Design of Oleanolic Acid-Based Hybrid Compounds as Potential Pharmaceutical Scaffolds

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Abstract:

Infectious disease as well as cancer are leading causes of death worldwide. Drug resistance usually results in treatment requiring a combination of two or more drugs. In this study, oleanolic acid was hybridized with selected known pharmaceutical scaffolds via the carboxylic acid functionality on oleanolic acid, in order to develop therapeutics with increased biological activity. The compounds were characterized by FTIR, NMR and MS spectroscopy, which confirmed their successful isolation. Antibacterial activity was determined using the micro-dilution assay against selected Gram-positive and Gram-negative bacteria. Compound **4** indicated antibacterial activity which was superior against *Staphylococcus aureus, Proteus vulgaris, Staphylococcus epidermidis, Escherichia coli* and *Klebsiella oxytoca* with MIC values of 0.078, 0.078, 1.25 and 1.25 mg/mL when compared to the control, oleanolic acid (MIC = 2.5 mg/mL). Furthermore, *in vitro* cytotoxicity, as determined using the SRB assay, against selected cancer cells revealed that compound **3** was the most cytotoxic to MDA, MCF-7 and DU145 cells with IC_{50} value of 69.87±1.04, 85.27±1.02 and 73.2±1.08 µg/mL when compared to oleanolic acid (IC_{50} >200 µg/mL). The results are indicative thereof that oleanolic acid is a potential precursor for the development of antibacterial and anticancer agents.

Introduction

The continuing increase in bacterial resistance to currently used antibiotics has led to huge concern in the public health sector (Dalrymple et al. 2010; Walvekar et al. 2019). This has been brought about among other by the misuse of these drugs (Taganna et al. 2011; Morgan et al. 2011). Yearly, approximately 700 000 people across the globe die due to antimicrobial infections that are resistant to treatment, and it is estimated that by 2050, the numbers will increase to 10 million (Ghosh et al. 2019; Zhang 2019; Mundy et al. 2016; Belete 2019). This clearly indicates the urgent need for the development of novel and effective antimicrobial agents.

Various efforts are currently underway to develop therapeutic drugs to counteract this resistance (Karagöz et al. 2019; Harvey AL 2008; Ganesan A 2008). Plant-based products have proven to be highly effective against a number of bacterial pathogens (Subbaiah et al. 2017; Arulmozhi et al. 2018). Most of these plant-derived compounds, such as pentacyclic triterpenoids are potential antibacterial agents (Kurek et al. 2012; Kuźma et al. 2007; Chouab et al. 2015). Their structural modification can enhance their biological activity which is of interest in medicinal chemistry and organic synthesis (Dinh et al. 2014; Khwaza et al. 2018).

Oleanolic acid (**1**) (3β-hydroxy-olea-12-en-28-oic acid; OA) (**Figure 1**), a pentacyclic triterpenoid, is found in various plant species. This compound as well as its derivatives have been reported to possess a wide range of biological properties, such antibacterial (Chouab et al. 2015; Hichri et al. 2003), antiviral (Zhu et al. 2001; Kong et al. 2013), anticancer (Zhang et al. 2018; Oprean et al. 2016; Chouaïb et al. 2016) and anti-inflammatory activity (Chouaïb et al. 2016; Rali et al. 2016). The mechanism of action with regards to anticancer activity includes inhibition of cell proliferation, nuclear factor-κB activation, mitochondrial dysfunction, apoptosis, angiogenesis, and modulation of MDR genes and proteins (Yan et al. 2014; Ghante et al. 2019).

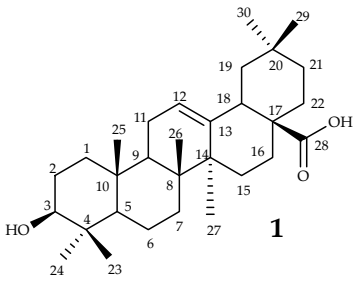

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Figure 1: Chemical structure of oleanolic acid.

Oleanolic acid has three active sites (i.e. C-3 hydroxyl ring A, ring-C double bond and carboxylic acid C-28), where chemical transformations can take place which may increase the compounds' biological activity (Khwaza et al. 2018). The carboxylic acid position C-28 has been used to prepare various types of esters, amides, alcohols, triazoles and nitriles (Cheng et al. 2015). Covalently linking two compounds to form a single compound may increase bioavailability, enhance the pharmacokinetic and pharmacodynamic properties of the compound, overcome drug toxicity, and improve drug uptake (Wang et al. 2016; Kucuksayan et al. 2017; Fortin et al. 2013).

Due to the advantageous characteristics of hybrid molecules, oleanolic acid was hybridized with selected pharmaceutical scaffolds via C-28. The structures of the synthesized hybrid compounds were studied using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR), ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS), Fourier Transform Infrared Spectroscopy (FTIR). *In vitro* cytotoxicity and antibacterial activity were assessed.

2. Materials and methods

2.1. Materials used in the chemical synthesis

All chemicals and reagents used were purchased from Sigma-Aldrich (South Africa) and used without further purification unless and otherwise specified. The solvents used for synthesis were of high-grade and they were dried over molecular sieves with the pore size of 4 Å and particle size of 4-8 Mesh. The identification of specific functional groups was achieved by assigning of group frequencies (cm^{-1}) of particular peaks within the IR spectra. The IR spectra

were obtained using a Perkin Elmer spectrometer (model 100 Hz; UK) and the percentage transmittance was recorded against the wavenumber (cm^{-1}) for the reported signals in the range of 4000-400 cm^{-1}. The ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker Nuclear Magnetic Resonance Spectrometer (400 MHz) at room temperature using deuterated chloroform ($CDCl_3$). The chemical shifts (δ) were reported in ppm relative to the internal solvent peaks and the coupling constants (*J*) were measured in Hertz (Hz). The high-resolution mass spectra were recorded on Bruker Compact Liquid Chromatography Mass Spectrometry (LC-MS).

2.2. Isolation of oleanolic acid and its confirmation

The dried flower buds of *Syszygium aromaticum* (L.) Merr. & Perry (Family Myrtaceae) were purchased from the spice market in Durban, South Africa. The plant was authenticated by Mr Pravin Poorun, a senior plant taxonomist and a botanist from the School of Biological and Conservation Sciences (University of KwaZulu-Natal, Westville). A voucher specimen (OO4) is deposited at the University Herbarium.

Oleanolic acid was isolated from the flower buds of *Syzygium aromaticum* using a standard protocol validated as previously reported (Nkeh-chungag et al. 2015). Briefly, 500 g of the dried, powdered sample was sequentially extracted with 2000 mL organic solvents of different increasing polarity, namely; n-hexane, ethyl acetate, dichloromethane and methanol. The collected mixture was placed on a rotating shaker for a period of approximately two weeks. The supernatant was filtered (Whatman), and concentrated using a rotary evaporator at reduced pressure. The concentrated extracts were air dried in a fume hood. The ethyl acetate crude extract containing mixtures of eugenol (EU), OA and maslinic acid (MA) was purified by column chromatography using silica gel (Merck, silica gel 60 F_{254}: 0.063-0.200 mm) with hexane:ethyl acetate solvent systems, 7:3 for OA and 6:4 for MA to afford EU (0.42%), OA (0.17%) and MA (0.04%), respectively which were further purified by recrystallization from methanol.

The obtained compounds were confirmed by spectroscopic analysis using mass spectrometry (MS), melting point (m.p.), FT-IR, and ^{1}H- and ^{13}C-NMR. Isolated OA ($C_{30}H_{48}O_3$), was obtained as a white amorphous powder (7.68 g; 17.3%) with; R_f 0.45, Mz+ 456, m.p. 240 °C. IR (ν_{max} cm^{-1}): 3443 (-OH), 2941 - 2870 (aliphatic -CH), 1693 (-C=O), 1468 (-C=C), 1031-998 (-C-O), respectively. ^{1}H-NMR (400 MHz, $CDCl_3$): showed signals of an olefinic proton

H-12 at 5.35 cm^{-1} , carbonyl proton H-3 at 2.19 and 7 methyl groups at 0.87, 0.78, 0.91, 0.81, 1.21, 0.89 and 0.89 ppm which confirmed the characteristics of the oleanane skeleton (Hossain and Zhari 2013) [38]. ^{13}C-NMR (C1–C30, ppm): 33.64, 27.48, 79.03, 41.25, 55.24, 22.97, 35.3, 39.9, 49.8, 37.03, 25.88, 123.07, 143.22, 41.25, 29.71, 27.48, 55.24, 41.78, 47.64, 30.67, 39.43, 31.45, 18.43, 18.32, 23.46, 17.18, 23.63, 172.93, 27.19, and 27.48.

*2.3. Synthesis of hybrid compounds **2-4***

2.3.1. Hybrid compound (***2***)

Oleanolic acid (200 mg, 0.40 mmol) and ergocalciferol (162.09 mg, 0.44 mmol) were dissolved in 5 mL dichloromethane (DCM). 4-Dimethylaminopyridine (DMAP) (48.87 mg, 0.40 mmol) and N.N'-dicyclohexylcarbodiimide (DCC) (90.78 mg, 0.40 mmol) were added in portions. The resultant mixture was stirred at room temperature for 10 min, after which it was heated to reflux at 120 ^{o}C for 24 h. The reaction was monitored by thin layer chromatography (TLC) using hexane: EtOAc (7:3) to monitor the completion of the reaction. Thereafter the solution was diluted with DCM (20 mL x 3) and the mixture was washed with 30 mL of ice water to afford an organic and aqueous layer. The organic layer was collected and dried over anhydrous sodium sulphate, filtered and concentrated on the rotary evaporator to obtain white crystals. The crude compound was purified by column chromatography using silica gel (Merck, silica gel 60 F_{254}: 0.063-0.200 mm) (**Figure 2**). The column was eluted with hexane: EtOAc (7:3) to afford white crystals (Yield 77%, m.p 120-122 ^{o}C, R_f value 0.64). IR ν_{max} (cm^{-1}): 3553-3462 (OH), 2930-2854 (CH aliphatic), 1709 (C=O), 1626 (C=C alkene) 1488 (C=C aromatic), 1214 (C-O ester). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 6.22-6.20 (d, *J*=8.0 Hz, 1H, H-41), 5.86-5.84 (d, *J*= 8.0 Hz, 1H, H-42), 5.44-5.42 (d, *J*=8.0 Hz, 1H, H-53), 5.24 (t, *J*=4.0 Hz, 1H, H-13), 4.94 (s, 2H, H-40), 4.32 (m, 1H, H-35), 3.16-3.12 (dd, J=4.0 Hz. 1H, H-2), 2.78-2.73 (dd, J=4.0 Hz, 1H, H-18), 2.19(s, 1H, H-33). ^{13}C-NMR (400 MHz, $CDCl_3$): δ (ppm) 172.92 (C27), 145.12 (C38), 144.20 (C43), 143.92 (C12), 141.17 (C39), 134.99 (C54), 130.25 (C53), 126.42 (C41), 124.05 (C42), 123.37 (C13), 111.12 (C40), 79.02 (C2), 74.75 (C35). MS (ESI^{+}) 834.69 calculated for $C_{58}H_{90}O_3$ $[M+H]^{+}$, found 834.69.

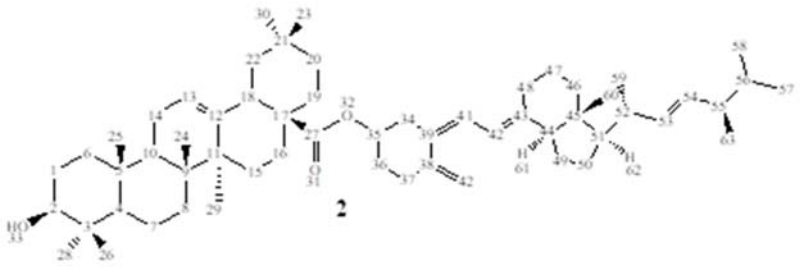


Figure 2: Structure of compound **2**.

*2.3.2. Hybrid compound (**3**)*

Oleanolic acid (200 mg, 0.4 mmol) and curcumin (162.09 mg, 0.44 mmol) was dissolved in 5 mL of DMSO. DMAP (48.87 mg, 0.4 mmol) and DCC (90.78 mg, 0.4 mmol) were added in portions and the resultant mixture was stirred at room temperature for 10 min after which the mixture was heated to reflux at 120 ^{o}C for 24 h. The reaction was monitored by TLC to ensure the completion of the reaction, after which the obtained mixture was diluted with DCM (20 mL) and the mixture was washed with 30 mL of ice cold water to afford an organic and aqueous layer. The organic layer was collected and dried over anhydrous sodium sulphate, filtered and concentrated on the rotary evaporator to obtain a light brown powder. The crude compound was purified by column chromatography using silica gel (Merck, silica gel 60 F_{254}: 0.063-0.200 mm). The column was eluted with EtOAc:MeOH (9:1) to afford a pale yellow powder (Yield 53%, m.p 132–133 ^{o}C, R_f = 0.62) (**Figure 3**). IR ν_{max} 3354 (OH), 2929-2898 (CH aliphatic), 1735 (C=O), 1469 (C=C), 1156- 1031(C-O ester). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 7.34-7.32 (d, *J*=8.0 Hz, 1H, H-46), 6.82-6.80 (d, *J* = 8.0 Hz, 1H, H-53), 6.78 (s, 1H, H-50), 6.72-6.70 (d, J=8.40 Hz, 1H, H-54), 6.65-6.63(d, J=8.0 Hz, 1H, H-46), 5.24(t, J=4.0 Hz, 1H, H-13), 4.99(s. 1H, H-57), 4.65(s, 1H, H-43), 3.45(s, 6H, H-56 & 59), 3.16-3.12(dd, J=4.0, 1H, H-2), 2.83-2.79 (dd, J=4.0 Hz, 1H, H-18). ^{13}C-NMR (400 MHz, $CDCl_3$): δ (ppm) 196.14 (C44), 196.13 (C42), 177.63 (C27), 158.34 (C51), 152.02 (C36), 143.58 (C12), 142.25 (C46), 141.51 (C40), 139.04 (C52), 133.52 (C47), 129.25 (C38), 127.61 (C45), 127.60 (C41), 122.52(C13), 114.13 (C34), 113.24(C37), 113.23(C50), 79.02(C2), 67.96(C59), 58.47(C56). MS (ESI^{+}) 806.48 calculated for $C_{51}H_{66}O_8$ $[M+H]^{+}$, found 801.14.

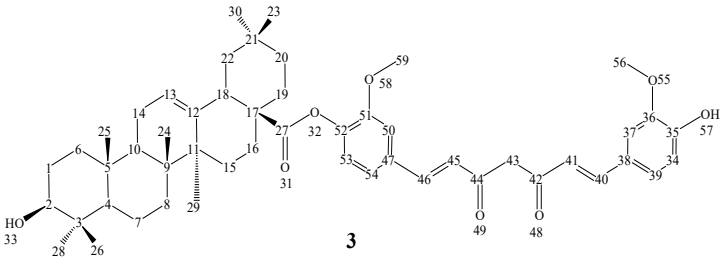
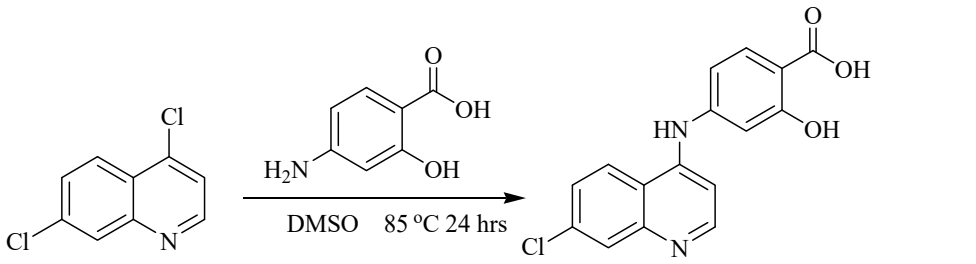


Figure 3: Structure of compound **3.**

*2.3.3. Hybrid compound (**4**)*

Hybrid compound **4** (**Figure 4**) was prepared by the synthesis of 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid as shown in **Scheme 1**, followed by esterification with oleanolic acid.

2.3.3.1 Synthesis of 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid



4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid

Scheme 1: Synthesis of 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid.

4-Aminosalicylic acid (70 mg, 0.45 mmol) was dissolved in 5 mL DMSO, after which 4.7-dichloroquinoline (100 mg, 0.45 mmol) was added. The reaction was allowed to stir for approximately 10 min until the solute was completely dissolved. This was followed by the addition of DMAP (55 mg, 0.45 mmol) and the reaction was allowed to stir for 10 min, followed by the addition of DCC (103 mg, 0.50 mmol) in portions over a time interval of 5 min. The reaction was allowed to stir overnight at 85°C and was monitored by TLC using 6:4 toluene/ethyl acetate, and R_f = 0.24. The obtained product was extracted thrice with 20 mL

DCM and 20 mL cold distilled water. The organic layer was dried over anhydrous sodium sulphate, filtered and then concentrated on the roti-evaporator. A viscous liquid was obtained which was further purified by column chromatography (6:4:1 Toluene/Ethyl acetate/Methanol). (0.108 g), Yield: (68%). FT-IR (cm^{-1}): 3382 (N−H), 2981 (C−H), 15621 (C=C aromatic), 1695 (C=O), 1288 (N−H bending) and 1176 (C−O) (Jama et al. 2020).

Oleanolic acid (200 mg, 0.4 mmol) and 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid (162.09 mg, 0.44 mmol) were dissolved in 5 mL of DMSO. DMAP (48.87 mg, 0.4 mmol) and DCC (90.78 mg, 0.4 mmol) was added in portions and the resultant mixture was stirred at room temperature for 10 min before been heated to reflux at 120ºC for 24 h. The reaction was monitored by TLC to ensure the completion of the reaction, after which the obtained solution was diluted with DCM (20 mL) and the mixture was washed with 30 mL of ice cold water to afford an organic and aqueous layer. The organic layer was collected and dried over anhydrous sodium sulfate, filtered and concentrated on the rotary evaporator to obtain a light brown powder. The crude compound was purified by column chromatography using silica gel (Merck, silica gel 60 F_{254}: 0.063-0.200 mm). The column was eluted with hexane: EtOAc (7:3) to afford a white powder (Yield 57%, m.p 134–135 ºC, R*f* value 0.25) (**Scheme 2**). IR ν_{max} 3535 (NH), 3391 (OH), 2932-2855 (CH aliphatic), 1704 (C=O), 1626 (C=C alkene), 1451 (C=C aromatic), 1274 (C-N), 628 (C-Cl). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.96-7.94 (d, *J*=8.0 Hz, 1H, H-39), 7.80 (s, 1H, H-34), 7.56-7.54 (d, *J*=8.40 Hz, 1H, H-48), 7.43-7.41 (d, *J*=8.0, 1H, H-37), 7.34- 7.32 (d, *J*=8.0 Hz, H-32), 5.74 (s, 1H, H-45), 5.64-5.63 (d, *J*=4.0 Hz, 1H, H- 40 & 49), 5.28 (t, *J*=4.0 Hz, 1H, H-13), 3.40 (s, 1H, H-43), 3.17-3.13 (dd, J=4.0 Hz, 1H, H-13), 2.48-2.44(dd, J=4.0 Hz, 1H, H-18), 2.10 (s, 1H, H-31). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 176.34 (C26), 171.44 (C51), 157.22 (C46), 151.74 (C39), 149.21 (C41), 148.99 (C44), 148.58 (C35), 144.76 (C12), 136.42 (C33), 128.61 (C48), 127.42 (C34), 122.50 (C13), 118.40 (C37), 112.72 (C40), 112.71 (C49), 111.23 (C47), 10 5.40 (C45), 78.96 (C2). MS (ESI^+) 752.4 calculated for $C_{46}H_{57}ClN_2O_5$ $[M+H]^+$, found 755.08.

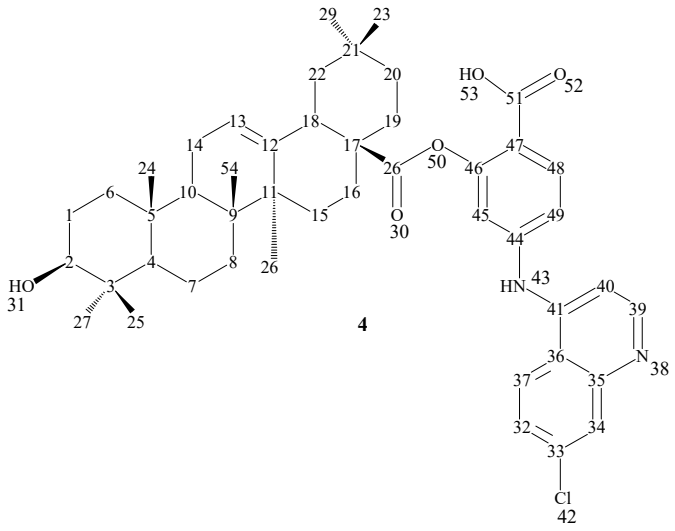
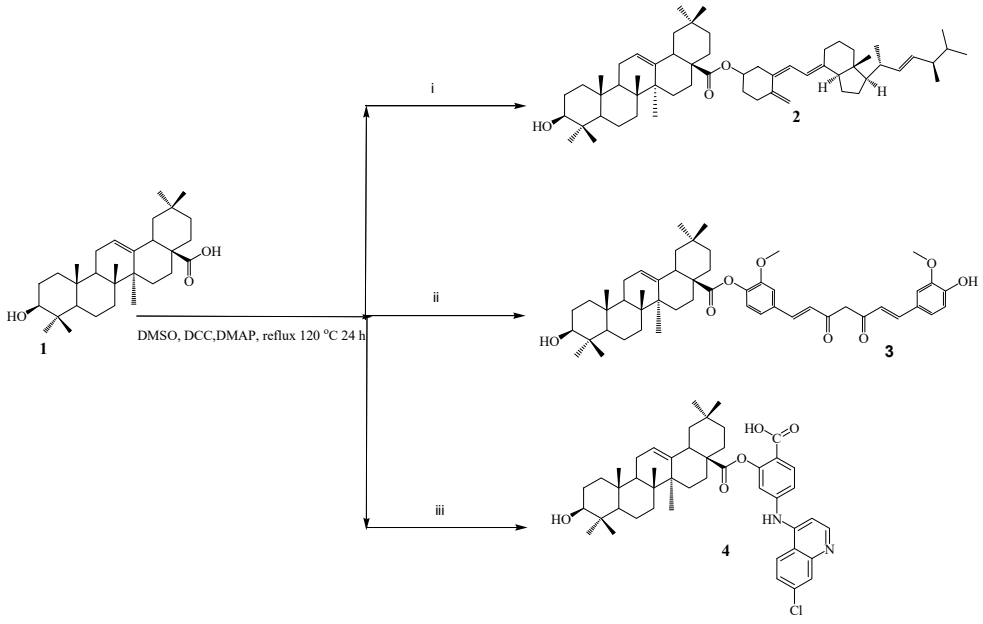


Figure 4: Structure of compound **4**.



Scheme 2. Reagents and conditions: (i) ergocalciferol (ii) curcumin, and (iii) 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid.

2.7. *In vitro* studies

2.7.1. Antimicrobial activity

The synthesized hybrid compounds were tested against 11 reference bacterial strains, which included Gram-positive bacteria: *Bacillus subtilis* (ATCC19659), *Enterococcus faecalis* (ATCC13047), *Staphylococcus epidermidis* (ATCC14990), *Staphylococcus aureus* (ATCC25923), and Gram-negative bacteria: *Enterobacter cloacae* (ATCC13047), *Proteus vulgaris* (ATCC6380), *Klebsiella oxytoca* (ATCC8724), *Proteus vulgaris* (ATCC6380), *Pseudomonas aeruginosa* (ATCC27853), *Proteus mirabilis* (ATCC7002) and *Escherischia coli* (ATCC25922) as well as the mycobacterium, *Mycobaterium smegmatis* (MC2155).

The minimum inhibitory concentration (MIC) of the compounds was determined using the method of Fonkui (2018). Stock solutions were prepared by adding 4 mL of DMSO to 20 mg of the synthesized compounds. These solutions were then serially diluted in nutrient broth to the working concentrations (2.5, 1.25, 0.625, 0.3125, 0.1562, 0.0781 mg/mL). To the wells of a 96-well plate was added, 100 µL working solution and 100 µL of a bacterial culture (0.5 O.D. McFarland standard in nutrient broth). Plates were incubated for 24 h at 37°C. Streptomycin, OA and nalidixic acid were used as positive control. Experiments were conducted in triplicate on three occasions.

2.7.2. Cytotoxicity

Cytotoxicity was measured by cell density determination using the sulforhodamine B (SRB) staining assay on four different cell lines as described by (Vichai et al. 2006) with minor modifications. DU 145 (ATCC® HTB-81™), MDA-MB-231 (ATCC® HTB-26™), MCF-7 (ATCC® HTB-22™) and MCF-12A (ATCC® CRL-10782™) cells were used in experiments. DU 145, MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% non-essential amino acids, 1% L-glutamine, penicillin (100 U/mL), streptomycin (100 U/mL) and 10% heat-inactivated foetal calf serum (FCS). MCF-12A cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/mL human epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin and 500 ng/mL hydrocortisone, 10% heat-inactivated foetal calf

serum. All cell cultures were incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere until confluent. Confluent cells (80%) were washed with sterile phosphate-buffered saline (PBS) and chemically detached with TrypLE™Express dissociation solution. Cells were harvested and centrifuged at 200 g for 5 min and the pellet re-suspended in 1 mL supplemented medium. Cells were counted using the trypan blue exclusion assay (0.1% w/v) and re-suspended to a cell concentration of 5 x 10^4 cells/mL. Cells (100 µL) were seeded into sterile, clear 96-well plates, and incubated overnight to allow for attachment. Blank wells contained 200 µL FCS (10%)-supplemented media without cells to account for background noise and sterility. Attached cells were exposed to 100 µL medium (negative control), experimental compounds (0.001-200 µM) or saponin (1%; positive control) prepared in 10 % FCS supplemented medium for 72 h. Cells were fixed using 50 µL trichloroacetic acid (50%) overnight at 4°C. Fixed cells were washed three times with tap water, air dried and stained using 100 µL SRB solution (0.057% in 1% acetic acid) for 30 min. Stained cells were washed four times with 150 µL acetic acid (1%) and air-dried. The bound dye was eluted using 200 µL Tris-buffer (10 mM, pH 10.5) and the absorbance measured a 510 nm (reference 630 nm) using an EL-X 800 microplate reader (Biotek Inc, USA). Compounds were tested in triplicate on three occasions. All values were adjusted by subtracting the blank and the cell density relative to the negative control expressed as a percentage. IC_{50} values were calculated with the use of Graphpad software.

3. Results and discussion

The compounds were prepared by esterification reaction of OA and pharmaceutical scaffolds such as ergocalciferol, curcumin and 4-aminosalicylic acid which resulted in the isolation of compounds **2**, **3** and **4**, respectively. The compounds were isolated in yields which ranged between 53 and 77%. Compound **4** was prepared by amination of 4,7-dichloroquinoline with 4-aminosalicylic acid, which resulted in the formation of 4-(7-chloroquinolin-4-ylamino)-2-hydroxybenzoic acid, followed by esterification reaction with OA. The molecular masses of the compounds were confirmed by UHPLC-HRMS. The molecular mass of compounds **2**, **3** and **4** was 834.69, 801.14 and 755.08 g/mol, respectively. The melting points of the compounds were in the range of 120-135°C. The compounds were characterized by the presence of an ester linker. Oleanolic acid with ester linker between OA and zidovudine has been reported by Thi and Tuyet (2014). Ester linkers are cleavable at physiological pH, which makes them useful

in hybrid molecules (Jornada et al. 2016; Pawełczyk et al. 2018). Esters contain carbonyl (C=O) and ether (O-C) dipoles due to the covalent bond between the electronegative oxygen atoms and the electronically neutral carbon atoms. The presence of these dipoles make esters good hydrogen-bond acceptors, which enables them to participate in hydrogen bonding with water or protic solvents, resulting in their enhanced water solubility (DeRuiter J 2005). ^{13}C-NMR spectra revealed ester carbons at 172.93, 177.63 and 176.34 ppm, for hybrid compounds **2**, **3** and **4**, respectively. Furthermore, the C-O stretch of the ester for the compounds was noted in the range of 1031-1214 cm^{-1} confirming the successful synthesis of the compounds.

Antibacterial activity of the compounds was evaluated against selected Gram-positive and Gram-negative strains of bacteria (**Table 1**). The compounds all inhibited growth of the bacteria. Compound **4** inhibited the growth of Gram-positive bacteria; *S. aureus* (0.078 mg/mL) and Gram-negative bacteria; *P. vulgaris* (0.078 mg/mL), at a lower concentration than OA.

Table 1: Antibacterial activities of synthesized compounds.

Minimum inhibitory concentration (MIC) (mg/mL)											
Test compounds	***Gram-positive***					***Gram-negative***					
	BS	***EF***	***SE***	***SA***	***MS***	***ECL***	***PV***	***KO***	***PA***	***PM***	***EC***
2	2.5	1.25	1.25	2.5	1.25	2.5	2.5	1.25	2.5	1.25	1.25
3	2.5	1.25	1.25	2.5	1.25	1.25	2.5	1.25	1.25	1.25	2.5
4	1.25	2.5	2.5	0.078	1.25	1.25	0.078	1.25	1.25	2.5	1.25
OA	2.5	2.5	1.25	2.5	1.25	1.25	2.5	2.5	0.078	1.25	2.5
STM (μg/mL)	***16***	***128***	***8***	***256***	***4***	***512***	***128***	***16***	***16***	***128***	***64***
NLD (μg/mL)	***16***	***>512***	***64***	***64***	***512***	***16***	***128***	***8***	***256***	***32***	***512***

STM: *Streptomycin*, **NLD:** *Nalidixic acid*, **OA:** Oleanolic acid, ***BS:*** *Bacillus subtilis*, ***EF:*** *Enterococcus faecalis*, ***SE:*** *Staphylococcus epidermidis*, ***SA:*** *Staphylococcus aureus*, ***MS:*** *Mycobacterium smegmatis*, ***ECL:*** *Enterobacter cloacae*, ***PV:*** *Proteus vulgaris*, ***KO:*** *Klebsiella oxytoca*, ***PA:*** *Pseudomonas aeruginosa*, ***MS:*** *Mycobaterium smegmatis*, ***EC:*** *Escherichia coli, and* **PM:** *Proteus mirabilis*

The selective antibacterial activity of the compounds **2, 3** and **4** is attributed to the synergistic effect of hybridizing the selected drugs with OA. Hybridizing two antibacterial compounds can result in a compound with improved antibacterial activity due to synergistic activity (Shavit et al. 2017; Domalaon et al. 2017). Hybridizing oleanolic acid with 4-aminosalicylic acid resulted in compound **4** with enhanced antibacterial activity against certain strains of bacteria. 4-Aminosalicylic acid has been reported to inhibit *E. coli* growth (Saifullah et al. 2014). Furthermore, the latter compound has been reported to inhibit *Mycobacterium tuberculosis* growth via inhibition of dihydropteroate synthase (DHPS) in the folate biosynthetic pathway. The folate biosynthetic pathway generates tetrahydrofolate in prokaryotes and eukaryotes, thereby initiating protein synthesis in bacteria. Inhibition of dihydrofolate reductase (DHFR) is the target of several important anticancer and antibacterial drugs (Zheng et al. 2013).

The antibacterial activity of compound **2** indicated the efficacy of hybridizing ergocalciferol with antibacterial agents. Antibacterial activity of vitamin D-based compounds against Gram-positive and Gram-negative strains of bacteria is well known (Hewison 2011; Hosoda et al 2015). It has also been noted that the combination of vitamin D with antibiotics results in sustained antibiotic activity in efflux-associated resistant strains of *S. aureus* (Tintino et al. 2016).

The reduced ability of compound **3** to inhibit bacterial growth compared to OA may result in antagonistic activity when hybridizing curcumin with OA. Although curcumin has been reported to exhibit good antibacterial activity against Gram-positive and Gram-negative strains of bacteria, derivatives of curcumin prepared by condensation with substituted aromatic aldehydes, and urea/thiourea were found to be inactive against *S. aureus, E. coli* and *P. aeuriginosa* (Khaldi-Khellafi 2019).

Furthermore, some synthesized oleanolic acid-based hybrids have demonstrated good antibacterial activity when compared to OA (Blanco-Cabra et al. 2019; Gu et al. 2015). Some of the derivatives exhibited synergistic activity when combined with antibiotics (Do Nascimento et al. 2014).

In vitro cytotoxicity evaluation revealed that compound **4** and OA were not cytotoxic to the cancer cell lines at the highest concentration tested (**Table 2**). Compounds **2** displayed moderate cytotoxic effect against DU145 cell line, indicating cell line specific cytotoxicity. Vitamin D has been reported to exhibit anticancer activity by inducing cell cycle arrest and apoptosis, suppressing pro-proliferative signalling molecules, and inhibiting angiogenesis (Donald and Jeanny 2018). Combining Vitamin D with selected anticancer drugs has been reported to result in synergistic or additive activity (Beer et al. 2007; Schwartz et al. 2009).

Table 2: *In vitro* cytotoxicity expressed as IC_{50} values (µg/mL).

Compounds	**IC_{50} (µg/mL)**			
	MDA	**MCF-7**	**MCF-12A**	**DU145**
2	> 200	> 200	> 200	100.7±1.1
3	69.87±1.04	85.27±1.02	91.95±1.1	73.2±1.08
4	> 200	> 200	> 200	> 200
Oleanolic acid	> 200	> 200	> 200	> 200

Compound **3** was noted to induce cytotoxicity in a narrow concentration range against all the cell lines against which activity was tested. The anticancer activity of compound **3** may be attributed to the presence of its curcumin scaffold. Curcumin has been reported to exhibit additive cytotoxic activity when combined with anticancer drugs (Nagy et al. 2015; Allegra et al. 2017). The inhibition of cell growth and increased occurrence of apoptosis in breast cancer cells induced by curcumin and its derivatives is attributed to their uptake by the cancer cells (Chang et al. 2012). Curcumin has been reported to inhibit the proliferation of both androgen-dependent and androgen-independent prostate cancer cell lines (Shehza et al. 2010), as well as DU145 prostate cancer cells (Yang et al. 2013). Derivatives of OA have also been reported to exhibit anticancer activity *in vitro* in cervical cancer cells via apoptosis and the production of reactive oxygen species (Song et al. 2015).

4. Conclusion

The synthesis of compound **4**, an oleanolic acid-based hybrid compound, with enhanced antibacterial against selected strains of bacteria, compared to the parent compound, OA, proved successful. Furthermore, hybrid compound **3** displayed cytotoxic activity against all of the cancer cell lines. These findings therefore indicate that these hybrid compounds have the potential to be developed further.

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