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Original Research Article

Evaluation of Bacterial Activity *in Vitro* on *Salmonella Enterica* of *Typhi* Stereotype of Drugs of Medicinal Plants, *Annickia Chlorantha* (Oliv.) Setten & Maas, *Alstonia Boonei* De Wild and *Costus Afer* Ker Gawl Used In the Treatment of Typhoid Fever

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Abstract

The general objective was to evaluate the *in vitro* activity on *Salmonella enterica* of *typhi* stereotype of plant drugs used in the treatment of typhoid fever. The crop drugs harvested were processed and dried for three weeks. After maceration in ethanol, the crude extracts were concentrated using a rotary evaporator and a phytochemical screening was carried out. The antimicrobial activity of the ethanolic extracts was evaluated *in vitro* on *Salmonella enterica* of *typhi* stereotype, by the method of microdilution in liquid medium on microplates of 96 wells in U bottom. Seven possibilities were offered for this evaluation in the microplates: three first (E_1, E_2 and E_3) concerned the isolated extracts and the four others (E_1 + E_2 + E_3, E_1 + E_2, E_1 + E_3 and E_2 + E_3) combinated extracts. The observation of the turbidity of the microplate liquid media after 24 h of incubation allowed the determination of the minimal inhibitory concentrations (CMI) and the seeding of the wells on solid media HEKTOEN and EM allowed the determination of the minimal bactericid concentrations (CMB). Characterization of the different groups of secondary metabolites in isolated ethanolic extracts revealed the presence of phenols, steroids, triterpenes, coumarins and alkaloids. Isolated extract of *Costus afer* showed a very low CMI and CMB with values of 0.07 and 0.15 mg/mL and was more active on Salmonella enteric typhi stereotype than the other two isolated extracts. Combination of *Costus afer* and *Annickia chlorantha* (E_1 + E_3) stems showed low CMI and CMB with values of 0.15 mg/mL and were more active on *S. enterica* of *typhi* stereotype. The combinated extracts showed CMB values between 0.15 and 0.60 mg/mL versus 0.15 and 1.25 mg/mL for the isolated extracts. Analysis of CMI and CMB has shown that combinated extracts have more effective antibacterial activities than isolated extracts because of the positive interactions between bioactive contents involved, and should be considered in *in vivo* pharmacological activities in rats.

Keywords: *Annickia chlorantha*, *Alstonia boonei*, *Costus afer*, phytochemical screening, antibacterial activity, *Salmonella enteric typhi* stereotype.

INTRODUCTION

In developing countries, traditional medicine is the first resort for over 80% of the population, due to its ease of geographical, economic and cultural access [1, 2]. This situation prevails especially in rural areas, characterized by a shortage of doctors, with an average ranging from 0.5 to 10 doctors per 100,000 inhabitants [3, 4]. The importance of this medicine depends on the specific diversity of the local flora present and the knowledge held by the tradipratricians on the medicinal use of the concerned plants [5]. In 2011, an ethnobotanical survey carried out in the markets of Douala revealed the importance of polytherapy in the traditional management of typhoid fever. Three plants reappeared in the recipes proposed by traditional healers: *Annickia chlorantha* (Annonaceae), *Alstonia boonei* (Apocynaceae) and *Costus afer* [6]. Typhoid fever, caused by *Salmonella enteric typhi* sterotype, is endemic in developing countries and poses a major public health problem (540 cases/100,000 inhabitants) [7]. The considerable improvement in hygiene conditions in developed countries has made it possible

to virtually disappear from this infection, which has become, for the most part, an important pathology [8-10]. However, Africa is not immune to typhoid fever, given the climatic conditions and standard of living of its inhabitants [11]. Typhoid fevers are common in all parts of Cameroon. Its incidence is 16 to 20 cases/100,000 inhabitants. In the Far North of the country, in the health area of Doualare its incidence was about 400 cases from 2009 to 2012 [12].

Treatment with modern medicine uses antibiotherapy as an essential element with chloramphenicol, which remains the reference antibiotic in the treatment of typhoid fever. Amoxicillin, cotrimoxazole, ceftriaxone and fluoroquinolones have been shown to be very effective in reducing the duration of treatment and the frequency of relapses. But their high cost remains an obstacle to wider use [13]. In traditional medicine, the proposed treatments concern recipes based on several plants, hence the term of polyherbal medicine used to designate this method of treatment. To contribute to the valorization of this therapy, the present work was undertaken. The general objective was to evaluate the *in vitro* activity on *Salmonella enterica* of *typhi* stereotype of herbal medicines used by traditional healers (herbal drugs) during the traditional management of typhoid fever. The specific objectives were to: identify the different groups of secondary metabolites of the ethanolic extracts of the *Annickia chlorantha* barks, *Alstonia boonei* barks and the *Costus afer* stems; *in vitro* determination of minimal inhibitory concentrations (CMI) and minimal bactericid concentrations (CBM) of the isolated extracts and combinated extracts and their activity on *Salmonella enterica* of *typhi* stereotype.

METHODS AND MATERIAL

Material

Plant material

Plant material harvested from a degraded tropical rain forest in the Region of Coast included *Annickia chlorantha* barks, *Alstonia boonei* barks and *Costus afer* stems.

Technical Material

Technical equipment consisting of extraction equipment and active ingredients included: a SF-400 A brand precision balance, for mass gain; plastic buckets with lid, for maceration; a grinder, for the transformation of drugs into fine powder; filter papers, to separate the filtrates from the marc; the plastic funnel, to help with filtration; 500 mL glass jars, to collect the filtrate before concentration; 125 mL flasks, to collect concentrated extracts; spatulas, for the homogenization of the powders intended, for the preparation of the culture mediums, and the transport of the samples; 50 mL Erlenmeyer flasks, 125 mL, 250 mL, to homogenize the samples without splashing and transfer them to the flasks; 25 mL, 100 mL, 200 mL beakers, to collect the solvent, and to heat the samples; the BÜCHI R-205 rotary evaporator, to concentrate the extracts; plastic bottles for the recovery of evaporated solvents; 500mL flasks, to homogenize the environment and promote evaporation; 1000 mL, to collect the condensed solvent and the solvent, ethanol at 96 °, for extraction.

Material of Bacterial Activity

The material of bacterial activity included a bacterial strain of *Salmonella enterica* of typhi stereotype from the Pasteur Center of Yaounde was characterized at the Douala General Hospital. The culture media included LIOFILCHEM laboratories EMB, LIOFILCHEM laboratories' Muller Hinton broth of LIOFILCHEM Laboratories and KEKTOEN culture medium of LIOFILCHEM Laboratories; an autoclave manufactured by JOUAN ASTEL set at 37 °C, to sterilize the culture mediums and soiled samples; a refrigerator manufactured by FACIS with a frequency of 50 to 60HZ, to preserve culture media and samples; micropipettes, for the preparation of inoculums, the filling of wells; the petri dishes, for the transplanting of the strain, their cultivation; culture tubes for suspending germs; test tubes, to measure turbidity, collect culture broths, inoculums and extracts; the densitometer, to measure the turbidity of the bacterial suspension; U-bottom microplates to test the inhibitory activity of the samples; the calibrated loop, for sowing; the bunsen burner, to provide a sterile work environment on a specific radius.

METHODOLOGY

The study carried out was of the experimental type and was carried out from November 2016 to May 2017. The extraction and the concentration were carried out respectively at the Laboratory of Biology and Physiology of Plant Organisms of the Faculty of Science, the University of Douala. The antimicrobial activity of the extracts was made at the Laboratory of Bacteriology of the General Hospital of Douala.

Extraction

Botanical identification

The samples were confirmed at the Cameroon National Herbarium in Yaounde, where the specimens were confronted with identification numbers: *Annickia chlorantha* (32065/HNC) trunk bark, *Alstonia boonei* (43364/HNC) trunk bark, *Costus afer* (66605/HNC) stems.

Preparation of Plant Material

The bark fragments of *Annickia chlorantha* and *Alstonia boonei* were cleaned and then cut, while the stems of *Costus afer* were peeled and cut into slices to facilitate drying. It was done in lasted for three weeks, sheltered from the sun. At the end of it, the plant material was ground into a fine powder, using an electronic mill.

Extraction

The powders obtained were subjected to extraction by successive macerations using ethanol at 96 ° as a solvent. Each of these drugs was macerated in a container, for 48 hours with manual and constant agitation, at room temperature (25 °C) of the Laboratory of Biology and Physiology of Plant Organisms, the University of Douala and at the shelter of the light. The extraction was repeated three times for each drug. After each maceration, the whole was filtered in order to separate the marc from the filtrate.

Extracts Concentration

The filtrates were evaporated using a rotary evaporator to obtain almost dry extracts. The yield of each extraction was calculated by the following formula:

$$R = \frac{\mathrm{m}}{\mathrm{M}} \times 100$$

Where,

m: mass of the extract

M: mass of initial powder

Phytochimical Screening

Different groups of secondary metabolites were investigated by the method described by Harbone [14].

Antibacterial Test

The antibacterial test determined the minimal inhibitory and minimal bactericid concentrations (CMI and CMB) of the extracts.

Preparation of Sample Extracts and the Microplates Sterility Test

Each crude extract was diluted in distilled water and then inoculated in the same poly Vitex solid culture medium (PVX), which is a non-selective medium allowing all the germs to grow. The extracts thus seeded were put into the incubator.

Obtention of Samples for the Tests

The weighing of 62.5 mg of each crude plant extract was followed by their dilution in 25 mL of distilled water associated with 10% dimethylsulfoxide (DMSO). Thus, for each extract, samples were obtained at 2.5 mg mL. The latter were packaged in glass bottles of 30 mL capacity with polystyrene stopper. The preservation was done at a temperature of 4 °C.

Bacterial Strain

The bacterial strain was streaked and incubated at 37 °C for 18-24 hours to obtain young and well isolated colonies which were subsequently used for the preparation of the inoculum.

Preparation of the inoculum

From a pure culture of 18 to 24 hours seeded on a selective solid medium (HEKTOEN ENTERIC AGAR), 1 to 3 well isolated colonies were removed with a loop and suspended in 4 mL of physiological saline solution. The turbidity of the suspension was adjusted to 0.5 Mac Farland, using a densitometer. This *Samonella enterica* of *typhi* stereotype value corresponded to 1.5 × 108 CFU/mL. Then 100 µL of the culture solution of *Salmonella enterica* of *typhi* stereotype was removed and introduced into a tube containing 10 mL of BMH. Then the second-order dilution of the samples in the wells was performed. The 100 µL volums of BMH were placed in wells 1, 9, 11 and the distilled water was placed in well 12 with the samples. Then, 100 µL of sample was introduced into well 1 and then successive dilutions were made (100 µL of sample well 1 to well 9). Volums of 100 µL of bacterial inoculums (whose concentration is 104 bacteria/mL) were placed in wells 1 to 9 and also in well 1L to obtain a total volume of 200 µlL (Table-1).

Table-1: Second order dilution of samples in wells

	Extract (mg/mL)	1	2	3	4	5	6	7	8	9	10	11	12
A	E_1	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-	CC	SC
B	E_2	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		
C	E_3	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		
D	$E_1 + E_2$	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		
E	$E_1 + E_3$	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		
F	$E_2 + E_3$	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		
G	$E_1 + E_2 + E_3$	2.50	1.25	0.60	0.30	0.15	0.07	0.04	0.02	9.3×10^{-3}	-		

CC: culture control; SC: sterility control; E_1: ethanolic extract of *Costus afer*; E_2: ethanolic extract of *Alstonia boonei*; E_3: ethanolic extract of *Annickia chlorantha*

When the inoculation was completed, the microplate was incubated in the oven at 37 °C for 24 hours after observing the starting clarity. The experiment was done three (03) times.

Data Analysis

The data was entered in Excel 2007 (Microsoft Office, USA) and analyzed with SPSS software version 16 (SPSS, Inc., Chicago, IL, USA). The descriptive statistics concerned the presentation of data as a percentage and mean ± standard deviation (ds) in graphs (2D histograms). The Mann-Whitney test was used to compare the CMI and CMB of different plant extracts. The significance level was set at a p-value <0.05.SPSS = Statistical Package for Social Sciences.

RESULTS AND DISCUSSION

Results

Extraction yield

The masses, yields, appearance and color of the ethanolic extracts of the crude *Annickia chlorantha*, *Alstonia boonei* barks and *Costus afer* stems were determined (Table-2).

Table-2: Mass, yield, appearance and color of crude extracts *of Annickia chlorantha* barks, *Alstonia boonei* barks and *Costus afer* stems

Drogues	Mass of extract (g)	Yield (%)	Appareance	Color
Annickia chlorantha trunk bark	11.58	1.30	Pasty	Honey color
Alstonia boonei trunk bark	6.8	0.70	Pasty	Brown
Costus afer stem fragment	0.9	1.25	Tighting	Olive green

Phytochimcal Screening

Phytochemical screening allowed to characterize the different secondary metabolites (Table-3).

Table-3: Qualitative chemical analysis of some secondary metabolites of crude extracts

Secondary metabolites secondaires	*Annickia chlorantha* barks	*Alstonia boonei* barks	*Costus afer* stems
Triterpenes/ stéroids	_	+	+
Phenols	+	_	++
Coumarins	_	+	_
Alcaloids	++	+	_

(-): absence; (+): trace; (++): Abundance

Determination of CMI

The CMI of the isolated extracts and the CMI of the combined extracts were determined in the graph of the CMI of the samples (Figure-1).

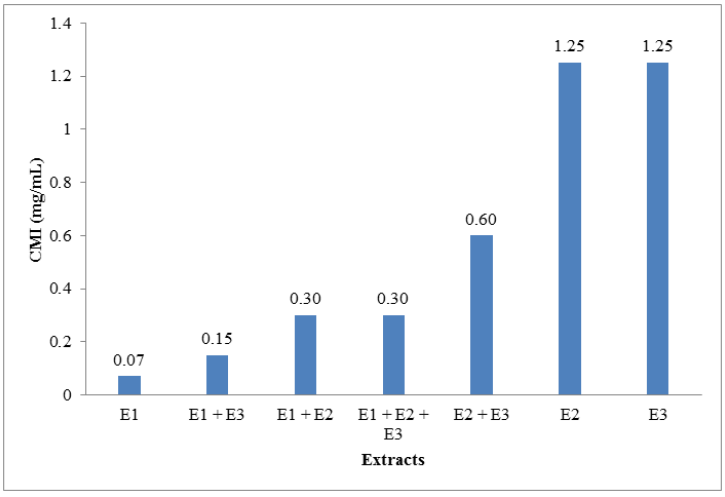

Fig-1: Graph of CMI of samples

The CMI of the combined extracts are lower than the CMI of the isolated extracts with values between (0.15; 0.60) against (0.07; 1.25), so the combined extracts would be more active than the isolated extracts. The CMI values of the isolated and combined samples were compared using the Mann-Whitney test (Table-4).

Table-4: Comparison of CMI of isolated extracts and combined extracts

	E_1	E_2	E_3	$E_1 + E_2$	$E_1 + E_3$	$E_2 + E_3$	$E_1 + E_2 + E_3$
E_1	1						
E_2	0.0253	1					
E_3	0.0253	1	1				
$E_1 + E_2$	0.0253	0.0253	0.0253	1			
$E_1 + E_3$	0.0253	0.0253	0.0253	0.0253	1		
$E_2 + E_3$	0.0253	0.0253	0.0253	0.0253	0.0253	1	
$E_1 + E_2 + E_3$	0,0253	0.0253	0.0253	1	0.0253	0.0253	1

The Mann-Whitney test was used to make the comparisons; p-value <0.05 are considered statistically significant

The samples E_2 and E_3 have been presented as having equivalent CMI values, just as this has the combinations $E_1 + E_2$ and $E_1 + E_2 + E_3$.

Determination of CMB

Experimental wells were seeded on HEKTOEN and EMB media. The purpose of this manipulation was the determination of minimal bactericid concentrations (CMB). The last solid medium seeding trait with no bacterial outbreak corresponded to CMB. The CMB of the isolated extracts and those of the combined extracts were summarized in the graph of CMB comparisons (Figure-2).

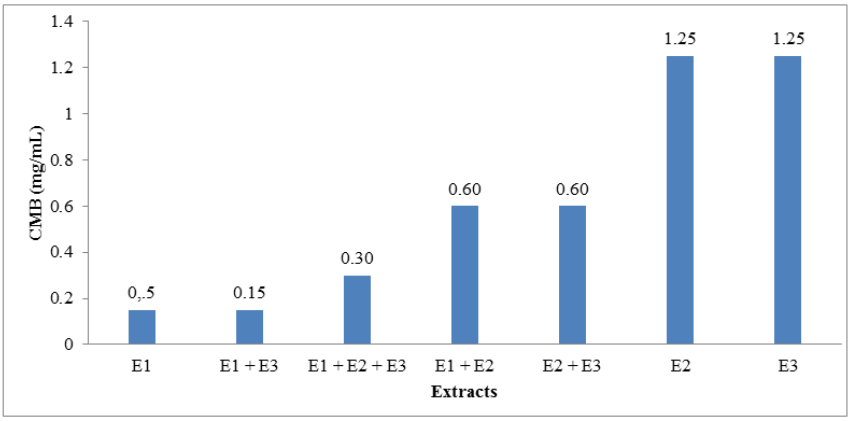

Fig-2: Graph of CMB comparisons of isolated exacts and combined extracts

The graph of CMB comparisons of isolated extracts and combined extracts showed, like that of the CMI graphs, that the combined extracts had the lowest CMB between (0.15; 0.60) against (0.15; 1.25) for the isolated extracts. The combined extracts would be more active than the isolated extracts. The CMB from the isolated and combined extracts were summarized using the Mann-Whitney test (Table-5).

Table-5: Comparison of CMB from isolated extracts and combined extracts

	E_1	E_2	E_3	$E_1 + E_2$	$E_1 + E_3$	$E_2 + E_3$	$E_1 + E_2 + E_3$
E_1	1						
E_2	0.0253	1					
E_3	0.0253	1	1				
$E_1 + E_2$	0.0253	0.0253	0.0253	1			
$E_1 + E_3$	1	0.0253	0.0253	0.0253	1		
$E_2 + E_3$	0.0253	0.0253	0.0253	1	0.0253	1	
$E_1 + E_2 + E_3$	0.0253	0.0253	0.0253	0.0253	0.0253	0.0253	1

The Mann-Whitney test was used to make the comparisons; p-value <0.05 are considered statistically significant. The isolated extracts E_1 and E_3 had equivalent CMB values, this was also the case of the isolated extract E_1 and the combination E_1+ E_3 and finally the combinations $E_1 + E_2$ and $E_2 + E_3$. The summary summarizes the different CMI and CBM values of the isolated extracts and combined extracts (Table-6).

Table-6: CMI and CMB obtained

Samples (mg/mL)	CMI	CMB
E_1	0.07	0.15
E2	1.25	1.25
E_3	1.25	1.25
$E_1 + E_2$	0.30	0.60
$E_1 + E_3$	0.15	0.15
$E_2 + E_3$	0.60	0.60
$E_1 + E_2 + E_3$	0.30	0.30

E_1: Isolated extract of *Costus afer*; E2: Isolated extract of *Alstonia boonei*; E3: Isolated Extract of *Annickia chlorantha*; E1 + E2: Combinated extract of *Costus afer* and *Alstonia boonei*; E1 + E3: Combinated extract of *Costus afer* and *Alstonia boonei* ; E2 + E3: Combinated extract of *Alstonia boonei* and *Annickia chlorantha* ; E1 + E2 + E3: Combinated extract of the three of *Costus afer*, *Alstonia boonei* and *Annickia chlorantha*

DISCUSSION

Phytochemical screening of the crude extracts tested revealed the presence of alkaloids, coumarins, phenols, triterpenes and steroids. Many molecules belonging to these groups of compounds are responsible for several biological activities, including antibacterial activity [15].

The extracts of *Annickia chlorantha*, and *Alstonia boonei* had a positive reaction to alkaloid tests [16, 17]. Quaternary alkaloids are often present in plants in the form of salts which may be dyeing principles of basic yellow dyes (the honey color of the raw extract of *Annickia chlorantha* bark). These include berberine, jatrorrhizin, columbamine and palmitin. Alkaloids with indole and steroid nuclei were considered as chemotaxonomic markers of Apocynaceae). The presence of this group of compounds is justified in *Alstonia boonei* extract [17]. The crude extracts of *Annickia chlorantha* and *Costus afer* contain phenolic compounds including tannins, which confer astringent, antidiarrheal, healing and anti-haemorrhagic properties [18].

The E_1 isolated extract from the *Costus afer* sample had the CMB of 0.15 mg/mL lower than the CBM of the E_2 and E_3 isolated extracts respectively of the *Alstonia boonei* and *Annickia chlorantha* samples which were 1.25 mg/mL. This could be due to the high phenol contents in the *Costus afer* extract revealed by phytochemical screening. The compounds with the highest antimicrobial efficacy and the broadest spectrum are phenols (thymol, carvacrol and eugenol). Phenols cause irreversible lesions on the membranes and are useful in bacterial, viral and parasitic infections whatever their location [19].

The most active combinated extract was (E_1+E_3), in other words the one that corresponded to the combinated extract of *Costus afer* and *Annickia chlorantha*. The increase in the CBM of the other combinations would be due to the combination of three extracts (E_1 + E_2 + E_3) to the decrease in the bioactive contents of one of them, despite the persistence of interactions between bioactive contents. For combinated extracts (E_1+E_2) and (E_2+E_3), there would also be a halving of the content of bioactive contents, thus limiting the important interactions for biological activity [20, 13]. Combinated extracts provided better antibacterial activity than isolated extracts. This could be explained by the diversity of intermolecular interactions offered by the combinated extracts [21]. Polyherbal therapy is the method used by traditional healers in the traditional management of typhoid fever. Of the plants used in a recipe, one or more are often primary and act on the causative agent, while the other associated plants deal with the major symptoms of the pathology. In the recipe of this study, the main plants could be *Costus afer* and *Annickia chlorantha* because of their too low CMB equivalent to 0.30 mg/mL. *Alstonia boonei* would then be the accessory plant that would contribute in the management of major symptoms. Sometimes the symptoms of typhoid fever are not very specific, this has often been the cause of a confusion of diagnosis with malaria, leptospirosis or viral hepatitis [13]. *Alstonia boonei* barks have been shown to have antidiarrheal and antimalarial activities, however with a lower effect than cinchona. *Alstonia boonei* the barks have been also used for the relief of rheumatic pains and several other pains [6]. *Annickia chlorantha* stems have been widely used in traditional herbal medicine for the treatment of jaundice, malaria, fever and viral hepatitis [6]. All this could justify the use of the combination of the three plants: *Annickia chlorantha*, *Alstonia boonei* and *Costus afer* in the traditional management of typhoid fever caused by *Salmonella enteric* of *typhi* stereotype.

CONCLUSION

The overall objective was to evaluate the *in vitro* activity of *Salmonella enterica* of *typhi* stereotype of medicinal plant drugs used in the treatment of typhoid fever, in traditional medicine. Characterization of different groups of secondary metabolites in the ethanolic extracts of *Annickia chlorantha*, *Alstonia boonei* and *Costus afer* stems revealed the presence of phenols, steroids, triterpenes, coumarins and alkaloids. Phenols and alkaloids have been found to have high antimicrobial potential. Analysis of CMI and CBM showed that the combinated extracts have more effective antibacterial activities than isolated extracts because of the positive interactions between bioactive contents involved and should be considered in *in vivo* pharmacological activities in rats.

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