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In vitro Assessment of Antibacterial Activity of *Citrus aurantifolia* Extracts

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Abstract

Leaf extracts of *Citrus aurantifolia*, was investigated for antibacterial activity against *Salmonella typhi*, *Salmonella* Paratyphi A and *Salmonella* Paratyphi B using agar-well diffusion and gradient serial dilution methods. Phytochemical screening was conducted in order to detect secondary metabolites. The in vitro antibacterial activity of the extracts showed antibacterial activity against *S. typhi* and *S.* Paratyphi B. Watersoluble leaf extracts demonstrates higher zone of inhibition (19mm) against *S.* Paratyphi *A.S.* Typhi was found resistant to both water and chloroform soluble leaf extracts but sensitive to ethanolic leaf extracts at 50µm/mlconcentration. Both *S.* Paratyphi A and *S.* Paratyphi B were found resistant to ethanolic leaf extract. The occurrence of alkaloids, flavonoids, tannins, saponins, steroids, terpenoids and phenols in the aqueous leaf extracts of *C. aurantifolia* justifies its use in the folklore medicine.

Keywords: Antibacterial activity, *Salmonella typhi, Salmonella* Paratyphi A*, Salmonella* ParatyphiB, Resistance, Susceptibility, *Citrus aurantifolia*.

INTRODUCTION

C. aurantifolia alleviates anxiety and nervousness, relieves stress related disorders such as insomnia or nervous originated digestive disorders, also possesses anti-inflammatory potential, has antispasmodic virtues that are being experienced during spasm of the digestive system (distension, diarrhoea), has an anticoagulant property, which renders it very valuable for people with cardiovascular risks. It is also used against fever, headaches and cold (Chellaiah *et al.*, 2006).

Citrus aurantifolia belongs to the family Rutaceae (orange family) (Bakare *et al.,* 2012). *Citrus aurantifolia* is a Perennial Tree, with evergreen leaves, thorny stem, whitish flowers, globase fruits with many seeds, green when unripe and greenish-yellow when ripe, with sour taste (Ikeyi and Omeh, 2014).*Citrus* plants are medicinal due to the high level of flavonoid content. Flavonoids are of particular importance in the human diet as there is evidence that they act as free radical scavengers, antioxidants, diuretic, antiviral, antibacterial, antimicrobial, anti-inflammatory, anti-tumor and anti-platelets agents (Njoku and Obi, 2009; Sofowora, 1993).

Juice extract of *C. aurantifolia* is mixed with honey for catarrh, cough and respiratory problems, stomach aches, flatulence, nausea and anemia(Ikeyi and Omeh, 2014).*C.aurantifolia* contains alkaloids, vitamin C, flavonoids, tannins, phenols, saponins, niacin, carotenoids as well as P, K, Mg, Na, and Cawhich were reported to possess antimicrobial properties (Ikeyi and Omeh, 2014).

C. aurantifolia is used in the folklore medicine as an antiseptic, anthelmintic, mosquito bite repellent, for stomach ailments, tonic, antiscorbutic, astringent, diuretic, headache, arthritis, digestive and appetite stimulant, and for colds, coughs and sore throats (Morton, 1987; Aliyu, 2006).

This study was aimed to determine antibacterial activity of *C. aurantifolia* leaf extracts.

MATERIALS AND METHODS

Sample Collection and Handling

The leaves of *C. aurantifolia* were collected from staff quarters in Bayero University, Old campus, Kano, Nigeria. The identity of the study plant was confirmed by a Botanist at the Department of Plant Science, Bayero University, Kano.Voucher specimen numbered; BUKHAN 0113 was deposited in the departmental herbarium. The leaves were air-dried under shade for 3 weeks. The air dried leaves werepulverized into fine powderusingmortar and pestle (Aliyu, 2006).

Extraction

Fifty grammes (50g) of *C. aurantifolia* dried leaves powder was weighed using electric weighing balance and put in a conical flask (500ml). Two hundred and fifty ml of ethanol was added and allowed to percolate for one week, with regular shaking. Similar method was employed to extract the leaf using chloroform (250ml)and water (500ml). The extracts were filtered using Whatman No. 1 filter paper. The filtrates were concentrated by complete evaporation of solvent in a water bath at 100^{o}C. The crude extracts of the *C. aurantifolia* leaf were stored appropriately until used (Fatope *et al.,* 1993).

Physical properties of *C. aurantifolia* leaf extracts

Physical properties observed in the case of this study were colour, odour and texture as well as percentage yields of the water, ethanolic and chloroform extracts of the *C. aurantifolia.*

Phytochemical Screening

Test for Flavonoids

To 4 mg/ml of each of the fractions, a piece of magnesium ribbon was added this was followed by drop wise addition of concentrated HCl. A colour change ranging from orange to red indicated flavones while red to crimson indicated flavonoids (Sofowora, 1993).

Test for Tannins

Two milliliters of each of the extract was diluted with distilled water in separate test tube and 2 to 3 drops of 5% ferric chloride ($FeCl_3$) solution was added to it. A green – black or blue colouration indicated the presence of tannins (Ciulci, 1994).

Test for Steroids

Two milliliters of the extracts were evaporated to dryness in separate test tubes and the residues dissolved in acetic anhydride followed by addition of chloroform. Concentrated sulphuric acid was added by means of a pipette via the side of the test tubes. Formation of brown ring at the interface of the two liquids and violet colour in the supernatant layer denoted the presence of steroids (Ciulci, 1994).

Test for Cardiac Glycosides

Ten millilitre (10mL) of 50% H_2SO_4 was added to each 1ml of the*C. aurantifolia* leaf extracts (for both water, ethanol and chloroform fractions) in separate test tubes and the mixture heated for 15minutes followed by addition of 10ml of Fehling's solution and boiled. A brick red precipitate indicated the presence of Glycosides (Sofowara, 1993).

Test for Alkaloids

Samples aliquots of extract (0.1ml) were added in test tubes and then 2 to 3 drops of Dragendoff's reagent were added. An orange red precipitate indicated the presence of alkaloids (Ciulci, 1994).

Test for Saponins

5ml of distill water was added to 2ml of the extract and shaken vigorously. Formation of foam following shaking indicates the presence of saponins (Sofowara, 1993).

Test for Terpenoids

To 0.5g of the plant extracts 2ml of chloroform was added followed by additional 3 ml of concentrated sulphuric acid resulting in the formation of two layers. A reddish brown colouration at the interface indicated the presence of terpenoids (Ayoola *et al*., 2008).

Test for Phenols

A millilitre of each plant extract filtrate was shaken in 10 ml of distilled water for 30seconds. Persistent frothing indicated the presence of saponins (Sowofora, 1993).

BIOASSAY

Test isolates

Isolates comprising *Salmonella typhi*, *Salmonella paratyphi* A, and *Salmonella paratyphi* B, were collected from Department of Microbiology, Aminu Kano Teaching Hospital, Kano, Nigeria.The test bacterial isolates were characterized by observing their cultural growth characteristics and various biochemical tests for identification of catalase, oxidase, indole, motility, citrate utilization, urease production, hydrogen sulfide production as well as acid and gas production, according to standard procedures (Cheesbrough, 2006).

Standardization of inocula

Each of the test bacteria were cultured onto Mueller Hilton agar plates and incubated for 18 – 24hours at 37^0C to obtain colonies. After overnight incubation, 4 colonies were selected with a sterile disposable inoculating loop and transferred to a glass tube of sterile physiological saline and vortex thoroughly until the turbidity of the suspension matched the turbidity of the 0.5 McFarland standardcontaining about 1.5×10^8 CFU/mL (NCCLS, 2008).

Sensitivity Testing

Agar well diffusion method was used for the antibacterial susceptibility test. Using sterile swab stick, standardized inocula ($1.5x10^8$ cells/mL) of each isolate was swabbed onto the surface of Mueller Hinton Agar in separate Petri dishes. Wells of 6mm diameter were made with cork borer. Into each well 0.15 mL of the plant extract was dispensed. The extracts were allowed to diffuse into the medium for 30minutes at room temperature. This was then incubated at 37^0C for 24 hours after which the zones of growth inhibition were measured and recorded in millimeter. Standard antibiotic was used as positive control while sterile distilled water was used as the negative control (Dahiru *et al.,* 2008).

Determination of Minimum Inhibitory Concentration

MIC was determined by preparing various concentrations of the extracts by serial doubling dilution using Dimethyl Sulfoxide (DMSO) and incorporated into test tubes containing 2 ml nutrient broth. Specifically 0.1 ml of standardized inocula was added to each of the test tubes and incubated at 37°C for 24 hand observed for the least concentration without turbidity.Tube containing broth and extracts without inocula served as a positive control while tubes containing broth and inocula without extract served as negative control (Fatope *et al.,* 1993).

Determination of Minimum Bactericidal Concentration

MBC was determined by inoculating samples from the MIC tubes that showed no bacterial growth on Mueller Hilton agar plates separately and then incubated at 37^oC for 24 hours. After the incubation the plates were observed for presence or absence of growth. The least concentration of the extract that showed no bacterial growth was considered as the MBC (Fatope et al., 1993).

RESULTS AND DISCUSSION

The physical properties and percentage yield of the aqueous, ethanolic and chloroform extract of *C. aurantifolia* are shown in (Table 1). The highest percentage yield of the extract was observed in aqueous extract (9.96%w/w), chloroform extracts (6.18% w/w) and ethanolic extracts with (2.70% w/w). Table 2 shows the result of phytochemical screening.Alkaloids, tannins, flavonoids, and phenols are the main phytochemical constituents of *C. aurantifolia*. These phytochemicals have been reported for antimicrobial activity (Singh and Bhat 2003).

This finlding corroborates the work of Ikeyi and Omeh, (2014) who reported that *C. aurantifolia* contains a lot of phytochemical constituents including alkaloids, flavonoids, tannins, phenols which were reported to possess antimicrobial properties. The metabolism and pharmacokinetics of flavonoids has been an area of active research in the last decade (Crozier *et al*., 2006).Harborne and Williams (2000) revealed that, Terpenoids are attributed for analgesic and anti-inflammatory activities and flavonoids have been reported to possess many useful properties, including anti-inflammatory, estrogenic, enzyme inhibition, antimicrobial, antiallergic, antioxidant, vascular and cytotoxic anti tumour activity. Pritesh and Zara (2015) reported that although alkaloids sometimes help in important cure, they have pain killing and poisonous effect.

Table 3 shows antibacterial activity of *C. aurantifolia.*The aqueous extract produces highest zone of inhibition of (19mm) against *S. paratyphi A*, followed by chloroform extract with zone of inhibition of 13mm against the same bacteria. *S. typhi* was resistant to both aqueous and chloroform extract of*C. aurantifolia* while been sensitive to ethanolic extract of *C. aurantifolia* even at lowest concentration. Both *S. paratyphi A* and *S. paratyphi B* showed resistance toethanolic extracts of *C. aurantifolia*.

Odunbaku *et al.*(2012)reported that *Citrus aurantifolia* and *Mangifera indica* plant extracts have considerable inhibitory effects on *Staphylococcus albus, Pseudomonas aeruginosa, Aspergillus terreus, Aspergillus niger* and *Penicillium oxalicum.*

Table 4 shows that water extracts of *C. aurantifolia*had MIC of (12.5µg/ml - 25µg/ml), ethanolic extracts had (12.5µg/ml - 50µg/ml) while chloroform extracts had MIC range of (25µg/ml - 100µg/ml).Both the extracts had MBC range of (25µg/m-100µg/m).While the standard antibiotic (amoxicillin) had MIC and MBC ranges of (6.25µg/m-12.5µg/m) and (12.5µg/m-25µg/m) respectively.

Table 1: Some Physical Properties of *C. aurantifolia* Extracts

Solvent	Color	Odor	Texture	WSU (g)	Amount Recovered(g)	% Yield
Chloroform	Dark green	Lemony	Thick and Oily	50	3.09	6.18
Ethanol	Yellowish Green	Fragrant	Slightly slimy	50	1.35	2.70
Aqueous	Reddish Brown	Pungent	Gummy	50	4.98	9.96

Key: WSU= Weight of Sample Used

Table 2: Phytochemical composition of *C. aurantifolia* Extracts

Extracts	Alk	Sap	Tan	Flavonoids	Steroids	Glycosides	Terpenoids	Phenols
Aqueous	+	+	+	+	+	-	+	+
Ethanolic	+	+	+	+	-	-	-	+
Chloroform	+	-	+	+	-	-	-	+

Key: += present, -= absent, Alk = alkaloids, Sap = saponins, tan = tannins

Table 3: Antibacterial Activity of *C. aurantifolia* Extracts

Test Organisms	AE (µg/ml)				EE (µg/ml)				CE (µg/ml)				Amx (µg/ml)			
	50	100	200	400	50	100	200	400	50	100	200	400	50	100	200	400
	Zone of Inhibition Diametre (mm)															
S. typhi	0	0	0	0	9	11	12	12	0	0	0	0	12	12	15	18
S. par. A	8	9	11	19	0	0	0	0	10	11	12	13	9	11	11	14
S. par. B	0	0	10	12	0	0	0	0	0	0	0	8	8	9	14	16

Key:AE = Aqueous Extract, EE = Ethanolic Extract, CE = Chloroform Extract, Amx = Amoxicillin

Table 4: MIC and MBC of *C. aurantifolia* Against the Test Bacteria

S/N	Test Organisms	Aqueous Extract		Ethanolic Extract		Chloroform Extract		Amoxicillin	
		MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml
1	*S. typhi*	25	50	50	100	50	100	12.5	25
2	*S. paratyphi A*	12.5	50	12.5	25	50	100	6.25	25
3	*S. paratyphi B*	25	50	12.5	25	25	50	6.25	12.5

Key: MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration

CONCLUSION

From the result of this research it can be concluded that *C. aurantifolia* presents potential of producing new drugs for the treatment of typhoid fever. Further research should be carried out on this plant to determine its safety for consumption.

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