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Research Article
Antibacterial and Cytotoxic Activities of Three Medicinal Plants from Cameroon (*Alstonia boonei*, *Cassia alata* and *Garcinia lucida*) Against Diarrhea

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Abstract

Background and Objective: Diarrhea is a bacterial disease classified as the third leading cause of death for children under 5. Due to the frequent setbacks that surround the use of first line antibacterials, this thematic outline was designed with the aim of finding future alternative means of treatment on the basis of *Cassia alata*, *Garcinia lucida* and *Alstonia boonei*, medicinal plants used traditionally for the treatment of diarrhea. **Materials and Methods:** The various plant extracts were obtained by methanol and hydroethanol maceration and their inhibitory potential was evaluated on 5 diarrheal-causing enterobacteria by the broth microdilution method. Phytochemical screening was performed by colorimetric methods. Cytotoxicity was assessed on Vero cell line by the spectrophotometric method. The effects of the extracts on bacterial (*S. flexneri*) membrane destabilization and nucleotide leakage was evaluated spectrophotometrically, while the effect on loss of salt tolerance and time kill kinetics was done by enumerating colonies after treatment. **Results:** MIC values ranged from 500-1000 μg mL^{-1}. Phytochemical screening revealed the presence of flavonoids, phenols, anthocyanins, glycosides, quinones and steroids. The CC_{50} values ranged from 357.9±10.818 to 161.7±65.195 μg mL^{-1}. Methanolic extract of *C. alata* leaves showed its ability to destabilize the *S. flexneri* membrane as well as foster loss of salt tolerance. The time kill kinetics of *S. flexneri* showed a bacteriostatic effect up to 8h with the methanolic extracts of leaves of *C. alata*. **Conclusion:** The overall results support the traditional use of these plants and show that they could serve as potent sources of non-cytotoxic antidiarrheal phytomedicines.

Key words: *C. alata, G. lucida,* antibacterial activities, modes of action, cytotoxicity, antidiarrheal, phytochemicals

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infectious diarrhea is a microbial pathology caused by several germs, which together with their produced endotoxins have the ability to destabilize the membranes of the epithelial cells of the intestines. The direct consequence is the non-adsorption of water, nutrients and solutes, leading to diarrhea[1]. This is the second leading cause of death for children under five, especially in developing countries, with 2.5 million deaths per year[2]. However, several factors contribute to the development of this pathology, namely non-compliance with hygiene rules, difficulties in accessing uncontaminated drinking water, immunodeficiency and certain chronic diseases[3]. The management of this disease is based on prophylaxis and curative treatment. The latter is based on the use of rehydration solutions and chemotherapy. However, these different strategies have several limitations such as resistance phenomena and undesirable side effects. These limitations raise the need for new therapeutic substances to address this problem[4]. Traditional medicine through the use of medicinal plants is one of the promising alternatives.

Cassia alata (Caesalpiniaceae) is a shrub with a wide range of uses. The different parts of *C. alata* are used in mouthwash and lotions of eczema and scab. Decoctions of wood are used for the treatment of liver infections, rhinitis and loss of appetite due to gastrointestinal problems[5]. In Cameroon, *C. alata* is used for the treatment of many infections including typhoid fever and diarrhea[6]. *Garcinia lucida* (Clusiaceae) is a small tree used in food and traditional medicine. The barks are used against gastroenteritis infections, in gynecology and also as an aphrodisiac stimulant. In the South Region of Cameroon it is considered antidote (against venom of the snake). In the South West Region it is used in combination with some plants for the treatment of typhoid[6]. *Alstonia boonei* (Apocynaceae) is a tree used in Ivory Coast for the treatment of malaria, typhoid fever, gonorrhea, asthma, dysentery. It is also applied to ulcers, snake bites, rheumatic pains and toothaches[7]. Due to limited and at times shallow scientific works on the titled species, this study was designed to evaluate the antibacterial and cytotoxic properties of *C. alata*, *G. lucida* and *A. boonei*.

MATERIALS AND METHODS

This research was carried out in the Laboratory for Phytobiochemistry and Medicinal Plants Studies of the Antimicrobial and Biocontrol Agents Unit (AmBcAU), Department of Biochemistry, University of Yaoundé I, Cameroon, from January, 2018-April, 2019.

Plant material: The samples of different parts of *C. alata, G. lucida* and *A. bonei* plants were harvested from Kumba (South West Region, Cameroon) in January, 2018. Botanical identification was done by Mr. Nana Victorat the National Herbarium of Cameroon by comparing the specimens (roots, leaves, bark) to those previously registered under reference numbers 62835HNC, 45146HNC and 43368HNC, respectively.

Extraction: The extracts were obtained by macerating each plant powder in methanol (1:10, w/v) for 72 h at room temperature according to Tchakam *et al.*[8], with slight modifications. The mixtures were stirred twice a day and the macerates obtained were filtered using a hydrophilic cotton wool and then evaporated using a rotary evaporator (Buchi, 011) at 60°C. The process was repeated 3 times in order to deplete the plant material and the crude extracts were obtained.

Bacterial species: The *in vitro* antibacterial activity of the extracts was evaluated on 5 bacterial isolates including *Shigella flexneri*, *Salmonella typhimurium*, *Salmonella enteretidis*, *Staphylococcus aureus* from the Pasteur center of Cameroon (CPC) and *Salmonella typhi* from the 'Centre Hospitalier Universitaire' (CHU) of Yaoundé-Cameroon. These isolates were stored in the laboratory in tubes containing Muller Hinton agar by slant culture at 4°C.

Preparation of bacterial Inocula: The different bacterial inocula were prepared according to the standard 0.5 McFarland. For this purpose, a mother suspension was prepared at 0.5 McFarland turbidity (corresponding to a concentration of approximately 1.5×10^8 CFU mL^{-1}) from 24 h young cultures and then diluted to 1.5×10^6 CFU mL^{-1} for the tests[9].

Preparation of stock solutions of extracts and reference antibacterial: The stock solutions of extracts were prepared at 100 mg mL^{-1} by dissolving 100 mg of extract in 1 mL of absolute DMSO. As for ciprofloxacin, it was prepared under the same conditions, at 2 mg mL^{-1} in sterile distilled water and served as a positive control during the tests.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC): The microdilution method on Muller Hinton broth was used to test the susceptibility of bacteria. The tests were carried out on 96-well microplates according to the M07 A9 protocol described by CLSI[9]. For this, two-folds serial dilutions of

extracts were carried out in the Muller Hinton broth to obtain volumes of 100 µL per well. One hundred microliters of a bacterial suspension (1.5×10^6 CFU mL^{-1}) were added into each well containing the test substances to obtain final concentration range of 1000-62.5 µg mL^{-1}. The percentage of DMSO in the first wells was 1% and showed no effect on bacterial growth. Ciprofloxacin was used as a positive control and the plates were covered and incubated at 37°C for 24 h. MICs were determined by the addition of 50 µL of INT (0.2 mg mL^{-1}) to the wells and re-incubated at 37°C for 30 min. Membrane dehydrogenases from viable cells reduce the yellow-colored dye (INT) to formazan pink. The MIC was determined as the lowest concentration of test substance which hindered bacterial growth, marked by no change in color of the medium.

MBCs were determined by sub-culturing 50 µL aliquots of the inhibitory cups (not having received INT) into the wells of sterile plates containing 150 µL of Muller Hinton broth. The plates were then covered and incubated at 37°C for 48 h. Cell viability was determined by the INT colorimetric method as above. The lowest concentration of the test substances showing no color change was considered MBC. The tests were performed in triplicate at two different occasions.

Evaluation of the phytochemical profile of the most active extracts: The extracts of the various plants were freshly prepared and subjected to a qualitative evaluation of the presence of phytochemical groups of secondary metabolites. This was done by colorimetric and/or complexation reactions as described by Bruneton[10] and Harborne[11].

Evaluation of the cytotoxic activities of the extracts: Vero ATCC CRL 1586 cells from the 'Centre Pasteur' of Cameroon (CPC) were used to evaluate the safety of extracts.

Determination of median cytotoxic concentrations (CC_{50}): The determination of the median cytotoxic concentrations was evaluated by the MTT colorimetric method as described by Mosmann[12]. The mitochondrial enzymes of viable cells reduce the yellow MTT to formazan violet. Accordingly, 100 µL cell suspension titrated at 5×10^3 cells/well was introduced into the wells of a microplate 4 h before exposure to the extracts for adhesion and cell confluence. Subsequently the extracts (100 µL) prepared at different concentrations were brought into contact with these adherent cells and incubated at 37°C, 5% CO_2 for 48 h. DMSO at 0.2-10% were used as negative and positive controls respectively, while sterility control wells contained only culture medium. At the end of the incubation time, 20 µL of MTT were introduced into all the wells and re-incubated at 37°C for 5 h. The formed formazan was dissolved in absolute DMSO and the optical densities (ODs) were measured at 570 nm using the TECAN Infinite M200 plate reader. These ODs were used to calculate cell viability percentages:

$$\text{Viability (\%)} = \frac{\text{OD test}}{\text{OD negative control}} \times 100$$

Non-linear regression curves of percentage viability against sample concentrations led to the determination of the CC_{50} values. The tests were carried out in triplicate.

Evaluation of the effect of extraction the destabilization of the outer membrane: The evaluation of the potential effect of the extracts on the destabilization of the bacterial membrane of *S. flexneri* was carried out according to the protocol previously used by Oliveira *et al.*[13] with some modifications. Cells from 24 h cultures titrated at 1.5×10^6 CFU mL^{-1} were incubated with extracts at MIC, 2MIC and 4MIC in 96-well microplates at 37°C for 24 h. The optical densities were measured at 405 nm (wavelength at which the complex form between Lipopolysaccharides and membrane stabilizing divalent cations absorbs) via a Tecan Infinite M200 plate reader. These ODs made it possible to calculate the percentage of membrane destabilization. The tests were performed in triplicate:

$$\text{MD (\%)} = \frac{\text{DO negative control} - \text{DO test}}{\text{DO negative control}} \times 100$$

Evaluation of the effect of extract on nucleotide leakage: The test was carried out according to the protocol previously used by Oliveira *et al.*[13]. Briefly, an overnight culture of *S. flexneri* was washed in sterile physiologic water (2 mL of 0.9% NaCl) and the resulting solution centrifuged at 10 000 rpm for 10 min. After this time, the supernatant was discarded and the resulting pellet re-suspended in 10 mM PBS (pH 7.4) and the turbidity adjusted to 0.5 McFarland ($1.5x10^8$ CFU mL^{-1}). The inoculum (100 µL) was introduced into 100 µL of MHB containing each extract at varying concentrations (MIC, 2MIC, 4MIC) and incubated at different times intervals (0, 2, 4, 6, 8 and 12 h). Following each incubation period, the cell suspension was centrifuged at 10 000 rpm for 10 min, the supernatant appropriately diluted and the optical densities were recorded at 260 nm. The test was performed in triplicate and simultaneously for positive

(ciprofloxacin), negative (PBS+cell suspension), sterility control (MHB alone) and blank (MHB+extract). The optical densities were plotted against the different times to determine the time dependent degree of leakage of the different extract concentrations.

Evaluation of the effect of extraction the loss of salt tolerance: The ability of *S. flexneri* to form colonies in the presence of extracts on NaCl-supplemented MHA was evaluated according to the protocol previously used by Etame *et al.*[14]. In effect, a preliminary test was carried out by culturing the bacterium at 37°C for 24 h on MHA supplemented with NaCl at different concentrations (10-100 mg mL^{-1}). At the end of this incubation period, the number of colonies was counted and NaCl concentrations that did not affect the growth of the microbe were selected. Subsequently the cells (0.5 McFarland) were mixed with the *C. alata* methanol leaf extract at different concentrations (MIC, 2CMI and 4MIC) followed by incubation for 1 h at 37°C. The content of each well was subcultured on MHA supplemented with NaCl at the selected concentrations (60, 70 and 80 mg mL^{-1}). The petri dishes were incubated at 37°C for 24 h and the number of colony forming units (CFU) was plotted against the extract and NaCl concentrations. The tests were carried out in triplicate.

Evaluation of the time kill kinetics of *C. alata* methanol leaf extract on *S. flexneri*: The time killing kinetic of the *C. alata* methanol leaf extract was performed according to the method described by Klepser *et al.*[15] with slight modifications. Here, extract concentrations of MIC, 2MIC, 4MIC and 8MIC were prepared by serial two-fold dilution in a 96 well micro-plate. One hundred microliters (100 µL) of *S. flexneri* suspension (1.5×10^6 CFU mL^{-1}) were added and the plate incubated at 37°C for different time intervals (0, 1, 2, 4, 6, 8, 12 and 24 h). Following each incubation period, the cell suspensions were appropriately diluted (in NaCl 0.9%) and the resulting solution sub-cultured on SS agar plates for further 24 h at 37°C. Ciprofloxacin was used as positive control. Wells containing the bacteria incubated with MHB were used as growth controls. The test was performed in triplicate and results were presented as Mean±SD.

Statistical analysis: Data were analyzed by the One-way analysis of variance (ANOVA) using the Statistical package for social science (SPSS) software version 16.0. The GraphPad Prism.7 software was used to calculate the CC_{50} using the nonlinear regression curve. The results were expressed, where appropriate, as Mean±SD. The differences between the means were compared by the Waller Duncan test at 95% confidence ($p\leq0.05$).

RESULTS

Extraction yields: The extraction yields of the extracts according to the plant part and the solvent are grouped in Table 1. The leaves of *C. alata* (34.2%) and leaves of *A. boonei* (49.29%) contain more secondary metabolites extractable by methanol.

Anti-bacterial activity of the methanolic and hydroethanol extracts of *C. alata, G. lucida and A. boonei*: The Minimum inhibitory concentration values vary from 500-1000 µg mL^{-1}, while the minimal bactericidal concentrations was 500 µg mL^{-1} for the methanolic extracts of *G. lucida* bark on 40% of the pathogens (Table 2). *G. lucida* extracts were active on all bacterial isolates tested. The best MIC (500 µg mL^{-1}) was obtained with the bark methanolic extracts, on *S. typhi, S. aureus, S. typhimurum and S. enteritidis while S. flexneri* was the least sensitive with a MIC of 1000 µg mL^{-1}. Extracts of *C. alata* were less active than those of *G. lucida*, with best activities on *S. typhi* (MIC of 500 µg mL^{-1}). However, MBCs of the methanolic and hydroethanolic extracts of the leaves, barks and stems of *C. alata* were higher than the tested concentrations (1000 µg mL^{-1}). The activity of the various extracts tested was less important than that of ciprofloxacin (reference antibiotic) with MICs between 0.039-0.078 µg mL^{-1} depending on the isolates.

Phytochemical profiles of active extracts: The results of the phytochemical screening of the various extracts show the presence of three classes of secondary metabolites, phenolic compounds, terpenes and glycosides (Table 3). Flavonoids and phenols were present in all extracts. In addition to the above two groups of metabolites, *G. lucida* extracts contained quinones and tannins, on the other hand, the hydroethanolic extracts of *C. alata* were rich in anthocyanins, glycosides and tannins. In addition, anthocyanins, glycosides, quinones and

Table 1: Extraction yield as a function of plant part and solvent

Extracts	Yield (%)
A. Boonei leaves (MeOH)	49.29
A. Boonei roots (MeOH)	03.46
A. Boonei twigs (H_2O/EtOH)	13.42
C. Alata stems (H_2O/EtOH)	10.81
C. Alata twigs (MeOH)	30.02
C. alata leaves (MeOH)	34.02
G. lucida stems (MeOH)	17.06

Table 2: Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) in (μg mL^{-1}) of selected extracts

Microbes	*G. lucida* stems (MeOH)	*C. alata* stems (H_2O/EtOH)	*C. alata* leaves (MeOH)	*C. alata* twigs (MeOH)	Ciprofloxacin
Staphylococcus aureus					
MIC	500	1000	1000	1000	0.078
MBC	*	*	*	*	0.156
Salmonella typhi					
MIC	500	500	500	1000	0.156
MBC	500	*	*	*	0.156
Salmonella typhimurium					
MIC	500	>1000	1000	1000	0.078
MBC	*	*	*	*	0.312
Salmonella enteretidis					
MIC	500	1000	1000	1000	0.078
MBC	500	*	*	*	0.078
Shigella flexneri					
MIC	1000	1000	1000	1000	0.078
MBC	*	*	*	*	0.156

*Not determined (MBC>1000 μg mL^{-1})

Table 3: Qualitative phytochemical constituents of extracts of *G. lucida* and *C. alata*

Group of Secondary metabolites	Extracts: *G. lucida* stems (MeOH)	*C. alata* stems (H_2O/EtOH)	*C. alata* leaves (MeOH)	*C. alata* twig (MeOH)
Phénols	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	-	-
Anthocyanins	-	+	+	-
Quinones	+	-	+	+
Alkaloids	-	-	-	-
Glycosides	-	+	+	-
Saponins	-	-	-	-
Steroids	-	-	+	+
Triterpenes	-	-	-	-

+: Present, -: Absent

Table 4: Median cytotoxic concentrations of extracts

Extracts	CC_{50} (μg mL^{-1})
Stems *G. lucida* (MeOH)	357.95±10.81^{c}
Stems *C. alata* (H_2O/MeOH)	232.95±09.82^{ab}
Leaves *C. alata* (MeOH)	227.85±25.66^{ab}
Twigs *C. alata* (MeOH)	161.07±65.19^{a}

Values carrying the same letter superscripts are not significantly different (p>0.05), Waller Duncan

steroids were present in the methanol extracts of the leaves of *C. alata* unlike those of the stems that contained quinones and steroids. A critical observation of these results showed that either a combination of quinones and tannins is vital to the antibacterial activities of these extracts or specific active principles are found in the *G. lucida* extracts.

Cytotoxic activity of active extracts: The results of the cytotoxic activity showed that the median cytotoxic concentrations (CC_{50}) range from 357.9±10.81 to 161.7±65.19 μg mL^{-1} (Table 4). The methanol extracts from *C. alata* twigs showed lower CC_{50} compared to methanolic and hydroethanolic extracts of leaves and stems of *C. alata*. The methanolic extract of *G. lucida* stem bark had significantly higher ($p \leq 0.05$) CC_{50} and therefore less cytotoxic compared to the rest of the extracts.

Effect of extracts on the destabilization of the outer membrane of *S. flexneri*: Figure 1 shows the variation of destabilization percentages of the outer membrane of the bacterium as a function of the concentrations of different extracts. It can be noted that only the methanolic extracts of *C. alata* leaves showed a mechanism of membrane destabilization by the chelation of divalent cations. The degree of destabilization of the outer membrane of the bacterium was concentration-dependent and was significantly higher ($p \leq 0.05$) than that of polymyxin at the corresponding highest concentrations (4 MIC).

Effect of *C. alata* methanol leaf extract on membrane lysis: The period variation of the optical densities of supernatants collected after treatment of *S. flexneri* isolates with different concentrations of the methanolic extract of the

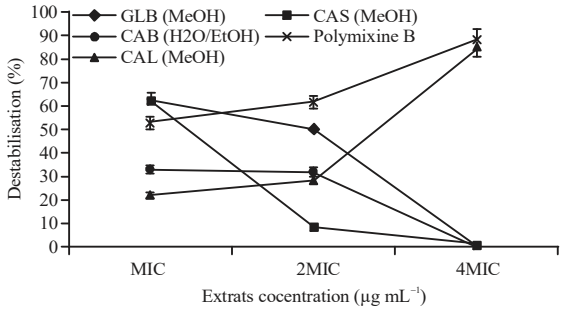

Fig. 1: Curve of the destabilization (%) of the outer membrane of *S. flexneri* by the active extracts

GLB (MeOH): Methanolic extract of *G. lucida* bark, CAB (EtOH/H2O): hydroethanolic extract of *C. alata* bark, CAL (MeOH): Methanolic extract of leaves of *C. alata*, CAS (MeOH): Methanolic extract of *C. alata* stems, Polymyxin: Positive control, MIC: Minimum Inhibitory concentration

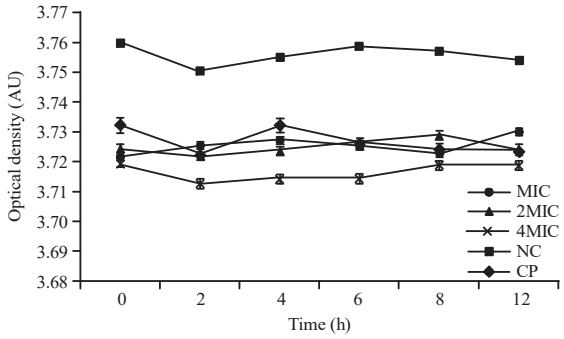

Fig. 2: Variation of the optical density as a function of the concentration of the methanolic extract of the leaves of *C. alata* with time

MIC: Minimum inhibitory concentration, CN: Negative control, CP: Positive control (Ciprofloxacin)

leaves of *C. alata* is presented in Fig. 2. It shows that the extract did not cause any membrane damage materialized by the absence of the release of the nucleic acids (DNA) in the extracellular medium. Indeed, an increase in the optical density at 260 nm would translate a release of nucleic acids (DNA) in the medium. Compared with growth control, no variation was observed, meaning that this extract would not exert its antibacterial activity by this mechanism.

Effect of extract on loss of salt tolerance: The potential of salt tolerance of *S. flexneri* in the presence of *C. alata* leaf extract at different concentrations is shown in Fig. 3. These results showed that the number of colony forming units (CFU) of the bacterium simultaneously depends on the extract as well as the salt concentrations. For a given salt concentration, this number CFU decreases significantly ($p \leq 0.05$) with increase in extract concentration. Likewise, for a given extract concentration the CFU decrease significantly ($p \leq 0.05$) with the salt concentration. Thus, the *C. alata* leaf extract at 2MIC and 80% salt concentration reveals the best inhibitory activity on the *S. flexneri* isolate. This shows the inability of *S. flexneri* to tolerate the presence of NaCl in its growth medium.

Time kill kinetic effect of methanolic extract of leaves of *C. alata* on *S. flexneri*: Due to the fact that ethanolic extract of leaves of *C. alata* was the only extract with mechanism of action on the destabilization of the outer membrane of *S. flexneri*, its effect on the kinetics of mortality was evaluated on this isolate. For different extract concentrations, the number of colonies was evaluated at different times and

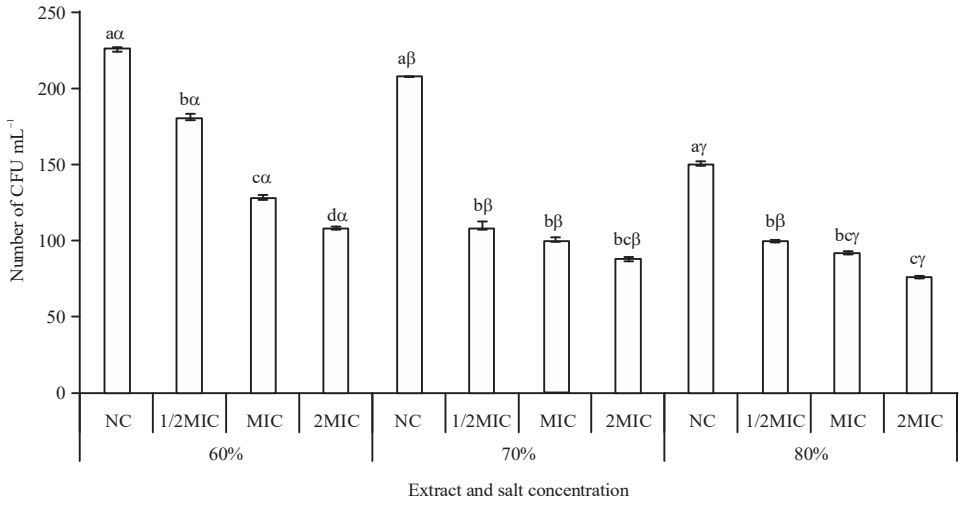

Fig. 3: Variation of the number of *S. flexneri* colonies as a function of the concentration of extract and NaCl

For the same salt concentration, bars with same letters are not significantly different (p 0.05), for the same extract concentration, bars with same Greek alphabet are not significantly different (p 0.05), Waller Duncan test, MIC: Minimum inhibitory concentration, NC: Negative control

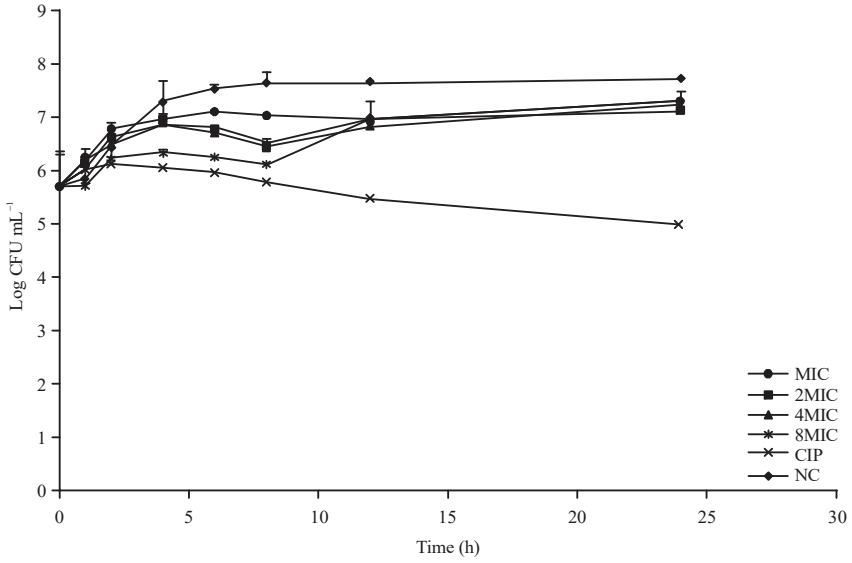

Fig. 4: Time kill kinetics of *S. flexneri* as a function of the concentration of methanolic extract of the leaves of *C. alata*
MIC: 1000 µg mL^{-1}, CP: Positive control (ciprofloxacin 0.078 µg mL^{-1}), NC: Negative control

presented in Fig. 4 to determine the time when the destabilization of the membrane by the extract was maximal. With regards to this figure, it was found that the methanol extract of the leaves of *C. alata* at concentrations of MIC, 2MIC, 4MIC and 8MIC affected bacterial growth during first 8 h following initial contact with the microbe whereas the effect of ciprofloxacin (0.039 µg mL^{-1}) was felt throughout the experimental period (Fig. 4). The membrane destabilization effect of the extract was concentration-dependent and was maximum at 8 h, after which there was resurgence of the bacterial growth. After this resurgent time, the activity of the extract was no longer concentration-dependent, as there was no significant difference in the rate of increase of the colonies. This suggested that this resurgent time could be considered as the time interval for the re-administration of the extract in a subsequent *in vivo* evaluation of its therapeutic efficacy.

DISCUSSION

The antibacterial parameters obtained show the inhibitory potential of the methanolic extract of the bark of *G. lucida* and the methanolic and hydroethanolic extracts of the leaves, stems and barks of *C. alata*. According to the classification scale defined, all the tested pathogens were susceptible to the different methanolic extracts of *G. lucida* barks as well as ethanolic and hydroethanolic bark, leaves and stems of *C. alata*. Ethanolic extracts of the barks of *G. lucida* as as well as extracts from different parts of *C. alata* presented MIC values varying from 500-1000 µg mL^{-1}. The antimicrobial activity of a plant extract is considered to be highly active if the MIC<100 µg mL^{-1}, significantly active when 100≤MIC ≤512 µg mL^{-1}, moderately active when 512<MIC≤2048 µg mL^{-1}, weakly active if MIC> 2048 µg mL^{-1} and not active when MIC[16]>10 000 µg mL^{-1}. Therefore, the extracts of *G. lucida* bark exhibited significant activities on all the tested microbes, while those of *C. alata* showed significant to moderate activities as a function of the bacterial species. These results are in agreement with those of Momo *et al.*[17] who obtained a MIC range of 128-256 µg mL^{-1} respectively, on *S. typhi* and *S. aureus* strains with methanolic extracts of *G. lucida* bark. In contrast, Dzoyem *et al.*[18] showed that the methanolic extracts of leaves of *G. lucida* have a MIC of 500 µg mL^{-1} on a strain of *S. aureus*. Pissang *et al.*[19] obtained a MIC of 1250 µg mL^{-1} with alcohol extracts from *C. alata* leaves on *S. aureus* and Promgool *et al.*[20] obtained substantially the same results on *S. typhimurum* strain with MICs ranging from 620-1280 µg mL^{-1} depending on the parts.

The presence of total phenols, flavonoids, tannins, quinones, glycosides and terpenoids in the methanolic extracts of *G. lucida* bark and all the methanolic and hydroethanolic extracts of *C. alata* may explain the source of antibacterial activities of these extracts. The variability of these groups of secondary metabolites which may be influenced by environmental factors[21] as well as the modes of action could justify the differences in activities recorded with the extracts. The richness of these extracts in secondary metabolites having diversified modes of action justifies the activities obtained. Indeed, phenolic compounds such as tannins, flavonoids,

simple phenols, quinones have been shown to possess inhibitory effects on the alteration of membrane structures[22]. Quinones have the ability to irreversibly complex with nucleophilic amino acids and proteins, leading to the loss of their functions[23]. Furthermore, phenols have chemical and biological effects known to be due to the redox system, phenol/semiquinone/quinone and the semiquinone intermediate is therefore the basic molecule responsible for chemical and biological effects[24]. The antimicrobial mechanisms of several flavonoids could be attributed to the inhibition of nucleic acid synthesis[25-27]. Tannins have the ability to induce chelation with proteins[4]. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, membrane enzymes and membrane transport proteins[28]. These results are in agreement with those obtained by Karthika *et al.*[29] who showed the presence of phenolic compounds in the methanolic extracts of leaves of *C. alata.* Sylvie *et al.*[30] demonstrated the presence of phenols and flavonoids in the methanolic extracts of fruits and barks of *G. lucida*, while Owoyale *et al.*[31] showed that anthocyanins were absent in extracts of *C. alata*. Sylvie *et al.*[30] and Ramaraj *et al.*[32] showed the presence of steroids in leaf extracts of *C. alata* and methanolic extracts of bark and fruit of *G. lucida*, respectively.

The search for new drugs for the treatment of pathologies is increasingly critical because of undesirable side effects such as the cytotoxicity of isolated, semi-synthetic or synthetic substances. Thus, the methanolic extracts of *G. lucida* bark and all the methanolic and hydroethanolic extracts of the bark, leaves and stems of *C. alata* were evaluated for cytotoxicity on Vero cell line and their CC_{50} values varied from 357.95±10.81 to 161.7±65.19 µg mL^{-1}. According to the American National Cancer Institute[33], all the extracts tested were not cytotoxic since their CC_{50} values were greater than 30 µg mL^{-1}. To our knowledge, no scientific research has been done on the cytotoxicity of these plants with the Vero cell line. Nevertheless, Arthanari *et al.*[34] showed that the CC_{50} of the methanolic extract of *C. grandiflora* flowers was greater than 20 µg mL^{-1}.

The resistance of most Gram-negative bacteria is due in part to the outer membrane which is a semi-permeable barrier to different antibiotics[35]. The measurement of membrane permeability of bacteria is therefore essential in the study of the mode of action of antibiotics[36]. The literature shows that the most lipophilic flavonoids can disrupt bacterial membranes by destabilizing them[23]. In addition, phenolic compounds are known as chelators of bivalent cations for bridging LPS. This chelation induces the release of LPS thus destabilizing the outer membrane of bacteria[35,37]. Thus, the presence of flavonoids in the leaves of *C. alata* could explain the destabilization of the outer membrane of *S. flexneri* as presented above. These results are in agreement with those obtained by Puupponen-Pimia *et al.*[38], which showed that gallic acid isolated from *Caesalpiniamimosoides* has the ability to permeabilize the outer membrane of the *Salmonella typhimurium* strain by chelating the divalent cations. Although, the other extracts were rich in flavonoids, tannins, quinones and phenols capable of complexing with membrane proteins and divalent cations[28], they did not show destabilization of the bacterial membrane, probably due to low concentrations and/or presence of non-destabilizing specific metabolites of the above classes. They would therefore certainly act by other mechanism(s) such as intracellular efflux of K^+, chelation with nucleic acids, lysis of cellular proteins.

As food is the main source of diarrhea-causing bacteria, some food industries use saline solutions for food preservation. However, several studies have shown a development of salt tolerance by these bacteria. The loss of salt tolerance by the bacterium *S. flexneri* after treatment with methanolic extracts of leaves of *C. alata* may explain the ability of the extracts to disrupt the expulsion of salts from the cell, which is associated with the alteration bacterial membrane[14,39]. This alteration could induce the permeability of the membrane; affect cellular exchanges and the inadequate regulation of cell osmosis as well as the exclusion of toxic elements. The consequence of loss of salt tolerance and other toxic molecules can be used to demonstrate damage to the bacterial membrane[40]. This induction of loss of salt tolerance by *C. alata* leaf extract can be beneficial for the treatment of ailments caused by bacteria ingested from salt-preserved foods.

Membrane damage can induce permeability of the membrane, thereby promoting the release of cellular content such nucleic acids. The release of nucleic acids thus makes it possible to evaluate the degree of damage of the membrane. From the results, no continuous increase of optical density was recorded with concentration and time, indicating that the extract does not act by membrane lysis.

Time-kill assays allow antibacterial agents to be classified as bacteriostatic or bactericidal and characterize the relationship between agent concentration and activity over time. Measurement of bacterial kinetics showed a concentration-dependent retardation in cell growth with extract. The extract's effect lasted during the first 8 h, after which a resurgence of the microbe was observed indicating its bacteriostatic effect, which in turn gives the immune system of the host the time needed to clear the microbes from the

system[41]. Keeping aside toxicological studies, a phytomedicinal preparation of this plant could be administered at 8 h intervals.

This study shows that these extracts are not toxic to human cells, provides scientific data in support of the traditional use of these plants in the treatment of bacterial infections and suggests that doses should be repeated at 8 h intervals (3 times daily). However, more work needs to be carried out to demonstrate their *in vivo* therapeutic efficacies as well as their toxicological profiles.

CONCLUSION

The study revealed that the extracts of *G. lucida* and *C. alata* possess phytochemicals exhibiting significant (MICs ranging from 500-1000 μg mL^{-1}) bacteriostatic activities within the first 8 h following administration and are non- cytotoxic. In addition, the mode of action of the *C. alata* methanol leaf extract was achieved through the destabilization of the outer membrane of bacteria and/or prevention of salt expulsion from the bacterial cells. These results showed that the title species could offer great perspective in the development of antibacterial phytomedicines.

SIGNIFICANCE STATEMENT

This study discovers the outer membrane destabilization effect of *C. alata* methanol leaf extract on human pathogenic bacteria that could be very beneficial in the elucidation of the mechanisms of action of its phytochemicals. It also demonstrates the inability of *S. flexneri* to tolerate the presence of NaCl in its growth medium when supplemented with the extract that many researchers were not able to explore. This study will help researches to unveil new strategies in the incorporation of salt in phytomedicinal formulations. Thus a more efficient way of preparing phytomedicines may be arrived at.

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