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In vitro Antimicrobial Activity of Crude Extracts of *Citrus aurantifolia* Linn and *Tithonia diversifolia* Poaceae on Clinical Bacterial Isolates

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Abstract: The aim of this study is to investigate the antibacterial effects of two plant extracts, *Citrus aurantifolia* linn (lime) and *Tithonia diversifolia* poaceae (sunflower) commonly used as traditional medicines in Nigeria. The antibacterial activities of aqueous extracts of these two plants were evaluated using disk diffusion susceptibility testing on 53 fresh human bacterial pathogens isolated in Ladoke Akintola University Teaching Hospital, Osogbo, Nigeria. These isolates included 27 *Staphylococcus* sp., 15 *Escherichia coli*, 3 *Klebsiella* sp., 4 *Proteus* sp. and 4 *Pseudomonas* sp. The Mean Zone Diameter (MZD) of inhibitions to 5 μL of *Citrus aurantifolia* linn extracts are 10, 12, 11, 17 and 16 mm for *Staphylococcus* sp., *E. coli, Klebsiella* sp., *Proteus* sp. and *Pseudomonas* sp., respectively. The MZD of inhibitions to 5 μL of *Tithonia diversifolia* poaceae extract are 1, 1, 0, 8 and 0 mm and for 5 μg ciprofloxacin (control) disk are 29, 19, 20, 25 and 15 mm for these respective organisms. Forty four percent (12 of 27) of the Gram positive and 69% (18 of 26) of the Gram negative pathogens showed Zone Diameter (ZD) of inhibition = 10 mm to 5 μL extract of *Citrus aurantifolia* linn. Four percent (1 of 27) of the Gram positive and 8% (2 of 26) of the Gram negative pathogens showed ZD of inhibition = 10 mm to 5 μL of *Tithonia diversifolia* poaceae. In comparison, 93% of the Gram positive (25 of 27) and 65% (17 of 26) of Gram negative pathogens showed zone diameter of inhibition = 10 mm to 5 μg ciprofloxacin (control disk). When compared to ciprofloxacin, 5 μL extract of *Citrus aurantifolia* linn possess approximately 33% antistaphylococcal activities of 5 μg ciprofloxacin and approximately the same activity on the Gram negative bacterial isolates tested ($p = 0.2767$). However, 5 μL extract of *Tithonia diversifolia* poaceae does not possess any appreciable antibacterial effect on any of the isolates when compared to 5μg ciprofloxacin disc ($p = 0.0045$) or when compared to 5 μL extract of *Citrus aurantifolia* linn ($p = 0.0149$). *Citrus aurantifolia* linn showed promising broad spectrum antibacterial effects on human pathogens.

Key words: Antibacterial, citrus, tithonia, extracts, antimicrobial activity crude

INTRODUCTION

Infectious diseases have remained a leading cause of death worldwide but particularly in developing countries. In recent times, epidemics of infections due to drug resistant and hitherto unknown microbial organisms have posed enormous health concern. Infectious diseases are the underlying cause of death in approximately 80% of cases even in the United States of America (Pinner *et al*., 1996). This situation has called for renewed strategies on treatment and prevention, of which the development of new antimicrobial agents is one of the strategies adopted by the Center for Disease Control and Prevention (Fauci, 1998).

Extracts of plants have been used for treatment of many ailments in folklore medicinal practice. In Nigeria, more than 70% of the populace depends on various forms of plant extracts used as herbal concotions for treatment of many ailments including infectious diseases (Kimbi and Fagbenro, 1996). Some of these plants have been shown by a number of researchers (Akobundun and Agyakwa, 1987; Almagboul *et al*., 1988; Rocio and Rion, 1989; Mistral *et al*., 1992; Hable-Miriam *et al*., 1993) to possess antimicrobial effects.

Citrus aurantifolia Linn popularly known as lime is one plant that has been in use for ailments such as common cold, depressive illness and alcoholism and has been acclaimed to also possess anti-inflammatory, anti-rheumatic, anti-scorbutic, anti-coagulant, anti-spasmodic and anti-infective properties. Oboh *et al.* (1992), Oboh and Abulu (1997) and Onyeagba *et al.* (2004) have at various times demonstrated anti-microbial effects of this plant

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on such bacteria as *Bacillus* sp., *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. with varying results. *Tithonia diversifolia* poaceae (sunflower) considered as weeds in farmland is one of the 75 sp. in 34 plant families used as sources of traditional medicines to treat 59 ailments in Central Kenya (Njoroge *et al*., 2004). It has been acclaimed to possess antimalarial and anti-helminthic properties and also useful for the treatment of cold/flu, pimples and stomachache. This plant is also commonly used in Nigeria along with *Citrus aurantifolia* linn to prepare herbal concotions for treatment of many ailments believed to be caused by microbes.

In this study, the anti-microbial effects of these two plant extracts were tested against some human bacterial pathogens. This is an attempt to provide scientific basis for the use of these extracts in traditional African medicinal practice and the possibility of using this knowledge to produce new antimicrobial agents that may be effective against some of the emerging and re-emerging bacterial pathogens.

MATERIALS AND METHODS

Bacterial isolates: The bacterial organisms were recovered from fresh clinical specimens routinely processed at the Microbiology Laboratory of Ladoke Akintola University Teaching Hospital, Osogbo and identified using standard microbiological methods (Barrow and Feltham, 1993; Cheesborough, 2000) between September and December 2005. The isolates are 27 *Staphylococcus* sp., 15 *Escherichia coli,* 3 *Klebsiella* sp., 4 *Proteus* sp. and 4 *Pseudomonas* sp. (Table 1). Each of the organisms is a clinically significant bacterial isolate recovered in pure cultures from an infective focus. The isolates were colony purified and kept on nutrient slopes until use.

Preparation of extract of *citrus aurantifolia* linn (lime): Lime fruits were brought from Igbona market in Osogbo, Southwest Nigeria and identified by a Botanist in Ladoke Akintola University of Technology, Ogbomoso also in Southwest Nigeria. The fruits were washed with sterile water and then cut open with sterile knife and the juice pressed out into a sterile conical flask. The juice was sieved using net sieve to remove the seeds and poured into a conical flask with foil plug. The juice was stored at 4°C until use.

Preparation of aqueous extract of *tithonia diversifolia* poaceae (sunflower): The sunflower leafs were plucked from various locations in Osogbo, Nigeria and identified by a Botanist in Ladoke Akintola University of Technology, Ogbomoso. Four grams of the leaf was washed with sterile water and then blended with an electric blender. The contents of the blender was dissolved in 4 litres of sterile water in a sterile container and left for 2 weeks, after which it was sieved using sterile net sieve. The filtrate (extract) was poured into a sterile conical flask with foil plug and stored at 4°C until use.

Table 1: Fresh clinical bacterial isolates tested against the plant extracts and ciprofloxacin

Bacterial isolates	Gram positive	Gram negative
Staphylococcus sp.	27	-
Escherichia coli	-	15
Klebsiella sp.	-	3
Proteus sp.	-	4
Pseudomonas sp.	-	4
	27	26

Preparation of wet antimicrobial disks from the extracts: Wet disks for antimicrobial sensitivity tests were prepared in the Microbiology Laboratory of the Ladoke Akintola University Teaching Hospital, Osogbo, by the modification of the method of Gould and Bowie (1952). First, a 6.25 mm-diameter plunger was used to punch a Whatman No 1 absorbent filter paper to obtain a 6.25 mm diameter paper disks. The disks for *Citrus aurantifolia* linn were marked 'C' and that for *Tithonia diversifolia* poaceae 'T' for identification. The paper disks were dispensed in batches of 200 in screw-capped bottles and sterilized at 160°C for 1 h. One mililitre of each of the extract was added to the bottle of 200 disks, each disk containing 0.005 mL (5 μL) of the extract. These were then stored in wet condition in sterile screw-capped container, with cap tightly screwed. The container was dated and kept in the refrigerator at 4°C until use.

Susceptibility testing procedure for the extract and ciprofloxacin: Susceptibility testing was done on each isolate (in triplicate) for each of the extract and ciprofloxacin using the single disk principle previously described (Bauer *et al*., 1966; Onyeagba *et al*., 2004). Briefly, fresh subculture of pure colonies of each bacterial isolate was made on nutrient agar and incubated overnight aerobically at 37°C. A sterile straight wire was used to touch 5 discrete colonies of each isolate on the culture plate. This was inoculated into 2 mL peptone water inside a Bijou bottle. The turbidity of the inoculum was standardized with 0.5 McFarland standards to give 8×10^{8} CFU mL^{-1}. The inoculum was then poured onto a freshly prepared Mueller-Hinton agar plate and excess fluid decanted into a disinfectant jar. The plates were allowed to dry. A disk of *Citrus aurantifolia* linn (C) and *Tithonia diversifolia* poaceae (T) and a commercially supplied 5 μg

ciprofloxacin disk (Swipha Nig Ltd) were placed on each plate with the aid of a sterile forcep. The plates were incubated at 37°C for 24 h. Zone diameter of inhibitions (in mm) to each extract and to ciprofloxacin disk was measured using a calibrated ruler and recorded.

The antimicrobial activity of each extract was inferred from the zone diameters of inhibition of each organism and this was compared with that for ciprofloxacin.

RESULTS

The antibacterial effects of the two plant extracts and ciprofloxacin were tested against 53 bacteria isolates; 27 Gram positives and 26 Gram negatives. These isolates included 27 *Staphylococcus* sp., 15 *Escherichia coli*, 3 *Klebsiella* sp., 4 *Proteus* sp. and 4 *Pseudomonas* sp. (Table 1).

The Mean Zone Diameter (MZD) of inhibitions to 5 μL of *Citrus aurantifolia* linn extracts are 10, 12, 11, 17 and 16 mm for *Staphylococcus* sp., *E. coli, Klebsiella* sp., *Proteus* sp. and *Pseudomonas* sp., respectively. The MZD of inhibitions to 5 μL of *Tithonia diversifolia* poaceae extract are 1, 1, 0, 8 and 0 mm and to 5 μg ciprofloxacin (control) disk are 29, 19, 20, 25 and 15 mm for these respective organisms (Table 2).

Forty four percent (12 of 27) of the Gram positive and 69% (18 of 26) of the Gram negative pathogens showed Zone Diameter (ZD) of inhibition = 10 mm to 5 μL extract of *Citrus aurantifolia* linn. Only 4% (1 of 27) of the Gram positive and 8% (2 of 26) of the Gram negative pathogens showed ZD of inhibition = 10 mm to 5 μL of *Tithonia diversifolia* poaceae. In comparison, 93% of the Gram positive (25 of 27) and 65% (17 of 26) of Gram negative pathogens showed zone diameter of inhibition = 10 mm to 5 μg ciprofloxacin (Table 3 and 4). When compared to ciprofloxacin, 5 μL extract of *Citrus aurantifolia* linn possess approximately 33% antistaphylococcal activities of 5 μg ciprofloxacin and approximately the same activity on the Gram negative bacterial isolates tested (p= 0.2767).

Table 2: Mean Zone Diameters (MZD) of inhibition of the isolates by the plant extracts and ciprofloxacin

Organisms	Ciprofloxacin (mm)	Tithonia (mm)	Citrus (mm)
Staphylococcus sp. (n = 27)	29	1	10
Escherichia coli (n = 15)	20	1	12
Klebsiella sp. (n = 3)	19	0	11
Proteus sp. (n = 4)	25	8	17
Pseudomonas sp. (n = 4)	15	0	16
Mean±SD	21.6±5.459	2±3.391	13.2±3.114
p value	p>0.10	p = 0.0049	p>0.10

Table 3: Zone diameter of inhibition of Gram positive isolates by the plant extracts and ciprofloxacin

Isolate	Isolates no	Ciprofloxacin	Tithonia	Vitrus
Staphylococcus sp.	1	38	0	22
	2	32	0	8
	3	11	0	0
	4	25	0	10
	5	30	0	11
	6	34	8	7
	7	21	0	0
	8	29	0	15
	9	11	0	12
	10	28	8	9
	11	26	0	10
	12	31	0	8
	13	34	0	9
	14	46	0	0
	15	40	0	8
	16	37	0	0
	17	37	0	13
	18	43	0	23
	19	25	0	0
	20	46	0	19
	21	20	0	15
	22	37	0	0
	23	0	0	13
	24	38	11	18
	25	21	0	20
	26	7	0	12
	27	32	0	7
	Mean ZD (mm)	28.9	1.0	9.96

*Susceptibility test for each extract and ciprofloxacin was conducted on each isolate in triplicate and an average of the three tests taken for that isolate.
**MZD is the mean of the inhibition zones for all the isolates in a group

Table 4: Zone diameter of inhibition of Gram negative isolates by the plant extracts and ciprofloxacin

Isolate	Isolate no	Ciprofloxacin	Tithonia	Citrus
E. coli (n = 15)	1	18	0	12
	2	9	0	10
	3	33	0	9
	4	28	0	0
	5	10	0	10
	6	8	0	9
	7	21	0	12
	8	16	0	12
	9	38	0	14
	10	35	0	15
	11	14	0	13
	12	0	0	16
	13	7	0	10
	14	30	7	16
	15	28	10	20
	MZD (mm)	19.7	1.1	11.9
Kleb sp.	1	40	0	18
	2	17	0	5
	3	0	0	10
	MZD (mm)	19	0	11
Proteus sp.	1	27	0	15
	2	0	0	17
	3	35	16	17
	4	37	16	17
	MZD (mm)	24.8	8	16.5
Pseudomonas sp.	1	27	0	15
	2	33	0	16
	3	0	0	17
	4	0	0	14
	(MZD (mm)	15	0	15.5

*Susceptibility test for each extract and ciprofloxacin was conducted on each isolate in triplicate and an average of the three tests taken for that isolate.
**MZD is the mean of the inhibition zones for all the isolates in a group

However, 5 μL extract of *Tithonia diversifolia* poaceae does not possess any appreciable antibacterial effect on any of the isolates when compared to 5μg ciprofloxacin disk ($p = 0.0045$) or when compared to 5 μL extract of *Citrus aurantifolia* linn ($p = 0.0149$).

DISCUSSION

The clinical success of quinine and quinidine isolated from the *Cinchona* tree bark and recently artemisinin from *Artemisia annua* in the treatment of malaria have rekindled interest in medicinal plants as potential sources of novel drugs (DiFlumeri, 2000). Also, one of the renewed strategies on treatment and prevention of infectious diseases by the Center for Disease Control and Prevention is the development of new antimicrobial agents (Fauci, 1998).

Our study was aimed at investigating the antimicrobial activity of two plant extracts that are popularly used in Nigeria for medicinal purpose. The results of this study revealed that *Citrus aurantifolia* linn fruit juice had broad spectrum antibacterial effects but more on Gram negative bacteria with 69% of these producing ZD of inhibition = 10 mm while 44% of Gram positive bacteria produced ZD of inhibition = 10 mm. This finding which agrees with that of Onyeagba *et al.* (2004) and others (Oboh *et al*., 1992; Oboh and Abulu, 1997) shows that lime juice contain active ingredient that justify its use in traditional African medicinal practice.

In this study, the antimicrobial effect of crude lime juice on Gram negative bacteria is comparable to that of ciprofloxacin (a highly potent synthetic fluoroquinolone) though the latter possess a comparatively higher antibacterial effect on Gram positive bacteria. Identification and characterization of the active antibacterial component of *Citrus aurantifolia* linn may be necessary to produce a new drug in view of the fact that resistance to the currently available antibiotics including the fluoroquinolones by micro-organisms is increasingly being reported (Montefiore *et al*., 1989; Ogunsola *et al*., 1997; Threfall *et al*., 1997; Martinez-Martinez *et al*., 1998; Livermore *et al*., 2002; Daini *et al*., 2005).

Tithonia diversifolia poaceae on the other hand does not appear to possess appreciable antimicrobial effect on Gram positive and Gram negative bacteria as only 4 and 8%, respectively exhibited zone diameter of inhibition = 10 mm. By implication, it means the plant contain little or no appreciable active antibacterial ingredients or that the aqueous extraction method used did not yield enough active ingredients that can inhibit the organism. *Tithonia diversifolia* has been shown to be an important weed used for treatment of some ailments in Central Kenya (Njoroge *et al*., 2004). It has also been acclaimed to possess anti-malarial and anti-helminthic properties and is commonly used as herbal concotion when mixed with *Citrus aurantifolia* in Nigeria.

Our study did not, however, demonstrate any significant antibacterial effect of *Tithonia* and it does appear that the antibacterial effects of the combined *Citrus* and *Tithonia* herbal concotions is probably due to the antibacterial effect of only the *Citrus* component. *Tithonia* usefulness as herbal remedy may be limited to the acclaimed anti-helminthic or anti-malarial properties, which we did not investigate in this study. There may be the need in a separate study, to investigate the anti-helminthic and anti-parasitic properties of *Tithonia* and also to use other alternative extraction method such as ethanolic acid which has been shown to yield more active ingredients from medicinal plants (Ezenroye *et al*., 2005) before a conclusion could be drawn about the anti-microbial properties of *Tithonia*.

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