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Synergistic effect of *Murraya koenigii* and *Telfairia occidentalis* aqueous leaf extract on some bacteria

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Abstract
The phytochemical and antibacterial properties of aqueous leaf extract of *Murraya koenigii* and *Telfairia occidentalis* and their synergy were determined against five bacterial isolates: *Klebsiella pneumoniae, Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Shigella dysenteriae*. Saponins, flavonoids, sugar terpenoids, alkaloids, steroids, glycosides and tannins were detected. Antibacterial activity was determined by the disc diffusion method. Larger zones of inhibition were observed for *M. Koenigii* extract than *T. occidentalis* extract, and larger zones of inhibition were observed by their synergy than on their separate use. Synergistic antibacterial activity of the extract ranged from 0 mm to 20.0 ± 0.03 mm, zone of inhibition of *M. koenigii* extract ranged from 0 mm to 16.0 ± 0.03 mm while that of *T. occidentalis* ranged from 0 mm to 12.0 ± 0.30 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the two separate extracts and their synergy were also evaluated. These ranged from 62.5 mg/ml to 500 mg/ml for *M. koenigii*, 62.5 mg/ml to 500 mg/ml for *T. occidentalis* and 31.25 to 500 mg/ml for their synergy (least MIC to highest MBC). The test organisms were assessed for their antibiotic susceptibility pattern and were observed to be multi-drug resistant. The synergy showed higher zone of inhibition against the test bacteria than the separate extracts and the standard antibiotics. It is therefore, recommended to use the synergy to treat infections caused by the test organisms.

Keywords: Synergy, *Telfairia occidentalis, Murraya koenigii*, Antimicrobial activity, Aqueous extract

INTRODUCTION

Microbial infections constitute a major public health problem in developing countries (Adwan *et al*., 2010) where the high cost of antibiotics makes them unaffordable to the majority of the population. Therefore, the discovery of new antimicrobial agents is still relevant nowadays. Despite the impressive scientific progress in vaccination and chemotherapy, infectious diseases remain a serious health issue. Following the massive and inappropriate use of antibiotics, bacteria have developed various mechanism of resistance; consequently, infectious diseases remain one of the leading causes of morbidity worldwide (Ahluwalia and Sharma, 2007). Among the bacterial resistance mechanisms, efflux of antibiotics plays an important role. In fact, it is widely recognized that the expression of efflux pumps encoded by housekeeping genes in bacteria is largely responsible for the phenomenon of intrinsic antibiotic resistance (Poole, 2005). Also, the shortcomings of the drugs available today and the scarcity of novel antibiotics propel the discovery of new chemotherapeutic agents

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from medicinal plants (Ates and Erdogru, 2003).In the last two decades, antibiotic resistance has been an emerging problem worldwide (Walsh, 2000; Cohen, 2002). This has led to the search for new, safe and effective antimicrobial agents from alternative natural resources like plant products. At the same time, there is a growing demand among consumers for natural preservative or additives in processed foods (Gautierrez *et al*., 2008). In comparison to chemical or synthetic additives herbal additives from medicinal plants are preferred as these are safer, flavour enhancer and without any side effects (Brull and Coote, 1999).

Medicinal plants contains biologically active chemical substances such as saponins, tannins, essential oil flavonoids, alkaloids and other chemical (Sofowora, 1996) which have curative properties. These complex chemical substances of different composition are found as secondary plant metabolite in one or more of these plants (Kayode and Kayode, 2011). A medicinal plant is any plant which is one or more of its organs contain substances that can be used for the synthesis of useful drugs (WHO, 1977). According to the World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al.*, 2000). There are several published reports describing the antimicrobial activity of various crude plant extracts either in single or in combinations (Igoli *et al.*, 2005). It is estimated that there are about 2.5 million species of higher plants and the majority of these have not yet been examined for their pharmacological activities. Herbal extracts are fast becoming popular as natural antimicrobial preservatives or additives (Cox *et al.*, 2010).

Telfairia occidentalis, commonly called fluted pumpkin has been reported by many investigators, as having many medicinal uses. In Nigeria, the herbal preparation of the plant has been employed in the treatment of anaemia, chronic fatigue and diabetes (Alada, 2000; Dina *et al*., 2006; Kayode and Kayode, 2011). The leaves contain essential oils, vitamins; root contains cucubitacine, sesquiterpene, lactones (Iwu, 1983). The young leaves sliced and mixed with coconut water and salt are stored in a bottle and used for the treatment of convulsion in ethno medicine (Gbile, 1986). The leaf extract is useful in the management of cholesterolemia, liver problems and impaired defense immune systems (Eseyin *et al*., 2005). *Telfairia occidentalis* is popularly used in soup and folk medicine preparation in the management of various diseases such as diabetics, anaemia and gastrointestinal disorder (Oboh *et al*., 2006). A study has shown that the ethanol root extract of *T. occidentalis* possess antiplasmodial potential (Okonko *et al*., 2007) and an inhibitory effects on some enterobacteriacae (Odoemena and Onyeneke, 1998) while Oluwole *et al*. (2003), reported *Telfairia occidentalis* anti-inflammatory activities (Kayode and Kayode, 2011). *T. occidentalis* occurs in the forest zone of West and central Africa most frequently in Benin, Nigeria and Cameroon (Kayode and Kayode, 2011). It is a popular vegetable all over Nigeria and has been suggested that it originated in south-east Nigeria and was distributed by the Igbos, who have cultivated this crop since time immemorial (Badifu and Ogunsina, 1991). Studies have also reported the medicinal activities of curry leaf (*Murraya koenigii*).

Murraya koenigii belongs to the family Rutaceae, it is found throughout India and it is cultivated for its aromatic leaves. The leaves are pinnate, with 11-21cm broad and flowers are small, white with pleasant fragrance. The leaves are used extensively as a flavouring agent curries and chutreys (Gopalan *et al*., 1984). The green leaves were chewed raw for the cure of dysentery (Gopalan *et al*., 1984), while the leave paste were used eternally to treat bites of poisonous animals (Kesari *et al*., 2005), bruises and eruption (Kumar *et al*., 1999). The plant had reported to possess positive inotropic effect (Rahman and Gray, 2005), antidiabetic, cholesterol reducing property antibacterial and microbiological activity (Manfred *et al*., 1985) antiulcer activity (Xie *et al*., 2006) antioxidative property and cytotoxic activity (Shah and Juvekar, 2006). It has been reported to contain acytotoxic coumarin murraya compound and flavonoids which has constantly being screened for anti-tumour activity (Ruby *et al*., 1995). The main constituents reported were alkaloids volatile oil, amino acids, glycosides, proteins, monoterpenoids, and sesquiterpenoids. Furthermore, the microbial mutations and appearance of new recombinant pathogenic microorganisms necessitate the continuous assessment of new antimicrobial activities of different medicinal plants. Currently, researchers have focused on the synergistic effects of various plant extracts such as (Dawoud *et al*., 2013). However, there are no study on the antimicrobial synergy of *Murraya koenigii* and *Telfairia occidentalis* aqueous leaf extract, hence the interest of the authors in this research.

EXPERIMENTAL

Collection of plant materials. Fresh *M. koenigii* and *T. occidentalis* leaves were purchased from New Benin market in Benin City, Edo State, Nigeria and identified in Department of Plant Biology and Biotechnology of the University of Benin, Benin City, identification was confirmed with appropriate literature (Hutchinson and Dalziel, 1954; Odugbemi and Akinsulire, 2006). The leaves were air dried, grinded and made into a fine powder using laboratory mortar and pestle. The powdered leave was kept in a sterile air-tight container to avoid contamination.

Preparation of aqueous extract: Fifty grams each of dried pulverized leaf powder was dissolved in 500ml each of distilled water for 24hrs and centrifuged at 3000rpm to enable paper diffusion of the active ingredients into the extraction medium. Filtration was later carried out using Whatman's (No. II) filter paper. The filtrate was evaporated to dryness using steam water bath at 100 ^{o}C. The extracts were now stored at 4^{o}C in a refrigerator. Combination of both extracts was used in the synergistic assessment.

Collection of test bacteria. The test bacteria were collected from the Department of Medical Microbiology, University of Benin Teaching Hospital, (UBTH), Benin City, Nigeria. Their identity was confirmed using standard biochemical tests as prescribed by Jolt *et al*., 1994 and Cheesbrough, 2006. The test bacteria were maintained on nutrient agar slants at 4 ^{o}C.

Description of research bacteria. The test organisms: *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Bacillus subtilis* and *Shigella dysenteriae*, have been previously described (Prescott *et al*., 2005; Akinnibosun *et al*., 2008a, 2008b).

Phytochemical screening of extracts. Phytochemical screening of the extracts was carried out according to methods described by Odebiyi and Sofowora, (1978) and Evans, (1989). The component analysed for were: flavonoids, tanins, glycosides, reducing sugars, terpenoids, saponins, anthraquinones, alkaloids, steroids.

Determination of antimicrobial activity. The inocula were prepared by enriching the test organisms in nutrient broth and in incubating them at 37 ^{o}C for 24 hrs. Antimicrobial activity of the extracts was evaluated against the test organisms using the disc diffusion method (Newman *et al*., 2003). Nutrient agar plates were seeded with the suspension (diluted cultures) of the test bacteria. Sterilized Whatman (No. I) filter paper was used to prepare the disc (6mm) and impregnated with the different concentration of the extracts (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml 31.25 mg/ml), dried and placed aseptically on seeded plates with the help of sterile forceps. The discs were spaced to prevent overlapping of zones of inhibition. The plates were incubated at 37 ^{o}C for 24 hrs. The resulting zones of inhibition were measured and recorded. Standard antibiotics were used as positive control and for antibiotic susceptibility testing of the test organisms.

Determination of Minimum Inhibitory Concentration (MIC). The nutrient agar was prepared and sterilized, then poured into sterile Petri dishes and allowed to solidify. The surface of the medium was inoculated with the test isolates. The disks soaked in different concentrations of the extract were placed on the surface of the seeded nutrient agar. The plates were incubated at 37 °C for 24 hours, after which they were examined for the presence of growth inhibition. The MIC was taken as the lowest concentration that prevented the growth of the test microorganisms.

Determination of Minimum Bactericidal Concentration (MBC). A loopful of the content of each plate in the MIC determination above, which did not show any visible growth after the period of incubation was streaked unto freshly prepared Nutrient agar to determine their MBC and then incubated at 37 °C for 24 hours after which it was observed for visible growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration.

Antibiotic susceptibility test. Antibiotic susceptibility tests of the isolates was performed according to the recommendations of the National Committee Laboratory Standards (NCCLS), (2002) using the following antibiotic discs: tetracycline (20 µg), ampiclox (30 µg), zinnacef (20 µg), amoxicillin (30 µg), rocephin (25 µg), ciprofloxacin (l0 µg), nitrofurantin (20 µg), streptomycin (30 µg), erythromycin (l0 µg), gentamicin (I0 µg), septrin (30 µg), chloramphenicol (25 ug), perfloxacin (l0 µg), and ofloxacin (30 µg). The antibiotics resistance was interpreted by diameter of inhibition zones around the antibiotic discs.

RESULTS AND DISCUSSION

Antibacterial activities of extracts of different plants against various microorganisms have been reported by many scientists (Sagdic and Ozcar, 2003; Shan *et al*., 2007; Gautierrez *et al*., 2008). But there are few reports on their synergistic effects, extract on some pathogenic organisms and comparing it with the activity of standard antibiotics.

Table 1 shows the phytochemical components of the extracts of *M. koenigii* and *T. occidentalis*. The results indicated the presence of flavonoids, tannins, glycosides, reducing sugars, terpenoids, saponins, alkaloids, anthraquinones and steroids. This agrees with the works of Akande and Yahaya, 2010 and Mohar *et al*., 2011, who isolated the compounds from the leaves. These compounds have been found to inhibit bacterial growth and are capable of protecting certain plants against bacterial infections (Oyewole and Abalaka, 2012; Clark, 1981; Gonzala and Matler, 1982).

The antibacterial activity of the aqueous leaf extract of *M. koenigii*, *T. occidentalis* and their synergy is shown in tables 2, 3 and 4 respectively. The extract showed varying antimicrobial activity against the test organisms as indicated in the tables. The highest concentration of 500 mg/ml was the most effective is inhibiting the organisms. The antibacterial activity was measured as zones of inhibition in mm and it was shown in this study, to be concentration dependent. That is, increase in concentration of the extract resulted in increased antimicrobial activity. This agrees with the finding of Kurosaki and Nishi, (1933) and Akinnibosun and Akinnibosun, (2011) who reported that higher concentration of antimicrobial substances showed appreciably more growth inhibitions being both bacteriostatic and bacteriocidal.

The antibacterial activity of *T. occidentalis* was less than that of *M. koenigii*, but it was also concentration dependent. At 500 mg/ml, 10 ± 0.01mm inhibition zone was recorded against *K. pneumoniae*, with the extract of *T. occidentalis*, compared to 16.5 ± 0.03mm against *K. pneumoniae* observed by *M. koenigii* leaf extract. More so, it was observed that the extracts were active against Gram positive and Gram negative organisms (Tables 2, 3 and 4). This indicates that the plant extracts contained active principle with broad antibacterial spectrum (Bankole, 1992).

The synergy of *M. koenigii* and *T. occidentalis* leaf extracts gave higher zones of inhibition (Table 4) neither *M. koenigii* extract nor *T. occidentalis* extract could give. This showed that both leaf extracts acted synergistically against the test isolates (Ates and Erdogrul, 2003; Adwan *et al.*, 2010). This synergy is supported by Prekesh *et al.*, 2006a and Dawoud *et al.*, 2013.The additive and synergistic effects of phytochemicals enhanced the antibacterial effect of the synergy extract (combined) extract (Matchimuthu *et al.*, 2008) According to Cain *et al*. (2003), synergistic activity suggest different mode of action of the combining components.

The MIC and MBC of individual plant extract and their synergy were evaluated. The extract synergy (showed lower MIC and MBC values against the test organisms (Tables 5, 6 and 7). This observation was supported by Dawoud *et al.*, 2013). The MIC and MBC of *M. koenigii* (Table 5) on *K. pneumoniae* were 62.5 mg/ml and 500 mg/ml respectively, the MIC and MBC of *T. occidentalis* (Table 6) on *K. pneumoniae* were 62.5 mg/ml and 500 mg/ml. The other organisms had similar results and this further proved that *M. koenigii* was more potent than *T. occidentalis* agrees with those of Mohar *et al.* (2011); and Akande and Yahaya (2010).

Antibiotic susceptibility test was carried out on all the test bacteria employed in this work and the results have been displayed in table 8. The microorganisms were found to be resistant to many of the standard antibiotic used. The resistant nature of these microorganisms may have been acquired via plasmid transfer or chromosomally mediated (Walsh, 2000; Cohen, 2002; Coutinho *et al.*, 2010). Drug abuse and indiscriminate misuse of antibiotics among the general population has favoured the emergence of resistant strains. Multidrug resistance was observed for most of the test bacteria as they were resistant to more than one drug (Wasfy *et al.*, 2000; Akomie and Akpan, 2013). The worldwide escalation in both community and acquired antimicrobial resistant bacteria is threaten the ability to effectively treat patients, emphasizing the need for continued surveillance, more appropriate antimicrobial prescription, prudent infection control and new treatment alternatives (Mulvey, 2014; Rhomberg *et al.*, 2006; Chikere *et al.*, 2008; Okonko *et al*., 2009a). In comparison to the extracts, the synergy of both extracts was more potent than the standard antibiotics, since all the test organisms had higher zones of inhibition than the commonly used standard antibiotics. The susceptibility of antibiotic resistant bacterial strains to the plant extract is quite interesting and these plant extract can be used as an alternative in the treatment of diseases caused by these microorganisms (Cohen, 2002).

Conclusion. This study has shown that combinations of extracts demonstrated synergistic and additive effects on microorganisms.

Table 1: Phytochemical analysis of *M. koenigii* and *T. occidentalis* leaf extracts

Phytochemicals	*M. koenigii*	*T. occidentalis*
Flavonoids	+	+
Tannins	+	+
Glycosides	+	+
Reducing sugars	++	+++
Terpenoids	+	+
Saponins	+	+
Anthraquinones	+	++
Alkaloids	+	+
Steroids	++	++

Key: + = less abundant, ++ = abundant, +++ = highly abundant

Table 2: Antibacterial activity of *M. koenigii* leaf extract (zone of inhibition in mm)

	Concentration				
Test organisms	500mg/ml	250mg/ml	125mg/ml	62.5mg/ml	31.25mg/ml
S. aureus	15.0 ± 0.01	14.0 ± 0.05	10.5 ± 0.01	5.0 ± 0.05	0
K. pneumonia	16.5 ± 0.03	10.0 ± 0.01	7.0 ± 1.10	6.0 ± 0.06	0
B. subtilis	0	0	0	0	0
E. coli	14.0 ± 0.30	12.0 ± 1.10	7.0 ± 0.05	5.0 ± 0.05	0
S. dysenteriae	15.0 ± 0.10	13.0 ± 0.25	9.0 ± 0.30	6.0 ± 0.01	6.0 ± 0.01

Table 3: Antibacterial activity of *T. occidentalis* leaf extract (zone of inhibition in mm)

	Concentration				
Test organisms	500mg/ml	250mg/ml	125mg/ml	62.5mg/ml	31.25mg/ml
S. aureus	5.0 ± 0.03	0	0	0	0
K. pneumonia	10.0 ± 0.01	7.0 ± 0.01	3.0 ± 0.10	0	0
B. subtilis	8.0 ± 0.20	**0**	0	0	0
E. coli	12.0 ± 0.10	8.0 ± 0.10	6.0 ± 0.01	5.0 ± 0.01	0
S. dysenteriae	8.0 ± 0.10	8.5 ± 0.25	6.0 ± 0.30	0	0

Table 4: Antibacterial activity of the synergy of *M. koenigii* and *T. occidentalis* leaf extract (zone of inhibition mm)

	Concentration				
Test organisms	500mg/ml	250mg/ml	125mg/ml	62.5mg/ml	31.25mg/ml
S. aureus	20.0 ± 0.03	15.0 ± 0.02	13.0 ± 0.20	10.0±0.03	7.0 ± 0.10
K. pneumonia	15.0 ± 0.02	13.5 ± 0.01	12.0 ± 0.30	7.0 ±0.07	0
B. subtilis	14.0 ± 0.22	12.0 ± 0.10	8.0 ± 0.20	0	0
E. coli	20.0 ± 0.30	17.0 ± 0.20	13.0 ± 0.05	10.0±0.03	5.0±0.01
S. dysenteriae	10.0 ± 0.20	7.0 ± 0.10	0	0	0

Table 5: MIC and MBC of *M. koenigii* leaf extract

Test organisms	MIC(mg/ml)	MBC(mg/ml)
S. aureus	62.5	250.0
K. pneumonia	62.5	500.0
B. subtilis	ND	ND
E. coli	62.5	500.00
S. dysenteriae	62.5	250.00

Key: ND= Not detected

Table 6: MIC and MBC of *T. occidentalis* leaf extract

Test organisms	MIC(mg/ml)	MBC (mg/ml)
S. aureus	500.0	ND
K. pneumoniae	125.0	500.0
B. subtilis	62.5	ND
E. coli	62.5	500.0
S. dysenteriae	125.0	500.0

Key: ND= Not determined

Table 7: MIC and MBC of the synergy of *M. koenigii* and *T. occidentalis* leaf extracts

Test organisms	MIC (mg/ml)	MBC (mg/ml)
S. aureus	31.25	250.00
K. pneumoniae	62.50	500.00
B. subtilis	125.00	ND
E. coli	31.25	250.00
S. dysenteriae	250.00	ND

Key: ND= Not determined

Table 8: Antibiotic susceptibility testing (Positive control) (Zone of inhibition in mm).

Organisms	CPX	S	SXT	E	PEF	CN	APX	Z	AM	R
S. aureus	13.0	0.0	0.0	10.0	15.0	13.0	0.0	0.0	0.0	0.0
Bacillus subtilis	14.0	0.0	0.0	10.0	0.0	13.0	0.0	0.0	0.0	0.0
Gram –ve	TE	NB	OF	CPX	C	CN	AM			
E. coli	13.0	10.0	25.0	13.0	10.0	16.0	10.0			
K. pneumonia	10.0	10.0	15.0	17.0	0.0	10.0	0.0			
S. dysenteriae	11.0	0.0	8.0	0.0	0.0	0.0	0.0			

Key: CPX = Ciprofloxacin R = Rocephin S = Streptomycin TE = tetracycline
SXT = Septrin NB = Nitrofurantin E = Erythromycin C = Chloramphenicol
PEF = Pefloxacin OF = Ofloxacin CN = Gentamicin AM = Amoxicillin
APX = Ampiclox Z = Zinnacef

This synergy is performed as microbial tolerance is less likely to develop against substances having more than one type of modes of action. Differential antimicrobial activity of the extract against different bacteria was due to the presence of different active phyto-compounds which made the antibiotic-resistant organisms to be susceptible. It is therefore recommended that the synergistic use of medicinal plant extract be encouraged to prevent drug resistance and treat the emerging and re-emerging diseases caused by the organisms.

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