RESEARCH ARTICLE

Hybrid Compounds Containing Carvacrol Scaffold: *In Vitro* Antibacterial and Cytotoxicity Evaluation

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Abstract: ***Background*:** The design of hybrid compounds is a distinct approach for developing potent bioactive agents. Carvacrol, an essential oil, exhibits antimicrobial, antifungal, antioxidant, and anticancer activity, making it a good precursor for the development of compounds with potent biological activities. Some patents have reported carvacrol derivatives with promising biological activities.

***Objective*:** This study aimed to prepare hybrid compounds containing a carvacrol scaffold with significant antibacterial and anticancer activity.

***Methods*:** Esterification reactions between carvacrol and known pharmacophores were performed at room temperature and characterized using ^{1}H-NMR, ^{13}C-NMR, and UHPLC-HRMS. *In vitro* antibacterial study was determined using the microdilution assay and cytotoxicity evaluation using sulforhodamine B staining assay.

***Results*:** The FTIR spectra of the carvacrol hybrids revealed prominent bands in the range of 1612-1764 cm^{-1} and 1014-1280 cm^{-1} due to (C=O) and (C-O) stretching vibrations, respectively. The structures of the carvacrol hybrids were confirmed by ^{1}H-NMR, ^{13}C-NMR, and UHPLC-HRMS analysis, and compound **5** exhibited superior activity when compared to the hybrid compounds against the strains of bacteria used in the study. The *in vitro* cytotoxicity evaluation showed that compound **3** induced cytotoxicity in all the cancer cell lines; MDA (16.57 ± 1.14 μM), MCF-7 (0.47 ± 1.14 μM), and DU145 (16.25 ± 1.08 μM), as well as the normal breast cells, MCF-12A (0.75± 1.30 μM). Compound **7** did not induce cytotoxicity in the cell lines tested (IC_{50} > 200 μM).

***Conclusion*:** The modification of carvacrol through hybridization is a promising approach to develop compounds with significant antibacterial and anticancer activity.

Keywords: Gram-positive bacteria, Gram-negative bacteria, antibacterial, carvacrol, hybrid compounds, anticancer.

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1. INTRODUCTION

The World Health Organization (WHO) lists microbes as one of the ten most common causes of death globally [1]. Antimicrobial resistance results in at least 700,000 deaths each year. In 2017, over 3 million cases of antibiotic-resistant infections were reported in the USA, with 48,000 deaths. The global death toll is expected to increase drastically by 2050 [2]. Due to the pressing need to develop effective antibacterial agents, researchers have adopted different approaches. One such approach is the combination of two drugs to form a single hybrid compound while maintaining the properties of the individual drugs, thus allowing for the possibility of synthesizing a compound with increased therapeutic efficacy. These compounds should be developed with the focus being to ensure targeted delivery, enhance pharmacodynamic and pharmacokinetic properties, enhance drug uptake, and reduce toxicity [3]. Hybridized compounds have the potential to overcome drug resistance by being effective in cases where a single compound displays inactivity [3].

Carvacrol, a terpenoid compound, is an essential oil that is found in perennial herbs such as oregano and thyme. It is reported to exhibit antimicrobial, anti-inflammatory, antifungal, antioxidant, and anticancer activity [4-7]. The antibacterial activity of carvacrol is well known [8, 9]. The increase in drug-resistant strains of microorganisms has necessitated the development of novel antibacterial compounds that overcome these resistance mechanisms [10]. Furthermore, the derivatives of carvacrol have been reported to possess anticancer activity against selected cancer cell lines *in vitro* [11-14]. There are some patent reports on carvacrol and its biological activity [15, 16].

Due to the efficacy of carvacrol-based hybrid compounds, they were synthesized containing ester linkers. The compounds were characterized using FTIR, UHPLC-HRMS, ^{1}H- and ^{13}C-NMR. *In vitro* antibacterial and cytotoxicity analyses were carried out to determine the biological activity and safety of the synthesized compounds.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Chemicals and reagents were purchased from Sigma-Aldrich (Johannesburg, South Africa). The solvents were of the highest grade and were dried over molecular sieves with a pore size of 4 Å and particle size of 4-8 Mesh. The reactions were monitored using thin-layer chromatography (TLC; silica gel 60 F254), which was procured from Merck Chemicals (Johannesburg, South Africa). A Perkin Elmer Spectrum Fourier Transform Infrared Spectroscopy (FTIR) spectrometer was used to determine the functional groups present in the carvacrol-based hybrid compounds. ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) spectra were recorded on a Bruker ultra-shield 400 NMR spectrometer using $CDCl_3$ as a solvent at a temperature of 303 K functioning at frequencies of 400 MHz for ^{1}H- and ^{13}C-NMR. The mass spectra were obtained using Ultra High- Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) spectrometer (Kyoto, Japan). The TLC plates were viewed at 254 nm with a UV lamp (MiniMAXTM UV lamp, LASEC, South Africa).

2.2. Synthesis of Hybrid Compounds

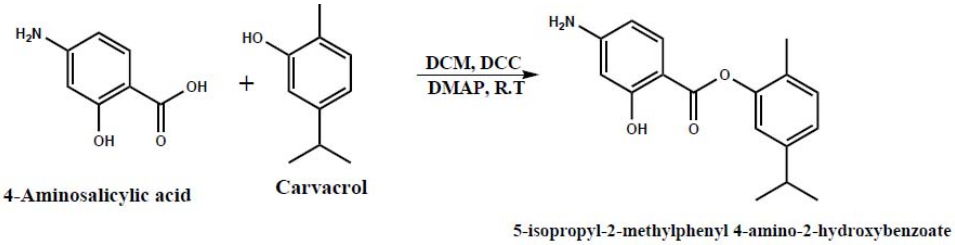
2.2.1. 5-Isopropyl-2-methyl phenyl 4-amino-2-hydroxybenzoate (Compound 1)

Compound **1** (Scheme **1**) was prepared by dissolving 4-aminosalicylic acid (1.02 g, 6.66 mmol) and carvacrol (1.00 g, 6.66 mmol) in 10 mL of dried dichloromethane (DCM). The resultant solution was stirred at room temperature, and N,N′-dicyclohexylcarbodiimide (DCC; 1.40 g, 1.46 mmol), dissolved in 10 mL of dimethylformamide (DMF), was added drop-wise, and the reaction mixture was stirred for 10 min in an ice-cold bath. 4-Dimethylaminopyridine (DMAP) (0.89 g, 6.66 mmol) was added, the resultant mixture was stirred at room temperature overnight, and the progress of the reaction was monitored by TLC using hexane/ethyl acetate (7:3; Rf = 0.5). A white precipitate of dicyclohexylurea (DCU), a by-product formed, was washed in cold ethyl acetate and filtered off. The conventional work-up process was performed by adding 10 mL of 1.13 M citric

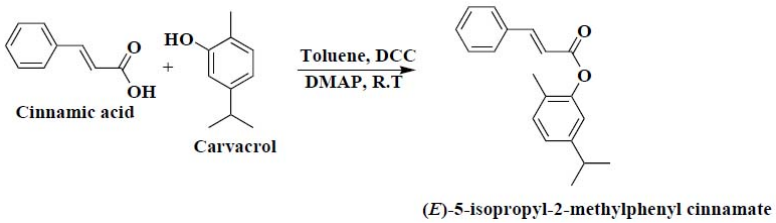
acid to the filtrate followed by the addition of 10 mL of sodium bicarbonate ($NaHCO_3$) and extraction with 20 mL of DCM. The organic layer was collected and dried over anhydrous magnesium sulphate. The organic solution was concentrated on a roti-evaporator followed by column chromatography to obtain yellow solids (compound **1**) (Yield: 1.7 g, 84 %), melting point: 70-74°C. HRMS (ESI): $C_{17}H_{19}NO_3$ [M+H]+: calculated 285.1365; measured 286.1449. Compound **1**, selected IR (ATR, cm^{-1}) vmax: 3400 (N-H), 3100 (=C-H), 2967 (-C-H), 1680 (C=O), 1575 (Ar-H) and 1153-1200 (C-O). ^{1}H- NMR (400 MHz, $CDCl_3$, ppm): 7.89-7.87 (d, J=8 Hz, ^{1}H), 7.20-7.18 (d, J=8 Hz, ^{1}H), 7.08-7.06 (d, J=8 Hz, ^{1}H), 6.97 (s, ^{1}H), 6.60-6.50 (m, 2H), 2.96-2.85 (m, ^{1}H), 2.18 (s, 3H), 1.26-1.24 (d, J=8 Hz, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 168.70, 164.34, 154.10, 149.01, 148.32, 132.26, 131.09, 127.69, 124.44, 120.10, 107.23, 102.43, 100.88, 33.72, 24.06, 15.94.

2.2.2. Carvacryl Cinnamate (Compound 2)

Compound **2** was prepared by dissolving cinnamic acid (0.19 g, 1.33 mmol) and carvacrol (0.10 g, 0.66 mmol) in 10 mL of dried toluene (Scheme **2**). An aliquot of EDC.HCl (0.28 g, 1.46 mmol) was added, and the mixture was stirred in an ice-cold bath for 15 min, followed by the addition of DMAP (0.19 g, 0.66 mmol). The reaction mixture was stirred overnight at room temperature, and the progress of the reaction was monitored by TLC using hexane/ethyl acetate (7:3, Rf = 0.7). A white precipitate of DCU was formed, which was washed in cold ethyl acetate and filtered off and the conventional work-up process was performed. The organic layer was collected and dried over anhydrous magnesium sulphate. The solvent was removed under reduced pressure on a roti-evaporator to afford a yellow viscous liquid. Yield: (0.22 g, 75 %), HRMS (ESI): $C_{19}H_{20}O_2$ [M+H]+: calculated 280.1463; measured 281.1549. The structure of the compound was confirmed by IR (ATR, cm^{-1}) vmax: 3000 (=C-H) 2900-2800 (C-H), 1720 (C=O), 1697 (Ar-H), 1200-1148 (C-O). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.19-7.17 (d, J=8 Hz, 2H), 7.06-7.04 (d, J=8 Hz, 2H), 6.95 (s, 2H), 6.69-6.67 (d, J=8 Hz, 2H), 2.95-2.85 (m, ^{1}H), 2.19 (s, 3H), 1.24-1.26 (d, J=8 Hz, 6 H). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 165.24, 149.32, 148.10, 146.45, 134.25, 130.91, 129.01, 128.31, 127.37, 124.17, 119.98, 117.27, 29.72, 21.94, 15.87.



Scheme 1. Synthesis of 5-Isopropyl-2-methylphenyl 4-amino-2-hydroxybenzoate (**1**).



Scheme 2. Synthesis of Carvacryl cinnamate (**2**).

2.2.3. Carvacryl Artesunate (Compound 3)

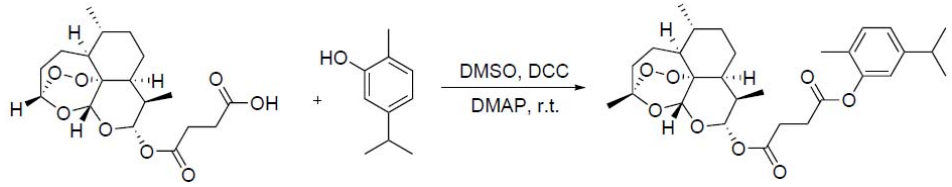
Compound **3** was prepared according to Scheme **3**. Artesunate (0.13 g, 1.67 mmol) was dissolved in 5 mL of dimethyl sulfoxide (DMSO) and the solution was stirred at 30°C in a water bath. Carvacrol (0.50 g, 1.67 mmol) was added, followed by the addition of DCC (0.076 g, 0.37 mmol). After 10 min, DMAP (0.040 g, 1.67 mmol) was added. The reaction mixture was stirred overnight and the progress of the reaction was monitored by TLC using DCM/MeOH (1:1; Rf = 0.3). The DCU that was formed was filtered off followed by the conventional workup. The organic layer was collected and dried over anhydrous magnesium sulphate followed by the removal of the solvent on a roti-evaporator to afford a yellow viscous liquid. Yield: (0.09 g, 60 %), HRMS (ESI): $C_{30}H_{42}O_7$ [M+H]+: calculated 514.2931; measured 515.3350. The structure of the compound was confirmed using selected IR (A-TR, cm^{-1}) vmax: 2947 (C-H), 1710 (C=O), 1546 (Ar-H), 1200-1018 (C-O). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.12-7.10 (d, J = 8.0 Hz, ^{1}H), 7.01-6.99 (d, J = 8.0 Hz, ^{1}H) and 6.67 (s, ^{1}H), 5.60 (s, ^{1}H), 5.40 (s, ^{1}H), 2.80 (m, ^{1}H), 2.60 (m, 2H), 2.40 (m, 2H), 2.20 (s, 3H), 2.1 (s, 3H), 2.0 (m, 3H), 1.9 (m, 3H), 1.80 (m, 5H), 1.24 (d, J= 8Hz, 6 H), 0.90 (d, J= 8 Hz, 3H), 0.85 (d, J= 8Hz, 3H). ^{13}C- NMR (400 MHz, $CDCl_3$, ppm): 172.21, 171.00, 153.94, 130.68, 124.15, 119.70, 118.18, 113.09, 104.49, 87.76, 80.40, 80.12, 52.51, 51.55, 49.98, 45.20, 40.73, 37.27, 36.22, 34.08, 26.18, 25.94, 24.79, 23.89, 21.99, 20.21, 15.47, 12.02.

2.2.4. Carvacryl Zidovudine Succinate (Compound 4)

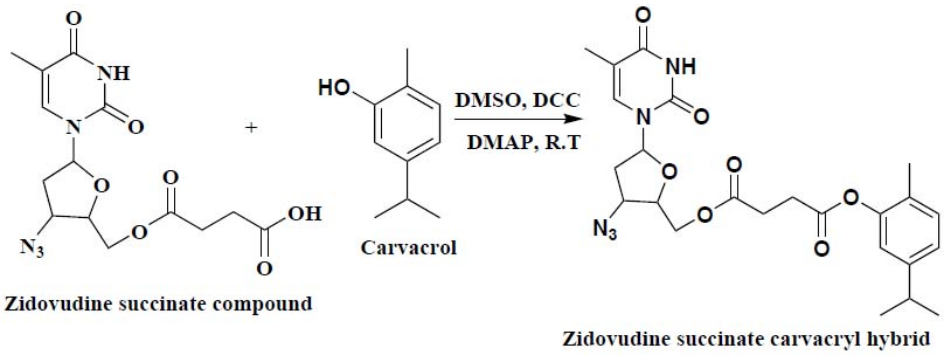
Compound **4** was prepared according to Scheme **4**. Zidovudine succinate (0.24 g, 0.67 mmol) was dissolved in 5 mL of DMSO, followed by the addition of carvacrol (0.10 g, 0.67 mmol). The reaction mixture was stirred at room temperature after which DCC (0.15 g, 0.73 mmol) and DMAP (0.08g, 0.67 mmol) were added. The resultant mixture was stirred at room temperature for 24 h and the progress of the reaction was monitored by TLC using Hexane/TEA/MeOH (6:3:1, Rf = 0.5) to confirm the completion of the reaction. The DCU was filtered and the conventional workup process was performed. The solvent was evaporated and a yellow viscous liquid compound was obtained. Yield: (0.17 g, 50 %), HRMS (ESI): $C_{24}H_{29}N_5O_7$ [M+H]+: calculated 499.2067; measured 500.2134. The structure of the compound was confirmed using selected IR (ATR, cm^{-1}): vmax: 2969 (C-H), 1700 (C=O), 1570 (Ar-H), 1023-1000 (C-O). ^{1}H-NMR (400.1 MHz, $CDCl_3$, ppm): 6.84-6.82 (d, J = 8.0 Hz, ^{1}H), 6.52 (s, 2H), 6.49-6.47 (d, J = 8.0 Hz, ^{1}H), 3.21 (s, 2H), 2.68 (m, ^{1}H), 2.58 (s, 3H), 2.30 (m, 2H), 2.00 (s, 3H), 1.75 (s, 2H), 1.10-1.08 (d, J=8 Hz, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 172.50, 154.89, 147.52, 136.32, 130.11, 121.25, 117.03, 112.18, 110.49, 84.90, 81.80, 63.05, 60.41, 39.02, 36.44, 33.61, 28.62, 23.11, 14.41.

2.2.5. Synthesis of 2-(7-Chloroquinolin-4-ylamino)ethyl 5-isopropyl-2-methyl phenyl Succinate (Compound 5)

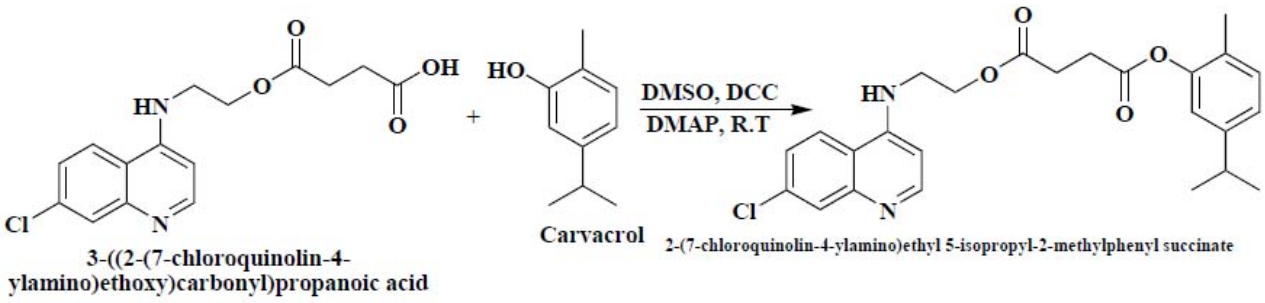
The synthesis of compound **5** is provided in Scheme **5**. The reaction was initiated by dissolving (7-chloroquinolin-4-ylamino) succinate (0.11 g, 0.33 mmol) and 0.50 g (0.33 mmol) of carvacrol in 5 mL of DMSO. The reaction mixture was stirred at room temperature until the complete dissolution of the solutes. The DCC (0.75 g, 0.37 mmol) was dissolved in 5 mL of DMSO and added drop-wise to the reaction mixture, and the



Scheme 3. Synthesis of Carvacryl artesunate (**3**).



Scheme 4. Synthesis of Carvacryl zidovudine succinate (**4**).



Scheme 5. Synthesis of 2-(7-chloroquinolin-4-ylamino)ethyl 5-isopropyl-2-methylphenyl succinate (**5**).

resultant mixture obtained was stirred for 10 min. DMAP (0.40 g, 0.33 mmol) was added, and the reaction mixture was stirred at room temperature overnight and monitored by TLC using hexane/ethyl acetate (7:3; Rf = 0.3) to confirm the completion of the reaction. A white precipitate of DCU was formed, which was washed in cold ethyl acetate and filtered off. The filtrate was washed with 10 mL of ice-cold water and 10 mL of DCM, and the organic layer was dried over anhydrous magnesium sulphate. A brown viscous liquid was obtained after removal of the solvent on a roti-evaporator. Yield: (55 %) HRMS (ESI): $C_{25}H_{27}ClN_2O_4$ [M+H]+: calculated 454.1659; measured 455.3344, Compound **5** selected IR (ATR, cm^{-1}) vmax: 2936 (C-H), 1667 (Ar-H), 1174 (C-O).

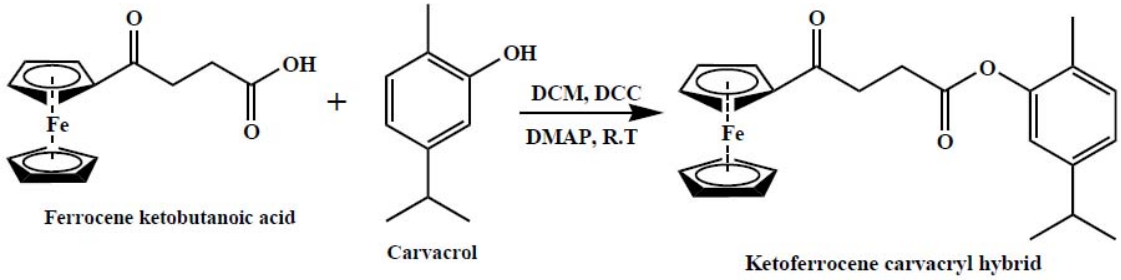
2.2.6. Synthesis of Keto-ferrocene Carvacryl Hybrid (Compound 6)

This compound was prepared as indicated in Scheme **6**. Ferrocene ketobutanoic acid (0.38 g, 1.33 mmol) was dissolved into 5 mL of dried DCM, followed by the addition of carvacrol (0.20 g, 1.33 mmol). DCC (0.30 g, 1.46 mmol) was added to the aforementioned reaction mixture, followed by the addition of DMAP (0.16 g, 1.33 mmol). The reaction mixture was stirred at room temperature for 24 h and monitored by TLC until the reaction was complete using Toluene/Ethyl acetate (7:3; Rf = 0.9). A white precipitate of DCU that formed was washed with cold ethyl acetate and filtered. The filtrate was washed with 10 mL of citric acid and 10 mL of $NaHCO_3$, followed by extraction with 20 mL of DCM. The organic layer was collected and dried over anhydrous magnesium sulphate followed by concentration using a roti-evaporator. A brown viscous oil was obtained, Yield: (0.55 g, 81 %). HRMS (ESI): $C_{26}H_{32}FeO_3$ [M+H]+: calculated 448.1701; measured 449.3841. Selected IR (ATR, cm^{-1}) vmax: 2930 (C-H), 1461 (=C-H), 1146 (C-O), 1676 (C=O). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.08-7.06 (m, ^{1}H), 6.95 (m, ^{1}H), 6.63 (m, I H), 4.70 (s, 2H), 4.40 (s, 2H), 4.20 (s, 5H), 3.12 (t,

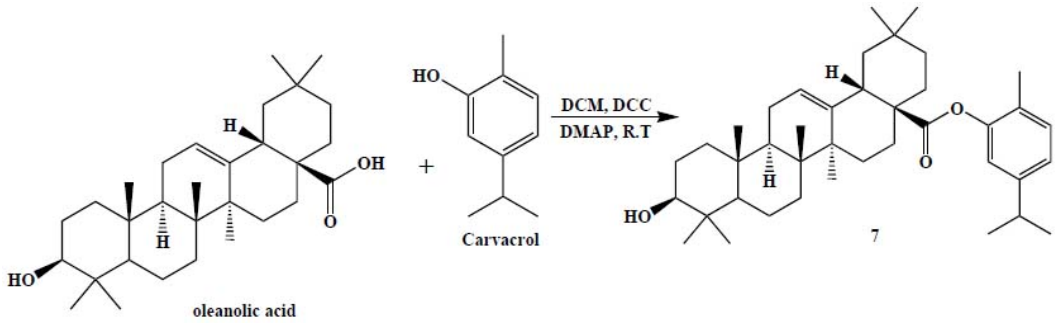
J=4 Hz, 2H), 2.91 (t, J=4 Hz, 2H), 2.14 (s, 3H), 1.24 (d, J=8 Hz, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 201.99, 171.55, 153.79, 130.89, 124.10, 119.84, 118.62, 113.03, 77.29, 69.97, 69.24, 34.20, 33.57, 29.72, 24.03, 15.89.

2.2.7. Synthesis of (4aS,6aS,6bR,10S,12aR,12bR, 14bS)-5-isopropyl-2-methylphenyl 1,2,3,4,4a,5,6, 6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydro-10-hydroxy-2,6a,6b,9,9,12a-hexamethyl-picene-4a-carboxylate (Compound 7)

Compound **7** was prepared according to Scheme **7**. Oleanolic acid (1.00 g, 2.189 mmol) was dissolved in 10 mL of dried DCM followed by the addition of carvacrol (0.33 g 2.189 mmol). DCC (0.49 g, 2.4079 mmol) was added drop-wise for 5 min while stirring. DMAP (0.27 g, 2.189 mmol) was added and the reaction mixture was stirred for 24 h and monitored by TLC until completion using hexane/ethyl acetate (7:3 Rf = 0.25). The DCU formed was filtered and the filtrate was washed with 10 mL of citric acid and 10 mL of $NaHCO_3$. 20 mL of DCM was used for extraction and the organic layer was collected and dried over anhydrous magnesium sulphate. The solvent was removed on a roti-evaporator to obtain a white solid. Yield: ((1.16 g, 88 %), m.p. 244-246°C. HRMS (ESI): $C_{40}H_{60}O_3$ [M+H]+: calculated 588.4542; measured 589.4528. Selected IR (ATR, cm^{-1}) vmax: 2944 (C-H), 1468 (Ar-H), 1047 (C-O). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.17 (s, ^{1}H) and 7.09 (d, J = 8 Hz, 2H), 5.24 (s, IH), 3.20 (m, ^{1}H), 2.77 (d, J=16 Hz, ^{1}H), 2.28 (s, 3H), 1.89 (t, J=8Hz, 2H), 1.75 (m, 4H), 1.50 (m, 6H), 1.25 (m, 6 H)1.19 (s, 3H), 1.07 (s, 6 H), 0.92 (s, 6H), 0.86 (t, J= 8Hz, 6 H), 0.73 (d, J=8 Hz, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 172.93, 143.22, 129.05, 128.24, 125.31, 123.07, 79.03, 55.25, 48.38, 47.64, 41.79, 41.26, 39.44, 38.77, 38.49, 37.03, 33.03, 28.13, 27.48, 27.21, 25.89, 23.64, 23.46, 22.00, 18.10, 17.18, 15.60, 15.29.



Scheme 6. Synthesis of keto-ferrocene carvacryl hybrid (**6**).



Scheme 7. Synthesis of (4aS,6aS,6bR,10S,12aR,12bR,14bS)-5-isopropyl-2-methylphenyl 1,2,3,4,4a,5,6, 6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydro-10-hydroxy-2,6a,6b,9,9,12a-hexamethylpicene-4a-carboxylate (**7**).

2.3. Determination of Antibacterial Activity

Antibacterial activity was assessed against four strains of Gram-positive bacteria: *Bacillus subtilis* (ATCC19659), *Enterococcus faecalis* (ATCC13047), *Staphylococcus epidermidis* (ATCC14990), *Staphylococcus aureus* (ATCC25923), and six Gram-negative bacteria: *Enterobacter cloacae* (ATCC13047), *Proteus vulgaris* (ATCC6380), *Klebsiella oxytoca* (ATCC8724), *Klebsiella aeruginosa* (ATCC27853), *Proteus mirabilis* (ATCC7002), *Escherischia coli* (ATCC25922) as well as a mycobacterium, *Mycobaterium smegmatis* (MC2155). Streptomycin and nalidixic acid were used as positive control against Gram-positive and Gram-negative bacteria strains, respectively. The negative control consisted of 50 % nutrient broth in DMSO. The minimum inhibitory concentration (MIC) of the synthesized compounds was determined using the method reported by Fonkui *et al.* [17]. Stock solutions were prepared by adding 20 mg of the synthesized compounds to 3 mL of DMSO. The stock solutions were diluted in nutrient broth to obtain the desired concentrations (6.66, 3.33, 1.66, 0.83, 0.42, 0.208, 0.104 mg/mL). Thereafter, 100 µL of each of these solutions was pipetted in duplicate into the wells of a 96-well plate. The solution was added to 100 µL of bacteria which was diluted to a 0.5 McFarland standard in nutrient broth, and the plates were incubated overnight at 37°C. The analysis was performed in duplicate for qualitative evaluation.

2.4. Cytotoxicity Determination

Cytotoxicity was measured by cell density determination using the sulforhodamine B (SRB) staining assay on four different cell lines as described by Vichai and Kirtikara with minor modifications [18]. DU 145 (ATCC® HTB-81™), MDA-MB-231 (ATCC® HTB-26™), MCF-7 (ATCC® HTB-22™), and MCF-12A (ATCC® CRL-10782™) cell lines were purchased from the American Type Culture Collection. DU 145, MCF-7, and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1 % nonessential 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, 500 ng/mL hydrocortisone, and 10 % heat-inactivated foetal calf serum. All cell cultures were incubated at 37°C in a humidified incubator with a 5 % CO_2 CO_2 atmosphere until confluent. Confluent cells (80 %) were washed with 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 was re-suspended in a 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 104 cells/mL. Cells (100 µL) were seeded into sterile, clear, flat bottom 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 a 200 µL Tris-buffer (10 mM, pH 10.5), and the absorbance was measured at 510 nm (reference 630 nm) using an EL-X 800 microplate reader (Biotek Inc, USA). Experiments were carried out in triplicate on three different°Ccasions. 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 Prism 5 software.

3. RESULTS

3.1. FTIR, NMR, and UHPLC-MS Analysis

The compounds were isolated in good yields in the range of 50-88 %. The synthesis of the compounds was performed at room temperature, mak-

ing it an affordable approach to develop promising compounds. The by-product that was formed, DCU, was easily removed *via* filtration. The compounds were characterized by FTIR, NMR, and UHPLC-MS. Most of the compounds were isolated as oil (compounds **2-6**), and compounds **1** and **7** were obtained as solids. The melting points of compounds 1 and 7 were 70-74°C and 244-246°C, respectively. The FTIR spectra of the compounds revealed significant signals of C-H stretching at 2967-2930 cm^{-1}, =C-H stretching at 3100-3000 cm^{-1}, C=O signal at 1720-1680 cm^{-1}, C=C stretching at 1697-1461 cm^{-1}, C-O stretch at 1200-1000 cm^{-1} confirming the successful synthesis of the compounds. Furthermore, the molecular masses of the hybrid compounds were confirmed using UHPLC-MS, and the molecular masses of the compounds were 286.1449, 281.1549, 515.3350, 500.2134, 455.3344, 449.3841, and 589.4528 (Table **1**). For compound **1**, the six aromatic protons were visible at the range of 7.89-6.50 ppm, and the protons of the methyl group on the carvacrol scaffold were visible as a singlet signal at 2.18 ppm for $-CH_3$ and as a doublet signal at 1.26-1.24 ppm for $-C(CH_3)_2$ on the ^{1}HNMR spectrum. ^{13}CNMR spectrum of compound 1 showed ester, O-C=O carbon at 168.70 ppm, and the aromatic carbons (Ar-C) were found at 120.10-164.39 ppm. The signals due to CH_3 and CH_2 were observed at 33.72 ppm, 24.06 ppm, and 15.94 ppm.

Table 1. UHPLC-HRMS results of carvacrol analogues.

Hybrid Compounds	Expected Molecular Formula	Expected Molecular Mass	Found Molecular Mass
1	$C_{17}H_{19}NO_3$	285.1365	286.1449
2	$C_{19}H_{20}O_2$	280.1463	281.1549
3	$C_{30}H_{42}O_7$	514.2931	515.3350
4	$C_{24}H_{29}N_5O_7$	499.2067	500.2134
5	$C_{25}H_{27}ClN_2O_4$	454.1659	455.3344
6	$C_{26}H_{32}FeO_3$	448.1701	449.3841
7	$C_{40}H_{60}O_3$	588.4542	589.4528

Compound **2** revealed aromatic protons at 7.19-6.67 ppm for eight protons and $-CH_3$ signals at 2.19 ppm and 1.24-1.26 ppm on the ^{1}HNMR spectrum. ^{13}CNMR spectrum showed O-C=O carbon at 165.24, and Ar-C signals showed between 149.32-117.27 ppm, confirming the successful synthesis of the compound. Compound **3** ^{1}HNMR spectrum confirmed that the compound was synthesized with characteristics aromatic proton signals in the range of 7.12-6.67 ppm, signals for the protons of CH_3 on the artesunate scaffold at 1.90 ppm, 0.90 ppm, and 0.85 ppm, and the protons of CH_3 on the carvacrol moiety at 1.24 ppm and 2.20 ppm. On the ^{13}CNMR spectrum of compound **3**, O-C=O was visible at 172.21 and 171.00 ppm. Ar-C was observed between 153.94-113.09 ppm and CH_2=COO was visible at 104.49 ppm. The signal due to C-O is observed at 87.76 ppm, the signals assigned to CH_2 were found in a range of 80.12-40.73 ppm, C was visible at 37.47 ppm, and CH_3 was observed at 12.02 ppm. ^{1}H and ^{13}CNMR spectra confirmed the successful synthesis of compound **3**.

For compound **4**, the HNMR spectrum showed signals for three aromatic protons on the carvacrol ring at 6.84-6.52 ppm, and the =C-H proton at 6.49-6.47 ppm appeared as a doublet signal. The signal of the protons CH_3 on the carvacrol ring was visible at 2.00 and 1.10-1.08 ppm. ^{13}CNMR spectrum also displayed a prominent signal of O-C=O at 172.50 ppm, and the Ar-C was found at 154.86-110.49 ppm. Compound **6** contained a ferrocene scaffold. ^{1}HNMR spectrum showed peaks for the aromatic protons on the carvacrol ring at 7.08-6.63 ppm. The protons on the ferrocene ring were visible as singlets at 4.40 ppm and 4.20 ppm. The CH_3 peaks on carvacrol were found at 2.14 ppm and 1.24 ppm. The protons on the linker between the ferrocene and carvacrol moieties were significant and found as triplets at 3.12 ppm and 2.91 ppm. On the ^{13}CNMR spectrum, the signals of the ketone (C=O) and ester (O-C=O) carbons were visible at 201.99 and 171.55 ppm, respectively. The aromatic carbons (Ar-C) were found at 153.79-113.03 ppm, and the signal between 72.29-69.24 ppm was assigned to cyclopentadiene carbon. Compound **7** was composed of oleanolic acid and carvacrol moiety. The aromatic protons on carvacrol were present at 7.17-7.09 ppm, while

the =C-H proton on the oleanolic acid ring was present at 5.24 ppm. The signal of the ester carbon O-C=O was visible at 172.93 ppm.

3.2. *In Vitro* Antibacterial and Cytotoxicity Analysis

All of the carvacrol hybrid compounds inhibited the growth of bacteria to varying degrees (Table **2**). Compounds **3** and **4** displayed significant antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis*, respectively. Furthermore, compounds **4** and **7** were effective against *Proteus vulgaris* and *Staphylococcus aureus*, respectively. Compound **5** was the most effective hybrid compound in inhibiting the growth of both Gram-positive and Gram-negative bacteria used in the study. However, this was not superior to carvacrol. Carvacrol was used as a control for comparison with the synthesized compounds.

Cytotoxicity evaluation was performed on selected hybrid compounds **1, 2, 3, 6**, and **7**, which contain pharmacophores with known antiproliferative activity. Compound **3** was the most cytotoxic compound against the cancerous cell lines (Table **3**). Compound **2** was not found to be cytotoxic on both the MCF-7 and DU145 cancer cell lines. However, it displayed moderate toxic effects against the MCF-12A cell line, which is a normal cell line (Table **3**). Compounds **6** and **7** displayed a moderate cytotoxic effect against the MDA-MB-231 cells. Compounds **1, 2, 6**, and **7** did not inhibit the proliferation of MCF-12A cells, indicating that they were not cytotoxic. The IC_{50} values of the compounds against MDA-MB-231 were 60.90 ± 1.04, 159.8 ± 1.14, 16.57 ± 1.14, 90.24 ± 1.17, and 196.3 ± 1.16 for compounds **1, 2, 3, 6**, and **7**, respectively.

4. DISCUSSION

4.1. FTIR, NMR, and UHPLC-HRMS

All the compounds were prepared by esterification reaction using DCC and DMAP (Scheme 1-7). The by-product, dicyclohexylurea (DCU), was formed, which was filtered after column chromatography, and the compounds were obtained in moderate to good yields. Compounds **1-7** were all characterized by the presence of an ester linker. The ester linker is found in many potent pharmaceutical drugs due to its ability to cleave in the physiological environment [19, 20]. The metabolism of a drug after administration when it is in contact with enzymes alters its chemical structure, and its stability in the physiological environment can increase the drug's potency. The nature of the functional groups within a drug molecule influences its susceptibility to metabolism [21]. Ester-containing prodrugs have been reported to exhibit improved bioavailability [22].

Table 2. Antibacterial activity of carvacrol and its synthesized hybrid compounds.

Minimum Inhibitory Concentration (MIC, mg/mL)											
Tested Compound	**Gram-positive**					**Gram-negative**					
	BS	***EF***	***SE***	***SA***	***MS***	***ECL***	***PV***	***KO***	***KA***	***PM***	***EC***
Compound 1	1.25	0.63	0.63	0.63	1.25	0.63	1.25	1.25	1.25	1.25	0.63
Compound 2	1.66	1.66	1.66	1.66	3.33	1.66	1.66	1.66	3.33	1.66	1.66
Compound 3	3.33	1.66	1.66	0.208	3.33	0.83	1.66	3.33	3.33	1.66	1.66
Compound 4	1.66	0.42	1.66	1.66	1.66	1.66	0.83	1.66	1.66	1.66	1.66
Compound 5	0.44	0.22	0.44	0.22	0.68	0.22	0.44	0.44	0.44	0.44	0.10
Compound 6	1.66	1.66	3.33	3.33	1.66	3.33	3.33	3.33	1.66	1.66	3.33
Compound 7	1.25	1.25	0.63	0.63	1.25	1.25	1.25	1.25	1.25	2.50	1.25
Carvacrol	0.13	0.27	0.13	0.03	0.27	0.54	0.03	0.02	0.54	0.02	0.03
Streptomycin (µg/mL)	16	128	8	256	4	512	128	16	16	128	64
Nalidixic acid (µg/mL)	16	>512	64	64	512	16	128	8	256	32	512

Bacillus subtilis **(BS)**, *Enterococcus faecalis* **(EF)**, *Staphylococcus epidermidis* **(SE)**, *Staphylococcus aureus* ***(SA)***, *Mycobaterium smegmatis* **(MS),** *Enterobacter cloacae* **(ECL)**, *Proteus vulgaris* **(PV)**, *Klebsiella oxytoca* **(KO)**, *Pseudomonas aeruginosa* **(KA)**, *Proteus mirabilis* **(PM)**, *Escherischia coli* **(EC)**.

Table 3. Cytotoxicity activity of selected hybrid compounds, expressed as IC_{50} values.

Compounds	IC_{50} (μM)			
	MDA-MB-231	MCF-7	MCF-12A	DU145
1	60.90 ± 1.04	55.44 ± 1.05	58.86 ± 1.04	50.01 ± 1.03
2	159.8 ± 1.14	> 200	116.2 ± 1.11	> 200
3	16.57 ± 1.14	0.47 ± 1.14	0.75 ± 1.30	16.21 ± 1.08
6	90.24 ± 1.17	63.34 ± 1.14	> 200	59.78 ± 1.06
7	196.3 ± 1.16	> 200	> 200	> 200
Oleanolic acid	> 200	> 200	> 200	> 200
Carvacrol	> 200	> 200	> 200	> 200

Note: The experiment was performed in triplicate.

The FTIR spectra of the compounds revealed peaks indicating C=O stretch in the range of 1742-1630 cm^{-1} and a C-O band in the range of 1174-1014 cm^{-1}, which confirmed the successful synthesis of the compounds. C=C bands were also visible in the spectra of all the hybrid compounds. Compound **1** revealed the N-H stretch band at 3492-3389 cm^{-1}. Similar findings were reported by Zhao *et al.* for 4-aminosalicylic acid derivatives in which the N-H stretch was visible at 3385 cm^{-1} and C=O stretch at 1744 cm^{-1} [23]. The hybrid compounds all contained an ester functional group which was visible on the ^{13}CNMR spectra at 165-172 ppm. The aromatic protons were also visible at 110-170 ppm on the ^{13}CNMR spectra for all the compounds. ^{1}HNMR and ^{13}CNMR spectra of the compounds revealed signals for the aromatic protons, which further confirmed the successful synthesis of the compounds (Supplementary figures). The ^{1}H and ^{13}CNMR spectra confirmed the successful synthesis of compounds **1-7** [24-26]. UHPLC-HRMS confirmed the successful isolation of compounds **1-7** (Table **1**).

4.2. Antibacterial Activity

The synthesized carvacrol ester hybrids, compounds **1-7**, were found to exhibit antibacterial activity, and compound **5** displayed the best antibacterial activity of all the hybrid compounds synthesized (Table **2**). The superior activity of compound **5** is ascribed to the 4-aminoquinoline scaffolds. The latter has been reported to be a potent scaffold for the synthesis of antimicrobial agents [27-29]. Furthermore, the hydroxyl group on carvacrol is reported to contribute to its antibacterial activity. Some reports have shown that the modification of the hydroxyl group on carvacrol results in compounds with decreased antibacterial activity [8, 26, 30-32]. The importance of the hydroxyl group on carvacrol is believed to be the inhibition of the growth of micro-organisms. The absence of the free hydroxyl group on some carvacrol derivatives prevented them from exchanging a proton, which resulted in low hydrophobicity, thereby reducing membrane permeability and micro-organism growth inhibition effects [5, 30, 31, 33, 34].

Compound **5** inhibited the growth of *E. cloacae* at a concentration of 0.22 mg/mL. This bacteria has developed resistance to many antibiotics [35]. Compound **5** also inhibited *K. aeruginosa* growth. This microorganism is associated with infections that result in a high mortality rate, specifically in patients with compromised immune systems [36]. Previously, some researchers developed analogues of carvacrol with selective antibacterial activity [24, 37, 38]. Ester derivatives of carvacrol were found to have significant antibacterial activity against *B. polymyx*, *B. subtillis*, *B. japonicum*, and *B. megaterium* [38]. Analogues of carvacrol with selective antibacterial activity have been reported [37]. Nikumbh *et al*. [38] synthesized ester analogues of carvacrol by reacting it with selected alkyl halides and acid chlorides which were isolated in good yields in the range of 83-90 % and 85-92 %, respectively. The antibacterial activity of the esters revealed the synergistic effect of hybridizing carvacrol with selected pharmacophores [38]. Mathela *et al*. also prepared carvacrol ester analogues, and most of the compounds exhibited

moderate antibacterial activity against all the tested strains of bacteria [24]. The nature of the compounds hybridized with carvacrol *via* the O-H group on carvacrol influenced the antibacterial outcome of the hybrid analogues. Compound **6** did not display any significant inhibitory effect when compared to carvacrol. However, the obtained antibacterial results revealed that carvacrol-hybrid compounds are promising therapeutics for the treatment of bacterial infections.

4.3. Cytotoxicity Activity

Compound **3** was the most cytotoxic of the three derivatives tested against MDA-MB-231, MCF-7, and DU-147 cancer cell lines with IC_{50} values of 16.57±1.14, 0.47±1.14, and 16.21±1.08 μM, respectively (Table **3**). Research on the anticancer activity of carvacrol using breast cancer cell lines is scarce. Carvacrol is reported to induce apoptosis which is mediated by cell cycle arrest during the S phase. Furthermore, carvacrol has been reported to reduce mitochondrial membrane potential, leading to an increase in cytochrome c release from these organelles [39]. Previously, carvacrol was reported to display low toxicity in breast cancer cell lines *in vitro* [39-42]. The IC_{50} value of carvacrol has been reported as 100 μM in MDA-MB 231 cells [39]. Variable IC_{50} values have been reported in MCF-7 cells; 244.7 ± 0.71 μM [40], 305.58 ± 14.87 μM [41] and 200 μM [42]. Compounds **1**-**3** displayed high cytotoxicity in the MCF-7, estrogen receptor-positive breast cancer cell line, which is not metastatic, as well as the MDA-MB-231 cell line, a highly invasive and metastatic human cancer cell line (a triple-negative breast cancer) when compared to the reported cytotoxic effect of carvacrol, which was found to be >200 μM. These findings revealed the efficacy of hybridizing carvacrol with other anticancer agents.

The high cytotoxic activity of compound **3** is attributed to the incorporation of artesunate, a derivative of artemisinin. The antiproliferative effect of artesunate against MDA-MB-231 cells has been attributed to the induction of cell cycle arrest in the G2/M phase [43]. Artesunate has been reported to induce apoptosis in MCF-7 cell lines [44] and inhibit the growth of prostate cancer cell lines by promoting apoptosis and suppressing the migration of tumour cells [45]. The latter indicates its potential to prevent the formation of metastases in the bone, which is a common occurrence in an advanced stage of prostate cancer [45-47]. Compound **1** was prepared from the hybridization of carvacrol with 4-aminosalicylic acid, an anti-tuberculosis drug [48]. The cytotoxic effect of compound **1** in MCF-7 and MDA-MB-231 cancer cell lines suggest that 4-aminosalicylic acid acts as a potentiating agent when hybridized with carvacrol. Antimicrobial agents are reported to exhibit antiproliferative activity *via* intercalation to DNA, production of free radicals, DNA cross-linking, DNA binding and alkylation, and inhibition of topoisomerase II [49]. Compound **2** indicated cytotoxic activity in MDA-MB-231 cancer cell lines, which may be due to the efficacy of hybridizing carvacrol with cinnamic acid. Cinnamic acid has been reported to exhibit anticancer activity by inducing apoptosis *via* inhibition of nuclear factor-κB (NF-kB). Furthermore, it acts as a prooxidant, generates reactive oxygen species (ROS), and can mediate DNA fragmentation [50].

The synthesized hybrid compounds were characterized by the presence of an ester linker which is prone to hydrolysis, suggesting that they will be readily cleaved at a biological pH such as the acidic pH of the cancer environment [51]. The ester linker can be cleaved by enzymes resulting in the hybrid compound disintegrating into two molecules that can operate independently at different sites [52]. *In vitro* cytotoxicity evaluation was also performed on compounds **6** and **7** as the anticancer activity of ferrocene [53, 54] and oleanolic-based compounds [55, 56] has been reported. Compounds **6** and **7** were not cytotoxic to the normal cell line, MCF-12A. The latter revealed their safety and thus suitability for therapeutic application. Compound **6** displayed cytotoxic effect on the breast cancer cell lines, MDA-MB-231 and MCF-7, with an IC_{50} of 90.24 ± 1.17 μM and 63.34 ± 1.14 μM, respectively. Ferrocene scaffolds exhibit good redox activity, making them suitable precursors for drug development. Its anticancer activity is ascribed to DNA damage caused by the formation of ROS in the cell [53, 54]. Wang *et al.* synthesized hybrid compounds con-

taining ferrocene that were effective against MDA-MB-231 cancer cell lines, and the compounds contained an ester linker [57].

Similarly, Perez *et al.* also reported hybrid compounds containing ferrocene with an ester linker, which inhibited the proliferation of MCF-7 cell lines [58]. Carvacrol has been reported to be cytotoxic to breast cancer cell lines [39]. Hybridizing carvacrol with active pharmacophores is expected to result in compounds with good anticancer effects. Compound **6** was also cytotoxic to DU145, prostate cancer cell lines, with an IC_{50} value of 59.78 ± 1.06 μM, which is noteworthy when compared to the cytotoxic activity of carvacrol reported by other researchers against this cell line. Previous reports revealed carvacrol with an IC_{50} value of 430.6 ± 21.9 μM against DU145 cell lines [59]. Furthermore, 80.52 % of cell death°Ccurred in DU145 cells when treated with 500 μM of carvacrol for 48h [60]. The mode of action of carvacrol in these cells is believed to be *via* reduction of p-Akt and phosphorylated extracellular-signal-regulated kinase (pERK) levels [59] as well as the induction of apoptosis [60]. Furthermore, compound **7** displayed a cytotoxic effect on MDA cell lines but not on MCF-7 and DU145 cancer cell lines, revealing its selective anticancer activity.

CONCLUSION

Carvacrol-based hybrid compounds **1-7** were successfully synthesized in a yield ranging between 50 and 88 %. NMR, FTIR, and MS analysis confirmed the successful synthesis of these compounds. Compound **5** displayed promising antibacterial activity, whereas compound **3** exhibited significant cytotoxic activity against MCF-7, MDA, and DU145 cancer cell lines. The modification of carvacrol through hybridization was found to result in compounds with promising therapeutic potential.

CURRENT & FUTURE DEVELOPMENTS

Most infectious and chronic diseases are treated by combination therapy using two or more therapeutic agents in a fixed-dose combination. However, this approach is limited by factors such as the development of drug resistance, drug-drug interaction, *etc*. The hybridization of two or more drugs into one compound has the potential to overcome the development of drug resistance, lower toxicity, and result in improved therapeutic outcomes.

In this study, hybrid compounds containing carvacrol scaffold were prepared. Some of the hybrid compounds displayed significant anticancer activity compared to the control, carvacrol, which was used as a precursor. Compound **5** displayed potent antibacterial activity, while compound **3** exhibited significant cytotoxic activity against MCF-**7**, MDA, and DU145 cancer cell lines. The modification of carvacrol through hybridization was found to result in compounds with promising therapeutic potential.

The findings obtained in this study suggest that the hybridization of carvacrol with known compounds is a promising approach for developing new antibacterial and anticancer agents. In the future, there will be a need to hybridize other classes of compounds with carvacrol followed by more vigorous *in vitro* and *in vivo* studies. There is no doubt that continuous research in the development of carvacrol hybrid compounds will result in potent molecules.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study has been approved by the University of Fort Hare's Research Ethics Committee (UREC), Grant number: FER001-21.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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None.

CONFLICT OF INTEREST

The author(s) declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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