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Antibacterial Effects of Chemical Constituents Isolated from the Roots of *Cordia myxa* (Boraginaceae) on *Salmonella typhi* (Enterobacteriaceae)

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Abstract: *Cordia myxa* is a Cameroonian pharmacopoeia traditional plant. It belongs to the Boraginaceae family, which different parts are used in the treatment of various bacterial infections. The phytochemical screening carried out on the *Cordia myxa* species indicates the presence of alkaloids, triterpenes, steroids, carbohydrates, flavonoids and saponins. Our investigations on the roots of this plant allowed us to isolate two triterpenes from the ethyl acetate extract, which crystallize in the Hex/AcEt [2/8] solvent system; (23R) campesta-9 (11), 24 (30) diene-18,23-diol 3-O-α-rhamnopyranoside (compound 1) and alphitolic acid (compound 2). The isolation and characterization of these compounds were carried out respectively by silica gel chromatography and by NMR (1D, 2D) spectral analysis. Compound 1and 2 were tested on the in vitro growth of *E.coli* and *Salmonella typhi* using the Muller-Hinton solid medium disc method. The results revealed that these compounds have a dose-dependent antibacterial activity on *Salmonella typhi*. However, compound 2 had a better antibacterial potential on the *Salmonella* strain (18.6±0.6mm, 30mg/ml) with the MIC=0.25mg/ml than the compound 1 (14.5±0.4mm, 30mg/ml,) with the MIC=0.44mg/ml. Few classes of antibiotics have been tested, to look for those with the best results and compare the different doses of our compounds. The two compounds did not have any effect on *E. coli*.

Keywords: *Cordia myxa*, Antibacterial Effect, *Salmonella typhi*, Triterpene

1. Introduction

Typhoid fever is a serious foodborne illness caused by *Salmonella typhi*, a bacterium found in faecally contaminated water and food. The disease is transmitted through contaminated water/food and occurs mainly in areas where hygiene conditions are poor [1]. The enterohaemorrhagic *E.coli* strains are responsable for a variety of infections ranging from watery diarrhea to haemorrhagic colitis and can develop into a haemolytic-ureenic syndrome in children, mainly under three years of age, or thrombotic microangiopathy in adults [2], they can acquire particular virulence factors and give rise to either extra-intestinal pathologies (meningitis, urinary tract infections) or intestinal pathologies [3, 4]. Faced with the appearance of resistant forms of several bacteria to certain antibiotic, and the high cost of these products leading to the search for new active molecules with a broad spectrum of action has become a

necessity [5]. One of the strategies for this research is to explore plants used in traditional medicine [6]. *Cordia myxa* is a plant from the tropical and subtropical region; it belongs to the Boraginaceae family. The phytochemical screening carried out on the *Cordia myxa* species indicates the presence of alkaloids, triterpenes, steroids, carbohydrates, flavonoids and saponins [7]. Traditionally, the different parts of this plant are used against urinary tract infections, antihelminthic, antidiuretic; their fruits have anti-inflammatory properties [8]. In Africa, the pulp of the fruit of *Cordia myxa* is also used to treat diarrhea, dysentery, tuberculosis and ulcers [9]. In Cameroon, *Cordia myxa* is used to treat diarrhea, stomachaches, fever and also urinary tract infections. Thus, the objective of this study is to isolate, characterize and evaluate the antimicrobial activity of secondary metabolites isolated from the root of *Cordia myxa*.

2. Experimental

2.1. Plant Materials

The roots of *Cordia myxa* were collected in the northern region of Cameroon, in Mayo Louti division, in Kossel Danneel village. The plant was identified by the botanist Dr Froumsia Mouksia from the Department of Biological Sciences, University of Maroua, Maroua, Cameroon. One Voucher specimen (Nº 6410/HEFG) was deposited at the Herbarium of School for the Training of Specialists in Wildlife Management of Garoua, Garoua, Cameroon.

2.2. Extraction and Isolation

SOME 3.50 kg powders from the roots were subjected to successive extraction with the hexane and ethyl acetate, after total evaporation of the solvent, a black ethyl acetate extract was obtained with a mass of 75 g. This extract was submitted to the silica gel column chromatography (SiO_2, 0.063-0.200) and the elution was carried out by gradient of increasing polarity of solvents (hexane, hexane-ethyl acetate). The elution of the extract with hexane/EtOAc [2: 8] polarity gradient yielded compounds 1 and 2.

2.3. Bacterial Strains

The microorganisms used in this study are Gram negative bacteria *Escherichia coli* and *Salmonella typhi* (Enterobacteriaceae). These are clinical isolates provided by the "Centre Pasteur", Yaounde, Cameroon. The biochemical and serological tests were used for confirmation of both bacterial strains.

2.4. Study of Antibacterial Activity

First, the discs are prepared and then the different concentrations of compounds 1 and 2 (60mg/ml, 30mg/ml, 15mg/ml). These compounds were dissolved in methanol before preparing the concentrations. These solutions are coated and sterilized in an autoclave (121°C for 15mn) [1]. Blotting paper discs of 6mm diameter are impregnated with these different concentrations and even methanol (negative control disc). All the discs prepared are dried in an oven at 37°C. Some classes of antibiotics are used to search for the best reference antibiotics [1].

2.5. Agar Diffusion Method

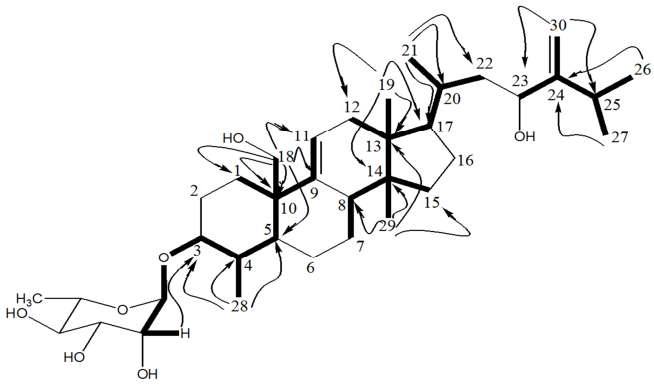
The antibacterial activity of the different compounds was evaluated by the agar diffusion method as described by Bauer and collaborators [10] and taken up by Barry and collaborators [11]. From 18 to 24-hour young colonies, a bacterial suspension is made in sterile distilled water for each strain. The turbidity of this suspension was adjusted to 0.5Mc Farland and then diluted to 1/100. An estimated inoculum of 10^6 colony-forming units per milliliter (cfu/ml) was then obtained. This inoculum was inoculated by flooding on petri dishes containing Muller-Hinton agar [12]. The discs impregnated with the different concentrations of compounds 1and 2, methanol and antibiotics were then delicately deposited on the surface of the agar. The petri dishes were first left for 1 hour at room temperature for pre-diffusion of the substances, before being incubated at 37°C in an oven for 24 hours [13]. Antibacterial activity was determined by measuring the diameter of the inhibition zone around each disc [14]. The minimum inhibition concentration (MIC) was determined by the dilution method described by Haddouchi and his collaborators [5].

3. Results and Discussion

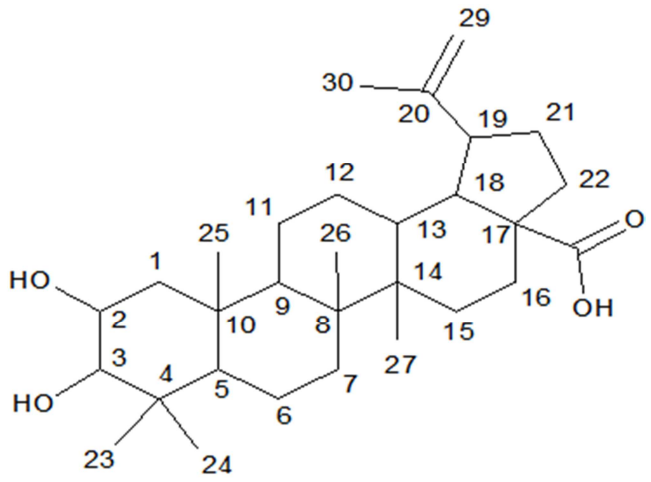
3.1. Identification of the Compounds

Compounds 1 and 2 are all white powders. Compounds 1 and 2 crystallize in the Hex/AcEt [2/8] solvent system. These compounds are all soluble in methanol. The Libermann-Buchard test of componds 1 and 2 is positive. This information confirms that the compounds belong to the triterpene family. The RMN spectrum proton of Compound 1 reveal six (06) methyl between δ 0.67-1.74ppm; five (05) singlets at δ 0.81ppm (3H,s, Me-19); 0.97ppm (3H,s, Me-21); 1.09ppm (3H, s, Me-26); 1.07ppm (3H,s, Me-27); 0.67ppm (3H,s, Me-28); a broad singlet at δ 5.34ppm which corresponds to a proton of the double bond; two (02) singlets at δ 5.06ppm and 4.88ppm, which are the protons of methylene at the C-30 position. These informations confirm the basic structure of the genin. The proton at δ 3.07ppm (H-3), which is a deflated proton, and the presence of an anomeric proton at δ 4.82ppm, suggest that compound 1 has a sugar. Its COSY spectrum shows the follow correlations: the anomeric proton correlates with the signal at δ 3.94ppm ($H_{2'}$), and which correlates with the proton at δ 3.74ppm (H_3'). The proton H_3'correlates with the signal at δ 3.43ppm ($H_{4'}$) which correlates with the signal at δ 3.80ppm ($H_{5'}$). The proton $H_{5'}$ correlates with the methyl proton at δ 1.29ppm ($H_{6'}$). The spectral analysis of the ^{13}C NMR of compound 1 confirms the presence of a steroid. Its ^{13}C-NMR exhibit the signals at δ 160.5ppm (C-24); 105.6ppm (C-30); 124.4ppm (C-11) and 50.6ppm (C-18). These chemical shifts of the carbon confirm the basic structure of the genin.

The chemical shift of the carbon at δ 85.3ppm shows that the carbon is linked to a heteroatom, which unshielded it. The chemical shift at δ101.9ppm is of an anomeric carbon which confirm the presence of sugar in the compound 1. Its ^{13}C-NMR spectrum contain six (06) methyl carbons δ 15.9ppm (C-19); 18.0ppm (C-21); 23.2ppm (C-26); 22.4pmm (C-27), 16.5ppm (C-28); 15.6ppm (C-29); eight (08) methylene δ 40.2 (C-1); 28.7 (C-2); 50.6 (C-6); 26.5 (C-7); 38.3 (C-12); 32.9 (C-16); 50.6 (C-18); five (05) sugar methine δ 101.9 (C-1'); 71.0 (C-2'); 71.6 (C-3'); 73.2 (C-4'); 67.9 (C-5'); Nine (09) genin methine δ 85.3 (C-3); 39.5 (C-4); 52.3 (C-5); 48.4 (C-8); 124.4 (C-11); 51.5 (C-17); 33.3 (C-20); 43.8 (C-22); 71.0 (C-23) and five (05), quarternary carbons δc 136.4 (C-9); 71.7 (C-10); 44.0 (C-13); 48.7 (C (-14); 160.5 (C-24). The DEPT 135 spectrum combined with the ^{13}C NMR spectrum confirm six (06) methyl groups δc 29.1 (C-23); 19.5 (C-30); 17.8 (C-24); 17.2 (C-25); 16.6 (C-26); ten (10) methylene δc 19.5 (C-6); 22.2 (C-11); 26.8 (C-12); 30.8 (C-16); 31.7 (C-15); 35.4 (C-7); 38.2 (C-21); 46.4 (C-22); 48.6 (C-1), seven (07) methine δc 69.7 (C-2); 84.2 (C-3); 56.8 (C-5); 51.9 (C-9); 48.5 (C-18); 39.6 (C-13), seven (07) quaternary carbons δc 36.5 (C-4); 40.5 (C-10); 57.5 (C-17); 152.0 (C-20); 180.4 (C-28). All these informations combined with the literature permitted to identify compound1 as (23R) campesta-9 (11), 24 (30) diene-18, 23-diol 3-O-α-rhamnopyranoside isolated from *Cordia lutea Lam* [15, 16]. The ^{1}H NMR spectrum of compound 2 show (06) methyl, δ 0.77-1.67ppm which characterizes the pentacyclic; five (05) singlets δ 0.77ppm (3H,s, Me-26); 0.91ppm (3H,s, Me-24); 0.96ppm (3H,s, Me-25); 0.98ppm (3H,s, Me-23); 1.00ppm (3H,s, Me-27); 1.67ppm (3H,s, Me-29); two (02) ethylenic protons at δ 4.68ppm and 4.56ppm (H-29) which are the exomethylene protons; a multiplet at 2.31ppm (1H,m, H-19). These spectral analyses correspond to the chemical displacements of dihydroxylup-20 (29)-ene which is a basic structure of pentacyclic triterpene of the lupane type [17]. A singlet at δ 1.67ppm corresponds to the C-30 protons. The chemical shift at 3.60ppm (1H, ddd) corresponds to that of the proton in position 2 [18]. The DEPT 135 spectrum combined with the ^{13}C-NMR spectrum exhibit: six (06) methyl groups δ 29.ppm (C-23); 19.5ppm (C-30); 17.8ppm (C-24); 17.2ppm (C-25); 16.6ppm (C-26); 15.0ppm (C-27); ten (10) methine at δ 19.5ppm (C-6); 22.2ppm (C-11); 26.8ppm (C-12); 30.8ppm (C-16); 31.7ppm (C-15); 35.4ppm (C-7); 38.2ppm (C-21); 46.4ppm (C-22); 48.6ppm (C-1); seven (07) methylene δ 69.7ppm (C-2); 84.2ppm (C-3); 56.8ppm (C-5); 51.9ppm (C-9); 48.5ppm (C-18); 39.6ppm (C-13); 39.6ppm (C-19); seven (07) quaternary carbons δ 39.6ppm (C-4); 40.5ppm (C-10); 57.5ppm (C-17); 152.0ppm (C-20); 180.4ppm (C-28). The spectral data are shown in table 2. All the data compared to the literature confirms that compound 2 is an alphitolic acid [19].



(A) Compound (1) and selected HMBC correlations



(B) Compound (2)

Figure 1. *Chemical structure of compound 1and 2 and selected HMBC correlations of compound 1.*

Table 1. *The ^{13}C, ^{1}H NMR (MeOD, 500MHz), COSY, HMBC spectrum of compound 1.*

Positions	Compound1 δ_C (ppm)	Compound 1 δ_H (m, J en Hz)	COSY	HMBC	Genin δ_C (ppm)	Genin δ_H (ppm)
1	40.2	1.53 (sl,)	H6	C6, C9, C5	40.2	1.53
2	28.7	1.80/1, 88 (d)	H3, H1	/	28.7	1.80/1.88
3	85.3	3.07 (d, 10, 7)	H4, H2	C1’	85.3	3.07
4	39.5	1.47 (td, 4, 6)	H29, H3, H5	C29	39.5	1.47
5	52.3	1.00 (d, 4, 6)	H4	C6, C1	52.3	1.00
6	50.6	2.28/2.58 (d)	H8, H6	C1, C8	50.6	2.28/2.58
7	26.5	1.35/1.53 (d)	H8, H7, H16	/	26.5	1.35/1.53
8	48.4	2.20	/	C6, C28, C7	48.4	2.20
9	136.4	/	/	C6, C7	136.4	/
10	71.7	/	/	C6	71.7	/
11	124.4	5.34 (s)	H12	C6	124.4	5.34
12	38.3	2.02 (s)	H11	C19	38.3	2.02
13	44.0	/	/	C28, C19, C21	44.0	/
14	48.7	/	/	C28, C19	48.7	/
15	32.9	1.37	/	/	32.9	1.37
16	32.9	1.23/2.14 (dd)	H16	/	32.9	1.23/2.14
17	51.5	1.54 (s)	H15, H16	C19, C21	51.5	1.54
18	50.6	2.28/2.58 (dd)	H6, H6	C1, C8, C5	50.6	2.28/2.58
19	15.9	0.81 (s)	/	C12, C13, C17	15.9	0.81
20	33.3	1.74 (s)	H22	C21	33.3	1.74
21	18.0	0.97 (d, 6, 7)		C12, C22, C17	18.0	0.97
22	43.8	1.15/1.57 (s)	H20, H22	C21,	43.8	1.15/1.57
23	71.0	4.18 (d, 9, 9)	H22, H22	C30,	71.0	4.18
24	160.5	/	/	C30, C27	160.5	/
25	30.5	2.25 (s)	/	C24, C27	30.5	2.25
26	23.2	1.09 (d, 7, 0)	H25	C25, C24	23.2	1.09
27	22.4	1.07 (d, 7, 0)	H25	C25, C24	22.4	1.07
28	16.5	0.67 (s)	/	C13, C14, C15	16.3	0.67
29	15.6	0.95 (d, 6, 6)	H4	C5, C3, C4	15.6	0.95
30	105.6	5.06/4, 88	H30	C24, C24, C23	105, 6	5, 06/4, 88
1’	101.9	4.82 (s, 1, 4)	H2’	C5’, C4’, C3,	/	/
2’	71.0	3.94 (dd, 3, 6)	H3’, H1’	C4’, C3’	/	/
3’	71.6	3.74 (d, 9, 6)	H2’, H5’H4’	C4’	/	/
4’	73.2	3.43 (dd, 9, 6)	H5’, H3’	C6’, C5’, C3’	/	/
5’	67.9	3.80 (dd, 6, 2)	H6’H4’	/	/	/
6’	17.2	1.29 (d, 6, 2)	H5’	C5’	/	/

Table 2. *^{13}C, ^{1}H NMR (MeOH, 500Hz) and HMBC spectrum of compounds 2.*

Position	Compound2 δ_C (ppm)	Compound 2 δ_H (ppm)	Compound 2 (HSQC)
1	48.6	0.79; 1.97 (dd)	CH2
2	69.7	3.60 (td,5. 1)	CH
3	84.4	2.87 (dl, 9.6)	CH
4	39.5	/	/
5	56.8	0.75 (dl, 6.5)	CH
6	19.5	1.37 (t, 8,5)	CH2
7	35.4	1.33 (m); 1,36 (m)	CH2
8	41.9	/	CH
9	51.9	1.31 (dd, 15.7)	CH
10	40.5	/	/
11	22.2	1.24 (dd, 3.5)	CH2
12	26.8	0.99 (dd, 4.6)	CH2
13	39.6	2.20 (td, 3.4)	CH
14	43.6	/	/
15	31.7	1.13 (dt); 1.46 (td)	CH2
16	30.8	1.37 (tl); 2.22 (dt)	CH2
17	57.5	/	/
18	48.5	3.01 (m)	CH
19	50.4	2.31 (td, 4.9)	CH
20	152.0	/	/
21	38.2	1.89 (m); 1.42 (m)	CH2
22	46.4	2.41 (t); 1.73 (td)	CH2
23	29.1	0.98 (3H, s)	CH3
24	17.8	0.91 (3H, s)	CH3
25	16.6	0.96 (3H, s)	CH3
26	17.2	0.77 (3H, s)	CH3
27	15.0	1.00 (3H, s)	CH3
28	180.4	/	/
29	110.1	4.70 (d, 2.1)	CH2
30	19.5	1.67 (3H, s)	CH3

3.2. Results and Discussion on Antimicrobial Activity

The evaluation of the antimicrobial activities of compounds 1 and 2 was carried out by the Muller-Hinton agar diffusion method, as proposed in the recommendations of the Antibiogram committee of the French Society of Microbiology [20]. The antibacterial ability was classified according to the DIZ (diameters of the inhibition zone) as follows: not sensitive (DIZ < 8.0mm) moderately sensitive (8.0 < DIZ <14.0mm); sensitive (14.0 < DIZ < 20.0), and extremely sensitive (DIZ > 20.0mm) [21, 22]. The results obtained on the antibacterial activity of our compounds varied significantly depending on the concentration on *Salmonella typhi*. For the compound 2, the highest concentration (30mg/ml) at an inhibition zone diameter of 18.6±0.6mm (Table 3 and figure 2), at this dose was sensitive

because it is between (14.0<DIZ<20.0mm), we can say that, at this concentration, compound 2 has the same effect as the antibiotic CAZ10 (Ceftazidim) which belongs to the β-lactam class, the diameter of its inhibition zone was 19.2±0.2 mm, it was also sensitive because it belongs to the same range. Compound 2 showed the sensitive effect at a concentration of 15mg/ml, as its inhibition zone was 16.5±0.7mm (14.0<DIZ<20.0mm) (Table 3 and figure 2). Our results are similar to that of Xiao and his collaborators, who tested dihydromyricetin on*Salmonella typhi*, at a dose of 11.34mg/ml, the diameter of the inhibition zone obtained is 13.1±0.5mm, this is a moderately sensitive effect (8.0<DIZ<14.0mm) [22].

Table 3. *Effect of compound 2 on E.coli and Salmonella typhi.*

Bacteria	Negative Control		DIZ (mm) compound 2		CMI Compound 2
Gram negative	MeOH	30mg/ml	15mg/ml	7.7mg/ml	
E.coli	ND	ND	ND	ND	0.25mg/ml
Salmonella tiphy	ND	18.6±0.6	16.5±0.7	8.6±0.6	

ND=not detected, DIZ=diameter of the inhibition zone, MIC=minimum inhibitory concentration. Values represent means of three independent replicates ± standard deviation (SD)

At these two concentrations of the compound 2, the larger effect on *Salmonella typhi* than the reference antibiotic C30 chloramphenicol was shown. Chloramphenicol had a moderate sensitive effect because its inhibition zone diameter was 10.2±0.1mm (8.0<DIZ<14.0mm) (Figure 2 and Table 3), as of compound 2 at a dose of 7.7mg/ml (8.6±0.6mm).

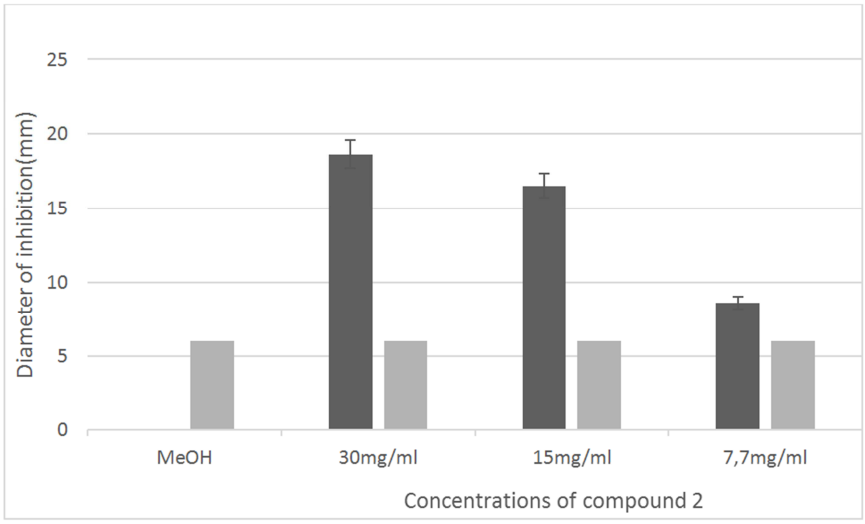


Figure 2. *These histograms illustrate the diameter of inhibition as a function of compound 2 concentrations on Salmonella typhi (grey) and E. colis (black)(N=3), methanol as negative control.*

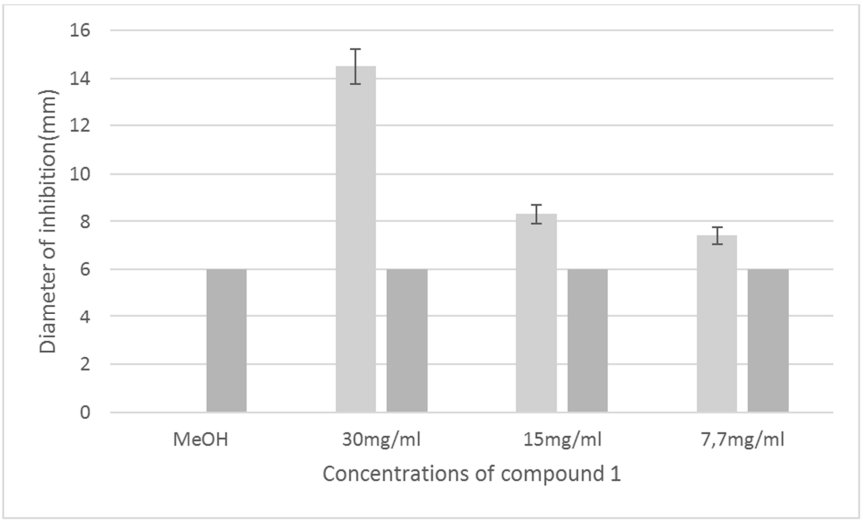


Figure 3. *Diagram determining inhibition diameters as a function of compound 1 concentrations on Salmonella typhi (light grey) and E. coli (dark grey) bacterial strains (N=3), with methanol as a negative control.*

Table 4. *Effect of compound 1 on E. coli and Salmonella typhi.*

Bacteria	Control negative		DIZ (mm) Compound 1		CMI Compound 1
Gram negative	MeOH	30mg/ml	15mg/ml	7.7mg/ml	
E.coli	ND	ND	ND	ND	0.44mg/ml
Salmonella typhi	ND	14.5±0.4	8.3±0.5	7.4±0.6	

ND=not detected, DIZ=diameter of the inhibition zone, MIC=minimum inhibitory concentration. Values represent means of three independent replicates ± standard deviation (SD)

Similarly, for compound 1, at doses of 30mg/ml and 15mg/ml we observe respectively the diameter of the inhibition zone, 14.5±0.4 and 8.3±0.5mm. For the 30mg/ml dose, it has the sensitive effect because its inhibition zone belongs to the interval of 14.0<DIZ<20.0mm and it has the moderately sensitive effect (8.0<DIZ<14.0mm) for 15mg/ml dose (Table 4 and figure 3) [21].

These different maximum concentrations of compound 1 had the same effect as the reference antibiotic C30. The lowest concentration of compound 1 (7.7mg/ml), inhibits *Salmonella typhi* at an inhibition diameter of (7.4±0.6mm), it is not a sensitive effect.

Table 5. *Effect of β-lactam antibiotics class on Salmonella typhi.*

	DIZ (mm)		
Antibiotiques	CXM30	CAZ10	IPM10
Concentrations	30µg/ml	10µg/ml	10µg/ml
Salmonella typhi	20.9±0.1	19.2±0.2	22.9±0.1

Among the antibiotics belonging to the β-lactam class, the best that has had an effect on *Salmonella typhi* is CXM30 (cefuroxim), followed by CAZ (ceftazidim). Their inhibition diameter were respectively, 20.9±0.1mm and 19.2±0.2mm, CXM30 had an extremely sensitive effect (DIZ>20.0mm), but CAZ10 had a sensitive effect (14.0<DIZ<20.0mm) (Table 5 figure 3).

Table 6. *Effects of Aminoside and phenicol antibiotics classis.*

	DIZ (mm)		
Antibiotiques	CN10	TOB10	C30
Concentrations	10µg/ml	10µg/ml	10µg/ml
Salmonella typhi	23.1±0.1	18.9±0.4	10.2±0.1

The compounds 1 and 2, had no effect on *E. coli*. Among the reference antibiotics belonging to the Aminoside and phenicol classes, CN10 (Gentamicin) had an extremely sensitive effect (DIZ>20.0mm) with an inhibition diameter of 23.1±0.1mm and TOB10 (Tobramycin) with an inhibition diameter of 18.9±0.4mm, these references antibiotics were sensitive on *Salmonella typhi* (Table 6 figure 4).

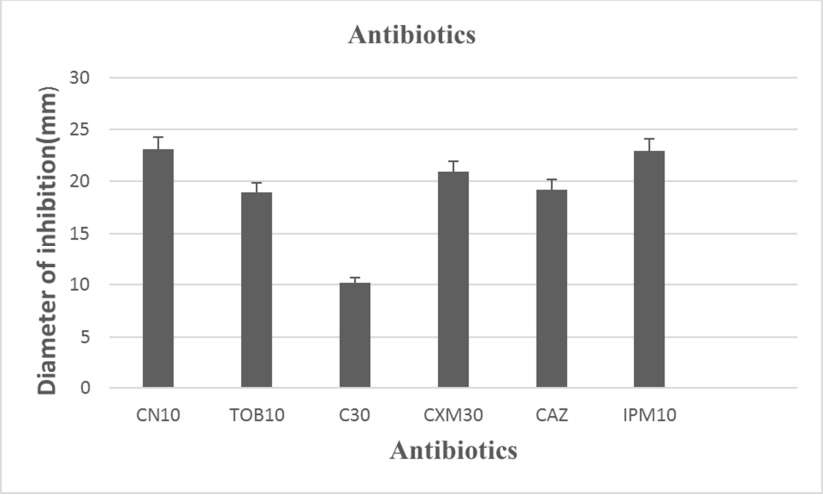


Figure 4. *Diameter inhibition of antibiotics on Salmonalla typhi.*

Antimicrobial substances are defined as substances used to kill or inhibit the growth of microorganisms, including antibiotics and other antibacterial agents [23]. *Salmonella typhi* and *E. coli* are both gram (-) bacteria, the effect of compound 1 and 2 on *Salmonnella typhi* was dose-dependent, their MIC were respectively 0.25mg/ml and 0.44mg/ml. Our two compounds did not have any effect on *E. coli*. The process of the high resistance of *E.coli* to our compounds could probably be attributed to their outer membranes, which surround the cell wall and limit the diffusion of hydrophobic compounds by the covering lipopolysaccharides [23].

4. Conclusion

The investigations on the roots of *Cordia myxa* leaded to isolation of two triterpenes from the ethyl acetate extract, the (23R) campesta-9 (11), 24 (30) diene-18,23-diol 3-O-α-rhamnopyranoside and the alphitolic acid-The effect of these compounds on *Salmonnella typhi* was dose-dependent, their MIC were respectively 0.25mg/ml for (23R) campesta-9 (11), 24 (30) diene-18,23-diol 3-O-α-rhamnopyranoside and 0.44mg/ml for 2α,3β-dihydroxybetulinic acid. The two compounds did not have any effect on *E. coli*. Therefore, it is

suggested that *Cordia myxa* could be a potential source of natural antimicrobial that could have great importance as therapeutic agent in the treatment of *Salmonella typhi* infections. Further research on others parts of the plant is recommended for characterization of important active constituents, responsible for antibacterial activity.

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