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Antibacterial activity of four Cameroonian medicinal plants against MDR bacteria and study of mode of action

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Abstract

Background: The increasing number of resistant bacteria to commonly used antibiotics makes the search for new active molecules a real challenge. The aim of this study is to evaluate the antibacterial activities of crude extracts and fractions from four Cameroonian medicinal plants against multi-resistant bacteria as well as their mechanisms of action.

Methods: The crude extracts were prepared by maceration in methanol. The fractionation was carried out by successive depletion in hexane and ethyl acetate. The qualitative phytochemical analysis of the extracts and fractions was performed using standard methods. The antibacterial activities of extracts alone and their synergistic effect with amoxicillin and serum were evaluated using the broth microdilution method. The effect of extracts on the red blood cells and bacterial cell membrane was determined by spectrophotometric method. The bacteriolytic activity was evaluated by the time-kill kinetic method.

Results: The results showed that all the crude extracts contain phenols, alkaloids, sterols, triterpenes and tannins. The extracts of *Curcuma longa, Rubus idaeus, Centella asiatica* and *Taraxacum officinale* displayed variable antibacterial activities (MIC= 64-2048 µg/mL) confirming their traditional use in the treatment of infectious diseases. The fractionation of methanol extracts of *C. longa* and *R. idaeus* has distributed the antibacterial activities in different fractions. Some synergistic effects between amoxicillin and methanol extracts of *C. longa* and *R. idaeus* were observed. The antibacterial activities of plant extracts and tetracycline increased under osmotic stress conditions (2.5% NaCl) while those of vancomycin decreased under these conditions. A loss of nucleic material and a decrease in the optical density from *S. aureus* suspension treated with the methanol extracts of *C. longa* and *R. idaeus* were observed. The serum resulted in a concentration-dependent increase in the antibacterial activity of the methanol extracts of *C. longa* and *R. idaeus*. The tested plant extracts showed less haemolytic activity, indicating their good selectivity to the bacterial cell.

Conclusions: Overall, the present results show that the studied plant extracts possess antibacterial activity that can justify their traditional use in the treatment of infected diseases. The antibacterial activity mechanism is due to cell lysis and disruption of the bacterial cytoplasmic membrane.

Keywords: Medicinal plants; extracts; Phytochemical analysis; antibacterial; synergy; mode of action.

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Background

Infectious diseases are pathologies resulting from the aggression of an organism by pathogens such as viruses, fungi, parasites and bacteria. They are responsible for relatively high morbidity and mortality in children and adults but mainly in immunocompromised person worldwide [1,2]. In developing countries such as Cameroon, recession and poverty are partly responsible for the upsurge of these infectious diseases. They are the second leading cause of death in the world, with more than 17 million deaths each year, nearly 90% of them in developing countries [3]. Bacterial infections in particular, account for 70% of the mortality [4]. Examples included typhoid fever, which attacks about 21.5 million of people, diarrheal diseases that kill 5-8 million of children each year worldwide [5], and *Staphyococcus aureus* infections which cause 7-10% of deaths [6].

The treatment of bacterial infections is generally based on the use of synthetic antibiotics. The resurgence of these infections is due in part to the emergence of new multi-resistant strains following the abusive and often inappropriate use of these drugs [7]. To this first problem is added the cost of treatment which remains very high compared to the purchasing power of most patients, especially in poor countries where health insurance is almost non-existent. Moreover, the limited spectrum of action and high toxicity of certain antibacterial drugs [8] constitute handicaps to the eradication of these infections [9]. All these difficulties justify the need for a permanent search for new antimicrobial substances.

Faced with these problems, the real challenge would be to find new antibacterial substances that are effective, available and less toxic. As a result, the use of medicinal plants with antimicrobial properties is a more interesting way to explore, since they have a variety of secondary metabolites endowed with pharmacological properties [7]. Thus, several varieties of plants encountered in Cameroon could constitute a source of antibacterial agents. Hence, the aim of this study is to evaluate the antibacterial activity of extracts and fractions from four Cameroonian medicinal plants against multi-resistant bacterial strains as well as their mechanisms of action.

Methods

Plant materials

Plant materials were consisted of rhizomes of *Curcuma longa*, collected at Bamenda (North-West Region of Cameroon); leaves of *Rubus idaeus*, harvested in the locality of Santa (North-West Region of Cameroon); whole plants of *Taraxacum officinale* and *Centella asiatica* collected at Dschang (Western Region of Cameroon). These plants were harvested in May 2016. They were selected on the basis of their traditional uses (Table 1). These plants were identified and authenticated at the Cameroon National Herbarium, where the voucher specimens were kept under the reference numbers (Table 1). The plant material was washed thoroughly under running water and dried under room temperature. It was crushed to powder using mixer grinder and stored in air tight bottles.

Plant extraction

One hundred grams (100 g) of finely powdered plant parts were separately weighed out in a weighing balance and macerated in 1000 mL of methanol for 48 hours. The mixture was stirred 4 times per day to maximize the yield. After 48 hours, the mixture was filtered using Wattman No. 1 paper. The filtrate was evaporated using a rotary evaporator (BÜCHI R-200) at 65 °C to give the extract. The extraction yield was calculated and the dried extract was stored in well closed containers under refrigeration conditions and dilutions of the plant extract in DMSO were used for antimicrobial studies.

Preparation of fractions

Hexane fraction: The methanol extracts of *C. longa* (11.53 g) and *R. idaeus* (11.96 g) were dissolved separately in 100 mL of hexane. The whole was stirred and then left to stand for 30 min. The hexane phase was decanted. We repeated the operation until the solvent collected was as light as possible. The hexane phase was concentrated on the rotary evaporator at 68 °C to give the hexane fraction which was dried in an oven at 40 °C for complete evaporation of the hexane.

Ethyl acetate fraction: A volume of 100 mL of ethyl acetate was added to the hexane residue, stirred and allowed to stand for 30 min. The upper phase with ethyl acetate was decanted. The operation was repeated until the solvent collected was as light as possible. The ethyl acetate phase was concentrated on a rotary evaporator at 78 °C to give the ethyl acetate fraction which was dried in an oven at 40 °C for complete evaporation of the solvent.

Residual fraction: It's constituted of the remainder from the ethyl acetate fraction. It was then dried in an oven at 40 °C for complete evaporation of the solvent residues.

Table 1. Botanical identification, parts used, extraction solvent/yield and traditional therapeutic indications of studied medicinal plants.

Scientific name (Family)	Voucher specimen	Part used; extraction solvent and yield	Traditional therapeutic indications
Curcuma longa L. (Zingiberaceae)	42173/HNC	Roots; methanol; 6.27%	Anti-bacterial, antioxidant, antifungal, antispasmodic, antiasthmatic, antiviral, hypoglycemiant, immunostimulant, anti-ulcer, anti-inflammatory, anti-cancer [10]. Coughs, ageing processes, diabetes, diarrhoea, liver diseases, stomach disorders, wound healing, cholesterol-lowering effect [11].
Rubus idaeus L. (Rosaceae)	52342/HNC	Leaves; methanol; 5.89%	Wounds, colic, diarrhea, kidney infection, childbirth, antibacterial, antiproliferative, antioxidant, anti-inflammatory and anti-obese activities [12,13].
Taraxacum officinale F.H.Wigg. (Asteraceae)	35489/HNC	Aerial part; methanol; 11.43%	Digestion, liver disorder, diuretic, anti-rheumatic and anti-inflammatory properties [14].
Centella asiatica (L.) Urb. (Apiceae)	7042/SRF/Cam	Aerial part; methanol; 9.58%	Albinism, anemia, asthma, bronchitis, cellulitis, cholera, diarrhea, smallpox, constipation, dermatitis, dizziness, dysentery, dysmenorrhea, epilepsy, hemorrhoids, hypertension, nephritis, neuralgia, rheumatism, toothache, and chickenpox. The plant is also used as anti-inflammatory, antioxidant and immunostimulant [15,16].

Phytochemical screening of extracts

The phytochemical screening of the crude extract was carried out according to the methods described by Trease and Evans [17]. The plant extract was screened for the presence of different classes of compounds including alkaloids, flavonoids, steroids, triterpenes, anthraquinones, tannins, anthocyanins, saponins and polyphenols.

Antibacterial assay

Microorganisms

The microorganisms used in this study were consisted of five Gram-positive bacteria namely *Staphylococcus aureus* ATCC 25923, methycillin sensitive *S. aureus* (MSSA1), methycillin resistant *S. aureus* (MRSA3 and MRSA4) and *Bacillus subtilis*. Also included were six Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Shigella flexneri* SDINT, *Vibrio cholerae* NB2, SG24(1) and CO6. These bacteria were collected from the Department of Biochemistry, University of Calcutta in India and from the Institute of Medical Mycology**,** Teikyo University in Japan. Among the clinical strains of *Vibrio cholerae* used in this study, strains NB2 and SG24 belonged to O1 and O139 serotypes, respectively. All these strains were able to produce cholera toxin and hemolysin and multi-drug-resistants (MDR). The other strains used in this study were *V. cholerae* non-O1 and non-O139 (strain CO6). The MDR *V. cholerae* non-O1 and non-O139 strain CO6 isolated from aquatic environment was positive for hemolysin production but negative for cholera toxin production [18]. The bacterial strains were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar plates 24 h prior to any antibacterial test. The Mueller Hinton Agar (MHA) was used for the activation of bacteria. The Mueller Hinton Broth (MHB) and nutrient agar (Hi-Media) were used for the MIC and MBC determinations respectively.

Inocula preparation

Suspensions of bacteria were prepared in MHB from cells arrested during their logarithmic phase growth (4 h) on MHB at 37 °C. The turbidity of the microbial suspension was read spectrophotometrically at 600 nm and adjusted to an OD of 0.1 with MHB, which is equivalent to 1×10^{8} CFU/mL. From this prepared

solution, other dilutions were made with MHB to yield $1x10^6$ CFU/mL.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of extracts and their fractions were assessed using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute [19,20] with slight modifications. Each test sample was dissolved in dimethylsulfoxide (DMSO) to give a stock solution. The 96-well round bottom sterile plates were prepared by dispensing 180 µL of the inoculated broth ($1x10^6$ CFU/mL) into each well. A 20 µL aliquot of the stock solution of extract/fractions was added. The concentrations of tested sample were 16, 32, 64, 128, 256, 512, 1024 and 2048 µg/mL. The final concentration of DMSO in each well was <1% [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms]. Dilutions of amoxicillin (Sigma-Aldrich, Steinheim, Germany), chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and ciprofloxacillin (Sigma-Aldrich, Steinheim, Germany) served as positive controls, whereas broth with 20 µL of DMSO was used as negative control. The ATCC strain *Staphylococcus aureus* ATCC 25923 was included for quality assurance purposes. Plates were covered and incubated for 24 h at 37 °C. The assay was repeated three times. After incubation, the MIC values of samples were determined by adding 50 µL of a 0.2 mg/mL *p*-iodonitrotetrazolium (INT) violet solution followed by incubation at 37 °C for 30 min. Viable bacteria reduced the yellow dye to a pink color. MIC values were defined as the lowest sample concentrations that prevented this change in color indicating a complete inhibition of microbial growth.

For the determination of MBC values, a portion of liquid (10 µL) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar (MHA) and incubated at 37 °C for 24 h . The lowest concentrations that yielded no growth after this subculturing were taken as the MBC values.

Evaluation of the antibacterial activity of the associations between extracts and amoxicillin

The determination of the synergistic effect was performed with amoxicillin and the most active extracts by using the broth microdilution method as described above. The synergistic interaction between amoxicillin and MeOH extracts of *C. longa* and *R. idaeus* (the most active extracts) was performed. The antibacterial activity of the extract in the presence amoxicillin (1/8xMIC and ½xMIC) and that of amoxicillin in the presence of the extract (1/8xMIC and ½xMIC) were evaluated as described above. The preliminary tests allow the selection of MIC/8 and MIC/2 as the sub-inhibitory concentrations of the samples. The fractional inhibitory concentration (FIC) index for combinations of two antibacterial agents was calculated according to the following equation: FIC index = FIC A + FIC E; where FIC A = MIC of antibiotic in combination / MIC of antibiotic alone; FIC E = MIC of the extract in combination / MIC of the extract alone. The FIC indices were interpreted as follows: ≤ 0.5, synergy; > 0.5 to 1, addition; > 1 and ≤ 4, indifference and > 4, antagonism [21]. All the experiments were performed in triplicate.

Study of mode of action

Antibacterial assay under osmotic stress (2.5% NaCl) condition

Osmotic stress condition was prepared by adding 2.5% NaCl (w/v) to MHB. The MHB supplemented with 2.5% NaCl was then sterilized and used for the determination of the new MIC and MBC values of the samples as described above. The incubation time was increased from 24 hours to 48 hours at 37 °C.

Evaluation of the effect of methanol extracts of C. longa and R. idaeus on the cell membrane of S. aureus MSSA1, MRSA3 and MRSA4

The alteration of the cell membrane of *S. aureus* was evaluated by measuring the optical densities at 260 nm of bacterial suspensions in the presence and absence of the MeOH extracts of *C. longa* and *R. idaeus* using the method described by Carson et al. [22]. For this purpose, the extracts were tested at their MIC value using 1 mL of the bacterial suspension (at 10^8 CFU/mL). The mixture was incubated at 37 °C in an incubator. At different time intervals (0: immediately after addition of the extract; 15; 30; 60 min), 50 µL of the mixture was removed and mixed with 1.95 mL of PBS buffer. Absorbance was measured on the spectrophotometer at 260 nm against the blank (PBS). For the negative control, 1 mL of bacterial suspension was incubated at 37 °C and 50 µL of suspension were removed at the end of the various incubation times and melted at 1.95 mL of PBS buffer. The optical densities were read as before.

Bacteriolytic assay

The bacteriolytic activity of extracts from *C. longa* and *R. idaeus* was determined using the time-kill kinetic method as described by Ooi et al. [23] with slight modifications. Full growth of *S. aureus* MSSA1 in MHB was diluted 100 times and incubated at 37 °C to produce an OD_{600} of 0.8 as starting inoculum. Extract solutions were added to the starting bacterial suspension to give a final concentration of 2 × MIC and incubated at 37 °C with shaking, then 100 µL

were removed from each tube at 0, 15, 30, 60, and 120 min and the optical density measured at 600 nm. Amoxicillin was used as a positive control and the tubes without extracts served as a negative control.

Haemolytic assay

Whole blood (10 mL) from albino rats was collected by cardiac puncture into a conical tube containing EDTA as an anticoagulant. The study was conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals (Registration no. 173/CPCSEA, dated 28 January, 2000), Government of India, on the use of animals for scientific research. Erythrocytes were harvested by centrifugation at room temperature for 10 min at 1,000 x *g* and were washed three times in PBS solution [24]. The cytotoxicity was evaluated as previously described [24]. Non-haemolytic and 100% haemolytic controls were the buffer alone and the buffer containing 1% Triton X-100, respectively. Cell lysis was monitored by measuring the release of hemoglobin at 595 nm with a spectrophotometer (Thermo Scientific, USA). Percent hemolysis was calculated as follows:

[(A595 of sample treated with extract - A595 of sample treated with buffer)/(A595 of sample treated with Triton X-100 – A595 of sample treated with buffer)] x 100.

Antibacterial assay in the presence of serum

The antibacterial activity of all the extracts was also performed in the presence of serum. The Muller Hinton broth (MHB) was prepared, sterilized and cooled to room temperature; then supplemented with rat serum at concentrations of 2.5% and 5%. The supplemented MHB was used to determine the new MIC and MBC values as previously described.

Results and discussion

Phytochemical analysis of extracts and fractions

The phytochemical analysis of plant extracts and their fractions was carried out with the aim of highlighting the different classes of secondary metabolites that can explain their antibacterial properties. The results of the chemical analysis of the methanol extract of *C. longa* revealed the presence of phenols, alkaloids, flavonoids, steroids, triterpenes, tannins, anthrocyanins, coumarins, saponins and anthraquinones (Table 2). These results partially corroborate those of Gupta *et al.* [25] who showed the presence of alkaloids, tannins, phenolic compounds, glycosides, carbohydrates, flavonoids and the absence of steroids and triterpenoids in the methanol extract of rhizomes of this plant species. The phytochemical screening also revealed the presence of phenols, alkaloids, flavonoids and tannins and the absence of saponins in the methanol extract of *T. officinale*. These results are partially different of those obtained by Jassim et al. [26] who showed the presence of glycosides, phenolic compounds, tannins, flavonoids and proteins as well as the absence of resins, saponins, alkaloids in aqueous and alcoholic extracts of *T. officinale* leaves. The phytochemical analysis of the *C. asiatica* extract revealed the presence of phenols, alkaloids, steroids, triterpenes and tannins, and the absence of flavonoids, anthocyanins, coumarins, anthraquinones and saponins. These results are in agreement with those obtained by Arumugam *et al.* [27], who demonstrated the presence of alkaloids, glycosides, flavonoids, terpenoids, steroids and reducing sugars as well as the absence of saponins in the methanol extract of the leaves of this plant. The results of the phytochemical analysis of the methanol extract of *R. idaeus* are partially different of those found by Okuda et al. [28] who showed the presence of phenolic acids and esters, flavonoids, anthocyanins, and tannins in the leaves of *R. idaeus*. The other classes of compounds are selectively distributed in fractions. The differences in the chemical compositions of crude extracts and those of the literature may be due to the extraction solvents, the place and the harvest period of the plant or the variety of plant species. Moreover, the differences in chemical composition between the various fractions of extract should be linked to the difference in polarity of extraction solvents. Indeed, each extraction solvent selectively extracts groups of compounds which are soluble to it.

Antibacterial activity of crude extracts and fractions

The plant extracts and their fractions possess antibacterial activities which vary according to the bacterial species (Table 3). MICs of plant extracts range from 64 µg/mL to 2048 µg/mL whereas those of fractions vary between 32 µg/mL and 2048 µg/mL. The methanol extract of *C. longa* (MIC = 64 - 1024 µg/mL) was the most active followed in decreasing order by *R. idaeus* extract (MIC = 64 - 2048 µg/mL) > *C. asiatica* extract (MIC = 512 - 2048 µg/mL) > *T. officinale* extract (MIC = 1024 - 2048 µg/mL). The fractionation of MeOH extracts of *C. longa* and *R. idaeus* enhanced their antibacterial activities in some fractions. In general, the ethyl acetate fractions of *C. longa* (MIC = 32-512 µg/mL) and *R. idaeus* (MIC = 256-512 µg/mL) were the most active when compared with other fractions. On the basis of the MBC values (Table 4), the antibacterial activities of the studied plant extracts also varied according to the extracts, fractions and tested bacteria.

Differences in the antibacterial activity were noted between the crude extracts and fractions. These

differences may be due to the different classes of secondary metabolites contained in these extracts. Indeed, the antimicrobial activity of medicinal plants is correlated with the presence in their extracts of one or more classes of bioactive secondary metabolites [29]. This could justify the case of the methanol extract of *C. longa*, which was the most active and contains all groups of secondary metabolites. Nevertheless, early reports showed that the antimicrobial activity of plant extracts does not necessarily depend on the number of active compounds present in these extracts [30]. This observation could also justify the antibacterial activity of methanol extract of *R. idaeus*, which contains the same number of secondary metabolites as *T. officinale* and was more active than the latter. All these observations suggest that the antibacterial activity of a plant extract depends not only on the presence of secondary metabolites, but also on the types present, their quantity and the possible interactions between the constituents.

Effect of the association between extracts and amoxicillin

The synergy effect between plant-derived compounds and antibiotics makes it possible to use antibiotics when their efficacy alone is reduced [30]. These observations could explain the evaluation of the antibacterial activity of the combination between amoxicillin and plant extracts. The MIC values of *C. longa* and *R. idaeus* extracts in combination with amoxicillin at ½ MIC are smaller than those of their respective extract used alone; suggesting an increase in the activity of these extracts in combination with amoxicillin (Table 5). Also, the MIC values of amoxicillin in combination with *C. longa* and *R. idaeus* extracts at ½ MIC are smaller than those of amoxicillin alone; indicating an increase in the activity of amoxicillin in combination with the extracts at their ½MICs (Table 6). The MeOH extract of *C. longa* has a synergistic effect against *B. subtilis*, *E. coli* S2 (1), *P. aeruginosa*, *S. flexneri* SDINT, *V. cholerae* CO6, *S. aureus* ATCC, MSSA1, MRSA3 and MRSA4 as well as an additive effect against *V. cholerae* NB2 and *V. chorae* SG24 in the presence of amoxicillin (Table 7). Amoxicillin and the MeOH extract of *R. idaeus* exert in combination indifference effects against *B. subtilis* and *S. aureus* ATCC; synergistic effects on *E. coli* S2 (1), *P. aeruginosa*, *S. flexneri* SDINT, *V. cholerae* CO6, *S. aureus* MSSA1, MRSA3 and MRSA4 and additive effects against *V. cholerae* NB2 and *V. chorae* SG24 (Table 8). Indeed, in addition to substances having direct antibacterial activity, it has been demonstrated that within the plants other substances can act as adjuvants by modulating the activity of antibacterial agents [31]. The polyphenols, as well as the flavonoids detected in most of these extracts, would be responsible to the potentiating activity observed. Indeed, several studies have shown that polyphenols and flavonoids may improve antibiotic activity against multi-drug-resistant bacterial strains [32-33].

Table 2. Secondary metabolites present in the extracts and fractions of the studied plants

Extracts	Secondary metabolites									
	Phe	**Alc**	**Flav**	**Ster**	**Trit**	**Antho**	**Cou**	**Anthr**	**Tan**	**Sapo**
MeOH extract of *C. longa*	+	+	+	+	+	+	+	+	+	+
Hexane fraction of *C. longa*	-	+	-	+	+	-	-	-	-	-
Ethyl acetate fraction of *C. longa*	+	+	+	-	-	+	+	+	+	-
Residual fraction of *C. longa*	+	+	+	-	-	+	+	+	+	+
MeOH extract of *R. idaeus*	+	+	-	+	+	-	+	+	+	+
Hexane fraction of *R. idaeus*	-	+	-	+	+	-	-	+	-	-
Ethyl acetate fraction of *R. idaeus*	-	+	-	+	+	-	+	-	-	-
Residual fraction of *R. idaeus*	+	+	-	-	-	-	+	-	+	+
MeOH extract of *T. officinale*	+	+	+	+	+	+	-	+	+	-
MeOH extract of *C. asiatica*	+	+	-	+	+	-	-	-	+	-

+ : presence ; - : absence ; Phe : Phenols ; Alc : Alkaloids; Flav : Flavonoids ; Ster : Steroids ; Trit : Triterpenes ; Antho : Anthocyanins ; Cou : Coumarins ; Anthr : Anthraquinones ; Tan : Tannins ; Sapo : Saponins

Mode of action of extracts

Effect of osmotic stress on antibacterial activity of extracts

Under osmotic stress (at 2.5% NaCl), the MIC values of plant extracts are generally smaller than those obtained under normal conditions (at 0% NaCl) (Table 9). In the presence of 2.5% NaCl, the *C. longa* MeOH extract was the most active, followed in decreasing order by *R. idaeus*, *T. officinale* and *C. asiatica*. With the exception of the MIC values on *E. coli* S2 (1), *P. aeruginosa*, *V. cholerae* NB2, *V. cholerae* SG24 and *V. cholerae* CO6, the MIC values

of chloramphenicol determined under osmotic stress conditions are smaller than those determined under normal conditions. However, the MIC values of vancomycin determined under osmotic stress are higher than those obtained under normal conditions. As the MIC values, the MBCs of MeOH extracts of *C. longa* and *R. idaeus* determined under osmotic stress are smaller than those obtained under normal conditions (Table 10). With the exception of MBC values of the MeOH extract of *T. officinale* against *E. coli* S2 (1) and *S. flexneri* SDINT, the MBC values of MeOH extracts of *T. officinale* and *C. asiatica* have not been improved under osmotic stress.

The antibacterial activities of plant extracts and chloramphenicol increased under osmotic stress (2.5% NaCl) whereas those of vancomycin decreased under these conditions. The current findings have key implications in disinfectants, antiseptics and wound-dressing formulations for topical treatments since *C. longa*, *R. idaeus*, *T. officinale and C. asiatica* are traditionally used as wound healing in Cameroonian medicinal system. Previous reports have demonstrated that some bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *V. cholerae*) could survive and develop under osmotic stress conditions [34]. Under these conditions, the salt will induce the accumulation of osmotic protectors such as glycine, betaine and proline from the growth medium [35] as well as the increase in the synthesis of stress proteins such as Chaperones [36] and the alkyl hydroperoxide reductase C [37] for adapting the bacteria to stress [34]. A previous study has shown that at low water activity, the lipid composition of the bacterial cell membrane is modified [38]. This phenomenon is likely to lead to the appearance of a greater number of antibacterial binding sites at the cellular membrane of the bacteria; which may explain the increased susceptibility of bacteria to the extracts and chloramphenicol. Thus, the presence of the salt in the medium can cause changes in the lipid composition of the membrane [38]; making it more permeable to plant extracts and chloramphenicol: a synthetic antibiotic inhibiting protein synthesis by inhibition of the polymerase. This may explain the increased antibacterial activity of these samples. However, the mechanisms that make bacteria more sensitive to certain antibiotics/extracts under osmotic stress conditions are still unknown. The results on vancomycin activity are similar to those of McMahon *et al.* [39] who demonstrated a decrease in the activity of amikacin, ceftriaxone and trimethoprim against *E. coli* and *S. aureus* under osmotic stress conditions. Unlike vancomycin, a synthetic antibiotic acting on the synthesis of the bacterial wall by blocking the polymerization of peptidoglycan, plant extracts contain a multitude of compounds that can act individually or interact on several targets [40]. This could make it difficult to develop mechanisms of resistance by bacteria to the tested extracts [40].

Effect of serum on the antibacterial activity of extracts

The MIC and MBC values of MeOH extracts of *C. longa* and *R. idaeus* determined in the presence of the serum are generally smaller than those determined in the absence of the serum; suggesting an increase in the antibacterial activity of these extracts in the presence of the serum (Table 11). In most cases, these activities increase with the serum concentration in the medium. The determination of the serum effect on the antibacterial activity of an extract may be useful in defining optimal therapy [41]. In our study, we noted a concentration-dependent increase in the antibacterial activity of MeOH extracts of *C. longa* and *R. idaeus* in the presence of serum. This result suggests, firstly, a synergistic effect between the constituents of the extract and those of the serum and secondly, that the chemical constituents of these extracts bind weakly to the serum proteins. It is generally accepted that antibiotics which bind serum proteins have a reduced antibacterial activity when tested *in vitro* in the presence of serum proteins because only the free drug is available for antibacterial activity [42]. *In vitro* synergy between antibiotics and serum components, including antibodies and complement has been reported [43,44]; and can also be expressed in patients' responses to antimicrobial chemotherapy. Early report demonstrated that the antibacterial activity of cetrimide, sodium hypochlorite and chlorhexidine against *Enterococcus faecalis* was improved by the association with bovine serum compared to the activity of antiseptics alone [45].

Effect of methanol extracts of C. longa and R. idaeus on the loss of S. aureus MSSA1, MRSA3 and MRSA4 nucleic material

After treatment of *S. aureus* suspensions with the methanol extracts of *C. longa* and *R. idaeus* at MIC values, the OD_{260} of filtrates of all tested strains increased and most of the leakage occurred during the initial period (≤ 15 min), followed by a slight increase with prolonging the incubation period. At the same time, the OD_{260} of control without extract was not changed (Figure1). After 60 minutes, the extracts of *C. longa* and *R. idaeus* induce an increase in the optical density of 70.69% and 50.43% for the MSSA1; 62.78% and 52.43% for the MRSA3, and finally 69.66% and 60.54% for MRSA4, respectively. Many antimicrobial compounds (α-pinene, chlorhexidine, polymyxin, tetracyclines,) that act on the bacterial cytoplasmic membrane induce loss of genetic material [22]. In the present study, the loss of absorbent material at 260 nm was observed and the greatest loss was recorded during the first incubation periods, followed by a slight increase as a result. Plant phenols are known for their interaction with

DNA [46,47]. The loss of the genetic material of the bacterial cells treated with the extracts of *C. longa* and *R. idaeus* may be due to an alteration of the bacterial cell membrane caused by the presence of the phenolic compounds found in these plants. This finding is in agreement with the results of Stojković et al. [48] who showed that protocatechuic acid, a main phenolic compound in the aqueous extract of *Veronica montana*, inhibits the growth of *Listeria monocytogenes* by causing changes in permeability of the cell membrane.

Bacteriolytic activity of the extracts of C. longa and R. idaeus against S. aureus SASM1

The results of the bacteriolytic activity showed a decrease in the optical density of the *S. aureus* suspension (SAAM1) treated with the extracts of *C. longa* and *R. ideaus* (Figure 2). After 120 minutes, the extracts of *C. longa* and *R. idaeus* induced a reduction in the turbidity of the bacterial suspension of 90.82% and 94.53% respectively with respect to time zero incubation; indicating lysis of bacterial cells. The analysis of results also showed that the *C. longa* extract is more able to cause lysis of bacterial cells compared to that of *R. idaeus* and amoxicillin.

Haemolytic activity of extracts and fractions from the studied plants

No haemolytic activity was observed with the ethyl acetate fraction of *C. longa*, extracts and residual fraction of *C. longa* and *R. idaeus* (the results are not presented). In contrast, *C. asiatica* extract (34.24%) had the highest percentage of haemolytic activity followed in decreasing order by the extract of *T. officinale* (28.49%), ethyl acetate fraction of *R. idaeus* (18.88%), hexane fractions of *R. idaeus* (10.78%) and *C. longa* (5.55%). In all, the extracts and fractions of the studied plants showed little or no hemolytic activity; suggesting their good selectivity against the bacterial cell (Figure 3).

Table 3. Minimal inhibitory concentrations (MIC) of crude extracts and fractions from the studied plants according to the tested bacteria

Bacteria	MeOH extract of *C. longa*	Hexane fraction of *C. longa*	Ethyl acetate fraction of *C. longa*	Residual fraction of *C. longa*	MeOH extract of *T. officinale*	MeOH extract of *C. asiatica*	MeOH extract of *R. idaeus*	Hexane fraction of *R. idaeus*	Ethyl acetate fraction of *R. idaeus*	Residue extract of *R. idaeus*	Amo.	Chlo.	Cip.
B. subtilis	1024	512	256	512	1024	1024	256	512	256	256	32	16	1
E. coli S2(1)	512	1024	128	256	1024	512	256	256	512	512	64	4	1
P. aeruginosa	512	512	512	512	2048	2048	1024	512	256	1024	128	64	2
V. cholerae NB2	128	1024	256	1024	1024	512	512	512	512	512	128	64	16
S. flexneri SDINT	256	512	64	256	2048	2048	1024	512	512	256	1	64	4
S. aureus	64	512	64	512	1024	1024	512	512	512	512	1	32	0.5
V. cholerae SG24	128	2048	64	128	2048	2048	2048	1024	512	256	128	4	32
V. cholerae CO6	512	512	32	256	1024	1024	512	512	256	256	8	16	4
S. aureus MSSA1	128	/	/	/	2048	1024	64	/	/	/	4	2	1
S. aureus MRSA3	128	/	/	/	2048	2048	256	/	/	/	16	64	2
S. aureus MRSA4	128	/	/	/	2048	2048	128	/	/	/	16	64	2

Amo : Amoxicillin; Chlo. : Chloramphenicol; Cip. : Ciprofloxacin; /: Not determined

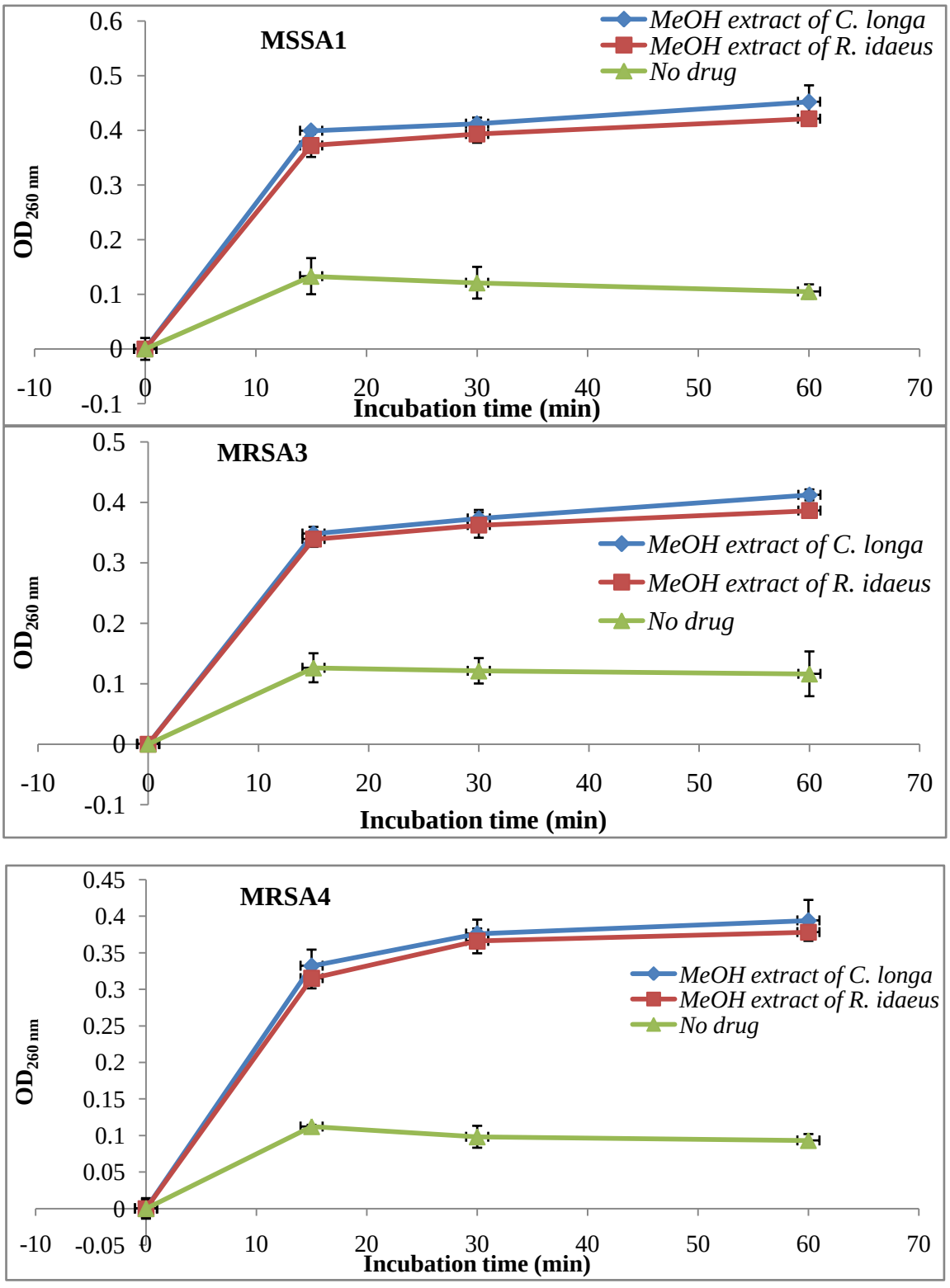

Figure 1. Effect of methanol extracts of *C. longa* and *R. idaeus* on the loss of *S. aureus* SASM1, SARM3 and MRSA4 nucleic acid. Results represent the mean ± standard deviation of the triplicate $OD_{260\ nm}$ at each incubation time.

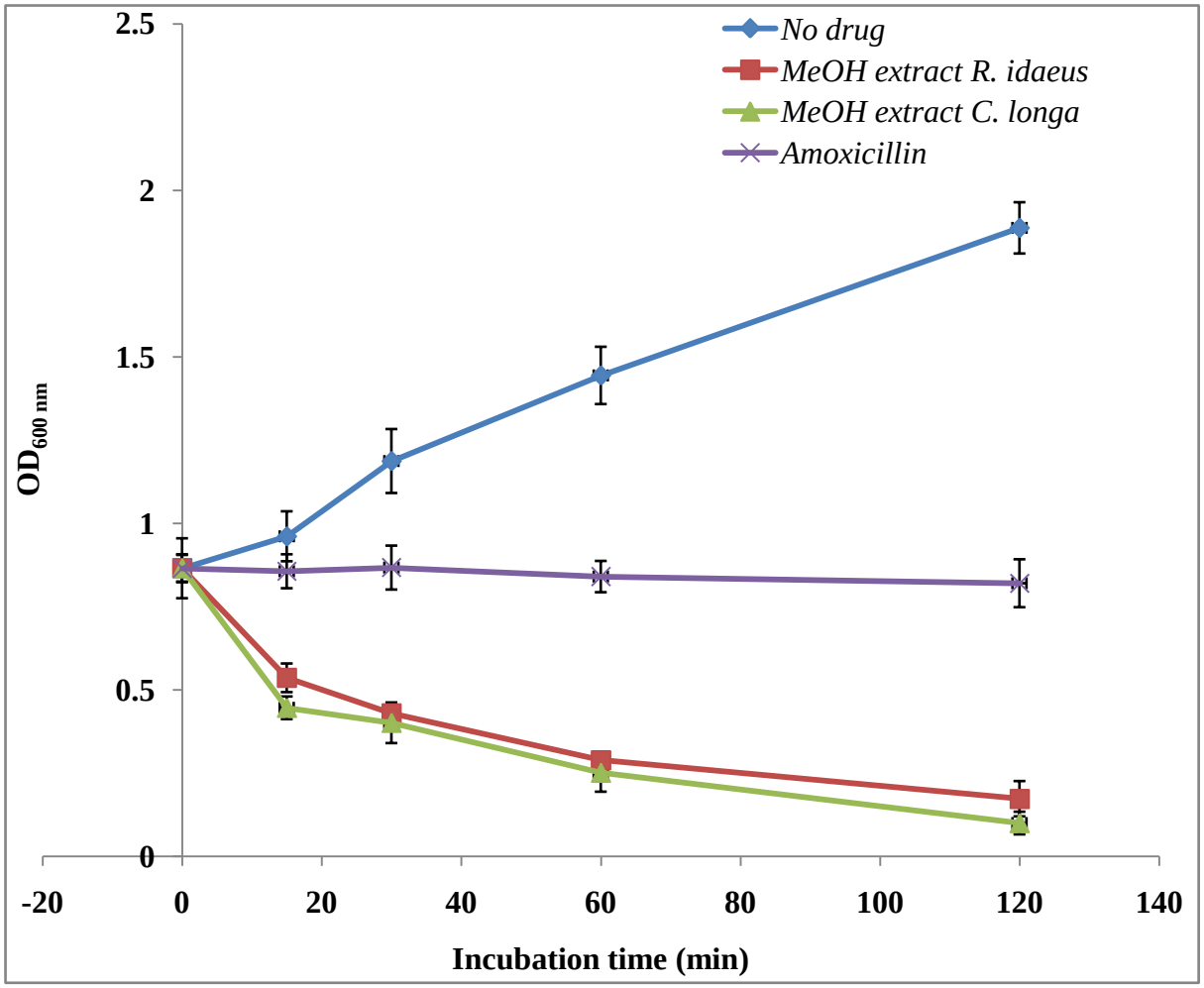

Figure 2. Bacteriolytic effect of methanol extracts of *C. longa* and *R. idaeus* on *Staphylococcus aureus* MSSA1. Results represent the mean ± standard deviation of the triplicate $OD_{600\ nm}$ at each incubation time.

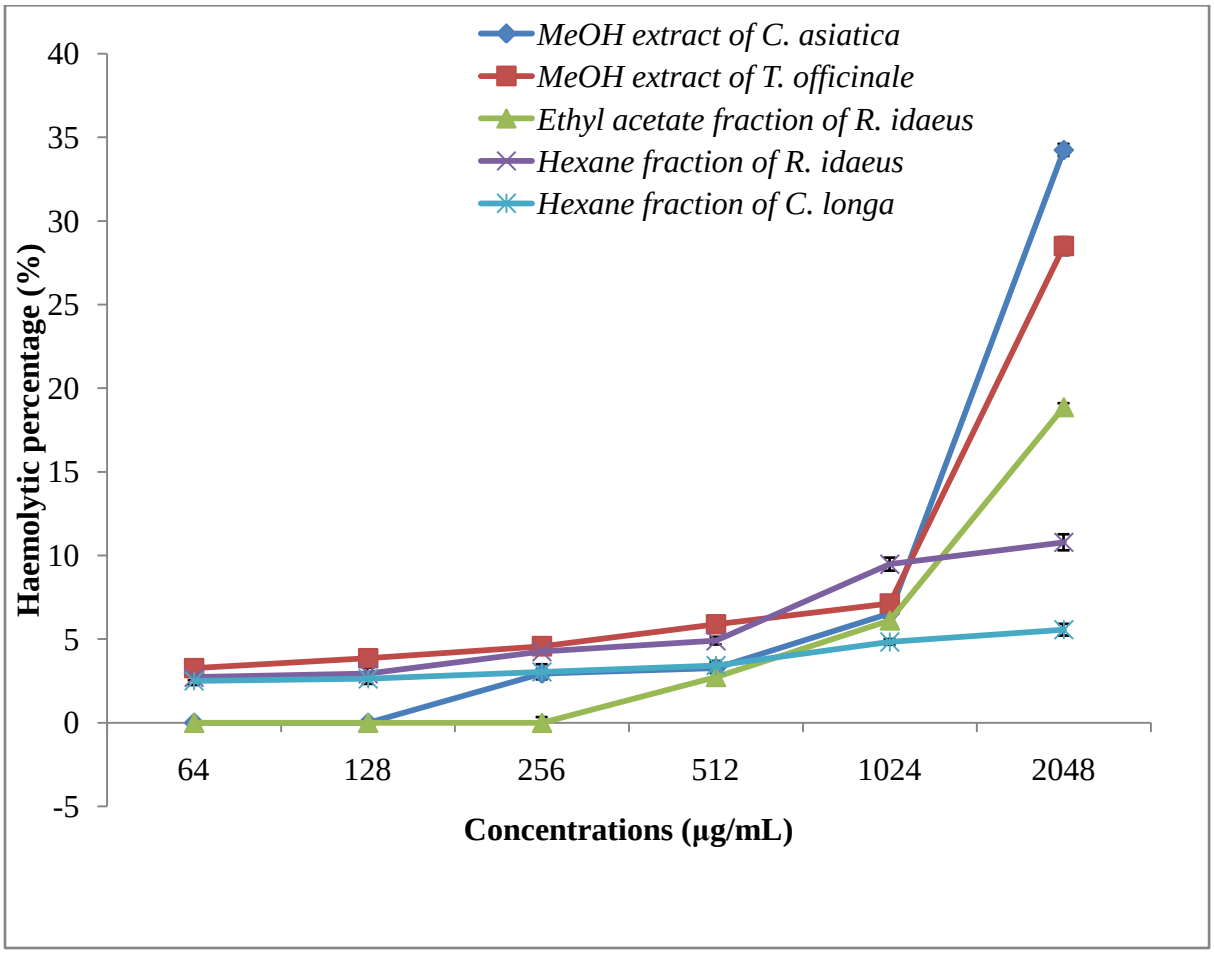

Figure 3. Haemolytic activity of extracts and fractions of plant extracts studied as a function of concentrations. Results represent the mean ± standard deviation of the triplicate hemolytic activity at each concentration.

Table 4. Minimum bactericidal concentrations (MBC) of crude extracts and fractions from the studied plant according to the tested bacteria

Bacteria	MeOH extract of *C. longa*	Hexane fraction of *C. longa*	Ethyl acetate fraction of *C. longa*	Residual fraction of *C. longa*	MeOH extract of *T. officinale*	MeOH extract of *C. asiatica*	MeOH extract of *R. idaeus*	Hexane fraction of *R. idaeus*	Ethyl acetate fraction of *R. idaeus*	Residual fraction of *R. idaeus*	Amo.	Chlo.	Cip.
B. subtilis	>2048	2048	512	2048	>2048	>2048	1024	1024	512	512	32	128	1
E. coli S2(1)	1024	>2048	1024	1024	1024	2048	1024	2048	1024	1024	64	64	1
P. aeruginosa	2048	>2048	2048	1024	>2048	>2048	2048	1024	1024	>2048	256	256	2
V. cholerae NB2	>2048	>2048	2048	>2048	2048	1024	2048	1024	512	2048	256	256	16
S. flexneri SDINT	512	512	128	512	2048	>2048	2048	1024	1024	2048	4	128	4
S. aureus	256	1024	256	>2048	>2048	>2048	1024	2048	1024	2048	1	64	0.5
V. cholerae SG24	512	>2048	256	2048	>2048	>2048	>2048	>2048	>2048	>2048	256	16	32
V. cholerae CO6	2048	2048	512	2048	>2048	>2048	>2048	2048	2048	>2048	16	64	4
S. aureus MSSA1	512	/	/	/	>2048	1024	1024	/	/	/	4	2	1
S. aureus MRSA3	/	/	/	/	>2048	>2048	2048	/	/	/	16	64	2
S. aureus MRSA4	2048	/	/	/	2048	>2048	>2048	/	/	/	16	64	2

Amo : Amoxicillin; Chlo. : Chloramphenicol; Cip. : Ciprofloxacin; /: Not determined

Table 5. Antibacterial activities of plant extracts in the presence of amoxicillin at ½ and 1/8 of MIC as a function of bacteria.

Bacteria	MeOH extract of *C. longa*	MeOH extract of *C. longa* with amoxicillin at ½ MIC		MeOH extract of *C. longa* with amoxicillin at 1/8 MIC		MeOH extract of *R. idaeus*	MeOH extract of *R. idaeus* with amoxicillin at ½ MIC		MeOH extract of *R. idaeus* with amoxicillin at 1/8 MIC	
	MIC	MIC	FIC	MIC	FIC	MIC	MIC	FIC	MIC	FIC
B. subtilis	1024	32	0.031	128	0.125	256	32	0.125	256	1.00
E. coli S2(1)	512	8	0.015	32	0.062	256	32	0.125	64	0.25
P. aeruginosa	512	64	0.125	128	0.25	1024	128	0.125	512	0.5
V. cholerae NB2	128	64	0.5	128	1.00	512	256	0.5	1024	2
S. flexneri SDINT	256	32	0.125	128	0.50	1024	32	0.031	512	0.5
S. aureus	64	32	0.5	128	0.50	512	64	0.125	512	1.00
V. cholerae SG24	128	64	0.5	256	2	2048	256	0.125	1024	0.5
V. cholerae CO6	512	32	0.062	128	0.25	512	64	0.125	512	1.00
S. aureus MSSA1	128	32	0.25	64	0.5	64	16	0.25	32	0.5
S. aureus MRSA3	128	16	0.125	32	0.25	256	8	0.031	32	0.125
S. aureus MRSA4	128	16	0.125	32	0.25	128	16	0.125	32	0.25

MIC: minimal inhibitory concentration; FIC: fractional inhibitory concentration

Table 6. Antibacterial activities of amoxicillin in the presence of plant extracts at ½ and 1/8 of MIC as a function of bacteria.

Bacteria	Amoxicillin alone	Amoxicillin in the presence of the *C. longa* MeOH extract at ½ MIC		Amoxicillin in the presence of the *C. longa* MeOH extract at 1/8 MIC		Amoxicillin in the presence of *R. idaeus* MeOH extract at ½ MIC		Amoxicillin in the presence of *R. idaeus* MeOH extract at 1/8 MIC	
	MIC	MIC	FIC	MIC	FIC	MIC	FIC	MIC	FIC
B. subtilis	32	8	0.25	32	1	32	1	64	2
E. coli S2(1)	64	2	0.031	4	0.062	2	0.031	4	0.062
P. aeruginosa	128	8	0.062	64	0.50	16	0.125	64	0.50
V. cholerae NB2	128	16	0.125	32	0.25	64	0.50	64	0.50
S. flexneri SDINT	1	0.0312	0.031	0.50	0.50	0.50	0.50	1	1
S. aureus	1	0.0312	0.031	0.125	0.125	1	1	4	4
V. cholerae SG24	128	64	0.50	128	1	64	0.50	128	1
V. cholerae CO6	8	.0312	0.004	1	0.125	0.50	0.062	1	0.125
S. aureus MSSA1	4	0.50	0.125	1	0.25	0.25	0.062	0.50	0.125
S. aureus MRSA3	16	4	0.25	2	0.125	1	0.062	2	0125
S. aureus MRSA4	16	2	0.125	4	0.25	0.50	0.031	1	0.062

MIC: minimal inhibitory concentration; FIC: fractional inhibitory concentration

Table 7. Fractional Inhibitory Concentration (FIC) indices calculated for the combination amoxicillin and MeOH extract of *C. longa* as a function of bacteria.

Bacteria	∑ FIC	Interpretation
B. subtilis	0.281	Synergy
E. coli S2(1)	0.046	Synergy
P. aeruginosa	0.187	Synergy
V. cholerae NB2	0.625	Additive
S. flexneri SDINT	0.156	Synergy
S. aureus ATCC	0.531	Synergy
V. cholerae SG24	1.00	Additive
V. cholerae CO6	0.066	Synergy
S. aureus MSSA1	0.375	Synergy
S. aureus MRSA3	0.375	Synergy
S. aureus MRSA4	0.25	Synergy

Σ FIC: sum of fractional inhibitory concentrations

Table 8. Fractional Inhibitory Concentration (FIC) indices calculated for the amoxicillin combination and MeOH extract of *R. idaeus* as a function of bacteria.

Bacteria	∑ FIC	Interpretation
B. subtilis	1.125	Indifference
E. coli S2(1)	0.156	Synergy
P. aeruginosa	0.25	Synergy
V. cholerae NB2	1.00	Additive
S. flexneri SDINT	0.531	Synergy
S. aureus	1.125	Indifference
V. cholerae SG24	0.625	Additive
V. cholerae CO6	0.187	Synergy
S. aureus MSSA1	0.312	Synergy
S. aureus MRSA3	0.093	Synergy
S. aureus MRSA4	0.156	Synergy

Σ FIC: sum of fractional inhibitory concentrations

Table 9. Effect of osmotic stress on the Minimum Inhibitory Concentrations of plant extracts according to the tested bacteria

Bacteria	MeOH extract of *C. longa*		MeOH extract of *T. officinale*		MeOH extract of *C. asiatica*		MeOH extract of *R. idaeus*		Chloramphenicol		Vancomycin	
	0% NaCl	2.5% NaCl	0% NaCl	2.5% NaCl	0% NaCl	2.5% NaCl	0% NaCl	2.5% NaCl	0% NaCl	2.5% NaCl	0% NaCl	2.5% NaCl
B. subtilis	1024	32	1024	128	1024	256	256	32	16	8	8	16
E.coli S2(1)	512	32	1024	128	512	512	256	64	4	4	8	32
P. aeruginosa	512	128	2048	256	2048	256	1024	128	64	64	8	32
V. cholerae NB2	128	128	1024	512	512	512	512	256	64	128	16	64
S. flexneri SDINT	256	32	2048	32	2048	128	1024	64	64	1	16	32
S. aureus ATCC	64	128	1024	512	1024	512	512	256	32	1	0.25	1
V. cholerae SG24	128	32	2048	256	2048	1024	2048	256	4	128	32	64
V. cholerae CO6	512	32	1024	256	1024	256	512	512	16	128	32	128
S. aureus MSSA1	128	64	2048	1024	1024	512	64	32	32	8	0.50	1
S. aureus MRSA3	128	32	2048	1024	2048	2048	256	128	64	16	0.50	1
S. aureus MRSA4	128	32	2048	1024	2048	512	128	128	64	16	1	2

Table 10. Effect of osmotic stress on the minimum bactericidal concentrations of plant extracts according to the tested bacteria

Bacteria	MeOH extract of *C. longa*		MeOH extract of *T. officinale*		MeOH extract of *C. asiatica*		MeOH extract of *R. idaeus*		Chloramphenicol		Vancomycin	
	0%	2.5%	0%	2.5%	0%	2.5%	0%	2.5%	0%	2.5%	0%	2.5%
B. subtilis	>2048	256	>2048	>2048	>2048	>2048	1024	32	128	16	16	16
E. coli S2(1)	1024	128	1024	512	2048	>2048	1024	64	64	64	16	32
P. aeruginosa	2048	512	>2048	>2048	>2048	>2048	2048	128	256	256	16	32
V. cholerae NB2	>2048	1024	2048	>2048	1024	>2048	2048	256	256	256	32	64
S. flexneri SDINT	512	256	2048	256	>2048	>2048	2048	64	128	4	16	32
S. aureus	256	128	>2048	>2048	>2048	>2048	1024	256	64	32	0.50	1
V. cholerae SG24	512	256	>2048	>2048	>2048	>2048	>2048	256	16	16	64	128
V. cholerae CO6	2048	1024	>2048	>2048	>2048	>2048	>2048	512	64	64	64	128
S. aureus MSSA1	512	64	2048	2048	1024	2048	1024	32	64	16	1	1
S. aureus MRSA3	2048	64	2048	2048	2048	2048	2048	256	64	32	1	2
S. aureus MRSA4	2048	128	2048	2048	2048	2048	>2048	128	64	16	2	4

Table 11. Effect of serum on the antibacterial activity of MeOH extracts of *C. longa* and *R. idaeus* according to the studied bacteria

Bacteria	Extracts											
	0% of serum				2.5% of serum				5% of serum			
	MeOH extract of *C. longa*		MeOH extract of *R. idaeus*		MeOH extract of *C. longa*		MeOH extract of *R. idaeus*		MeOH extract of *C. longa*		MeOH extract of *R. idaeus*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	1024	>2048	256	1024	32	>2048	128	>2048	8	1024	32	2048
E. coli S2(1)	512	1024	256	1024	32	1024	256	1024	8	1024	128	1024
P. aeruginosa	512	2048	1024	2048	64	2048	256	2048	64	512	128	512
V. cholerae NB2	128	>2048	512	2048	64	2048	512	512	64	1024	512	1024
S. flexneri SDINT	256	512	1024	2048	64	1024	256	1024	32	512	128	1024
S. aureus	64	256	512	1024	128	512	256	1024	32	512	128	1024
V. cholerae SG24	128	512	2048	>2048	64	1024	256	2048	64	512	128	2048
V. cholerae CO6	512	2048	512	>2048	64	1024	512	2048	32	512	128	1024
S. aureus MSSA1	128	512	64	1024	128	256	32	128	4	128	8	128
S. aureus MRSA3	128	2048	256	2048	128	512	64	256	8	256	32	128
S. aureus MRSA4	128	2048	128	>2048	256	512	64	256	8	256	16	128

Conclusions

The results of the present study show that the studied plant extracts possess antibacterial activity that can justify their traditional use in the treatment of infected diseases. The serum resulted in a concentration-dependent increase in the antibacterial activity of the MeOH extracts of *C. longa* and *R. idaeus*. The antibacterial activities of the crude extracts of the four studied plants and chloramphenicol increased under osmotic stress conditions (2.5% NaCl) whereas those of vancomycin decreased under these conditions. The synergistic effects (ΣFIC <0.5) and additives (ΣFI> 0.5 and <1) between amoxicillin and MeOH extract of *C. longa*, as well as indifference (ΣFIC> 1 and <2) and synergy (ΣFIC <0.5) effects between amoxicillin and MeOH extract of *R. idaeus* were observed. The antibacterial activity mechanism of the plant extracts is due to cell lysis and disruption of the bacterial cytoplasmic membrane. The tested plant extracts showed less haemolytic activity, indicating their good selectivity to the bacterial cell. So, clinical investigations are warranted.

Authors' Contribution

ICK did the biological assays and helped in manuscript writing. JDT designated the study, contributed to the data collection and analysis, supervised and revised the manuscript critically for important intellectual content. All authors read and agreed on the final version of the manuscript.

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Conflict of interest

The authors declare no competing interests with regard to the publication of this article.

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