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Modes of action of the methanol extract and 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid from *Acacia polyacantha* against multidrug-resistant Gram-negative bacteria

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Abstract

Background: Bacterial infections, especially those caused by multidrug-resistant (MDR) Gram-negative bacteria, remain a global health concern. Previous studies have reported the antibacterial activities of the methanol extract of the leaves of *Acacia polyacantha* Willd. (Fabaceae) (APL) as well as some of its phytoconstituents. In this study, APL and its constituent, 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid (GGOA) were evaluated on growth kinetics, bacterial membrane, and H^+-ATPase proton pumps. The acute toxicity of APL was also studied.

Methods: The effects of APL and GGOA on bacterial growth kinetics and the bacterial membrane was determined using spectrophotometric methods. Their effects on the bacterial H^+-ATPase proton pumps were evaluated through the acidification of the bacterial external environment using a pH-meter. The acute toxicity of APL was determined using the standard limit test method described by the Organization for Economic Cooperation and Development (OECD) code 425.

Results: APL and GGOA modified the bacterial kinetics of *Providencia stuartii* ATCC29916 with emphasis on the latent phase. They blocked the proton pumps of this bacterium and destroy the bacterial membrane. The medium lethal dose (LD_{50}) of APL was greater than 5000 mg/kg body weight (b.w.).

Conclusion: The results of the present study provide important and complementary information on APL and GGOA as good antibacterial candidates for combating infections due to MDR Gram-negative bacteria.

Keywords: 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid; *Acacia polyacantha*; acute toxicity; Gram-negative bacteria; mode of action; multidrug resistance.

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Background

Globally, bacterial infections, particularly those caused by multidrug-resistant Gram-negative bacteria, are a real public health problem. Despite the abundance of various classes of antibiotics, the emergence of increasingly resistant bacterial strains is on the rise. The latter complicates treatment and consequently increases mortality rates from infectious diseases worldwide [1]. This phenomenon of resistance is responsible for more than 700,000 deaths each year worldwide, 33,000 in Europe, and could result in an additional 10 million deaths per year by 2050 [2]. To resist the toxic action of antibiotics, bacteria have developed several adaptation mechanisms, including the efflux mechanism. The latter is used by more than 15 Gram-negative pathogenic species via their resistance-nodulation cell division efflux pump (RND) [3, 4]. This efflux pump simultaneously affects several classes of antibiotics and leads to high-level resistance when expressed simultaneously with other resistance mechanisms. If urgent measures are not taken, we will soon enter a post-antibiotic era in which common infections and small wounds will again be fatal [5, 6]. The search for new molecules capable of effectively combating multidrug-resistant bacteria has become an absolute necessity. In the same light, the World Health Organization (WHO) through its global partnership for research and development, is encouraging the introduction of new drugs [6]. A plethora of medicinal plants and their constituents are a great source of potentially active molecules that are largely untapped [7-26]. It is estimated that 80% of the African population relies on medicinal plants for treatment, and 30% of the drugs sold worldwide are derived from medicinal plants [27-29]. Plants are an alternative for this research as they are rich in bioactive compounds with antibacterial properties. In recent years, several plants in the Cameroonian pharmacopoeia and their molecules have proven their ability to inhibit the growth of most strains of Gram-negative MDR bacteria [30-33] and to potentiate the activity of common antibiotics [34-36]. Controlling the different pathways by which they induce bacterial death, and their safety is important for effective control of infections caused by multidrug-resistant Gram-negative bacteria. *Acacia polyacantha* Willd. (Fabaceae) is a plant in the Cameroonian pharmacopoeia used for the treatment of various bacterial, parasitic, and fungal infections [37, 38]. The antibacterial activity of the methanolic extract of the leaves (APL) of this plant, as well as that of its constituent, 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid (GGOA), have been previously reported [38, 39]. Although *Acacia polyacantha* (*A. polyacantha*) is used as traditional medicine by indigenous populations, it can be harmful to human health. Studies over the past 20 years have shown that not all natural substances are systematically good for health [40]. Although the frequency of plant poisoning is poorly known in tropical and intertropical areas, due to the lack of poison control centers, the incidence of mortality due to plant poisoning is 1.5% in France, 5% in Belgium, 6% in Turkey, 6.5% in Italy and 7.2% in Switzerland [41]. The evaluation of the acute toxicity of *A. polyacantha* leaf extract is important to ensure its safety. The present study was designed to evaluate the effects of APL and GGOA on growth kinetics, the bacterial membrane, and the bacterial proton pumps. This study also investigated the acute toxicity of APL.

Methods

General procedure

For the characterization of the compound, electrostatic ionization mass spectrometry (ESI-MS), nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C), column chromatography (CC), and UV spectra were performed according to the protocols described previously [39].

Plant material, extraction, and purification of bioactive compound

The description of the plant material, the extraction protocol, as well as the isolation and purification process of GGOA (Figure 1) from the methanol extract of *A. polyacantha* leaves have been described previously [39]. Elucidation of the chemical structure of this compound using NMR data (^{1}H and ^{13}C), in comparison with the literature, has also been reported previously [39].

Chemicals for antimicrobial assay

The chemicals used included GGOA, reference antibiotics (RA), and the microbial growth indicator. The microbial growth indicator used in this study was piodonitrotrazolium chloride ≥97% (INT, Sigma-Aldrich). The reference antibiotics used were chloramphenicol (CHL) and polymyxin B (Poly B). A 2.5% dimethylsulphoxide (DMSO) was used to dissolve our test samples. These molecules and reagents were obtained from Sigma Aldrich (St. Quentin Fallavier, France).

Bacterial strains and culture media

The reference strain (American Type Culture Collection, ATCC) of *Providencia stuartii* (ATCC29916) was used in this work. The bacterial culture was maintained on 4°C agar plates and subcultured onto suitable fresh agar 24 hours prior to any antimicrobial testing. The activation of the bacteria prior to testing was performed in Mueller Hinton agar (Sigma), while antibacterial testing was performed with Mueller Hinton broth (MHB; Sigma) [40].

Experimental animals

Nulliparous (never given birth) and non-pregnant (not carrying embryos) young female rats, aged 8-12 weeks with an average weight of 189.4 ± 21.9 g were obtained from the animal house of the Department of Biochemistry, University of Dschang, Cameroon. The animals were acclimatized in the experimental animal room for 7 days with a cycle of 12 hours of light and 12 hours of darkness before the start of the experiment. Water and standard rodent food were provided to all experimental animals.

Antimicrobial mechanisms of action

Effect of test samples on bacterial growth

The effect of APL and GGOA on the bacterial cell cycle was evaluated using the spectrophotometer by reading optical densities at 600 nm following the protocol described [42] with some modifications. It was carried out with the *P. stuartii* strain ATCC29916 as these samples simultaneously showed good antibacterial activities [40]. The bacterial suspensions were prepared at a concentration of 10^8 CFU/mL in the corresponding vials. These were treated with the substances of interest at concentrations equivalent to 0.5 × MIC, MIC, and 2 × MIC. The flasks were incubated at 37°C under an orbital shaker (REMI) at 200 rpm.

The negative and positive controls consisted of the vials containing CHL at MIC/2, MIC, and 2 MIC as well as the vial containing MHB and the bacterial suspension. After incubation times corresponding to t = 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, and 18 h; 500 µL were withdrawn from each vial and introduced into the spectrophotometric cell for reading of the optical densities (OD) at 600 nm Each test was repeated 3 times. Optical density values were used to plot OD versus time curves [43] using Microsoft Office Excel 2016 (Figures 2 and 3).

Effect of test samples on the bacterial membrane

The effect of APL and GGOA on the membrane was performed by the method described [44]. Briefly, a bacterial suspension of *P. stuartii* ATCC29916 with optical density (OD_{600}) equal to 2 was prepared from an activated bacterial culture as previously described. Bacterial cells were separated from the medium by centrifugation (Compact Cooling Centrifuge, REMI) at 400 g for 15 min. These were washed twice with PBS buffer (pH 7.4) and re-suspended in the same buffer. The suspensions were treated with APL and GGOA at concentrations of 0.5 × MIC, MIC, and 2 × MIC. The culture was incubated at 37°C for 60 min with agitation. Samples taken at times 0, 30, and 60 min of the experiment were centrifuged at 13400 g for 15 min. For each time point and concentration, the optical density was measured at 260 nm with a Thermo Scientific spectrophotometer (Langenselbold, Germany). Polymyxin B was used as a positive control. The test was repeated three times and the data obtained from the OD reading was used to plot histograms [OD = f (time)] using GraphPad Prism version 5.01 software (Figures 4, 5, and 6).

Effect of test samples on bacterial H+-ATPases

The evaluation of the effect of APL and GGOA on ATPases pumps was performed *via* the acidification of the bacterial external environment using a pH meter. For this purpose, a bacterial colony of 18 h was taken and introduced into 20 ml of MHB contained in a conical flask. The pre-culture was prepared by maintaining the whole mixture at 37°C for 18 h under agitation at a speed of 130 rpm using a magnetic stirrer. Aliquots of this bacterial pre-culture were then taken and introduced into conical flasks containing MHB at 1/100 v/v dilution. After 18 h incubation at 37°C (with agitation), 100 ml of bacterial cells were centrifuged at 4000 rpm for 30 min. The pellet was washed with distilled water, then with 50 mM KCl and re-suspended in 50 ml of 50 mM KCl. The suspension was then stored at 4 °C for 18 h (for glucose deprivation) and the pH was adjusted to 6.4 by adding HCl and/or NaOH. To 4 ml of this solution, 0.5 ml of APL and GGOA dissolved in DMSO was added to obtain a concentration of 0.5 × MIC, MIC, and 2 × MIC. After 10 min of pre-incubation at 37°C, the acidification of the medium was triggered by adding 0.5 ml of a 20% glucose solution whose rapid catabolism will be accompanied by the release of protons into the medium. Subsequently, the pH of the medium was measured every 10 min for 1 h. For the negative control, the extract was replaced by DMSO. The pH values recorded allowed the plotting of pH variation curves against time. Any inhibition of the acidification of the medium in the presence of a plant extract was attributed to an inhibitory effect of the operation of ATP-H^+-pumps by these extracts [45]. Each test was repeated 3 times. The pH values were used to plot the pH versus time curves using Microsoft Office Excel 2016. (Figures 7 and 8).

Oral acute toxicity study

The acute oral toxicity study was accessed according to OECD Guideline 425, which stipulates the use of a maximum of five animals. Thus, 10 rats weighing 189.4 ± 21.9 g were divided into two groups of 5 rats each numbered 1 to 5, and fasted. A single dose of 5000 mg/kg body weight (b.w.) of APL was administered by gavage to one group of 5 rats by means of a stomach tube. To the control group of 5 other rats, drinking water was administered. The animals were kept fasting for 4 hours. During the first 4 hours, the rats were observed individually with particular attention, then regularly during the first 24 h and daily for 14 days. Pain sensitivity (tail pinching), motor activity (observation of movements within the cage), noise sensitivity, teeth grinding, the appearance of feces, tail condition, tremors, drowsiness, and occurrences of convulsions were observed. After 14 days of observation, each animal was necropsied for macroscopic pathological changes in organs such as the liver, kidneys, lungs, spleen, and heart. The organs of the animals that received the extract were compared with those of the control animals [46].

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences were significant at the 5% probability level ($P<0.05$) and the degree of significance noted as * p-values 0.05; ** p-values 0.01; *** p-values 0.001 and **** p-values 0.0001. Using GraphPad Prism version 5.01 software, data were analyzed using analysis of variance (*one-way* ANOVA) followed by Dunnett's test for multiple comparisons.

Results

Effect of APL and GGOA on bacterial growth

Figures 2 and 3 show that the tested samples interfere in a concentration-dependent manner with the bacterial growth kinetics. In the absence of APL and GGOA, the growth curve (inoculum alone) shows a regular pattern of bacterial growth. The *P. stuartii* strain ATCC29916 shows three phases: a latent phase, an exponential phase, and a stationary phase. After 10 hours of incubation, GGOA at 0.5 x MIC shortens the exponential phase (Figure 3). At MIC, crude leaf extract and GGOA prolongs the lag phase by 4 h, followed by very slow growth of the microorganism. At 2 × MIC, APL and GGOA prolong the lag phase until the end of the experiment. At this same concentration, the observed effect was similar to that of the positive control (Figures 2 and 3).

Effect of APL and GGOA on the bacterial membrane

Figures 4, 5, and 6 indicate that the increase in the optical density of the culture medium of the treated cells was proportional to the number of cytoplasmic elements. This reflects the presence of cytoplasmic elements in the culture medium due to the damage to the plasma membrane. The figures show that the destruction of the bacterial membrane changes with time and the concentration of the test substance. Compared to untreated cells (negative control), at t = 0 minutes, no significant difference was observed. Indeed, a significant difference is observed at the 0.01 and 0.001 probability levels for the crude leaf extract from the 30^{th} and 60^{th} minute, respectively. For GGOA, Figure 5 shows a significant difference at the 0.05 probability level at MIC and 2 × MIC concentrations from

the 30th minute until the end of the experiment. APL exerts a higher activity on the plasma membrane than GGOA. It is also evident from these figures that crude leaf extract (Figure 4) and GGOA (Figure 5) significantly alter bacterial membranes from the 30th minute onwards in contrast to polymixin (positive control), which only acts at the 60th minute (Figure 6).

Effect of APL and GGOA on bacterial H^+-ATPases

The ability of APL and GGOA to block proton pumps was evaluated and the results are shown in Figures 7 and 8. It was found that, in the absence of APL and GGOA, there is rapid acidification of the medium induced by glucose metabolism from the tenth minute to the end of the experiment. However, in the presence of APL at concentrations of 0.5 x MIC, MIC, and 2 × MIC this acidification was not observed. This is shown by a linear trend in the curves corresponding to these three concentrations, in contrast to the curve for the negative control, which tends to decrease with time (Figure 7). Furthermore, in the presence of GGOA at 0.5 × MIC, there is rapid acidification of the culture medium from the tenth minute to the twentieth minute, followed by slow acidification until the end of the experiment. At MIC and 2 × MIC we did not observe any acidification of the growth medium, as at these two concentrations the curves are linear in contrast to the negative control which decreases with time (Figure 8). Figures 7 and 8 also show that at the 2 × MIC concentration, the substances of interest exert an action on the ATPase pumps similar to that of the positive control.

Acute toxicity

The Hodge and Stener toxicity scale (Table 1) for a chemical-based on medium lethal dose (LD_{50}) was considered in the acute toxicity of APL. Oral administration of APL at 5000 mg/kg b.w. did not induce any abnormal signs of toxicity (Table 2). After single-dose administration of the APL, all animals showed similar behavior, no signs of toxicity, and no mortality was recorded after 14 days. The extract appeared to be safe at a dose level of 5000 mg/kg body weight, and the LD_{50} was >5000 mg/kg b.w.

Body weight change

The results of the weight gain obtained after oral administration of APL at the dose of 5000mg/Kg b.w. are shown in Table 3. From this table, no significant difference ($P \geq 0.05$) was noted between the body weight of the control and test groups.

Relative organ weights

The results of the relative organ weights are shown in Table 3. No significant ($P \geq 0.05$) variation in relative organ weights was observed.

Metabolic activity of the liver and hepatic cytolysis

The effects of the extract on liver metabolic activity and liver cytolysis are shown in Table 4. Compared to the control group, the test group showed no significant difference in total protein and serum transaminase activity.

Discussion

Bacterial growth kinetics are specific to each bacterium. In a non-renewed medium, the bacterial growth curve comprises 6 phases: the latent phase, the acceleration phase, the exponential phase, the deceleration phase, the stationary phase, and the decline phase [47]. Each phase corresponds to biochemical processes necessary for the proper development of the bacterial cell. Inhibition of these biochemical processes leads to a prolongation of these phases, thus inhibiting the growth of the bacterium in question. In the present study, the prolongation of the lag phase indicates the inhibitory action of APL and GGOA at MIC and 2 × MIC concentrations (Figures 2 and 3). APL and GGOA act similarly on the growth of *P. stuartii* ATCC29916. This indicates that they probably act by inhibiting the biosynthesis of enzymes necessary for metabolism and therefore block the entry into the exponential phase [48]. Thus, in case of infection, these molecules can prevent the colonization of the infection site by bacteria.

The proton pump is a transmembrane protein (active membrane transporter), which moves protons against their concentration gradient using the energy released by the hydrolysis of an adenosine triphosphate (ATP) molecule. Its operation results in the enrichment of the extracellular medium of the bacteria with H+ ions, leading to the acidification of this extracellular medium. Proton-ATPase pumps play varying roles in bacterial cells. One of the most important roles is to regulate the pH of the cytoplasm of Gram-negative bacteria. Inhibition of these pumps also inhibits the growth of bacteria [49]. Blocking of these pumps by GGOA and APL indicated by constant pH of the culture medium at MIC and 2 × MIC concentrations (Figures 7 and 8) shows that these pumps are possible targets of these substances [50]. The bacterial strains used in this study are resistant mainly through the RND efflux pump. Blockage of the ATPase pumps lead to the cessation of the flow of proton (H^+) necessary for the operation of the RND pump, which results in the death of the bacteria.

Investigating the different pathways by which APL and GGOA inhibit bacterial growth, we noted that they also affect the integrity of the plasma membrane (Figures 4 and 5). This could be due to the presence of saponins in APL following the example of GGOA. Indeed, saponins are heterosides (glucosides) with surface-active (surfactant) properties and cause membrane dissolution (haemolytic activities). They act by inserting themselves between the outer phospholipids of the membrane. This insertion can lead to an abnormal increase in membrane permeability, which would lead to leakage of intracellular substances through the plasma membrane, resulting in the death of the bacteria [51]. The difference in membrane action noted between APL and GGOA is thought to be since the crude extract contains a mixture of compounds that act synergistically including phenolic compounds. It has been reported that the presence of the hydroxyl group confers to the phenolic compounds the ability to destroy the cytoplasmic membrane of bacterial cells [52]. The accumulation of hydrophobic phenolic groups in the lipid bilayer can disrupt the lipid-protein interaction and increase the permeability of membranes. This permeability causes alterations in the membrane structure and accelerates the leakage of intracellular constituents [53,54].

The acute toxicity *of* APL was assessed to prevent human exposure to potential risks associated with its consumption and to valorize the crude extract of its leaves. Throughout the study, no mortality of rats was observed after oral administration of 5000 mg/kg crude extract. According to Hodge and Stener's LD50 and route of administration toxicity scale (Table 1), this extract has a toxicity index equivalent to 5, indicating that it is almost non-toxic

[55, 56]. Furthermore, no signs of toxicity were observed during the first 4 h after administration of the extract, including decreased sensitivity to the stimulus (pain and noise), decreased mobility (motor activity) or softening of the feces, teeth grinding, tail condition, tremors, drowsiness and convulsion (Table 1). Concerning the relative weight of the organs in general, no significant difference was found between the control and the test group (dose of 5000 mg/kg of the crude extract) as well as on total protein. The liver is the central organ of metabolism. The activity of transaminases (ALAT, ASAT) increases when this organ is damaged [57]. This was not the case in this study because, compared to the control group, there was no significant variation in serum transaminase activity. This extract is therefore relatively non-toxic as substances with an oral LD50 greater than 5000 mg/kg are of low toxicity but may be hazardous to vulnerable populations [46].

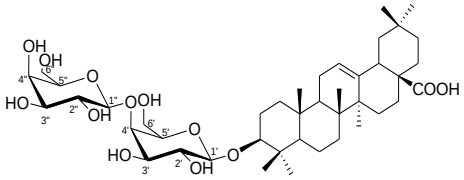

Figure 1. Chemical structure of 3-*O*-[*β*-galactopyranosyl-(1→ 4)-*β*-$_D$-galactopyranosyl]-oleanolic acid isolated from the leaves of *Acacia polyacantha*

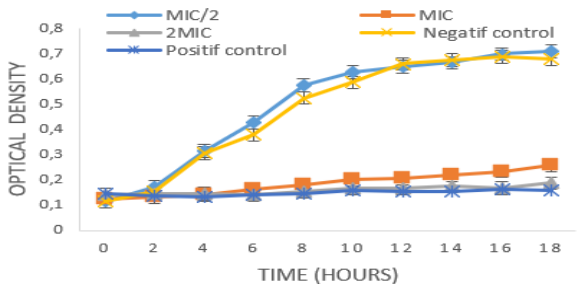

Figure 2. Effect of crude extract of *Acacia polyacantha* leaves on the growth kinetics of *Providencia stuartii* ATCC29916
Each point represents the mean ± SD; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one demi; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

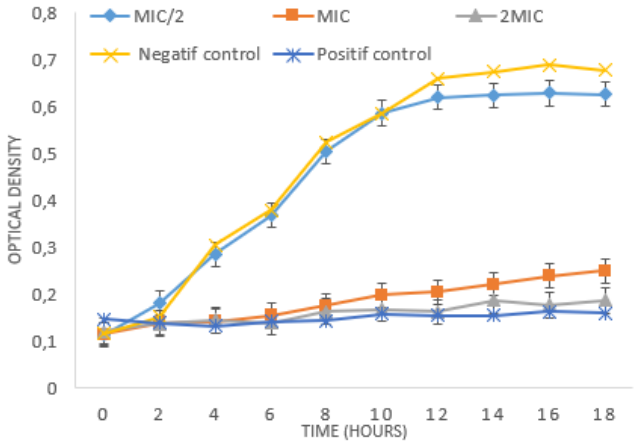

Figure 3. Effect of 3-*O*-[*β*-galactopyranosyl-(1→ 4)-*β*-$_D$-galactopyranosyl]-oleanolic acid on the growth kinetics of *Providencia stuartii* ATCC29916
Each point represents the mean ± SD; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one half; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

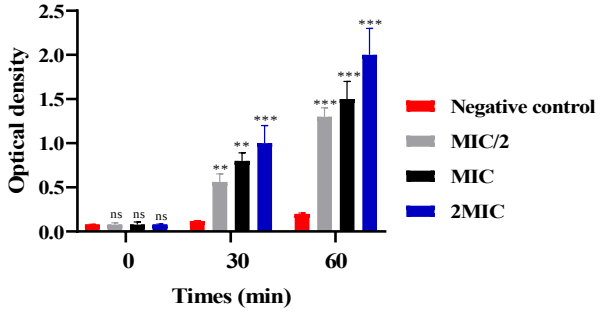

Figure 4. Effect of crude extract of *Acacia polyacantha* leaves on the plasma membrane of *Providencia stuartii* ATCC29916
The p-values 0.05 were considered statistically significant and their degree of significance was noted as * p-values <0.05; ** p-values <0.01; *** p-values <0.001 and **** p-values <0.0001. ns: not significant; Each point represents the mean ± SD; MIC/2 = Minimum Inhibitory Concentration multiplied by one; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

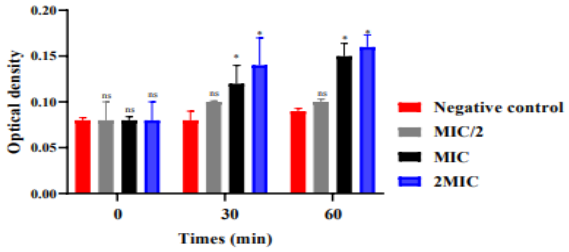

Figure 5. Effect of 3-*O*-[*β*-galactopyranosyl-(1→ 4)-*β*-$_D$-galactopyranosyl]-oleanolic acid on the plasma membrane of *Providencia stuartii* ATCC29916.
The p-values 0.05 were considered statistically significant and their degree of significance was noted as * p-values <0.05; ** p-values <0.01; *** p-values <0.001 and **** p-values <0.0001. ns: not significant; Each point represents the mean ± SD; MIC/2 = Minimum Inhibitory Concentration multiplied by one; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

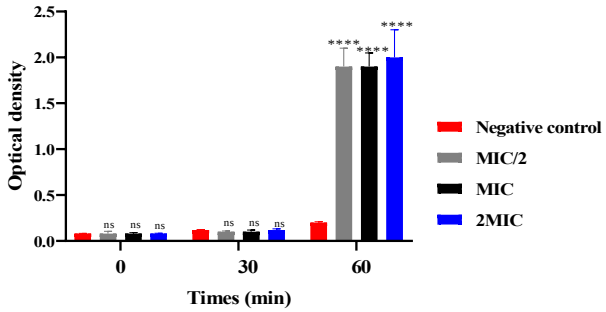

Figure 6. Effect of polymixin on the plasma membrane of *Providencia stuartii* ATCC29916.
The p-values 0.05 were considered statistically significant and their degree of significance was noted as * p-values <0.05; ** p-values <0.01; *** p-values <0.001 and **** p-values <0.0001. ns: not significant; Each point represents the mean ± SD; MIC/2 = Minimum Inhibitory Concentration multiplied by one; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

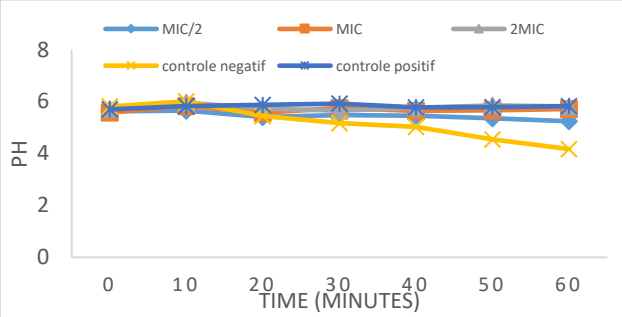

Figure 7. Effect of crude extract of *Acacia polyacantha* leaves on proton pumps of *Providencia stuartii* ATCC29916.
Each point represents the mean ± SD; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

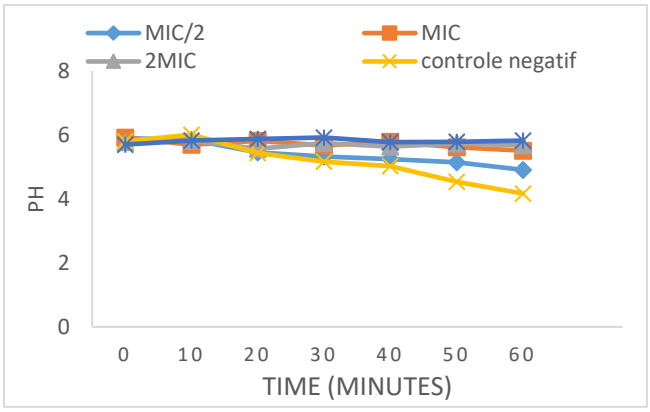

Figure 8. **Effect of 3-*O*-[*β*-galactopyranosyl-(1→ 4)-*β*-$_D$-galactopyranosyl]-oleanolic acid on proton pumps of *Providencia stuartii* ATCC29916**
Each point represents the mean ± SD; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two.

Table 1. Hodge and Stener toxicity scale for a chemical based on LD_{50} and route of administration

Toxicity indices	Commonly used terms	Routes of administration			Mount probably lethal in humans
		Oral LD_{50}	LD_{50} inhalation	Dermal LD_{50}	
		A single dose to rats (mg/kg)	Exposure of rats for 4 Hours (ppm)	A single application on the skin of rabbits (mg/kg)	A speck, a pinch, a drop
1	Extremely toxic	1 or less	10 or less	5 or less	4 mL
2	Highly toxic	1-50	10-100	5-43	30 mL
3	Moderately toxic	50-500	100-1000	44-340	600 mL
4	Slightly toxic	500-5000	1000-10000	350-2810	1 L
5	Almost non-toxic	5000-15000	10000-100000	2820-22590	1 L
6	Relatively harmless	15,000 or more	100,000 or more	22,600 or more	1 L

Table 2. General appearance and behavioral observations of acute toxicity study

Observation	Normal group	Test group
Sensitivity to pain	Normal	Normal
Sensitivity to noise	Normal	Normal
Motor activity	Normal	Normal
Teeth grinding	Absent	Absent
Appearance of feces	Normal	Normal
Condition of the tail	Normal	Normal
Trembling	Absent	Absent
Drowsiness	Absent	Absent
Convulsion	Absent	Absent

Table 3. Animal weight and weight gain during the experiment

Group of rats	Parameters		
	Initial weight	Final weight	Weight gain
Control (0mg/kg)	213.8 ± 3.059	227.2 ± 3.187	13.5 ± 3.261
Group test: methanol extract of *Acacia polyacantha* (5000 mg/kg)	174.8 ± 8.280^{ns}	191.2 ± 7.110^{ns}	16.2 ± 5.678^{ns}

ns: not significant.

Table 4. Effects of acute administration of methanol extract of *Acacia polyacantha* (APL) on relative organ weights in rats

Organ	Control (0 mg/Kg)	Methanol extract of *A. polyacantha* (5000 mg/Kg b.w.)
Liver	3.235 ± 0.16	3.537 ± 0.328^{ns}
Heart	0.301 ± 0.005	0.308 ± 0.018^{ns}
Lung	0.580 ± 0.092	0.551 ± 0.069^{ns}
Spleen	0.402 ± 0.019	0.407 ± 0.050^{ns}
Kidney	0.659 ± 0.033	0.692 ± 0.059^{ns}

Results were expressed as mean ± standard error of the mean (SD); differences were significant at the 5% probability level (P<0.05); ns: not significant.

Conclusion

The present study reports for the first time the mode of action of the crude leaf extract and 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid from the leaves of *A. polyacantha*. Moreover, the acute oral toxicity of the crude extract of the leaves showed that it is relatively non-toxic and potentially safe for human health. Sub-acute, chronic toxicity studies, pre-clinical and clinical investigations will be performed to provide more data on the safety of this plant.

Abbreviations

A. polyacantha: *Acacia polyacantha*; APL. methanol extract of the leaves of *Acacia polyacantha* Willd. (Fabaceae); ATCC: American Type Culture Collection ; b.w., body weight; CC: column chromatography; CFU: Colony Forming Unit; CHL: Chloramphenicol ; DMSO: Dimethyl sulfoxide ; GGOA, 3-*O*-[*β*-galactopyranosyl-(1→4)-*β*-$_D$-galactopyranosyl]-oleanolic acid; INT: p-iodonitrotetrazolium chloride ≥ 97% (INT, Sigma-Aldrich); LD_{50}, lethal dose ; MDR: Multidrug resistant ; MHB: Mueller Hinton Broth ; MIC: Minimal Inhibitory Concentration ; NMR: Nuclear Magnetic Resonance; OECD : Organization for Economic Cooperation and Development; OD: Optical Density; *P. stuartii*: *Providencia stuartii*; PBS : Phosphate Buffer Solution; PolyB: Polymixin B; RA: Reference antibiotic; RND : Resistance-nodulation-cell division; SD: standard deviation; WHO: World Health Organization.

Authors' Contribution

FTM, BENW, PN, FA and HTM carried out the study; VPB and VK designed the experiments; CFT wrote the manuscript; VK and VPE supervised the work; VK provided the bacterial strain and facilities for antibacterial assays; all authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest

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