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Phytochemical screening and *in-vitro* evaluation of antimicrobial and antioxidant activities of ethanolic extracts of *Elephantopus mollis* Kunth. (Asteraceae)

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Abstract
Elephantopus mollis Kunth is a medicinal plant used in Cameroon and others African countries for the treatment of microbial diseases. This work aimed to evaluate the antimicrobial and antioxidant activities of the hydroalcoholic extracts of stems, leaves and whole plant of *E. mollis*. Phytochemical screening of different extracts has been performed and these were evaluated for their antimicrobial activity against ten bacteria and three fungal strains by dilution method while, antioxidant activity was examined using ABTS**,** DPPH and FRAP assays. Thus, flavonoids, tannins, coumarins, saponins, phenols and alkaloids were constituents detected in those extracts. The results of the antimicrobial tests revealed that, all the extracts were most actives against tested bacteria strains, except for *S. enterica* NR 13555, while higher antifungal activity was found for *C. andida albicans* strain. On the other hand, all extracts exhibited antioxidant activity using DPPH and ABTS and FRAP methods. Following, both stems and leaves extracts showed significant antioxidant activity with IC_{50} values of 2.53 and 4.13 µg/mL, respectively. The results provide justification for the use of this plant in folk medicine to treat various infectious diseases. This study might be considered as a prelude to discover new antibacterial agents.

Keywords: *Elephantopus mollis*, phytochemical screening, antimicrobial activity, antioxidant activity

Introduction
Plants form an integral part in traditional medicinal practices in all cultures worldwide and a sizeable portion of the world population uses plant for prevention and management of different kinds of ailments. The rural population in particular who do not have access to primary health care, either as a result of non-availability or inability to afford it depends solely on plant remedies for their health problems [1]. Many of the plants used in ethnomedicine have been found to contain useful therapeutic substances and a good number of them have found their way into orthodox medical practice. The search for new compounds which can be useful in the management of diseases that have defied current therapeutic options focuses majorly on plants as a reliable source of lead substances [2]. Many plants, which are used in ethnomedicine, have shown promising activity against a host of disease causing microorganisms and they have been documented [3]. *Elephantopus* genus consists of at least twelve species of perennial plants in the daisy family (Asteraceae). It includes *E. carolinianus*, *E. scaber E. tomentosus* and *E. mollis* among others. *Elephantopus* species are traditionally used for the treatment of rheumatism, stomach ache, cough, diarrhea, wounds, and dysentery [4, 5]. Previous studies on the genus showed the presence of sesquiterpene lactones, steroids and triterpenes, some of which possessed antitumor, antidiabetic, antioxidant anti-inflammatory and hepatoprotective activities [4-9]. *Elephantopus mollis* is a medicinal plant widely used in South America and Africa [10-13]. In Cameroon, it is known as "Ta`a bekone" by Pygmée Baka. Decoction of the leaves is used to treat dysentery, bronchial affections and pains caused by fever. In external application, leaves are also used to treat wounds, pimples and other skin affections. In the Centre region of Cameroon, maceration of the leaves is used as a purgative for children to treat intestinal disorders. Earlier studies reported the presence of sesquiterpene lactones, germacranolides and triterpenes from this plant, while its cytotoxic, antitumor, antioxidant and antileishmanial activities were also demonstrated [14-16]. Herein, we report the antimicrobial and antioxidant activities of the hydroethanolic extract of *Elephantopus mollis*.

Materials and Methods

Plant material

Elephantopus mollis was harvested from Esse sub-division, Centre region of Cameroon, (August 2017) and identified at the Cameroon National Herbarium (HNC), where a voucher specimen was deposited (9516/SFR-CAM).

Extraction of plant material

The powdered stems (300 g), leaves (201.8 g) and whole plant (308.8 g) of *E. mollis* were macerated with ethanol-water mixture (7:3, v/v) (4 L) at room temperature for 72 h. The macerate was filtered and evaporated under reduced pressure to obtain stems (EM1: 23.2 g), leaves (EM2: 50.3 g) and whole plant (EM3: 53.1 g) extracts, respectively.

Phytochemical screening

The extracts were subjected to phytochemical screening to detect the presence of alkaloids, anthocyanins, anthraquinones, cardiac glycosids, coumarins, flavonoids, glycosids, lipids, phenols, polyphenols, saponins, sterols, sugars, tannins, and triterpenes using protocols described by Pietta *et al*. [17] and modified by Karuppannan *et al*. [18].

Antimicrobial activity

Microorganisms Tests

Microorganisms used in this study were provided by the bacteriology laboratory of the University Hospital Center of Yaounde. It consisted of 6 Gram-negative strains (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Klebsiella pneumoniae* NR 41916, *Pseudomonas aeruginosa* HM 601, *Salmonella* enterica NR 13555, *Salmonella* typhi CpC); 4 Gram-positive strains (*Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 49619, *Staphylococcus aureus* NR 46374, *Shigella flexineri* NR 518) and *3 yeasts (Candida albicans NR-29445, Candida krusei ATCC 6258, Candida parapsilosis ATCC 22019).* These strains were stored at 37 °C on nutrient agar in the Galenic Pharmaceutical and Microbiology Laboratory Section of the Department of Galenic Pharmacy and Legislation of the Faculty of Medicine and Biomedical Sciences of the University of Yaounde I.

Inoculum preparation

Before any test, bacteria were subcultured on Mueller Hinton agar slants at 35EC for 18 h. Mature colonies were collected with inoculating loop and introduced into a tube with 5 mL of sterile saline (0.9% NaCl) and homogenized. The turbidity of the solution was adjusted at 0.5 McFarland standards.

Preparation of stock solution of plant crude extracts

Crude extract samples were prepared by weighing 5 mg and dissolving them in 1 mL of DMSO 10% for a final concentration of 5 mg/mL. 500 mg of standard control was dissolved in 250 mL of DMSO 10% for a concentration of 2 mg/mL. After preparation, the stock solutions were sterilized by heating at 60 °C for 30 min.

Determination of minimal inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

The minimum inhibitory concentration (MIC) was determined according to the National Committee for Clinical Laboratory Standards (NCCLS) M38 microdilution method using (12×8 wells) microtitre plates. This method was applied on extracts that demonstrated high efficacy against microorganisms using disc diffusion method. In the well of the first line (line 1), 100 µL of culture medium Mueller Hinton Broth (Mast Group Ltd.) was introduced and 100 µL in the remaining well of the plates. Later on, 100 µL of stock solution of crude extracts at 20 mg mLG1G were added to the first well. The medium and sample in the first well were mixed thoroughly before transferring 100 µL of the resulting mixture to the well of the second line. Then two-fold serial dilutions of the test samples were made from line 1 until line 11 and 20 µL of inoculum standardized at 0.5 McFarland standards were introduced in the entire well containing the tested substances except the columns of blank (Column C and F) which constitute the sterility control. The concentration was 20 mg/mL for crude extracts. In each microtiter plate, a column with broad-spectrum antibiotic (Ciprofloxacin) with the concentration was used as positive control. After incubating at 35EC for 24 h, turbidity was observed as indication of growth. Thus, the lowest concentration inhibiting the growth of bacteria was considered as MIC. The minimum bactericide concentration (MBC) was determined by transferring 25 µL aliquots of the clear wells into 100 µL of freshly prepared Muller Hinton Broth medium and incubating at 35EC for 24 h. The MBC is the lowest concentration of test sample which did not produce turbidity as above, indicating no microbial growth. All tests were performed in triplicate. Ciprofloxacin was used as positive control. The antibacterial effect of extracts was evaluated by calculating the ratio of MBC/MIC. If MBC/MIC was ≤ 4, the extracts was defined as bactericidal, or if MBC/MIC was > 4, the extract was defined as bacteriostatic [19].

Determination of minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) was determined according to National Committee for Clinical laboratory Standards (NCCLS) M27-A3 microdilution method using (12 x 8 wells) microtitre plates. In the well of the first line (1-12), 100 µL of culture medium Sabouraud Dextrose Broth was introduced and 100 µL in the other well of the plates. Later on, 100 µL of stock solution of crude extracts were added to the first well. The medium and extract in the first well were mixed thoroughly before transferring 100 µL of the resultant mixture to the well of the second line. Serial two-fold dilutions of the test samples were made and 20 µL of inoculum standardized 2.5 × 104 cells/mL were introduced in the entire well containing the test substances except the column of blank which constitute the sterility control. The concentrations ranged from 20 mg/mL for crude extracts. In each microtiter plate, a column with broad-spectrum antibiotic (Fluconazol) with the concentration was 2 mg/mL was used as positive control. After an incubation period of 48 h at 37 °C, turbidity was observed as indication of growth. Thus, the lowest concentration inhibiting the growth of yeast was considered as the MIC. The minimum fungicidal concentration (MFC) was determined by transferring 25 µL aliquots of the clear wells into 100 µL of freshly prepared broth medium and incubating at 37 °C for 48 h. The MFC was regarded as the lowest concentration of test sample which did not produce turbidity as above, indicating no microbial growth. All tests were performed in triplicate. The anticandidal effect of extracts was evaluated by calculating the ratio of MFC/MIC. If MFC/MIC was ≤ 4, the extract was defined as fungicidal, or if MFC/MIC was > 4, the extract was defined as fungistatic [19].

Antioxidant Activity

ABTS radical activity

The ABTS radical scavenging capacity of the samples was measured with modification to a 96-well microtitre plate format as described by Re *et al*. [20] with slight modifications. ABTS radical was generated by reacting 7 mM solution of ABTS and 2.45 mM solution of potassium persulfate at room temperature for 12 h. The ABTS radical stock solution was adjusted to 7.00 ± 0.02 at 734 nm before use. The test samples (40 µL) were made in a concentration range of 0.78 to 100 µg/mL by two fold serial dilutions and 160 µL of ABTS radical solution was added. Absorbance was measured after 6 min at 734 nm. Trolox and ascorbic acid were used as positive controls, methanol as negative control and compound without ABTS as blank. Percentage of $ABTS^{\bullet+}$ inhibition was calculated using formula:

Scavenging capacity (%) = 100 – [(absorbance of sample – absorbance of sample blank) × 100/(absorbance of control) – (absorbance of control blank)]. The IC_{50} values were estimated from the percent inhibition versus concentration plot derived from the percentage scavenging activity.

DPPH free radical assay

The DPPH free radical-scavenging activity was determined using the method proposed by Brand-Williams *et al*. [21], with some modifications to 96-well microtitre plate. Various concentrations of compounds in methanol were prepared (7.81 to 1000 µg /mL). Ascorbic acid and trolox were used as a positive control at concentration of 100 to 0.78 µg/mL. Then, 160 µL of DPPH (0.037 mg/mL) in methanol was added to 40 µL of the test solution, or standard, and allowed to stand at room temperature in a dark for 30 min. Methanol was used as a blank. The change in colour from deep violet to light yellow was then measured at 517 nm using a Versamax microplate reader. Results were expressed as percentage reduction of the initial DPPH absorption in relation to the control. The concentration of compound that reduced DPPH colour by 50% (IC_{50}) was determined as for $ABTS^{\bullet+}$.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain [22] with slight modifications. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was freshly prepared and was warmed to 37 ºC in a water bath prior to use. Fifty microliters of sample were added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The standard curve was constructed using $FeSO_4$ solution (0.1-2 mM), and the results were expressed as µM $FeSO_4$/g dry weight of extract. All the measurements were taken in triplicate and the mean values were calculated.

Results and discussion

Phytochemical screening

The preliminary phytochemical screening of different crude extracts revealed the presence of phenols, terpenoids, flavonoids, saponins, tanins, and alkaloid (Table 1). This result can be explained by the fact that during maceration, secondary metabolites are separated according to their affinity and solubility with extraction solvent [23].

Table 1: Phytochemical constituents of different extracts

Secondary metabolites	Extracts		
	EM1	EM2	EM3
Terpenoids	+++	-	+++
Steroids	++	++	++
Alkaloids	++	-	++
Free flavonoids	-	-	-
Phenols	++	+	++
Coumarins	-	-	-
carbohydrates	-	-	-
Tanins	++	++	++
Saponosids	++	-	++
Resins	-	-	-
Anthocyanes	-	-	-
Cardiotonicheterosids	-	-	-
Lipids	-	-	-

EM1: Stems extract; EM2: Leaves extract and EM3: Whole plant extract

Antimicrobial activity

The results of the MIC, MBC, MFC, MIC/MBC and MFC/MIC are summarized in Tables 2 and 3. The antibacterial and anticandidal effect of extracts was evaluated by calculating the ratio of MBC/MIC and MFC/MIC. If MBC/MIC or MFC/MIC was ≤ 4, the extracts was defined as bactericidal or fungicidal respectively, similarly, if MBC/MIC or MFC/MIC was > 4, the extract was defined as bacteriostatic or fungistatic [19]. Results showed that, the minimum inhibitory concentration ranged from 1.25 µg/mL and 10 µg/mL for all the tested microorganisms. Amongst the crude extract, the best activities were observed with EM3 extract for the tested microorganisms and it was considered as bactericidal and fongicidal (MBC/MIC≤ 4). All the extract are fungicidal on all the tested microorganisms (MBC/MIC≤ 4). Overall, Gram-negative bacteria were less sensitive than Gram-positive bacteria with the extracts. This observation is in accordance with a previous report in which, these facts were due to their outer membranes that contribute to the intrinsic resistance by acting as an efficient permeability barrier, Gram-negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than are Gram-positive [24]. Interestingly, EM1 extract had remarkable activity against one Gram-positive strain (*S. aureus* ATCC 49619) with MIC value of 1.25 µg/mL. The observed antimicrobial activity was certainly due to the presence of various classes of secondary metabolites within the crude extracts such as tannins, saponins, steroids, flavonoids and triterpenoids. These metabolites may exert their inhibitory effect through different mechanisms. In fact, it is known that tannins exert their antimicrobial activity by binding with proteins and adhesins, inhibiting enzymes, complexation with the cell wall and metal ions, or disruption of the plasma membrane [23]. On the other hand, saponins have the ability to cause leakage of proteins and certain enzymes from the ?????? [22].

Triterpenoids isolated from various parts of plants have been reported to have antiinflammatory activity [25] bactericidal analgesic, antiviral and antiallergic [26]. The presence of these phytochemicals in the plants suggest that the plant is pharmacologically active and supporting the claim by traditional healers.

Table 2: MIC, MBC and MBC/MIC of crude extracts

Mioorganisms tested	Sample/MIC, MBC (µg/mL)								
	EM1			EM2			EM3		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
E. coli ATCC 25922	5,0±0,0	10,0±0,0	2	5,0±0,0	10,0±0,0	2	5,0±0,0	10,0±0,0	2
K.pn ATCC 13883	>20	>20	1	20,0±0,0	>20	1	20,0±0,0	>20	1
K. pn NR 41916	7,5±0,0	>20	4	5,0±0,0	>20	4	5,0±0,0	>20	4
P. ae HM 601	5,0±0,0	10,0±0,0	2	5,0±0,0	5,00±0,00	2	5,0±0,0	20,0±0,0	4
S. enterica NR 13555	2,5±0,0	>20	8	0,93±0,0	>20	nd	5,0±0,0	>20	4
S. typhi CpC	5,0±0,0	>20	4	5,0±0,0	>20	4	5,0±0,0	>20	4
S. aureus ATCC 43300	2,5±0,0	10,0±0,0	1	7,5±0,0	10,0±0,0	1	2,5±0,0	5,0±0,0	2
S. aureus ATCC 49619	1.25±0,0	1.25±0,0	4	5,0±0,0	10,0±0,0	2	5,0±0,0	5,0±0,0	1
S. aureus NR 46374	2,5±0,0	5,0±0,0	2	2,5±0,0	10,0±0,0	4	2,5±0,0	5,0±0,0	2
S. flexine NR 518	>20	>20	1	>20	10,0±0,0	nd	>20	>20	1
Cipro	0,125±0,0	0,125±0,0	0,125±0,0	0,125±0,0	0,125±0,0	0,125±0,0	0,125±0,0	0,125±0,0	32.0±0,0

Table 3: MIC, MFC and MFC/MIC of crude extracts

Mioorganisms tested	Sample/MIC, MFC (µg/mL)								
	EM1			EM2			EM3		
	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
C. albic NR-29445	5,00±0,00	10,00±0,00	2	5,00±0,00	10,00±0,00	2	5,00±0,00	10,00±0,00	2
C. Krus ATCC 6258	>20	>20	1	20,00±0,00	>20	1	20,00±0,00	>20	1
C. para ATCC 22019	7,50±0,00	>20	4	5,00±0,00	>20	4	5,00±0,00	>20	4
C. albic NR-29445	5,00±0,00	10,00±0,00	2	5,00±0,00	5,00±0,00	2	5,00±0,00	20,00±0,00	4
Fluco	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00	0,125±0,00

Antioxidant activity

A meaningful antioxidant capacity cannot be fully described using a single method because antioxidant capacity is influenced by many factors. The antioxidant activity of extracts can be determined *in vitro* by hydrogen atom transfer (HAT) method and single electron transfer (SET) method. HAT method measures the capacity of an antioxidant to scavenge free radical by hydrogen donation to form a stable compound. SET method determines the ability of the antioxidant to transfer one electron to reduce compound including metals, carbonyls and radicals [27]. FRAP assay involves SET method, while DPPH and ABTS assay involve both method predominantly via SET method [28]. In this study, the antioxidant activity of extracts was determined using the free radical 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the ferric reducing antioxidant power (FRAP) assays. The use of at least two different assays in evaluating antioxidant activity of plant products has been recommended by Moon and Shibamoto [29]. The IC_{50} values were obtained and presented in Table 4. The IC_{50} values for the different extracts ranged from 0.117 ± 0.008 to 0.185 ± 0.002 µg/mL in DPPH assay, from 2.53±0.49 to 17.04±0.29 µg/mL in ABTS assay and from 16.00 ± 0.38 to 31.10 ± 0.035 µg/mL in FRAP assay (Table 4). The IC_{50} values of extracts were significantly different from the IC_{50} of ascorbic acid and trolox, standard antioxidant agents used as a positive control. The capacity of flavonoids to act as antioxidants in vitro has been previously studied [30]. Order hence, all extracts had antiradical activity in DPPH (0.117 to 0.185 µg/mL) assays and ABTS assay, EM1 and EM2 extracts showed significant antioxidant activity with IC_{50} values 2.53 and 4.13 µg/mL respectively. In FRAP assay, antioxidant extract react by donating an electron, thus converting Fe^{3+} to Fe^{2+}. Similar to DPPH and ABTS results, EM1 had the antioxidant potential. The action of these antioxidants is believed to be due to their ability to donate hydrogen or electron atoms derived mainly from the flavonoid cycle A hydroxyl and the study conducted by Ooi *et al*. [12] highlighted the presence of phenols in extracts of *Elephantopus mollis*.

Table 4: Antioxidant activity of crude extract of *E. mollis*

Extracts	IC_{50} (µg/mL)		
	DPPH	ABTS	FRAP
EM1	0.185±0.002	2.53±0.49	16.00±0.38
EM2	0.155±0.003	4.13±0.10	31.10±0.035
EM3	0.117±0.008	17.04±0.29	17.40±0.065
Trolox	5.36±0.10	3.71±0.21	Nd
Ascorbic acid	2.80±0.03	2.61±0.08	Nd

Conclusion

The results provide justification for the use of this plant in folk medicine to treat various infectious diseases. This study might be considered as a prelude to discover new antibacterial agents to the problematic pathogenic bacteria. Moreover, isolation of compounds from the plant will give an opportunity for a possible discovery of bioactive components for clinical development trials.

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Declaration of interest

The authors report no conflict of interest

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