International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 2 No. 2 Feb. 2013, pp. 138-145

http://www.issr-journals.org/ijias/

Phytochemical Screening and In Vitro Anti-Bacterial Studies of the Ethanolic Extract of Citrus Senensis (Linn.) Peel against some Clinical Bacterial Isolates

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ABSTRACT: Citrus senensis peel has many medicinal properties and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, cormunative, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. It is also used to treat and prevent vitamin deficiencies, colds, flu, and scurvy and helping to fight viral and bacterial infections. The aim of the study is to verify the ethnomedicinal use of the peel as anti-bacterial. The peels were air-dried and ground to powder using mortar and pestle, extracted with 95% ethanol. The extract was subjected to phytochemical screening using standard procedures. Agar diffusion method was employed to test the antibacterial activity of the extract and the MIC and MBC of the extract were determined by broth dilution technique. The results of the phytochemical screening indicated the presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids. The results of the antibacterial activity showed that the isolates were sensitive to the extract, with MIC of 0.25-2.5mg/ml and MBC of 0.5-5.0mg/ml. The antibacterial effects of the extracts suggest their possible use for the treatment of infections caused by the test bacteria. The chemotherapeutic potential of the fruit peel could be due to the presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids. The success of this study could lead to the development of cheap, easily available and relatively safe bactericides from a tropical plant.

KEYWORDS: ethnomedicinal, phytomedicine, antibacterial, phytochemical-screening, Citrus senensis.

1 INTRODUCTION

The mass scale productions of synthetic medicines by the western countries in the begging of the current century make the medicines and drugs of vegetables origin lost their significance. They were rejected by the medicinal practitioners all over the world, being referred to as 'old woman's remedy' [1]. In the recent years, in view of increasing awareness about adverse side effects of synthetic drugs, a sense of back to nature has been developed and the people in most of the developed countries now prefer raw carrot instead of carotene compound based tablets, instead of vitamin C tablet, they feel happy to

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use citrus fruits. It is mainly due to innumerable side effects being observed following the use of synthetics i.e. resistance to certain antibiotics and immune-suppressive activities of certain drugs. It is felt that there is a sense of revival in medicines from a vegetative source and it is once again gaining recognition [2].

Citrus senensis Peel: is derived from the fruit of *Citrus sinensis,* the plant is called sweet orange (English) and is locally called Lemon – zaki (Hausa). Though most people peel the *Citrus senensis* and eat only the fruit, the *Citrus senensis* peel, though not tasty on its own, is used medicinally [3]. *Citrus senensis* peel contains calcium, phosphorus, potassium, ascorbic acid, and vitamin A, as well as volatile oil and hesperidin. In Africa, *Citrus senensis* peel is used to treat colic, and in India, Citrus senensis peel is used to treat upset stomach [3]. The British pharmacopoeia has list *Citrus senensis* peel as aromatic compound for use as aroma and flavor enhancer. Furthermore the bioflavonoid constituents of this herb are reported to reduce the permeability of blood vessels, especially capillaries, so that extracts from *Citrus senensis* peel are also included in remedies for phlebitis. New studies on a monoterpene found in *C. senensis* peel called "Limonene" has shown that it can effectively prevents individuals from developing abnormal growths on their skin [3].

The limonene compound isolated from *C. senensis* peel has demonstrated prevention efficacy in preclinical models of breast research, which shows that the herb may help in reducing the occurrence of squamous cell skin cancer. The *C. senensis* peel is also used in treating diuretic, cormunative, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. Also used in Ayurvedic medicine to tonify liver, strengthening to blood vessels, help in relieving symptoms and discomfort of varicosa, peripheral circulatory system function. It increased circulation to the extremities. Used to treat and prevent vitamin deficiencies, colds, flu, and scurvy [4]. The high citric acid content in *C. senensis* peel has powerful health benefits in treating heavy-metal poisoning in people and helping fight viral and bacterial infections [4].

Previous reports show that fruit and vegetable peel extracts showed better antifungal activity than antibacterial activity; Gram-negative bacteria were more susceptible than Gram-positive bacteria which contradict the earlier reports that plant extracts are more active against Gram positive bacteria than Gram-negative [5]. The methanolic extract of *C. grandis* shows activity against *Bacillus Subtilis*, *B. cereus*, *Staphylococcus aureus*, *E. coli* and *Salmonella enteritidis* [6]. The flavonoid extract of *C. reticulate* Blanco showed activity on *E. coli*, *S. aureus*, *Streptococcus epidermidis*, *Enterococcus feacalis*, *S. typhimurium* and *Enterobacter cloace* [7]. The oil extract of *C. reticulata* Blanco showed activity against *Alternaria altanata*, *Rhizoctonia solani*, *Curvularia lunata*, *Fusarium oxysporum* and *Helminthosporium oryzae* [8]. The oil extract of *C. acida* Roxb. showed activity against *B. subtilis*, *B. cereus*, *E. coli*, *Enterobacter aerogenes*, *S. typhimurium*, *Aspergillus ficum*, *A. fumigatus*, *A. flavus Fusarium solani*, *F. Oxysporum*, *Penicillium digitatum* and *Candida utilis* [9]. The ethanolic fraction of *C. bergamia* Risso showed activity against *E. coli*, *Pseudomonas putida*, *S. enterica, Listeria, Innocua, Bacillus subtilis, S. aureus, Lactobacillus lactis* and *Sacharomyces cerevisae* [10]. The hexane and wax extract of *C. senensis* peel exhibited antibacterial activity against *S. aureus* (12mm and 20mm) and *E.coli* (12mm and 20mm) with MIC of 500µg/ml and 1000µg/ml respectively [11]. The essential oils from *C. senensis* peels shows antibacterial activity against *S. aureus* (16mm), *S. typhimurium* (15mm), *Enterobacter aerogenes* (12mm), *B. subtilis* (11mm), *E. coli* (10mm) and the tested oil has shown nearly equal antibacterial effect on both Gram negative and positive bacteria *B. subtilis*, *S. aureus* and *E. coli*, *S. typhimurium*, *E.aerogenes* [12]. Similar kind of observations was also made by [13] and [14] indicating that the essential oil of *Citrus* was active against all tested bacteria including Gram positive and Gram negative cultures ([15]-[16]). Also the aqoues extract of orange peel possessed antibacterial activity against *S. aureus*, [17], the ethyl acetate extract of *Citrus* peel showed antibacterial activity against *S. aureus* and *E. coli* [18], the antibacterial activity of lemon peel against *Pseudomonas aeruginosa*, *S. typhimurium* and *Micrococcus aureus* was also reported [19] and the antibacterial activity of essential oil from *C. senensis* against *E. coli, S. aureus, K. pneumoniae, B. cereus, Micrococcus luteus, Proteus vulgaris, Mycobacterium smegmatis, listeria monocytogenes, p. aeroginosa* [20]. The objective of the study is to identify the specific soluble extracts of *Citrus sinensis* peel that are active against *Salmonella species, A. hydrophila* at various concentrations.

2 MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION OF PLANT MATERIALS

Citrus senensis fruit was obtained from Yankaba Market in May, 2010, the peels were obtained by removing the pericarp. Its botanical identity was further confirmed, authenticated and voucher specimen (16) was deposited at the Herbarium section of the Botany unit of the former Department of Biological sciences, Bayero University, Kano, Nigeria, for future reference.

PREPARATION OF THE TREATMENT SAMPLES

The peels were air-dried and ground to powder using mortar and pestle as described by [21]. The content was then stored in air-dried container until required for further analysis.

EXTRACTION PROTOCOLS

This was carried out according to the method of [21]. The fine powder of the peels (100g) was weighed and percolated with 1000 cm^3 of 95% ethanol. It was allowed to stand for two weeks with shaking at regular intervals under room temperature. The percolate was then filtered and solvent (ethanol) evaporated at room temperature to obtain the ethanolic extract of the peels. The extract was stored in sterile bottle under refrigerated condition.

PHYTOCHEMICAL SCREENING OF THE PLANT EXTRACT

The extract was analysed for the presence of alkaloids, flavonoids, saponins, tannins, steroids, glycosides, triterpenoids, phytosterols and amino acids as follows:

(a) Test for alkaloids

This was carried out according to the method described by ([22]-[23]). A quantity of 5 cm^3 of the extract was added to 2 cm^3 of HCl. To this acidic medium, 1 cm^3 of Dragendroff's reagent was added. An orange or red precipitate /turbidity produced immediately indicated the presence of alkaloids.

(b) Test for flavonoids

This was carried out according to the method of ([22]-[23]). To 3cm^3 of the extract was added 1cm^3 of NaOH, a yellow colouration indicated a positive test for flavonoids.

(c) Test for saponins (frothing test)

This was carried out according to the method of ([22]-[23]). Two cm^3 of the extract was placed in a test tube and then 2cm^3 of distilled water was added. The tube was then shaken vigorously. A persistent froth that lasted for at least 15-minutes indicated a positive test for saponins.

(d) Test for tannins

(i). This was carried out according to the method describe by ([22]-[23]). Two drops of 5% $FeCl_3$ was added to 1cm^3 of the extracts. A green precipitate indicated a positive test for the presence of tannins.

(2). This was carried out according to the method described by ([24]-[25]). To 5cm^3 of the extract, a few drops of 1% lead acetate were added. Formation of a yellow precipitate indicated the presence of tannins.

(e) Salkwoski's Test for Steroids

This was carried out according to the method of ([22]-[23]). To 1cm^3 of the extract 5-drops of conc. H_2S0_4 was added. A red colouration indicates a positive test for steroids.

(f) Fehling's test for glycosides

This was carried out according to the method of ([22]-[23]). Ten cm^3 of 50% H_2S0_4 was added to 1 cm^3 of the extract in a test tube. The mixture was heated in a boiling water bath for 15 minute. Ten cm^3 of fehling's solution was added and the mixture was boiled. Formation of brick red precipitate indicated a positive test for glycosides.

(g) Test for Triterpenoids

This was carried out according to the method described by ([24]-[25]). Ten mg of the extract was dissolve in 1cm^3 of chloroform; 1cm^3 of acetic anhydride was added following the addition 2 cm^3 of conc. H_2S0_4. Formation of reddish violet colour indicated the presence of triterpenoids.

(h) Test for phytosterols

This was carried out according to the method described by ([24]-[25]). The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification took place. The mixture was diluted and extracted with ether. The ether was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3cm^3 of acetic anhydride was added followed by few drops of conc. H_2S0_4. Appearance of bluish green colour showed the presence of phytosterol.

(i) Test for amino acids

This was carried out according to the method described by ([24]-[25]). One cm^3 of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour showed the presence of amino acids.

PREPARATION OF SENSITIVITY DISCS

Whatman No. 1 filter paper was punched using puncher to obtain disc of 6.0mm in diameter. These were placed in a sterile screw-capped Bijou bottles and sterilized in an oven using a dry heat at 140^{o}C for 1-hour. The discs were allowed to cool; twenty five discs were dispensed into each solution with defined concentration by means of sterile forceps. Standard antibiotic (Oxoid, UK) discs were used as positive control.

PREPARATION OF EXTRACT CONCENTRATIONS

The stock solution of the plant extract was prepared in screw capped bijou bottle containing Dimethly sulphoxide (D.M.S.O). One gram of the extract was weighed on a metler balance, and dissolved in 1cm^3 of DMSO to arrive at 1000,000 µg/cm^3 (10^{6}µg/cm^3) concentration of stock solution. Twelve varied extract concentrations 1000 µg/ml - 4000 µg/ml, 10,000 µg/ml - 40,000 µg/ml, 100,000 µg/ml - 400,000 µg/ml, were prepared from the stock solution (1000,000 µg/ml) using 10-fold serial dilution.

TEST CULTURE

The test organisms were isolated from stools samples of patients presenting with diarrhoea attending Aminu Kano teaching Hospital (AKTH) Kano-Nigeria and Murtala Mohammad Specialists Hospital Kano-Nigeria, using standard methods [26]. The isolates included six *Salmonella paratyphi B*, one *Salmonella typhi* and three *Aeromonas hydrophila*. The isolates were maintained on a freshly prepared nutrient agar slant and kept at 4^{o}C until required for use.

PREPARATION OF MCFARLAND TURBIDITY STANDARD

Barium sulphate suspension at 1.0% w/v was prepared as follows. One percent (1% v/v) solution of sulphuric acid was prepared by adding 1ml of concentrated H_2SO_4 in 99cm^3 of water. One percent (1% w/v) solution of barium chloride was also prepared by dissolving 0.5g of dehydrated barium chloride in 50cm^{3}distilled water.

Barium chloride solution (0.6cm^3) was added to 99.4cm^3 of sulphuric acid solution to yield 1.0% w/v barium suspension. The turbid solution formed was transferred into a test tube as the standard for comparison [27].

STANDARDIZATION OF INNOCULUM

Using inoculating loop, enough material from an overnight culture of the test organisms were transferred into a tube containing 2.0cm^3 normal saline, until the turbidity of the suspension matched the turbidity of standard (1% barium sulphate), [27].

EXTRACT ANTIBACTERIAL ACTIVITY TESTING

Agar diffusion method [28] as modified by [29] was employed. The freshly prepared nutrient agar plates were dried; the plates were aseptically inoculated uniformly with test organism by streaking method. The impregnated paper disc containing *Citrus senensis* peel extract at different concentrations were arranged radially and pressed firmly onto the inoculated agar surface to ensure even contact and the plates were incubated at 37±1^{o}c for 18 – 24hrs.

DETERMINATION OF MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATIONS OF THE EXTRACTS

The MIC and MBC were determined in accordance with the methods of [30].

3 RESULTS

A total yield of 31.00g of the ethanolic extracts from the original weight of 100g was recovered from the peel of *C. senensis*. The physical characteristics were indicated in Table 1. Table 2 showed the phytochemical composition of the plant parts screen. Phytochemical analysis of the *C. senensis* peel revealed the presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids. Only glycosides and amino acids were absent in *C. senensis* peel. The antimicrobial activity pattern of the extract was shown in Table 3-4. The results of the study showed that the ethanolic extract of the peel of the plant demonstrated activity on all the tested isolates.

Table 1. Physical characteristic of Citrus senensis peel extract

Plant part	Solvent	Initial weight (g)	Final weight (g)	Colour	Odour	Texture
Peel	Ethanol	100.00	31.00	Orange	Fruity	Oily

Table 2. Phytochemical characteristics of Citrus senensis peel extract

Ingredient	*C.senensis*
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	+
Steroids	+
Glycosides	-
Triterpenoids	+
Phytosterols	+
Amino acids	-

Key: + = present; - = absent.

Table 3. Antibacterial activity of Citrus senensis peel extract against some clinical bacterial isolates

Test Bacteria		Average zone of inhibition (in mm)/Disc Potency in µg/disc													
		4000	3000	2000	1000	400	300	200	100	40	30	20	10	S.D	Zone
Salmonella B_1	*Paratyphi*	09	07	11	09	07	07	08	07	08	11	11	10	S	27
Salmonella B_2	*Paratyphi*	08	08	08	08	09	09	09	08	09	08	08	08	SXT	35
Salmonella B_3	*Paratyphi*	10	00	10	11	00	00	11	09	00	00	00	00	AMP	24
Salmonella B_4	*Paratyphi*	07	00	00	00	00	08	09	00	00	07	00	00	CPX	42
Salmonella B_5	*Paratyphi*	10	00	10	00	08	10	08	08	00	10	10	10	PN	21
Salmonella B_6	*Paratyphi*	00	00	00	00	00	00	00	00	00	07	00	07	S	12
Salmonella	*typhi*$_1$	10	10	10	10	12	12	10	10	10	10	10	10	PEF	30
Aeromonas	*hydrophila*$_1$	10	10	10	10	00	00	00	35	10	12	10	10	OFX	20
Aeromonas	*hydrophila*$_2$	15	10	15	10	07	10	07	12	00	00	08	10	APX	15
Aeromonas	*hydrophila*$_3$	14	07	07	07	08	08	08	08	00	00	00	00	OFX	25

KEY: PEF = PEFLACINE 10µg. CPX = CIPROFLOXACIN 10µg. PN= AMPHICILLIN 30µg. S= STREPTOMYCIN 30µg. PEF = PEFLACINE 10µg. OFX = TARIVID 10µg. AMP = AMPHICILLIN 10µg. APX=AMPHICLOXACIN10µg.

Table 4. Minimum Inhibitory and Bactericidal Concentrations of ethanolic extract of Citrus senensis peel against some clinical bacterial isolates

Test organism	MIC value (mg/ml)	MBC value (mg/ml)
S.paratyphi B_1	0.25	0.5
S.paratyphi B_2	0.25	0.5
S.paratyphi B_3	0.25	0.5
S.paratyphi B_4	0.25	1.0
S. typhi$_1$	0.25	0.5
A. hydrophila$_1$	0.25	1.0
A. hydrophila$_2$	0.25	0.0
S. paratyphi B_5	2.5	5.0
S. paratyphi B_6	2.5	0.0
A. hydrophila$_3$	2.5	5.0

4 DISCUSSIONS

The crude ethanolic extract of *C. senensis* peel was found to be active against the *Salmonella species* and *Aeromonas hydrophila*. The highest activity was seen against *A. hydrophila*$_1$ at a concentration of 100µg/disc with zone diameter of 35cm. The antibacterial activity of *C. senensis* peel extract was prominent against *A. hydrophila*$_1$, which was seen to be more sensitive to the peel extract at a concentration of 100µg/disc (10,000µg/ml) with zone of inhibition of 35mm, in comparison with standard antibiotic disc, Tarivid (OFX) 10µg which shows a zone diameter of 20mm. The lowest MIC was demonstrated at a concentration of 0.25mg/ml against *S. paratyphi* B_1, B_2, B_3, B_4, *S. typhi*$_1$, *A. hydrophila*$_1$ and *A. hydrophila*$_2$ and the highest was seen at a concentration of 2.5mg/ml against *S. paratyphi* B_5, B_6 and *A. hydrophila*$_3$. The lowest MBC was demonstrated at a concentration of 0.5mg/ml against *S. paratyphi* B_1, B_2, B_3 and *S. typhi*$_1$ and the highest was seen at a concentration of 5mg/ml against *S. paratyphi* B_4 and *A. hydrophila*$_3$.

The antibacterial activity recorded with the ethanolic extract of *C.senensis* peel in this study against *Salmonella* species and *Aeromonas hydrophila* is in close agreement with that of [31] which reported that the aqeous extracts of peel of *C. senensis* (L) has antibacterial activity against *S. paratyphi* A, *S. paratyphi* B and *S. typhi* the highest activity was seen at a

concentration of 100mg/ml with zone of inhibition of 30mm, 31mm and 24mm respectively with gradual increase in activity with increase in concentration indicating a concentration dependent pattern. In a similar study that involves the essential oil of the peel, it was reported that essential oils from *C. senensis* peels showed antibacterial activity against *S. aureus* (16mm), *S. typhimurium* (15mm), *Enterobacter aerogenes* (12mm), *B. subtilis* (11mm), *E. coli* (10mm) and the tested oil has shown nearly equal antibacterial effect on both Gram negative and positive bacteria *B. subtilis*, *S. aureus* and *E. coli*, *S. typhimurium*, *E.aerogenes* [12]. Similar kind of observations was also made [14] indicating that the essential oil of *Citrus* was active against all tested bacteria including both Gram positive and Gram negative cultures ([15]-[16]). In addition, [19] also reported the antibacterial activity of lemon peel against *Pseudomonas aeruginosa*, *S. typhimurium* and *Micrococcus aureus.*

5 CONCLUSION AND RECOMMENDATION

It is concluded that, some ethanol-extractable phytochemicals from *C. senensis* peel possess in-vitro antibacterial activity against the test bacterial isolates. The findings as a whole further support the fact that cheap, easily available and relatively safe bactericides could be developed from a tropical plant. It is therefore recommended for the detailed bioassay-directed fractionation to identify and elucidate the bioactive compounds responsible for the result observed.

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