).

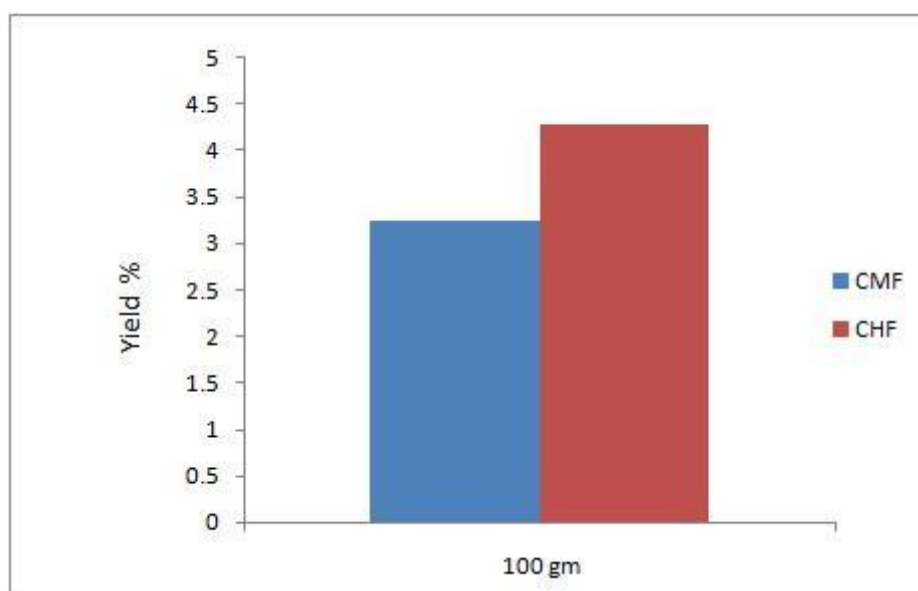


Fig. 1. The graph represents the extract yield of *Citrus medica* L. fruit (CMF) and *Citrus hystrix* D.C. fruit (CHF)

3.3 GC-MS Analysis

GC-MS study of CMF extract resulted presence of Pterin-6-carboxylic acid only with an area % of 1.500 and retention time of 9.018 min. The CHF extract reveals presence of 9-Octadecenamide, (Z) with an area % of 20.50 % and retention time of 20.920 min, Gamma-Sitosterol with an area % of 3.75 % and retention time of 28.074 min, n-Hexadecanoic acid with an area % of 3.14 % and retention time of 17.310 min and 2-Methoxy-4-vinylphenol with an area % of 2.56 % and retention time of 9.904 min.

3.4 LC-MS Analysis

On the other hand, the HR-LC-MS result of the CMF extract showed 12 metabolites while the CHF extract showed 9 metabolites with DB diff ranging between -0.1 to +1.58. The metabolites present in CMF and CHF are given in Tables 2 and 3. Similar metabolites present in both CMF and CHF are Tranylcyproamine glucuronide and Diosmin only.

Phytochemicals are non-nutritive plant chemicals, but they have defensive properties or are protective against many diseases; however, the dietary intake of phytochemicals resulted in numerous health benefits such as protection against chronic conditions diseases like cancer [20]. The phytochemical, either alone or with others (synergistic effects), gives tremendous therapeutic benefits to humans for curing many

diseases. *Citrus* fruits are well known for their beneficial properties, therefore not only used for pharmaceutical purpose but also utilized as nutraceuticals [21]. Thus, the study of phytochemical helps in knowing therapeutic benefits and could help promote nutraceuticals. In CMF, four bioactive compounds, i.e., Ranitidine, 4-Methylesculetin, Diosmin, and Avobenzon are present. Ranitidine and Diosmin had an inhibitory effect on gastric acid secretion [22] and protected against gastric injury [23]. At the same time, Avobenzon had antiinflammatory potential [24]. Thus, the effective use of CMF as an antiulcer might be due to the presence of Ranitidine and Diosmin. Besides the above three bioactive compounds, 4-Methylesculetin has antiinflammatory, antiarthritic activity, and diuretic properties [25]. 4-Methylesculetin presence in CMF might be the reason for the effectiveness of the treatment of uric acid stone due to its antiarthritic and diuretic potentials [25]. In the case of CHF, seven bioactive compounds, i.e., 9-Octadecenamide, Gamma-Sitosterol, n-Hexadecanoic acid, 2-Methoxy-4-Vinylphenol, Rhoifolin, Diosmin, and Phytosphingosine are present. The 9-Octadecenamide, (Z) has hypolipidemic [26] and antiinflammatory [27] potential. Gamma-Sitosterol showed cell proliferation inhibition [28]. The n-Hexadecanoic acid has antioxidant, antiandrogenic, hypocholesterolemic, hemolytic 5-Alpha reductase inhibitor [29,30], and antiinflammatory properties [31]. 2-Methoxy-4-vinylphenol has anticancer effects on cell lines of

pancreatic cancer [32]. Rhoifolin has antiproliferative effect on cancer cell lines [33], Diosmin has antidiabetic [34,35], anticarcinogenic [36] and protective effect of hepatic cells [37]. And, Phytosphingosine has an antiinflammatory effect on epidermal hyperplasia [38]. The compounds present in CHF highlight that they might be more suitable for cell proliferation inhibition or anticancer activity. The CMF and CHF phyto content is shown to be effective as a nutraceuticals.

Citrus medica juice is rich in flavonoids such as neoeriocitrin, naringin, neohesperidin, apigenin di-C-glucoside, diosmetin di-C-glucoside, rhoifolin and chrysoeriol 7-O-neohesperidoside [39]. In GC-MS and HR-LC-MS study of CMF no similar compounds were identified as compared with *Citrus medica* L. juice. The chemicals identified from GC-MS and HR-LC-MS of CMF are Pterin-6-carboxylic acid, Tranylcyromineglucuronide, Ranitidine, 2-Methoxyresorcinol, 4-Methylesculetin, Diosmin, O-Benzyl-L-Tyrosine, Fenamisol, 5H-Oxireno [4,5] furo [3,2-g] [1] benzopyran-5-one, 1a,8bdihydro-3-methoxy-, Citropten, Avobenzene and 12beta-Hydroxy-3-oxo-5betacholan-24-oic Acid. The flavonoid content in CMF is Diosmin. The difference in phytochemical contents between *Citrus medica* L. juice and CMF might be due to difference in extraction process. On the

other hand, phytochemical study of *Citrus hystrix* D.C. fruit is few and in this GC-MS and HR-LC-MS study shown content of 9-Octadecenamide, (Z), Gamma-Sitosterol, n-Hexadecanoic acid, 2-Methoxy-4-vinylphenol,4-Hydroxy-6-methylpyran-2-one, Tranylcyromineglucuronide, 2H-Indol-2-one, 1,3-dihydro-4-[2-hydroxy-3-[(1-methylethyl) amino]propoxy]-, Rhoifolin, Diosmin, Buddleofl avonoloside, 2-Ethoxycarbonyl-5,7-Dihydroxy-8,3',4',5'-Tetramethoxyisoflavone, Demethylm ethoxsalen and Phytosphingosine. The flavonoids present in CHF are Rhoifolin and Diosmin.

3.5 GF-AAS and ICP-OES

Elemental study of *Citrus medica* L. fruit revealed the presence of 20 elements namely Li, V, Sn, As, Ti, Mg, Mo, Ni, Sr, B, K, Co, Cu, Na, Zn, Fe, Mn, Cr, Al and Pb. Out of all the elements, Pb (4.26±0.120 ppm) had the highest concentration. On the other hand, in *Citrus hystrix* D.C. fruit 18 elements i.e. As, Cu, Fe, Na, Pb, Sn, Ni, Mn, Zn, Co, Al, Li, Sr, V, K, B, Mg and Cr are found present where Mo and Ti are absent. Cr (4.35±0.70 ppm) had the highest concentration in *Citrus hystrix* D.C. fruit. The graphical presentation of elements content in the *Citrus medica* L. and *Citrus hystrix* D.C. fruits are shown in Fig. 2.

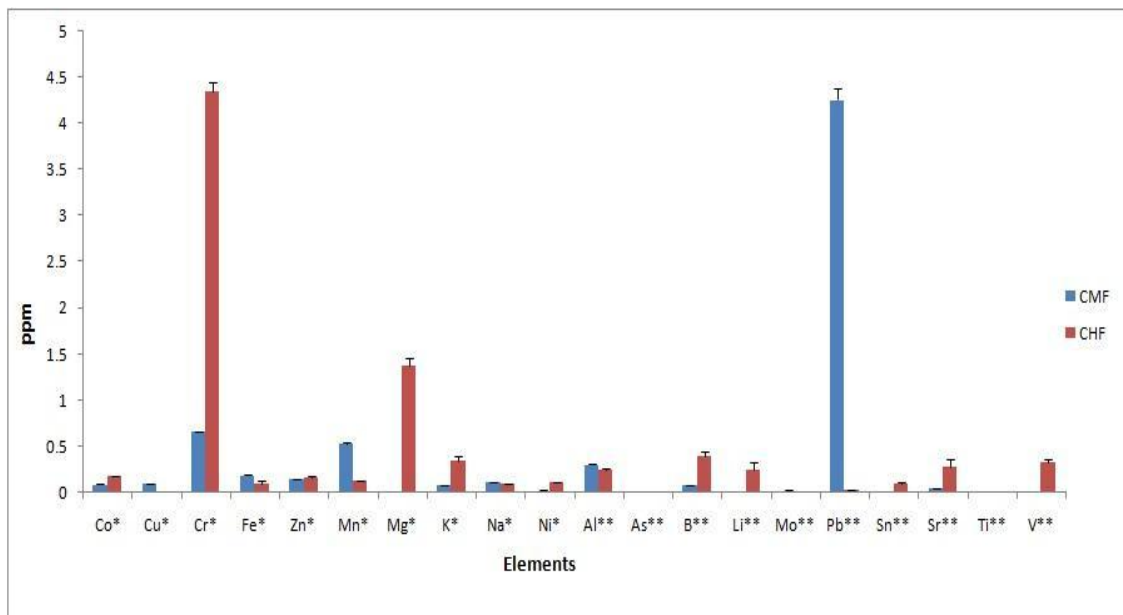


Fig. 2. The graph represents the elements content in *Citrus medica* L. fruit (CMF) and *Citrus hystrix* D.C. fruit (CHF). *GF-AAS and **ICP-OES

Table 2. Metabolite identified from the methanol extract of *Citrus medica* L. fruit by HR-LC-MS

Compound Name	RT	Molecular Formula	Molecular mass	Db diff (ppm)
Tranlycypromineglucuronide	2.321	C ₁₅ H ₁₉ N O ₆	310.12	0.76
Ranitidine	3.086	C ₁₃ H ₂₂ N ₄ O ₃ S	315.14	0.98
2-Methoxyresorcinol	5.78	C ₇ H ₈ O ₃	141.04	0.12
4-Methylesculetin	7.312	C ₁₀ H ₈ O ₄	193.04	0.95
Diosmin	9.022	C ₂₈ H ₃₂ O ₁₅	609.18	1.58
O-Benzyl-L-Tyrosine	9.457	C ₁₆ H ₁₇ N O ₃	272.12	0.27
Fenamisal	10.309	C ₁₃ H ₁₁ N O ₃	230.08	0.25
5H-Oxireno[4,5]furo[3,2-g][1]benzopyran-5-one, 1a,8bdihydro-3-methoxy-	10.852	C ₁₂ H ₈ O ₅	233.04	0.88
Citropten	13.518	C ₁₁ H ₁₀ O ₄	207.06	0.53
O-Benzyl-L-Tyrosine	16.843	C ₁₆ H ₁₇ N O ₃	272.12	-0.02
Avobenzone	26.704	C ₂₀ H ₂₂ O ₃	311.16	0.86
12beta-Hydroxy-3-oxo-5betacholan-24-oic Acid	32.567	C ₂₄ H ₃₈ O ₄	413.26	0.98

Table 3. Metabolite identified from the methanol extract of *Citrus hystrix* D.C. fruit by HR-LC-MS

Compound Name	RT	Molecular Formula	Molecular mass	Db diff (ppm)
4-Hydroxy-6-methylpyran-2-one	1.289	C ₆ H ₆ O ₃	127.03	-0.03
Tranlycypromineglucuronide	2.309	C ₁₅ H ₁₉ N O ₆	310.12	0.34
2H-Indol-2-one, 1,3-dihydro-4-[2-hydroxy-3-[(1-methylethyl) amino] propoxy]-	4.4 & 4.728	C ₁₄ H ₂₀ N ₂ O ₃	265.15	0.43 & -0.07
Rhoifolin	8.645	C ₂₇ H ₃₀ O ₁₄	579.17	0.85
Diosmin	8.897	C ₂₈ H ₃₂ O ₁₅	609.18	0.65
Buddleoflavonolside	9.863	C ₂₈ H ₃₂ O ₁₄	593.18	0.36
2-Ethoxycarbonyl-5,7-Dihydroxy-8,3',4',5'-Tetramethoxyisoflavone	10.507	C ₂₂ H ₂₂ O ₁₀	447.12	0.78
Demethylmethoxsalen	12.602	C ₁₁ H ₆ O ₄	203.03	0.33
Phytosphingosine	17.343	C ₁₈ H ₃₉ NO ₃	318.30	0.31

Studying elements in any medicinally used plants or fruits are an unavoidable step to avoid any adverse health effects. The elemental composition of indigenous plants is associated with the soil composition, climatic condition, agrochemical characteristics, rainfalls, atmosphere [40]. Elements in *Citrus medica* L. fruit with the highest concentration is Pb (4.26 ± 0.120 ppm) while the other elements are below 1 ppm. On the other hand, in *Citrus hystrix* D.C. fruit, Cr (4.35 ± 0.70 ppm) is found highest, followed by Mg (1.37 ± 0.08 ppm), and the other elements are below 1 ppm. The elements present in the *Citrus medica* L. and *Citrus hystrix* D.C. fruits are not beyond the average daily requirement as per the Dietary Reference Intakes (DRIs): Estimated Average Requirements and WHO permissible limit for heavy metals (for plants). However, in *Citrus hystrix* D.C. fruit, Pb is also present but at a low concentration. Other elements in *Citrus medica* L. and *Citrus hystrix* D.C. fruits are not beyond WHO's permissible limit. However, there is no safe limit of Pb as well, as there is no biological role of Pb in the human system, but its adverse effects have been known, such as damage to the kidney, nervous system, reproductive system [41]. Accumulation of heavy metals in plants, including in citrus leaves, fruits' peels, and fruits from the environment, had been reported already [42-44]. The element in plants and fruits depends on the soil and environment it grows, so it is indeed an important call to add a step to mitigate the toxic element to get the nutritional values from the fruits without any toxic elements.

Presence of Ca, P, Fe, Mg, K, Cu, Mn, Zn, Cr in *Citrus medica* L. fruit had been reported previously [39]. In this study Fe, Mg, K, Cu, Mn, Zn and Cr elements are also found present in *Citrus medica* L. fruit.

3.6 Antimicrobial Assay

Antimicrobial properties of CMF and CHF resulted in a wide range of inhibitory potentials. Gentamicin was used as the standard and had an antimicrobial effect against all six organisms; St (10.4 ± 0.55 mm), Ec (12 ± 0.71 mm), Kp (10.2 ± 0.44 mm), Sa (10.6 ± 0.89 mm), Ef (10.8 ± 1.3 mm), and Pa (10.8 ± 1.09 mm). The inhibition zone diameter was recorded in mm, and the MIC was noted and is given in Table 4. CMF was found to have antimicrobial activity against the Kp (13 ± 0.70 mm), Sa (17.8 ± 0.45 mm), Ef (10.4 ± 0.54 mm), and Pa (7.6 ± 2.19 mm);

where higher inhibitory zone was shown in Sa and Kp than the Gentamicin. CMF with the MIC value of 0.6 mg/mL had the most effective antimicrobial against the Sa. Whereas CHF was found to have antimicrobial activity against the St (10.4 ± 0.55 mm), Kp (12.4 ± 0.89 mm), Ec (10.4 ± 0.55 mm), Sa (22.2 ± 0.84 mm), and Pa (10.6 ± 0.55 mm); higher inhibitory zone was observed against Ec, Kp, and Sa than the Gentamicin. Here, CHF, also with the MIC value of 0.6 mg/mL, had the most effective antimicrobial against the Sa.

Antimicrobial inhibitory potentials of both the plant extract vary from one another; this could be due to the difference in phyto-chemical constituents. CMF has shown to have higher inhibitory potential than CHF against Kp, Sa, and Ef. In contrast, CHF had the highest inhibitory potential than CMF against Sa and Pa. The highest inhibitory antimicrobial activity of CMF and CHF was shown against Sa, i.e., CMF (17.8 ± 0.48 mm) and CHF (22.2 ± 0.84 mm). Thus, antimicrobial assay results in the idea that CMF might be more effective than CHF on struvite crystal growth due to its antimicrobial properties against Kp, Sa, Ef, and Pa, and the presence of 4-Methylesculetin, which have diuretic properties [25] that might resulted to reduce accumulation of bacterial growth by dilution. CMF exhibited higher antimicrobial inhibitory properties against Kp, Sa, and Pa at MIC values of 0.5 mg/mL and 0.6 mg/mL, respectively, than Gentamicin (5 mg). Therefore, both CMF and CHF might also be effective on urinary tract infection treatment, primarily caused by Ec, Kp, Sa, and Pa. On the other hand, CHF showed higher antimicrobial inhibitory activity against Ec, Kp, and Sa at MIC values of 0.5 mg/mL and 0.6 mg/mL, respectively, than Gentamicin (5mg). Ec, Kp, Sa, and Pa are well-known organisms for the cause of urinary tract infections [45]. Therefore, CMF and CHF might also effectively treat urinary tract infections caused by Kp, Sa, and Pa for CMF and Ec, Kp, and Sa for CHF.

Citrus medica L. fruit juice and ethanolic extract of *Citrus medica* L. fruit has shown to have antimicrobial property against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* [46]. This study shows that CMF is also known to have antimicrobial activity against Sa, Eg, Kp and Pa. However, CMF does not exhibit antimicrobial activity against Ec unlike that of fruit juice and ethanolic extract of *Citrus medica* L..

Table 4. Antimicrobial inhibition activity of Gentamicin, methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruit against *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*

Micro-organism Name	Inhibition zone (mm)			MIC (mg/mL)		
	CMF	CHF	G	CMF	CHF	G
<i>Salmonella typhimurium</i> (St)	Nil	Nil	10.4±0.55	Nil	Nil	5
<i>Escherichia coli</i> (Ec)	Nil	12.4±0.89**	10.4±0.9	0.6	0.5	5
<i>Klebsiella pneumonia</i> (Kp)	13±0.70**	10.4±0.55	10.2±0.44	0.5	0.5	5
<i>Staphylococcus aureus</i> (Sa)	17.8±0.45**	22.2±0.84**	10.6±0.89	0.6	0.6	5
<i>Enterococcus faecalis</i> (Ef)	10.4±0.54	Nil	10.8±1.3	0.6	Nil	5
<i>Pseudomonas aeruginosa</i> (Pa)	7.6±2.19**	10.6±0.55	10.8±1.09	0.6	0.6	5

**P <0.001 significant from Gentamicin. Inhibition zone values are represented as mean±standard deviation; n=5. (Abbreviation: CMF – Methanol extract of *Citrus medica* L. fruit, CHF – Methanol extract of *Citrus hystrix* D.C. fruit, G - Gentamicin. MIC - minimum inhibitory concentration.)

On the other hand, the methanol extract of dry pulp of *Citrus hystrix* D.C. revealed to have high antimicrobial potential against the *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Sarcina lutea*, *Salmonella paratyphi*, *Shigella dysenteria*, *Sh. boydii*, *Vibrio mimicus* and *Vibrio parahaemolyticus* [47] [48] [16]. CHF also exhibited antimicrobial properties against Ec, Kp, Sa, Ef and Pa.

3.7 Acute Toxicity Assay

The weight of all the mice recorded before and after the experiment remained constant. There were no changes of urine pH to those administered CMF while a slight change of urine pH of those 1000 mg/kg CHF administered mice were observed (6.17±0.29 to 6.33±0.29 pH); however, overall urine pH was between 6.1-6.2 pH, which lies within the normal urine pH range.

In the acute toxicity studies, no death was observed during the treatment period on both the CHF and CMF administered mice for all of the doses. All the mice showed no sign of toxicity at all the given doses and looked healthy even at the highest dose i.e., 5000 mg/kg body weight. Thus, this shows that LD₅₀ was more than 5000 mg/kg body weight.

In the genotoxicity study, no chromosome breakage was observed as well as no missing

chromosome was recorded. Thus, no chromosomal aberration occurred in each of the mice administered with CHF and CMF.

The serum calcium, sodium, urea, uric acid, creatinine, phosphorous, and albumin levels of mice after administration of CMF and CHF are shown in Figs 3, 4, 5, 6, 7, 8 and 9.

The serum calcium and sodium level of both extract administered mice showed significant differences compared with the control. The serum calcium level of mice administered with CMF and CHF was significantly higher than the control. Serum sodium level of mice administered with 1000 mg/mL of CMF, 1000 mg/mL of CHF, and 2000 mg/mL of CHF revealed a significantly higher level than control (146 ±1) while other doses resulted in having lower serum sodium level than control. However, serum urea levels of mice administered with CMF and CHF were significantly lower from that of the control, while in contrast, serum uric acid levels of mice administered with CMF and CHF extract were significantly higher than that of the control. Meanwhile, serum phosphate of mice administered with CHF was significantly higher than control CHF while lower than those administered with CMF extract. The serum creatinine level of mice administered with CMF was not significantly different from control, while those administered with CHF significantly vary from control. Moreover, serum albumin levels of mice administered with CMF and CHF were not significantly different from that of control.

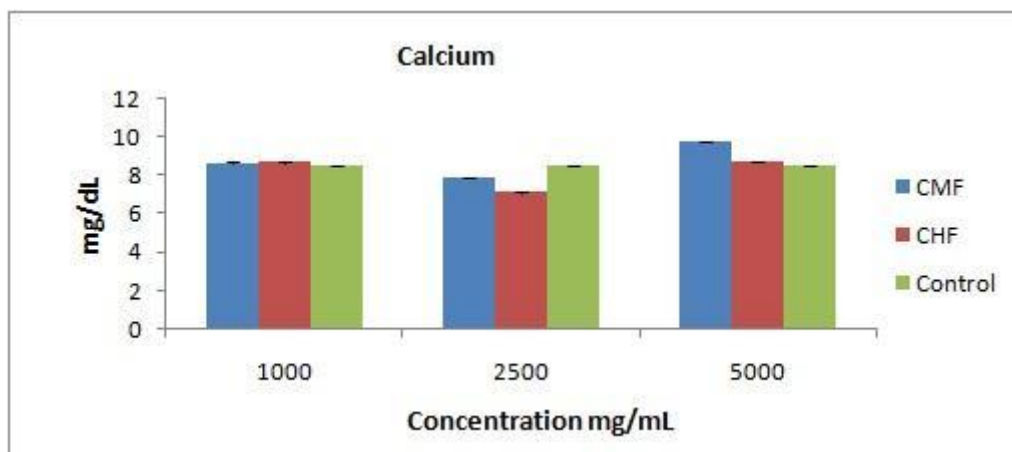


Fig. 3. Serum calcium level of mice: Serum calcium level of mice administered with methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruits are significantly different as compared to control, $P < 0.05$; $P < 0.001$

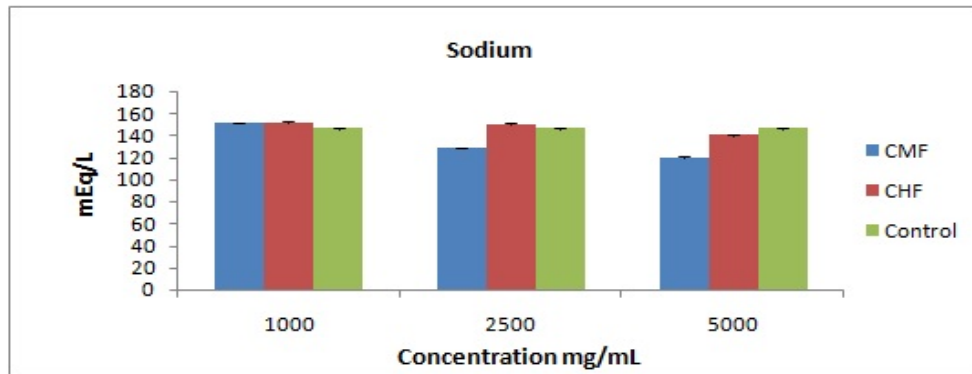


Fig. 4. Serum sodium level of mice: Serum sodium level of mice administered with methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruits are significantly different as compared to control, $P < 0.05$; $P < 0.001$

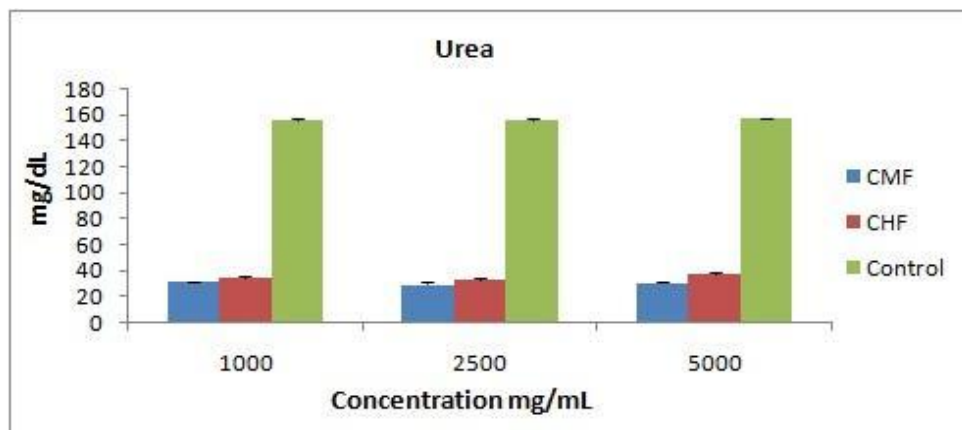


Fig. 5. Serum urea level of mice: Serum urea level of mice administered with methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruits are significantly different as compared to control, $P < 0.05$; $P < 0.001$

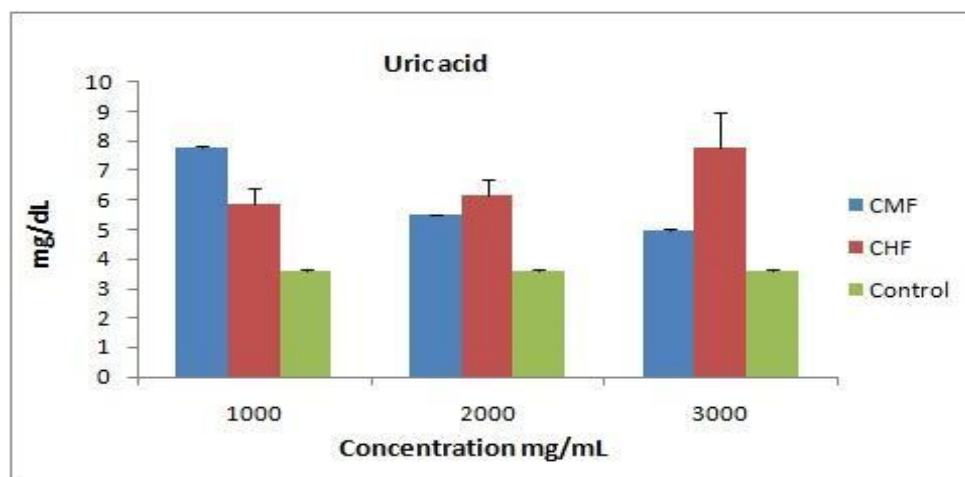


Fig. 6. Serum uric acid level of mice: Serum uric acid level of mice administered with methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruits are significantly different as compared to control, $P < 0.05$; $P < 0.001$

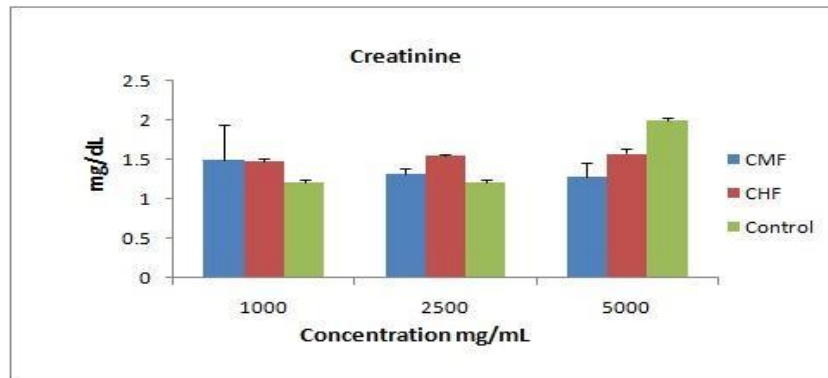


Fig. 7. Serum creatinine level of mice: Serum uric acid level of mice administered with methanol extract of *Citrus medica* L. fruits does not show significant difference from control however methanol extract of *Citrus hystrix* D.C. fruits shown to have significant differences as compared to control, $P < 0.05$; $P < 0.001$

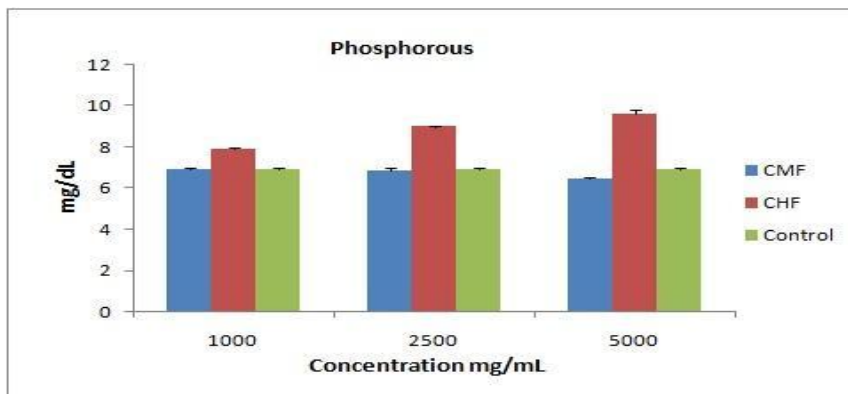


Fig. 8. Serum phosphorous level of mice: Serum phosphorous level of mice administered with methanol extract of *Citrus medica* L. fruits does not show significant difference from control however methanol extract of *Citrus hystrix* D.C. fruits shown to have significant differences as compared to control

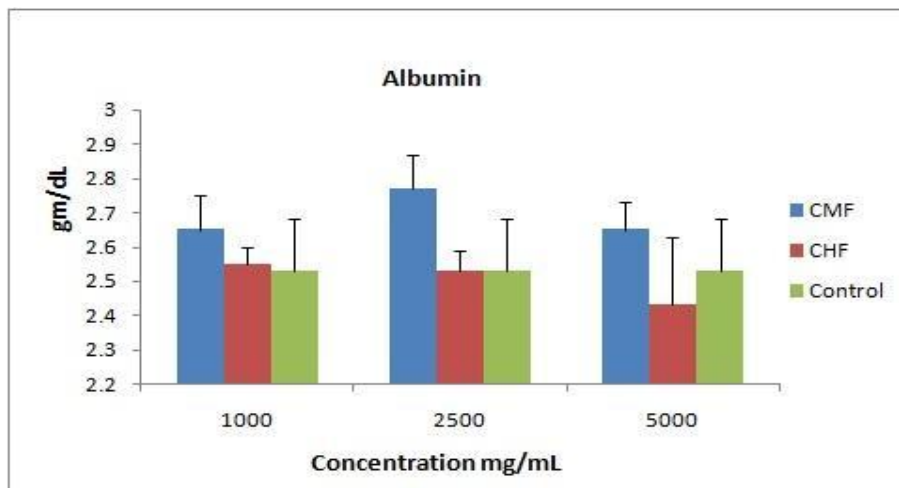


Fig. 9. Serum albumin level of mice: Serum albumin level of mice administered with methanol extract of *Citrus medica* L. and *Citrus hystrix* D.C. fruits do not show significant differences from control

High LD₅₀ and, no genotoxicity of the CHF and CMF extracts on the mice, show lesser or no toxic effect. The serum calcium level of mice administered with CHF and CMF revealed an increase of serum calcium level with increase of extract concentration. In contrast, the serum sodium level showed a significant decrease with the increase of extract concentration. Therefore, both the extract was revealed to have the potential to increase calcium and decrease serum sodium. The serum urea level of mice administered with CMF and CHF showed a decrease in serum urea level compared to control; thus, both extracts have a urea lowering effect. The serum uric acid level after administration of CMF was shown to decrease with the increased concentration of CMF, while for CHF administered mice, serum uric acid level increased with the increase in the concentration of CHF. However, serum uric acid levels of mice for all doses of CMF and CHF were significantly higher than control. Mice administered with CMF show no significant difference in serum creatinine, phosphorous and albumin levels from control. However, serum creatinine and phosphorus levels on mice administered with CHF were recorded in an increase from that of control, while serum albumin levels of mice administered with CHF were shown to decrease with an increase in CHF concentration. The result of serum analysis highlighted that CMF administered mice exhibited an increase in serum calcium, sodium (only at 1000 mg/mL administered mice), and uric acid level. At the same time, CHF administered mice showed increased calcium, sodium, uric acid, creatinine, phosphorous, and albumin levels (at 1000 mg/mL and 2000 mg/mL administered mice). Only serum urea levels of mice were observed lowering on administered CMF and CHF. However, for long-term usage of CMF and CHF, further study is critically needed to understand whether changes in serum constituent level for prolonged period usages as medicine or nutraceuticals would result in adverse health effects or not.

4. CONCLUSION

Citrus medica L. and *Citrus hystrix* D.C. fruits are commonly used because of their therapeutic potential for many diseases. However, prior examination of soil and environment before plantation is recommended to avoid Pb content in these fruits. The effective use of the *Citrus medica* L. in antiulcer, antiarthritic, and diuretic treatment could be correlated with the content of

compounds with antiulcer, antiarthritic, and diuretic potentials. Similarly, *Citrus hystrix* D.C. fruits for anticancer potentials. *Citrus medica* L. fruits can play an important role in the treatment of antiulcer, antiarthritic, and diuretic. *Citrus hystrix* D.C. fruits can also be employed in the treatment of cancer. In addition, both fruits were found to have antimicrobial potentials in this study; therefore, they could be effective in treating UTI. Therefore, both fruits can potentially be worked in the development of effective therapeutic agents.

Further research is needed for *Citrus medica* L. and *Citrus hystrix* D.C. fruits extract to shed extra light on the cellular and molecular mechanism underlying effects on antiulcer, antimicrobial, antiarthritic, diuretic treatment, antiinflammatory and anticancer. However, although the experimental and preclinical evidence suggesting significant effects of many *Citrus* species is compelling, preventive along with clinical studies directly point to the antiulcer, antimicrobial, antiarthritic, antiinflammatory, anticancer potential of *Citrus medica* L. and *Citrus hystrix* D.C. fruit extracts are still lacking. In addition, such studies will hopefully play the suppressive role that *Citrus hystrix* D.C. fruit extract plays in ulcer, arthritic, inflammatory and cancer. Moreover, the phytochemical evidence, acute toxicity assay suggesting *Citrus medica* L. and *Citrus hystrix* D.C. fruit extracts could show no toxic effects. Besides, evidence that the use of Citrus as potent nutraceuticals also points to the use of *Citrus medica* L. and *Citrus hystrix* D.C. fruits are also still lacking. Therefore, future *Citrus medica* L. and *Citrus hystrix* D.C. fruit extracts studies should focus on establishing a link between the Phyto-compounds content and antiulcer, antimicrobial, antiarthritic, diuretic, antiinflammatory, anticancer effects reported along with treatment/prevention in preclinical and clinical settings.

DISCLAIMER

The products used for this research are commonly and predominantly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Mice used in this experiment were provided from the animal house, Regional Institute of Medical Sciences, after the approval from the Institutional Animal Ethics Committee of Regional Institute of Medical Sciences, Imphal, Manipur, India (Registration No:1596/GO/a/12/CPCSEA).

RESEARCH SIGNIFICANCE

The study highlights the efficacy of "traditional medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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