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Evaluation of the antimicrobial activity of tannin extracted from the barks of *Erythrophleum guineensis* (Caesalpiniaceae)

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Abstract
Acute diarrheal diseases caused by food poisoning consitute serious public health problem in developing countries where they are the leading cause of death. WHO recommends research towards discovery of new anti-infective molecules that could be used in the treatment of bacterial and fungal infections such food poisoning, diarrhea. Literature reports that tannins are a very effective natural treatment. For example, in Democratic Republic of Congo, MEYAMICINE® was developed as very effective drug against food poisoning and diarrhea. The antimicrobial activity of condensed tannins from *Erythrophleum guineensis* stem barks (Caesalpiniaceae) was evaluated using the micro-dilution method in a liquid medium on seven reference strains, notably *Staphylococcus aureus* CIP 7625, *Salmonella enterica* NR 13555, *Shigella flexneri* NR 518, *Escherichia coli* ATCC 25922, *Candida albicans* NR 29450, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019.
The hydro-ethanolic crude extract of stem bark contains several classes of secondary metabolites such as polyphenols, tannins, flavonoids, terpenoids, alkaloids, saponins mucilage, coumarin, cardiac glycosides, etc. The partition of this crude extract with ethyl acetate resulted into the isolation of condensed tannins. The determination of condensed tannins via total proanthocyanidin assay yielded a concentration of 0.865 mg EC / mL with an optical density of 0.558. The minimum inhibitory concentrations (MIC) ranged from 1.25 to 2.5 mg / mL while the minimum bactericidal or fungicidal concentrations (MBC or MFC) ranged from 1.25 mg / mL to 5 mg / mL. The best bactericidal and absolute fungicidal activities were respectively recorded on *Staphylococcus aureus* and *Candida krusei* and *Candida parapsilosis* with results (MBC/ MIC or MFC / MIC = 1). A relatively bactericidal activity good was also recorded againat *Shigella flexneri* (MBC / MIC = 2). On the other hand, condensed tannins exhibited bacteriostatic or fungistatic activitities on *Salmonella enterica*, *Shigella flexneri*, *Escherichia coli* and *Candida albicans* with (MBC / MIC = 4).
This study has also led to isolation of a proanthocyanidol. On the basis of the antimicrobial activities of condensed tannins in this study, it can be concluded that condensed tannins from *Erytrhophleum guineensis* effectively exhibit antimicrobial properties and this may justify the use of this plant in the treatment of diarrhea caused by food poisoning.

Keywords: *Diarrhea - Erytrhophleum guineensis - Condensed tannins - Antimicrobial activity*

Introduction
The use of plants has always been a common practice in our societies, especially in Africa and Asia. [1] In Cameroon as elsewhere in Africa, traditional medicine is a very important component of the socio- cultural heritage [2]. According to WHO, more than 80% of the African population use traditional medicine to treat diseases including food-borne diseases that constitute public health problems in many parts of the world [3, 4]. Food-borne diseases such as diarrhea represent the leading cause of death with nearly 17 million deaths per year [5]. Foodborne diseases also impede socioeconomic development by straining health care systems, and harming national economies, tourism and trade [5]. At the beginning of the twenty-first century, 1407 microbial species were identified as human pathogens (38.2% were bacteria against 7.8% fungi) [6]. These microorganisms disseminate easily due to their ubiquitous nature through various vectors, notably food items. Indeed, food can be a serious health risk for the consumers when it is handled and/or consumed under undesirable hygienic conditions. Thus the consumption of contaminated foods with pathogenic microorganisms (or their toxins) at an infective dose is often responsible for cases of foodborne illness [7]. Food poisoning includes both foodborne infections due to the ingestion of live pathogens, causing intestinal balance and preformed microbial toxins already present in the food [7], generating many symptoms such as diarrhea. Diarrhea is ranked the third most deadly infections and accounts for approximately

2.2 million deaths per year, with nearly 2 million children under 5 alone [8]. The impacts of foodborne diseases are most pronounced in the tropics where environmental conditions favorable to the growth of microorganisms prevail.

Antibiotics and antifungals remarkably constituted efficient weapons in controlling acute infectious diarrhea caused by food poisoning [9]. Though the discovery of antibiotics and antifungal agents has significantly improved healthcare worldwide, the inappropriate use of these substances has favored the development of microbial resistance [10].

The phenomenon of antimicrobial resistance has led to the search of new alternative antimicrobials such as medicinal plants that are relatively cheap and readily available.

In continuation of the work undertaken on *E. guineense*, the purpose of the present research study was to characterize this plant extract and evaluate the antimicrobial activity of condensed tannins of the bark of the trunk of *E. guineense* used in the locality of AKOUNOU (Centre Region of Cameroon) for the treatment of syphilis.

Materials and Methods

Study materials

Erythrophleum guineensis was collected in July 2015 from AKOUNOU in the Mefou and Afamba Division (Central Region). The identification was carried out at the Cameroon National Herbarium in comparison with an existing referenced sample, coded 45750 HNC.

Fig 1: Barks of *E. guineensis*

Extraction

Extraction was performed following the method of Gedir *et al*. [11]. In fact, part of the resulting powder (2000 g) was soaked for 24 hours and repeated three times in a mixture of ethanol-water 70/30 (v / v). After filtration on Whatman No. 1 and evaporation under reduced pressure using a rotary evaporator (55 °C), the resulting solution was treated with NaCl brine to precipitate the condensed tannins of high molecular weight. After filtration, the filtrate containing tannins of small molecular weight was partitioned with ethyl acetate. The organic phase was treated with anhydrous sodium sulfate, filtered and concentrated using a rotary evaporator to give a tannin-rich extract. Different concentrated solutions obtained during the extraction were freeze-dried to remove residual water and obtain dried extracts. The latter were weighed, labeled, sealed and kept refrigerated at 4 °C.

Dtermination of condensed tannin content

The determination of the tannin content was based on a standard procedure described by Nakamura et *al* [12]. To a 0.5 mL of catechin solution (1mg/mL) were added 3 mL of vanillin -methanol solution (4%) and 1.5 mL of hydrochloric acid. The resulting mixture was vortexed and was allowed to settle for 15 min at room temperature. Absorbance was measured at 500 nm. The extract was evaluated at a final concentration of 2 mg / mL.

Assessing the antimicrobial activities of the extracts

Determination of Minimum Inhibitory Concentration (MIC)

This was performed according to the M27-A3 and M7-A9 protocols for yeasts and bacteria respectively [13]: 100 µL of liquid medium were added to each well on the plate. In the wells of the first line, 100 µL of the solution of the F3 fraction were introduced in order to obtain a total volume of 200 µL. From these wells, a series of 10 dilutions in decreasing order obeying a geometric progression of 2 was performed for each well. From the first term (5 mg / ml) and the last term (0.00489 mg / mL), 100 µL of solution were withdrawn from the first well and were introduced into the second well and so on. Thereafter, 100 µL of the microbial suspension of $2.5x10^3$ CFU / ml for yeasts and 0.5 Mac Farland ($0,5x10^8$ CFU / mL) for the bacteria were introduced into the wells on the plate except the control wells (blank). The plates were incubated for 24 and 48 hours at 37 °C. The tests were done in triplicate.

The positive control was prepared as above except that instead of extracts, chloramphenicol and fluconazole were introduced (5 mg / ml to 0.0048 mg / ml) into the control wells. For the negative control, each well was filled with 100 µL culture medium to which 100 µL of microbial suspension were added.

A column containing only the culture medium was the blank indicating the sterility of the culture medium. Another column consisted of wells containing the culture medium, the inoculum and the dilution solvent (10% DMSO). This column was to ensure that this solvent did not affect the viability of the microorganism.

Determination of minimum bactericidal concentrations (MBC) and fungicidal (CMF)

The assessment of this parameter was performed by subculturing in liquid medium preparations taken from the plates used for determining MIC.

Following the incubation of the plates used for the determination of MICs, fractions of 50 µL were collected from the well corresponding to the MIC and four previous wells and then transferred to five wells of another plate previously prepared each containing 150 µL of sterile broth liquid medium. Thus, concentrations of compound contained in these wells were diluted 3 times to remove the inhibitory effect of the test compound.

The plates were covered and incubated at 37 °C for 24 and 48 hours for bacteria and yeast respectively; the well with the lowest concentration of a compound showing no visible growth of the yeast or bacteria, has a MIC equivalent to the corresponding plate containing the fungicide or bactericidal concentration of the compound.

Interpretation

The smallest concentration at which no visible growth was observed was considered as the minimal bactericidal and fungicidal concentration.

Results

Phytochemical composition of the extract condensed tannin content

Table I: Phytochemical composition of the extract condensed tannin content

Secondary metabolites	Test method	condensed tannin
Catechic tannins	Stiasny	+++
gallic tannins	Sodium ferric acetate Perchloride	-

Legend: (+) positive reaction; (-): Negative reaction

It appears from the latter that only condensed tannins (catechic tannins) are present in the extract

Fig 2: Demonstration of catechic tannins

Condensed tannin content of the extracts

The total proanthocyanidins content in this extract was estimated using a calibration curve obtained with a reference extract (catechin) at different concentrations. The results are expressed in mg equivalent catechin per mL. The calibration curve was established with a correlation coefficient R^2 = 0.998 and an equation: Y = 0,6451X where Y is the absorbance and X is the concentration.

Table II: Results of the determination of total proanthocyanidins

	Optical density (OD)	Total proanthocyanidin content (EC mg / mL)
Extract	0.558	0.865

With reference to the calibration curve of catechin and according to the equation:
$y = 0,6451x$, $R^2 = 0.998$, where y will be absorbance and x the concentration. It appears that the total proanthocyanidin content of the extract is 0.865 mg catechin equivalents per mL with an optical density of 0.558.

Determination of MIC, MBC and MFC fraction F3

The results obtained following the tests of activity on bacteria and fungi were analysed and presented in Table III in terms of MIC, MBC (MFC) and the report MBC / MIC.

Table III: MIC, MBC (MFC) and MBC / MIC, MFC / MIC for each of the microorganisms tested on the extract

Microorganisms	MIC (mg/mL)	MBC /MFC (mg/mL)	R
Staphylococcus aureus	1.25	1.25	1
Salmonella enterica	1.25	5	4
Shigella flexneri	2.5	5	2
Escherichia coli	1.25	5	4
Candida albicans	1.25	5	4
Candida krusei	1.25	1.25	1
Candida parapsilosis	1.25	1.25	1

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC minimum fungicidal concentration; A: Report MBC / MIC or MFC / MIC

It was shown that the MIC ranged from 1.25 to 2.5 mg / mL based on the microorganisms tested. MIC values varied between 1.25 mg / mL (*Staphylococcus aureus, Salmonella enterica and Escherichia coli, Candida albicans*, *Candida krusei*, *Candida parapsilosis*) and 2.5mg / mL (*Shigella flexneri*). As for the values of MBC and MFC, they varied from 1.25 mg / mL (*Staphylococcus aureus, Candida krusei, Candida parapsilosis*) to 5 mg / mL (*Salmonella enterica, Shigella flexneri* and *Candida albicans, Escherichia coli*).

Discussion

In continuing the work undertaken in 2015 in our laboratory [14], condensed tannins were extracted from *E. guineense* extracts using Gedir method. The resulting extract gave a positive reaction with the formation of precipitates to Stiasny test and a negative reaction to sodium acetate and ferric perchloride tests, respectively, indicating the presence of catechic tannins and absence of gallic tannins. This indicates a rich content of condensed tannins in the test extract. The determination of condensed tannins via total proanthocyanidin assay yielded a concentration of 0.865 mg EC / mL with an optical density of 0.558. Condensed tannins (CTs) are common constituents of woody plants, but are often found in grains, fruit and beverages such as beer and wine. These polymeric flavonoids (flavan-3-ols) have broad protein-precipitating and antimicrobial activities [15, 16]. it has been proposed that some plants evolved condensed tannins production as a chemical defence, first against invasion by pathogenic microorganisms, then against being eaten by insects and finally against being eaten by grazing herbivores [17].

The results for the antimicrobial activity of the plant extracts indicated that MICs ranged from 1.25 to 2.5 mg / mL whereas MBCs and the MFCs ranged from 1.25 mg / mL to 5 mg / mL. *Staphylococcus aureus, Salmonella enterica, Escherichia coli, Candida albicans, Candida krusei* and *Candida parapsilosis* are relatively sensitive to low MIC values (1.25 mg / mL) compared to *Shigella flexneri* (2.5 mg / mL). MBC and MFC values recorded for *Staphylococcus aureus*, *Candida krusei*, *Candida parapsilosis* were equally low (1.25 mg / mL) in comparaison to those for *Salmonella enterica, Escherichia coli, Candida albicans, Shigella flexneri* (5 mg / mL). If MIC values seem not to depend on Gram status, on the contrary, MBC values appear relatively higher in Gram negative organisms. *Shigella, Salmonella,* and *E. coli* used in this study belong to Enterobacteriaceae are known to be implicated in food-borne infections [18].

Their main mode of transmission is via the consumption of contaminated food due to lack of hygiene. The antibacterial activity of *E. guineense* against these Gram-negative bacilli should also be tested on other bacterial strains that are

responsible of major human diseases such as typhoid fever and cholera being endemic in many parts of the world. Thus, the use of this plant in the traditional management of infectious diseases can be justified from the activity of condensed tannins in its extracts. In fact, the action of condensed tannin on these microorganisms, could be explained by the fact that the tannins inhibit bacterial growth by complexing enzyme (permeases) and proteins of the bacterial outer membrane (porins). This inhibition then impede many vital functions in microorganisms ranging from respiration, synthesis of the wall components to events related to functions of nucleic acids [16, 17].

Its inhibitory effects on microbial enzymes may strongly be associated with its antimicrobial activity. This activity has already been demonstrated in Staphylococcus producing β - lactamase PBP2 as epigallocatechic gallate restores the activity of penicillin resistance strains. Therefore, condensed tannins inhibit the synthesis of beta-lactamase produced by the bacteria [19]. The relatively lower activity of this plant on enterobacteria may be attributed to the presence of the outer membrane in the latter. This may signify that at least some target of condensed tannins are intracellular and that the porosity of the bacterial envelope does not allow the passage of some of these tannins (high molecular weight, for example) as they are water-soluble. Condensed tannins of small molecular weight such as oligomers can cross this barrier and bind to intra cytoplasmic proteins and enzymes.

Another hypothesis may be that condensed tannins borrow a mechanism of antibacterial action different from that of conventional antibiotics. This is because the varied chemical structures of condensed tannins affect its biological properties [17]. They are capable of binding metal ions involved in the metabolism of bacteria. Thus by combining with Ca^{2+} ions involved in the structure of the Gram -negative bacteria, condensed tannins affect the permeability of the bacterial cell wall ; disrupting the absorption of trace elements essential for bacterial growth [18].

Salmonella enterica, Shigella flexneri, Escherichia coli and *Candida albicans* are highly involved in foodborne infections and acute infectious diarrhea. The condensed tannins such as polymers due to their high molecular weight, would have difficulty to destroy the bacteria, for they would be unable to pass through the pores of the capsule and poring of bacterial outer membrane. Moreover, the formation of the polysaccharide-tannin complex also depends in part on the molecular size of the sugar, which should be high; and this is not the case for polysaccharides of the capsule of these organisms. MIC and MBC values recorded in this study may be probably attributed to condensed tannins of smaller sizes (oligomers) such as catechin or proanthocyanidol. Indeed, because of their low molecular weight, they could be able to pass through the pores of the capsule and the outer cell wall and inactivate the periplasm permeases involved in the transport of amino acids and carbohydrates.

On the basis of MIC and MBC or MFC values, our extract has been proved more bactericidal or absolute fungicidal than bacteriostatic or fungistatic to bacterial and fungal strains used in the study with MBC / MICor MFC ranging from 1 for *Staphylococcus aureus* and *Candida krusei* and *Candida parapsilosis,* 2 for *Shigella flexneri* to 4 for *Salmonella enterica*, *Shigella flexneri*, *Escherichia coli*, and *Candida albicans*. In testing the antimicrobial activity of condensed tannins, the choice of solvent is also a very important parameter. The hydroethanolic mixture (70% v / v), gives us background information on the affinity and selectivity solvents towards secondary metabolites solvents; this helps us to understand the role of the synergistic action of secondary metabolites in the antimicrobial mechanism. The condensed tannins of *E. guineensis* extract, taken alone provide antimicrobial activity much lower than the total ethanolic extracts of the same plant. Thus the total extracts have the advantage of containing groups of bioactive metabolites which act synergistically in killing bacteria and yeasts.

Conclusion

The objective of this work was to evaluate the antimicrobial activity of condensed tannins from the stem barks of the Cameroonian *Erythrophleum guineensis*; this study was done with seven strains of microorganisms including four bacteria and three yeasts known to be involved in food poisoning and acute infectious diarrhea.

Determination of condensed tannins via total proanthocyanidin assay gave a concentration of 0.865 mg EC/mL with an optical density of 0.558.

The evaluation of the antimicrobial activity of tannins by microdilution method in a liquid medium showed bactericidal and fungicidal effects of plant extracts on the majority of the test microorganisms. The best bactericidal and absolute fungicidal activity was observed against *Staphylococcus aureus*, *Candida krusei* and *Candida parapsilosis*, with MIC in the range of 1, 25 mg / ml and a ratio MBC / MIC or MFC equals to1; other microorganisms registered the following results: MIC of 2.5 mg / ml for *Shigella fexneri* with MBC / MIC ratio of 2. On the other hand *Salmonella enterica*, *Shigella flexneri*, *Escherichia coli*, *Candida albicans* recorded bacteriostatic / fungistatic activity, with MBC and MFC of about 5 mg / mL and a MBC / MIC ratio equals 4.

In perspective, given the fact that there is variation in the chemical structures of condensed tannins which, in turn affect their biological properties, it is therefore important to determine the chemical structure of the condensed tannins isolated from *E. guinensis* in view of knowing its molecular weight and size.

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