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Abstract

Carpolobia lutea (G. Don) (Polygalaceae) is a tropical medicinal plant putative in traditional medicines against gonorrhoea, gingivitis, infertility, antiulcer and malaria. The present study evaluated the antimicrobial, antifungal and antihelicobacter effects of extracts *C. lutea* leaf, stem and root. The extracts were examined using the disc-diffusion and Microplates of 96 wells containing Muller-Hinton methods against some bacterial strains: *Escherichia coli* (ATCC 25922), *E. coli* (ATCC10418), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 6571), *Enterococcus faecalis* (ATCC 29212) and *Bacillus subtilis* (NCTC 8853) and four clinical isolates: one fungi (*Candida albican*) and three bacteria (*Salmonella*, *Sheigella* and *staphylococcus aureus*). The Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 19659) and the Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Cândida albicans* (ATCC 18804) and *Helicobacter pylori* (ATCC 43504). Some of these extracts were found to be active against some tested strains but activity against *H. pylori* was >1000mg/ml and good fungistatic activity against *C. albican*. The MIC against *C. albican* is in the order n-HF > CHF > ETF= EAF. The order of potency of fraction was the ethanol root > n-HF leaf > ethanol fraction stem > chloroform fraction leaf = ethyl acetate fraction leaf. Polyphenols were demonstrated in ethanol fraction, ethyl acetate fraction, crude ethyl acetate extract and ethanol extract, respectively. These polyphenols isolated may partly explain and support the use of *C. lutea* for the treatment of infectious diseases in traditional Ibibio medicine of Nigeria.

Keywords: *Carpolobia lutea*, Polygalaceae, antimicrobial, antifungal, antihelicobacter, Polyphenols

Introduction

The shrub *Carpolobia lutea* G. Don (Polygalaceae) is a small tree which grows up to 15ft high (Hutchinson and Dalziel, 1954). It is widely distributed in West and Central areas of tropical Africa (Mitaine - Offer et al., 2002). It occurs as a dense overgrowth or an evergreen shrub or small tree, up to 5m high. The leaf is 2-7.5cm long, 1-2.8cm broad; branches and mid-rib densely pubescence; lamina variable in shape, ovate, ovate-elliptic, oblong, or narrowly elliptic, obtuse or rounded at the base, more or less parallel and rather close. The flower is zygomorphic, often brightly colored. The keel petal is about 16mm long, 3-4mm broad and broader than that of other petals; outer 3 sepals are 2-5mm long, 3-5mm broad and smaller than the two inner sepals which is 6-7.5mm long and 3-6mm broad. Racemes contain 1-2 flowers. The fruit is fleshly yellow or red; seed very densely villous with copious fleshy endosperm (Hutchinson and Dazel, 1968). The common names which the plant is known include cattle stick (English), Abekpok Ibhuh (Eket), Ikpafum, Ndiyan, Nyayanganga (Ibibio), Agba or Angalagala (Igbo) and Egbo oshunshun (Yoruba).

The stem is used as chewing stick (Kayode and Omotoyinbo, 2008); the root is also used as chewing stick because of its aphrodisiac potentials. Its shrubby and smallish stems give it a ornamental use as sweeping material or broom (indian) in rural areas among the Ibibio tribes of Akwa Ibom State, Nigeria (Etukudo, 2003). The resilience of the woody stem enhances its patronage by cattle herders as cane to control their cattle heads.

The decoction of the root is used in locally-made alcohol as an aphrodisiac. It is used in the treatment of genitourinary infections, gingivitis and waist pains (Etebong and Nwafor, 2009). The root decoction is also useful in the treatment of internal heat. The hot water extract of the root was reported to have antimicrobial activity (Malcolm and Sofowora, 1969). The plant (*C. lutea*) has been reported to possess anti-inflammatory and anti-arthritis properties (Iwu and Anyanwu, 1982), gastro-protective effects (Nwido and Nwafor, 2009); antinociceptive effects (Nwido et al.,

2011); antimicrobial activities (Ajibesin, 2005; Philip et al., 2005); anti-diarrhoeal and anti-ulcerogenic properties (Nwafor and Basse, 2007); antimalarial activity and moderate toxicity (Bero et al., 2009). The antimicrobial effects of the root have been established (Etebong and Nwafor, 2009). The root is used to facilitate childbirth, treat sterility, headache, worm infestation and as aphrodisiac and stimulant properties (Mitaine-Offer et al., 2002). It has analgesic, androgenic properties and it is reputed to cure rheumatism, fever and to combat sterility. The leaf is reported to be used in the management of fever accompanying diarrhoea, headache, leprosy, snakebite, venereal disease, wounds (Ajibesin et al 2007; Lewis and Elvin-Lewis, 1977).

Three new triterpene saponins were isolated from the root (Mitaine-Offer et al., 2002). Polyphenols have been isolated from the leaf of *C. lutea* (Nwidu et al., 2011). It has been suggested that polyphenols are responsible for the antibacterial effects upon *Streptococcus mutans* (Kakiuchi et al., 1986). This could be related to polyphenols action as mediators in cell membrane and oxidative phosphorylation at low concentrations (Scalbert et al., 2005). However, only recently, polyphenols in fruits and leaves have received attention regarding their antibacterial effect upon microorganisms in biofilms (Huber et al., 2003; Duarte et al., 2006; Percival et al., 2006). Hence, the aim of this study is to screen the gradient fractions (*n*-hexane, chloroform, ethyl acetate and ethanol) and crude ethyl acetate extract of the leaf, crude ethanol extract of the stem and crude ethanol extract of the stem for antimicrobial activity. Besides, submit the most active fraction to preparative TLC to isolate and characterized active compounds. The goal is to verify ethnomedical antimicrobial claim of the effectiveness of the extract of leaf, stem and root of *C. lutea* used by Efik people in Nigeria in the treatment of toothache, menstrual pain, urinary tract infection and stomach ache. This investigation will also confirm earlier antimicrobial report on the root (Etebong and Nwafor, 2009).

Materials and Methods

Plant Material

C. lutea leaves, stem and root were collected and supplied by Mr. Etefia, the traditional herbalist, attached to the Pharmacognosy Department, University of Uyo. The plant was identified and authenticated by Dr. (Mrs.) Margaret Basse of Department of Botany, University of Uyo, Akwa Ibom State, Nigeria. A voucher specimen (UOH 998) was deposited at the University Herbarium. The leaf, stem and root were air-dried, powdered with pestle and mortar. The pulverized materials were stored at room temperature.

Test organisms

Six organisms used in this study as test organisms were seven typed cultures of bacteria: *Escherichia coli* (ATCC 25922), *E. coli* (ATCC10418), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 6571), *Enterococcus faecalis* (ATCC 29212) and *Bacillus subtilis* (NCTC 8853) and four clinical isolates: one fungi (*Candida albicans*) and three bacteria (*Salmonella*, *Shigella* and *staphylococcus aureus*). The Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 19659) and the Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 18804) and *Helicobacter pylori* (ATCC 43504) were obtained from the Microbiology Department, Faculty of Pharmaceutical science, Sao Paulo University. The remaining typed organisms and clinical isolates were obtained from the Pharmaceutical Microbiology Unit of the Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, University of Uyo, Uyo. The typed cultures of bacteria and clinical isolates of fungi were sub-cultured on nutrient agar (Oxoid) and saboraud dextrose agar (Oxoid) slants respectively and stored at 4°C until used.

Antimicrobial agents

The following chemotherapeutic agents were included in the test as positive controls: ciprofloxacin 1mg/ml, nystatin 25,000 I.U/ml and fluconazole 65 µg/mL.

Preparation of extract

The plant leaf, stem and root were air-dried at 45°C and pulverized using harmer mill into fine powder. The leaf was sequentially extracted by cold maceration for 72 hr using 2.5L of *n*-hexane (*n*-HF), chloroform (CHF), ethyl acetate (EAF) and ethanol (ETF) as solvents. These were then filtered and the filtrates were concentrated under reduced pressure by the use of rotary evaporator (BUCHI, USA) at 40°C. The crude extracts: ethyl acetate extract of leaf (EAEL) and ethanol extract (ETEL) of the leaf, the ethanol extract (ETES) of the stem and ethanol extract of the root (ETER) were obtained after 72 hrs of maceration in respective solvent; each solvent was filtered and concentrated under reduced pressure as above. The dry weight of the fractions and extracts were obtained by allowing the evaporation of the solvent to take place in fume cupboard. These percentage yielded dry fractions of *n*-HF (3.66%),

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CHF (3.03%), EAF (3.63%), ETF (15.07%), EAEL (7.82%), ETEL (27.08%), ETES (21.72%), and ETER (54.09%) respectively. These fractions and extracts were subsequently kept in sterile bottles at -4°C pending use. *n*-HF, CHF, EAF, ETF, EAEL, ETEL, ETES and ETER were used to determine the concentration in mg/ml.

Extraction and Fractionation of *C. lutea*

Procedure of gradient solvent extraction is as described in earlier report (Nwidi and Nwafor, 2009). The chemical constituents present in *C. lutea* were analyzed according to Wagner et al. (1984) and this has been reported in Nwidi et al., 2011.

Evaluation of antibacterial activity

The evaluation of antibacterial and the determination of MIC were done by the micro-dilution technique based on the M7-A6 of the national committee for Clinical and standards with modification (NCCLS, 2003). Bacterial cultures were cultivated in caldo Muller-Hinton (Difco) and transferred to phosphate solution of P^H 7.2 and turbidity adjusted up to scale 0.5 of McFarland (10⁸ cells/mL), and in the second dilution was adjusted 1:10 concentration for 10⁷ cells/ mL which was therefore used in the tests. Microplates of 96 wells were utilized and each well filled with sterilized Muller-Hinton. The inoculums and the diluted extract of the solution were made up to 100 µL. The solution of the extract was prepared in concentration of 2000µg/ml and was diluted serially up to 3.75 µg /mL. For the positive control, a solution of ciprofloxacin 35 µg/mL was used. For the negative control, the environment, the solvent and the inoculums were sterilized. The results were evaluated based on turbidity observed in the test wells using colorimetric evaluation resazurina as the indicator. The wells with blue colored showed activity whereas the wells with no activity were pink in color. The Gram-positive bacteria used were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 19659) and the Gram-negative bacteria used were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

Evaluation of antifungal activity

The evaluation of the antifungal activity and the determination of the MIC were done by the technique of microdilution according to the methodology described by M27-A2 da Nacional Committee for Clinical Laboratory Standards (NCCLS, 2002) with some modifications. *Candida albicans* (ATCC 18804) cultures in sabouraud dextrose agar (Difco) were incubated for 48 hrs and transferred to 0.85% saline solution and turbidity adjusted to 0.5 de McFarland (10⁸ céls/mL) and the concentration made up to 2.5 x 10⁷ cells/mL which was therefore used for the test. Microplates with 96 wells were used and each well filled with RPMI 1640 liquid, adjusted to a pH of 7.0 by MOPS before sterilization through membrane filtration. The volume of the extract was first solubilised and the volume made up to 100µL in each well. The solutions of the extract were prepared in concentration of 2000 µg/mL to 3.75 µg/mL by serial dilution. The positive control drug used is fluconazole at a concentration of 65 µg/mL. For the negative control, the environment, the inoculums and the extract were sterilized. The evaluation of the results were done by observing the turbidity occurring in each well and also colorimetric evaluation was done using Triphenyl tetrazolium chloride (TTC) as an indicator. The wells with no color change had activity while the wells with fungal growth show red color.

The Evaluation of Anti-*Helicobacter pylori*

The *Helicobacter pylori* (ATCC 43504) cultures were incubated at -80 °C in Mueller-Hinton agar containing 5% bovine serum and 20% glycerol. The stock was replicated in Mueller-Hinton agar containing 10% sheep blood and incubated 37 °C for 72 hrs in anaerobic atmosphere with 10% CO₂ and 98% humidity. The *H. pylori* cells were suspended in a sterile saline solution until a turbidity of 0.5 de McFarland was obtained. Muller-Hinton agar was utilized to test the susceptibility. A Whatman paper disc of 6mm were prepared, sterilized and impregnated with each 25µL of extract or solvent. The bacterial inoculum was placed in the wells using sterile swab. The wells were dried for 3-5 mins. After drying the entire discs were placed in the wells maintaining a distance of 15mm to avoid overlaps in the zones of inhibition. Discs containing 35µg of ciprofloxacin and DMSO or methanol 25% were impregnated and utilized as controls. 15 mins after inoculation of the disc, the wells of *H. pylori* were incubated at 37 °C for 2-5 days in micro aerobic atmosphere of 98% humidity after this period, the diameter of zone of inhibition was measured (NCCLS, 2003; Hirumi-Lima et al., 2008).

Results and Discussion

The results of the antimicrobial activities when tested at the pharmacological doses are as shown in Tables 1 and 2.

Table 1: Antimicrobial activity of *C. lutea* leaf extract and fractions

Organisms	Conc/ml Mg/ml	Ethyl acetate crude	Ethanol fraction	Ethyl acetate fraction	Chloroform fraction	n-Hexane fraction	Cipro 1mg/ml
<i>E.coli</i> 10418	192.5	14.0		15.0			
	385.0	16.2		18.0			
	770.0	18.0	15.0	22.0	-	-	30.2
<i>Salmonella</i>	192.5	14.0		16.0			
	385.0	16.2		18.0			
	770.0	21.0	14.5	22.0	14.0	11.0	31.0
<i>Staphylococcus aureus</i> 25923	192.5	15.0		20.0			
	385.0	18.0		28.0			
	770.0	22.0	25.0	32.0	14.0	-	35.0
<i>Pseudomonas</i>	192.5	-		-			
	385.0	-		16.0			
	770.0	-	13.0	20.0	-	-	24.0
<i>B.subtilis</i>	192.5	14.0		18.0			
	385.0	18.0		21.0			
	770.0	20.0	18.0	30.0	16.0	10.0	41.0
<i>E.coli</i> 25922	192.5	10.0		16.0			
	385.0	16.0		18.0			
	770.0	18.0	18.0	21.0	-	-	30.0
<i>Candida albican</i>	192.5	12.0		16			
	385.0	16.2		21			
	770.0	18.0	14.0	24	-	-	22
<i>Staphylococcus aureus</i> Clinical isolates	192.5	14.0		20.0			
	385.0	16.0		26.0			
	770.0	19.5	18.0	32.0	15.0	-	31
Sheigella	192.5	10.5		15			
	385.0	14.0		18.0			
	770.0	16.0	20.0	22.0	18.0	15.0	35.0
<i>Staphylococcus aureus</i> 6571	192.5	12.0		16.0			
	385.0	16.5		17.5			
	770.0	18.0	26.0	20.0	18.0	14.2	32.0

Medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it for primary health care. The evaluation of antibacterial potentials of various plants is imperative as infections are known to be caused by microorganisms' especially genitourinary infections. The MIC of the various fractions and extract tested the order of susceptibility of the tested organism is *B. subtilis* > *C. albican* > *E. faecalis* > *E. coli* > *S.aureus* = *P. aurogenosa* = *H.pylori*. For *B.subtilis* the order of activity of MIC of the plant parts is root > stem > leaf. The commonest cause of infertility in women in Nigeria is infection, particularly pelvic inflammatory disease (Abudu, 1985; Otubu, 1985). *B. subtilis*, is known to be responsible endocarditis, fatal pneumonia, bacteremia, septicemia and infection of a necrotic axillary tumor. *B. subtilis* have been isolated from surgical wound-drainage sites, from a subphrenic abscess from abreast prosthesis, and from two ventriculo-atrial shunt infections (Logan, 1988).

The plant extract and fractions were very effective against gram positive organism than gram negative organism and this has been corroborated for the root fractions by Ettebong and Nwafor, (2009). The MIC of the plants fractions and extract for gram negative organisms were greater than 1000µg/ml. The gastroprotective effectiveness of

Table 2: Minimum inhibitory concentration ($\mu\text{g/mL}$) of different extract and fractions of *C. lutea*

Extracts/ Fractions	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>H. pylori</i>
EAF Lf	-	1000	-	1000	-	-	-
ETF Lf	-	-	-	-	-	-	-
ETE Stem	-	250	-	1000	-	1000	-
ETE Lf	-	-	1000	-	-	-	-
EAE Lf	-	500	-	-	-	1000	-
ETE rt	-	31.25	-	-	-	-	-
<i>n</i> -HF Lf	-	-	-	-	-	125	-
CHF Lf	-	-	-	-	-	500	-

Positive control: ciprofloxacin (35 $\mu\text{g/mL}$)(-): CIM > 1000 $\mu\text{g/mL}$ Solvent utilized: *n*-HF = DMSO 20 %; CHF = DMSO 20 %; Demais extratos = Methanol 25%

the leaf extract (Nwido and Nwafor, 2009) may not be related to anti-helicobacter activity. However the plants parts demonstrate good fungistatic activity against *C. albican*. The MIC against *C. albican* is in the order *n*-HF > CHF > ETF = EAF. The order of potency of fraction was the ethanol root > *n*-HF leaf > ethanol fraction stem > chloroform fraction leaf = ethyl acetate fraction leaf. The chloroform fraction of the root has been reported the most potent against Gram negative organism (Etebong and Nwafor, 2009). The high doses of the plant leaf fractions and extract used in the pharmacological studies demonstrate proven antimicrobial activity. The ethyl acetate fraction and the crude ethyl acetate extract were more effective than the other fractions. The polyphenolics compounds have been implicated as potent antibacterial agents in some studies (Huber et al., 2003; Duarte et al., 2006; Percival et al., 2006). The quantification of total phenolics for the polar extract and fractions revealed that the percentage of total phenolics content increased from 61.33, 78.67, and 90.78 to 136.22 $\mu\text{g/ml}$ for ethanol fraction, ethyl acetate fraction, crude ethyl acetate extract and ethanol extract, respectively (Nwido, 2010). The high concentration of polyphenols in the leaf fraction isolated might lend credence to observed activity. The results of this study lend acceptance to the ethnomedical use of the root, stem, and leaf in the treatment of sterility, genitourinary infection and gingivitis among Effik people of Nigeria.

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