Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.29, 2015

The Antimicrobial Potential and Phytochemical Composition of *Aristolochia ringens* Vahl.

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Abstract
Aristolochia ringens, a plant called Ako-igun in Yoruba, is widely distributed in southwestern Nigeria. Traditionally, it is used for the treatment of cancerous sore, lung inflammation, dysentery and dermatitis. The antimicrobial activity of *A. ringens* was tested on six pathogenic bacteria viz., *Shigella sonnei, Salmonella typhii, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Proteus mirabilis* and five fungi viz., *Fusarium* sp*, Sporotrichum* sp*, Pichia* sp, *Penicillium camemberti* and *Aspergillus niger*. Extracts from powdered stem and root barks of *A. ringens* were prepared using sterile water, ethanol, petroleum ether and acetone. The extracts were tested on each of the pathogens for their antimicrobial properties. The stem and the root barks were also screened for presence of secondary metabolites following standard procedures. The aqueous extracts of the stem and the root barks were not effective against all the bacteria and fungi tested except *Shigella sonnei* with Minimum Inhibitory Concentration (MIC) of 40mg/ml. Petroleum ether extracts of the stem and root barks were equally not active against all the bacteria isolates used but the root bark extracts reacted effectively against the fungal isolates, showing good potency at 40mg/ml. Ethanol extract of the stem bark was very potent against all the bacteria isolates at MIC of 33.3mg/ml except *Shigella sonnei* while the root bark ethanol extract only showed little effectiveness against the fungi. A similar result was obtained when acetone was used as solvent of extraction. Comparatively, ethanol and acetone stem and root bark extracts were less potent on the fungi isolates than the petroleum ether extract. The plant parts studied showed the presence of alkaloids, flavonoids, tannins, terpenoids and cardiac glycosides. Thus, the root and stem bark extracts from *Aristolochia ringens* may be broad based in their antimicrobial activities. The extracts, if purified and crystallized, may serve as alternatives to antimicrobial drugs.
Keywords: *Aristolochia ringens*, Microorganisms, Ethnomedicine, Phytochemicals, Plant extracts

Introduction

Plants are known in ancient and modern civilizations for their healing properties. They remain the sole source of healing principle to man's ailments until the 19th century when development of chemistry, particularly organic compounds led to the experimental and clinical validation of their efficacy (Edeoga *et al.*, 2005; Natarajan *et al.,* 2011). *Aristolochia ringens*, a related local species, resembles over 500 species of *Aristolochia* (family Aristolochiaceae) that spread across tropical and sub-tropical regions of Asia and Africa, and used traditionally for cancerous sore, dysentery, lung inflammation, scorpion and snake bite (Kumar and Suryanarayana, 2008; Vermal *et al.*, 2008; Abhijit and Jitendra, 2011; Thirumal *et al.*, 2012). *Aristolochia* species are herbaceous vine with woody stock and widely distributed throughout the southwestern Nigeria (Kanjilal *et al.,* 2009; Kumar *et al*., 2011). The leaves are variably glabrous, obovate - oblong with sub-pandurate entire and inflated pale green perianth up to 4cm long (Das *et al.,* 2010). Like other species, *Aristolochia ringens* has been studied to contain essential oil, aristolochia acid and other phytochemicals that are of biological importance (Ashokkumar *et al.,*2010; Sinha and Choudhury, 2010; Tajkarimi *et al.,* 2010; Abhijit and Jitendra, 2011; Samy *et al.,* 2011).

According to an ethnomedicobotanist's oral interview conducted at Bode, a popular herbal market in Ibadan metropolis within Nigeria, *A. ringens* is used to treat various ailments such as wounds, dysentery, throat infections and skin problems of which are linked to microbial infestations. In Asian countries, especially India, over 2,500 plants have been studied to have provided alternative medicine and curative properties to the available synthetic drugs (Sarmiento *et al.*, 2011; Thirumal *et al.*, 2012). Extracts from *Aristolochia* sp, especially, phytochemicals and essential oil have been receiving earnest *in-vitro* investigations for their numerous activities. Among the documented activities traced to such phytochemical properties of *A. ringens* are antimicrobial, anti-inflammatory, anti-venom, antipyretic, antiseptic, abortifacient, emmenagogues, storage stability (preservative), foaming (lather), curative, taste, flavours and aroma on one hand and potent nephrotoxic, anti fertility and antispermatogenic on the other (AshokKumar *et al.,* 2010; Sinha and Choudhury, 2010; Tajkarimi *et al.,* 2010; Abhijit and Jitendra, 2011; Kumar *et al.,* 2011). In recent years, the traditional application of natural compounds of plant origin has been receiving a lot of attention as an alternative source of remedy for the treatment of diseases coupled with the belief of their better safety nature and of less or non toxicity. This has led to the increase in laboratory (*in-vitro*) research into herbal medicine to establish their acclaimed efficacy and their therapeutic applications. This study aimed at ascertaining the acclaimed antimicrobial property possessed by *A. ringens* as an ingredient in the production of herbal medicine that serves as effective therapeutic agent against pathogenic microorganisms and their associated infections.

Materials and Methods

Collection of Plant Materials

The plant materials used for the work were the barks of the stem and root of *Aristolochia ringens* (Ako-igun in Yoruba). The materials were purchased at Bode herbal market in Ibadan, southwestern Nigeria. They were thoroughly washed with sterile distilled water and air dried before milling into powder for antimicrobial *in-vitro* analysis.

Collection of fungal and bacterial isolates

The bacteria used in this study were obtained from the Medical Microbiology and Parasitology Department, University College Hospital, Ibadan, Nigeria. They are: *Shigella sonnei, Salmonella typhii, Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Proteus mirabilis*. The fungal isolates obtained from the Microbiology Department, Lead City University, Ibadan, Nigeria were: *Fusarium* species, *Sporotrichum* species, *Pichia* species, *Penicillium camemberti* and *Aspergillus niger*. Sub-culturing of both the bacterial and fungal isolates was done into sterile Petri plates and were stored in agar slants and kept in the refrigerator at 4^0C for subsequent use.

Plant Extract Preparation

Fifty grams each of powdered bark of stem and root of *A. ringens* were separately measured into 500ml of each of the four solvents: Aqueous, Ethanol, Petroleum Ether and Acetone. These were allowed to soak for 24 hours. The supernatants were filtered into separate conical flasks using Whats'man No. 1 filter paper. The extracts were used immediately, and the remaining were stored in the refrigerator for further studies (Harrigan and McCane, 1986; Fawole and Osho, 1989)

Inhibitory Tests on the *Aristolochia ringens* extracts

Sterile 8mm diameter size cork borer used for creating well cavity was used to cut and pick each fungus culture with the agar. This was then inoculated into the 4mm deep well of Malt Extract Agar (MEA). Petri plates of Nutrient Agar were also streaked with the bacterial isolates before the wells were bored and the prepared extracts in different concentrations poured into them. The various concentrations were calculated as follows: 50mg of powdered sample of *Aristolochia ringens* was added into 500ml of each of the various solvents (Aqueous, Ethanol, Petroleum Ether and Acetone) to give an undiluted extracts of 100mg/ml. These were further diluted serially by mixing 10ml of extract with 5ml of each solvent; 10ml of extract with 10ml of solvent; 10ml of extract with 15ml solvent and 10ml of extract with 20ml solvent to finally assume the respective concentrations of 66.6 mg/ml, 50mg/ml, 40mg/ml, 33.3mg/ml while each solvent was used alone as control. The plates were later incubated for 72h at 30^oC and 24h at 37^oC for fungal and bacterial growth respectively. The experiment was done in triplicates.

Data collection

The diameter of growth inhibition for each bacterial colony and each fungus was determined by direct meter rule measurement (mm). The average measurement of inhibition zone for each triplicate was then calculated. Diameter of inhibition zone for the commercially sold antibiotics disc set side-by-side was equally determined (Guptee, 2001; Nester *et al*., 2004).

Potency calculation

Potency for the extract was calculated according to Tajkarimi *et al.* (2010) and Nand *et al.* (2012) by deducting values of control from treated values, while the concentration of extracts of resulting values above 10ml was taken as potent.

Potency = (Control values – treated values) above 10ml

Phytochemical Screening

The powdered plant was subjected to various chemical tests using standard procedures to identify the secondary metabolites as described by Harbone (1973), Trease and Evans (1989) and Sofowora (1993).

Results

Table 1 shows the Minimum Inhibitory Concentration (MIC) obtained for the various solvent extracts (aqueous, ethanol, petroleum ether and acetone) of *A. ringens* stem bark on the microbial isolates - *Shigella sonnei, Salmonella typhi, Escherichia coli, Staphylococcus aureus, Bacillus subtilis*, *Proteus mirabilis, Fusarium* sp*, Sporotrichum sp, Pichia sp, Penicillium camemberti* and *Aspergillus niger*. Potency of various solvent extracts from the stem and root barks on the microbial` isolates are shown in Figs. 1 and 2 respectively while Fig 3 shows the effect of the Standard Antibiotics Disc sold commercially on microbial isolates.

The aqueous extract from stem bark was found to be effective only against *Shigella sonnei* at MIC of 40mg/ml. It was also observed that acetone extracts was effective against *S. sonnei*, *E. coli, S. aureus and B. subtilis* at a stable MIC of 50mg/ml. Although, ethanol extracts was more effective against all the bacteria isolates used at various concentrations ranging from 40mg/ml against *S. typhii* to 66.6mg/ml against *P. mirabilis* (Table 1). A contrary result was obtained when the petroleum ether extract was used on the microbial isolates. The petroleum ether extract was non-effective on the bacterial isolates used except the fungal and at a varying MIC, ranging from 40mg/ml against *A. niger* and *Fusarium sp* to 66.6mg/ml and 100mg/ml against *P. camemberti, Pichia sp* and *Sporotrichum sp* respectively.

Table 2 shows aqueous extract of *A ringens* root bark effectiveness only against a bacterium, *Shigella sonnei* at MIC of 40mg/ml. The ethanol extract showed an effective reaction against all the organisms used at a varying MIC of 40 - 100mg/ml except against *Pichia sp*. Petroleum extract showed effective reactions against the microbial isolates used at a varying MIC of 40 -100mg/ml except for *Sh. sonnei*, *Salmonella typhii* and *E. coli*. Acetone extract was effective against *E.coli*, *Staphylococcus aureus*, *Fusarium* sp, *Pichia* sp and *P. camemberti* at 66.6mg/ml while its effectiveness for *A. niger* was at 40mg/ml.

Table 1: Growth inhibition zones (mm) for microbial isolates cultured with *Aristolochia ringens* stem bark extracts at different concentrations (Conc.)

S/N	Microbial isolates	Extraction solvents	Undiluted extract (100mg/ml)	Conc.1 (66.6mg/ml)	Conc.2 (50mg/ml)	Conc.3 (40mg/ml)	Conc.4 (33.3mg/ml)	Control (Solvent only)
1	*Shigella sonnei*	Aqueous	28	28	22	20	-	-
		Ethanol	41	40	38	36	-	35
		Pet. Ether	-	-	-	-	-	-
		Acetone	33	32	28	24	-	24
2	*Salmonella typhii*	Aqueous	-	-	-	-	-	-
		Ethanol	12	11	10	8	8	-
		Pet. Ether	-	-	-	-	-	-
		Acetone	-	-	-	-	-	-
3	*Escherichia coli*	Aqueous	-	-	-	-	-	-
		Ethanol	28	28	26	14	-	14
		Pet. Ether	-	-	-	-	-	-
		Acetone	20	18	16	-	-	10
4	*Staphylococcus aureus*	Aqueous	-	-	-	-	-	-
		Ethanol	32	30	30	28	18	8
		Pet. Ether	-	-	-	-	-	-
		Acetone	11	9	7	-	-	-
5	*Bacillus subtilis*	Aqueous	-	-	-	-	-	-
		Ethanol	40	35	33	23	-	20
		Pet. Ether	-	-	-	-	-	-
		Acetone	31	26	25	-	-	8
6	*Proteus mirabilis*	Aqueous	-	-	-	-	-	-
		Ethanol	20	16	-	-	-	5
		Pet. Ether	-	-	-	-	-	-
		Acetone	10	10	-	-	-	10
7	*Fusarium* sp	Aqueous	-	-	-	-	-	-
		Ethanol	-	-	-	-	-	-
		Pet. Ether	9	8	8	8	-	7
		Acetone	-	-	-	-	-	-
8	*Sporotrichum* sp	Aqueous	-	-	-	-	-	-
		Ethanol	-	-	-	-	-	-
		Pet. Ether	9	8	8	8	-	8
		Acetone	-	-	-	-	-	-
9	*Pichia* sp.	Aqueous	-	-	-	-	-	-
		Ethanol	-	-	-	-	-	-
		Pet. Ether	10	8	8	-	-	8
		Acetone	-	-	-	-	-	-
10	*Penicillium camemberti*	Aqueous	-	-	-	-	-	-
		Ethanol	15	8	-	-	-	8
		Pet. Ether	17	11	8	8	-	8
		Acetone	-	-	-	-	-	-
11	*Aspergillus niger*	Aqueous	-	-	-	-	-	-
		Ethanol	14	13	-	-	-	9
		Pet. Ether	18	17	16	16	14	14
		Acetone	-	-	-	-	-	-

Figures are means of triplicate values

Table 2: Growth inhibition zones (mm) for microbial isolates cultured with *Aristolochia ringens* root bark extracts at different concentrations (Conc.)

S/N	Microbial isolates	Extraction solvents	Undiluted extract (100mg/ml)	Conc.1 (66.6mg/ml)	Conc.2 (50mg/ml)	Conc.3 (40mg/ml)	Conc.4 (33.3mg/ml)	Control (Solvent only)
1	*Shigella sonnei*	Aqueous	12	10	6	6	-	-
		Ethanol	15	13	12	9	-	8
		Pet. Ether	-	-	-	-	-	-
		Acetone	-	-	-	-	-	-
2	*Salmonella typhii*	Aqueous	-	-	-	-	-	-
		Ethanol	10	9	-	-	-	8
		Pet. Ether	-	-	-	-	-	-
		Acetone	-	-	-	-	-	-
3	*Escherichia coli*	Aqueous	-	-	-	-	-	-
		Ethanol	10	8	8	7	-	7
		Pet. Ether	-	-	-	-	-	-
		Acetone	18	16	-	-	-	-
4	*Staphylococcus aureus*	Aqueous	-	-	-	-	-	-
		Ethanol	12	10	9	-	-	6
		Pet. Ether	10	9	9	-	-	8
		Acetone	9	7	-	-	-	-
5	*Bacillus subtilis*	Aqueous	-	-	-	-	-	-
		Ethanol	6	5	-	-	-	-
		Pet. Ether	11	10	10	8	-	8
		Acetone	-	-	-	-	-	-
6	*Proteus mirabilis*	Aqueous	-	-	-	-	-	-
		Ethanol	13	13	9	8	-	8
		Pet. Ether	11	11	-	-	-	7
		Acetone	10	10	-	-	-	10
7	*Fusarium sp*	Aqueous	-	-	-	-	-	-
		Ethanol	24	23	20	-	-	16
		Pet. Ether	70	52	52	41	33	33
		Acetone	16	15	-	-	-	14
8	*Sporotrichum sp*	Aqueous	-	-	-	-	-	-
		Ethanol	36	29	-	-	-	-
		Pet. Ether	70	56	41	33	-	32
		Acetone	-	-	-	-	-	-
9	*Pichia sp*	Aqueous	-	-	-	-	-	-
		Ethanol	-	-	-	-	-	-
		Pet. Ether	50	46	30	-	-	27
		Acetone	9	8	-	-	-	-
10	*Penicillium camemberti*	Aqueous	-	-	-	-	-	-
		Ethanol	50	45	-	-	-	45
		Pet. Ether	70	66	60	49	-	39
		Acetone	29	25	24	-	-	24
11	*Aspergillus niger*	Aqueous	-	-	-	-	-	-
		Ethanol	46	38	-	-	-	29
		Pet. Ether	80	75	60	50	46	46
		Acetone	26	24	24	20	-	19

Figures are means of triplicate values

The aqueous stem extract was only observed to be potent against *S. sonnei* at 40mg/ml (Fig.1) while acetone stem extract was not potent against the microbial isolates except for *E. coli*, *S. aureus* and *B. subtilis* at 100mg/ml, 100mg/ml and 50mg/ml

respectively. Ethanolic stem extract equally showed potency against *S. aureus* at 33.3mg/ml, *B.subtilis* (50mg/ml), *E.coli* (50mg/ml), *Pr. Mirabilis* (66.6mg/ml) and *Salmonella typhii* (50mg/ml).

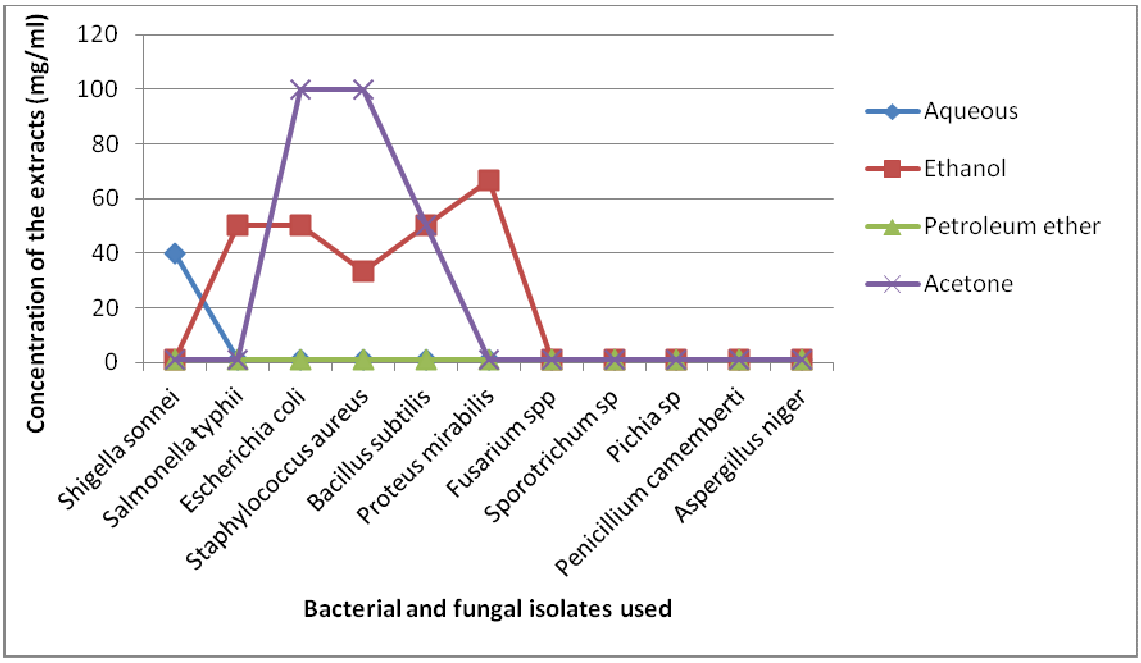

Fig.1: Potency of *Aristolochia ringens* stem bark extracts against microbial isolates

Fig. 2 showed that ethanolic root extract was only potent on *Sporotrichum* sp at 66.6mg/ml and *A. niger* at 100mg/ml while petroleum root extract was observed to be less potent on bacterial isolates used but very potent on the fungal isolates at 40mg/ml for *Fusarium* sp and P. *camemberti*, 50mg/ml (*A. niger*) and 66.6mg/ml (*Sporotrichum* and *Pichia* species).

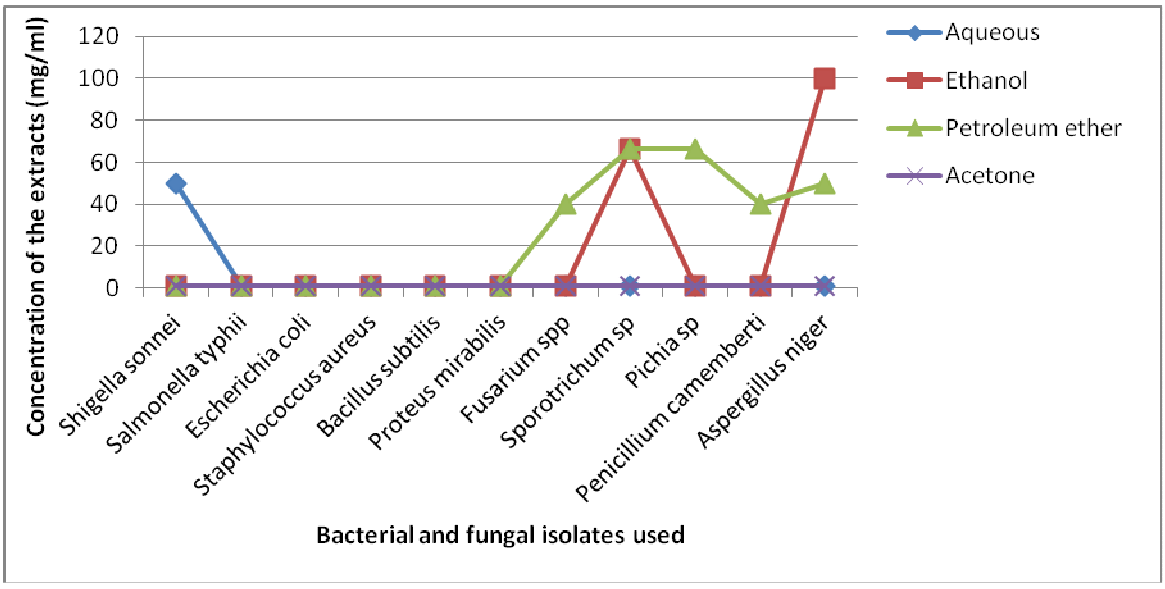

Fig. 2: Potency of *Aristolochia ringens* root bark extracts against microbial isolates

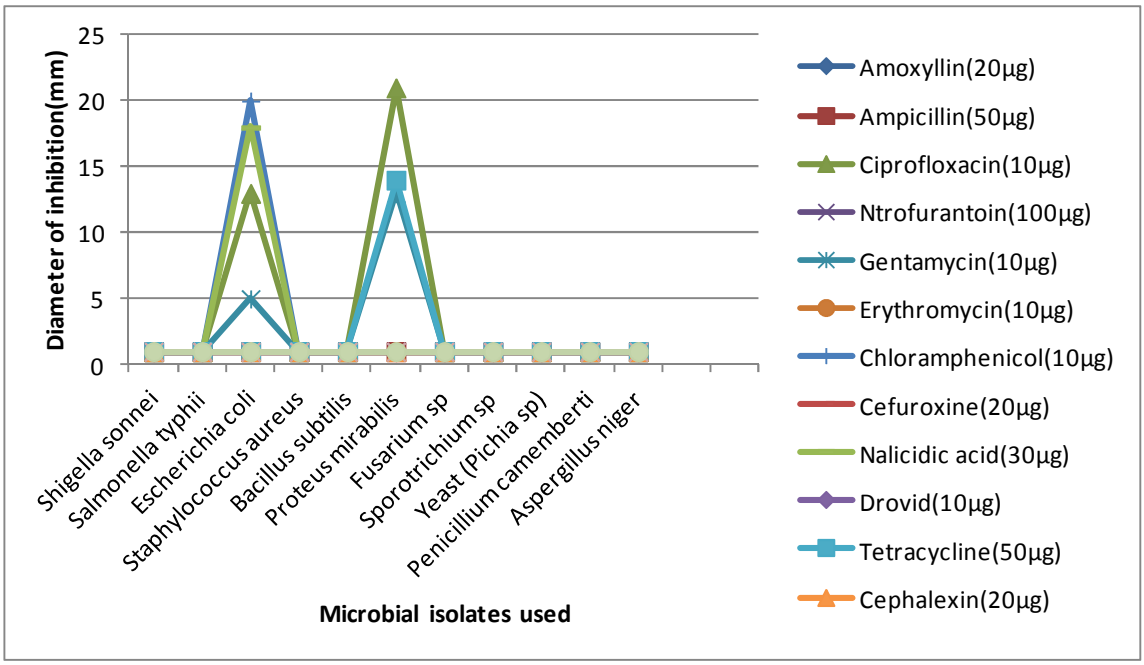

Fig. 3: Reaction of Microbial Isolates with the Standard Antibiotics Disc sold commercially

Table 3: A preliminary phytochemical screening of *Aristolochia ringens* stem and root barks

Plant parts	Phytochemical compounds						
	Alkaloids	Tannins	Flavonoids	Saponins	Cardiac glycosides	Steroids	Terpenoids
Stem	+	++	+	-	+	-	++
Root	+++	+++	++	++	+ ++	++	+++

Key: + = slightly present; **++ =** moderately present; **+++ =** highly present; - = absent

Escherichia coli and *Proteus mirabilis* were the only bacteria that showed sensitivity to the commercially sold antimicrobial discs with varying concentrations and zones of inhibition as shown in Fig.3. The discs' effectiveness are as follows: Ciprofloxacin (21mm), Chloramphenicol (20mm), Nalicidic acid (18mm), Tetracycline (14mm) Gentamycin (13mm). The plant parts showed the presence of the following phytochemicals: alkaloids, flavonoids, tannins, cardiac glycosides in both the stem and root of *A. ringens*. However, saponins and steroids were absent in the stem of the same plant (Table 3).

Discussion

According to Tajkarimi *et al.* (2010) *in- vitro* analysis of plant extract for antimicrobial activities are nowadays being expressed by researchers in two ways: Minimum Inhibitory Concentration (MIC) and Minimum Antimicrobial Concentration (MAC). The MIC required to inhibit < 90% inoculums viability is a measure of extract "effectiveness" determined by inhibitory clearing zone of <10mm while MAC is the concentration required to completely destroy 99.9% inoculums load. Though extract "POTENCY" is determined by inhibitory zone of > 10mm, the wider the zone of inhibition the better the potency according to Tajkarimi *et al.* (2010) and Nand *et al.* (2012).

The result showed that acetone stem bark extract and ethanol root extract were reactive and potent on all the bacteria isolates, but not on the fungi at MIC 50mg/ml with inhibitory clearance of 17mm diameter (Table 1). This might be due to varied solubility of active principle in different analytical solvents or that the presence of active components was insufficient to show activity at the dose level employed as reported by Kamal *et al.* (2010). In a similar report, Akharaiyi and Boboye (2010) revealed the presence of bioactive properties in plant parts at various degrees that also reflects in their therapeutic efficacy. In this study, the root bark extracts especially petroleum ether extract were found to be potent on the fungi isolates

and not the bacteria. The observation also revealed that root bark petroleum extract was a better fungicide at MIC 40mg/ml (10mm in diameter) than the acetone and ethanol extracts. The petroleum ether extract had 38mm as minimum inhibitory zone of clearance (Table 2). Besides the use of different solvent of extraction, effectiveness of plants as antimicrobial agents is hinged on their mode of action in organisms as plant extracts have demonstrated affinity for specific systems with resultant multiple effects (Abubakar, 2010).

Extracts from *A. ringens* stem and root bark using various solvents were noted to differ significantly in their biocides activities. Water extracts have been noted to have very poor activity by some workers (Nzeako *et al*., 2006; Kamal *et al.*, 2010). Corroborating these findings, this study found that the aqueous extracts from both parts of the plant (stem and root barks of *A. ringens*) were observed to be very potent only against *Shigella soonnei*. The bacterium *S. sonnei* belongs to the same genus *Shigella dysentariae* that produces lethal toxin clinically known as bacillary dysentery. The disease can be lethal and endemic if not treated on time. In the same vein, the biocidal activity of acetone extract studied is considered low, thus making its antimicrobial properties insignificant. However, on the contrary, the ethanolic extracts of both stem and root bark were very potent on the bacterial isolates used but not on the fungi. Though petroleum stem extract was not potent on all the microbial isolates in Fig.1, the petroleum root extract was very potent on all the fungal isolates as shown in Fig. 2. This study therefore supports herb users of the acclaimed antimicrobial property possessed by *A. rigens.* In other to corroborate this finding, Ramasubramania and NiranjanBabu (2011) reported the antifungal activity of a similar species *A. bracteole*. Likewise, Alviano *et al.* (2008) discussed the antibacterial activities of *A. cymbifera* extract as the highest bactericidal effect against all tested bacteria. *Aristolochia ringens* may therefore be used for the production of herbal medicine that will serve as an effective medicinal plant drug against some pathogenic microorganisms and their associated infections.

Aristolochia rigens in this work was analyzed to contain flavonoids, alkaloids, tannins, cardiac glycosides and steroids. The traditional use of these plant parts for treatment of various illnesses and curative properties against several diseases can be traced to this biocomposition. The importance of alkaloids, tannins and saponins in various antibiotics used in treating common pathogenic strains has been reported by Kubmarawa *et al.* (2007) and Mensah *et al.* (2008). Del-Rio *et al.* (1997) and Okwu (2004) reported that flavonoids and phenols are potent water soluble anti-oxidants which prevent oxidative cell damage having antiseptic, anti-cancer and anti-inflammatory effects with mild anti-hypertensive properties. Okwu and Okwu (2004) reported that alkaloids are known to possess anti-microbial, anti-fungal and anti-inflammatory effects; and also act as anti-hypertensive agents (Sofowora 1993). Leverin and Mc Matron (1999) reported that cardiac glycosides have been effective in the treatment of congestive heart failure and regulation of heart beats. Thirumal *et al.* (2012) reported the phytochemical and pharmacological properties of *A. bracteolata* that were of shared similarity with *A. ringens*. In the same vein, Leon- Diaz *et al.* (2010) was found to have its hexane extract of *A. taliscana* to be antimycobacteria.

This work shows that solvent extracts from the parts of *A. rigens* possess antimicrobial (bacteriostatic and fungistatic) properties. Hence, concerted efforts to produce safe and potent biocides from its parts should be encouraged. It is recommended that pharmacological properties and the bioactive ingredients from these extracts should be studied, and scientifically evaluated.

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(Received: 3rd May, 2014; Accepted: 28th July, 2014)