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Antimicrobial Activity of Single And Combined Extracts of Medicinal Plants From Cameroon

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Abstract: *Selected Cameroonian plants have been investigated for their antifungal and antibacterial activity against five species: four bacteria, namely S. aureus, S. pyogenes, S. mutans, Pseudomonas aeruginosa and the yeast C. albicans. The solvents used for plant extraction were either ethanol or water. The in vitro antimicrobial activity was performed by disk diffusion and microdilution method. The aqueous extracts showed no activity whereas the ethanolic extracts showed a significant antibacterial activity, which may be associated to the high content in tannins shown by some of the extracts. In conclusion, this work adds to the accumulating evidences supporting the development of new alternatives to antibiotics based on the use of natural products.*
Keywords: *antibiotics, MIC, phytochemical investigation, Streptococcus mutans*

I. Introduction

According to the world health organization (WHO), medicinal plants represent an essential source drugs. In developed countries, it is estimated that 80% of individuals use traditional medicine [1]. In African societies, medicinal plants have played an important role and their value is appreciated to date. In most African countries there is a long tradition in applying plants as medicines and such practice has been transmitted from generation to generation. Medicinal plants are often prescribed by traditional healers in their homes and sold in marketplaces [2]. In Cameroon medicinal plants are traditionally used by local population and herbalists to treat infectious diseases. They may be used as infusions for systemic infections or directly applied on the skin to cure local infections [3]. It has been reported that during the last 20 years plants, which are claimed to possess antimicrobial activity, have gained bigger interest. This is mainly due to the consternating increase of human infectious diseases rate, especially in tropical and subtropical area [4]. An additional concern is related to the increasing use of antibiotics and the associated phenomenon of antibacterial resistance. Moreover several other clinical problems may also appear during treatment due to associated side effects, such as allergic reactions [5]. Hence the search for alternatives to classical antimicrobial drugs is an urgent topic. The purpose of this study was to investigate the antimicrobial activities of single extracts of Cameroonian plants selected on the basis of their traditional medical use and determine the antimicrobial activity of some extract combination thereof. The rationale behind the combination studies is that medicinal plants are often prescribed by local healers in preparations that contain more than one plant. Even different formulations available in African local markets consist of a mix of several plants. Most scientific reports focused on the antimicrobial activity of single plant extracts or plants-antibiotics combinations. Only few studies have considered combinations of different plant extracts [6,7]. Based on this evidence it seemed opportune to investigate whether the combination of plant extracts might be synergistic, additive or antagonistic. This study has also evaluated the phytochemical composition of the plant extracts in searching for classes of components giving a major contribution to the antibacterial activity.

II. Material and methods

2.1 Plant collection

The selected plants used in this work were purchased from Cameroonian local markets (under the recommendation of the local traditional healer). The collected plants are listed in Table 1.

Table 1. Plant Species and their uses

Plant species	Family	Part used	Therapeutic use
Aframomum citratum	Zingiberaceae	Fruits	Fever, intercostals pains, tonic, aphrodisiac [8]
Ocimum gratissimum	Labiatae	Leaves	Treatment of fungal infections, fever, cold and catarrh [9]
Ficus exasperate	Moraceae	Leaves	Haemostative ophthalmia, coughs and heamorrhoid. It is also used for treating various infections and as sand paper for polishing woods [10]
Commelina benghalensis	Commelinaceae		Bitter, laxative, beneficial in leprosy [11]
Moringa oleifera	Moringaceae	Leaves	Treatment of infectious diseases of the skin and mucosa such as spots and ringwarm rush; fever, influenza and

			stomach pains [12]
Alchornea cordifolia	Euphorbiaceae	Leaves	Washing sores, urinary tract infections and dysentery. Antibacterial, antimalarial and antitumor activities of leaf methanol extracts [13]
Tithonia diversifolia	Asteracaea	Flowers	Applied externally on wounds, treatment of skin eczema [14]
Cymbopogon citratus	Poaceae	Leaves	Treatment of nervous and gastrointestinal disturbances, and as an antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative [15]

2.2 Extraction procedure

Extraction of plant material was performed using either ethanol or water (20g of plant material in 250 mL of solvent). The mixture was stirred for 2h at room temperature, paper filtered (Whatman filters). The supernatant of the ethanolic extracts were evaporated to dryness under reduced pressure on a rotavapor (Büchi Rotavapor R-200) at 40 °C whereas those from aqueous extraction were lyophilized. The final material was then resuspended in the same solvent used for the extraction at a final concentration of 20 mg/mL and kept frozen at -20°C until use.

2.3 Antimicrobial activity

2.3.1 Bacterial strains

Plant extracts were tested against five species including *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 700294), *S. mutans* (ATCC 700610), *Pseudomonas aeruginosa* (ATCC 27853) and the yeast *Candida albicans* (ATCC 24433). They were maintained on agar Petri dishes at 4°C and sub-cultured on a fresh appropriate agar plates 24 h prior to susceptibility testing.

2.3.2 Disk diffusion method

The antimicrobial activity was assessed by the paper disk diffusion method as indicated by the international guidelines of the CLSI [16]. Briefly, a suspension of 10^8 cells per mL prepared in saline solution (10^6 per mL for *C. albicans*) was spread on the solid media plates using a sterile cotton swab. Sterile filter paper discs (6 mm in diameter) were placed onto the surface of inoculated plates and spotted with 10 µL of extract. The plates were incubated at 35±2°C in normal atmosphere (*Streptococcus spp.* in 5% CO_2 atmosphere). A diameter higher than 6 mm was indicative of growth inhibition. Positive controls were Ciprofloxacin (5µg disk) against *S. aureus*, *S. pyogenes*, *S. mutans*, and *P. aeruginosa* and Nystatin (110 Units disk) against *C. albicans*. Ethanol and water were used as negative controls.

2.3.3 Microdilution method

The MIC of the ethanolic/aqueous extracts was determined by microdilution test following CLSI guidelines. Extracts to be tested were dissolved in ethanol or water (20mg/mL) and then diluted in medium to reach a concentration of 2048 µg/mL. Extracts were serially two fold diluted and one hundred microlitres (100 µL) of inoculum (1.5×10^6 CFU/mL) prepared in medium was then added. The total volume in each well was 200 µL. The medium used was the Muller Hinton broth in the case of *S. aureus*, S. *pyogenes*, and *P. aeruginosa*; RPMI broth was used for *C.albicans.* The endpoint was 24h incubation time (48h for *C. albicans*) and the MICs were determined as the lowest concentration of extract able to inhibit visible growth of the microorganism.

2.4 Phytochenìmical investigations

Qualitative phytochemical tests for the detection of tannins, saponins, steroids/triterpenoids, cardiac glycosides, anthraquinones, alkaloids, phenols and flavonoids were performed following the common methods with some modifications [17,18]. For each phytochemical, a brief description class follows as a list:

Tannins (1 g plant material in 10 mL distilled water); a 2 mL filtrate + 2 mL of 1% $FeCl_3$, blue-black precipitate indicates the presence of tannins.

Saponins (frothing test: 0.5 mL filtrate + 5 mL distilled water); frothing persistence indicates the presence of saponins.Cardiac glycosides (1g plant material in 10 mL ethanol); 2 mL of alcoholic filtrate + 1mL glacial acetic acid + 1-2 drops of 1% $FeCl_3$ were added followed by 1 mL of conc. H_2SO_4. A brown ring at the interface indicates the presence of cardiac glycosides.

Phenols (1 g plant material in 10 mL ethanol); 2 mL filtrate + 1 mL 1%$FeCl_3$. Green or blue color indicates the presence of phenols.Steroids and triterpenoids (1 g plant material in 10 mL chloroform); a 2 mL filtrate + 2 mL acetic anhydride + few drops of conc. H_2SO_4. Blue-green ring indicates the presence of steroids or triterpenoids. Anthraquinones (1 g plant material in 10 mL methanol); a 2 mL filtrate + 2 mL ether-chloroform 1:1 v/v + 4 mL NaOH 10 % (w/v). Red color indicates the presence of anthraquinones. Flavonoids (1 g plant material in 10 mL ethanol); a 2 mL filtrate + few drops of conc HCl followed by the addition of zinc turnings. Magenta red or pink color indicates the presence of flavonoids. Alkaloids (1 g plant material in 10 mL

methanol); a 2 mL filtrate + 1, 5 mL of 1% HCl. After heating the solution in water bath, 6 drops of Mayor's reagent were added. Turbidity or precipitate indicated the presence of alkaloids.

III. Results and Discussion

3.1 Phytochemical composition of plant extracts

Qualitative phytochemical analysis revealed the presence of phenols, cardiac glycosides, alkaloids, steroids and triterpenoids (Table 2). Other secondary metabolites like tannins, saponins, and anthraquinones were present in small traces in some of the plant extracts. A positive correlation was found between the content of tannins and phenols (Pearson correlation coefficient = 0.67; P = 0.017) and between phenols and cardiac glycosides (Pearson correlation coefficient = 0.58; P = 0.049). In general, phytochemical composition differs among plant species. Thus it is not surprising that there are differences in the antimicrobial activity shown by the various plant species. A possible explanation may be that the extraction method influenced the quantity of secondary metabolites. A finding confirming this hypothesis has been reported for *Afromomum citratum* [18]. Conversely, some plants may contain low amount of components with antibacterial activity.

Table 2. Phytochemical composition of selected medicinal plants

Plant species	**Tannins**	**Saponins**	**Phenols**	**Cardiac glycosides**	**Steroids/Triterpenoids**	**Anthraqui-nones**	**Flavonoids**	**Alcaloids**
A. citratum	-	-	-	+	-/-	+	-	-
O. gratis simum	+	-	+		+/+	-	-	+
F. esasperate	++	-	+	++	+/+	-	-	+
C. benghalensis	-	-	+	+	+/+	-	-	+
M. oleifera	++	+	++	++	+/+	-	-	+
A. cordifolia	++	++	++	++	-/+	+/-	-	+
T. diversifolia	+	++	+	+	-/-	-	-	+
C. citratus	+	++	+	++	+/+	-	-	+

+ = trace amount; ++ = appreciable amount; − completely absent

3.2 Antimicrobial activity of plant extracts

The antimicrobial activity of the selected plant species are summarized in Table 3 and Table 4. None of the aqueous extracts showed antimicrobial activity. The differences of solvent related activity could be speculatively explained by the insolubility of active compounds in water. The antimicrobial activity of the identified active components from plants, are aromatic or saturated compounds which are usually obtained by ethanol or methanol extraction [19]. Overall, a correlation between the presence of tannins and antimicrobial activity of some of the extracts was found (Pearson correlation coefficient = 0, 7 – 0, 8; $P < 0.05$). Indeed, it has been previously reported that tannins are able to inhibit bacterial growth causing precipitation of proteins which serve as nutrients for bacteria [20].

Table 3. Ranges of minimum inhibitory concentrations (MIC) of ethanolic extracts from three independent experiments

Plant species	**MIC (µg/mL)**				
	S. aureus **ATCC 25923**	***S. pyogenes*** **ATCC 700294**	***S. mutans*** **ATCC 700610**	***P. aeruginosa*** **ATCC 27853**	***C. albicans*** **ATCC 24433**
Aframomum citratum	**128**	>1024	>1024	>1024	>1024
Ocimum gratis simum	>1024	>1024	>1024	>1024	>1024
Ficus esasperata	>1024	>1024	>1024	>1024	>1024
Commelina benghalensis	>1024	>1024	>1024	>1024	>1024
Moringa oleifera	>1024	>1024	>1024	>1024	>1024
Melaleuca alternifolia	>1024	>1024	>1024	>1024	>1024
Dioscorea bulbifera	>1024	>1024	>1024	>1024	>1024
Alchornea cordifolia	**256-512**	**64-128**	>1024	>1024	>1024
Tithonia diversifolia	>1024	>1024	>1024	>1024	>1024
Cymbopogon citratus	>1024	>1024	>1024	>1024	>1024
Ciprofloxacin	0.5	0.25	n.a.	0.5	n.a.
Nystatin	n.a.	n.a.	n.a.	n.a.	4.0

[a] n.a.: not appropriate

Table 4. Inhibition zone diameter (IZD = mm) of ethanolic extracts

Plant species	***S. aureus* ATCC 25923**	***S. pyogenes* ATCC 700294**	***S. mutans* ATCC 700610**	***P. aeruginosa* ATCC 27853**	***C. albicans* ATCC 24433**
	Mean IZD[a] ± SD	**Mean IZD ± SD**	**Mean IZD ± SD**	**Mean IZD ± SD**	**Mean IZD ± SD**
Aframomum citratum	7.67±0.58	7.67±1.53	8.33±1.53	–	–
Ocimum gratis simum	–[b]	–	–	–	–
Ficus esasperata	–	–	–	–	–
Commelina benghalensis	–	–	–	–	–
Moringa oleifera	–	–	–	–	–
Melaleuca alternifolia	–	–	–	–	–
Dioscorea bulbifera	–	–	–	–	–
Alchornea cordifolia	8.33±0.58*	11.00±1.00*	7.33±1.53	–	6.67±0.58
Tithonia diversifolia	–	–	–	–	–
Cymbopogon citrates	–	–	–	–	–
Ciprofloxacin	20.67±1.15	22.33±0.58	15.33±0.58	28.5±1.25	
Nystatin					16.33±0.58

[a] IZD represents mean values of three different experiments ± standard deviation (SD)
[b] No activity.
* $p \leq 0.05$ = statistically significant if compared to the no activity control.

In particular, it appeared that only the ethanolic extracts of *Aframomum citratum* and *Alchornea cordifolia* prevented the growth of two bacterial strains (*S. aureus* and *S. pyogenes*). *Aframomum citratum* showed antibacterial activity only against *S. aureus* (MIC = 128 µg/mL) whereas *Alchornea cordifolia* inhibited the growth of *S. aureus* and *S. pyogenes* with an MIC = 256 µg/mL and 128µg/mL, respectively. Antibacterial activities of *Aframomum citratum* have been already documented, but tested bacterial species were different [10]. Antibacterial activity of crude extracts was considered to be significant if MIC values were below 100 µg/mL and moderate when in the range between 100 and 625 µg/mL [18]. Consequently antibacterial activities of A. *citratum* (128µg/mL) and *A. cordifolia* (64-128 µg/mL) against *S. aureus* and *S. pyogenes* respectively can be considered almost significant. If the criterion proposed by Fabri and coll. is considered, where MIC values below 8000 µg/ mL are indicative of a significant antibacterial activity [21], the antibacterial activity of *A. citratum* and *A. cordifolia* can be considered significantly high.

Plant combination	***S. aureus ATCC 25923***	***S. pyogenes* ATCC 700294**	***S. mutans* ATCC 700610**	***P. aeruginosa ATCC 27853***	***C. albicans ATCC 24433***
	Mean IZD ± SD	***Mean IZD ± SD***	***Mean IZD ± SD***	***Mean IZD ± SD***	***Mean IZD ± SD***
Aframomum citratum + A. cordifolia	*7,00*±0,00*	*9,67*±0,58*	*10,67**±1,15*	–[a]	–

Table 5. Antimicrobial activity of combination of two ethanolic extracts (50% each)

IZD = inhibition zone diameter expressed in mm
Results are mean values of three different experiments ± standard deviation (SD);*$p \leq 0.05$; ** GIIs > 0, 5
[a] No activity

Combinations of plant extract were performed based on the antibacterial activity of the single extracts summarized in Table 3 and Table 4. *A. citratum* and *A. cordifolia* (50% v/v each) were mixed to evaluate in the antibacterial activity potency. Interpretative criteria for the activity of plant extracts combination were from the work of Mandal and coll. [6]. They proposed to consider as synergistic, additive, and antagonistic the activity of combinations resulting in a growth inhibitory index (GIIs) > 0.5, = 0.5, and < 0.5, respectively. The determination of the GIIs for the combination of *A. cordifolia* and *A. citratum* ethanolic extracts is reported in Table 5. It may be concluded that it is synergistic only in the case of *S. mutans*.

IV. Conclusion

In conclusion the results of the present work provide additional information on the possible use of plant extracts, alone or in combination, in the treatment of infectious diseases. The unraveled synergistic effect of the mixture of *A. cordifolia* and *A. citratum* ethanolic extracts is a proof of concept that a combination may potentiate the antimicrobial activity of each single plant extracts. In the specific case, the demonstrated improved anti-*S. mutans* activity of *A. cordifolia* and *A. citratum* ethanolic extracts' combination may find a possible application in the development of products for oral hygiene.

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