

Original article

Phytochemical screening, antibacterial potentials and gas chromatography-mass spectrometry analysis (GC-MS) of *Citrus sinensis* leaves extracts

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ABSTRACT

Background: Antibiotic resistance to commonly used antibiotics has made man to resort to the use of their ancestral medicine, by exploiting the numerous potentials in plant parts. Plants were the foremost materials used in folklore medicinal practice and other traditional practices. Studies have indicated important metabolites in plant which have antibacterial potentials. **Aim:** This descriptive cross-sectional study was conducted during dry season, to determine the phytochemical constituent, antibacterial property and gas chromatography mass spectrometry (GC-MS) analysis of the plant extract. **Methods:** Extraction of *Citrus sinensis* (*C. sinensis*) leaves was performed successively with water and methanol using percolation method. Phytochemical screening to determine the metabolites present in the extract was done. The extracts obtained were tested in vitro for antibacterial activity against clinical isolates of *Salmonella typhi* (*S. typhi*) and *Salmonella paratyphi* (*S. paratyphi*) using agar well diffusion procedure. Extracts were further analyzed using GC-MS to reveals compounds present in the extracts. **Results:** Phytochemical screening indicates the presence of metabolites such as alkaloids, tannins, sterols, terpenoids and flavonoids in the extracts. Antimicrobial screening revealed that the extracts exhibited little or no activities against the different isolates with zones diameter of 5mm. Hence, minimum inhibitory concentration (MIC) of the extract and minimum bacteriocidal concentration (MBC) of the extracts were not determined. The GC-MS revealed the identity of compounds when matched with National Institute of Standard and Technology (NIST) library. **Conclusion:** Important metabolites are present in the extracts, and extracts showed no activity against the test organism at varying extract concentration.

Introduction

One of the alternatives left for man is to resort back to harnessing the potentials of plants parts in treatment of infections which are resistance to the commonly antibiotics. Plant products have been and will continue to be important candidate of new pharmaceutical compound [1]. Already some developing countries have incorporated herbal

medicinal practices in their health care system. *Citrus sinensis* (*C. sinensis*) are grown worldwide and commonly known as sweet orange. The leaves of *C. sinensis* are commonly used in treatment of infections one which is the typhoid fever. It has anti-inflammatory, antibacterial, antifungal, antiparasitic, antiproliferative activity and

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antioxidant properties [1]. *Citrus* flavonoids possess biological and healthy effects as antioxidants as reported [2]. *Citrus* flavonoids can prevent cancer through selective cytotoxicity, antiproliferative actions and apoptosis. Acetone and hexane extracts of *C. sinensis* leaf showed inhibition zones of 27mm towards *Helicobacter pylori* [1]. Aqueous, ethanol and petroleum ether extracts of *C. sinensis* L. (Osbeck) showed activity against *Candida albicans* [3]

The leaf extract of *C. sinensis* shows activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumonia* only they are found to be inactive against organism like *Salmonella typhi* (*S. typhi*), *Streptococcus faecalis*, *S. pyogenes*, *E. coli*, *Moraxella catarrhalis* and *Proteus spp.* [4]. The peels of *C. sinensis* have remarkable activity against *Proteus spp.*, *S. typhi*, *P. aeruginosa*, *S. pyogenes* and *K. pneumoniae*. Interestingly the juice of *C. sinensis* have activity against *S. typhi* and *P. aeruginosa*. [4]. The leaves of *C. sinensis* are most commonly used in treatment of typhoid fever, most often in combination with other plants parts especially *Cassia occidentalis* and *Eucalyptus camaldulensis* which are reported to have activity against *Salmonella* species [5,6]. This study is therefore meant to justify the inclusion of *C. sinensis* leaves in the makeup of the concursion.

Materials and Methods

Collection and processing of plant leaves

This descriptive cross-sectional study was conducted during dry season, between the months of January to June. *C. sinensis* are grown as tree plant, and found abundantly in the middle belt and southern part of Nigeria. However, in northern part of the country (where the study was carried out) with annual rainfall record which is always low, this plant is grown mainly in garden. The leaves were collected in different gardens of Hadejia Jigawa state Nigeria. The leaves were aseptically rinsed with water and dried for six days under shade. The dried leaves were then pulverized and homogenized using a blender. The powdered leaves was kept in cellophane bag for further usage.

Extraction of the crude *C. sinensis* extracts

The pulverized part of the plant was extracted using the method of Gupta et al. [7]. One hundred grams (100g) of the dried pulverized leaves were weighed into 2 glass containers and successfully extracted with 500ml each of methanol and distilled water by percolation. This involves shaking the mixture at

regular intervals. The mixtures were then filtered through filter paper. The filtrates were then obtained and concentrated evaporating the solvent using rotary evaporator. However, the water extract was evaporated using water bath at 45°C [6].

Percentage yield of the extract was calculated from the each weight of the extracts using the formula below:

$$\text{Percentage yield} = \frac{\text{weight of the extract}}{\text{Total weight of sample extracted}} \times 100\%$$

Other physical parameters such as colour and texture of the extracts were also recorded [6].

Preparation of extract stock concentration for antimicrobial screening

Extract concentrations of 30mg/ml, 60mg/ml, 90mg/ml and 120mg/ml for aqueous and methanol were prepared by dissolving 0.3g, 0.6g, 0.9g and 1.2g respectively of each extract in 10mls of distilled water in separate test tubes. The same concentrations were made for the control antibiotic (amoxicillin) [7].

Phytochemical screening of the extract

Standard procedures were employed as reported for determining the presence of secondary metabolites in the pulverized plant material [8,9].

Antimicrobial screening

Organism source

The clinical isolates were obtained from stool samples of presumptive typhoid patients at the Department of Medical Microbiology Aminu Kano Teaching Hospital (AKTH) and Department of medical Microbiology, Hadejia general hospital Jigawa, Nigeria. The test organisms were *S. typhi*, *S. paratyphi* (*S. paratyphi*) A and *S. paratyphi* B. Their susceptibility pattern to common antibiotics was determined and found to be susceptible to the commonly used antibiotics. The test organisms obtained were confirmed using the methods of Cheesbrough [10] by observing their cultural growth characteristics each. Biochemical confirmatory tests were also performed to further confirm the identity of each of the test organisms. The organisms were checked for any form of impurity and then stored at 4°C in slants of nutrient agar till use.

Preparation of the bacterial inoculum

Using wire loop the test organisms were taken from the agar slant and then sub-cultured into test tubes containing nutrient broth which were incubated for 24hrs at 37°C. The organisms' suspensions in the broth were then standardized by addition of normal saline to obtain a turbidity and population density, equivalent to a 0.5 McFarland standard. Exactly 99.5ml of 1%

BaCl₂ was added to 0.5ml of 1% H₂SO₄ to obtain 100ml of BaSO₄ which is equivalent to 0.5 McFarland's turbidity standard equivalent to 1.0 X 10⁸ cfu/ml population for bacterial isolates.

▪ Preparation of media

The media used were prepared as directed by the manufacturer. A Cork borer of 5mm diameter was used to cut a well into the media, which was already seeded with the test organisms. 0.01ml of the well dissolved extract was then transferred into the well. The plates were immediately incubated at 37°C for 24hrs, and observed for the zone of inhibition of growth. The zones were measured, and the result recorded in millimeters. The antibacterial screening was done in triplicates. Same concentration of amoxicillin was used as control.

▪ Determination of activity index of the extracts

The activity index of the crude plant extract was determined using the following relation [7];

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Quantitative and qualitative analysis of the extract using gas chromatography mass spectrometry (GC-MS) technique

The different extracts were subjected to quantitative analysis using the GC-MS analyser to quantify the compounds contained in each of the plants extracts and determine the proportion as well as to identify the biochemical constituent of the extract. These extracts were analysed using GC-MS analyser. Mass spectra of the compounds identified in the extract were matched with that of National Institute of Standard and Technology (NIST) Library.

Results

The filtrates appeared greenish in colour, while the dried extracts appeared as deep green in

Table 2. Phytochemical constituents of *C. sinensis* leaf extract.

Extract	Alkaloids	Saponins	Tannins	Flavonoids	Steroids	Glycosides	Terpenoids	anthraquinones
Aqueous	+	-	+	+	+	-	+	-
Methanolic	+	-	+	+	+	-	+	-

+ =presence, - = absent

colour with soft and gummy textures. The highest percentage yield of the extract was observed in aqueous extract which was 14.2% of the total sample extracted, 7.4% of the extract was obtained after drying of the methanolic extract, hence the least yield of the two different extracts as shown in **table (1)**. Phytochemicals identified from the aqueous and methanolic extract of *C. sinensis* leaves include the following secondary metabolites; terpenoid, alkaloids, tannins, flavonoid, and steroids as shown in **table (2)**. Phytochemicals identified were found in both extracts.

Antimicrobial activity of *C. sinensis* against clinical isolates

Table 3 showed the antimicrobial activities of the extracts and that of standard antibiotic (amoxicillin) at different concentrations of (120mg/ml, 90mg/ml, 60mg/ml, and 30mg/ml for each extract) against the test organisms. Extracts of *C. sinensis* showed no activity against the test organisms at all concentration tested.

Gas chromatography mass spectrometry

The GC-MS analysis of the extracts reveals the compounds present when matched with the NIST library. Terpene, and sterols compounds such as eucalyptol, vitamin E, carveol, squalene, mrycenol were identified (**Tables 4&5**). Many terpenoids were found in the extracts which are used in cosmetics as fragrance.

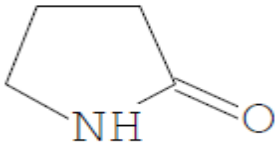
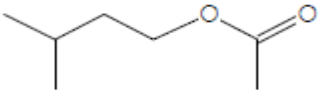
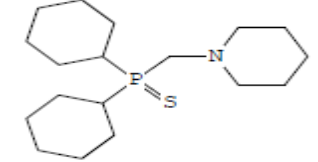
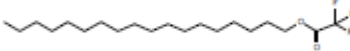
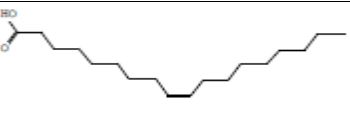
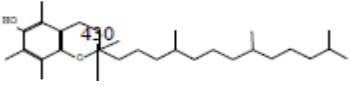
Table 1. Physical characteristics of *C. sinensis* leaf extracts.

Extracts	Colour	Texture	% yield (g)
Methanol	Green	Soft and gummy	7.9
Aqueous	Green	Soft	14.2

Table 3. Antimicrobial activity of the various *C. sinensis* leaves extract against the test organisms using agar well diffusion method.

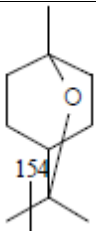
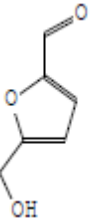
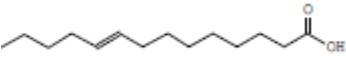
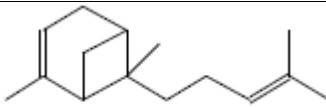
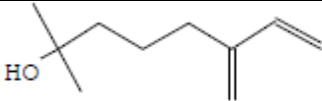
		Mean zone of inhibition in (mm) produced against the test organism													
N.	Test organism	Methanolic extract (mg/ml)				Aqueous extract (mg/ml)				Amoxicillin (mg/ml)				Activity index	
		120	90	60	30	120	90	60	30	120	90	60	30	Met	Aqu
1	<i>S. typhi</i>	5	5	5	5	5	5	5	5	23	19	15	15	0.00	0.00
2	<i>S. paratyphi A</i>	5	5	5	5	5	5	5	5	21	19	15	13	0.00	0.00
3	<i>S. paratyphi B</i>	5	5	5	5	5	5	5	5	21	17	15	15	0.00	0.00

Table 4. Gas chromatography mass spectrometry analysis on *C. sinensis* water extract. Nineteen (19) compounds were identified in the preliminary GC-MS of this extract and the major compounds include.

N.	Retention time	Area %	IUPAC nomenclature	Molecular formula	Structural formula
1	5.631	23.24	2-pyrrolidinone (Aminobutyrolectam)	C ₄ H ₇ NO	
2	6.511	24.82	1-butano, 3 methyl acetate	C ₇ H ₁₄ O ₂	
3	9.098	8.73	1(Diclohexylphosphorothioyl -methyl) piperidine	C ₁₈ H ₃₄ NPS	
4	25.790	6.28	Trifluoroacetic acid, n- octadecyl ester	C ₂₀ H ₃₇ F ₃ O ₂	
5	20.586	5.96	9-octadecenoic acid (oleic acid)	C ₁₈ H ₃₄ O ₂	
6	26.680	1.45	Vitamin E	C ₂₉ H ₅₀ O ₂	
7		29.52	Others (remaining 13 compounds)		

Other important compounds identified from the extract includes; Piperidine, acetic acid, linalool.

Table 5. Gas chromatography mass spectrometry analysis on *C.sinensis* leaves methanolic extract. Twenty two (22) compounds were identified in the preliminary GC-MS analysis with the major ones being.

N.	Area %	Retention time	IUPAC nomenclature	Molecular formula	Structural formula
1	21.00	4.669	2-oxabicyclo (2.2.2) octane (Eucalptol)	C ₁₀ H ₁₈ O	
2	23.44	7.555	2-Furancarboxaldehyde, 5-(Hydroxymethyl)	C ₉ H ₁₂ O	
3	9.38	20.497	E-9-Tetradecenoic acid	C ₁₄ H ₂₆ O ₂	
4	5.93	10.310	Trans-alpha-bargomotene	C ₁₅ H ₂₄	
5	5.43	6.877	2-Methyl-6-methylene-7-octen -2-ol (Mrycenol)	C ₁₀ H ₁₈ O	
6	34.82		Others (remaining 17 compounds)		

Other important compounds identified from the extract includes; Isopinocarveol.

Discussion

From the result obtained water can be said to be the best solvent (used in the study) for extraction of these leaves extract as it gives higher yield of the extract, though both solvents have the potentials of extracting secondary metabolites from the leaves. However, all the solvents contains the same phytochemicals compounds based on the phytochemical screening. Saponin, glycoside and anthraquinones were absent in all the *C. sinensis* extract. Presence of steroid in the leaves of *C. sinensis* was reported also by **Rani et al.** [11]. Flavanoid were found in the extracts which concur with the findings of **Escudero-López et al.** [12]. **Babayi et al.** reported the

presence of similar phytochemicals identified in this study [13]. Based on the antimicrobial testing conducted, methanolic and water extracts of *C. sinensis* showed no activity against the test organisms at all concentration tested. The inactivity of *C. sinensis* leaves extract on the test organisms as seen in this study confirms earlier finding by **Nada and Zainab** [4]. Also **Uchechi and Edeha** [14] reported the inactivity of *C. sinensis* crude leaf extract. This may be due to absence of important phytochemicals like saponins which have antibacterial property and the nature of the Gram negative cell wall. *Citrus sinensis* extracts have activity index of 0 against the test organisms. Terpene such as carveol in form of isopinocarveol found in the methanolic extracts are

reported to have anti-parasitic, neuromodulatory and anticancer activity, and are commercially used as a food additives and fragrance [15,16]. Sterols such as squalene which has pharmacological, cosmetic and nutritional potentials [17].

Limitation of the study

The study has limitations which includes;

1. Only few group of bacteria were tested hence the result cannot be used to conclude the potentials of the plant on other organisms.
2. The inactivity of the extract according to the antibacterial study using methanol or water as the solvent do not disqualify *C. sinensis* as a potential product of antibacterial study since other solvent like hexane and acetone may extract other secondary metabolite.
3. Further research is needed to exploit the potentials of the compound identified in the GC-MS analysis.

Conclusion

Methanol and water have the potentials of extracting metabolites from *C. sinensis* leaves. All extract tested against the test organisms showed no activity in accordance with the extract concentration. Antibacterial screening indicates that *C. sinensis* leaves should not be used alone in the treatment of infections caused by *Salmonella* species. From the GC-MS analysis carried out, compounds of important property from the various extracts were revealed. In this regard, considering the health benefits of *C. sinensis* leaves, it presents excellent options for treating or management of an infection due to its bioactive secondary metabolites (drug candidates) that show important activities or for developing new pharmaceutical products.

Conflicts of interest: None.

Financial disclosure: None.

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