Semi-synthesis of Nitrogen Derivatives of Oleanolic Acid and Effect on Breast Carcinoma MCF-7 Cells

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Abstract. *Background: Oleanolic acid is a triterpenoid that has shown in vitro cytotoxic activity against human tumour cells and is known to be present in many higher plants. Materials and Methods: Oleanolic acid is known to have some biological potential including anticancer property. Oleanolic acid was isolated from the ethyl acetate fraction of Syzygium aromaticum seed with an aim of dervitatising the functional group and evaluating the biological activities of the semi-synthesised compounds. Acylation of the alcohol functional group of the oleanolic acid afforded the opportunity of hydrazine reaction to give 3-acetoleanolic hydrazide. Further reaction of 3-acetoleanolic hydrazide with benzyladehyde, glacial acetic acid and methanol resulted in the synthesis of the corresponding 3-acetoxyoleanolic hydrazone. Results: The semi-synthetic oleanolic acid derivatives did not exhibit enhanced cytotoxic activity over oleanolic acid itself. Conclusion: 3-acetoxyoleanolic hydrazide has a potent anticancer activity.*

Syzygium aromaticum (syn. *Eugenia caryophyllus*, *E. aromatica*, *Caryophyllus aromaticus*) belongs to the Myrtaceae family (1). It is an evergreen tree that grows up to 10-20 m in height which is indigenous to India, Zanzibar, Indonesia, and Sri Lanka. The tree is best known for its buds which are often referred to as cloves (2, 3). Cloves are used traditionally to treat asthma (4), respiratory and digestive disorder (1, 5). Cloves are also reported to be used in treating dental conditions, respiratory ailments, headaches, and sore throat complaints (6). They are also used as an antidote against oral bacteria often associated with dental caries and periodontal disease (7, 8). It has been reported that *S. aromaticum* is among the major ingredients in toothpaste and mouth fresheners in India (9). It is a common spice used in food preparation among certain tribes and believed to have anti-carcinogenic properties (5, 10). The aqueous infusion of cloves has been reported to inhibit germination of spores of Bacillus subtilis and other bacteria (9), thus it is often used as antiseptic (1). *S. aromaticum* has also been reported to have anti-inflammatory, cytotoxic and anaesthetic activity (11), anti-fungal activity (12, 13), anti-viral activity, also against hepatitis virus (14-16). Eugenol and some other active components of the essential oil from cloves are used to prevent lipid peroxidation and have strong antioxidant properties (1, 17-19). Isolation of two flavonoid apigenintriglycosides from the seed of *S. aromaticum* has been reported by Nassar (20). Eight compounds, namely gallic acid, ellagic acid, biflorin, kaempferol, rhamnocitrin, myricetin, D-glucopyranoside and oleanolic acid (compound **1**, Figure 1) were isolated from methanolic extract of cloves (7).

Previous literature reports have described the synthetic derivation of oleanolic acid at positions 3, 28 and 12, in which the functional groups are modified. Such derivatives include 3-O-(3',3'-dimethyl)-succinyloleanolic acid (21), acetyloleanolic acid and 3-acetyl-11-oxo-oleanolic acid. Oxidation of acetyloleanolic acid yielded 12α-hydroxyl-δ-lactone and 12-oxo-α-lactone (22). Li, *et al*. reported the synthesis of two acetyl derivatives of oleanolic acid (23). Phosphate derivatives of acetyloleanoic acid, diethyl-3-acetylolean-12-en-28-oxyphosphate and diethyl-3-hydroxyolean-28-oxyphosphonate were synthesised and reported by Hirch *et al*. (22). Three esters were also synthesised by reaction at position 3 by Hirch *et al*. (22). These reports have demonstrated that functional

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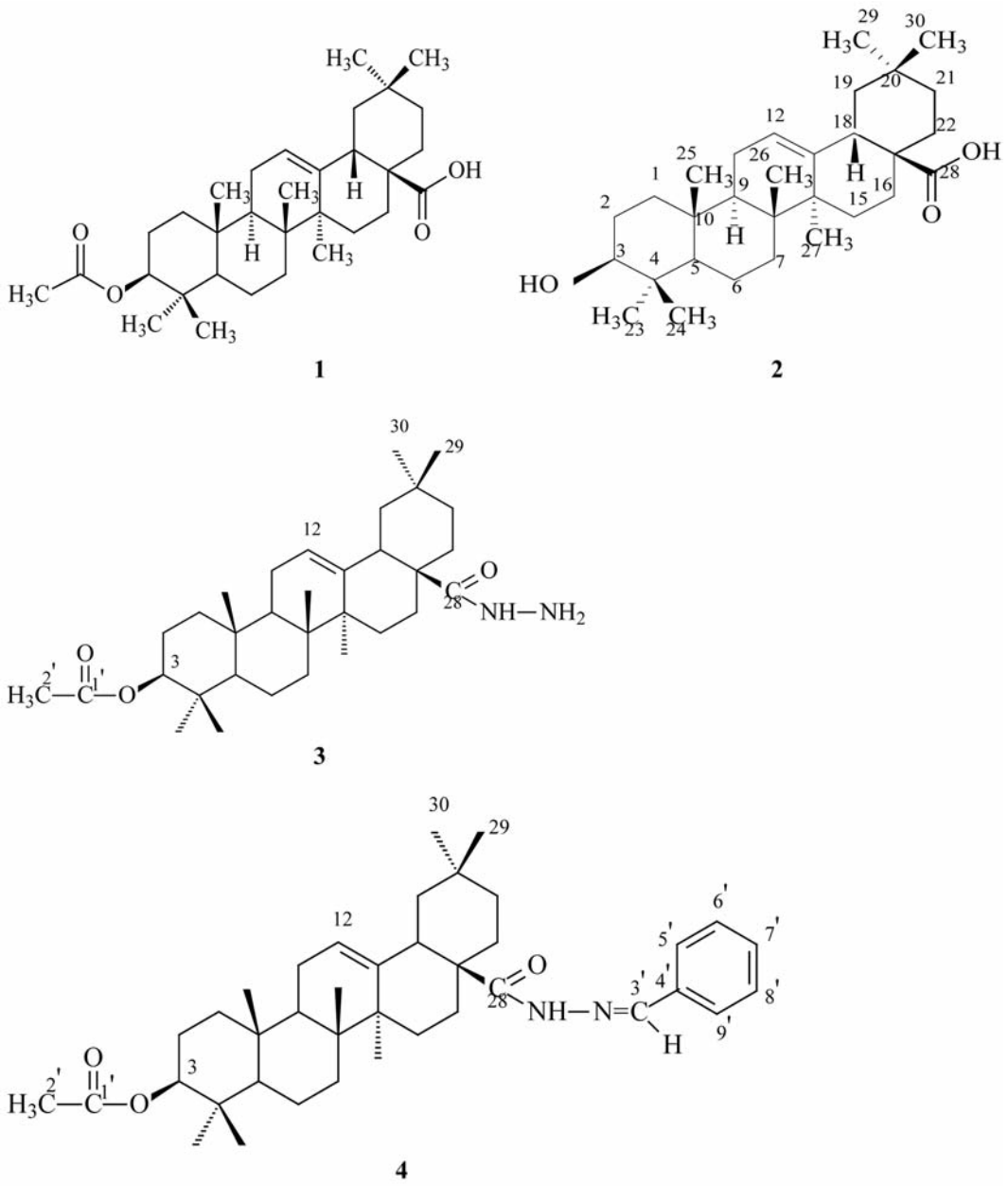

Figure 1. *Structure of oleanolic acid (**1**), 3-acetoxyoleanolic acid (**2**), 3-acetoxyoleanolic hydrazide (**3**), and 3-acetyloleanolic hydrazone (**4**).*

group modifications can result in enhancement of bioactivities in several bioassays, including those for anticancer, anti-HIV, anti-sickling and anti-diabetic properties (21, 24-26). To the best of our knowledge, the hydrazide and hydrazone derivatives have not been reported. We, therefore, report further semi-synthetic derivatives of acetoxyoleanolic acid (compound **2**, Figure 1) (27) at position 28 with hydrazine and hydrazone moieties.

Materials and Methods

All chemicals used were purchased from Merck (Pty) Ltd. South Africa. 3-Acetyloleanolic acid (0.4 g) (1), which was derivatised from oleanolic acid (27), and triethylamine (0.1 g) were dissolved in dichloromethane (20 ml). A solution of thionyl chloride (0.1 g) in dichloromethane (10 ml) was added to the mixture dropwise while cooling under ice and continuous stirring. Approximately 0.25 g of hydrazine hydrate was then added to the mixture. A solution of

Table I. *^{1}H and ^{13}C Nuclear Magnetic Resonance data for oleanolic acid (**1**), 3-acetoxyoleanolic acid (**2**), 3-acetoxyoleanolic hydrazide (**3**), and 3-acetyloleanolic hydrazone (**4**).*

	δ_H Compound				δ_C Compound			
Position	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	1.05(1H,*m*)	0.87(1H,*t*)	1.05(1H,*m*)	1.05(1H,*m*)	38.41(*t*)	38.07(*t*)	38.11(*t*)	38.17(t)
	1.58(1H,*m*)	1.58(1H,*t*)	1.60(1H,*m*)	1.58(1H,*m*)				
2	1.06(1H,*m*)	1.69(1H,*m*)	1.09(1H,*m*)		27.18(*t*)	27.66(*t*)	27.20(*t*)	27.21(t)
	1.70(1H,*m*)	1.84 (1H,*m*)	1.48(1H,*m*)					
3	4.24(1H,*m*)	3.25(1H,*s*)(OH)	4.47(1H,*m*)	4.46(1H,*m*)	79.03(*d*)	80.93(*d*)	80.81(*d*)	80.81(d)
		3.90 (1H,*m*)						
4	-	-	-	-	38.76(*s*)	39.28(*s*)	39.35(*s*)	39.50(s)
5	0.79(1H,*m*)	0.76(1H,*t*)	0.85(1H,*m*)		55.21(*d*)	55.29(*d*)	55.18(*d*)	55.13(d)
6	1.30(1H,*m*)	1.32(1H,*m*)	1.46(1H,*m*)		18.30(*t*)	18.18(*t*)	18.13(*t*)	18.11(t)
	1.51(1H,*m*)	1.57(1H,*m*)	1.73(1H,*m*)					
7	1.45(1H,*m*)	1.73(1H,*m*)	1.49(1H,*m*)		34.63(*t*)	32.55(*t*)	32.95(*t*)	
	1.51(1H,*m*)	1.62(1H,*m*)	1.54(1H,*m*)					
8	-	-	-	-	39.27(*s*)	36.97(*s*)	36.83(*s*)	36.82(s)
9	1.58(1H,*m*)	1.57(1H,*m*)	1.58(1H,*m*)		47.63(*d*)	47.55(*s*)	47.45(*d*)	47.46(d)
10	-	-	-	-	37.07(*s*)	37.69(*s*)	37.68(*s*)	37.67(s)
11	1.94-1.95 (2H,*m*)	0.97(1H,*m*)	1.63(1H,*m*)		23.57(*t*)	23.52(*t*)	23.48(*t*)	23.49(t)
		1.68(1H,*m*)	1.91(1H,*m*)					
12	5.15 (1H,*m*)	5.25(1H,*m*)	5.35(1H,*m*)	5.53(1H,*m*)	122.65(*d*)	122.57(*d*)	123.32(*d*)	123.47(d)
13	-	-	-	-	143.58(*s*)	143.59(*s*)	144.73(*s*)	145.49(s)
14	-	-	-	-	41.63(*s*)	41.59(*s*)	41.89(*s*)	42.05(s)
15	1.09(1H,*m*)	1.07-1.11(2H,*m*)			27.68(*t*)	28.04(*t*)	27.99(*t*)	28.01(t)
	1.63(1H,*m*)							
16	1.56(1H,*m*)	1.59(1H,*m*)	1.60(1H,*m*)		23.40(*t*)	23.39(*t*)	23.50(*t*)	23.69(t)
	1.86(1H,*m*)	1.85(1H,*m*)	1.90(1H,*m*)					
17	-	-	-	-	46.49(*s*)	46.51(*s*)	46.39(*s*)	46.69 (s)
18	2.79(1H,*m*)	2.80(1H,*m*)	2.47-2.43(1H,*m*)	2.79(1H,*m*)	41.04(*d*)	41.00(*d*)	41.41(*d*)	42.17(d)
19	1.55(1H,*m*)	1.23(1H,d)	1.18(1H,*m*)		45.88(*t*)	45.84(*t*)	45.86(*t*)	46.47(t)
	1.69(1H,*m*)	1.26(1H,d)	1.72(1H,*m*)					
20	-	-	-	-	30.67(*s*)	30.67(*s*)	30.71(*s*)	30.73(s)
21	1.30(1H,*m*)	1.23(1H,*m*)	1.20(1H,*m*)		33.80(*t*)	33.79(*t*)	33.93(*t*)	34.01(t)
	1.38(1H,*m*)	1.18(1H,*m*)	1.38(1H,*m*)					
22	1.54(1H,*m*)	1.23(1H,*m*)	1.24(1H,*m*)		32.43(*t*)	32.43(*t*)	32.12(*t*)	32.07(t)
	1.76(1H,*m*)	1.80(1H,*m*)	1.75(1H,*m*)					
23	0.89(3H,*m*)	0.92(3H,*s*)	0.87(3H,*s*)		28.10(*q*)	22.98(*q*)	21.05(*q*)	21.31(q)
24	0.72(3H,*s*)	0.98(3H,*s*)	0.72(3H,*s*)	0.98(3H,*s*)	15.55(*q*)	16.66(*q*)	16.65(*q*)	16.96(q)
25	0.99(3H,*s*)	0.89(3H,*s*)	0.98(3H,*s*)	0.89(3H,*s*)	15.32(*q*)	15.38(*q*)	15.39(*q*)	15.44(q)
26	0.84(3H,*s*)	0.77(3H,*s*)	0.82(3H,*s*)	0.82(3H,*s*)	17.11(*q*)	17.13(*q*)	16.64(*q*)	16.63(q)
27	1.16(3H,*s*)	1.13(3H,*s*)	1.15(3H,*s*)	1.16(3H,*s*)	25.92(*q*)	25.88(*q*)	25.78(*q*)	25.79(q)
28	-	-	-	-	182.27(*s*)	182.75(*s*)	179.17(*s*)	174.82(s)
29	0.87(3H,*s*)	0.90(3H,*s*)			33.06(*q*)	33.05(*q*)	32.95(*q*)	32.93(q)
30	0.90(3H,*s*)	0.91(3H,*s*)	0.89(3H,*s*)		22.96(*q*)	23.57(*q*)	23.74(*q*)	24.16(q)
1'	-	-	-	-	-	171.04(*s*)	171.04(*s*)	171.06(q)
2'	2.06(3H,*s*)	-	2.08(3H,*s*)	2.06(3H,*s*)	-	22.93(*q*)	21.30(*q*)	
O=C-NH								
3'				8.98(1H,*s*)				147.69(d)
4'				-				134.19(s)
5'				7.71(1H,d)				128.62(d)
6'				7.37(1H,d)				127.73(d)
7'				7.36(1H,*m*)				127.71(d)
8'				7.37(1H,*m*)				127.73(d)
9'				7.71(1H,d)				128.62(d)
O=^{28}C-NH	-	-	7.02(1H,*m*)		-	-	-	
O=^{28}C-NH-NH_2	-	-	2.6-4.0 (2H,*brm*)		-	-	-	

triethylamine (0.1 g) in 10 ml of dichloromethane was prepared and added to the mixture dropwise under ice with continuous stirring for 3 h. On completion of the reaction, the dichloromethane-soluble fraction was separated and the solvent was distilled off to give a crude reaction product. The product was purified using column chromatography with silica gel as the stationary phase. Forty-six fractions were collected and analysed with thin layer chromatography (TLC); fractions 1-33 were combined as unreacted acetoxyoleanolic acid, while fractions 34-46 contained the expected product (95 mg) which was labelled as compound **3** (Figure 1). Compound **3** was subjected to a full 1D and 2D Nuclear Magnetic Resonance (Table I), Infra-Red Spectroscopy (Perkin Elmer ATR FT-IR, Durban, South Africa) and melting pointing analyses.

3-Acetoxyoleanolic hydrazone. A mixture of 3-acetoxyoleanolic hydrazide (200 mg), benzaldehyde (0.05 g), glacial acetic acid (2 drops), and methanol (20 ml) in a round-bottom flask was refluxed for 2 hduring which the desired product crystallized out. The crystals were filtered by suction from the solution, washed, and dried. The yield was 165 mg (82.5%). The NMR data of the product, compound **4**, are presented in Table I.

Cytotoxicity screening. Human MCF-7 breast adenocarcinoma cells (ATCC no. HTB-22) (28) were grown in a 3% CO_2 environment at 37˚C in RPMI-1640 medium, supplemented with 10% foetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per litre of medium, 15 mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 μl. After 48 h, supernatant fluid was removed by suction and replaced with 100 μl growth medium containing 1.0 μl of Dimethyl sulfoxide (DMSO) solution of the test compound (1% w/w in DMSO), giving a final concentration of 100 μg/ml for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 μl DMSO/ml) were used. Tingenone was used as a positive control (29). After the addition of compounds, plates were incubated for 48 h at 37˚C in 5% CO_2; medium was then removed by suction, and 100 μl of fresh medium was added to each well. In order to establish percentage kill rates, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cell viability was carried out (30). After colorimetric readings were recorded at 570 nm (using a Molecular Devices SpectraMAX Plus microplate reader, Sunnyvale, CA), average absorbances, standard deviations, and percentage kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated. Cytotoxicity determinations were repeated for compounds with greater than 50% killing using two-fold dilutions of the compounds. LC_{50} (lethal concentration, 50%) values were determined using the Reed-Muench method (31).

Results and Discussion

3-Acetyloleanolic hydrazide (3). A colourless powdery solid with percentage yield of 23.8% (95 mg) was obtained from the column chromatography fraction after further purification. The melting point was 210-212˚C. The IR spectra gave the following peaks indicating the functional groups present vC=O1729, vN-H 3308, vC-N 1251, vC-H 2939,vC=O1618, vC-O 1075, vC=C1655 cm^{-1}. The molecular weight of the compound was 512.

Table II. *Bioassay of compound cytotoxicity towards MCF-7 breast adenocarcinoma cells.*

Compound	LC_{50}
Oleanolic acid (**2**)	61.8±9.7 μg/ml (0.135±0.002 μM)
3-Acetoxyoleanolic hydrazide (**3**)	51.7±3.4 μg/ml (0.137±0.007 μM)
3-Acetyloleanolic hydrazone (**4**)	>100 μg/ml (0.10±0.007 μM)

LC_{50}: Lethal concentration, 50%.

The 1D and 2D NMR spectra along with the IR spectra and mass spectra confirmed the transformation of the C 28 from a carboxylic acid to a hydrazide as presented when compared with the NMR data of compound **1** which has been reported earlier by our study and those in literature (22, 24). Compound **3** was therefore confirmed to be a synthesized hydrazide of 3-acetyloleanolic acid. Table I presents the ^1H and ^{13}C NMR data of the synthesized 3-acetyloleanolic hydrazide.

*3-Acetoxyoleanolic hydrazone (**4**)*. White powdery solid with a yield of 165 mg was obtained. The melting point was 168-170˚C while, the molecular weight (LC-MSD-Trap-VL) was 587. IR Spectroscopy (Perkin Elmer ATR FTIR) gave v_{C-H} 2945,v_{C-N} 2163, $v_{C=O}$1661, $v_{C=O}$(COOH) 1728, and v_{C-O} 1071cm^{-1}.

Comparison of the ^1H and ^{13}C NMR of the synthesized 3-acetoxyoleanolic hydrazone with those of oleanolic acid and the hydrazine derivative (Table I) above confirmed the product as being 3-acetoxyoleanolic hydrazone (Figure 1).

Oleanolic acid showed *in vitro* cytotoxic activity toward MCF-7 human breast tumour cells, consistent with previous reports on cytotoxicity of this triterpenoid (32-35). However, 3-acetoxyoleanolic hydrazide (**3**) had comparable cytotoxic activity towards MCF-7 cells, but the hydrazone derivative (**4**) was inactive (Table II). Esterification at the C(3) –OH group generally seems to have little effect on cytotoxicity, whereas derivatization of the carboxylic acid moiety often reduces cytotoxic activity (32, 35).

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