Phytochemical Analysis, Antioxidant and Antimicrobial Properties of the Leaves and Stem Bark of Scyphocephalium ochocoa Warb (Myristicaceae)

Feuya Tchouya GR1,2*, Foundikou H1, Lebibi J1, Choudhary MI1, Menkem EZ2, Nantia EA3, Kezetas JJB3 and Tchouankeu JC4

1Department of Chemistry, Faculty of Science, Scientific and Technical University of Masuku, Box. 223(Potos), Franceville, Gabon
2International Center for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan
3Department of Biochemistry, Faculty of Science, University of Yaounde 1, Box. 812, Yaounde, Cameroon
4Department of Organic Chemistry, Faculty of Science, University of Yaounde 1, Box. 812, Yaounde, Cameroon

Abstract

Aim: This study aimed to evaluate the pharmacological importance of Scyphocephalium ochocoa Warb. (Myristicaceae), through screening of phytochemical constituents and isolation of biomolecules, in vitro antioxidant and antimicrobial activities of the aqueous alcohol extracts of the stem bark and leaves of this species.

Method: The phytochemical constituents were identified in the stem bark and leaves extracts of S. ochocoa using standard procedures described in the literature. Subsequently, the stem bark extract was fractionated by means of silica gel column chromatography, and the isolated biomolecule characterized through extensive spectroscopic analyses. Antimicrobial activities were determined using both disc diffusion and broth micro dilution methods against different bacteria and fungi. The free radical scavenging activity and the total phenolic content were determined using the DPPH free radical and the Folin-Ciocalteu assays respectively.

Results: Phytochemical analysis revealed the presence of flavonoids and tannins in both parts of the plant. Coumarins and terpenoids were present only in leaves while anthocyanins, saponosides and steroids were found in stem bark. S. ochocoa stem bark and leaves extracts showed significant 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (IC50 = 0.169 ± 0.019 µg/ml) and polyphenol content (153.57 ± 0.63 mg/g of extract). S. ochocoa extracts significantly inhibited microbial growth of Echerichia coli and a gentamycin resistant Staphylococcus aureus strains. The chromatographic separation of the stem bark afforded the bioactive compound isopregomisin.

Conclusion: This study showed that S. ochocoa stem bark and leaves extracts contain various phytochemicals, with important amount of phenolic compounds, and possess antioxidant and antimicrobial activities. Isopregomisin can be considered as the antioxidant active principle of S. ochocoa.

Keywords: Scyphocephalium ochocoa; Phytochemistry; Antimicrobial activity; Antioxidant activity; Isopregomisin

Introduction

Antimicrobial resistance (AMR) within a wide range of infectious agents is a growing public health threat of broad concern to countries and multiple sectors. Increasingly, governments around the world are beginning to pay attention to a problem so serious that it threatens the achievements of modern medicine. A post-antibiotic era - in which common infections and minor injuries can kill - far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century [1]. With such impact in the use of available antimicrobials, it is essential to look for new sources of cheaper and efficient drugs with broad spectrum of action. One strategy of this research is to explore the plants used in traditional medicine. Indeed, the use of therapeutic plants (herbal medicine) is very old and currently WHO estimates that

*Corresponding author: Guy Raymond Feuya Tchouya, Department of Chemistry, Faculty of Science, Scientific and Technical University of Masuku, Box. 223(Potos), Franceville, Gabon, Tel: (241) 08043682 / 03107733; Fax: (241) 01677578; E-mail: gfeuya@yahoo.fr

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Materials and Method

Materials

**Plant material**: Stem bark and leaves of *Scyphocephalium ochocoa* (2 kg each) were collected in May 2012 near Franceville, in the Haut Ogooue Province, south-east of Gabon and identified by Mr Yves ISSEMBE, a botanist of the National Herbarium of Gabon. A specimen was kept in the Scientific and Technical University of Masuku, Franceville, Gabon (N° So 083/UM). The plant material were then dried for two and four days respectively for leaves and stem bark at room temperature and finely powdered. Powders obtained were extracted aqueous alcohol solution (ethanol/water: 1/1) for two days and the extracts were freeze-dried to yield 56 g and 75 g of stem bark and leaves aqueous alcohol extracts respectively.

**Microbial strains**: The bacterial strains including Gram-positive bacteria *Staphylococcus aureus* (SA) and Gram-negatives, *Escherichia coli* ATCC 25922 (EC) and *Klebsiella pneumoniae* (KP) were obtained from the Central Hospital of Yaoundé (Cameroun). The strains were grown and maintained on Mueller Hinton agar slants at 35°C until used. Four *Candida albicans* strains ATCC126 (CA1), ATCC126 (CA2), ATCC37039 (CA3), and ATCC37037 (CA4) were obtained from BEI Resources, NAID, NIH. Yeasts were grown and maintained on Sabouraud Dextrose agar slants at 37°C until used. Before any test, yeasts were cultured 48 hours in Sabouraud Dextrose agar.

Method

**Phytochemical screening**: The plant extracts were screened for their qualitative chemical composition, using standard methods described in the literature [4-6]. The identification of the following groups was considered: alkaloids, anthocyanins, coumarins, flavonoids, reducing sugars, saponosides, sterols-triterpenes and tannins.

**Alkaloids**: 0.5 g of each extract was agitated with 5 ml of hydrochloric acid in a steam bath, then 1 ml aliquots of filtrate were treated with a few drops of Mayer's reagent or Dragendorff’s reagent. The presence of a precipitate after treatment with either reagent was a preliminary indicator of the presence of alkaloids. To remove non-alkaloid compounds that could lead to false-positive reactions, part of the extract was alkalinized with 40% ammonia solution then treated twice with chloroform. The second chloroform extract was concentrated and then restetted with the Mayer and Dragendorff reagents.

**Anthocyanins**: 1 ml of each extract was dissolved in 1 ml of water contained in a test tube, then, a few drops of a NaOH solution was added in the mixture. The appearance of a blue green color, that eventually fades, indicates the presence of anthocyanins

**Coumarins**: Examined in ultraviolet light, the TLC of drugs with coumarins present spots whose colouring, in presence of ammonia atmosphere, varies from blue to yellow and purple.

**Flavonoids**: Flavonoids were detected by using the Shibata reaction or cyanide test. Briefly, 3 ml of extract was evaporated and the residue was dissolved in 2 ml of 50% methanol, then a few magnesium shavings and a few drops of concentrated hydrochloric acid were added. The development of a red-orange or purplish color indicates the presence of flavones aglycones.

**Reducing sugars**: One milliliter of extract was dissolved in 2 ml of distilled water and 1 ml of Fehling liquor and boiled for 30 min. The formation of a brick-red precipitate indicates the presence of reducing sugars. Saponosides: 1% of each sample decoction was returned gradually in 10 ml test tubes for a final volume of 10 ml. After two vigorous shakes, the tubes were left to stand for 15 min and the height of foam was measured. The tube in which the height of the foam was at least 1 cm, showed the presence of saponosides. However, the height of the foam indicated the value of the foam index.

**Sterols and triterpenes**: These families of compounds were identified by using the Lieberman-Burchard reaction. Briefly, 0.5 g of extract was dissolved in 0.5 ml of chloroform with 0.5 ml of acetic anhydride, and cooled on ice before carefully adding sulfuric acid. A change in color from purple to blue indicates the presence of sterols, while a green or purple-red color indicates the presence of triterpenes.

**Tannins**: Initially, the Styasny reagent was used to detect the presence of tannins. A drop of the extract was placed on a slab of silica gel and eluted in an atmosphere saturated with chloroform/acetonic acid/formic acid (5:4:1), thereafter, the plates were sprayed with 10 ml of a methanol solution at 5% nitrous acid and heated in an oven at 80°C for 10 min. The presence of tannins was revealed by the appearance of blue spots. For the classes of tannins, boiled aqueous extract (1 ml) was mixed with 1% ferric chloride. A black-blue color indicated the presence of gallic tannins and a dark green color, condensed tannins.

For all the samples tested, according to the precipitation or color intensity of each tube, following evaluations were given: (+++); (++); (+).

Determination of free radical scavenging activity: The antiradical activity of *S. ochocoa* extracts was determined according to the method described by Nantia et al. (2013) [7]. Briefly 980 µl of freshly prepared DPPH solution (40 µg/ml) was introduced in tubes and the extract or standard vitamin C (0.02, 0.2, 2, 20, and 200 µg/ml) were added. After 30 min, the change from the radical to the non-radical form leads to the disappearance of the purple coloration of DPPH, which was recorded by spectrophotometry at 517 nm using a UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.). The inhibitory potential of extracts was expressed through their inhibitory concentration fifty (IC$_{50}$).

Determination of total phenolic content: The amount of total phenolics in *S. ochocoa* extracts was determined with Folin-Ciocalteu reagent according to the method of Singleton and Rossi [8] with slight modification using gallic acid as a standard. Briefly, in 200 µl of extract (2 µg/ml) was added 500 µl of 1/10 diluted Folin reagent and 20% Na$_2$CO$_3$. The mixture was allowed to stand for 30 min with intermittent shaking, and the absorbance was measured at 730 nm using a UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.). The total phenolic content was determined as mg of gallic acid equivalent per gram of plant extracts using an equation obtained from the standard gallic acid calibration graph.

Antimicrobial activity

**Inoculum preparation**: Before any test, bacteria strains were subcultured on Mueller Hinton agar slants at 35°C for 18 h. Mature colonies were collected with inoculating loop and introduce in a tube with 5 ml of sterile saline (0.9% NaCl) and homogenize. The turbidity of the solution was adjusted at 0.5 McFarland standards [9]. A stock inoculum suspension of yeast was prepared from a 2 days old culture on Sabouraud Dextrose Agar at 37°C. The colonies were collected with inoculating loop and introduced in a tube with 5 ml sterile normal saline. The suspension was quantified using the Malassez counting chamber under a microscope and adjusted to 2.5 x 10$^7$ cells/ml using sterile 0.9% sodium chloride (normal saline) solution [9].
Disc diffusion method: In vitro antibacterial and antifungal activity were screened by disc diffusion method on Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) respectively. The MHA and SDA plates were prepared by pouring 15 ml of molten media into sterile petri dishes (90 mm). The plates were allowed to solidify for 5 min and 0.1 ml of inoculum suspension was poured and the inoculum was allowed to dry for 5 min. The different extracts at 20 mg/disc were loaded on 5 mm sterile individual discs. The loaded discs were placed on the surface of medium, then the extracts were allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 hours and 48 hours for bacteria and fungi respectively. Negative control was prepared using respective solvent of dilution of extracts (10% DMSO). Amoxicillin, ampicillin, chloramphenicol and gentamycin (2 mg/disc) were used as positive control for bacteria and fluconazole and nystatin (2 mg/disc) were used for fungi. At the end of incubation, inhibition zones formed around the disc were measured with Vernier Calliper in millimeter. These studies were performed in triplicate [9].

Microbroth dilution method: The Minimum Inhibitory Concentration (MIC) was determined according to M38-A and M27-A3 protocols for bacteria and yeast respectively [10]. This method was applied on extracts that showed some efficacy against microorganisms by the agar well diffusion method (inhibition diameters above or equal to 7 mm). In the well of the first line, 100 μl of culture medium (Mueller Hinton Broth for bacteria and Sabouraud broth for fungi) was introduced and 100 μl in the remaining wells of the plates. Later on, 100 μl of stock solution of crude extracts and reference antibiotics at concentration range was 0.0081 to 8.3 mg/ml for extracts. In each stepwise gradient elution by CH₂Cl₂/MeOH mixtures (100:0; 98:2; 95:5; 90:10; 80:20; 0:100). Seventy column fractions, each containing (95:05), showed a precipitate that was filtered under vacuum to give the six fractions obtained, Fraction F3, after evaporation under vacuum. From this fraction, a beige crystalline solid that, after spectroscopic analysis and comparison of its spectroscopic and mass fragmentation patterns with that of isopregomisin [11], was purified and its purity was confirmed by TLC. The isolated substance was identified as isopregomisin [11].

Isolation and identification of active substance: 30 g of the stem bark aqueous alcohol extract obtained (56 g, 2.8%) was submitted to flash chromatography on a 70-230 mesh silica gel column (400 g) with stepwise gradient elution by CH₂Cl₂/MeOH mixtures (100:0; 98:2; 95:5; 90:10; 80:20; 0:100). Seventy column fractions, each containing 300 ml, were collected and combined according to their TLC profiles on pre-coated silica gel 60 F₂₅₄ plates developed with n-hexane/EtOAc and CH₂Cl₂/MeOH mixtures to give 6 groups of fractions: F₁ (1-10); F₂ (11-22); F₃ (23-40); F₄ (41-50); F₅ (51-63) and F₆ (64-70). Among the six fractions obtained, Fraction F3, after evaporation under reduced pressure and dissolution in an n-hexane/EtOAc mixture (95:05), showed a precipitate that was filtered under vacuum to give a pinkish powder. The recrystallization of the obtained powder in an n-hexane/OAcEt mixture afforded a beige crystalline solid that, after spectroscopic analysis and comparison of its spectroscopic and physical data with those reported in the literature, was identified to isopregomisin [11].

Data analyses

Data were expressed in mean ± standard deviation. For the anti-scavenging activity, the inhibitory concentration fifty (IC₅₀) value is the amount of the antioxidant required to decrease the initial DPPH radical concentration to 50% of extract was determined using Graph Pad Prism software. Differences between fractions were assessed by one factor ANOVA followed by the Student-Newman-Keuls test.

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**Table 1:** Qualitative analysis of phytochemical constituents of the stem barks and leaves of *S. ochocoa*.

| Phytochemical constituents | Plant parts extracts |
|---------------------------|----------------------|
|                           | Stem bark            | leaves        |
| Alkaloids                 | ++                   | -             |
| Anthocyanin               | +++                  | -             |
| Coumarins                 | -                    | +++           |
| Flavonoids                | +++                  | +++           |
| Reducing sugars           | -                    | -             |
| Saponosides               | +                    | -             |
| Sterols/Triterpenes       | ++ (sterols)         | +++ (triterpenes) |
| Tannins                   | +++ (gallic)         | +++ (gallic)  |

**+++** Very intense, ++ intense, + weak, − absent
Results

Phytochemical screening

Phytochemical screening using qualitative analysis on aqueous alcoholic extracts from different parts of *S. ochocoa* showed the presence of following constituents: flavonoids and tannins present in both parts of the plant; coumarins and terpenoids in leaves; and anthocyanins, saponosins and sterols only in the stem bark (Table 1).

Antimicrobial analysis

The antimicrobial activities of *S. ochocoa* extracts were evaluated on some pathogenic strains using the disc diffusion and the microbroth dilution methods (Tables 2 and 3). In general, activities exhibited by the extracts were lower than those of reference antibiotics. Leaves extract was more active than the stem bark extract, especially against bacteria. Compared to other microbial strain, *S. aureus* was more sensitive to both bark and leaves extracts of *S. ochocoa* (Table 2). However the leaves extract of *S. ochocoa* showed the lowest Minimum inhibitory concentrations (Table 3) for *S. aureus* and *E. coli* compared to the stem bark extract.

Total phenolic content and radical scavenging effect

The total phenolic content of the aqueous alcohol extract of *S. ochocoa* leaves (153.57 ± 0.63 mg/g extract) was significantly higher (P=0.0008) than the stem bark extract (102.01 ± 2.84 mg/g extract). Both extracts displayed comparable DPPH radical scavenging activity (IC50=0.169 ± 0.019 µg/ml and 0.175 ± 0.020 µg/ml for leaves and bark extracts, respectively) which was significantly higher (P=0.0012) than the reference compound vitamin C (IC50=0.267 ± 0.009 µg/ml).

Identification of the active substance

The chromatographic separation of the stem bark aqueous alcohol extract of *S. ochocoa* afforded the known biomolecule isopregomisin. Isopregomisin, C22H30O6 (0.0047 % from the starting material), beige crystals in hexane-EtOAc (v/v 95:05), mp 111-113°C (litt. 110-112°C), was identified on the basis of its NMR spectra [Figures 1-3] and compared with reported data in the literature [11]. The isopregomisin structure is shown in Figure 4. NMR spectra were recorded on a Brucker-500 spectrometer. 1H NMR (500 MHz, CDCl3): δ 0.83 (d, J=6.4 Hz, 6 H, 2 x CH3), 1.75 (d, J=6.4 Hz, 2 H, 2 x CH), 2.3±2.7 (m, 4 H, 2 x CH2), 3.80 (s, 12 H, 4 x OCH3), 6.33 (s, 4 H, ArH). 13C NMR (125 MHz, CDCl3): δ 146.82 (C); 132.87 (C); 132.72 (C); 105.59 (CH); 56.25 (OCH3); 39.40 (CH2); 39.05 (CH2); 16.23 (CH3).

Discussion

The phytochemical screening of *S. ochocoa* revealed molecules that may potentially be active against some bacterial and fungal pathogenic strains. These included flavonoids, tannins and triterpenes that have been shown to be active against diarrhea, dermal ulcers, skin rashes and abdominal pains [12-15]. Tannins also act as antifungals [16]. Similarly, alkaloids are stimulants, antibiotics, antifungals and pest-destroying [16]. The saponins have antitussive, expectorant, analgesic, immunomodulatory and cytoprotective properties [16]. The presence of these phytochemicals in *S. ochocoa* extracts sustains the common usage of this plant in traditional medicine for the treatment of several diseases. From the compared analysis of Tables 1 and 3, we realize that there is accordance between the chemical composition of the extracts and their antimicrobial activities. Hence, the leaves of *S. ochocoa* which are rich in flavonoids, tannins and triterpenes that have been shown to be active against diarrhea and skin rashes among others, are active against
S. ochocoa in the central African traditional medicine for the inhibition of microbial growth of some microorganisms, including E. coli. The antiradical activity of S. ochocoa demonstrated in many studies [29,30]. This is consistent with the isolation of ocholignan A, a compound found to possess significant antiproliferative effect of alcoholic extracts of some Gabonese medicinal plants on human colonic cancer cells. Afr J Tradit Complement Altern Med 6: 112-117.

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Conclusion

These findings demonstrated the presence of various phytochemicals including phenolics in S. ochocoa extracts. Among phenolic compounds, lignans, especially isopregomisin was isolated from the stem bark aqueous alcohol extract and identified through its Mass and NMR spectral data. These extracts also possessed antiradical activities above that of the reference compound vitamin C, and inhibited microbial growth of some microorganisms, including E. coli and a gentamycin resistant S. aureus strains. This study supports the use of S. ochocoa in the central African traditional medicine for the treatment of infectious diseases, and also justifies the predominance of the use of leaves in traditional medicine due to their interesting biological activities.
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