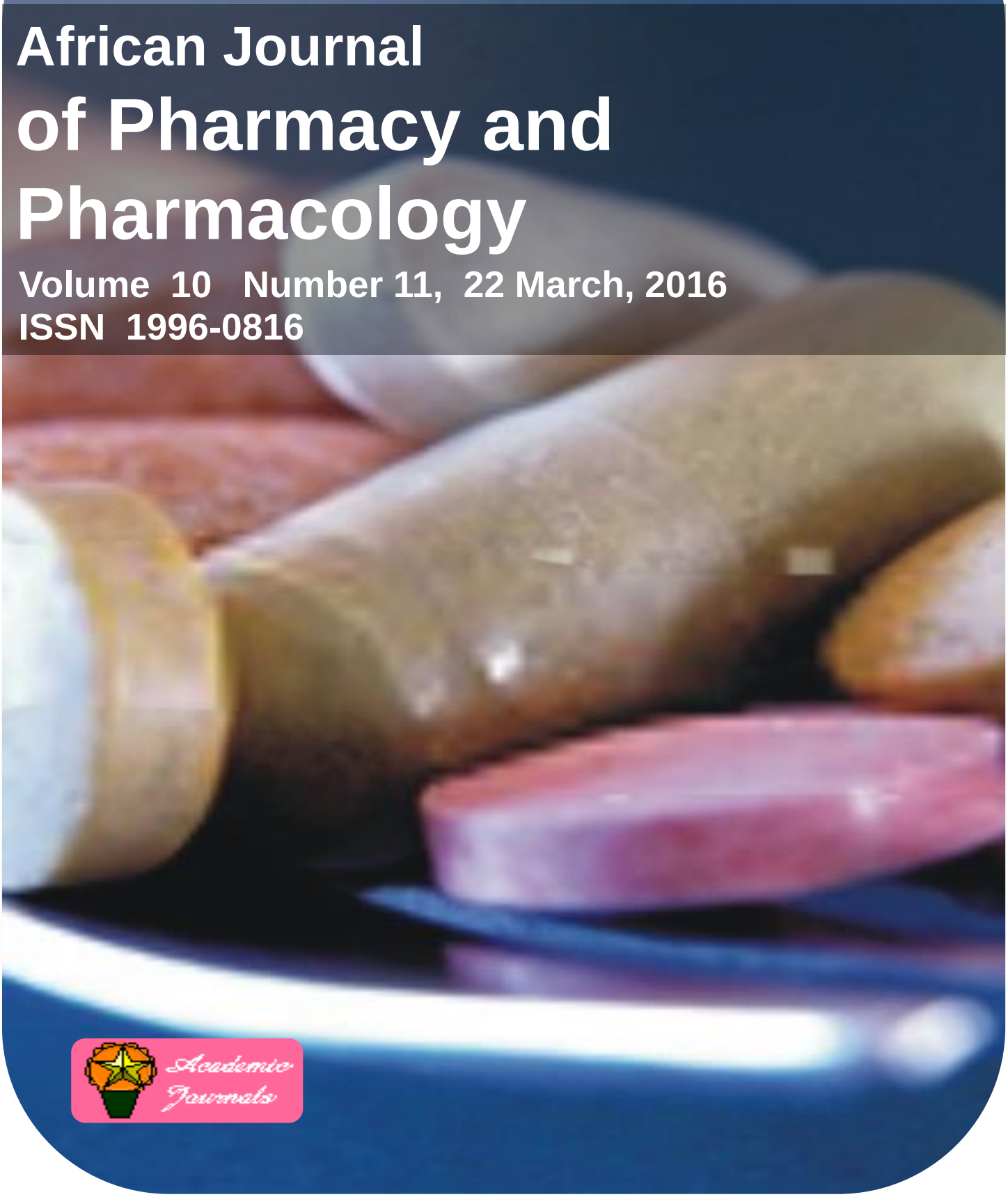
African Journal of Pharmacy and Pharmacology

Volume 10 Number 11, 22 March, 2016
ISSN 1996-0816



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African Journal of Pharmacy and Pharmacology

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Vol. 10(11), pp. 179-184, 22 March, 2016
DOI: 10.5897/AJPP2015.4444
Article Number: 0570DA457495
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antimicrobial activity of essential oil of *Cordia globosa*

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Received 1 September, 2015; Accepted 19 November, 2015

Cordia globosa **is used to treat gastrointestinal diseases in San Rafael Coxcatlan, Puebla, Mexico; however, its medicinal properties have not been investigated. This study reports the chemical composition and the antimicrobial effect of *C. globosa* essential oil. The essential oil of aerial parts was obtained by steam distillation and 25 compounds were identified by gas chromatography-mass spectrometry (GC-MS). The major constituent was α-pinene (38.4%). Antimicrobial activities were tested on 6 bacterial and 9 fungi strains. The most sensitive strain was *Vibrio cholera* (minimum inhibitory concentration [MIC], 0.060 mg/ml). These results show the chemical composition and biological properties of essential oil of *C. globosa*. The results validate the medicinal use of *C. globosa*.**

Key words: *Cordia globosa*, Boraginaceae, antimicrobial activity, essential oil.

INTRODUCTION

Cordia globosa (Jacq.) Kunth (Boraginaceae) is a shrub that grows in tropical lands of America. This plant is known as “blood herb” in San Rafael Coxcatlán, Puebla, Mexico and the infusion of aerial parts is used for diseases like skin fungal infections, gastrointestinal and throat of posible infectious origin as well as for its antitussive, astringent, hemostatic and tonic (Hernández et al., 2003).

Chemical analysis of many plants of the genus *Cordia*, including *Cordia alba, Cordia alliodora* (Manners and Jurd, 1977; Ioset et al., 2000a), *Cordia millenii* (Moir and Thomson, 1973), and *Cordia curassavica* (Ioset et al., 2000b; Hernández et al., 2007) have demonstrated they contain sesquiterpenes, triterpenes, flavanoids, chromens, quinones, and hydroquinones. Phytochemical studies of *C. globosa* include the isolation of meroterpenoid benzoquinones with cytotoxic activity against several cancer cell lines (Menezes et al., 2005)

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and flavonoids (Souza et al., 2004).

Searching for new drugs with high activity is very important, especially considering that in México infectious diseases constitute a serious public health problem (INEGI, 2011). The biosphere is a potential source of many unknown bioactive molecules, especially with the great biodiversity found in México. All these factors justify continued research for antimicrobial substances from plant origin.

There are few studies on the chemical composition of the volatile compounds of *Cordia* species. The aim of this study was to elucidate the composition of the essential oil of *C. globosa* as well as to determine their antimicrobial activity against pathogenic bacteria and fungi.

MATERIALS AND METHODS

Plant

C. globosa were collected in August 2009, in San Rafael, Coxcatlán, Puebla. A voucher specimen was deposited in the IZTA herbarium (Voucher no. HCM60/2009).

Isolation of essential oils

The essential oils were obtained from aerial parts of *C. globosa* by steam distillation (2730 g of fresh plant) for 4 h in a Cleavenger-type apparatus (Cassel et al., 2009) and stored at 4°C until tested and analyzed. The yield of the essential oil was 0.067% (w/w), d^{25} = 0.79 g/ml.

Gas chromatograph-mass spectrometry (GC-MS) analysis conditions

The essential oil was analyzed in an Agilent Technologies 6850 gas chromatograph equipped with a HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The temperature of the column was 325°C. Injector and detector temperatures were set at 230 and 280°C, respectively. Oven temperature was kept at 70°C for 2 min, and then programmed to 280°C at a rate of 8°C/min. Helium was the carrier gas at a flow rate of 1 ml/min. A volume sample of 1 µl was manually injected in the split mode. Peak areas were measured by electronic integration. The relative amount of the individual components was based on the peak areas. Mass analysis was performed on an Agilent Technologies 5975C mass spectrometer. The temperature of the column and the injector were the same as those of the GC. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention indices and mass spectra with the NIST08.L Mass Spectral of the internal device library (Match ≥ 90%). Retention indices were calculated by linear interpolation relative to retention times, of a series of *n*-alkanes (alkane's standards Sigma-Aldrich) and through the determination of the respective Kovats retention indices (KI). The KI were compared with those reported in literature (NIST, 2011).

Microbial strains

The following strains of bacteria were used: *Staphylococcus aureus* ATCC 12398, *Bacillus subtilis* donated by FES-Cuautitlán, *Streptococcus pneumoniae* isolated from a clinical case, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Vibrio cholera* isolated from a clinical case. These strains were maintained at 4°C in Mueller Hinton agar (Bioxon), and were subcultured every month.

The yeasts tested were: *Candida albicans* ATCC 10231, *C. albicans* ATCC 14065, *C. albicans* isolated from a clinical case donated by the Clinical Analysis Laboratory of FES-Iztacala, *Candida glabrata, Candida tropicalis* isolated from a clinical case donated by Hospital Angeles (Metropolitano) and *Cryptococcus neoformans* donated by FES-Cuautitlán. The filamentous fungi pathogens used were: *Trichophyton mentagrophytes* CDBB-H-1112, *Aspergillus niger* CDBB-H-179, *Fusarium sporotrichoides* NRLL3299 and *Rhizoctonia lilacina* CDBB-H-306. The stock culture was maintained on CzapekDox agar (Sigma).

Antibacterial activity

Antibacterial activity was measured by the disk-diffusion method (Van der Berghe and Vlietinck, 1991). Microorganisms were grown overnight at 37°C in 10 ml of Muller Hinton Broth (Bioxon). The cultures were adjusted to turbidity comparable to that of McFarland No. 0.5 standard (1.0×10^8 CFU/ml) with sterile saline solution (Christoph et al., 2000). Petri dishes containing Muller Hinton agar were inoculated with these microbial suspensions. Disks (Whatman No. 5) of 5 mm diameter were impregnated with 4 µl (3.16 mg) of essential oil. Disks with chloramphenicol (25 µg) were used as positive controls. The plates were incubated overnight at 37°C and the diameters of any resulting zones of growth inhibition (mm) were measured. Each experiment was made three times.

The estimation of the minimal inhibitory concentration (MIC) and the minimal bactericide concentration (MBC) were carried out by the broth dilution method (Van der Berghe and Vlietinck, 1991). Dilutions from 1.5 to 0.062 mg/ml of essential oil were used. Tubes were inoculated with 10^5 CFU/ml of microorganism suspension. MIC values were defined as the lowest extract concentration that prevents visible bacteria growth after 24 h of incubation at 37°C. Chloramphenicol was used as reference, and appropriated controls with no essential oil were used. Each experiment was repeated at last three times. The inactivation broth death kinetic method was performed using appropriate concentrations of essential oil (corresponding to ½ MIC, MIC and MBC). Death kinetics was expressed in $\log_{10}$ reduction time kill plots (Christoph et al., 2000).

Antifungal activity

For *Candida* strains and *C. neoformans,* the same protocol used for bacterial strains was followed in PDA agar. Nystatin (30 µg/disk) was used as a positive control against yeast. The assay of antifungal activity (filamentous fungi) was carried out in Petri dishes containing CzapekDox agar (20 ml). After the mycelia colony had developed, disks impregnated with 4 µl (3.16 mg) of essential oil, were placed at a distance of 0.5 cm away from the rim of the mycelia colony. Petri dishes were incubated at 23°C for 72 h until mycelia growth had developed. Disks containing crescents of inhibition were considered to contain antifungal activity (Wang and Ng, 2007). Ketoconazole (25 µg/disk) was used as a positive control.

For quantitative assays, a culture plate of 24 wells was used. Six dilutions of essential oil (1.50, 1.00, 0.50, 0.25, 0.125, and 0.0625 mg/ml) were added to CzapekDox agar (5 ml) at 45°C, these being mixed rapidly and poured into three wells of a culture plate. After the agar had cooled down to room temperature a small amount (1 × 1 mm) of mycelia was inoculated. After incubation at 23°C for 72 h, the area of the mycelia colony was measured and the inhibition of fungal growth was determined in percentage; concentration-response curves were constructed with the data to calculate the

Table 1. Chemical composition of essential oil of *Cordia globosa*.

No	Compounds	RI	RIr	Percentage
1	α-Pinene	917	917	38.4
2	Camphene	932	933	9.0
3	β-Pinene	956	964	1.3
4	α-Phellandrene	982	985	0.9
5	Limonene	1005	1014	4.6
6	γ-Terpinene	1059	1060	0.4
7	α-Terpinolene	1086	1093	0.4
8	Bornylacetate	1287	1287	0.5
9	α-Cubebene	1335	1339	0.6
10	α-Funebrene	1377	1399	0.6
11	β-Elemene	1437	1432	2.4
12	β-Bourbonene	1394	1391	0.3
13	γ-Elemene	1437	1432	8.0
14	Caryophyllene	1441	1444	4.3
15	γ-Cadinene	1456	1493	0.6
16	Calamenene	1465	1496	1.1
17	α-Caryophyllene	1470	1477	1.3
18	α-Gurjunene	1478	1408	0.9
19	β-Bisabolene	1496	1489	4.0
20	α-Selinene	1499	1494	3.1
21	Valencene	1503	1496	1.0
22	δ-Cadinene	1511	1519	2.3
23	*cis*-α-Bisabolene	1520	1511	3.1
24	3,7(11)-Selinadiene	1543	1542	1.9
25	α-Bisabolol	1690	1688	1.0
	Total	-	-	92.0

[a]Compounds listed in order of elution from a non-polar HP-5 MS capillary column. RI: Retention indices relative to *n*-alkanes on non-polar HP-5MS column. RIr: Kováts Index references (NIST, 2011).

medial fungicidal concentration (FC_{50}). Ketoconazole was used as reference and appropriate controls with no essential oil were used. Each experiment was repeated three times (Wang and Ng, 2007).

Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation of three experiments were determined. Statistical analysis of the differences between mean values obtained for experiment was compared using multifactorial analysis of variance (ANOVA) and the Tukey honest significant difference (HSD) test. The FC_{50} values were calculated by linear model.

RESULTS AND DISCUSSION

Constituents (25), comprising 92.0% of the oil from aerial parts of *C. globosa* were characterized by GC-MS (Table 1). α-Pinene was the major monoterpene constituent in the essential oil comprising 38.4% of the total. Hydrocarbon monoterpenes as well as camphene and limonene constituted 9.0%, and 4.6% of the oil, respectively. Essential oils are characterized by 56.1% of monoterpenes and 35.9% of sesquiterpenes. Generally, these compounds determine the biological properties of the oils (Bakkali et al., 2008). This is the first report of the composition of the essential oils of *C. globosa* collected in México.

Bioassays of antimicrobial activity showed that the essential oil of *C. globosa* was active and inhibited the growth of most of the microbial strains evaluated (Table 2). The more sensitive bacterial strains were *V. cholera, S. pneumoniae* and *P. aeruginosa* with a MIC of 0.060, 0.250, and 0.750 mg/ml, respectively. *S. pneumoniae* and *P. aeruginosa* infect the respiratory tract. *V. cholerae* is the responsible agent of severe gastrointestinal infections (cholera). The fungus most sensible to essential oil of *C. globosa* was *T. mentagrophytes* (IC_{50} 0.350 mg/ml). This microorganism causes athlete's foot. The results of bioassays with the essential oil of *C. globosa* are consistent with the popular use of this herb for the treatment of diarrheal diseases, respiratory tract and epidermal fungi infections. Clinical studies will be required to confirm the efficacy of essential oil of *C. globosa*.

Table 2. Antimicrobial activity of essential oil of *Cordia globosa.*

Organism	Positive controls					Essential oil		
	Inhibition zone (mm)			MIC (mg/ml)	FC_{50} (mg/ml) Ketoconazole	Inhibition zone (mm)	MIC (mg/ml)	FC_{50} (mg/ml)
	Chloramphenicol (25µg/disk)	Nystatin (30µg/disk)	Ketoconazol (25µg/disk)			3.16 mg/disk		
Sa	24.00±0.82	--	--	0.001	--	10.00±0.58	1.000	--
Spn cc	8.33±0.57	--	--	0.016	--	7.00±0.00	0.250	--
Bs	28.00±1.63	--	--	0.002	--	15.00±1.41	3.000	--
Ec	21.67±0.50	--	--	0.004	--	7.00±0.000	3.000	--
Vch cc	27.67±0.47	--	--	0.001	--	13.00±2.83	0.060	--
Pae	22.60±0.11	--	--	0.008	--	8.50±0.70	0.750	--
Ca 14065	--	11.83±2.02	--	0.011	--	NA	NA	--
Ca 10231	--	9.67±0.58	--	0.004	--	7.00±0.00	3.00	--
Cg	--	7.67±0.58	--	0.008	--	8.50±0.70	3.00	--
Ct	--	9.00±1.00	--	0.008	--	7.00±0.00	3.00	--
Cneo	--	8.67±0.58	--	0.004	--	7.00±0.00	1.00	--
An	--	--	++	--	0.0153	++	--	1.650
Fs	--	--	++	--	0.0040	++	--	0.760
Tm	--	--	++	--	0.0012	++	--	0.350
Rl	--	--	++	--	0.0215	++	--	0.820

Bacteria (MICs values); Fungi (IC_{50} values); Sa: *Staphylococcus aureus* ATCC12398; Spn cc: *Streptococcus pneumoniae* (clinical isolate); Bs: *Bacillus subtillis*; Vch cc: *Vibrio cholerae* (clinical isolate); Pae: *Pseudomonas aeruginosa* ATCC 27853; Ca 14065: *Candida albicans* ATCC 14065; Ca 10231: *Candida albicans* ATCC 10231; Cg: *Candida glabrata* (clinical isolate); Ct:*Candida tropicalis* (clinical isolate); Cneo: *Criptococcus neoformans* (clinical isolate); An: *Aspergillus niger*CDBB-H-179*;* Fs: *Fursariumsporotrichoides* NRLL3299; Tm: *Trichophytonmentagrophytes* CDBB-H-1112; Rl: *Rhizoctonia lilacina* CDBB-H-306; Na: no activity, -- No determinated, ++ radial growth inhibition ≥ 10 mm.

The essential oil molecules are lipophilic, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and rendering them more permeable (Dorman and Deans, 2000; Guynot et al., 2003; Bakkali et al., 2008). Essential oil exerts its antimicrobial effect at cytoplasmic membrane by altering the structure and function of microorganisms (Holley and Patel, 2005). Fei et al. (2011) demonstrated by electron microscopy, that essential oil of various plants disrupt the cellular membranes of *E. coli, S. aureus, B. subtilis* and *S. cerevisiae*.

Results of the effect of *C. globosa* essential oil at ½ MIC, MIC and CBM on cell viability (kill time) of *V. cholera* and *P. neumoniae* are shown in Figures 1 and 2, respectively. The oil caused a drop in viable cell number (CFU) in comparison to the control treatment at all assayed concentrations. In *V. cholerae*, a bactericidal effect was found after 10 min of exposure to the oil at MIC and CBM doses. After 10 min, the oil at ½ MIC decreased the cell count to <5 log CFU, the oil provided a bacteriostatic effect along the evaluated intervals (Figure 1). In *P. pneumoniae* the oil caused a significant decrease in the bacterial count in comparison to the control assay at 2 and 3 hours of exposure with MIC and CBM doses, in both cases the oil occurring bactericidal effect; at ½ MIC decreased the viable cells count to <3 log CFU (Figure 2). These findings show an interesting inhibitory effect of *C. globosa* essential oil toward the cell viability of *V. cholera* and *P. pneumoniae*, with a fast and steady bacterial kill rate. Time kill curve showed a clear relationship of the extent of inhibition and the oil concentration and time of exposure.

The antibacterial activity showed by the essential oil can be attributed to the presence of some components such as α-pinene, β-pinene, limonene, etc. (Rivas et al., 2012), which are

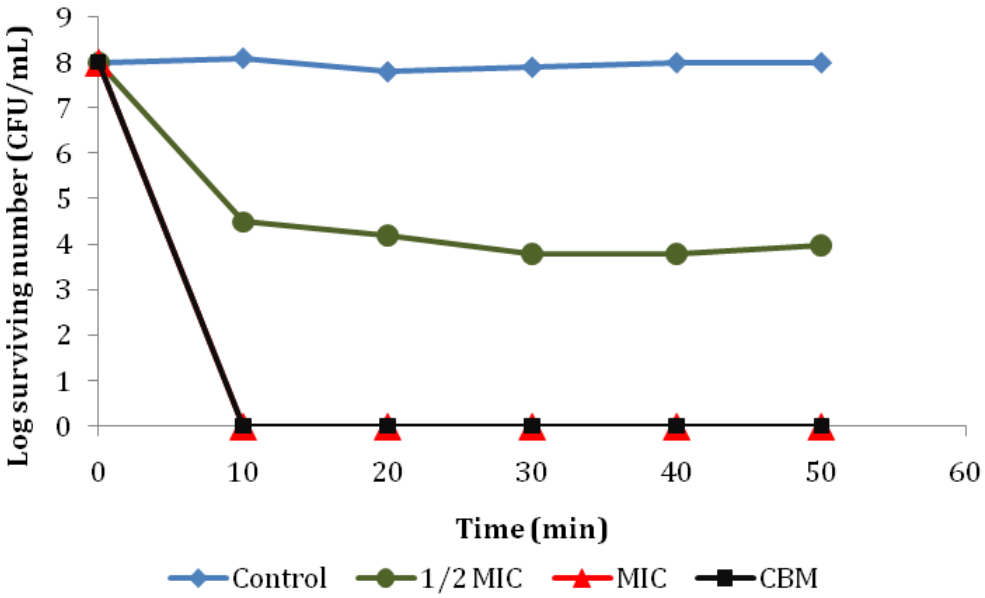

Figure 1. Survival curve of *Vibrio cholerae* exposed to essential oil of *C. globosa* collected in San Rafael Coxcatlán. The essential oil was added to each culture at time zero. The concentrations used were: 0.030 mg/ml (½MIC), 0.060 mg/ml (MIC) and 0.120 mg/ml (MBC). The control did not contain essential oil.

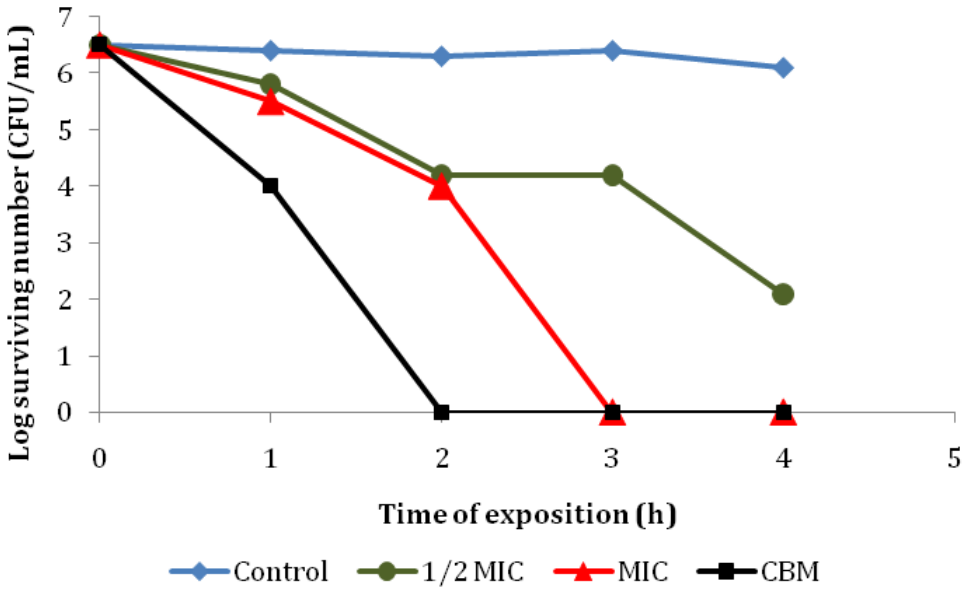

Figure 2. Survival curve of *Streptococcus pneumoniae* exposed to essential oil of *C. globosa* collected in San Rafael Coxcatlán. The essential oil was added to each culture at timezero. The concentrations used were: 0.125 mg/mL (½MIC), 0.250 mg/mL (MIC) and 0.500 mg/mL (MBC). The control did not contain essential oil.

already known to exhibit antibacterial activity and also several studies have demonstrated that whole essential oil usually have higher antibacterial activity than the principal components, suggesting that the mixes of the compounds are critical to synergistic activity (Solórzano-Santos and Miranda-Novales, 2012).

Conclusion

In summary, the major constituent of the essential oil of the aerial parts of *C. globosa* was α-pinene. The essential oil had antimicrobial activity on bacterial and fungi strains of medical importance. The oil presents antimicrobial

activity against Gram positive and Gram-negative bacteria and five fungal strains. The present study confirms the rational use in folk medicine of the aerial parts of *C. globosa* in gastrointestinal, respiratory, and dermatological diseases.

Conflict of interest

The authors have not declared any conflict of interest

ACKNOWLEDGEMENTS

This research has been supported by project useful San Rafael Coxcatlán Plants (MGU/Useful Plants Project México) Royal Botanical Gardens Kew, UNAM-PAPCA 2010-2011(3). The authors are grateful to Héctor Cervantes Maya and M en C. Luis Barbo Hernández Portilla for their technical assistance.

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Vol. 10(11), pp. 185-191, 22 March, 2016
DOI: 10.5897/AJPP2015.4456
Article Number: 21D4BF957501
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

DPPH radical scavenging and lipoxygenase inhibitory effects in extracts from *Erythrina senegalensis (Fabaceae) DC*

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Received 4 October, 2015; Accepted 9 November, 2015.

Erythrina senegalensis **DC (Fabaceae) is a plant used in traditional medicine in Burkina Faso (West Africa) to cure several diseases such as malaria, rheumatism, abdominal pain, fibroma and infections which are always accompanied by oxidative stress. This study aimed to highlight the antioxidant activities in dichloromethane (DCM) and ethyl acetate (EtOAc) extracts of *E. senegalensis* stem bark and roots. We used the 1, 1 diphenyl-2-picryl hydrazyl (DPPH) radical scavenging test and the 12-lipoxygenase I-B inhibitory method. We previously characterized the phytochemical groups by thin layer chromatography and colored reactions in tubes. The extracts in DCM (IC_{50}-root: 5.18 ± 0.06 and IC50-bark: 5.76 ± 0.68) showed strong scavenging activity. However, as a 12-lipoxygenase inhibition, the DCM extracts were almost inactive. The EtOAc extracts from root (IC_{50}-LOX: 7.21 ± 2.31; IC_{50}-DPPH: 7.27 ± 0.13) and from stem bark (IC_{50}-LOX : 4.95 ± 1.12; IC_{50}-DPPH : 11.4 ± 1.3) presented both the radical scavenging and the 12-lipoxygenase inhibitory effects. Polyphenols (flavonoids, tannins), steroids and terpenoids characterized in all extracts may be involved in the observed 12-lipoxygenase inhibition and radical scavenging.**

Key words: *E. senegalensis*, antioxidant, 12-lipoxygenase I-B inhibitory.

INTRODUCTION

Erythrina senegalensis DC (Fabaceae) is a plant used in traditional medicine in Burkina Faso (West Africa) to cure several diseases such as malaria, rheumatism, abdominal pain, fibroma (Nacoulma et al., 1999), skin

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diseases and amenorrhea in Mali (Togola et al., 2008). Aqueous stem bark extracts are effective against venereal and pulmonary infectious diseases (Iwu, 1993). The antioxidant properties of extracts from *E. senegalensis* are rarely assessed while phytochemicals are good antioxidants because they are able to scavenge free radicals or inhibit oxidative enzymes such as cycloxygenase and lipoxygenase. And then, phytochemicals are also less mutagenic and teratogen than the synthetic antioxydants.

Lipoxygenases (LOXs EC1.13.11.12) are nonheme iron-containing dioxygenases that catalyze the formation of corresponding hydroperoxides from polyunsaturated fatty acids such as linoleic and arachidonic acids. They are mainly called 5-, 12-, and 15-LOX based on their ability to insert molecular oxygen at the 5-, 12-, or 15-carbon atom of arachidonic acid (Wisastra and Dekker, 2014). LOX enzymes expressed in immune, epithelial, and tumor cells are an important source of reactive oxygen species (ROS) that display a variety of functions, including inflammation, skin disorder, and tumorigenesis (Mashima et al., 2015). So, implicated in the pathogenesis of inflammatory and hyperproliferative diseases, the LOXs represent potential targets for pharmacological intervention.

Free radicals increase in the body during inflammation, exercise or after exposure to exogenous sources such as pollution, smoking, certain medications and radiations (Lobo et al., 2010). An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions (Lobo et al*.*, 2010). Free radicals may oxidize and modify the DNA's genes or the cellular regulatory proteins and lipids leading to many metabolic and cellular disturbances such as cancer, asthma, atherosclerosis, cataract and inflammatory diseases (Lobo et al*.,* 2010).

The antioxidant system of the body, essentially enzymatic (superoxide dismutase, catalase, glutathione peroxidase) is often swamped and free radicals become dangerous for the health without an appropriate treatment. For all these reasons, it is very important to find vegetal antioxidant compounds from *E. senegalensis* that are able to reduce oxidative damage. The aim of this study is to characterize main phytochemical groups in the extracts of *E. senegalensis* and to show their free radical scavenging and 12-lipoxygenase inhibitory effects.

MATERIALS AND METHODS

Plant materials

Fresh roots and stem bark of *Erythrina senegalensis* (Fabaceae) DC, after locating the plant (30P0641573 UTM132 7922), were collected in June 2009 from their natural habitat in Saponé, at 50 km from Ouagadougou, Burkina Faso (West Africa). The specimen was certified by Dr. Souleymane GANABA, Department of Forestry of the National Centre for Scientific Research and Technology of Ouagadougou. A voucher specimen was deposited at Burkina National Herbarium (HNBU) and attributed No. 8709.

Collected plant materials were dried at room temperature under shade to prevent the direct effect of sun. The resultant dried plant parts were individually reduced to powder with mortar and pestle, sieved and kept in a clean dried cupboard before use.

Extraction of plant materials

The extraction of phytochemicals is made by successive exhaustion with increasing polarity solvents. Three hundred grams of powder (300 g) were initially defatted with ether, dried and successively exhausted with dichloromethane and ethyl acetate. The exhaustion of the drugs by a solvent is continued until the percolating liquid becomes limpid. Each liquid obtained by filtration was freeze-dried in a rotavapor to yield a solid residue. Appropriate concentrations of the extract were made and used in experiments. Dried plant drugs powder was weighed (M). After a complete exhaustion, the dry extract obtained from the rotavapor was also weighed (m).

Extraction r (%) performance is given by the formula:

$r = [m / M] \times 100$

Phytochemical screening

Standard screening colorimetric tests of the extract were carried out for various constituents according to Ciulei et al*.* (1982) and completed by thin layer chromatography. The extracts were screened for the presence of alkaloids, flavonoids, saponins, coumarins, tannins, steroids and triterpens.

DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging activity

The test was carried out according to Kim et al. (2003) with a slight modification. The reaction mixture contained test sample (extract or quercetin) in dimethyl sulfoxid and DPPH in methanol (101 µM, Sigma, Germany).The reaction mixture was incubated at 37°C for 30 min. The absorbance was measured at 520 nm. The percentage of radical scavenging activity was determined by comparison with a DMSO-containing control. Inhibitory concentration 50 (IC_{50}) values represented the concentration of compounds to scavenge 50% of DPPH radicals. Quercetin was used as a positive control. All the chemicals used were of analytical grade (Sigma, Germany).

In vitro lipoxygenase inhibition assay

Lipoxygenase inhibiting activity was measured by the spectrometric method developed by Lyckander and Malterud (1992) and adapted according to our working conditions. Lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (Germany). All other chemicals were of analytical grade. 400 µL of sodium borate buffer (0.2M, pH 9.0) containing lipoxygenase (167 U/mL) and 100 µL of test compound solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 500 µL linoleic acid (substrate) solution. This reaction led to the formation of (9Z, 11E)-(13S)-13-hydroperoxyoctadeca- 9,11-dienoate, and the change of absorbance at 234 nm for 10 min. Test compounds and the control were dissolved in methanol. Measurements of increase in absorbance at 234 nm for 30–90 s give extract inhibitory activities. All the reactions were performed in quadriplicate.

Treatment of data

All experiments were performed in quadruplicate (n = 4). The data

Table 1. Extracts obtained by successive extraction with different increasing polarity solvents.

Plant material	% of extraction per solvent	
	Dichloromethane	Ethylacetate
Stem bark	4.24	1.16
Roots	1.56	0.30

were given as mean ± SEM. Values of 50% inhibitory concentration (IC_{50}) were determined from the dose curves / effects obtained using the PRISM version 5.0 software.

RESULTS

Yields of extractions

Successive exhaustion by permeation of plant to obtain extracts masses whose catches in tests are presented in Table 1. It showed that by percolation of the plant, the masses of extracts vary according to the solvents used and according to the parts of the plant. *E. senegalensis* stem bark was the richer source of lipophilic extracts, more than roots. Dichloromethane gave higher yields of extraction than ethyl acetate extracts.

Colored reactions in tubes

Steroids, triterpens and coumarins were detected in all the parts of the plant. Flavonoids and tannins were found more in stem bark than in roots (Table 2).

Thin layer chromatography

TLC (Figure 1) has indicated a predominance of catechic tannins over gallic tannins in all the parts of the plant. Flavonoids were the most abundant in the stem bark extracts. Root extracts contained more phenolic acids than flavonoids. Triterpens and steroids have been characterized in all the parts of plant.

Radical scavenging activity in extracts of *E. senegalensis*

Extracts have shown dose-dependent effects, as in Figure 2. DCM extracts of root and stem bark have globally shown the best dose-dependent radical scavenging effect (IC_{50}-root: 5.1 µg/mL and IC_{50}-bark: 5.7 µg/mL).

Lipoxygenase (LOX) inhibitory activity in extracts of *E. senegalensis*

Extracts of *E. senegalensis DC* showed a dose-dependent inhibition of 12-LOX (Figure 3). EtOAc extracts were the most active (IC_{50}: 4.95 ± 1.12). These presented inhibitory potentials far exceeding those of DCM extracts (Table 3).

Inhibitory effects of DPPH and 12 - LOX in extracts of *E. senegalensis* DC

We have observed (Table 4) that stem bark extract in EtOAc presented the best LOX inhibitory effect (IC_{50}-LOX: 4.95 ± 1.12) and a moderate radical-scavenging effect (IC_{50}-DPPH: 11.4 ± 1.3). However, root extract in EtOAc was as well radical-scavenging (IC_{50}-LOX: 7.21 ± 2.31) as inhibitory of the 12-LOX of soybean *(*IC_{50}-DPPH: 7.27 ± 0.13).

DISCUSSION

Extractions and phytochemicals

Dichloromethane has given higher yields of extraction than ethyl acetate extracts. The exhaustion of the stem bark by dichloromethane gave 4.24% as extraction yields higher than those found by Togola et al. (2009) (3.9%) who used soxhlet.

Steroids, triterpens and coumarins were detected in all parts of the plant. Flavonoids and tannins were most found in stem bark than in roots. Our results are consistent with those of many authors.

Wanji et al*.* (1994) isolated many phenylisoflavones and many triterpen compounds in methanolic extracts. Nacoulma et al*.* (1996) indicated the presence of coumarins, flavonoids, saponins and steroids in the leaves, roots and bark of *E. senegalensis.* Saidu et al. (2000) characterized flavonoids, tannins and saponoside in macerated aqueous stem bark.

Our study did not highlight the presence of alkaloids in the studied parts of the plant. These results contrast with those of Nacoulma et al*.* (1996) and Saidu et al*.* (2000) who indicated the presence of alkaloids in the bark, roots, leaves and flowers. The differences may be explained by the plant material and the method used. Indeed, for the screening of phytochemicals, Saidu et al*. (2000)* used the tests according to Trease and Evans (1983) on macerate aqueous stem bark. Likewise, our method of extraction (successive exhaustion with 2 solvents) probably contributed to spread the small proportion of these alkaloids in different solvents, so that diluted; they were undetectable in our working condition as described by Ciulei (1982). In addition, our plant materials were not collected in the same geographic area. The variation in composition of phytochemicals may be a response of plants to factors such as climate conditions (Reynolds, 2002), the geological environment of crops sites (Gomes et al., 2007), the period of harvest, the enzyme responsible for equipment of the metabolic

Table 2. Phytochemicals characterized by colored method according to Cuilei et al.

Extracts	Phytochemicals	Stem bark	Roots
Dichloromethane	Steroids and triterpens	+	++
	Flavonids	++	++
	Coumarines	++	++
Ethylacetate	Flavonoids	++	+
	Tannins	++	

Note: (+): low quantities; (++): high quantities.

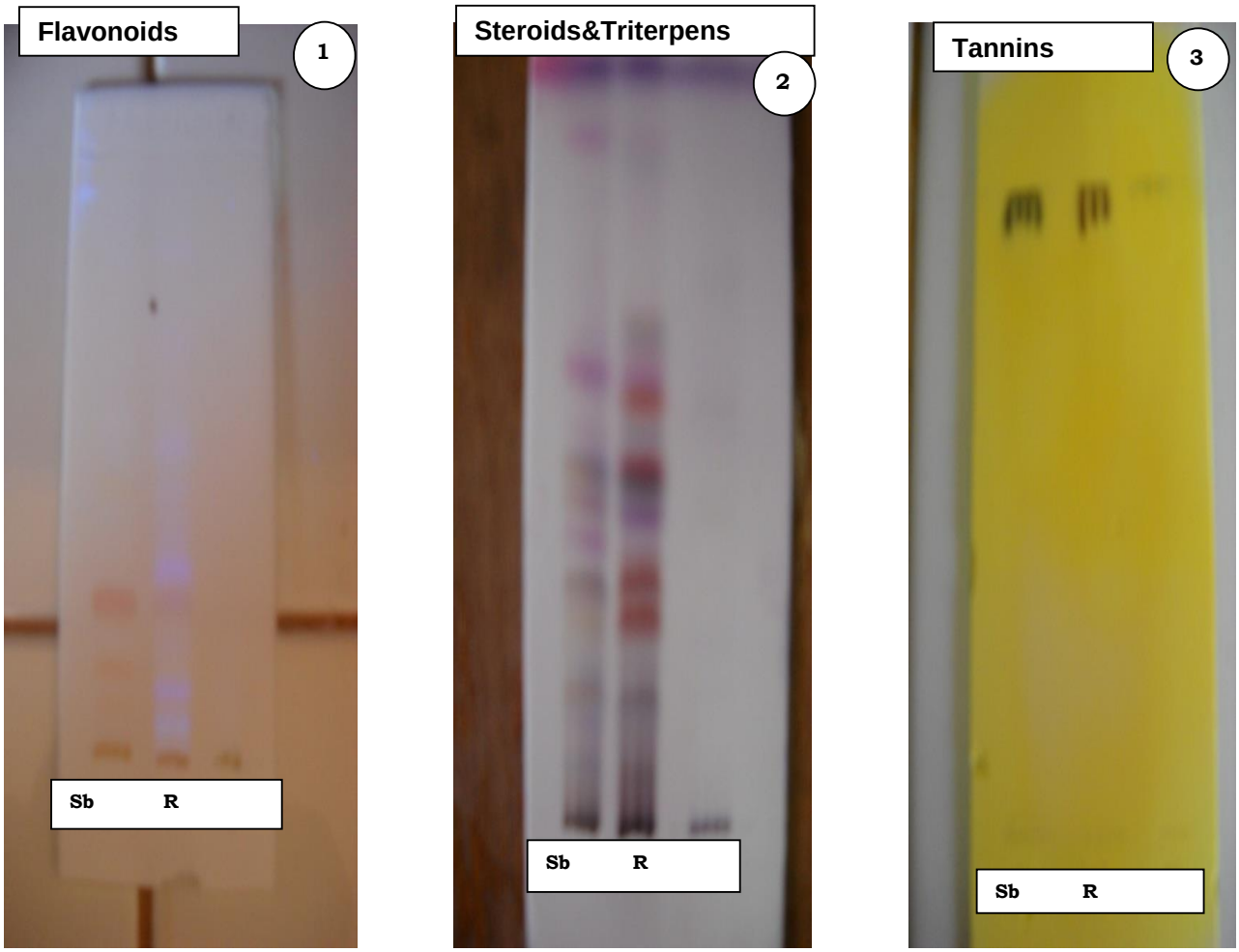

Figure 1. Tannins, flavonoids, triterpens and steroids characterized by Thin Layer Chromatography method. ***Sb****: stem bark;* ***R****: roots.*

pathways of biosynthesis (Pieters et al., 2005) and the regulation of the expression of genes (Boudet, 2007). TLC indicated a predominance of catechic tannins over gallic tannins in all the parts of the plant. Flavonoids were the most abundant in stem bark extracts. Roots extracts contained more phenolic acids than flavonoids. Triterpens and steroids were characterized in all the parts of the plant.

Radical scavenging activity in extracts of *E. senegalensis*

DCM extracts of root and stem bark have globally shown the best dose-dependent radical scavenging effect (IC_{50}-root: 5.1 µg/ml and IC_{50}-bark: 5.7 µg/ml). These results are consistent with Soro et al (2005) who found (by autobiography to DPPH) that radical-scavenging effect

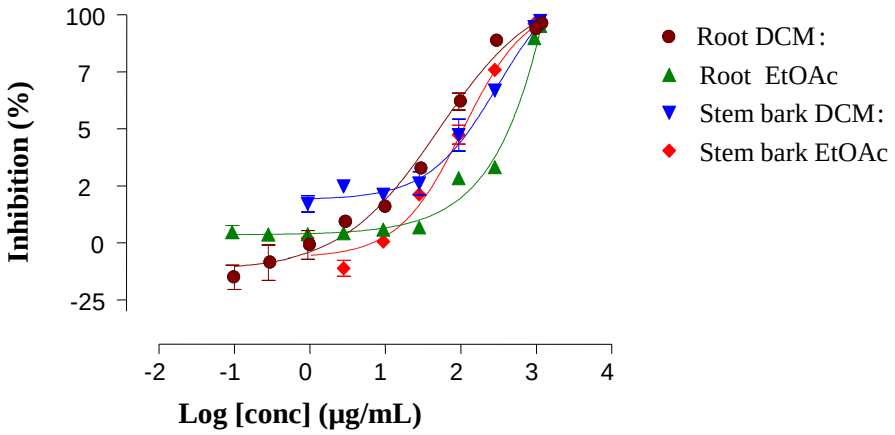

Figure 2. Concentration-dependent radical scavenging activities of dichloromethane (DCM) and ethyl acetate (EtOAc) extracts.

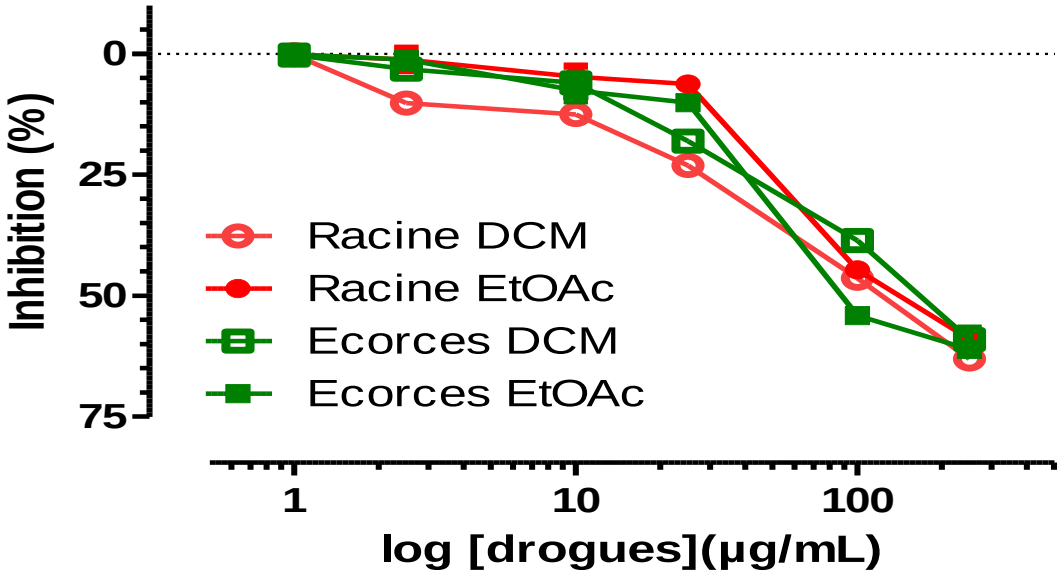

Figure 3. LOX inhibitory effects in extracts of stem bark and root.

was more marked with extracts in DCM. Extracts in DCM are lipophilic; these might contain many fat-soluble polyphenols (flavonoids, tannins) or their aglycones, triterpens and sterols responsible for the strongest radical scavenging activity. In many studies (Karou et al., 2005; Brewer, 2011); authors link the radical-scavenging effect of plant extracts to polyphenols. The radical-scavenging activity of polyphenols is due to their redox property which would play an important role in the adsorption, the capture, the neutralization of free radicals or in the decomposition of peroxide (Karouet al., 2005). The flavonoid heterocycle (aglycone) contributes to antioxidant activity by the presence of a free 3-OH, and permits conjugation between the aromatic rings. The closed C-ring itself may not be critical to the activity of flavonoids, given that chalcones are active antioxidants (Brewer, 2011).

The analysis of the IC_{50} values indicates that root extracts in EtOAc (7.2 ± 0.13µg/mL), despite containing less various polyphenolic compounds had a radical-scavenging potential. There is no relationship between the radical-scavenging effect and the quantitative composition in total polyphenols (Boudet, 2007). The ability to trap free radicals and radical-scavenging activity of polyphenols would depend on the arrangement of functional groups in relation to the structure of the nucleus. For more, according to (Brewer, 2011), the number and configuration of the hydroxyl group of donors

Table 3. LOX inhibitory effects in extracts of stem bark and root.

Extracts	Lipoxygenase inhibitory Effects (IC_{50}±SEM) µg/mL	
	Stem bark	Roots
DCM	21.46±3.8	22.03 ± 1.30
EtOAc	4.95 ±1.12	7.21 ± 2.31

Table 4. 12-LOX inhibition and DPPH radical scavenging effects in extracts of *E. senegalensis*

Extracts	Inhibitory effects (IC_{50} ± SEM) µg/mL			
	Stem bark		Roots	
	12-LOX	DPPH	12-LOX	DPPH
DCM	21.46 ± 3.8	5.76 ± 0.68	22.03 ± 1.30	5.18 ± 0.06
EtOAc	4.95 ± 1.12	11.4 ± 1.3	7.21 ± 2.31	7.27 ± 0.13

of hydrogen would constitute the major influences of the radical-scavenging potential of polyphenolic compounds.

Our best results (7.2 ± 0.13, 5.76 ± 0.68 and 5.18 ± 0.06 µg/mL) compared to the effect of quercetin (IC_{50}: 2.25 ± 0.001 µg/mL) taken as reference in our study remained low. Nevertheless, our crude extracts possess a non-negligible radical-scavenging potential, since quercetin is a purified compound.

Free radicals are highly reactive forms. Oxidative damage to lipids (lipid peroxidation) disrupts the functioning of the membranes, causes deposits of oxidized fat in vessels (formation of atheroma plates) and will generate carcinogens derivatives. The oxidation of proteins can damage cell proliferation or defense signals, inhibit enzymes and be responsible for deposits of amyloidosis and fibrosis (Lobo et al*.,* 2010). Radical attacks of the DNA will be sources of rupture and cell death, but especially of carcinogenic mutations (Lobo et al*.,* 2010).

From the foregoing, our potentially anti-radical extracts may be interesting in the prevention or reduction of the pathogenesis of these diseases.

Lipoxygenase (LOX) inhibitory activity in extracts of *E. senegalensis*

EtOAc extracts were the most active. These have presented inhibitory potentials far exceeding those of DCM extracts. Togola et al. (2009), for their part, have found the best LOX inhibitory effect in DCM extract from roots. Phytochemicals screening in EtOAc extracts has reported an abundance of flavonoids and tannins in stem bark and a scarce presence of flavonoid in roots. Thus, it would be possible that the presence in the bark of flavonoids and tannins, in a contributory way, is responsible for the strong inhibitory activity of lipoxygenase (synergy of action). These results are in line with other authors who have highlighted the inhibitory effects of polyphenols (tannins, flavonoids) on LOXs (Hu et al. 2006; Togola et al*.,* 2009).

LOXs are a family of enzymes with a non-heme iron responsible for stereo and region specific dioxygenation of polyunsaturated fatty acids that contain a 1, 4-pentadiene motif (Mashima et al*.,* 2015). Derivatives such as 12-hydroperoxy-éicosatétraénoic acid (12-HPETE), 12-Hydroxy-écosatétraénoique (12-HETE), leukotrienes and lipoxins are involved in cancer, cardiovascular disease, asthma, rheumatism (Wisastra et al*.*, 2014). Thus, inhibition of these enzymes can help reduce the occurrence of these diseases.

Inhibitory effects of DPPH and 12 - LOX in extracts of *E. senegalensis* DC

The extract of stem bark in EtOAc in which abundant flavonoids have been characterized, presented the best LOX inhibitory effect without radical-scavenging effect (IC_{50}-LOX: 4.95 ± 1.12*versus* IC_{50}-DPPH: 11.4 ± 1.3). Wangensteen et al*. (*2006) have shown that the DPPH scavenging and the LOX inhibition were not linked. The involvement of proton donation from these active compounds may be of less importance for LOX inhibition than for DPPH radical scavenging. In fact, this lack of correlation may suggest that LOX inhibitory compounds act by directly binding to the enzyme.

The root extract in EtOAc was as well radical-scavenger as 12-LOX inhibitor (IC_{50}-LOX: 7.21 ± 2.31 versus IC_{50}-DPPH: 7.27 ± 0.13). These results suggest that LOX inhibition would have required a donation of hydrogen atom. Indeed, in the reaction of peroxidation by LOX, polyunsaturated fatty acid must lose a hydrogen atom then rearrange before setting the radical oxygen. The gift of proton would be to compensate for this loss of hydrogen and then block the stage of dioxygenation. TLC had shown that this extract contained phenolic acids that could explain this activity.

Conclusion

Our study focused on *Erythrina senegalensis DC (Fabaceae)*, a medicinal plant of Burkina Faso. Roots and stem bark of the plant concentrate flavonoids, tannins, coumarins, and sterols and triterpens.

DCM extracts showed powerful anti-radical (DPPH) effects without 12-LOX inhibitory effect. However, EtOAc extracts possess both types of effects with a predominance of the inhibitory effect of 12-LOX of soybean.

We show in this study that extracts of *E. senegalensis DC (Fabaceae)* have an antioxidant potential and provide interesting data for the research of purified phytochemicals or for the development of phytomedicines for the prevention of cancer diseases, cardiovascular diseases or chronic inflammatory diseases.

Conflict of interest

The authors have not declared any conflict of interest

ACKNOWLEDGEMENTS

This study was supported by Conférence Episcopale Italienne (CEI), Union Economique Monétaire Ouest Africaine (UEMOA) for FS, CG, JBN, JS and Health's Ministry of Burkina Faso for FS and IPG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Vol. 10(11), pp. 192-199, 22 March, 2016
DOI: 10.5897/AJPP2015.4464
Article Number: 3E8649D57503
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Orthogonal test design for optimization of the isolation and purification of total favonoids from *Artemisia frigida* Willd using macroporous resin chromatography

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Received 17 October, 2015; Acceptrd 9 November, 2015

In the present study, the authors aimed to use an orthogonal test design to optimize conditions for isolating and purifying total flavonoids from *Artemisia frigida* Willd (*A. frigida*) using macroporous resins. An orthogonal L9(3)[4] test design was applied to select the optimum isolation and purification parameters, including the volume of sample solution, flow rate of adsorption and eluent concentration and volume. The efficiency of isolation and purification was evaluated by measuring the concentration of total flavonoids with an ultraviolet (UV) spectrophotometer. The qualitative analysis of the extracted flavonoids was carried out using infrared absorption spectroscopy (IR) and nuclear magnetic resonance (NMR). The crude extract from *A. frigida* was successfully isolated and purified by macroporous resin chromatography under the optimum conditions (the volume of sample solution, 40 mL; the flow rate of adsorption, 3.0 mL/min; the eluent concentration, 90% ethanol; the eluent volume, 40 mL). The positive reactions to Molish and HCl~Mg tests suggested that the extracted compounds were flavonoids. Furthermore, FTIR and NMR measurements also confirmed the presence of flavonoids in the extracts. The D101 macroporous resin is the most effective one for large-scale isolation and purification of flavonoids from *A. frigida*, and it meets industrial needs.

Key words: *Artemisia frigida* Willd, total flavonoids, orthogonal experiment design, ultraviolet (UV) spectrophotometer, macroporous resin.

INTRODUCTION

Artemisia frigida, Agi in Mongolian, which belongs to the composite family, is a medical material commonly used in Mongolian folk medicine (Wang et al., 2012). It is distributed throughout Inner Mongolia, occupying 10.38% of its steppe (Wang et al., 2011a). The aerial parts of *A. frigida* are used as a folk medicine to treat joint swelling, renal heat, abnormal menstruation and sore carbuncle (Wang et al., 2013a). Sesquiterpenoids (Liu and Mabry, 1981a), coumarins (Greger et al, 1983) and flavonoids (Liu and Mabry, 1981b; Liu and Mabry, 1981c) have been

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isolated from *A. frigida*. Recently, a series of flavonoids have been identified from the aerial parts of *A. frigida* (Wang et al., 2009; 2011b; 2010; 2013b) and these flavonoids (Shin et al., 2005) possess antioxidant and free radical scavenging activity. Epidemiological studies have indicated that consumption of these compounds is associated with a reduced risk of cancer and cardiovascular disease (Wang et al., 2009).

It will be of great significance to research the feasibility of extracting flavonoids from *A. frigida* since this plant is widely distributed in Mongolia. However, flavonoids from *A. frigida* are often isolated and purified by some conventional extraction and separation techniques, which involve the use of organic solvents and column chromatography including a silica gel and polymide. The organic solvents are unfriendly to our environment and the conventional separation methods have disadvantages like tediousness, time consuming and complex steps (Ramirez et al., 2004).

Macroporous resins (MRs) are made of porous high molecular material, which has porosity and biggish specific surface area, and adsorbs organic substance selectively from solutions through physical action (Liu et al, 2006). In recent years, MRs have become an effective means to extract and separate natural active constituents (Gao et al., 2012). The purification method using MRs is a new promising technology in pharmaceutical industry. It has the advantages of no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity and low cost, and has been successfully applied in the extraction, separation and purification of flavonoids, saponins and alkaloids (Liu and Guo, 2003; Xiang et al., 2003). However, it has never been reported on how to use macroporous resin chromatography (MRC) to isolate total flavonoids from *A. frigida*. Thus, it is imperative to investigate the possibility of isolation and purification of flavonoids from *A. frigida*.

In the present study, we aimed to use an orthogonal test design to optimize conditions for isolating and purifying total flavonoids from *A. frigida* using macroporous resins.

MATERIALS AND METHODS

The aerial parts of *A. frigida* were used as the experimental material and collected in Tongliao, Inner Mongolia of China in July 2013. They were identified by Prof. Buhebateer (Inner Mongolia University for Nationalities). A voucher specimen (No. 20130623) was deposited in the School of Traditional Mongolian Medicine of Inner Mongolia University for Nationalities.

Instrumentation and reagents

UV spectra were recorded by a Shimadzu UV-2201 spectrometer (Shimadzu, Japan). IR spectra were recorded in KBr discs by a Thermo Nicolet 200 double beam spectrophotometer (Shimadzu, Japan). NMR spectra were measured by a Bruker AVAIVCE Ш—500 NMR spectrometer with tetramethylsilane (TMS) as the internal reference, and chemical shifts were expressed in δ (ppm). MR AB-8, D101, NKA-9, ADS-7, ADS-17, HPD-100, D201 and HPD826 were provided by the Tianjin Bohong Rerin Technology Co., Ltd. Other chemicals used here were of analytical grade or better and purchased from Tianjin agent Co., Ltd., China. Rutin was purchased from National Institute for Control of Pharmaceutical and Biological Products, Beijing, China (No: 100080-200707).

Preparation of *A. frigida* extracts

The air-dried aerial parts of *A. frigida* (500 g) were crushed and extracted twice with 70% ethanol in a 10 L of solvent volume for 6 h under reflux. The ethanol solution was vacuum evaporated at 60°C. About 350 g of the residual ethanol was obtained. The residue was stored in a refrigerator (0-4°C) for further use.

Determination of total flavonoids in *A. frigida* extracts

Rutin was accurately weighed and dissolved in 250 mL volumetric flasks with 70% ethanol as stock standard solution (0.8 mg/mL). A series of standard solutions with a final concentration of 16, 32, 48, 64 or 80 μg/mL were prepared with the stock solutions as described previously (Hua et al., 2007). The absorbance of standard solutions was measured with a UV spectrophotometer at 350 nm. The calibration curves were constructed by the absorbance of the analytes (y) against the concentrations of the calibration standards (x). Equation $y = 11.156\ x + 0.0206$ ($r = 0.9997$) expressed the relationship between absorbance and concentration. All samples were quantified by using the same method and all measurements were performed in triplicate.

Screening of macroporous resins

Static adsorption and desorption: 20 mL of *A. frigida* extract solution (1.20 mg/mL) was added into each pretreated MR (400 mg) (Hou et al., 2013), followed by mixing in a thermostatic oscillator for 20 h at the speed of 180 revolutions/min. The resins were then filtered and the obtained filtrate was collected. In the end, the resins were desorbed with 20 mL of 70% aqueous ethanol.

Dynamic adsorption

Six grams of three kinds of pretreated resins were put individually into a glass column (1 × 20 cm) whose capacity was 3 mL. Next, the *A. frigida* extract solution (3.72 mg/mL) was added for adsorption dynamically at 3 mL/min. 3 mL of the eluting solution was each collected as one fraction. The content of total flavonoids was determined with a UV spectrophotometer at 350 nm.

Dynamic desorption

The resins were put into glass columns according to the method of dynamic adsorption and then 60 mL of the *A. frigida* extract solution was added for adsorption dynamically at 3 mL/min. The elution and desorption were performed with 200 mL water and 70% aqueous ethanol at 3 mL/min, respectively. The eluting solution (3 mL) was each collected as one fraction and detected as described above.

Revolutions

Optimization of A. frigida extraction

An orthogonal experiment $L9(3)^4$ test design was used to optimize

Table 1. The orthogonal experiment design (four factors and three levels).

Level	Factor			
	A, Volume of sample solution (mL)	B, Flow rate of adsorption (mL/min)	C, Concentration of the eluant (%)	D, Volume of the eluent (mL)
1	20	1	50	20
2	40	3	70	40
3	60	5	90	60

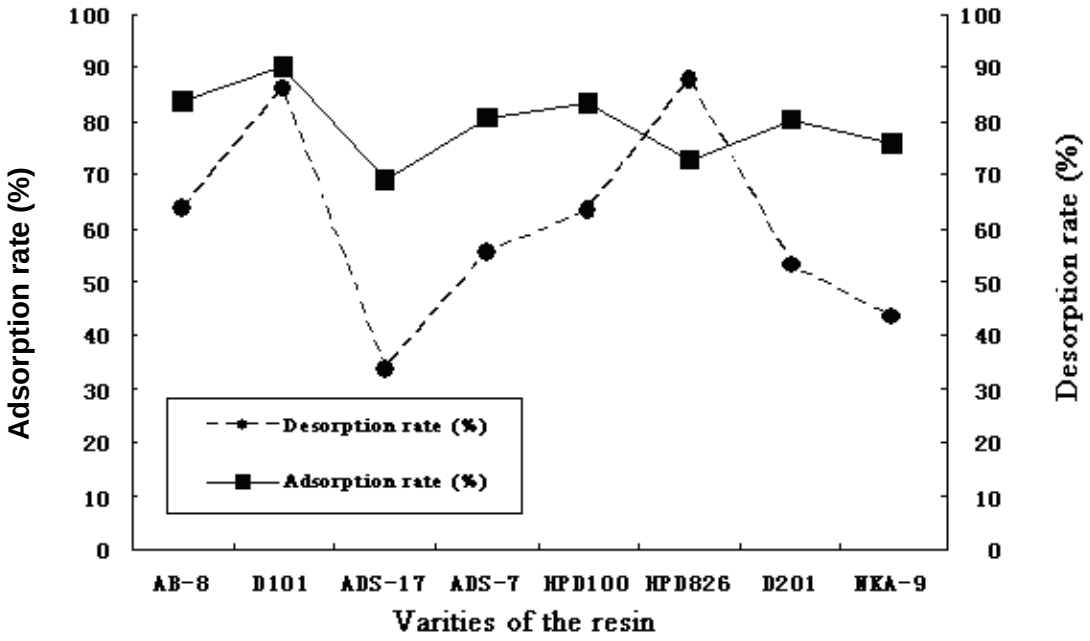

Figure 1. The adsorption and desorption rates of macroporous resins for total flavonoid extraction.

the conditions for isolating and purifying total flavonoids from *A. frigida* extracts. In this study, the isolation and purification was accomplished with MRs. The key parameters that influenced the flavonoid yield were analyzed, including the volume of sample solution (A), flow rate of adsorption (B), concentration of the eluant (C) and volume of the eluant (D). The factors and levels of L9(3)4 are listed in Table 1.

RESULTS AND DISCUSSION

Screening of macroporous resins

Adsorption followed by desorption of the filtrates was done to obtain total flavonoids from *A. frigida* extracts, and the flavonoid content was measured. As shown in Figure 1, the results indicated that eight MRs, including MR AB-8, D101, NKA-9, ADS-7, ADS-17, HPD-100, D201 and D101, showed an adsorption rate higher than 90% and a desorption rate higher than 80%. Among the tested resins, MR HPD826 had the best adsorption efficiency for total flavonoids and the D101 resin was the second (Figure 2). However, the desorbing process of MR D101 was better than that of HPD826 (Figure 3). The D101 resin was eventually selected for further study based on its regeneration property and price.

Optimized conditions for isolating and purifying total flavonoids from *A. frigida* extraction

Various parameters potentially affect the isolation and purification process using MRs, so it is critical to optimize experimental conditions when developing a MRC method. Hence, the related parameters in the present study were optimized to get efficient isolation and purification of total flavonoids from *A. frigida.* Generally, the volume of sample solution, flow rate of adsorption and eluent concentration and volume are all considered to be the most important factors. Optimization of the suitable isolation and purification conditions in a MRC method can be carried out step-by-step or by using an experimental design. In the present study, all selected

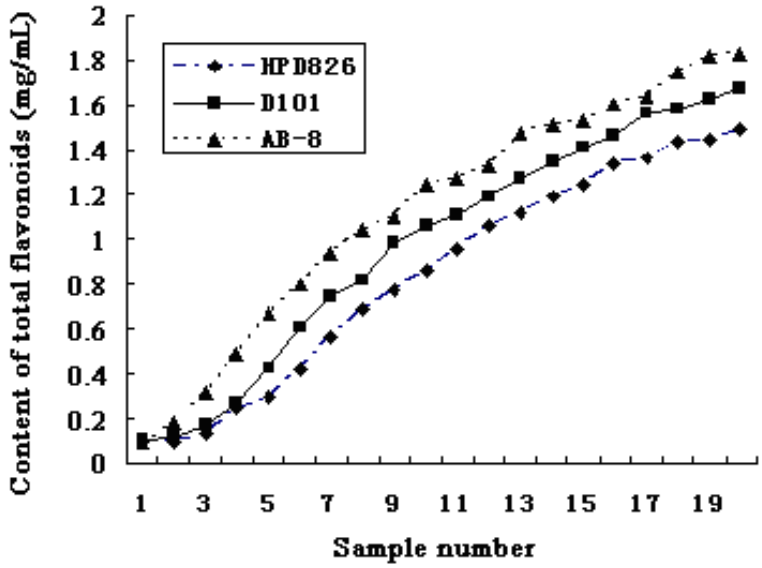

Figure 2. Results of dynamic absorption.

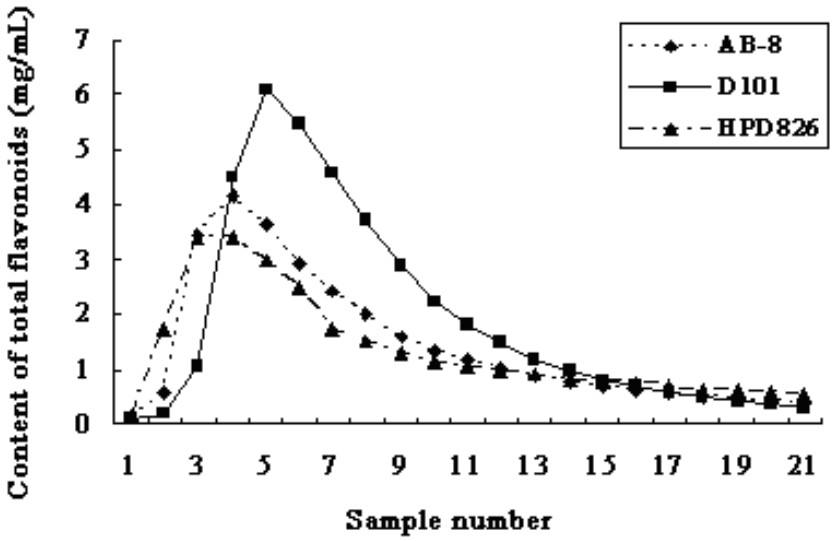

Figure 3. Results of dynamic desorption.

factors were examined using an orthogonal $L9(3)^4$ test design.

The results of the orthogonal test and the extreme difference analysis are presented in Table 2. However, the best extraction conditions cannot be determined only based on the outcomes in Table 2 and further orthogonal analysis is needed. Thus, the K, k and R values were calculated and listed in Table 3. As shown in Table 3, the yield and purity of total flavonoids were decreased in the order of B>C>D>A according to the R values. The flow rate of adsorption was found to be the most important factor determining the yield. In other words, the maximum yield of the flavonids was obtained, when the flow rate of adsorption was 3 mL/min, the eluent concentration was 90% aqueous ethanol, the eluent volume was 40 mL and the volume of sample solution was 40 mL, respectively.

Verification test

The isolation and purification was scaled up by 50 times using a preparative-scale MRC system. The yield of total

Table 2. Orthogonal array of the experiments extracting flavonids from *A. frigida.*

Experiment No.	Factor				Yield of total flavonoids (%)
	A	B	C	D	
1	1	1	1	1	40.41
2	1	2	2	2	90.63
3	1	3	3	3	84.54
4	2	1	2	3	71.59
5	2	2	3	1	87.95
6	2	3	1	2	70.91
7	3	1	3	2	69.70
8	3	2	1	3	67.73
9	3	3	2	1	60.76

Table 3. Analysis of $L9(3)^4$ test results.

*	Yield of total flavonoids (%)			
	A	B	C	D
K1	215.58	181.7	179.05	189.12
K2	230.47	246.31	222.98	231.24
K3	198.19	216.21	242.19	223.86
k1	71.86	60.57	59.68	63.04
k2	76.82	82.10	74.33	77.08
k3	66.06	72.07	80.73	74.62
R	10.76	21.53	21.05	14.04
Optimal level	A2	B2	C3	D2

* K1: Σ A1, Σ B1 and Σ C1; K2: Σ A2, Σ B2 and Σ C2; K3: Σ A3, Σ B3 and Σ C3. k1: K1/3; k2: K2/3 and k3: K3/3. R: Range.

Table 4. The purification results of total flavonoids from *Artemisia frigida* by different methods.

Method No.	70% ethanol extract (g)	Total flavonoids purified (g)	Purity of the total flavonoids (%)	Yield of the total flavonoids (%)
Macroporous resin chromatography (1)	5.0	1.48	65.54	85.46
Silica gel chromatography (2)	5.0	1.17	66.02	67.55
Polyamide chromatogtaphy (3)	5.0	0.98	71.24	56.58

flavonoids reached 85.34% (n = 5) and the purity of total flavonoids in the purified products was 65.32% (n = 5), indicating that this method was reliable and could be used for the industrialized production.

Check test

The 70% ethanol extract of *A. frigida* were isolated, purified and determined by the reported methods in the literature (Xu et al, 2011; Cai et al., 2009) and the presented optimized method, respectively. The results are listed in Table 4. The results show that the yield of the total flavonoids with the method 1 is more than that of the methods 2 and 3, and the purity of the total flavonoids is similar to that of methods 2 and 3.

UV/Vis absorption and FT-IR spectra of total flavonoids after the resin treatment

The positive reactions to Molish and HCl-Mg tests suggested that the extracted compound was flavonoid. UV and FTIR spectroscopy are effective tools for the semi-quantitative estimation of structural information on functional groups in complex solids (Cai et al., 2011;

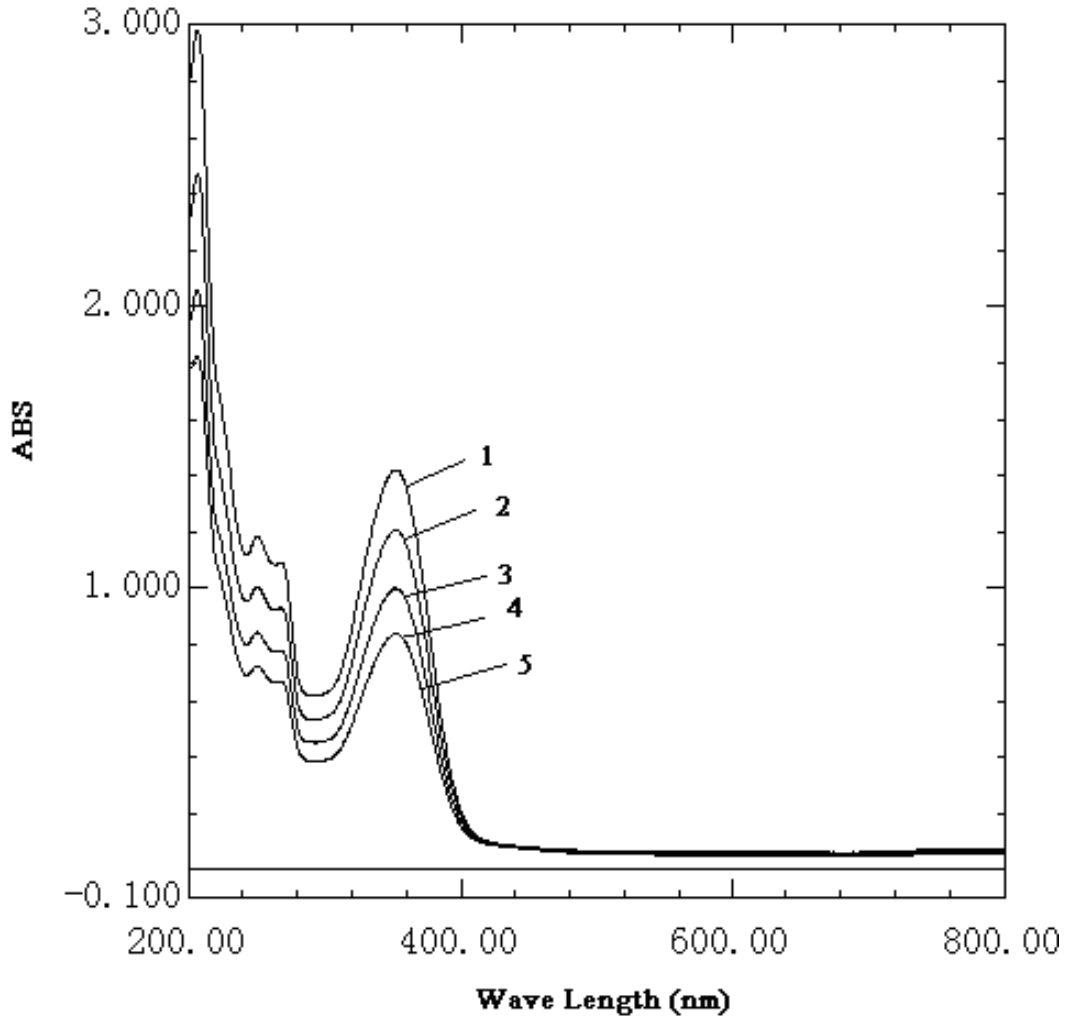

Figure 4. UV spectra of total flavonoids from *A. frigida* extraction (1. 50 µg/mL; 2. 40 µg/mL; 3. 30 µg/mL; 4. 20 µg/mL; 5. 10 µg/mL).

Huang et al., 2007). They were used to investigate the functional groups on the flavonoids after the treatment. The UV and FTIR spectrum of the flavonoids are shown in Figures 4 and 5. Two broad ultraviolet absorption peak appeared (Figure 4). One band indicated cinamyl (300~400 nm), and the other one was benzoyl (240~285 nm). As shown in Figure 5, the strong band appearing at 681.3 cm^{-1} was related to the expansion vibration of two isolated hydrogens in a benzene ring (Cai et al., 2011). The band at 822.4 cm^{-1} was related to the ortho-position hydrogen. Two bands at 1324.3 and 1263.1 cm^{-1} showed the anti-symmetry and the symmetrical expansion vibration related to the C-O-C ether. Two wide and strong absorption peaks appearing at 1725.6 and 1685.4 cm^{-1} were mainly produced by the C=O stretching vibration. The IR spectrum of total flavonoids also indicated the presence of aromatic groups (1601.5 and 1497.4 cm^{-1}). Based on the UV–VIS spectra of flavonoids at different concentrations in Figure 4, they all have the same UV absorption peaks of 350 nm, so that the content of total flavonoids was determined with UV spectrophotometer at 350 nm.

^{1}H-NMR and ^{13}C-NMR spectra of total flavonoids after the resin processed

The ^{1}H-NMR (500MHz, DMSO-d6) and ^{13}C-NMR (125MHz, DMSO-d6) spectra of the flavonoids are shown in Figure 6. In the ^{1}H-NMR spectrum, the characteristic resonances for HO-5 of the flavonoids exhibited at δH 13.6-12.0, and the downfield signals of aromatic protons at δH 8.0-6.0 belonged to the aromatic rings (A, B and C rings). Furthermore, the 1H-NMR spectrum showed signals for the methoxyl groups at δH 3.75-3.90 and for anomeric protons at δH 4.80-5.00. The ^{13}C-NMR signals also proved the presence of the flavonoids. Moreover, the characteristic resonances for C-4 of the flavonoids were

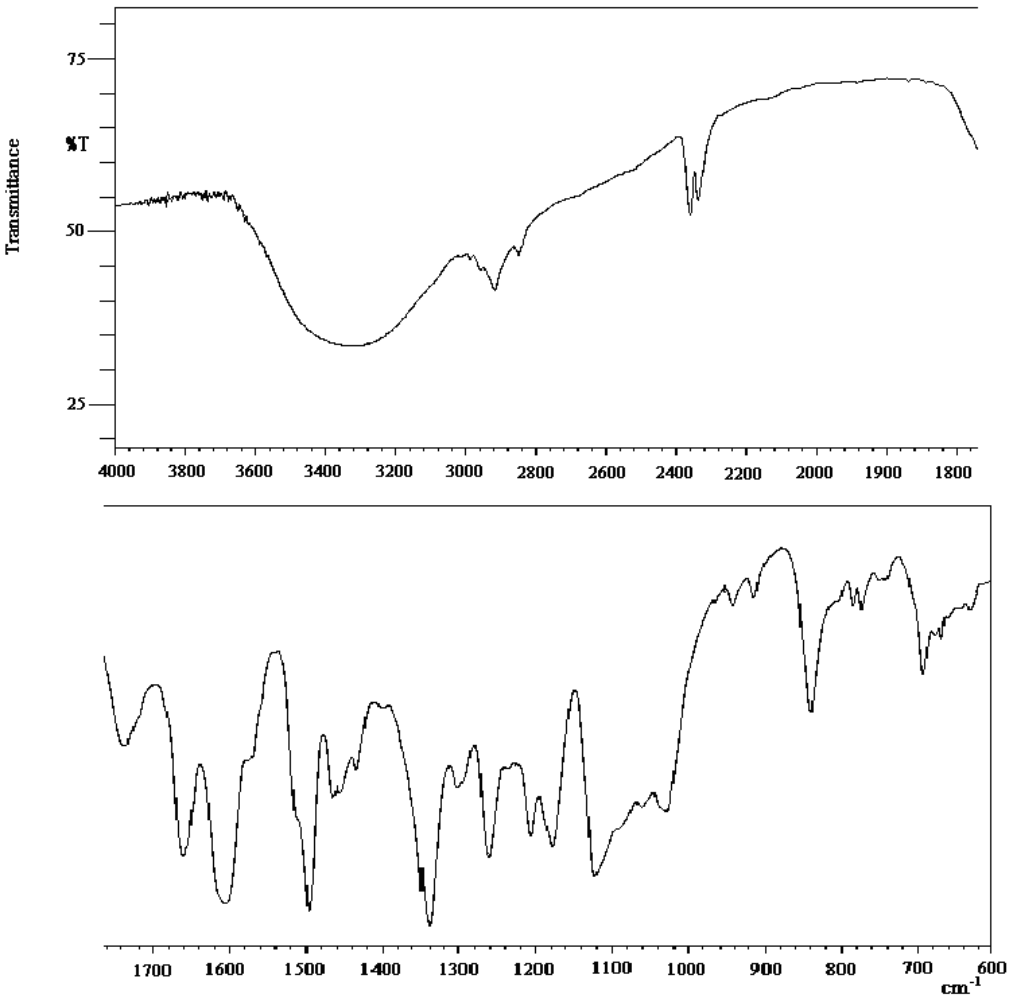

Figure 5. FTIR spectra of total flavonoids from *A. frigida* extraction.

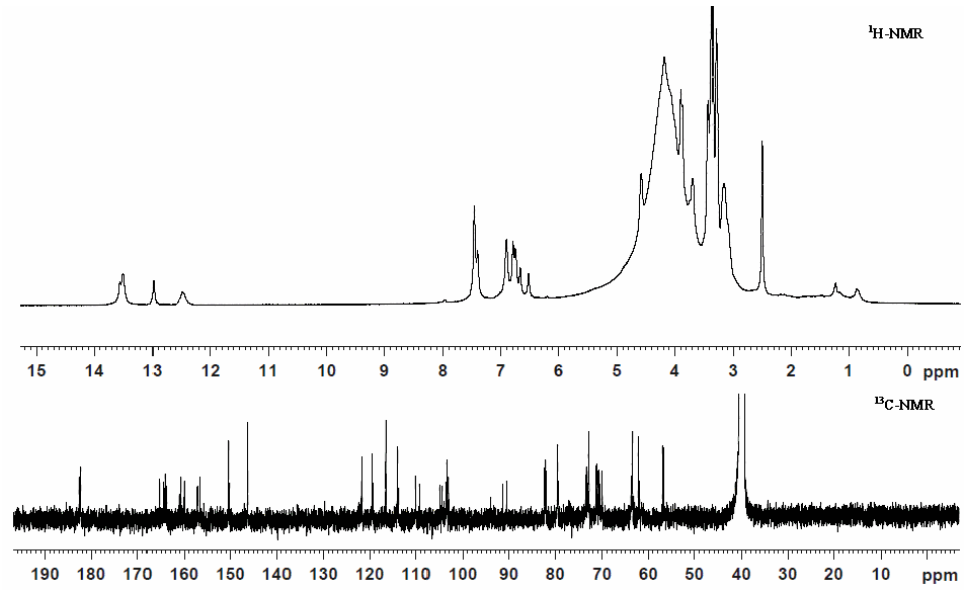

Figure 6. ^{1}H-NMR and ^{13}C-NMR spectra of total flavonoids from *A. frigida* extraction.

exhibited at δC 185.0-181.0, and the aromatic carbon signals of the aromatic rings (A, B and C rings) were displayed at δC 168.0-100.0. In addition, the remaining carbon signals belonged to the sugar moieties and methoxyl groups (Figure 6).

Conflict of interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Tongliao City and Inner Mongolia University for Nationalities Cooperation Program (SXZD2012010) and the National Natural Science Foundation of China (No. 81460654). The authors are grateful to Ning Xu and Narenchaoketu for the measurements of NMR spectra.

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Vol. 10(11), pp. 200-205, 22 March, 2016
DOI: 10.5897/AJPP2015.4386
Article Number: 496BA3257505
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antibacterial and antifungal activities of indican (indoxyl β-D-glucoside)

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Received 20 June, 2015; Accepted 28 January, 2016

Indican (Indoxyl β-d-glucoside), plant pigments found in true Indigo, are present in Indigofera genus plants, fungi and human urine. This study evaluated the antibacterial and antifungal activities of this pigment using the microdilution method. Antibacterial and antifungal activities were performed against *Staphylococcus aureus*, *Staphylococcus epidermidis, Pseudomonas aeruginosa*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum gypseun*. Minimum inhibitory concentrations (MICs) were measured according to the broth microdilution protocols by the Clinical and Laboratory Standards Institute (CLSI). The Indican showed only antifungal activity. It had MIC values of 128 µg/mL for yeasts and 512 µg/mL for most of the dermatophytes. The minimum fungicidal concentration (MFC) values of the Indican ranged between 512 µg/mL: *C. tropicalis* (LM-6) and *C. krusei* (LM-6 and LM-8) and 1,024 µg/mL: *C. albicans* (ATCC-76645, LM-108, LM-P20). Further studies are needed to clarify the potential fungicidal activity of the Indican and the possibility for topical applications.

Key words: Antibacterial activity, antifungal activity, indican.

INTRODUCTION

Brazilian medicinal plants have been used by the local population in the treatment of tropical diseases such leishmaniasis, malaria, schistosomiasis, fungal and bacterial infections (Duarte et al., 2005). In this context, the study of products from plant or synthetic substances with antimicrobial activity is gaining high prospects in the medical and pharmaceutical fields (Menezes and Lima, 2013). Plants have several chemical compound classes, including alkaloids. Indigo alkaloids belong to the bis-indole alkaloid class, being natural products used by mankind in dyes production and for medicinal purposes, in addition to being widely used in traditional Chinese medicine (Calvo, 2007). These pigments are present in Indigofera genus plants, fungi and human urine (Santos

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and Torres, 2012). Indican is the precursor of indigo pigments (indigo and indirubin), thus being starting material of these metabolites biosynthesis (Maugard et al., 2002).

Superficial fungal infections affect approximately 20 to 25% of the world population and they are classified into inflammatory and non-inflammatory infections. Disease control precautions involve hygiene and proper treatment, but drugs used to treat these diseases are toxic, and some microorganisms are already resistant to them. Drugs based on natural compounds, isolated or synthetic, appear as a possible solution to cope with these microorganisms resistance to conventional treatments (Oliveira and Silva, 2008; Filho et al., 2010; Dias et al., 2013).

The indigo biosynthesis pathway varies among plant species, but has indican (indoxyl β-D-glucoside) as a common precursor. This compound is mainly located in young leaves and can be extracted into aqueous solution. Indican undergoes hydrolysis through β-glucosidase enzyme action, leading to indoxyl (Sandoval et al., 2011).

Indigo is used in popular medicine, having anti-inflammatory, antipyretic, antiviral and antimicrobial activities, and recent studies have proved its anticancer property and efficacy in psoriasis treatment (Chiang et al., 2013). Studies showed that the indirubin is one potent cyclin-dependent kinases (CDKs) with activities such as anti-inflammatory, immunomodulatory, antifungal and antileukemic, in addition to inhibiting several tumoral cell lines (Suzuki et al., 2005). Considering the vast potentiality of plants as sources for antimicrobial drugs, the objective of this study was to assess the antibacterial and antifungal activities of the main precursor of indigo, the indican (indoxyl β-D-glucoside).

MATERIALS AND METHODS

Microorganisms

Strains used in the antimicrobial assay were obtained from the archival collection of the Federal University of Paraíba Laboratory of Mycology (LM). They include *Staphylococcus aureus* (ATCC-6538, LM-17, LM-197), *Staphylococcus* epidermidis (ATCC-1288), *Pseudomonas aeruginosa (*ATCC-9027, ATCC-25853), *P. aeruginosa* (LM-606), *Candida albicans* (ATCC-76645, LM-108, LM-P20), *Candida tropicalis* (ATCC-13803, LM-6), *Candida krusei* (LM-6, LM-08), *Trichophyton rubrum* (LM-640, LM-600), *Trichophyton mentagrophytes* (LM-02, LM-202) and *Microsporum gypseun (*ATCC-189).

Suspension was standardized by the 0.5 Mc Farland scale tube, set through spectrophotometric reading (Leitz-Photometer 340-800) to 90% T (530 nm), corresponding to approximately 10^6 colony-forming units (UFC) per mL. The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (Sahin et al., 2004).

Chemistry

Indican (Indoxyl-β-D glucoside), Cloranfenicol, Nistatin and Fluconazole were obtained from Sigma Aldrich, Brazil. The drugs were dissolved in dimethylsulfoxide (DMSO), and sterile distilled water was used to obtain solutions of 1,024 µg/mL for each. The concentration of DMSO did not exceed 0.5% in the assays.

Culture media

To test the biological activity of the products, the strains were maintained in appropriate culture media: Nutrient agar (NA) (Sigma-Aldrich, São Paulo, SP, Brazil) for bacteria, Roswell Park Memorial Institute (RPMI-1640) for yeasts, Sabouraud Dextrose Agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA) for filamentous. They were prepared and used according to the manufacturers' instructions.

Minimum inhibitory concentration (MIC)

MICs were measured according to the broth microdilution protocols by the Clinical and Laboratory Standards Institute (CLSI, 2008). The determination of MIC was performed in duplicate using 96 "U" bottomed well microplates. In each plate orifice, 100 µL of double concentrated nutrient broth (CN) was added for bacteria, with 100 µL solubilized Indican subsequent addition, which was also double concentrated. Through serial dilution, concentrations of 1.024 to 8 µg/mL were obtained, and plates were sealed and incubated at 35°C for 24 h. Bacterial growth control was done with chloramphenicol (100 µg/mL).

In antifungal activity was carried out by wells microdilution technique. Microplates were sealed and incubated at 35°C for 24-72 h for yeasts and at 28°C room temperature for 7-10 days for filamentous fungi. Negative controls (without drugs) were used to confirm strains viability, and sensitivity controls (for DMSO) were also included in the studies.

The minimum fungicidal concentration (MFC) was determined as the lowest concentration capable to inhibit total growth or promote less than three UFC, resulting in 99.9% fungicidal activity (Ernst et al., 1996; Espinel-Ingroff et al., 2002). The antimicrobial activity of the products was interpreted and considered active or not, according to the criteria proposed by Morales et al (2008).: strong/good activity (MIC: <100 µg/mL); moderate activity (MIC: 100-500 µg/mL); weak activity (MIC: 500-1000 µg/mL); and inactive product/no antimicrobial effect (MIC: >1000 µg/mL).

To determine the MFC, the authors subcultured 1 µL aliquots of MIC, MIC×2, and MIC×4 of Indican, Fluconazole, and Nistatin the control yeast growth onto Petri dishes containing SDA. After 24-48 h of incubation at 35°C, a reading was to to evaluate the MFC, based on the growth of the controls. The MFC was defined as the lowest product concentration that inhibited growth of the yeast or permitted less than three CFUs to occur, resulting thus in 99.9% fungicidal activity (Espinel-Ingroff et al., 2002). Biological activity assays were performed in duplicate, and the results were expressed as the arithmetic mean of the MIC and MFC.

RESULTS AND DISCUSSION

Bacterial resistance is currently one of the most important problems. In clinics, this problem is related to inadequate treatment and indiscriminate antibiotics use. This causes high mortality rates, especially when infections are caused by etiological agents, such as multidrug resistant *S. aureus*, *P. aeruginosa* and *Acinetobacter baumannii* (Gómez et al., 2008).

In vitro antimicrobial activity of indican synthetic

Table 1. Indican compound antibacterial activity.

Compounds	*S. aureus* ATCC-6538	*S. aureus* LM-17	*S. aureus* LM-197	*S. epidermidis* ATCC-12228	*P. aeruginosa* ATCC- 9027	*P. aeruginosa* LM-606
Indican	*	*	*	*	*	*
Chloramphenicol	*	*	**	**	*	**
Sensitivity control	*	*	*	*	*	*

*: Microorganism Growth; **: growth inhibition of the microorganism.

Table 2. Indican compound antifungal activity.

Compounds	*Candida albicans* ATCC-7664	*Candida albicans* LM-108	*Candida albicans* LM-P20	*Candida tropicalis* ATCC-13803	*Candida tropicalis* LM- 6	*Candida krusei* LM-6	*Candida krusei* LM-08
Indican	**	**	**	*	**	**	**
Nystatin	**	**	**	**	*	**	*
Fungal strain	*	*	*	*	*	*	*

*: Microorganism growth; **: growth inhibition of the microorganism.

Table 3. Indican MIC (µg/ml) on *Candida* genus.

Microbial strain	*MIC (µg/mL)	Nystatin	Fungal strain
C.albicans ATCC-76645	128	**	*
C.albicans LM – 108	256	**	*
C.albicans LM – P20	256	**	*
C. tropicalis LM- 6	512	NI	*
C.krusei LM-13	128	**	*
C. krusei LM-08	128	NI	*

*Microorganism Growth. **: Growth inhibition of the microorganism; NI: no inhibition.

compound was determined in this study. Indican showed no inhibition, although having growth on Gram-positive and negative strains (Table 1). In this test, it was possible to observe strains of bacterial growth in indican and Cloranfenicol standard antibiotic, demonstrating these microorganisms resistance to the compounds.

Bacteria use several mechanisms to ensure their growth in the presence of antibiotics, among them are: enzymatic inactivation, cell wall permeability changes, cell wall precursor changes, among others (Calvo et al., 2006). The difficulty in finding active drugs has led to a search for more potent drugs (Castro et al., 2006). Multidrug resistant microorganism infections complicates treatment, reduces the number of effective antibiotics, and creates the need for broad activity spectrum drugs use, configuring as a public health problem (Dutra, 2015).

I. suffruticosa leaf extracts (aqueous, methanolic, ethyl acetate and hexane) phytochemical screening showed alkaloid, steroid, triterpene, flavonoids, carbohydrates and coumarins presence. The aqueous extract showed antimicrobial activity against the strains *S. aureus*, *T. rubrum* (LM-09, LM-13) and *M. cani* (Leite et al., 2006).

Indican, indoxyl β-D-glucoside, is the main indigo and indirubin formation precursor, which is located in Indigofera genus plant young leaves, and is also obtained by synthesis. This study data related to synthetic Indican antifungal activity with Candida genus yeast-formed strains are shown in Table 2.

The compound, indican inhibited *C. albicans* (ATCC-7664; LM-108; P20), *C. tropicalis* (LM-6) and *C. krusei* (LM-6; LM-08), and did not inhibit *C. tropicalis* (ATCC-13803). In the study, it was observed that the *C. tropicalis* (ML-6) and *C. krusei* (LM-08) yeasts strains were resistant to Nistatina and sensitive to Indican.

Antifungal resistance is a challenge to clinical practice, with infectious agent isolation and MIC of potential drugs used for its treatment being suggested when possible (Colombo, 2003). The determination of MIC for Indican is shown in Table 3. Beginning with 128 µg/mL concentration, there was inhibition of growth and with the 256 µg/mL concentration, there was inhibition of most microorganisms.

The Candida genus, which is responsible for local and systemic infections, showed *C. tropicalis, C. parapsilosis,*

Table 4. Minimum fungicides concentrations (MFC, µg/mL) of indican on Candida genus

MFC of Indican (µg/mL)	Species of the genus *Candida*
512	*C. tropicalis* (LM-6) *C. krusei* (LM-6) *C. krusei* (LM-08)
1,024	*C. albicans* (ATCC-76645) *C. albicans* (LM-108) *C. albicans* (LM-P20)

Table 5. Minimum inhibitory concentration (MIC, µg/mL) of Indican on dermatophytes.

Microbial strain	MIC (µg/mL)*	Fluconazole	Fungal strain
T. rubrum (LM-640)	512	**	*
T. rubrum (LM-600)	1,024	**	*
T. mentagrophytes (LM-02)	512	**	*
T.mentagrophytes LM-202	512	**	*
M. gypseun ATCC-189	512	**	*

*Microorganism growth; **: growth inhibition of the microorganism.

C. glabrata, C. krusei, C. lusitaniae and *C. guillermondii* species as the most pathogenic agents. These microorganisms have also developed antifungal agent resistance. Taking into account mutations or increased genes expression, these pathogens have become resistant to azole agents (Menezes et al., 2009).

The minimum fungicides concentrations (MFC) are shown in Table 4. This study showed that from 512 µg/mL concentration, *C. tropicalis* LM-6 and *C. krusei* (LM-13;LM-08) strains were totally inhibited, and 1,024 µg/mL concentration inhibited *C. albicans* (ATCC-7664; LM–108; LM-P20) too.

C. tropicalis and *C. krusei* yeasts are opportunistic pathogens, having the ability to colonize different body parts (skin, gastrointestinal and genitourinary tract and respiratory system), and being resistant to several drugs (Pappas et al., 2009; Praneenararat, 2014). *C. albicans* exhibit polymorphism that makes species capable of changing its shape according to environment conditions, in addition to favoring different tissues colonization (Modrzewska and Kurnatowski, 2013).

There are few available drugs for candidiasis treatment. In addition to low supply, the drugs used have high toxicity and are not too efficient due to microorganisms acquired resistance (Wong et al., 2014).

Indican MIC determination on Trichophyton and Microsporum genus dermatophytes is shown in Table 5. At the 512 µg/mL concentration, only *T. rubrum* LM-600 was not inhibited. The 1,024 µg/mL concentration showed all strains growth inhibition (*T. rubrum* LM-640; 600; *T. mentagrophytes* LM-02; LM-202 and *M. gypseun* ATCC-189).

Antifungal agents frequent use combined with inadequate treatment are responsible for promoting these pathogens resistance to drugs used in medical routine. The difficulty in finding active drugs has led to a search for more potent drugs (Castro et al., 2006). Researches involving synthetic or natural (extracted from medicinal plants) compounds that have active ingredients against multidrug resistant strains are necessary (Gonçalves et al., 2009).

Results showed Indican antifungal potential on yeasts and dermatophytes. According to Sartoratto et al. (2004) and Houghton et al. (2007), Indican MIC can be considered to have good biological activity against *C. albicans, C. krusei, T. rubrum, T. mentagrophyte* and *M. gypseun*. *S. aureus*, *S. epidermidis* and *P. aeruginose* bacteria and *C. tropicalis* ATCC-13803 yeast showed resistance to the compound.

Chiang et al. (2013) proved indigo naturalis antimicrobial activity, which comes from *Strobilanthes formosanus* Moore, on *Staphylococcus* spp. strains and non-dermatophytes onychomycosis. *Isatis costata* indole alkaloids also showed antifungal activity over ten strains, showing best results on *Trichophyton simii* and *Trichophyton Schoen leinii* dermatophytes, having 80% growth inhibition, while *Candida albicans* yeast had approximately 69% growth inhibition (Fatima et al., 2007). *I. suffruticosa* leaves aqueous extract showed growth inhibition against dermatophytes and bacteria (Leite et al., 2006).

Studies on Indican synthetic are scarce in the literature.

However, studies on indican isolated from *I. suffruticosa* leaves showed cell lines growth inhibition in mice with Sarcoma 180 (Maranhão, 2008). Research by Lima et al. (2014) demonstrated that indican has anticancer and hepatoprotective activities.

Conclusions

Indican showed antibacterial activity in the tested strains. However, this compound showed good antifungal activity against different species, genus, candida and moderate activity against *Trichophyton* and *Microsporum* genera filamentous fungi, thus suggesting that this compound could be used in antifungal topic agent development. However, further investigation would be necessary on the use for the treatment of superficial mycoses.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) and Universidade Federal de Pernambuco (UFPE) for financial support.

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academicJournals

Vol. 10(11), pp. 206-211, 22 March, 2016
DOI: 10.5897/AJPP2015.4417
Article Number: 26AA7A857507
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Bioinorganic elemental content of the Ghanaian aphrodisiac medicinal plant, *Paullina pinnata* Linn. (Sapindaceae)

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Received 24 July, 2015; Accepted 12 January, 2016

Macro and micro element content of dried roots sample of *Paullinia pinnata* Linn. (Sapindaceae) in Ghana were analyzed using energy-dispersive x-ray fluorescence (ED-XRF) technique. The aim was to study the possible quantitative correlation between the measured elements and the traditional usage of the plant in the treatment of sexual dysfunction or enhancing/sustaining penile erection. The analyses yielded forty-five (45) elements, of which the concentrations of four (4) elements- calcium, magnesium, potassium and zinc were significantly high. These elements are considered to play a significant role in the physiology of sexual activity or promote penile erection. They may serve as bio-markers and also support the traditional use of the roots of *P. pinnata* as an aphrodisiac in some Ghanaian communities. Heavy metals such as arsenic, cadmium, lead and mercury were also measured and quantified. The quantities of these elements were below detection limits to warrant any toxicity concerns when the plant is used as aphrodisiac.

Key words: Aphrodisiac, *Paullinia pinnata* Linn. (Sapindaceae), ED-XRF, macro and micro element, sexual activity, penile erection, heavy metals.

INTRODUCTION

Ethno medicinal usage of plants in the Ghanaian culture dates back to antiquity. There are herbal medicines often made from combinations of more than one plant species for the treatment or management of diseases such as malaria, diarrhea, dysentery, menstrual pain, waist pain and erectile dysfunction.

Erectile disorders, characterized by the inability to develop or attain and/or maintain penile erection sufficient for sexual performance (AUA, 2005), are a major health concern among men. Male sexual function,

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however diminishes with age (Enzlin et al., 2004) and life style. Aging is associated with loss of libido or impotence, which may be a resultant of various complicated conditions such as atherosclerosis, high blood pressure, diabetes, depression or medications. The prevalence rate of erectile dysfunction in Ghana is between 10 – 52% among men (Amidu et al., 2010). Besides loss of sexual function, erectile dysfunction has both negative social and psychological effects on men in the Ghanaian community, because a man's self-image and identity is associated with his sexual virility. Ghanaian males desire to stay sexually virile throughout their life. Their two main core health concerns are therefore, to maintain optimal prostate and sexual health throughout life. These they pursue through the adaptation of quasi healthy lifestyle and eating habits and use of orthodox medicines, with their associated adverse effects. The desire to avoid the adverse effects as well as high cost of orthodox medicines has resulted in the patronage of complementary and alternative therapy, including herbal medicines such as those prepared from *Paullinia pinnata*, an important option for supporting male sexual function in some communities in Ghana.

Various research activities on medicinal plants to ascertain their pharmacological activities have focused largely on the activity of secondary metabolites or organic compounds (Zamble et al., 2006; Dipankar et al., 2013; Kalimuthu and Prabakaran, 2013). Few of these studies have examined the macro/micro elements and their physiological effects although a number of them have significant impact on human health. Some of these elements are vital in numerous metabolic functions in the human body (Prasad, 2008). Their biological significance is dependent on their concentration and their physiological interactions. For example, micro/macro elements such as zinc, selenium, magnesium, potassium and calcium, are known to be involved in the physiological processes that promote penile erection (Jeon, 2005; Ghofrani et al., 2006; Adrogue and Madias, 2007; Mike, 2013). Levels of magnesium and zinc in the prostate are known to be high in seminal fluid and assist in male fertility (Zieve and Chen, 2010). Zinc on the other hand is reported to stimulate the pituitary glands to release hormones that stimulate testosterone production (Myatt, 2012). Similarly, the M site of the phosphodiesterase-5 enzyme, implicated in erection sustenance, is reported to contain zinc and magnesium ions (Jeon, 2005).

According to reports, magnesium facilitates production of androgen, estrogen and neurotransmitters that regulate sex drive and hence acts as an efficient testosterone enhancer (Myatt, 2012). Potassium is also implicated in vasodilation due to hyperpolarization of vascular smooth muscle (Ghofrani et al., 2006). Reports indicate that 50% of selenium found in the testicles and seminal ducts of males is required for healthy sperm production (Myatt, 2012). Calcium ions also stimulate neuronal nitric oxide synthase through calmodulin path-way to affect erectile function.

Roots of the traditionally acclaimed Ghanaian aphrodisiac plant, *P. pinnata* (Linn) (Sapindaceae) – also known as Sweet gum or "Toantini" (among the Akans in Ghana), a woody or sub-woody climbing plant, commonly found in secondary forests in Ghana (GHP, 2007) have been used traditionally not only as an aphrodisiac among other uses (Annan et al., 2013, Chabra et al., 1991; Gill, 1992). In view of the important physiological roles of micro/macro elements in sexual function, we sorted to profile *P. pinnata* roots samples for these elements with a view of establishing whether they may be either physiologically or pharmacologically significant in managing erectile dysfunction.

EDXRF was used to analyze the roots samples for micro and macro element contents. Instrumental analytical methods used to qualify and quantify micro and macro elements in plants include neutron activation analysis (NAA), optical emission spectroscopy (OES), atomic absorption spectroscopy (AAS), mass spectroscopy (MS), inductively coupled plasma mass spectroscopy (ICPMS) and total reflection x-ray fluorescence (TXRF). Energy dispersive x-ray fluorescence is important to biologist, environmentalist, geologist, clinician, pharmacologist, biochemist and drug regulatory bodies for quality control. This is due to the non-destructive nature of analysis, rapid, sensitive, relatively cost effective sample analysis and its potential to enable simultaneous, qualitative, semi-quantitative and quantitative analysis without chemical pretreatment of samples of any size or number (Anzelmo and Lindsay, 1987; Jenkins et al., 2000; Ashok, 2014; Metz et al., 1994; Revenko, 2002; Sieber, 2000). This has opened doors for many research works on plant tissues by different researchers (Margui et al., 2005; Vazquez et al., 2003).

MATERIALS AND METHODS

Sample collection and preparation

Roots of *P. pinnata* were collected from Sekyere-Kwamang in the Ashanti region, Ghana. These were washed and room dried for two weeks. The roots were soaked in liquid nitrogen (-346 and -320.44°F, that is, 63 – 77K) for 10 min, powdered and sieved with a mesh size (aperture) of 180 µm into a fine powder and kept in a dry well-labelled container. Plant cells easily disintegrate if pretreated with liquid nitrogen and can be easily milled or blended into powder (Tim, 2014). Before pelletation, the sample was kept in an oven at 60°C overnight. Due to their morphology and the loose nature (Queralt et al., 2005), triplicate weighed samples – 4000 mg/sample - were added separately to 900 mg Fluxana H Elektronic BM-0002-1 (Licowax C micropowder PM-Hoechstwax) as binder, the mixture was homogenized using the RETSCH Mixer Mill (MM301) for 3 min and pressed manually with SPECAC hydraulic press for 2 min with a maximum pressure limit of 15 tons (15000 kg) into pellets of 32 mm in diameter and 3 mm thickness for subsequent XRF measurements. Time between pelletation and measurement was kept short to avoid deformation of the flat surfaces of the pellets (Anjos et al., 2002). Spectro X-Lab 2000 spectrometer (Geological Survey Department, Accra, Ghana) enhanced with three-axial

Table 1. Content of selected macro elements in *P. pinnata* (mg/4000 mg sample).

Element	Mean	Content (%)	CV (%)
Mg	15.99 ± 1.12	0.40	7.00
Al	7.66 ± 0.02	0.19	0.31
Si	14.10 ± 0.34	0.35	2.39
P	3.68 ± 0.12	0.09	3.35
Cl	1.55 ± 0.03	0.04	2.10
K	31.27 ± 0.82	0.78	2.63
Ca	101.83 ± 3.49	2.55	3.43
Ti	0.34 ± 0.04	0.009	12.46
Mn	0.25 ± 0.02	0.006	8.72
Fe	2.89 ± 0.14	0.07	4.89

CV: Coefficient of variance.

Table 2. Content of micro elements in *P. pinnata* (mg/4000mg sample).

Element	Mean	Content (%)	CV (%)
Cr	0.05 ± 0.01	0.001	24.81
Ni	0.01 ± 0.00	0.0003	2.94
Cu	0.03 ± 0.00	0.0008	9.23
Zn	0.03 ± 0.00	0.0007	12.37
As	<0.4	NQ	NQ
Rb	0.02 ± 0.00	0.0006	3.33
Sr	0.65 ± 0.01	0.02	2.12
Ba	0.11 ± 0.02	0.003	18.39
Cd	ND	ND	ND
Se	ND	ND	ND
Hg	ND	ND	ND

CV: Coefficient of variance; NQ – not quantified; ND – not detected.

geometry to reduce background noise due to radiation polarization and its monochromatic radiations emitted from the x-ray tube to excite the atoms of the samples was used for simultaneous analysis and measurement of the elemental content of the samples. This spectrometer is equipped with Rh anode and 400W Pd x-ray tube, a 0.5 mm Be end window tube, a Si (Li) detector (resolution of 148 eV – 1000 cps Mn Kα), available targets (Al_2O_3 and B_4C used as a BARKLA polarizer), an HOPG (High Oriented Pyrolitic Graphite) as a BRAGG polarizer, Al, Mo and Co as secondary target and a 0.5 mm Be side window. It has a carousel (circular rotating sample changer) inside a sample chamber with a capacity of 20 sample holder disc (32 mm) for sequential sample analyses. The radiation chamber was cooled using liquid nitrogen. Its computer-based multi-channel analyzer– SPECTRO X-Lab Pro Software package (Turboquant) controlled and computed spectral analysis, collected, evaluated and stored data. Combination of these different targets gave a typical detection limit for light elements (Si, Al, Mg and Na) in the range of 25-50 ppm. For heavy metals, 1-5 ppm were the limits of detection. The spectrometer was factory calibrated using a number of international rock standards.

RESULTS AND DISCUSSION

A total of forty-five (45) micro and macro elements were detected with the ED-XRF. From these, twenty-two (22) elements, eleven (11) macro and eleven (11) micro elements were identified and quantified. The macro elements as presented in Table 1 were sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), potassium (K), chlorine (Cl), calcium (Ca), titanium (Ti), manganese (Mn) and iron (Fe).

The microelements in Table 2 were chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), rubidium

Table 3. Levels of selected elements implicated in the aphrodisiac potential of *P. pinnata.*

Element	Mean (mg/4000 mg sample)	Content (%)	CV (%)
Mg	15.99 ± 1.12	0.40	7.00
K	31.27 ± 0.82	0.78	2.63
Ca	101.83 ± 3.49	2.55	3.43
Zn	0.03 ± 0.00	0.0007	12.37

CV: Coefficient of variance.

Table 4. Levels of some toxic heavy metals detected in *Paullina pinnata* (%/4000 mg sample in ppm).

Element	A_1	A_2	A_3
As	<0.4	<0.4	<0.4
Cd	<0.9	<0.8	<0.7
Hg	<0.5	<0.9	<0.9
Pb	<0.8	<0.8	<0.8

(Rb), strontium (Sr), barium (Ba), cadmium (Cd), selenium (Se) and mercury (Hg).

Elements which are implicated in the physiology of male penile erection evaluated in this study were Mg, Ca, K, and Zn listed in Table 3. Though selenium in Table 2 is reported to assist in the production of healthy sperms, the value recorded was below detectable limit.

The four gram (4g) samples were converted into milligrams (mg). Simple statistics (mean, standard deviation and coefficient of variation - CV) of the results were calculated to gain a better understanding of the results. The CV, that is, standard deviation versus mean ratio, expressed as a percentage, is reported to be a better way to express the goodness of a variable to be used for quality control and classification in environmental systems are shown in Tables 1, 2 and 3 (Queralt et al., 2005).

It could be observed from Table 3 that the levels of Ca, Zn, Mg and K in the four gram samples were prominent. Quantities of these elements may be sufficient as adjuvants in effecting penile erection, sustenance or triggering sexual desire in males. These observed levels may therefore support the traditional usage as an aphrodisiac.

The recommended average daily allowance for calcium is given as 1300 mg/day and the upper tolerable intake as 2500 mg/day (DRI, 2004). The high concentration of Ca (101.83 mg/4000 mg) in plant sample may account partly for its aphrodisiac effect. Ca^{2+} ion concentration plays a prominent role in Ca^{2+} – calmodulin pathway in the initiation and sustenance of erectile function (Tom, 2000). Literature reviews point to potassium as a vasodilator, inducing hyperpolarization of vascular smooth muscle thereby potentiating erectile function in males (Adrogue and Madias, 2007).

The recommended daily intake of potassium is 2300 mg/day (DRI, 2004). Thus, the relatively high concentration of K in *P. pinnata* (31.27 mg/plant material) may be associated with the aphrodisiac effect of the plant.

Magnesium is implicated in the production of androgen, estrogen and neurotransmitters effecting sexual drive (Edor et al., 2003). The 15.99 mg/ concentration of Mg detected in the plant material, though in a relatively smaller quantity, cannot be overruled to enhance levels of neurotransmitters and hormones through various mechanisms to help enhance sex drive and hence the aphrodisiac action of *P. pinnata*.

Though 0.03 mg/4000 mg concentration of zinc is recorded in plant sample, Zn is required for proper testosterone and sperm development (Yates, 2013). The prostate glands stores Zn in high concentrations and low concentrations are found in men with erectile difficulties. It also causes stimulation of the pituitary glands to release hormones responsible for testosterone production.

Selenium though below detectable limits, about 50% of selenium in males is said to be found in the testicles and seminal ducts. Reduction or loss of selenium levels contribute to low sperm count which may lead to erectile dysfunction (Mike, 2013).

Arsenic, cadmium, lead and mercury values recorded are shown in Table 4 were below detectable limits. The site of sample collection was in a forest, far from regular anthropogenic activities and this may account for the observed results (WHO, 2008).

Conclusion

Elements like Ca, Zn, Mg, Se and K are essential for penile erection and/or sustenance. The results indicated that the quantities implicated in penile erection or sustenance was within recommended levels except selenium.

Arsenic, cadmium, mercury and lead even below detectable limits, may pose as a threat to human health if consumption is not controlled.

XRF, aside being non-destructive, rapid, sensitive, relatively cost effective measurement, is suitable for simultaneous qualitative, semi-quantitative and quantitative analysis without prior chemical pretreatment of samples irrespective of the size and number.

The levels (concentrations) of micro and macro elements in the roots of *P. pinnata* in this study support the aphrodisiac use of the plant in the management of erectile disorders in Ghana. These micro and macro elements may serve as bio-markers and are recommended for analysis of plants used as aphrodisiacs.

Conflict of interest

This is an independently prepared paper. Authors have not declared any conflict of interest either commercially or otherwise in the publication of this article.

ACKNOWLEDGEMENTS

The authors are grateful to the technical team at the Ghana Geological Survey Department, Accra, and the geochemistry laboratory for the assistance offered during the XRF measurement.

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Vol. 10(11), pp. 212-215, 22 March, 2016
DOI: 10.5897/AJPP2015.3904
Article Number: B9308F957509
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

The prevalence of malaria and its therapeutic implication: A case study of Katcha Community

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Received 29 September, 2013; Accepted 29 October, 2015

Malaria is highly endemic in tropical and subtropical regions of the world, including Nigeria. In view of this, the prevalence of malaria in Katcha community of Nupeland, was studied. The data presented were collated from Munawwarat Hospital. A total of 6,193 registered cases were treated. The medical cases were registered between January, 2000 and December, 2004 and malaria cases were identified using clinical signs and microscopy. Blood samples were collected by finger prick and the method of cheesebrough for the laboratory identification of malaria parasites was employed. Out of 6,193 treated medical cases, 3,014 (48.7%) had suffered from malaria and 3,179 (51.3%) had suffered from other medical cases. However, 138, 374, 808, 1,239, and 455 people suffered from malaria in the years 2000, 2001, 2002, 2003, and 2004, respectively. Out of 1,775 treated malarial cases, 1451 (80.9%) were treated with chloroquine, while 228, 53, and 10 cases were treated with sulfadoxine-pyrimethamine, quinine, and proguanil/sulfadoxine-pyrimethamine, respectively. The malarial cases were not influenced by other medical cases (P>0.05), and there was an association among therapeutic regimens (P<0.05). Therefore, the decreased prevalence rate observed may be attributed to polypharmacy adopted in the treatment of malaria cases among Nupes.

Key words: Resistance, prevalence, malaria, chloroquine, Katcha.

INTRODUCTION

Malaria is an infectious disease caused by a parasitic protozoan of the genus *Plasmodium* transmitted by the female mosquito of the genus *Anopheles* when it feeds by sucking blood and whose life cycle alternates between man and mosquitoes (Smyth, 1996). Malaria in man is caused by four species of *Plasmodium*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium falciparum* (Bertram, 2004) of which the last named is not only the most common in Africa, but is the most virulent and enjoys the reputation as the greatest killer of mankind, being particularly dangerous for children (Ukoli, 2003) and responsible for nearly all serious complications and deaths related to malaria (Bertram, 2004). Malaria is responsible for deaths before

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the age of 5 years in 1/5 and 1/3 of children in urban and rural areas, respectively (Akubue, 2006). In Nigeria, *P. falciparum* predominantes (75%), *P. malariae* (15%), and *P. ovale* (3%) with *P. vivax* not found (James and Gilles, 1985). Malaria may also be transmitted by transfusion of infected blood, contaminated syringe and through placenta of non-immune infected pregnant women to the fetus (Brabin, 1989; Bruce Chawtt, 1983). Repeated cycles of infection can lead to the infection of many erythrocytes and complications (Ukoli, 2003). Malnutrition, splenomegaly and anaemia are the expected complications of repeated attacks of malaria (Ukoli, 1984).

Nigeria accounts for a quarter of all malaria cases in the African region and almost all cases are caused by *P. falciparum* with 57,506,430 malaria cases and 225,426 deaths in 2006. In 2007, Nigeria was the 7th malaria country (WHO, 2008). In Nigeria, malaria affects more people than it did in the 1960 (Ukoli, 1992). Fifty percent (50%) of Nigerian population experience one episode of malaria every year and one in four suffer from malaria at one time or the other (Ukoli, 2003). Resistance of *Anopheles* mosquito to insecticides and *Plasmodium* parasite to antimalarials has been responsible for unchanging, or increasing malaria prevalence in Africa (WHO, 1999).

Funding for malaria control in Nigeria was increased from US $17 million in 2005 to US $60 million in 2007 provided by the government, Global Fund, and World Bank (WHO, 2008).

The case of Katcha Community was investigated, because of its proximity to River Niger and other network of streams, drainages, which favour the breeding of mosquitoes, the main reservoir of malaria parasites. Hence, the aim of the study was to determine the prevalence rate of malaria and its relation to antimalarial chemotherapy in Katcha Community.

MATERIALS AND METHODS

The present day Nupeland is essentially the old Nupe kingdom presently spread among three states (Niger, Kwara, and Kogi) of the Nigerian federation. Nupeland, predominantly occupied by Nupe speaking people falls within the low basins of the Rivers Niger and Kaduna, between latitudes 9°30' and 8°30' North (Nadel, 1942). It covers about 11,200 km^2 with River Niger dividing it into equal parts (Idrees, 1998). The economic life of the Nupes is predominantly agrarian with riverine communities combining agriculture with fishing (Mann et al., 2003).

The sampling station was Munawwarat Hospital, the only functional hospital located in Katcha, the headquarter of Katcha Local Government Area of Niger State. The study was carried out between January 2000 and December 2004. The medical cases were registered, including malarial cases which were identified and diagnosed using clinical signs, which include paroxysm of fever, headache, fatigue, loss of appetite, muscle pains, chills, thirst, nausea, vomiting, delirium and convulsion in children, as well as microscopy for identification of Plasmodium species. The treatment regimens were instituted using various antimalarial drugs. Blood samples were collected using sterile lancet (finger prick). Sterile cotton wool moistened with methylated spirit was used in cleaning the thumb. The method of cheesebrough (Cheesebrough, 1991) was employed to identify *Plasmodium* species and the blood sample collected from finger prick was used to prepare thick blood smears. A thick blood smear was prepared by placing a drop of blood on the center of a clean grease-free slide. This was allowed to air dry and stained with Giemsa stain for 10 min. It was then rinsed with distilled water, air-dried, and a drop of immersion oil placed on it, and examined at ×100. Stained thick blood smear was used to concentrate and indicate the presence or absence of malarial parasite. Urine, stool, mucus, and blood microbiology was adopted for detection of other infectious agents. Chi-square method was used to analyze the results (Frank and Althoen, 1995).

RESULTS

Out of 6193 medical cases presented at the hospital and registered between 2000 and 2004, 3014 (48.7%) suffered from malaria, while 3179 (59.3%) suffered from other diseases. One hundred and thirty-eight (138), 374, 808, 1239, and 455 suffered from malaria in the years 2000, 2001, 2002, 2003, and 2004, respectively.

On the other hand, a total of 3,179 had suffered from other diseases during the period of study. Two hundred and fifty-six (256), 178, 512, 1830, and 403 suffered from other illnesses in the years 2000, 2001, 2002, 2003, and 2004, respectively. The total number of cases registered in 2000, 2001, 2002, 2003, and 2004 were 394, 552, 1320, 3069, and 858, respectively (Table 1).

Out of 3,014 patients with malaria, the therapeutic records of 1239 for the year 2003 were missing. But of the remaining 1,775 malarial cases, 1,451 (81.7%) cases were treated with chloroquine translating to 80.9% treated malarial cases using chloroquine. While 228, 53, and 10 cases were treated with sulfadoxine-pyrimethamine, quinine, and proguanil/sulfadoxine-pyrimethamine, respectively. But dihydroartemisinin, halofantrine, and proguanil/sulfadoxine-pyrimethamine were used to treat one patient each. However, 7, 4, 3, and 2 cases were treated with sulphadoxine-pyrimethamine, proguanil, pyrimethamine, and quinine/sulfadoxine-pyrimethamine, respectively (Table 2). But 48.7% of malaria prevalence rate was recorded during the period. But a total of 72.5% of the recorded malarial cases was treated using chloroquine with malarial prevalence rate of 53% in 2004. The malarial cases were not influenced by other medical cases ($P>0.05$) as there was an association among therapeutic regimens ($P>0.05$).

DISCUSSION

The results revealed that 3,014 out of 6,193 registered medical cases had suffered from malaria giving a malaria prevalence rate of 48.7% during the period under review. These results are supported by the findings of Ruth and Fedel (1997) that each year 300 to 500 million people living in the tropics and subtropics become infected with

Table 1. Prevalence rate of malaria among Katcha people as categorized from 2000 to 2004.

Medical case	Years under review				
	2000	2001	2002	2003	2004
Malaria (%)	138 (35.0)	374 (67.8)	808 (61.2)	1239 (40.4)	455 (53.0)
Other cases (%)	256 (65.0)	178 (32.2)	512 (38.8)	1830 (59.6)	403 (47.0)
Total No. of cases (%)	394 (100.0)	552 (100.0)	1320 (100.0)	3069 (100.0)	858 (100.0)

Table 2. Therapeutic regimens instituted during the period under review.

Drug	2000 (%)	2001 (%)	2002 (%)	2004 (%)
Cotexcin (%)	0 (0)	0 (0)	0 (0)	1 (0.2)
Metakelfin (%)	0 (0)	0 (0)	3 (0.4)	4 (0.9)
Fansidar (%)	0 (0)	22 (5.9)	102 (12.6)	104 (22.9)
Chloroquine (%)	137 (99.3)	330 (88.2)	654 (80.9)	330 (72.5)
Quinine (%)	0 (0)	15 (4.0)	38 (4.7)	14 (3.1)
Chloroquine + Fansidar (%)	0 (0)	1 (0.3)	8 (1)	1 (0.2)
Quinine + Fansidar (%)	0 (0)	2 (0.5)	0 (0)	0 (0)
Paludrine (%)	1 (0.7)	2 (0.5)	1 (0.1)	0 (0)
Halofantrine (%)	0 (0)	1 (0.3)	0 (0)	0 (0)
Daraprim (%)	0 (0)	1 (0.3)	1 (0.1)	1 (0.2)
Paludrine + Fansidar (%)	0 (0)	0 (0)	1 (0.1)	0 (0)
Total (%)	138 (100)	374 (100)	808 (100)	455 (100)

malaria. The malaria prevalence rate of 48.7% among Nupes agrees with the report of Saganuwan and Adelaiye (2007) indicating that 55% malaria cases is prevalent among males in the middle belt. However, our finding disagrees with the report of (Ukpai and Ajoku, 2001) that high prevalence rates of malaria in Owerri and Okigwe are 75 and 85%, respectively. Hence, the prevalence rate of malaria in the Southeast is higher than that of the middle belt. Ukoli (1992) reported that 50% of Nigerian population experience at least one episode of malaria each year, the financial implication could amount to ₦400 million (2 million in US dollars) every year. Therefore, in Nigeria today, malaria affects more people than it did in the 1960s and one in four people suffer from malaria fever at one time or the other (Ukoli, 1984) and the numbers affected are growing (Knell, 1991). Nigeria accounts for a quarter of all malaria in WHO African region and that transmission is more seasonal (WHO, 2008). Almost all cases are caused by *P. falciparum*, but most are unconfirmed. There is no evidence of a systemic decline in malaria burden, the upward trend in numbers of cases and deaths is probably due to improvements in reporting (WHO, 2008), although in 2003, the low prevalence rate (40.4%) of malaria infection was experienced (Table 1). This might have resulted from 80.9% treated malaria cases in 2002 (Table 2). The incessant use of chloroquine in 2000, 2001, 2002, and 2004 might have led to the development of resistance by *Plasmodium* parasites against chloroquine. Therefore, in 2004, the prevalence rate of malaria increased to 53%. This agrees with the report of Ukpai and Ajoku (2001) indicating that in Eastern Nigeria, 40 to 60% of malaria cases do not respond to chloroquine. WHO (1999) reported that for decades, chloroquine was the main drug used but increasing resistance forced its replacement in African countries as from 1990. High prevalence of mutations in candidate genes conferring chloroquine resistance in *P. vivax* has been identified (Golassa et al., 2015). The increased prevalent rate observed in 2004 confirms the report of the Health Exchange (2001) indicating that the Roll Back Malaria (RBM) global initiative to reduce malaria deaths by 50% by 2010 amongst the most vulnerable population has never been greater. A high prevalence rate of malaria among Katcha people clearly confirms the report of Ukoli that malaria remains Nigerian and the world's worst health problem (Ukoli, 2003). Having noticed high prevalence rate of malaria in 2002, the emphasis on malaria chemotherapy was shifted from chloroquine to sulphadoxine-pyrimethamine and some other antimalarial drugs in 2004. Sulphadoxine-pyrimethamine efficacy for acute malaria treatment has been compromised by resistance, but it retains partial activity among pregnant women with asymptomatic parasitaemia and might be useful (Gutman et al., 2015). High prevalence of mutations in sulphadoxine-pyrimethamine associated

drug resistance genes have been reported indicating that it should be discontinued (Lau et al., 2013). Therefore, there is need for reinforced surveillance of drug efficacy (Zatra et al., 2012). Use of sulphadoxine-pyrimethamine for malaria treatment is common during pregnancy. This may be contributing to adverse pregnancy outcomes. Antenatal care providers should endeavor to emphasize pregnancy (Odongo et al., 2015). Prolonged time to recrudescence may occur in pregnancy, regardless of anti-malarial treatment. Long intervals to recrudescence are more likely with the use of artemisinin containing treatments and also observed with intercalated *P. vivax* infections treated with chloroquine. Accurate determination of drug efficacy in pregnancy requires longer duration of follow-up, preferably until delivery or day 63 (Laochan et al., 2015). Difference in preventive anti-malarial chemotherapy regimes used during pregnancy had limited impact of malarial-immunity in a low transmission region (Teo et al., 2015). There was no change in the prevalence rate despite immunity amongst malarial patients living in endemic area, racial differences in susceptibility, and certain genetic factors of haemoglobin S which is common amongst Africans with sickle cell anaemia trait with lethal effect confer resistance to *P. falciparum* malaria, just as in those whose erythrocytes are deficient in the enzyme G6PD (Ukoli, 2003). Though, malarial parasites were not characterized, the highest prevalence (67.8%) noticed in 2001 may be due to the infection of *P. falciparum*, the predominant species in Nigeria and known to be chloroquine- and pyrimethamine-resistant (Ukoli, 1992; Ilobachei et al., 1995).

The use of polypharmacy for the treatment of malaria in Nupeland was strongly recommended. To treat drug-resistant *P. falciparum*, WHO recommends artemisinin-based combination therapy (ACT), which is currently being used in many neighboring African countries. This together with the use of mosquito net, bush clearing, and good drainages of the water networks in the area would not only assist in reduction of malaria endemicity, but would also aid in lowering the malaria prevalence rate. A well-educated public could lead to a vast proportion of the disease disappearing.

Conflict of interest

The authors have not declared any conflict of interest

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