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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Journal of Medicinal Plants Research

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ARTICLES

Research Articles

Full Length Research Paper

Antimicrobial activity and phytochemical screening of various parts of *Ixora coccinea*

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Ixora coccinea L. (Rubiaceae) has been used traditionally for a variety of ailments and also cultivated for ornamental purposes. The present study investigated antimicrobial activity of methanolic extracts of various parts of *I. coccinea* and determined the chemical groups of the active constituents. Antimicrobial activity was assessed using agar disc diffusion, microdilution and thin layer chromatography (TLC) bioautography assays. Methanolic extracts of leaf, flower and stem of *I. coccinea* displayed good antimicrobial activity, with inhibition zone in the range of 6.7 to 11.3 mm. minimum inhibitory concentration (MIC) values for all three extracts ranged from 0.78 to 3.125 mg/ml. Leaf and stem extracts of *I. coccinea* showed broad-spectrum antimicrobial activity. Of interest, stem extracts had MIC values against *Staphylococcus aureus* that were only 62.4 times less potent than the vancomycin. Likewise, leaf and stem extracts displayed good antimicrobial activity of 62.4 and 31.2 times, respectively lesser than gentamycin against *Shigella flexneri*. Minimum bactericidal/ bacteriostatic concentration (MBC) values for active extracts ranged from 0.78 to 6.25 mg/ml. TLC bioautography and phytochemical screening of the leaf and stem extracts showed that the antimicrobial activity of these extracts may be attributed to compounds belonging to terpenoid, flavonoid, coumarin, alkaloid and phenolic groups.

Key words: Antimicrobial, *Ixora coccinea*, minimum inhibitory concentration, minimum bacteriostatic/bacteriocidal concentration, bioautography.

INTRODUCTION

Plants are the oldest source of pharmacologically active substances and have provided humans with many medically useful compounds (Cordell, 1981). Plants pro-duce a diverse array of secondary metabolites, many of which have antimicrobial activity. Hence, natural products in particular medicinal plants remain as a potential source of new antimicrobial agents (Cowan, 1999). The in-creasing prevalence of multidrug resistant strains of

bacteria and the recent appearance of strains with reduced susceptibility to clinically used antibiotics (Rubiaceae) is a small evergreen flowering shrub found throughout Asia (Latha and Pannikar, 1998). It has been raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting stra-tegies (Sieradzki et al., 1999). Therefore, there is a conti-nuous search for substances from plant sources with

proven antimicrobial activity. *Ixora coccinea* L. used traditionally for a variety of ailments and also cultivated for ornamental purposes. The leaves are used to treat diarrhoea, the roots are used to treat hiccup, fever, sores, chronic ulcers and skin diseases. The flowers have been used in catarrhal bronchitis and dysentery (Sivarajan and Balachanadran, 1941). It is reported that this plant contains anthocyanins in flowers; methyl ester of palmitic, stearic, oleic and linoleic acids in root oil; octadecadienoic acid, saponins and tannins from root bark (Chopra et al., 1956; Grainge and Ahmed, 1988), alkaloids, flavonoids, sapogenins, sterols, terpenes and phenols (Annapurna et al., 2003). The aqueous extract of the *I. coccinea* demonstrated antino-ciceptive, anti-inflammatory and antitumor effects in mice (Rathnasooriya et al., 2005). Annapurna et al. (2003) has reported the antimicrobial activity of *I. coccinea* leaf extract based on disc diffusion method. However to date, there is no in depth antimicrobial evaluation done on this plant. With this in view, the present study was undertaken to evaluate in detail the antimicrobial activity of metha-nolic extract of various parts of *I. coccinea* against series of microorganisms and to identify the chemical groups of the bioactive constituents.

MATERIALS AND METHODS

Chemicals and reagents

Chloramphenicol was purchased from Acros Organics (New Jersey, USA). Gentamycin and amoxicillin were purchased from Sigma Aldrich (Steinheim, Germany) while vancomycin, miconazole and *para* iodonitrotetrazolium (INT) were purchased from Sigma (St. Louis, USA). Amphotericin B was purchased from Himedia (Mumbai, India). Penicillin G, gentamycin, chloramphenicol, amoxicillin and tetracycline impregnated discs were purchased from Oxoid (Hampshire, England). Muller Hinton agar, Muller Hinton broth, Potato Dextrose agar and methanol were purchased from Merck (Darmstadt, Germany) while potato dextrose broth was purchased from Lab Scan Analytical Sciences (Bangkok, Thailand). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Leicestershire, UK).

Plant material and extraction

Fresh plant materials of *I. coccinea* (leaves, flowers and stem) were collected from Penang, Malaysia. A voucher specimen (Collection No. 11038) has been deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The plant parts were washed with water to remove dirt prior to the drying process at 40°C for a week. The dried plant materials were powdered (100 gm) and extracted repeatedly with methanol (500 ml) by maceration for a week. Fresh methanol was replenished every two days. The extracts were filtered and the filtrates were concentrated *in vacuo* using rotary evaporator at 45°C. The concentrated extracts were subjected to freeze drying to obtain dry powdered extracts.

Microorganisms and media

Test microorganisms were obtained from the School of Biological Sciences and School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia. The test microorganisms used in this study were: *Bacillus cereus* (ATCC 10876), *Streptococcus pneumoniae* (ATCC 6303), *S. aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (laboratory strain), *S. flexneri* (ATCC 12022), *Enterobacter aerogenes* (laboratory strain) and *Saccharomyces cerevisiae* (laboratory strain). The strains were maintained and tested on Muller Hinton agar (bacteria) and potato dextrose agar (fungus). For the antimicrobial tests, strains were grown overnight in Muller Hinton agar (bacteria) and Potato Dextrose agar (fungus) in an anaerobic chamber at 37°C under atmosphere consisting 10% CO_2, 10% H_2O and 80% N_2.

Antimicrobial evaluation

Agar disc diffusion assay

The antimicrobial activity of different parts of *I. coccinea* was initially evaluated by agar disc diffusion assay (Bauer et al., 1966). 20 ml Muller-Hinton agar for bacteria or potato dextrose agar for fungi, sterilized in a flask and cooled at 45 to 50°C, were transferred to petri dishes (diameter of 90 mm) and allowed to solidify. Inoculum were prepared by mixing a few microbial colonies with Muller Hinton broth (bacteria) or potato dextrose broth (fungi) and incubated at 37°C for 3 h to get an approximately standard 0.5 Mac Farland solution. Then, inoculum suspensions were streaked over the surface of the media on each plate using sterile cotton swab to ensure the confluent growth of the organism. For antimicrobial testing, a 50 mg/ml stock solution of each extract (leaf, flower and stem) was prepared in methanol. Whatman filter paper No.1 discs of 6 mm diameter were used in this assay. Each sterile disc was impregnated with 20 µl of various extracts of *I. coccinea* (concentration 1 mg/disc) and then placed onto the agar plates which had previously been inoculated with the test microorganisms with sterile forceps and pressed gently to ensure contact with the inoculated agar surface. Amoxicillin (30 µg/disc), penicillin G (10 µg/disc), vancomycin (10 µg/disc), chloramphenicol (30 µg/disc), gentamycin (10 µg/disc), tetracycline (30 µg/disc) and miconazole (30 µg/disc) were used as positive controls while methanol (20 µl) was used as the negative control.

Finally, the inoculated plates were incubated at 37°C for 18 h and at 28°C for 48 h for the bacteria and fungi, respectively. The diameters of the inhibition zones were measured in milimeters. All measurement was carried out in triplicate. Extracts showing inhibition zones of more than 8 mm were selected for subsequent determination of MIC, MBC and minimum fungicidal concentration (MFC).

Determination of minimum inhibitory concentration (MIC)

The MIC of *I. coccinea* extracts were determined by serial two-fold dilution method as described by Eloff (1998). The extracts were initially dissolved in 50% DMSO to make up a concentration of 50 mg/ml. Then stock solution was serially diluted two-fold by using Muller Hinton broth (MHB) as diluents. Each well was inoculated with 100 µl of suspension containing 1.5×10^8 CFU/ml (equivalent to McFarland 0.5) of the culture. Final concentration of extracts and positive controls ranged from 25 to 0.006 and 0.4 to 0.00039 mg/ml, respectively. The 96 well plates were incubated at 37°C for 18 h for bacteria and at 28°C for 48 h for fungus. Amoxicillin, chloramphenicol, vancomycin and gentamycin (for bacteria) and miconazole (for fungus) served as positive controls, respectively while 50% DMSO was used as negative control. Microbial growth was evaluated by addition of 50 µl of 0.2 mg/mL of freshly prepared solutions of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide) or INT (*para* iodonitrotetrazolium) dissolved in water into each of the microplate wells. The covered microplates

Table 1. Zone of inhibition (mm) by various extracts of *I. coccinea* and reference antibiotics.

Strain	Mean zone of growth inhibition (mm)[a] (n=3)									
	I. coccinea extracts (1mg/disc) Reference antibiotic (µg/disc)									
	Leaf	Flower	Stem	AMX 30	PEN G 10	VC 10	CMC 30	GM 10	MICO 30	M
Gram positive bacteria										
Bacillus cereus	7.0±1.0	7.0±0	6.0 ±0	12.7 ±0.6						-
Streptococcus pneumoniae	9.0 ±0	8.3 ±0.6	8.3±0.6		28.7±0.6					-
Staphylococcus aureus	8.0±0	9.0 ± 0	9.0±0			13.0±0				-
Gram negative bacteria										-
Escherichia coli	6.0±0	6.7±0.6	8.7±0.6				19.7±0.6			
Klebsiella pneumonia	8.7±0.6	6.0±0	8.7±0.6					19.0±0		-
Shigella flexneri	8.8±0.6	8.0±0	9.0±0				20.3±0			-
Pseudomonas aeruginosa	8.0±0	9.0±0	9.0±0				20.0±0			-
Enterobacter aerogenes	10.0±0.5	8.0±0	9.0±0					29±0		
										-
Fungi										
Saccharomyces cerevisiae	11.0±0	10.0±0	11.3±0.6						12.7±0.6	-

[a] Diameter of inhibition zone including disc diameter of 6.0 mm. Values for growth inhibition are presented as mean±SD; '-' no inhibition zone observed; AMX, amoxicilin; PEN G, penicillin G; VC, vancomycin; CMC, chloramphenicol; GM, gentamycin; MICO, miconazole; M, methanol.

were incubated further for 30 min. MIC was defined as the lowest drug concentration of extract inhibiting growth of microorganism and there was no colour changes observed in the wells from yellow to purple for MTT and colourless to pink for INT.

Determination of MBC and MFC

MBC or MFC was defined as the lowest concentration of extracts that showed complete inhibition of colonies of microorganisms on agar plates. A loopful of each bacterial or fungal culture in each microplate well was inoculated to the culture medium agar plate and incubated under same conditions as described earlier (Shin et al., 2004). Each assay was carried out in triplicate.

Bioautography

Bioautography was performed using bacterial cultures (*S.*

aureus and *S. flexneri*) which were found to be the most susceptible to leaf and stem extracts following method described by Masoko and Eloff (2005). The developed TLC plates were carefully dried and inoculated with fine spray of the concentrated suspension of actively growing micro-organisms containing 10^9 cfu/ml approximately. The plates were incubated overnight at 37°C and then sprayed with 2 mg/ml solution of INT and further incubated for 2 to 3 h. The presence of white spots indicates presence of compounds that inhibited the growth of tested microorganisms.

Phytochemical screening

The leaf and stem extracts of *I. coccinea* were found to have antibacterial effect, thus were selected for phytochemical screening by TLC. TLC was performed on aluminium-backed TLC plates (Merck, Silica gel 60 F_{254}). Plates (10 × 5 cm) were loaded with 10 spots (20 mg/ml) of each extract. The prepared plates were developed with

chloroform: methanol (9.5: 0.5). The components were visualized under visible and ultra violet light (254 and 365 nm), respectively. The developed plates were sprayed with the following reagents for detection of respective chemical groups: natural products reagent for flavonoid (Wagner et al., 1984), aluminium chloride for phenolic compounds, methanolic potassium hydroxide for coumarins (Harbone, 1973) and Dragendorff's reagent for alkaloids and Liebermann-Burchard reagent for terpenoids (Krebs et al., 1969).

RESULTS

Methanolic extracts of various parts of *I. coccinea* were evaluated for antimicrobial activity on nine pathogenic microorganisms by using agar disc diffusion assay and microdilution assay. The growth inhibition zones measured in agar disc diffusion assay are presented in Table 1. The disc

Table 2. Minimum inhibitory concentration (MIC) of various extracts of *I. coccinea* and reference antibiotics.

Strain	Minimum inhibitory concentration (mg/ml), n=3							% DMSO not inhibiting microorganisms
	I. coccinea extracts			Reference antibiotic				
	Leaf	Flower	Stem	AMX	VC	GM	MICO	
Gram positive bacteria								
Streptococcus pneumoniae	1.56 (500×)	3.125 (1000×)	0.78 (250×)	0.003125				12.5
Staphylococcus aureus	1.56 (125×)	12.5 (1000×)	0.78 (62.4×)		0.0125			25.0
Gram negative bacteria								
Klebsiella pneumoniae	1.56 (2000×)	3.125 (4000×)	1.56 (2000×)			0.00078		12.5
Shigella flexneri	1.56 (62.4×)	1.56 (62.4×)	0.78 (31.2×)			0.025		12.5
Entrobacter aerogenes	1.56 (125×)	3.125 (250×)	1.56 (125×)			0.0125		25.0
Fungi								
Saccharomyces cerevisiae	1.56 (250×)	1.56 (250×)	0.78 (125×)				0.00625	25

AMX, amoxicilin; VC, vancomycin; CMC, chloramphenicol; GM, gentamycin; MICO, miconazole; Values given in bracket is the number of times the extract is less potent than the reference antibiotics.

diffusion results showed the *I. coccinea* extracts have potential antimicrobial activity against all tested bacteria strains and fungi *S. cerevisiae* with zone of inhibition between 6.7 to 11.3 mm. However their antimicrobial effects were found to be less potent than the reference antibiotics. Methanol used as negative control showed no inhibitory effect against the microorganism's growth. In subsequent experiments, the MIC, MBC and MFC values of the *I. coccinea* extract were determined against selected susceptible microorganisms. The results are summarized in Tables 2 and 3. The MIC, MBC and MFC values of the test extracts of *I. coccinea* ranged from 0.78 to 6.25 mg/ml.

In general, stem extract of *I. coccinea* was found to be most active extract followed by the leaf extract. Flower extracts was found to be the least active. Interestingly, the stem extract was found to have good antimicrobial activity against

S. aureus and *S. flexneri* with MIC value of only 0.78 mg/ml. By comparing MIC values, the stem extract was found to be only 62.4 times less potent than vancomycin for *S.aureus* and 31.2 times less potent than gentamycin for *S. flexneri*. The stem extract of *I. coccinea* was also found to have good antimicrobial activity against *S. cerevisiae.* DMSO showed no toxic effect at 25% for *E. aerogenes, S. cerevisiae* and *S. aureus* and at 12.5% for the rest of the microorganisms. Based on MIC results, MBC and MFC were determined for the active extracts against selected microorganisms. Antimicrobial substances are con-sidered as bacteriostatic agents when the ratio MBC/MIC>4 and bactericidal agents when the ratio MBC/MIC≤4 (Gatsing et al., 2009). The summary of the microbicide and microbistatic effects were given in Table 4. In the present study, leaf and stem extract showed the ratio MBC/MIC≤4 for most of the test microbes,

suggesting that these extracts may act as bactericidal agent on these strains. Stem extract showed MBC/MIC ratio of more than 4 on *S. aureus* and *S. flexneri* which may be classified as bacteriostatic agent.

The leaf and stem extract were further subjected to bioautography and phytochemical screening. Bioautography results demonstrated strong inhibition zones of these extracts against the growth of *S. aureus* and *S. flexneri* (Figure 1a and b; Table 5). There are few inhibition spots noted suggesting that more than one compound may be responsible for the observed antimicrobial effect. Interestingly, the active compounds (spots label A and D) of leaf extract which inhibited both microorganisms have almost same R_f values. The active compounds of leaf extract may belong to terpenoids, flavanoids, coumarins and alkaloids groups of compounds. As for the stem extract, spot B (R_f value of 0.39) and C (R_f value of 0.18),

Table 3. Minimum bactericidal/bacteriostatic concentration (MBC) and minimum fungicidal/fungi static (MFC) concentration of various extracts of *I. coccinea*

Strain	MBC and MFC (mg/ml), n=3					
	I. coccinea extracts			Reference antibiotic		
	Leaf	Flower	Stem	VC	GM	MICO
Gram positive bacteria						
Staphylococcus aureus	6.25 (250×)		6.25 (250×)	0.025		
Gram negative bacteria						
Klebsiella pneumoniae	6.25 (500×)		6.25 (500×)		0.0125	
Shigella flexneri			6.25 (62.5×)		0.1	
Entrobacter aerogenes	3.125 (16×)		3.125 (16×)		0.2	
Fungi						
Saccharomyces cerevisiae			0.78 (62.4×)			0.0125

VC, vancomycin; GM, gentamycin; MICO, miconazole. Values given indicate bracket is the number of times the extract is less potent than the reference antibiotics.

Table 4. Summary of the microbicide and microbiostatic effects of *I. coccinea* extracts on selected strains.

Extracts of various part of *I. coccinea*	Microbicide effect on specific strains	Microbiostatic effect on specific strains
Leaf	*Entrobacter aerogenes, Staphylococcus aureus, Klebsiella pneumoniae*	-
Stem	*Saccharomyces cerevisiae, Entrobacter aerogenes, Klebsiella pneumonia*	*Staphylococcus aureus, , Shigella flexneri*

Table 5. Summary of the bioautography of *I. coccinea* extracts against selected micro-organisms.

Microorganism Extract	A	B	C	D	E	F
	Inhibition zone					
Terpenoid	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+
Coumarin	+	+	+	+	+	+
Alkaloid	+	-	-	+	-	-
Phenol	-	-	+	-	-	+
R_f value	0.37	0.39	0.18	0.33	0.38	0.16

Growth inhibition of microorganisms: '+' present; '-' absent.

which inhibited *S. aureus* were similar to the spot E (R_f value of 0.38) and F (R_f value of 0.16) which inhibited *S. flexneri*. These spots may consist of terpenoids, flavanoids, alkaloids, coumarins and phenolic compounds.

DISCUSSION

The antimicrobial activities of medicinal plants have been reported by many researchers (Cowan, 1999; Motamedi et al., 2010; Shariff, 2001; Shin et al., 2004). Most antibacterial medicinal plants are active against gram-positive strains while few are active against gram-negative bacteria (Herrera et al., 1996; Meng et al., 2000; Scrinivasan et al., 2001). In the present study, antimicrobial activity of methanolic extracts various parts of *I. coccinea* was evaluated against series of micro-organisms. Among all three extracts of *I. coccinea*, leaf and stem extracts were found to be more active than flower extract. In particular, the leaf and stem extracts were active against few pathogenic microorganisms such
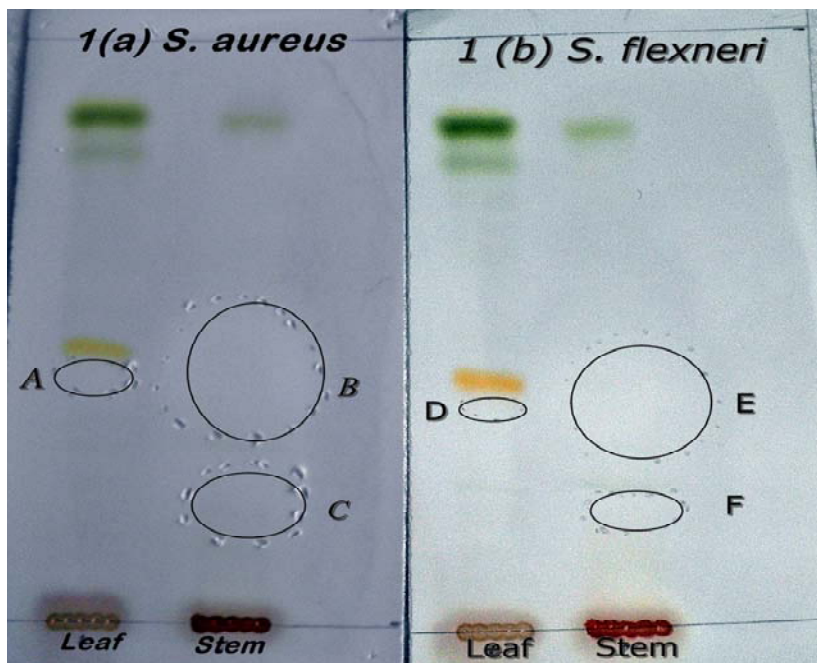
Figure 1. Bioautography of *I. coccinea* extracts against selected microorganisms. (Labelled spots indicate growth inhibition of microorganisms).

K. pneumonia, *S. aureus* and *S. flexneri*. Therefore, these extracts may be potential source leads for antimicrobial agents against these microorganisms. Annapurna et al. (2003) has previously reported on screening of antimicrobial activity of *I. coccinea* leaves extract using disc diffusion assay. The present findings were similar to the study whereby leaf extract was active against *S. aureus* with inhibition zone of 18 mm. Studies that used agar disc diffusion to detect antimicrobial activity of plant extract revealed poor accuracy and difficult nature of the method. According to Allen et al. (1991), low levels of antimicrobial activity of plant extracts are not detectable in agar disc diffusion method. MIC of *I. coccinea* extracts was carried out using microplate assay because this assay is based on correlation of bacterial growth with inhibitory effect of plant extract at certain concentration. This method has been identified as being more accurate than agar disc diffusion and less tedious (Eloff, 1998). *S. aureus* and *S. flexneri* was the most susceptible microorganisms against *I. coccinea* extracts (leaf and stem) with lowest MIC value 0.78 mg/ml.

In addition, these extracts were found to be bacteriostatic *S. aureus* and *S. flexneri*. According to Van and Viljoen (2009), as DMSO may exhibit antimicrobial efficacy, MIC values equivalent to or greater than that found for the DMSO control should be omitted from the data and considered not susceptible. In the present study, DMSO was found to have no inhibitory effect at the MIC values of the active extracts. Subsequently, the active constituents were identified by TLC bioautography and characterized by spraying with various reagents.

Bioautography is a useful technique for detecting bioactive compound(s) as well as indicator for separation technique during bioassay-guided isolation of active compounds (Masoko et al., 2007). For each extract, more than one active compound was identified. A group of compounds belonging to the terpenoids, flavanoids, alkaloids, coumarins and phenolics family may be responsible for the antimicrobial activity of stem and leaf extracts. These compounds are producing exciting opportunity for the expansion of modern chemotherapies against wide range of microorganisms (Lutterodt et al., 1999).

Conclusions

I. coccinea extracts have potential to be developed as antimicrobial agents, in particular against *S. aureus* and *S. flexneri*. Further studies on isolation and identification of the active principles and evaluation of possible synergism among these constituents for their antimicrobial activity are currently ongoing.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Anti-depression effect of *Chimonanthus salicifolicus* essential oil in chronic stressed rats

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The aim of this study was to investigate the interventional effect of the *Chimonanthus salicifolicus* essential oil on murine chronic stress-induced anxiety. Rats model of uncertainty empty bottle was established to induce anxiety. The exploratory change, grooming and attack behaviors were observed. After 7 days training period followed by continuous 14 days' stimulation, rats serum level of glucose (GLU), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were detected. The results showed that rat model of uncertainty empty bottle induced anxiety well. *C. salicifolicus* essential oil smelling could significantly appease the anxiety of rat which include the reduction of attacking behaviors. It also showed a reduced effect to the level of TC and GLU. The result also indicated that *C. salicifolicus* essential oil might have an anti-anxiety effect on rat uncertainty empty bottle model and could be used as a potential.

Key words: Anxiolytics, *Chimonanthus salicifolicus* essential oil, chronic emotional stress.

INTRODUCTION

The plants of calycanthaceae have a special odor and contain aroma compositions which can be extracted as essential oils. The *Chimonanthus salicifolicus* S. Y. Hu is a kind of calycanthaceae plant that is distributed mainly in Anhui, Zhejiang and Jiangxi Province, China. It is often used to treat cough, asthma and expectorant. It has been used for a long time by one of the Chinese minorities called She. The *C. salicifolius* S. Y. Hu oil mainly consists of 1,8-Cinele, myrcene, limonene, linalool, linalooloxide and pinocamphone (Li et al., 2008). It has been suspected to have an effect on releasing emotions because of its special aroma.

Anxiety is being explained as a psychological and physiological state marked by cognitive, somatic, emotional and behavioral elements. Together, these components provoke a disagreeable emotion associated with fear, worry, as well as uncomfortableness. Anxiety can appear suddenly without any triggering stimulus. Therefore, it can be an obstacle in everyday life. In a state of anxiety, the body prepares to cope with a menace. As a result, the symptoms are heart palpitations, tension, nausea, chest pain, shortness of breath, sweating, trembling, pale skin, papillary dilatation and so on. At this moment, the body is in "fight or flight" response. This can be experienced as panic in severe situations. Generalized and persistent anxiety often leads to the usage
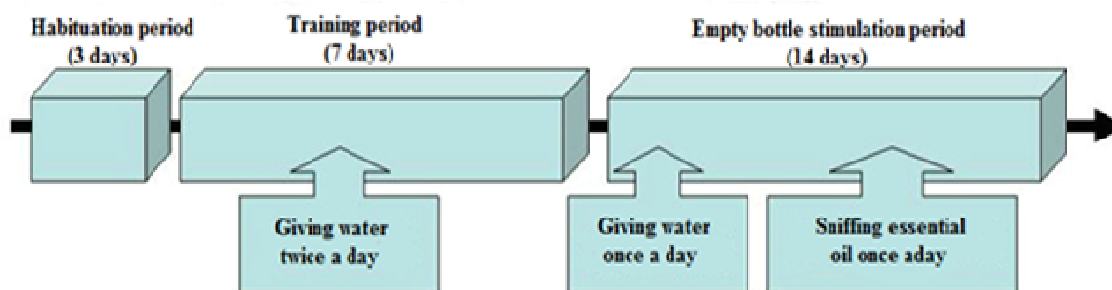
Figure 1. The process of establishing uncertainty empty bottle stimulation model.

of benzodiazepine, even these medications have several side effects, such as sedation. People who suffer from generalized and persistent anxiety often have high potential for drug abuse. Therefore, alternatives such as aromatherapy are needed (Woelk et al., 2010).

There are many medicinal plants with calmative effects on the central nervous system. In traditional Chinese medicine, the calycanthaceae plant has been recorded to have the function of relieving depression etc. Citrus is a popular anxiolytic oil which can help people in mitigating their anxiety and calming themselves down (Leite et al., 2008; Faturi et al., 2010; Giacinto et al., 2010; Lehrner et al., 2005). Lavender remedies are the most frequently used physiotherapeutic medications in the treatment of anxiety (Lehrner et al., 2005; Xu et al, 2008; Cline et al., 2008; Bradley et al., 2009; Braden et al., 2009; Kritsidima et al., 2010; Komori et al., 1995). Taking all of these into consideration, the aim of the presented work was to evaluate the potential anxiolytic effect of inhaled *C. salicifolius* S. Y. Hu essential oil on rats that were submitted to the uncertainty empty bottle by using the lavender and citrus essential oils as positive control.

MATERIALS AND METHODS

Subjects

Male Sprague Dawley rats, aged 35 days and weighing 120 to 130 g, were purchased from the Sino-British Sippr/BK lab. Animal Ltd. company. Upon arrival, the rats were weighed and maintained on a 12/12 h light/dark cycle (lights on 07.00 h) through artificial illumination. The rats were given food and water *ad libitum* except during the testing periods.

Plant collection

C. salicifolius were provided by the Institute of Chinese Medicine, Lishui of Zhejiang. Jade grape fruits were provided by Lishui, Zhejiang Academy of experimental base. Gannan navel oranges were bought from the market.

Extraction of essential oil

Leaves of plant were triturated in a common blender and placed in

boiling water (ratio 1 L water/100 g leaves).The essential oil was obtained by means of steam distillation. The oil was stored in the dark in a tightly closed bottle until used in the assays with animals.

Drugs

Lavender essential oil was purchased from Shanghai Apple Spice Co., Ltd.

Enzyme linked immunosorbent assay kit

The glucose (GLU), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) enzyme linked immunosorbent assay kits were purchased from Shanghai Langdun Biotechnology company.

Habituation and training

Rats were habituated to the laboratory environment for 3 days and being gently handled each day. After the habituation period, the rats were trained to drink water at 10.00 to 10.20 h and 20.00 to 20.20 h by allowing them to access water bottles only during these time periods for 1 week. The fluid intakes of the rats were measured during each time period (20 min).

Experimental procedure

From the day of stimulation (day 0), the rats were randomly assigned to one of seven groups (n = 10 for each group): control model group (M), physiological stress group (PS), blank emotional stress group (ES-M) and four essential oil sniffing groups-lavender, grapefruit, orange and *C. salicifolicus* groups (ES-L, ES-G, ES-O and ES-C). Rats in the M group were given water twice a day in the experimental procedure. Rats in the five ES groups (ES-M, ES-L, ES-G, ES-O, ES-C) were irregularly given empty water bottles during one of the two watering periods for 2 weeks to induce emotional stress. Rats in the PS group were given neither empty water bottles nor water during the same watering periods as for ES groups. This group was used to control the possible effect that an animal, which is trained to receive water at a particular time of day, is also emotionally stressed if it does not receive water at this time (Table 1). Rats in the ES-M groups sniffed the same dose water when the ES-L, ES-G, ES-O and ES-C groups sniffed essential oils. ES-L, ES-G, ES-O were positive controls; *C. salicifolicus* group (ES-C) was experimental group. According to the volume of the cage, a dose of 100 μl essential oils/water was added for every cage. The rats of experimental groups sniffed odor at 14:00 to

14:30. The rats of ES-L, ES-G, ES-O and ES-C group sniffed lavender oil, grapefruit oil, orange oil and *C. salicifolicus* oil, respectively at the same time period. Rats in ES-M group sniffed water as another control model. The process of establishing uncertainty empty bottle stimulation model is performed as Figure 1.

Behavior observation

The behaviors of all the rats in the seven groups during the experimental session of 20 min were observed. Fluid intakes of the rats were also evaluated during 20 min access to water. The behaviors observed included attacking (biting empty water bottles and cage shed), exploring (rearing and looking outside from the cage) and grooming (self-grooming, washing and scratching). According to the intensity of activity, the evaluation of each behavior item was recorded as a score of 0, 1, 2, 3 or 4, respectively, and a mean of all scores over the 14 days was used for statistical analysis.

Serology detection

After the completion of 14 experimental sessions, all rats were weighed and obtained a blood sample from the rats' tails. Blood was centrifuged to separate serum and plasma. Serum levels of TC, GLU, HDL-C and LDL-C were determined by enzyme-linked immunosorbent assay (ELISA).

Statistical analyses

All data were expressed as mean ± standard deviation (SD). For statistical evaluation of these results, a one-way analysis of variance (ANOVA) was used. If a significant main effect of a group was identified ($p < 0.05$), posthoc comparisons between groups were performed using the least significant difference (LSD) test. $p < 0.05$ was considered statistically significant.

RESULTS

The changes in fluid intake and body weight of the rats

During training procedure, the fluid intakes of the rats for each day were not significantly different among the three groups ($p > 0.05$). During experimental procedure, the fluid intakes of the rats in ES and PS groups reduced significantly for each day comparing to that of the rats in M group. However, there was no significant difference between the ES groups and PS group ($p > 0.05$). At the time of arrival and after training, there was no significant difference in body weight among the three groups. After experimental procedure, the body weights of the rats in ES and PS groups reduced significantly comparing to that in the M group (Figure 2).

The effect of emotional stress on the behavior of rats

From day 1 to day 14, attacking behaviors were exhibited strongly in the rats of ES-P group. However, this phenomenon did not appear among the rats of M and PS groups. This result indicated that the rats in the ES-P group were experiencing more stress than the other two groups (Table 2). Compared to the ES-P group, attacking behaviors from the other two groups were reduced significantly ($p < 0.05$) by sniffing either lavender oil or *C. salicifolicus* oil. The ES-O group reduced extremely significantly ($p < 0.01$). However, there was no difference in exploring and grooming behaviors between the rats in the ES groups and PS group.

The effect of emotional stress on the serology markers of rats

The TC and GLU levels increased significantly in the rats of the ES-M group compared to that in rats of the PS group ($p < 0.05$) (Table 3). However, compared to the ES-M group, only the group sniffing *C. salicifolicus* oil decreased significantly in TC and GLU ($p < 0.01$). There was no significant difference in the HDL-C and LDL-C levels among all the groups

DISCUSSION

The administration route of plant extracts is an important part of animal research. Most of the studies employ intraperitoneal administration route. However, the major method of aromatherapy in reducing levels of anxiety is by inhaling the essential oil. Considering that the inhalation-drug-deliver method allows a closer approach to the aromatherapy in use of essential oil, it is employed in this study to evaluate anxiety effects of essential oil.

The effect of emotional stress on the behavior of rats

The anxiolytic effects reflected by attacking behaviors were clearly shown in Table 2. In order to examine whether emotional stress influences the anxiety, a reliable animal model of emotional stress should be established in prior. The stimulation of uncertainty empty bottle model has a good physiological effect. It is commonly used in the studies of the mechanism of stress on ethological changes (Shao et al., 2003). According to the behavior observations, rats in the ES-M group that were irregularly given empty water bottles expressed significant attacking behaviors, such as biting bottles and cage shed, whereas rats in the M and PS groups did not. These behavioral results indicated that rats in the ES group were experiencing significant emotional stress during the stress procedure and rats in the PS group were less stressed comparatively. Combining the results of exploring and grooming behaviors as well as the results of the fluid intake and change of body weight, it was proven that the desperate dehydration had influences on both behaviors. The results indicated that bottle attacking behaviors could be a creditable standard to examine
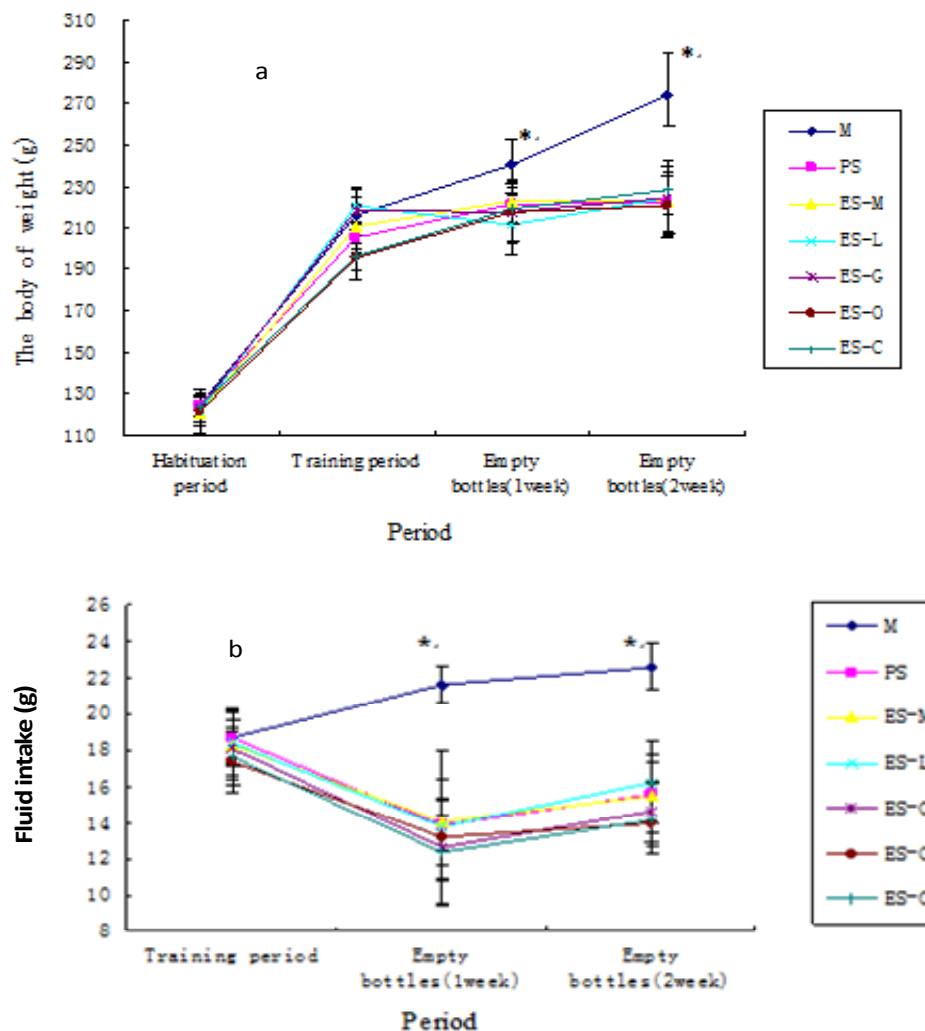
Figure 2. The body weight of the rats in every period (A) .The fluid intake of the rats for each day (B).
*p<0.05 compared with ES-M. M is the control model group, PS is the physiological stress group, ES-M is blank emotional stress group and ES-L, ES-G, ES-O and ES-C represent the four essential oil (lavender, grapefruit, orange and *C. salicifolicus*) sniffing groups, respectively.

anxiety.

The changes in fluid intake and body weight of the rats

According to the results, no difference among groups was detected when all rats received similar amount of fluid intake in two water access sessions in the training period. During the experimental period, rats in the ES and PS groups only randomly received water in one of the two watering periods everyday. Therefore, fluid intake in ES and PS groups were reduced significantly compared to the M group. However, there was no difference between all ES groups and PS group. This result indicated that rats in all the ES groups had the same dehydration as the

PS group. Fluid intake is associated with food intake, which can directly influence the body weight (Reed et al., 2004). During habituation and training periods, there was no difference in the changes of body weight among all the groups. However, in the experimental period, the body weight of rats in the ES and PS groups increased slower than the rats in the M group. These results demonstrated that the change in body weight of rats in the ES groups was similar to that in the PS group. The PS group had no difference when compared with ES-M group. Considering the results of the fluid intake, the desperate dehydration had limited the addition of body weight. There were no difference in all the inhaled odor experimental groups on both the fluid intake and body weight. It demonstrated that inhaling these essential oils had no influence to the fluid intake and change of body weight.

Table 1. Stress procedure.

Time	1	2	3	4	5	6	7
10:00-10:20	ES	ES	N	ES	ES	ES	N
20:10-20:20	N	N	ES	N	N	N	ES
Time	8	9	10	11	12	13	14
10:00-10:20	N	ES	N	N	N	ES	ES
20:10-20:20	ES	N	ES	ES	ES	N	N

ES, give empty water bottle; N, drink water at trained time.

Table 2. The effect of emotional stress on the behaviour of rats.

Parameter	Attacking behavior	Exploring behavior	Grooming behavior
M	0.08±0.214[*]	0.253±0.25*	0.287±0.487
PS	0.047±0.074[*]	1.52±0.493	0.273±0.153
ES-M	0.827±0.339	1.64±0.329	0.213±0.151
ES-L	0.44±0.272[*]	1.487±0.497	0.28±0.286
ES-G	0.613±0.416	1.38±0.598	0.32±0.254
ES-O	0.375±0.294[**]	1.727±0.663	0.341±0.366
ES-C	0.518±0.373*	1.437±0.566	0.279±0.233

*$p < 0.05$, compare with ES-M; $p < 0.01$, compared with ES-M.

The effect of emotional stress on the serology markers of rats

In terms of the results, inhaling lavender oil, orange oil and *C. salicifolicus* oil could reduce attacking behaviors significantly compared to the ES-P group, especially the orange oil. The results of serology detection also demonstrated that only the inhaling the *C. salicifolicus* essential oil could decrease the level of TC and GLU because of the anxiety caused by a stimulus bottles. There were many studies which proved that diabetes and depression negatively interact, in that depression leads to poor metabolic control and hyperglycemia exacerbates depression. The researchers found that depression by reducing adherence to treatment is partly related to a poor glycemic control (Talbot et al., 1999). It have been reported that some medicine have both effects of antianxiety and hypoglycemic like *Clonorchis sinensis. C. sinensis* has an antidepressant-like activity and they can attenuate the diabetes-induced increasing in blood glucose concentration (Guo et al., 2010). In this article, it is suggested that the *C. salicifolicus* may be a potential strategy for contemporary treatment of anxiety and hyperglycemia.

Anxiety may affect lipid metabolism and induce coronary atherosclerosis. Anxiety not only can influence the level of blood glucose, but also the TC, LDL-C, HDL-C and TG. Cholesterol levels and cardiovascular responses to emotionally arousing stimuli were examined in 60 healthy African males and females (Clark et al.,

1998). The results indicated that the total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) levels of plasma rose after acute stress. The study of McCann et al. (1999) found that short-term high-intensity workload stress make plasma apolipoprotein (apoB) and TG increase. In this ariticle, the results indicated that only the *C. salicifolicus* essential oil had effect on decreasing the level of triglycerides (TG) caused by anxiety. However, inhaling these kinds of essential oils had no influence on the level of HDL-C and LDL-C in stressed rats. The results indicated that the role of these essential oils was in different metabolic pathways.

All the findings represent the first evaluation of the traditional medicinal potential of *C. salicifolicus*, which is widely distributed in Zhejiang province and have a very special odor. Based on these results, the *C. salicifolicus* essential oils not only had an importance, per se, in treating antianxiety, but also considering its possible use as precursors in the development of new drugs as a contemporary treatment of anxiety, hyperglycemia and hypercholesteremia. The study proved that the administration of *C. salicifolicus* essential oil to Wistar rats reduces anxiety and verifies the anxiolytic effects of lavender and orange essential oils. The results provide a direction to extraction and separation of active ingredients. It is worth to be reminded that because of the reduction effect on glucose and total cholesterol, further research of *C. salicifolicus* ought to be launch on this aspect.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Review

New cultivation approaches of *Artemisia annua* L. for a sustainable production of the antimalarial drug artemisinin

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Artemisia annua **L. is an important medicinal plant from which artemisinin was extracted to treat malaria effectively. Artemisinin is isolated from the aerial part of the *A. annua* harvesting from the wild is causing loss of genetic diversity and habitat destruction. The use of controlled environments can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins. The aim of this review is to bring together most of the available scientific research papers about the cultivation and breeding conducted on the genus *A. annua*, which is currently scattered across various publications. Through this review the authors hope to attract the attention of all agronomist and breeders throughout the world to focus on the unexplored potential of *A. annua* species. Also, the future scope of this plant has been emphasized with a view of the importance of cultivation of *A. annua* for increasing of artemisinin content. New cultivation approach of *A. annua* offers the opportunity to optimize yield and achieve a uniform, high quality product.**

Key words: *Artemisia annua*, breeding, artemisinin, cultivation, medicinal plant.

INTRODUCTION

Since artemisinin was discovered as the active component of *A. annua* in early 1970s, hundreds of papers have focused on the anti-parasitic effects of artemisinin and its production (Bhattarai et al., 2007; Efferth et al., 2002; Singh and Lai, 2004; Utzinger and Keiser, 2004; Romero et al., 2005). The recent works of several groups show that artemisinin is currently the most effective malaria drug and is the World Health Organization's currently recommended medicine (Bhattarai et al., 2007)

in treating malaria. Artemisinin also shows promise as a potential therapeutic agent for other parasitic and viral diseases as well as for the treatment of certain cancers and the reduction of angiogenesis (Singh and Lai, 2004; Utzinger and Keiser, 2004; Romero et al., 2005). *A. annua* still remain one of the major sources of drug in the traditional and modern system of medicine throughout the world. Harvesting from the main source of raw material of *A. annua* is causing loss of genetic diversity and habitat

and habitat destruction. Cultivation of this plant is a viable alternative and can overcome cultivation difficulties of it.

At the present time, in the 21st century, malaria is still severely challenging people's health. Each year, more than one million people around the globe die of malaria and more than two billion people in over 100 countries are threatened by the disease (Bhattarai et al., 2007;WHO, 2008). In many developing countries, especially those in Africa, the mortality from malaria are still very high. The great majority of these are in children under the age of five years. Therefore, the world market for products including artemisinin derivatives is now growing rapidly, and the demand for artemisinin is increasing. At present, artemisinin compounds are derived from a raw substance extracted from the plant *A. annua* because artemisinin is very difficult to synthesize (Klayman, 1985; Singh et al., 1988). Enhanced production of artemisinin in the whole plant of *A. annua* is therefore highly desirable.

Many researchers have reported that the yields of extracted artemisinin is very poor (Singh et al., 1988; Charles et al., 1990) and much effort must be made to increase artemisinin content of *A. annua*. Because the plant material in wild stands is typically variable in its artemisinin content and plant biomass and this has an impact on drug extraction. Efforts are being made to increase its production in many ways such as plant tissue culture and biotechnological and agronomical practices (Jaziri et al., 1995). But artemisinin was not found to be accumulated in callus and cell suspension cultures, it is presumed that the biosynthesis of artemisinin to be restricted to the green part of the plant (Fulzele et al., 1991). At the present time, the biotechnological approach for the production of artemisinin remains disappointing, and the molecule must therefore still be extracted from *A. annua* plant grown outdoors.

GENUS *ARTEMISIA* AND GEOGRAPHICAL DISTRIBUTION OF *A. ANNUA*

The genus *Artemisia* belongs to a useful group of aromatic and medicinal plants. It is one of the largest and most widely distributed genera of the family Astraceae comprised over 450 diverse species. These species are perennial, biennial and annual herbs or small shrubs (Watson et al., 2002; Iranshahi et al., 2007; Torrell et al. 2003). In the literature, artemisinin has been reported in *A. annua*, *A. apiacea* L., *A. lancea* L., *A. cina* L., *A. sieberi* L., *A. absinthium* L., *A. dubia* L. and *A. indica* L. (Tan et al., 1998; Iranshahi et al., 2007; Temraz and El-Tantawy, 2008). But *A. annua* is suitable for cultivation and has been described as containing 0.5 to 1.2% artemisinin in the dried plant material (Ferreira et al., 1997) also, *A. annua* is economically the only natural botanical source for artemisinin production.

A. annua originated from China and is widely distributed in the Northern Hemisphere but poorly represented in the Southern Hemisphere. It grows mainly in the middle, eastern and southern parts of Europe, in the northern, middle and eastern parts of Asia and in North Africa (Simon et al., 1984; Ferreira and Janick, 1996). It is also distributed in the temperate, cool temperate, subtropical zones and Mediterranean region of the world. The plant is not grown the tropics because flowering will be induced when the plants are very small (Ferreira and Janick, 1996).

Botany *A. annua*

A. annua is shrub and usually single-stemmed reaching about 2 m in height with alternate branches. The plant is extremely vigorous and essentially disease and pest free. *A. annua* is a short-day plant and very responsive to short photoperiodic stimuli and flowers about two weeks after induction. They require about 1000 h of sunlight per year. Annual sunlight time is a critical factor for the growth of *A. annua* (Simon et al., 1984). The fruit of *A. annua* is an achene with a single seed inside. The seeds are approximately 1 mm in length, oblong and the 1000 seed weight is approximately 0.03 g. The seeds do not have a dormant phase and can be used in the same year or in the year following collection (Ferreira and Janick, 1996). During the vegetative growth of *A. annua* trichome numbers increased per unit area on the adaxial leaf surface until leaf expansion ceased, at which point trichome numbers began to decline, apparently as a result of their collapse (Lommen et al., 2006). Leaves had 89% of the total artemisinin in the plant with the uppermost foliar portion of the plant containing almost double that of the lower leaves (Charles et al., 1990).

Cultivation of *A. annua*

As we mentioned earlier, artemisinin cannot be synthesized, so it is still extracted from *A. annua* aerial parts. Therefore, the science of commercial cultivation of *A. annua,* to maximize artemisinin yields, should be well developed (Laughlin et al., 2002). The overharvesting of wild stands may restrict the ability for the plant to cross-pollinate and reseed naturally, eventually limiting the gene pool and genetic variability, which is critical to the development of improved seed lines. Another negative factor against application of wild stands is that transport distances often become uneconomic with a crop such as *A. annua*, which has relatively low artemisinin content and requires large biomass production.

In order to maximize the yield of artemisinin, the critical factor is day length, because the plant usually grows in the long summer days at high latitudes and flowers when the day length shortens. In the Tropics, where days are shorter than in Northern summers, flowering occurs earlier,

reducing the biomass achieved. However, yields can be maximized at higher altitudes and with late-flowering varieties (Laughlin et al., 2002; Ferreira et al., 1995). In wild-type plants, the greatest concentration of artemisinin is found in the inflorescence, although it occurs in all other aerial parts of the plant, except the seed (Ferreira et al., 1997). Seed varieties have been adapted by breeding for lower latitudes, and cultivation has been successfully achieved in many tropical countries, for example, in Congo (Mueller et al., 2000), India (Mukherjee, 1991) and Brazil (Milliken, 1997). The range of artemisinin content of *A. annua* harvested from different production areas is wide. The highest content of artemisinin that can be reached is up to 0.5 to 1.2% expressed as dry weight of leaves of *A. annua*. Although, the content of artemisinin is affected by numerous factors such as geographical conditions, harvesting time, temperature and fertilizer application, harvesting at the appropriate time is critically important to ensure optimum content of artemisinin in *A. annua*. Therefore, the best time for harvesting of *A. annua* should be determined by a study of the weather conditions, artemisinin accumulation and local harvesting experience. The yield of *A. annua* leaves and the content of artemisinin are reduced if harvesting is too early or is delayed.

For cultivation of this medicinal plant, it is important to plan the crop establishment for the beginning of the rainy season, which will enable fast growth at the crop's early stages and the production of higher biomass before flowering. In Switzerland, good biomass production was obtained from planting in late spring (Delabays et al., 1993) and early summer in Germany (Liersch et al., 1986) and the USA (Charles et al., 1990). If supplies of seed were freely available, direct sowing would be the most economical method of plant establishment, provided the environmental factors were suitable. For sowing of *A. annua*, the soil needs to be ploughed to a fine tilth and consolidated by rolling where appropriate. Because *A. annua* seed is small and it needs to be mixed either with some inert material. Depth of sowing is also critical for *A. annua*. A depth of drilling of 5 mm below the surface of finely prepared soil resulted in good emergence and establishment (Laughlin et al., 2002). Also, it is important to irrigate soon after sowing so that young seedlings do not suffer water stress. The irrigation frequency will depend on soil type, climate and season.

Plant population density and its components of inter- and intra-row spacing are important in determining yield and the practicability of both weed control and harvesting (Willey and Heath, 1969; Ratkowsky, 1983). If inter-row cultivation is intended for the control of weeds before the rows close, then inter-row spacing of 0.5 to 1.0 m may be appropriate. Similarly, wide intra-row spacing may also be appropriate. In earlier studies, low densities of 1 plant/m^2 (WHO, 988) and 2.5 plants/m^2 (Delabays et al., 1993) gave yields of 1 to 4 t/ha of dried leaf.

Very little published work exists on the vegetative growth responses of *A. annua* to the nitrogen, phosphorus and potassium or of their effects on the concentration of artemisinin and related compounds. Significant increase of total plant and leaf dry matter (1 to 3 t/ha) was obtained in Mississippi, USA, where a complete fertilizer mixture containing 100 kg N, 100 kg P and 100 kg K/ha was broadcast and worked uniformly through the soil (WHO, 1988). In China, a range of growing media and micronutrients were tested for their effect on the synthesis of artemisinin. There were no effects on artemisinin from any of these treatments (Chen and Zhang, 1987).

A. annua has been grown in a wide diversity of soils and latitudes, showing its potential for adaptation. If the *A. annua* cultivar and geographic region allow for a long vegetative cycle, more than one harvest can be performed to increase the final yield of leaves and artemisinin. In a study by Kumar et al. (2004) with the *A. annua* cultivar Jeevanraksha, carried out in a subtropical climate in India for 3 years the crops were harvested once, twice, three times and four times, respectively, during a 1-year growth cycle. *A. annua* can be grown under a wide range of soil pH (5.0 to 8.0), depending on the plant origin, but there are only a few studies on the effect of soil pH on the vegetative growth and artemisinin concentration in *A. annua*. It has been shown that some strains of *A. annua* are sensitive to soil pH below 5.0 to 5.5 (Laughlin, 1994). Weeds are a constant problem for crop production throughout the world and any system of *A. annua* cultivation must give careful thought to weed control. Weed control in the early stages of growth is critical for *A. annua*. Once plants become established, and with good early season weed control, the canopy shade will provide good weed control. There have been no serious pests or diseases yet reported to be a problem associated with *A. annua* (Simon and Cebert, 1988).

Many researchers used to think that sun and oven drying reduced the artemisinin content and that it was best to air-dry leaves in the shade (Laughlin et al., 2002; Ferreira and Luthria, 2010). However, comparisons between sun-drying, shade-drying and oven-drying at 60°C have shown that natural sun-drying is the best method (Ferreira and Luthria, 2010). Simonnet et al. (2001) found that sun-drying plants in the field increased the artemisinin content, but that if drying continued for more than a week, leaves were lost, decreasing the overall yield. The optimum would therefore seem to be drying in the field for 1 week, followed by air-drying in the shade. Harvesting time has to be established according to the cultivar of *A. annua* used because peak artemisinin can be achieved before or at full flowering, but it is generally accepted that the leaves should have no more than 12 to 13% relative humidity to realize optimum recovery of artemisinin. A drought a week before harvesting the plants could shorten the time needed to bring the cut plants to 12 to 13% relative humidity, but the effect of such a drought on artemisinin accumulation needs

to be investigated. The whole aerial part should be harvested, but leaves are the main source of artemisinin. These can be separated from the stems by threshing the whole plant over a plastic tarp. Leaves can then be sieved using a 5-mm mesh and then a 3-mm mesh (TechnoServe, 2004). Fine grinding of leaves is not necessary for the extraction of artemisinin because the compound is located in glandular trichomes found in both leaves and flowers (Ferreira and Janick, 1995).

In the agronomy point of view, novel production methods are required to accommodate the ever-growing need for this important drug. The future work should focuses on releasing high artemisinin content varieties and improved cultivation approaches. Cultivation of this plant requires at least 6 months, and extraction, processing and manufacturing of the final product require at minimum of 2 to 5 months depending on the product formulation. Research is also needed on the development of new cultivation practices methods to robust yield of new breeding lines. Such methods will require collaboration between Agronomists, breeders, farmers, and social scientists in developing techniques for initiating and sustaining farmer participation in yield improvement.

BREEDING FOR INCREASING ARTEMISININ CONTENT OF *A. annua*

A new breeding strategy of comprehensive integration of biotechnology and DNA marker applications with conventional backcross breeding techniques for *Artemisia* improvement should be developed. In designing breeding programs, germplasm collections are important reservoirs of genetic diversity and this diversity should exploit for artemisinin improvement. Artemisinin enhancing alleles may exist in wild *Artemisia* germplasms. Furthermore, this technique enables us to select potential parent lines of *A. annua*. Breeders should propose strategies for introgressing artemisinin alleles into elite cultivars. Three approaches are considered in this review. These are: 1. Conventional breeding programs; 2. Mutation breeding and 3. Molecular breeding approaches.

Conventional breeding programs

The scientific studies have shown that artemisinin content can vary widely among different genotypes of *A. annua* from different origins (Wallaart et al., 2001), so breeding for this trait may be feasible. The genetic basis of this variation has been investigated by many researchers. Additive genetic components were predominant, resulting in a high narrow-sense heritability estimate. This trait exhibits high heritability therefore it appears possible to breed *A. annua* with a higher level of artemisinin. For breeding purposes, crosses can be only made between

individuals of *A. annua* that those have high artemisinin content. Plant breeders must therefore select varieties capable of producing well in favorable and unfavorable conditions. Selection criteria to be used in order to obtain high yields under various environments should be determined by breeders. Authors believe that there are 4 major breeding targets that may result in improved artemisinin yield: increasing leaf yield potential, increasing number of branches, increasing artemisinin content and improving number of trichome in plants.

Since the plant *A. annua* is highly cross pollinated like the members of family Asteraceae the chemical character like 'artemisinin content' segregate like any other phenotypic characters as multigenic characters always segregate in the progeny population. Every individual of its population can be expected to be homozygous at many loci, but also heterozygous at many loci. As a consequence of the segregation and recombination alleles of many loci are reshuffled and regrouped into vast numbers of multilocus allelic configurations each generation. Due to this all the progeny plants of the high artemisinin containing plant may not yield same amount of the artemisinin. Some will be high and some very low. The authors believe that the pyramiding of independent artemisinin-promoting segments can lead to novel commercial varieties of *A. annua*. A hybrid variety of *A. annua* is made by cross-pollinating two specific parent varieties. This first generation of offspring is referred to as the F1 hybrid.

Mutation breeding

Induced mutations have great potentials and serve as a complimentary approach in genetic improvement of crops for greater yield and quality traits (Ahloowalia and Maluszynski, 2001). The generation of genetic variability by induced mutagenesis provides a base for strengthening plant improvement programs (Rekha and Langer, 2007). For any mutation-breeding program, selection of effective and efficient mutagen is very essential to recover high frequency of desirable mutations (Solanki and Sharma, 1994). The mutagenesis approach is attractive alternate method for enhancement of artimisinin *in vivo* as well as *in vitro*. The results of many studies showed that mutation breeding has the capacity to release mutants with high artemisinin content (Rekha and Langer, 2007). Also, there is evidence that mutagens (radiations) stimulate the metabolic activity of plants such as respiration glycolysis and oxidative phosphorylation (Mergen and Johnson, 1964) and cytochrome oxidase and catalase activity which may ultimately influence and enhance synthesis of plant products. The main advantage of mutation breeding is the possibility of improving one or two characters without changing the rest of genotypes. Induced mutation breeding, which has been recognized as a valuable supplement

to conventional breeding in crop improvement, has been least applied in *A. annua*. The authors believed that mutation breeding can induce enormous variability in the *A. annua* through physical and chemical mutagenesis, because genetic variation is the starting point of any breeding program. Produced new mutant plants have great potential to be incorporated in further breeding programmes for upgrading new productive cultivars.

Molecular breeding approaches

During the past 30 years, the continued development and application of plant biotechnology, molecular markers, and genomics has established new tools for the creation, analysis, and manipulation of genetic variation and the development of improved cultivars and new varieties (Sharma et al., 2002; Collard and Mackill, 2008). Presently, development of large numbers of molecular markers, high density genetic maps, and appropriately mapping populations are routined for many crop species. This technique, known as marker-assisted selection (MAS), is theoretically more reliable than selection based on phenotype. A vast majority of literature has considered the utility of molecular marker-assisted selection and its fit with different breeding methods (Dekkers and Hospital, 2002; Collard and Mackill, 2008). Molecular markers that are either within genes or tightly linked to QTL influencing traits under selection can be employed as a supplement to phenotypic observations in a selection index (Lande and Thompson, 1990). Use of molecular markers can significantly increase breeding efficiency and enhance genetic gain for traits where the phenotype is difficult to evaluate because of its expense or its dependence on specific environmental conditions.

Molecular marker technology has so far failed to be extensively used as a breeding tool by *Artemisia* breeders, because of the fact that no QTL responsible for a large enough effect on leaf yield has been discovered. In *A. annua*, there are now attempts to employ markers linked to leaf artemisinin content, trichom density and flowering delay. Only a very small fraction of the available *A. annua* germplasm has been assayed for alleles that might improve artemisinin content. It is likely that *A. annua* from many areas regarded as a wild plant and such plants have rarely been used as parents in QTL mapping studies. A more extensive survey of artemisinin content of *A. annua* germplasm will hopefully lead to the identification of lines carrying major genes conferring high artemisinin content. One of the objects of the molecular breeders is to develop an efficient marker system to be used in breeding *A. annua* for high artemisinin content. This marker system can distinguish plant tending to synthesize high amount of artemisinin when the biosynthetic system of the plant is functional. Other objective of the invention should generate a breeding and selection method using the marker assisted breeding to

increase the content of artemisinin in the plants. Individual loci with large effects on artemisinin content should be identified. The characterization of such genes and their anatomical, physiological, and molecular genetic effects, will be key factors in the application of molecular marker technology to the development of high artemisinin varieties.

PERSPECTIVES IN THE STUDY OF *A. annua*

In this review we discussed the possibility of increasing the amount of artemisinin in *A. annua* by cultivation and breeding approaches. To date progress in cultivation and breeding of *A. annua* focusing on artemisinin content has made little progress. This may be explained simply by the lack of investment. Research is needed on the development of new cultivation practices methods to robust yield of *A. annua*. Such methods will require collaboration between agronomist, breeders, farmers, and social scientists in developing techniques for initiating and sustaining farmer participation in cooperative testing networks. The research on *A. annua* is expected to require a substantial amount of resources. The improvement on *A. annua* will be faster and easier accomplished by an international and multidisciplinary collaboration among agronomists, breeders, traditional geneticist, molecular biologists, etc. If it is pursued by different groups, including academia, governmental and non-governmental organizations, interested in making a real contribution in the field of cancer, malaria, and other chronic and parasitic diseases, the result on *A. annua* will be better.

There is a need to develop high artemisinin content varieties that will produce acceptable yields under favorable and unfavorable environments. Many traits are known to contribute to improving leaf yield and artemisinin content, but the anatomical, physiological, and molecular pathways controlling them are not well understood. A better understanding of the genetic basis of artemisinin content will probably be achieved by using more diverse mapping populations and by precisely identifying the genes affecting variation in artemisinin content through fine-mapping, microarray analyses and proteomics. Germplasm collections of *Artemisia* are important reservoirs of genetic diversity, but very little of this diversity has been exploited for its improvement. Artemisinin enhancing alleles may exist in wild germplasm. A methodology for introgression of artemisinin enhancing loci from wild species to the cultivated germplasm should be developed. Scientists should propose strategies for simultaneously mapping and introgressing these alleles into elite cultivars. Development of new varieties of *Artemisia* with high artemisinin content will both reduce the costs of cultivation and the costs of extraction associated with more artemisinin production. It will also produce better returns to farmers who grow the crop. The development of artemisinin-rich genotypes of *A. annua*

has encouraged some to promote cultivation and local use of the plant to treat malaria.

It is hoped that the publication of this paper will be helpful for the prevention and treatment of malaria around the globe. The anti-malarial drug, artemisinin, production can be maximized by breeding of new varieties and new well developed cultivation systems. These species seem to hold great potential for in depth investigation for various biological activities. The future studies focused on the breeding of new varieties could be beneficial.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Assessment of the antimicrobial activity of aqueous and ethanolic extracts of *Piper guineense* leaves

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The antimicrobial activity of aqueous and ethanolic extracts of leaves of *Piper guineense* was determined on some bacteria and fungi, namely, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* and *Saccharomyces cerevisiae*, using agar well diffusion method and minimum inhibitory concentration (MIC). The ethanolic extract was found to show more activity than the aqueous extract on all the isolates. The diameter of zones of inhibition for the ethanolic extract ranged between 2 and 12 mm, while that of aqueous extract ranged between 5 and 8 mm. The MIC of the ethanolic extract was from 2.5 to 10 mg/ml, while for aqueous extract, the MIC was 10 to 20 mg/ml. *Escherichia coli* was found to show the greatest sensitity, while *P. aeruginosa* showed the least sensitivity of all the isolates. The phytochemical analysis carried out on *P. guineense* leaves revealed the presence of alkaloids, tannins, saponins, glycosides and flavonoids. The presence of these phytochemicals supports the use of this plant as antimicrobial agent. *P. guineense* can therefore be used as antimicrobial agent.

Key words: *Piper guineense*, antimicrobial, extracts, aqueous, ethanol, phytochemical.

INTRODUCTION

Plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well being. Plant extracts have been used for a wide variety of purposes for many thousands of years (Jones, 1996). The antimicrobial activity of plant oils and extracts has formed the basis of many applications, including raw and processed food preservatives, pharmaceuticals, alternative medicine and natural therapies (Reynolds, 1996; Lis-Balchin and Deans, 1997).

The use of traditional medicine is wide spread throughout the world. The term, traditional medicine, is interchangeably used with herbal medicine and natural medicine (Hazan and Atta, 2005). Since antiquity, man has used plants to treat common infectious diseases and even long before mankind discovered the existence of microbes; the idea that certain plants had healing potential was well accepted (Rios and Recio, 2005). A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purpose or which are precursors for the synthesis of useful drugs.

A number of plants have been used in traditional medicine for many years due to their antimicrobial properties (Sofowora, 1993). Specifically, the medicinal value of these plants lies in some chemical substances

that produce a definite physiological action on the human or animal body (Edeoga et al., 2005). The most important of these bioactive constituents which are mainly secondary metabolites are alkaloids, flavonoids, tannins and phenolic compounds. These phytochemicals are toxic to microbial cells. There is growing interest in exploiting plants for medicinal purposes especially in Africa. This stems from the fact that microorganisms are developing resistance to many drugs and as such created situation where some of the common and less expensive antimicrobial agents are losing effectiveness (Montefore et al., 1989). Medicinal plants generally contain a number of compounds which may be potential natural antibacterial for the treatment of common bacterial infections (Ratnasooriya et al., 2005). Plant derived medicines are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Kareem *et al.,* 2010). Herbal medicine which uses medicinal plants primarily presents as an alternative to such situation (Sofowora, 1993).

Medicinal plant such as *Piper guineense* has been asserted to provide various culinary and medicinal properties (Scott et al., 2005). These medicinal properties exert bacteriostatic and bactericidal effects on some microorganisms. These effects have been attributed to the peptides, alkaloids, essential oils, phenols and flavonoids which are major components in these plants (Okigbo and Igwe, 2007).

P. guineense, commonly referred to as African black pepper or Ashanti pepper, is very similar to *Piper nigrum* which is the true pepper of commerce from which black and white peppers are processed (Isawumi, 1984). *P. guineense* belongs to the family Piperaceae. It has more than 700 species throughout the tropical and subtropical regions of the world. It is known with different vernacular names in Nigeria: Igbo (Uziza) and Yoruba (Iyere). *P. guineense* has culinary, medicinal, cosmetic and insecticidal uses (Dalziel, 1955; Okwute, 1992). The insecticidal activity of *P. guineense* against *Zonocerus variegatus* is attributable to the piperine-amide component of the plant. The leaves are considered aperitive, carminative and eupeptic. They are also used for the treatment of cough, bronchitis, intestinal diseases and rheumatism (Sumathykutty et al., 1999).

In this study, the antimicrobial activity of crude extracts of the leaves of *P. guineense* has been studied as part of the exploration for new and novel bioactive compounds.

MATERIALS AND METHODS

Collections of samples

The samples of the leaves of *P. guineense* were bought from Ogige market in Nsukka, Enugu State, Nigeria. The variety was chosen because it is widely used in all parts of the country as spice, condiment and in soup making. The plant was identified and authenticated at the herbarium of the Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria. The samples were thoroughly washed with tap water and rinsed with distilled water. A voucher specimen was deposited at the herbarium for reference purposes.

Preparation of sample

The washed samples were dried at room temperature for one week after which they were finely ground. The ground samples were put into sterile screw capped container and stored under refrigeration condition in preparation for the extraction process.

Extraction procedure

The extraction was done by the soaking method using two solvents, namely, deionized distilled water and ethanol. Different 200 g portions of the powdered sample were differently extracted with 200 ml distilled water and 200 ml of 70% (v/v) ethanol in different 500 ml Erlenmeyer flasks. The samples were soaked for about 72 h with intermittent shaking after which they were filtered using Whatman No. 1 filter paper. The extracts were then concentrated by evaporating to dryness using rotary evaporator at a temperature of 40°C. A dark-green coloured mass of *P. guineense* was obtained and stored in airtight bottles at 4°C in a refrigerator until it is ready for use*.*

Reconstitution of extract

The stored extracts were reconstituted using the corresponding solvents to obtain stock solutions which were further diluted serially to obtain concentrations of 1.0, 2.5, 5.0, 10.0 and 20.0 mg/ml prior to determination of the minimum inhibitory concentration (MIC).

Preparation of test organisms

Microorganisms were obtained from the culture collections of the Department of Microbiology, University of Nigeria, Nsukka. Organisms were as follows: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* and *Saccharomyces cerevisiae*. The organisms were maintained on nutrient agar and Sabouraud dextrose agar (SDA) slants at refrigeration temperature. Overnight cultures were prepared by inoculating approximately 2 ml nutrient broth or Sabouraud dextrose broth with colonies of the appropriate organism taken from the agar slant. Broths were incubated overnight at 37°C for bacteria and 30°C for fungi. Inocula were prepared by diluting overnight cultures in saline to approximately 10^8 cfu/ml for each of the organisms.

Determination of antimicrobial activity of the extracts

The antimicrobial screening of the aqueous and ethanolic extracts was carried out using the agar well diffusion method as described by Lino and Deogracious (2006). Nutrient agar and SDA were, respectively, poured into sterile Petri dishes and allowed to solidify. About 1.0 ml of the test culture was dropped on the appropriate solidified agar and spread over the surface of the medium using a spreader. Wells of approximately 6 mm in diameter were made in the agar medium using sterile cork borer. Each well was filled with 0.2 ml of the appropriate concentration of each extract. The dishes were allowed to stand for 40 min at room temperature to allow for proper diffusion of the extract to occur. Control experiments were set up with 0.2 ml of 70% ethanol and 0.2 ml distilled deionized water in separate wells. The plates were incubated at 37°C for 24 h for the bacteria and at 30°C for 48 h for fungi. All tests were performed in duplicates and antimicrobial activity was expressed as the mean diameter of the clear zone (mm) produced by the plant extracts.

Determination of MIC of extracts

The MIC of the crude extracts was carried out using a modified method of Akinpelu and Kolawole (2004). Serial dilutions of the crude extracts were prepared and 2 ml aliquots of the different concentrations of the solution were added to 18 ml of pre-sterilized molten nutrient agar or SDA for bacteria and fungi, respectively, at 40°C to give final concentration regimes of 1.0, 2.5, 5.0, 10.0 and 20.0 mg/ml. The medium was then poured into sterile Petri dishes and allowed to set. The surface of the medium was allowed to dry before streaking with 18 h old bacterial and fungal cultures. The plates were later incubated at 37°C for 24 h and at 30°C for up to 48 h for bacteria and fungi, respectively, after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented the growth of the test microorganism.

Phytochemical analysis

The phytochemical analysis of the ground leaves of *P. guineense* was performed following the methods described by Trease and Evans (1989) and Harbone (1998). The phytochemicals analysed for were plant secondary metabolites which included flavonoids, tannins, saponins, alkaloids and glycosides.

RESULTS

Antimicrobial activity of the extracts

The results of the antimicrobial activity of the extracts against the test organisms, namely, *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, *Candida albicans* and *S. cerevisiae* are shown in Table 1. The extracts showed varying degrees of growth inhibition against the isolates. The mean zones of inhibition of growth of the isolates are a function of relative antimicrobial activity of the extracts. The ethanol extract showed higher growth inhibition (2 to 12 mm) than the aqueous extract (5 to 9 mm) against all the isolates. Ethanol and distilled deionized water which served as control showed no activity against the test organisms. The antimicrobial activities of the aqueous and ethanol extracts appeared to be broad spectrum as their activities were independent of Gram reaction. The inhibition zone for *P. aeruginosa* was the least and that for *E. coli* was the highest compared to the other isolates tested.

MIC of the extracts

The results of the MIC of the extracts against the tested isolates are shown in Table 2. The MIC of the ethanol extract for the different organisms ranged between 5.0 and 10.0 mg/ml, while that of the aqueous extract ranged between 10.0 and 20.0 mg/ml. Lower MIC values were obtained for the aqueous extracts. Higher concentrations of the *P. guineense* extracts were needed to inhibit *P. aeruginosa* and the fungi when compared with the other isolates. The MIC of the aqueous extract of *P. guineense* were 20.0 and 10.0 mg/ml for *P. aeruginosa* and *E. coli*,

respectively, while that of the ethanol extract were 10.0 and 5.0 mg/ml for *P. aeruginosa* and *E. coli*, respectively.

Phytochemical analysis

The phytochemical analysis of *P. guineense* leaves revealed the presence of flavonoids, tannins, saponins, alkaloids and glycosides.

DISCUSSION

The results of this study showed that the leaf extracts of *P. guineense* inhibited the growth of all the microbial isolates tested. This suggests that the leaf extracts have antimicrobial activity. The extracts are equally broad spectrum in activity as their activities were independent of Gram reaction. Higher antimicrobial activity of the extracts was observed against *E. coli* followed by *S. aureus*. The antimicrobial effect of *P. guineense* extracts is attributable to the phytochemical constituents present in it. The *P. guineense* leaves are rich in phytonutrients such as flavonoids, tannins, saponins, glycosides and alkaloids.

These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins have been found to form irreversible complexes with proline-rich proteins (Shimada, 2006) resulting in the inhibition of cell protein synthesis. Parekh and Chanda (2007) reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. Herbs that have tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery (Dharmananda, 2003) and they hasten the healing of wounds and inflamed mucous membrane (Okwu and Okwu, 2004).

The biological function of flavonoids includes protection against allergies, inflammation, free radicals, platelet aggregation, microbes, ulcers, hepatoxins, viruses and tumors (Okwu, 2004). These observations therefore support the use of *P. guineense* in herbal cure remedies. The plant, *P. guineense*, also contains alkaloids which are ranked the most efficient therapeutically significant plant substance. Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects. They exhibit marked physiological activity when administered to animals.

In the present study, the aqueous extract of *P. guineense*, generally, showed less antimicrobial activity than the ethanolic extract against the isolates. This observed difference may be due to insolubility of the active compounds in water or the presence of inhibitors to the antimicrobial components (Okigbo and Ogbonnanya, 2006). The less activity of the water extract than ethanol extract against most microbial strains investigated in this

Table 1. Antimicrobial activity of leaf extracts of *P. guineense*.

| Test organism | Zone of inhibition (mm) | | | | | | | | | |
| | Aqueous extract (mg/ml) | | | | | Ethanol extract (mg/ml) | | | | |
	1.0	2.5	5.0	10.0	20.0	1.0	2.5	5.0	10.0	20.0
S. aureus	-	-	-	5.0	8.0	-	-	2.0	5.0	10.0
E. coli	-	-	-	5.0	9.0	-	-	5.0	8.0	12.0
P. aeruginosa	-	-	-	-	5.0	-	-	-	4.0	8.0
B. subtilis	-	-	-	5.0	8.0	-	-	4.0	6.0	9.0
C. albicans	-	-	-	-	6.0	-	-	-	5.0	8.0
S. cerevisiae	-	-	-	-	6.0	-	-	-	5.0	9.0

Table 2. Minimum inhibitory concentration of *P. guineense* leaf extracts.

Test organism	Aqueous extract (mg/ml)	Ethanol extract (mg/ml)
S. aureus	10.0	5.0
E. coli	10.0	5.0
P. aeruginosa	20.0	10.0
B. subtilis	10.0	5.0
C. albicans	20.0	10.0
S. cerevisiae	20.0	10.0

investigated in this study is in agreement with previous works which showed that aqueous extracts of plants generally showed little or no antibacterial activities (Aliero et al., 2006; Ashafa et al., 2008). It has been reported by Okigbo and Ajale (2005) and Okigbo et al. (2005) that inactivity of plant extracts may be due to age of plant, extracting solvent, method of extraction and time of harvesting of plant materials.

There are variations in the degrees of antimicrobial activities of the extracts on the isolates. The variation is presumed to be due to differences in responses by the isolates to different active compounds present in the plant. Ethanol extracts of *P. guineense* showed more antimicrobial activity against *E. coli* and *Bacillus subtilis* than the other isolates. Moreover, *P. aeruginosa* showed the highest level of resistance to the extracts than all other bacterial isolates.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

It can be concluded from this study that the leaf extracts of *P. guineense* showed antimicrobial activity against the tested isolates. Therefore, the plant can be of use in phytomedicine and can be included in health care delivery system particularly in the developing economies. The chance to find antimicrobial activity was more apparent in ethanol than water extracts of the plant. Further studies on more effective methods and other solvents for extracting only the necessary constituents as well as other processing and purification measures would be necessary. The effect of the plant on more pathogenic organisms and toxicological investigations need to be carried out.

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