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THE ANTIBACTERIAL ACTIVITIES OF SOME PLANT-DERIVED TRITERPENES

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Abstract

Background: The increase in the prevalence of multi-drug resistant bacteria has necessitated the search for new antimicrobials from alternative sources such as traditional medicinal plants.
Materials and Methods: The agar well diffusion method was employed to determine the susceptibilities of four plant derived triterpenes namely, 3β-hydroxylanosta-9, 24-dien-21-oic acid (RA5), and methyl-3β-hydroxylanosta-9, 24-dien-21oate (RA3), a mixture of oleanolic acid and betulinic acid (SF1) and a mixture of 3β-acetonyloleanolic acid and 3β-acetonylbetulinic acid (SF2), at a concentration of 10 mg/ml against seven *Escherichia coli*, one *Bacillus cereus*, five *Enterococcus* and nine *Vibrio* bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined through the micro-broth dilution assay. The checkerboard method was used to determine the antibiotic-triterpene interactions while the cytosolic lactate dehydrogenase test was used to determine the membrane damaging potentials of the triterpenes in comparison to 3% Triton X-100.
Results: The triterpenes RA3, RA5, SF1 and SF2 had activities against 86.4%, 54.6%, 22.7% and 9.09% of the test bacteria respectively. SF1 had the lowest MIC values ranging 0.625-10 mg/ml with lower MIC values being noted against Gram negative bacteria in comparison to Gram positive bacteria; this trend was also noted among the activities of RA3 and RA5 although they had higher MIC value ranges of 1.25-10 mg/ml and 5- 10 mg/ml respectively. MBC studies proved the triterpenes to be mostly bacteriostatic. The interaction studies with ciprofloxacin were mainly ranging between indifference and antagonism. RA3 alone showed minimal membrane damaging potential with the levels of cytosolic lactate dehydrogenase released ranging from 1-36% in comparison to 3%Triton X-100 against *E.coli* (DSM-8695) and *V. vulnificus* (AL 042).
Conclusion: The results hereby show the potential that the test triterpenes have as antibacterial agents, especially against the Gram negative bacteria namely *E. coli* and *Vibrio* bacteria.

Key words: Plant triterpenes, bacteria, MIC, MBC

Introduction

Plants have evolved secondary biochemical pathways that allow them to synthesize chemicals known as secondary metabolites, often in response to specific environmental stimuli, such as herbivore-induced damage, pathogen attacks, nutrient depravation and abiotic stresses such as radiation (Kennedy and Wightman, 2011). These secondary metabolites can be unique to specific species or genera and do not play any role in the plants' primary metabolic requirements, but rather they increase their overall ability to survive and overcome local challenges by allowing them to interact with their environment (Cowan, 1999; Kennedy and Wightman, 2011).

The importance of plant secondary metabolites in the medical industry has increased with approximately 40% of medicines originating from them (Gershenzon and Kreis, 1999; Babalola and Shode, 2013). This has also seen an increasing research interest into their synthesis, biosynthesis and biological activities (Gershenzon and Kreis, 1999; Babalola and Shode, 2013). Secondary metabolites are usually classified according to their biosynthetic pathways, with three large molecule families being generally considered: phenolics (shikimate pathway or malonate/acetate pathway), terpenes and steroids (derived from the C5 precursor isopentenyl diphosphate), and alkaloids (derived from amino acids) (Bourgaud et al., 2001).

Terpenes broadly exhibit a range of toxicity from deadly to entirely edible, and this is in keeping with their broad range of ecological roles, which include antimicrobial properties and a range of properties that attract symbiotes for the purposes of pollination, seed dispersal and secondary protective roles (Kennedy and Wightman, 2011). Triterpenes which are the terpenes of interest in this study refer to three mono-terpenes and consequently to 30 carbons grouped in six isoprenyl units (Arthur and Hui, 1961; Sandjo and Kuete, 2013). Triterpenes are implicated in the mechanisms of action and pharmacological effects of many medicinal plants and they have been described as anti-inflammatory, antiviral, antimicrobial and antitumoral agents, as well as being immune-modulator compounds (Rios, 2010). In this study, two lupane triterpenes (mixture of oleanolic acid and betulinic acid (SF1) and a mixture of 3β-acetonyloleanolic acid and 3β-acetonylbetulinic acid (SF2)) isolated from *Melaleuca bracteata var* and two lanostane triterpenes (3β-hydroxylanosta-9, 24-dien-21-oic acid (RA5) and methyl-3β-hydroxylanosta-9, 24-dien-21oate (RA3)) isolated from *Protorhus longifolia* were studied for their antibacterial activities *in-vitro* against, *E. coli*, *Vibrio*, *Enterococcus* and *B. cereus* bacteria. *Protorhus longifolia* (Benrh) Engl. (Anacardiaceae), is a tree indigenous to Southern Africa and is traditionally used to cure various diseases including bleeding from the stomach, heart burn, hemiplegic paralysis (Hutchings et al., 1996), and to treat heart water and diarrhoea in cows (von-Teichman, 1991). *Melaleuca bracteata var* is also a traditional medicinal plant that is used to treat infected wounds and skin disorders and is also believed to aid in stimulating glandular secretions, and reducing vein congestion (Adesanwo et al., 2009).

The bacteria of choice in this study are of immense medical importance as they are causes of potentially fatal bacterial infections. *B. cereus* is normally associated with food poisoning that is characterized by either diarrhoea and abdominal distress or nausea and vomiting (Senesi and Ghelardi, 2010). It is also an opportunistic pathogen in immunocompromised patients causing severe endophthalmitis, bacteremia, septicemia,

endocarditis, pneumonia, meningitis, gastritis, and cutaneous infections (Senesi and Ghelardi, 2010); *Vibrio* bacteria is incriminated in mostly gastrointestinal and extra- intestinal diseases in man (Health protection agency, 2007); *Enterococcus* bacteria are common causes of nosocomial infections (Arias et al., 2010), such as catheter-associated urinary tract infections, endocarditis as well as surgical and burn wound infections (Abranches et al., 2013) ; *E. coli* bacteria, mostly the shiga toxin producing serotypes cause mild to bloody diarrhoea, haemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (Brooks et al., 2005). Treatment of these various bacterial infections is, however, being hindered by the prevalence of antibiotic resistant strains (Furtula et al., 2013; Banerjee et al., 2013; Ghaima et al., 2013; Costa et al., 2014), and this therefore necessitates the need for newer and effective antimicrobials of which the before mentioned plant triterpenes offer a viable alternative considering their vast beneficial therapeutic potentials (Mosa et al., 2011; Machaba et al., 2014; Penduka et al., 2014).

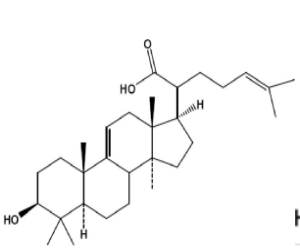
Material and Methods

Plant Triterpenes

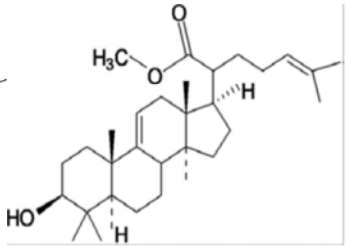
The triterpenes 3β-hydroxylanosta-9, 24-dien-21-oic acid (RA5) and methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA3) were previously isolated from the stem bark of *Protorhus longifolia* by Mosa et al. (2014a). The spectral details of 3β-hydroxylanosta-9,24-dien-21-oic acid have previously been given by Mosa et al. (2011): the compound was obtained as white flakes (paper-like solids), mp134 to 136°C, IR (KBr) vmax= 3360 cm-1, ^{1}H and ^{13}C NMR, molecular formula $C_{30}H_{47}O_3$, MW 455.77, with an estimated purity of more than 95%. Details of methyl-3β-hydroxylanosta-9,24-dien-21-oate have also been previously given by Mosa et al. (2014b): the compound was obtained as white crystals, estimated purity of more than 95% based on melting point, mp 204 to 205°C, IR (KBr) vmax= 3469, 1683 cm-1, ^{1}H and ^{13}C NMR molecular formula $C_{31}H_{50}O_3$, MW 470.736.

Oleanolic acid and betulinic acid mixture (SF1) was isolated from the defatted crude ethyl acetate extract of the leaf of *Melaleuca bracteata* var, subjected to chromatographic separation on silica gel (60-120 mesh) column (20 x 5.5 cm) using solvent system hexane/ethylacetate (8:2 to 7:3) and recrystallized with methanol. SF1 was obtained as a white powdered compound and the structure elucidation was done through NMR and IR spectra analysis and confirmed to be a mixture of oleanolic acid and betulinic acid. The data was in agreement with the literature values (Ibrahim et al., 2013). Betulinic acid and oleanolic acid are isomers and have Rf values of 0.62 and 0.68 respectively such that attempts to obtain each as a pure compound were unsuccessful (Ibrahim et al., 2013).

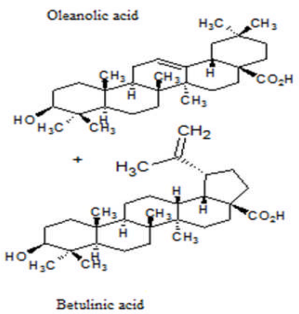
The acetylation of the mixture of oleanolic acid and betulinic acid (SF1) to obtain 3β-acetyloleanolic acid and 3β-acetylbetulinic acid (SF2) was done following the method described by Andrine et al. (2012). SF1 was dissolved in a solution containing a mixture of pyridine (10 ml) and acetic acid anhydride (12 ml). The mixture was refluxed for 3 h and stirred for 18 h at room temperature until a complete solution was obtained. The reaction was terminated by dissolving the mixture in distilled water (25 ml) and stirred for 45mins after which it was filtered under suction and washed with 12% HCl and this resulted in a white amorphous powdered compound containing a mixture of 3β-acetyloleanolic acid and 3β-acetylbetulinic acid (SF2). The compounds' structures were confirmed through NMR and IR spectral analysis and the results were in agreement with the literature values (Andrine et al., 2012). The chemical structures of the triterpenes RA5, RA3, SF1 and SF2 are as shown in Figure 1.



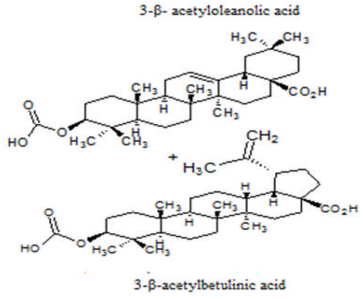
RA5



RA3



SF1



SF2

Figure 1: Chemical structures of the test triterpenes; 3β-hydroxylanosta-9,24-dien-21-oic acid (RA5), Methyl-3β-hydroxylanosta-9, 24-dien-21-oate (RA3), Mixture of oleanolic acid and betulinic acid (SF1), Mixture of 3-β-acetyloleanolic acid and 3-β-acetylbetulinic acid (SF2).

Test Organisms

The test organisms included seven *Escherichia coli* bacteria, one *Bacillus cereus*, five *Enterococcus* bacteria and nine *Vibrio* bacteria. All were obtained from the AEMREG culture collection at the University of Fort Hare Alice, South Africa and were kept in 20% glycerol at -80°C until use.

Preparation of the Inoculum

The inoculum was prior to use, prepared to match the 0.5 McFarland standard, by carrying out serial dilutions from an 18-24 h old bacteria culture grown in Mueller-Hinton broth.

Susceptibility Testing

The susceptibility test on the test triterpenes was determined using the agar well diffusion method as described by Okeke *et al*. (2001) with minor modifications. Freshly prepared Mueller-Hinton agar plates were streaked with each standardised test bacteria using a sterile cotton swab. Wells were bored into the agar and a 100 µl volume of the test triterpene, positive control (ciprofloxacin), solvent control (5% dimethyl sulfoxide (DMSO)) and negative control (sterile distilled water) were added into their respective wells in each plate. The plate was incubated for 18-24 h after which the zones of inhibition in mm were measured. The test was performed in triplicates and the mean zone of inhibition was recorded.

Determination of Minimum Inhibitory Concentration (MIC)

The MICs of the plant triterpenes were determined according to the method of Okoh and Penduka (2011) in a 96 well microtiter plate. Double strength Mueller-Hinton broth (100 µl) was pipetted into all the wells. The test organism was standardised to match the 0.5 McFarland-standard as mentioned before. The test wells contained 100 µl of test triterpene in broth, of which different concentrations were obtained through double fold serial dilutions in the Mueller-Hinton broth wells down the column before adding the 20 µl test bacteria. Sterility wells (containing broth only), positive control wells (containing 100 µl of ciprofloxacin serially diluted in Mueller-Hinton broth to make different test concentrations and 20 µl of the test bacteria), solvent control wells (containing 100 µl of 5% DMSO diluted in Mueller Hinton broth to make different test concentrations and 20 µl test bacteria) and negative control wells (containing 100 µl of broth and 20 µl test bacteria) were also added in each plate. The plates were then incubated at 37°C for 18-24 h, after which the MIC results were read visually by adding 40 µl of *p*- iodonitrotetrazolium violet (INT) into each well. A colour change from colourless to a pinkish or purple colour as viewed by the naked eye indicated growth of bacteria. The colour change is based on an oxidation-reduction reaction in which electrons are transferred from NADH (a product of the oxidation of threonine to 2-amino-3-ketobutyrate) to INT which then forms the red formazan (pink or purple in colour). The MIC was recorded as the lowest concentration of the triterpene that inhibited growth of the organism after 18-24 h of incubation. The test was performed in triplicates.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined as described by Penduka and Okoh (2013) by sub-culturing 10 µl from each well that did not show growth after 18-24 h of incubation from the MIC plate and streak inoculating it onto fresh Mueller-Hinton agar plates. The plates were incubated at 37^{o}C for 24-48 h, after which the number of viable colonies was counted. The MBC was defined as the lowest concentration killing more than or equal to 99.9% of the inoculum in comparison to the initial viable count. This experiment was done in triplicates.

Interactions

The interactions of the triterpenes and ciprofloxacin were interpreted by using the fractional inhibitory concentration (FIC) indices, which were determined using the checkerboard method according to the descriptions of Penduka *et al*. (2014). Serial dilutions of the antimicrobial combinations in double strength Mueller-Hinton broth were performed in 96 well microtiter plates to make different test concentrations. A 20 µl volume of the standardised test bacteria was added into the test wells which contained the antimicrobial combinations. Negative control wells (containing broth and test organisms); sterility wells (containing broth only), positive control wells (containing ciprofloxacin and test bacteria) and solvent control wells (containing 5% DMSO and test bacteria) were also added into each plate. The plates were incubated at 37 °C for 18-24 h after which MIC values of the combinations were read visually by adding 40 µl of *p*-iodonitrotetrazolium violet (INT) into each well. A colour change from colourless to a pinkish purple colour indicated the growth of bacteria established. The FIC index of the test triterpene (FIC_T) was calculated as the ratio of the MIC value of the triterpene in combination over the MIC value of the triterpene alone, and the FIC index of the antibiotic (FIC_A) was calculated as the ratio of the MIC value of the antibiotic in combination over the MIC value of the antibiotic alone. The overall FIC index (ΣFIC) was calculated as the summation of the FIC_T and the FIC_A. The interactions were interpreted as synergism when the ΣFIC index≤0.5, additive when ΣFIC index is between >0.5 and ≤1, and indifference when the ΣFIC index is >1 and ≤4 whilst antagonism was defined as when the ΣFIC index is >4.

Membrane Damage Determination

The cytosolic lactate dehydrogenase (LDH) release assay was used to determine membrane damage. It was carried out according to the method described by Korzeniewski and Callewaert (1983) following the descriptions of Soyingbe *et al*. (2013). Overnight grown test bacteria was

first standardised to match the 0.5 McFarland standard, and then grown for 18-24 h with either the 2× MIC value or the MBC value (if available) of the test triterpene. After incubation the microbial cultures were centrifuged at 5000xg for 5 min. A volume of 50 µl of the supernatant was mixed with 50 µl mixed reaction solutions that is 2µl substrate and 48µl buffer solution of the LDH activity assay kit (Sigma) at room temperature for 30 min. After which, the absorbance was measured at 450 nm using a 96 well microplate reader (BioTek). The total loss of membrane integrity resulting in complete loss of cell viability was determined by lysing the cells of untreated organisms with 3% Triton X-100 and using this sample as a positive control. The cytotoxicity in the LDH release test was calculated using the formula: cytotoxity =(E-C)/(T-C) × 100, where E is the experimental absorbance of the test bacteria incubated with the test triterpene, C is the control absorbance of the cell medium and T is the 3% Triton X-100 treated test bacteria supernatant. The test was performed in triplicates.

Results

Antibacterial Susceptibility Test

The results of the antibacterial susceptibilities of the test triterpenes against 22 bacteria strains, which include 16 Gram negative and 6 Gram positive bacteria are as shown in Tables 1a and 1b respectively. The percentage activities of the test triterpenes were, 86.4% for RA3, 54.6% for RA5, 22.7% for SF1 and 9.09% for SF2. The 5% DMSO solution which was the solvent control had 0% activity and ciprofloxacin; the positive control had 100% activity.

Table 1a: Zones of inhibition (mm) of the test triterpenes and ciprofloxacin against Gram negative bacteria (*E. coli* and *Vibrio*).

Organism	Test Triterpenes				Positive control
	SF1	SF2	RA3	RA5	Ciprofloxacin
E. coli (DSM-1089)	0	0	13± 1.5	14±1.1	24±5.7
E. coli (DSM-8695)	14±3	0	28± 2.5	19±3.5	23±3.5
E. coli (DSM-4618)	0	0	12±3.6	14±2	29±2
E. coli (DSM-10973)	0	0	11± 1.5	9±0.57	19±1.15
E. coli (DSM-10974)	0	0	12±1.5	0	25±3
E. coli (DSM-9025)	0	0	12±1.5	12±2	24±3.5
E. coli (ATCC-23922)	0	0	10±2.8	15±4.5	25±1.15
V. vulnificus (AL 042)	0	0	17 ± 2.12	7 ± 0.71	26 ± 1.41
V. fluvialis (AL 019)	16 ± 1.41	0	14 ± 0.71	7 ± 0.71	34 ± 0.71
V. fluvialis (AL 019)	0	0	15 ± 0.71	8 ± 0.71	29 ± 1.41
V. vulnificus (AL 044)	0	0	9 ± 0.71	13 ± 5.54	30 ± 1.41
V. fluvialis (AL 004)	0	0	16 ± 0.71	8 ± 0.71	25 ± 1.41
V. parahaemolyticus (AL032)	17 ± 1.41	0	11 ± 1.41	0	33 ± 1.41
V. parahaemolyticus (AL030)	0	7 ± 0.71	7 ± 0.71	0	18 ± 2.12
V. vulnificus (DSM 11507)	0	0	0	7 ± 0.71	30 ± 0.71
V. fluvialis (DSM 19283)	0	0	0	0	30 ± 0.71

Table 1b: Zones of inhibition (mm) of the test triterpenes and ciprofloxacin against Gram positive bacteria (*Enterococcus* and *B. cereus*)

Organism	Test Triterpenes				Positive Control
	SF1	SF2	RA3	RA5	Ciprofloxacin
E. avium (ATCC 1405)	12 ± 1.41	7 ± 0.71	33 ± 2.83	15 ± 4.95	33 ± 2.83
E. casseliflavus (ATCC 25788)	0	0	15 ± 1.41	7 ± 0.71	29 ± 1.41
E. hirae (ATCC 8043)	0	0	12 ± 0.71	0	25 ± 0.71
E. gallinarum (ATCC 49573)	0	0	16 ± 2.12	7 ± 0.71	28 ±0 .71
E. faecalis (ATCC 19433)	0	0	0	0	47 ± 2.83
B. cereus (ATCC-10702)	14±2.3	0	17±1.60	11±2	29±2.5

\Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the test triterpenes against both Gram positive and Gram negative bacteria are as shown in Tables 2a and 2b respectively. Lower MIC values were observed against Gram negative bacteria in comparison to Gram positive bacteria amongst all the active triterpenes. The MIC ranges for the triterpenes were 0.625-10 mg/ml for SF1, 1.25-10 mg/ml for RA3, 5- 10 mg/ml for RA5 and 10 mg/ml for SF2. The 5 % DMSO had no activity against the test bacteria while ciprofloxacin had MIC ranges of 0.156- 2.5 µg/ml.

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Membrane Damaging Potentials of the Test Triterpenes

Table 5 shows the results of the membrane damaging potentials of some of the test triterpenes in comparison to 3% Triton X-100. RA3 is the only triterpene that showed appreciable membrane damaging potential especially against *V. vulnificus* (AL 042), while SF1 and RA5 had no membrane damaging potentials noted.

Table 2a: Minimum inhibitory concentrations of the test triterpenes (mg/ml) and ciprofloxacin (µg/ml) against *E. coli* and *Vibrio bacteria*

Organism	Test triterpenes				Positive control
	SF1	SF2	RA3	RA5	Ciprofloxacin
E. coli (DSM-1089)	-	-	5	10	0.312
E. coli (DSM-8695)	5	-	2.5	5	0.312
E. coli (DSM-4618)	-	-	5	10	0.312
E. coli (DSM-10973)	-	-	2.5	10	0.625
E. coli (DSM-10974)	-	-	5	5	0.625
E. coli (DSM-9025)	-	-	2.5	10	0.312
E. coli (ATCC-23922)	-	-	2.5	10	0.312
V. vulnificus (AL 042)	-	-	1.25	10	2.5
V. vunificus (AL 018)	-	-	5	5	1.25
V. fluvialis (AL 019)	0.625	-	5	5	0.156
V. fluvialis (AL 004)	-	-	2.5	10	1.25
V. parahaemolyticus (AL032)	1.25	-	5	-	0.781

Keynote: - denotes not determined

Table 2b: Minimum inhibitory concentrations of the test triterpenes (mg/ml) and ciprofloxacin (µg/ml) against *B. cereus* and *Enterococcus bacteria*

Organism	Test Triterpenes				Positive control
	SF1	SF2	RA3	RA5	Ciprofloxacin
B. cereus (ATCC-10702)	10	-	5	10	0.625
E. avium (ATCC 1405)	10	10	10	10	2.5
E. casseliflavus (ATCC 25788)	-	-	5	10	5
E. hirae (ATCC 8043)	-	-	10	-	5
E. gallinarum (ATCC 49573)	-	-	10	10	1.25
E. faecalis (ATCC 19433)	-	-	-	-	1.25

Keynote: - denotes not determined

Minimum Bactericidal Concentration (MBC)

The MBC results showed all the test triterpenes to be bacteriostatic except for RA3 against *V. vulnificus* (AL 042) and RA5 against *V. fluvialis* (AL 004) which had bactericidal results. The results are as presented in Tables 3a and 3b. No bactericidal results were noted against the Gram positive bacteria tested.

Table 3a: Minimum bactericidal concentrations (mg/ml) of the test triterpenes and ciprofloxacin (µg/ml) against *E. coli* and *B. cereus* bacteria

Organism	Antibacterial Testing Agents Activity			
	SF1	SF2	RA3	RA5
E. coli (DSM-1089)	-	-	>5	>10
E. coli (DSM-8695)	>5	-	>5	>10
E. coli (DSM-4618)	-	-	>5	>10
E. coli (DSM-10973)	-	-	>5	>10
E. coli (DSM-10974)	-	-	>5	>10
E. coli (DSM-9025)	-	-	>5	>10
E. coli (ATCC-23922)	-	-	>5	>10
V. vunificus (AL 018)	-	-	>5	>5
V. parahaemolyticus (AL032)	>5	-	>5	-
V. fluvialis (AL 019)	>5	-	>5	>10
V. fluvialis (AL 004)	-	-	>5	10
V. vulnificus (AL 042)	-	-	1.25	>10

Table 3b: Minimum bactericidal concentrations (mg/ml) of the test triterpenes and ciprofloxacin (µg/ml) against *E. coli* and *B. cereus* bacteria

Organism	Test Triterpenes			
	SF1	SF2	RA3	RA5
B. cereus (ATCC-10702)	>10	-	>5	>10
E. avium (ATCC 1405)	>10	>10	>10	>10
E. casseliflavus (ATCC 25788)	-	-	>10	>10
E. hirae (ATCC 8043)	-	-	>10	-
E. gallinarum (ATCC 49573)	-	-	>5	>10

Interactions of the Test Triterpenes with Ciprofloxacin

Tables 4a, 4b and 4c show the results of the interactions of the triterpenes with ciprofloxacin against some selected bacteria. SF1 and ciprofloxacin's interaction against *Vibrio fluvialis* (AL019) was synergistic. All other interactions ranged from indifference to antagonism.

Table 4a: Interactions of RA3 and ciprofloxacin against *E.coli, V. fluvialis* and *Enterococcus avium* bacteria

Organism	FIC index of RA3	FIC index of ciprofloxacin	∑FIC index	Interaction
E.coli (DSM-8695)	1	1	2	Indifference
E. coli (DSM-10973)	2	1	3	Indifference
E. coli (DSM-9025)	2	8	10	Antagonism
E. coli (ATCC-25922)	2	6	6	Antagonism
V. fluvialis (A019)	1.4	4	5.4	Antagonism
Enterococcus avium (ATCC 1405)	0.5	3	3.5	Indifference

Table 4b: Interactions of 3β-hydroxylanosta-9, 24-dien-21-oic acid and ciprofloxacin against *E. coli, V. fluvialis and Enterococcus avium* bacteria

Organism	FIC index of RA5	FIC index of ciprofloxacin	∑FIC index	Interaction
E.coli (DSM-8695)	0.5	2	2.5	Indifference
E. coli (DSM-10973)	1	4	5	Antagonism
E. coli (DSM-10974)	0.5	2	2.5	Indifference
E. coli (DSM-9025)	1	4	5	Antagonism
E. coli (ATCC-25922)	1	4	5	Antagonism
V. fluvialis (A019)	2.3	5	7.3	Antagonism
Enterococcus avium (ATCC 1405)	2	4.2	6.2	Antagonism

Table 4c: The interactions of SF1 and SF2 with ciprofloxacin either *V. fluvialis* (AL019) or *Enterococcus avium* (ATCC 1405)

Organism	Combination	FIC index of test triterpene	FIC index of ciprofloxacin	ΣFIC index	Interaction
Vibrio fluvialis (AL 019).	SF1+ Cipro	0.1	0.4	0.5	Synergy
Enterococcus avium (ATCC 1405)	SF1+ Cipro	1.5	3.5	5	Antagonism
	SF2+ Cipro	0.5	1.5	2	Indifference

Keynote: Cipro denotes ciprofloxacin

Table 5: Membrane damaging activity (% cytosolic lactate dehydrogenase released) of the triterpenes against *E. coli* (DSM-8695) and *V.* ***vulnificus*** **(AL 042) bacteria in comparison to 3% Triton X-100**

Organism	SF1	RA3	RA5
E. coli (DSM-8695)	0	1	0
V. vulnificus (AL 042)	-	36	-

Keynote: - denotes not determined

Discussion

Plants and their secondary metabolites are a promising source of bioactive compounds that are potential therapeutic agents, including antimicrobials (Chung *et al*., 2011). In this study, the two lanostane triterpenes (RA3 and RA5) were found to exhibit broader spectrum activity in comparison to the two lupane triterpenes (SF1 and SF2). This can be attributed to the structural differences of the triterpenes; in a previous study oleanolic acid and betulinic acid were reported to be poor antibacterial agents (Fontanay et al., 2008). Medicines have also been formulated from the synthetic derivatives of most secondary metabolites (Kennedy and Wightman 2011) however, in this study the acetylated derivative of SF1, namely SF2, was not as potent as SF1, as evidenced by its lower percentage activity and higher MIC values in comparison to SF1 (Table 2a and b). This may imply that the antibacterial activity of SF1 is linked to the OH group on carbon number 3, although the possibility of an antagonistic effect from the combination of the acetylated oleanolic acid and betulinic acid cannot be ruled out also.

Results in this study showed lower MIC values against Gram negative bacteria in comparison to the Gram positive ones which contrast with the general norm that Gram negative bacteria are more resistant to antibacterial agents than their Gram positive counterparts. This difference is attributed to the presence of an outer membrane in Gram negative bacteria which combines a highly hydrophobic lipid bilayer with pore-forming proteins of specific size exclusion properties which act as a selective barrier (Delcour, 2009). However, hydrophobic antimicrobial agents are known to use the lipid mediated pathway to pass through the outer membrane (Delcour, 2009), which can possibly be the mechanism utilised by the test triterpenes since they are hydrophobic molecules.

The MIC values of the triterpenes in the present study were above the range of 100- 1000 µg/ml which is expected for pure plant compounds to be termed antibacterial agents (Tegos *et al*., 2002). However, previous antibacterial studies on RA3 and RA5 by Penduka et al. (2014) and Mosa et al. (2014b) showed some MIC values within the ranges of 100-1000 µg/ml. Interestingly, the study by Mosa et al. (2014b) similarly proved RA3 and RA5 to having a high MIC value of above 1000 µg/ml against *E. coli* bacteria. SF1 had an MIC value of 0.625 mg/ml against *V. fluvialis* (AL 019), but SF1 was a mixture of two compounds therefore it cannot also fall under the same category of pure plant compounds for it to be termed antibacterial.

The triterpenes were bacteriostatic against the test Gram positive bacteria. These findings are in line with studies by Penduka et al. (2014) on *Listeria* bacteria, however, studies by Mosa et al. (2014b) showed RA3 and RA5 to be bactericidal against some of the selected Gram positive and Gram negative bacteria. The MBC studies against the Gram negative bacteria in the present study were also mostly bacteriostatic except for RA3 against *V. vulnificus* (AL042) and RA5 against *V. fluvialis* (AL004) only, where bactericidal results were observed. The membrane damage test showed RA3 only to have appreciable membrane damaging potentials against *V. vulnificus* (AL042) with a 36% membrane damage in comparison to 3% Triton X-100. Previous studies have also shown RA3 to exhibit high membrane damaging potential against some other Gram negative bacteria namely *Proteus mirabilis* and *Escherichia coli* (Mosa et al., 2014b). However, both RA5 and RA3 were shown to not exhibit DNA damaging potential (Mosa et al., 2014b).

The results of the interactions of the triterpenes with ciprofloxacin proved to be mainly indifferent and antagonistic. Ciprofloxacin is a fluoroquinolone antibiotic, which acts through inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication (Hooper, 2000). In contrast, pent-acyclic triterpenes have been shown in some interaction studies to have synergistic activities with penicillin G (Penduka et al., 2014), vancomycin (Chung et al., 2011) and methillicin (Chung et al., 2011), which are all antibiotics that inhibit cell wall biosynthesis, which may suggest that pent-acyclic triterpenes work synergistically or favourably with antibiotics that inhibit cell wall biosynthesis in comparison to those that inhibit DNA replication.

The combined results of the present and previous (Penduka et al., 2014; Mosa et al., 2014b) studies support the postulations that the mode of action of triterpenoids is not yet fully understood (Chung et al., 2011). This then leads to the possible conclusion that the triterpenes work on

multiple target sites, such that their mechanism of action is possibly a combination of the blockage of cell division through DNA synthesis inhibition and the inhibition of macromolecular synthesis which may also be linked to cell membrane damage (de Leon and Moujir, 2008).

Conclusion

The present study revealed that the triterpenes could not be termed antibacterial against the test bacteria, but it has shown their antibacterial potentials which may be further exploited through other different chemical modification processes or through different antibiotic combination therapies. In addition, the test lanosteryl triterpenes have previously been reported to have therapeutic potentials such as antihyperlipidemic potential (Mosa et al., 2014; Machaba et al., 2014) and anti-platelet aggregation activity (Mosa et al., 2011). Potential pharmacological importance of oleanolic acid and betulinic acid as anti-inflammatory, antidiabetic, anticancer, immune modulatory, and antioxidant agents have also been reported (Laszczyk 2009; Lu et al., 2013).

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