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Antiseptic Activity of *Alchornea cordifolia* (Schumach & Thonn.) Mull.-Arg. (Euphorbiaceae) Aqueous Leaves Extract on Oral and Dental Bacteria

Ngene Jean Pierre[1*], Ngoule Charles Christian[1], Yinyang Jacques[1], Kidik Pouka Marie Cathérine[1], Eyetemou Miguel[1], Etame Loe Gisèle Marie Marguérite[1]

[1]Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box 2701 Douala, Cameroon

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***Corresponding author:** Ngene Jean Pierre
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box 2701 Douala, Cameroon

Abstract

Some infectious oral diseases are treated by medicinal plants in tropical areas, because they are abundant and cheap. The present work aimed to evaluate the antiseptic activity of the aqueous extract of *Alchornea cordifolia* leaves against three oral bacteria recognised as opportunistic pathogens, namely *Streptococcus mitis*, *Staphylococcus epidermidis*, and *Micrococcus luteus*. Fresh leaves of *A. cordifolia* were harvested, dried and pulverised. The extraction was performed by decocting in distilled water. The phytochemical screening was performed on the extract based on principles of colour change, and precipitation. Biological studies consisted in the *in vitro* antiseptic evaluation of the aqueous extract using the agar disc diffusion method, in comparison with a reference antiseptic mouthwash containing chlorhexidine. The extract had a 11.96% yield. The phytochemical screening revealed the presence of alkaloids, phenols, flavonoids, terpenes, tannins, glycosides, coumarins and saponins. The evaluation of the antiseptic activity revealed that the extract is active against *S. mitis* and *S. epidermidis*, and inactive against *M. luteus*. The diameters of inhibition zones were 16.9±1.1mm and 11.2±0.7mm at the concentration of 40mg/ml, and 7.7±0.3mm and 8.5±0.5mm at the concentration of 20mg/ml against *S. mitis* and *S. epidermidis*, respectively. Chlorhexidine did not inhibit *S. mitis* and showed diameters of inhibition of 13.0±1.0mm and 15.1±1.0mm against *S. epidermidis* and *M. luteus*, respectively. The minimal inhibitory concentration of the extract against the 2 sensitive bacteria was 20mg/ml.

Keywords: *Alchornea cordifolia*, phytochemical screening, antiseptic activity, oral bacteria.

INTRODUCTION

Oral health is an essential and integral component of health and well-being. Despite considerable progress in this area, problems remain in both developed and developing countries. Tooth decay and periodontal disease have always been considered the two main conditions in this field [1]. Scientific research has linked these diseases to dental plaque, which a microbial biofilm is made up of microorganisms strongly attached to a solid substrate and to each other using a protein matrix [2]. Reducing this plaque has become the hallmark of preventive dentistry, with the advent of oral antiseptics and the fact that bacteria are causal agents of dental disease [3].

Mechanical oral hygiene procedures, such as regular tooth brushing and the use of dental floss are key methods in controlling plaque growth. However, despite their potential, clinical experience and studies conducted in populations have shown that such methods are not sufficiently used by a large part of the population. Chemical plaque reduction methods, such as mouthwashes, are technically simpler alternatives [3]. Thus, various mouthwashes consist of antiseptic agents, in order, in particular, to prevent the bacterial adhesion which corresponds to the initial stage of dental plaque formation [4]. Of all these agents, chlorhexidine is the most studied and popular active substance, having been recognized as the benchmark against which the effectiveness of alternative anti-plaque agents should be compared [5]. But its use can lead to the development of unwanted side effects such as discoloration of the teeth, a burning sensation, and altered taste [6]. Other active substances have been isolated from antiseptic agents, such as thymol, eucalyptol, menthol, and methyl

salicylate and cetylpyridinium chloride. However, limitations were observed in their use in formulations, namely the discoloration also observed with chlorhexidine, as well as high alcohol content. An effective substitute for chlorhexidine is therefore highly desirable [7].

Many common oral infections are treated in the world in general and in Africa in particular with medicinal plants because of their availability and low cost [8]. Thus, in order to alleviate the side effects of chlorhexidine and to promote local medicinal plants, this work has focused on *Alchornea cordifolia* (Schumach. & Thonn), Müll.-Arg. The *Euphorbiaceae* family to which this species belongs is one of the most representatives of the thalamiflora series. The choice of this plant is based on its traditional use, its abundance in tropical Africa, chemo-taxonomic data, and previous work that recognizes this plant's anti-inflammatory, antibacterial, antifungal and antiparasitic properties, among others [9-14]. Species of the genus *Alchornea* possess a wide variety of biological activities, and, in this sense, several studies have validated their ethnopharmacological uses throughout the world, particularly in tropical regions of Africa (Nigeria, Congo and Côte d 'Ivory). Pharmacologically active molecules have also been identified in different parts of plants (leaves, bark, or root).

A. cordifolia is widely used in Africa, alone or in combination with other plants. All organs are used primarily in the fresh or dry state. The leaves decoction is used in Ivory Coast, Burkina Faso and the Central African Republic as an anti-dysenteric agent, against stomach aches in Ghana and as an emmenagogue. The leaf powder applied locally would produce rapid healing of wounds and ulcers [10].

The present work aimed to perform *in vitro* study of the antiseptic activity of the aqueous extract of *A. cordifolia* leaves against opportunistic oral pathogens.

MATERIALS AND METHODS

Harvest

Whole plant samples were collected and stored, then sent to the Cameroon National Herbarium for identification under number 40512/HNC. The fresh leaves of *Alchornea cordifolia* were harvested, then dried for two weeks in open air and protected from the sun.

Aqueous extraction

The extraction was done according to the procedure described elsewhere [15]. Briefly, the dried leaves were pulverized. The resulting fine powder was weighed, then decocted in distilled water. The resulting decoction was filtered and the filtrate was evaporated using a water bath to obtain the dry aqueous extract. The extraction yield (EY) was determined by the formula below:

$$EY = \frac{EM}{PM} \times 100$$

EM: mass of dry extract

PM: mass of dry leaves powder

Rd: rendement de l'extraction en pourcentage

Phytochemical screening. The phytochemical screening was carried out according to the Harborne method. Three principals were used: precipitation, turbidity and colorimetry.

For the identification of alkaloids, an amount of extract (0.5g) was dissolved in 1 ml of distilled water. A few drops of 2% sulfuric acid were added, followed by a few drops of Mayer's reagent. Obtaining a white precipitate or turbidity indicated the presence of alkaloids.

For the identification of phenolic compounds, a few drops of $FeCl_3$ were added to 5 ml of extract or compound solubilized in methanol. The formation of a blue or purple complex indicated the presence of the phenolic compounds.

In addition, for the specific case of flavonoids, a few drops of a 1/10 soda solution were added to the methanolic solution of dry extracts. The yellow-orange colour characterized the presence of the flavonoids.

In the case of tannins, 50 mg of extract were added to 5 ml of distilled water. The mixture was heated in a water bath for 5 min and filtered after cooling. Then 4 drops of 0.5% $FeCl_3$ were added to 2 ml of the filtrate. The presence of tannins resulted in the appearance of a navy-blue coloration.

A few drops of 10% potash were added to 5 ml of a methanolic extract solution. The appearance of a coloration varying from blue to purple yellow indicated the presence of coumarins.

Saponins were identified as follows: 50 mg of dry extract were dissolved in 5 ml of distilled water. The mixture was heated in a water bath for 5 min. After stirring, a foam index of less than 2 cm indicated the presence of saponins.

For triterpenes and sterols, an amount of product was dissolved using an appropriate solvent in a haemolysis tube. A few drops of Libermann-Buchard reagent (1 ml of concentrated H_2SO_4, 20 ml of acetic anhydride, 50 ml of $CHCl_3$) were added to the solution, giving a purplish colour indicating the presence of triterpenes, or a bluish-green colour indicating the presence of sterols.

As for the glucosides, 50 mg of the extract was dissolved in 1.5 ml of 5% HCl, and this mixture was neutralized with 2.5 ml of 5% NaOH, and then filtered after homogenization. Then, the hot Fehling (A and B) liquor test was carried out. The appearance of a brick red precipitate indicated the presence of the glucosides.

The identification of anthraquinones required the addition of 5 ml of 10% ammonia to 5 ml of the dry extract in aqueous solution. The appearance of a yellow or orange colour indicated the presence of bound quinones, while a purplish red colour indicated the presence of free quinones.

Finally, a few drops of dilute HCl and a few drops of ammonia were added to 5 ml of aqueous solution containing the extract. A turn of the solution to red after addition of HCl and subsequently to blue-purplish-greenish after addition of ammonia indicated that it contained anthocyanins.

Preparation of extract solutions for antiseptic test

The extract solutions were prepared to an initial concentration of 40 mg/ml (stock solution S_0), then filtered. The sterility of the extract solutions was verified by inoculating aliquots of extract on Mueller-Hinton (MH) agar. From the S_0, a series of 5 successive dilutions by geometric progression of ratio 2 was carried out so as to obtain a range of final solutions S_0 to S_5 at 40, 20, 10, 5, 2.5 and 1.25 mg/ml, respectively. The reference antiseptic solution was prepared according to its instructions for use indicated in the package leaflet, i.e. 20 ml of Eludril® solution (20% chlorhexidine), diluted to the top line of the stopper with distilled water.

Antiseptic tests

Antimicrobial testing was performed using the MH solid medium disc diffusion method. Each step was repeated 3 times to obtain a statistical average.

Preparation and inoculation of the inoculum

A few pure 24-hour colonies of 3 Gram-positive cocci, including two facultative anaerobes (*Streptococcus mitis* and *Staphylococcus epidermidis*), and one strict aerobic (*Micrococcus luteus*), all isolated from caries lesions and identified by biochemical tests, were collected using a sterile loop and inoculated in a small quantity of physiological water to obtain a turbidity of 0.5 McFarland (corresponding to $1\text{-}2x10^8$ CFU/ml). The inoculum was then seeded triply on MH agar in Petri plates.

Presumptive test

S_0, with the highest concentration tested, was used to assess the sensitivity of the sprouts to the aqueous extract of *A. cordifolia* leaves, as well as 20% chlorhexidine solution. Discs of 6 mm in diameter soaked in S_0 solutions and in chlorhexidine were applied to the surface of the culture medium and the plates were incubated at 37°C for 24 hours. The zone of inhibition around each disc was read using a calliper.

Determination of mean inhibitory concentrations (MIC)

Only bacterial strains that were inhibited by S_0 were retained in this part of the study. 6mm diameter discs soaked in *A. cordifolia* extract solutions S_0 to S_5 and in chlorhexidine were applied to the surface of the culture medium and the plates were incubated at 37°C for 24 hours. The zone of inhibition around each disc was read using a calliper, and MICs were determined for each bacterial strain.

DATA ANALYSIS

The method of analysis used was statistical. The data were recorded in Microsoft Excel 2013, then processed and analysed by the mean and standard deviation analysis test.

RESULTS AND DISCUSSION

Aqueous extraction

Alchornea cordifolia leaves extract was obtained from dried leaves decocted at 10% in distilled water for 30 minutes. The leaves were chosen for the relative stability of secondary metabolites, especially when they are intended to be used as antimicrobial agents [16]. The extract obtained was pasty in consistency with a dark brown colour. A dry extract of 119.6 g was obtained, for a mass of plant powder of 1000 g, i.e. a yield of 11.96% similar to that obtained by other authors ([10, 12]).

Phytochemical screening

The phytochemical screening consisted in revealing the presence or absence of major biochemical families contained in the extract obtained. The results obtained (Table 1) suggest that the leaves of *A. cordifolia* contain almost all of the secondary metabolites sought, anthraquinones and anthocyanins being the only families lacking.

Table-1: Phytochemical screening of *Alchornea cordifolia* leaves aqueous extract. (+) = presence; (-) = absence.

Secondary metabolites	Presence / Absence
Alkaloids	+
Terpenes	+
Sterols	+
Glucosides	+
Saponins	+
Phenols	+
Flavonoids	+
Tannins	+
Coumarins	+
Anthraquinones	–
Anthocyanins	–

Similar results were obtained in qualitative tests on leafy branches of *A. cordifolia* which showed the presence of the same classes of secondary metabolites, with the exception of saponins [10]. The presence of anthraquinones and anthocyanins, as well as that of sterols has been noted by other authors [17]. These discrepancies may be due to the various places of harvest, constituting the plant's immediate environment, and which could push it to synthesize certain secondary metabolites, especially during tissue damage or attacks by various pathogens [18].

The classes of secondary metabolites found are well-known antimicrobial agents. Flavonoids are synthesized by plants in response to microbial infection, so it is not surprising that they have been shown to be effective antimicrobial substances against a wide range of microorganisms *in vitro*. Tannins boost the immune system by stimulating macrophages and cell-mediated immunity [19]. The latter have been used traditionally for the protection of inflamed surfaces of the mouth and the treatment of wounds, haemorrhoids, and diarrhoea [20]. Coumarins stimulate macrophages, which would have an indirect negative effect on infections [21].

Evaluation of antiseptic activity

The presumptive test consisted in evaluating the sensitivity of the sprouts to *A. cordifolia* aqueous leaves extract, at a maximum tested concentration of 40 mg/ml, against a reference product containing chlorhexidine. The tests were carried out on 3 bacterial strains of Gram-positive cocci, including two facultative anaerobes (*Streptococcus mitis* and *Staphilococcus epidermidis*), and one strict aerobic (*Micrococcus luteus*), isolated from caries lesions and identified by biochemical tests. The sensitivity of the bacteria was reflected in the presence of zones of inhibition around the discs soaked in the 2 solutions, on Mueller-Hinton (MH) agar. The results (Table 2) suggest that 2 of the 3 bacterial strains tested were sensitive to the extract, namely *S. epidermidis* and *S. mitis*. The bacterial strain *M. luteus* was insensitive to the extract.

Table-2: Presumptive test. AI= Absence of inhibition

Bacterial strains	Inhibition diameter (mm)	
	S_0	Chlorhexidine
Streptococcus mitis	16,9±1,1	AI
Staphylococcus epidermidis	11,2±0,7	13,5±1,5
Micrococcus luteus	AI	15,1±1,0

The bacterial strain *S. mitis* was found to be the most sensitive to the *A. cordifolia* leaf extract, with an inhibition diameter of 16.9 ± 1.1 mm, but was unresponsive to chlorhexidine, with no inhibition. The *S. epidermidis* strain was sensitive to both the extract and chlorhexidine, with greater sensitivity noted to chlorhexidine. The *M. luteus* strain was insensitive to the extract, with no zone of inhibition, but sensitive to chlorhexidine, with an inhibition diameter of 15.1 ± 1.0mm.

The results obtained from the presumptive test suggest that 2 of the 3 bacterial strains tested are sensitive to *A. cordifolia* aqueous leaves extract. These are the 2 facultative anaerobic bacteria: *S. mitis* and *S. epidermidis*. The measured inhibition diameters were 16.9 ± 1.1mm and 11.2 ± 0.7mm respectively. The strictly aerobic *M. luteus* was found to be insensitive at the maximum concentration used (40 mg/ml). These results suggest that the extract is active on facultative anaerobic oral bacteria and inactive on strict aerobes, and are of interest, since most bacteria that colonize the tooth surface are facultative anaerobes [22].

These results are similar to those of other authors who have demonstrated the sensitivity of clinical strains of *Streptococcus* and *S. epidermidis* to the hydroalcoholic extract of *A. cordifolia* leaves [13]. On the other hand, these authors have demonstrated the sensitivity of *M. luteus*, which contrasts with our

results. This could be explained by the different origins of the strains used. In fact, these authors worked on a strain from the environment, while we performed our test on a clinical strain found on the dental surfaces and living within a biofilm. This confirms the theory that bacteria living within biofilms exhibit increased resistance to antimicrobial agents, compared to bacteria living in the planktonic state, since microorganisms organized in biofilms form a protective environment allowing them to perform metabolic and genetic exchanges [23].

The *S. mitis* strain showed to be sensitive to the extract but insensitive to chlorhexidine. It should be noted that *S. mitis* is a bacterial species found in both supragingival and subgingival plaque, and is considered one of the pioneer bacteria of dental biofilm. In fact, the adhesion of bacteria to the salivary film constitutes the first step in the colonization of dental surfaces. Oral streptococci have been consistently identified as the primary colonizers, and these organisms constitute 60-80% of dental plaque bacteria within the first hours of plaque formation [24, 25]. Pioneer bacteria are varieties of the *S. mitis* species, which adhere better than other streptococci, especially varieties of the *S. mutans* species [26]. Although a multitude of subsequent events are known, the importance of these initial events should not be overlooked in the development of dental biofilm. The early fixation on the enamel of streptococci of *S. mitis*, as well as the production, though weak, of acid by these species, make it possible to prepare the ground for the integration of the more acidogenic organisms in the biofilm (*S. mutans* and lactobacilli), which in the long term will lead to erosion and cavitation of the tooth enamel. The inhibition of *S. mitis* by our extract is therefore interesting and promising in the fight against dental plaque. In addition, the leaves of *A. cordifolia* inhibit many cariogenic bacteria such as *S. mutans* and *Lactobacillus caseii* [8, 27]. Compared to chlorhexidine, *A. cordifolia* leaves therefore prove to be an interesting antiplaque alternative.

The bacterial strain *S. epidermidis* is sensitive to both the extract and chlorhexidine, and a slightly lower inhibition was noted for the extract, with an inhibition diameter of 11.2±0.7mm, compared to chlorhexidine, with an inhibition diameter of 13.5±1.5mm. The extract would therefore have an activity almost comparable to chlorhexidine on this germ. This is interesting, since *S. epidermidis* is an opportunistic pathogen with the highest prevalence among staphylococci present in dental plaque [28, 29].

The 2 sensitive germs are recognized as opportunistic pathogens. Note that the leaves of *A. cordifolia* have already demonstrated their inhibitory effect on several other oral bacteria such as *S. mutans*, *L. caseii*, *Actinomyces viscosus* and *S. aureus* [8, 27]. The leaves of A. cordifolia therefore present as an interesting anti-plaque alternative.

Determination of MICs

Following the presumptive test, the 2 sensitive bacterial strains were selected to determine the minimum inhibitory doses. The extract from *A. cordifolia* leaves was tested at 6 different concentrations on these strains, with chlorhexidine as a reference. The inhibition diameters obtained were measured and noted (Table 3).

Table-3: Inhibition diameters of the extract at different concentrations. AI: Absence of inhibition

Bacterial strains		Inhibition diameter (mm)					
		Extract concentration (mg/ml)					Chlorhexidine
	40	20	10	5	2,5	1,25	
Streptococcus mitis	16,9±1,1	7,7±0,3	AI	AI	AI	AI	AI
Staphylococcus epidermidis	11,2±0,7	8,5±0,5	AI	AI	AI	AI	13,0±1,0

The inhibition diameters obtained were 16.9±1.1 mm and 11.2±0.7 mm at the extract concentration of 40 mg/ml, and 7.7±0.3 mm and 8.5±0.5 mm at the extract concentration of 20 mg/ml, for *S. mitis* and *S. epidermidis*, respectively. Chlorhexidine showed no inhibition against *S. mitis* and an inhibition diameter of 13.0±1.0 mm against *S. epidermidis*.

These results reveal that the aqueous extract of *A. cordifolia* leaves exhibits a MIC of 20 mg/ml on *S. mitis* and *S. epidermidis*, and does not inhibit the growth of *M. luteus*, even at the maximum concentration of 40 mg/ml. An MIC $\leq$ 2.5 mg/ml was noted for clinical strains of *S. epidermidis* and *Streptococcus spp*, with the hydroalcoholic extract of *A. cordifolia* [13]. This lower activity of the aqueous extract compared to the hydroalcoholic extract could result from the nature of the solvent used which impacts the type of secondary metabolites found in the extract. In fact, most of the antimicrobial substances that have been identified are not soluble in water, which suggests favouring the extraction by organic solvents, in particular ethanol, in order to obtain a better antimicrobial activity [30].

This study was the first to assess the antiseptic activity of *Alchornea cordifolia*. This is also the first study carried out on the activity of this plant on the 3 oral bacteria used. It therefore validates the use of the leaves of this plant in the treatment of infectious oral diseases, and suggests that these leaves be exploited for the production of an improved traditional medicine for oral use and for antiseptic purposes like a mouthwash.

Further studies on fractions and molecules isolated from *A. cordifolia* extracts will be used to produce a drug based on a pure active ingredient which can be incorporated into existing compositions.

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