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Araştırma Makalesi - Research Article

Design, Synthesis, and Biological Evaluation of Curcumin-β-sitosterol Conjugate a Potential Candidate for Breast Cancer Therapy

Meme Kanseri Tedavisi için Potansiyel Bir Aday Olan Kurkumin-β-Sitosterol Konjugatının Tasarımı, Sentezi ve Biyolojik Değerlendirmesi

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ABSTRACT

In this study, a novel steroidal conjugate was prepared via a convenient click chemistry technique. β-sitosterol (BS), a widely distributed phytosterol throughout the plant kingdom, was chosen as a steroidal component. It is known that BS uses in the stabilization of cell membranes and has beneficial effects in different diseases. On the other hand, curcumin (CUR), a phenolic compound, was used as a phytochemical agent with a variety of biological activities. The steroidal conjugate (BS-CUR) was achieved in high yield using azide-alkyne cyclization reaction. The structure of BS-CUR was elucidated by using FTIR, NMR, HRMS, and fluorescence spectroscopy techniques. *In vitro* cytotoxicity assays of the BS-CUR conjugate were evaluated against human breast cancer (MDA-MB-231) and healthy mouse fibroblast cell line (L929), respectively. The preliminary evaluation indicated that BS conjugate exhibited good cytotoxicity compare with the native compounds, CUR and BS. The BS-CUR conjugate could be considered a potential compound for further design and synthesis of highly effective anticancer agents.

Keywords- Curcumin, Cytotoxicity, Steroid, Steroidal conjugate, β-Sitosterol

ÖZ

Bu çalışmada, klik kimyası tekniği ile yeni bir steroidal konjugat hazırlandı. Bitki dünyasında yaygın olarak kullanılan bir fitosterol olan β-sitosterol (BS) steroidal bir bileşen olarak seçildi. BS'nin hücre zarlarının stabilizasyonunda kullanıldığı ve farklı hastalıklarda faydalı etkileri olduğu bilinmektedir. Fenolik bir bileşik olan kurkumin (CUR) ise çeşitli biyolojik aktivitelere sahip bir fitokimyasal ajan olarak kullanıldı. Steroidal konjugat (BS-CUR), azid-alkin halkalaşma reaksiyonu kullanılarak yüksek verimle elde edildi. BS-CUR'nin yapısı FTIR, NMR, HRMS ve floresans spektroskopi teknikleri kullanılarak aydınlatıldı. BS-CUR konjugatının anti-kanser ve biyouyumluluk analizleri, sırasıyla insan meme kanseri (MDA-MB-231) ve sağlıklı fare fibroblast (L929) hücre hatlarına karşı değerlendirildi. Ön değerlendirme, BS konjugatının, doğal bileşikler CUR ve BS ile karşılaştırıldığında iyi sitotoksisite sergilediğini gösterdi. BS konjugat, yüksek potansiyele sahip anti-kanser ajanlarının daha ileri tasarımı ve sentezi için umut verici bir ajan olarak düşünülebilir.

Anahtar Kelimeler- Kurkumin, Steroid, Steroidal Konjugat, β-Sitosterol, Sitotoksisite

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I. INTRODUCTION

Steroids are classes of natural products that play an important role in the development of novel drugs. The broad bioactivity spectrum of steroids has drawn the attention of researchers to develop new approaches and synthesize novel steroid derivatives [1, 2]. Phytosterols or plant sterols are a subgroup of the steroids found exclusively in plants, vegetable oils and foods such as peanut butter, pistachios and sunflower seeds [3]. Phytosterols have cholesterol-like structure from which they differ in their side chains and ability to reduce of cholesterol level in blood by inhibiting its absorption from the intestine in humans. β-sitosterol ((24R)-ethyl-5-cholestene-3β-ol) (BS) is the most abundant phytosterol and has a chemical structure similar to that of cholesterol [4]. It reduces the absorption of exogenous cholesterol in the intestine and is therefore used as an anti cholesteremic [5]. In vivo and in vitro studies have demonstrated that BS as a steroidal drug has the potential to inhibit tumour cell growth such as human colon, prostate, and breast cancer [6]. It has also anti-inflammatory effects, antioxidant, antidiabetic, antimicrobial, and antiviral activities [7]. BS has been found not toxic and is considered a safe and natural compound with many potential benefits [8]. Estrogen plays a role in the development of breast cancer, especially in its early stages. It has been shown in both clinical studies and epidemiologic data that BS inhibits in vitro proliferation of MCF-7, an estrogen receptor positive breast cancer cell line due to showing estrogen-mimetic activity. On the other hand, it has been reported that BS has a significant effect on inducing apoptosis in hormone-insensitive and metastatic MDA-MB-231 cells (estrogen receptor negative) [3, 9].

A current approach to the design of active molecular species relies on the conjugation of two or more biologically active compounds. With the combination molecules provided by this conjugation, a higher synergistic effect can be achieved on a single target molecule. Multifunctional conjugates may exert therapeutic effects at lower concentrations compared to single molecules [10]. In recent years, steroids have been widely used for conjugation with other biologically active molecules and have shown a variety of applications in biology, material sciences as well as pharmaceutical sciences [11]. Phytosterols have a therapeutic effect only at high concentrations, and it is therefore thought that combining steroids with other biologically active agents may be effective even at low concentrations [12]. Conjugation of an active agent to a steroid can enhance the antiproliferative activity and diminish the side effects and change its selectivity.

Natural products are promising bioactive compounds for bioconjugates in drug discovery. They show effective properties not only structural diversity but also many biological activities. Many research studies describe the bioconjugates derived from natural products having a range of biological activities [13]. Curcuma longa L. from the Zingiberaceae family is one of the famous natural products for its bioactive compound called curcumin as its main substance [14]. Curcumin (CUR), a natural diphenolic yellow-orange polyphenol, has various therapeutic properties such as anticancer, anti-inflammatory, antioxidant, antimicrobial, and antiviral properties [15]. It has been proven that curcumin is not toxic and safe by several in vitro and in vivo studies. CUR has three sites, two phenolic and one active methylene group, for attachment of the drugs or ligands and it is a good candidate to synthesize novel drugs [16].

Conjugation of biologically active compounds provides two benefits: First, conjugation of steroids with biologically active agents can have enhanced biological activity compared to unconjugated steroids. Second, the conjugation of biologically active agents with steroids can increase their ability to penetrate cells and achieve specific targets. Altogether, the conjugation of these two compounds provides a better therapeutic effect, more specific activity, and less toxicity. In this study, we reported to synthesis of an effective steroidal-conjugate by the combination of BS, an important phytosterol, and CUR, which has broad spectrum of pharmacological effects. We obtained this conjugation using the copper(I)-catalyzed "click reaction", between azide-terminated BS and alkyne-terminated CUR. We elucidated the structural analysis of the obtained target β-sitosterol-curcumin (BS-CUR) conjugate using FTIR, NMR, HRMS, and fluorescence techniques. We also evaluated the in vitro cytotoxicity of BS-CUR conjugate on MDA-MB-231 and L929cell lines. According to the results obtained, we can say that it is biocompatible and not toxic to L929 cells, but it is toxic to the MDA-MB-231 cell line.

II. MATERIALS AND METHODS

A. Materials

The starting compounds β-sitosterol (≥95%) and curcumin (from Curcuma longa (Turmeric), powder), Triethylamine (TEA) (≥99.5%), 4-Bromobutyryl chloride (95%), Sodium azide (NaN_3) (≥99.5%), Sodium sulfate (Na_2SO_4) (≥99.0%, anhydrous, powder), Propargyl bromide solution (~80% in toluene), Potassium carbonate (K_2CO_3) (≥99.0%), N,N,N′,N′′,N′′-Pentamethyldiethylenetriamine (PMDETA) (99%), and Copper (I) bromide (CuBr) (98%) were purchased from Sigma Chemicals, USA. All solvents used in this study were of analytical grade.

B. Characterization methods

Reactions were monitored in Merck aluminium sheet silica gel thin layer plates (TLC, 60F254), visualized in UV-cabinet (λ_{max} = 254 and 365 nm) and further charred with 1% p-anisaldehyde in 2% aqueous sulphuric acid with subsequent heating at 105 °C. Melting points were determined in open capillaries in E-Z Melt melting point apparatus, Stanford Research System, USA. Solvents were evaporated under reduced pressure at 50 °C in Buchi Rotavapor. ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) experiments were performed on NMR Bruker Avance DRX 500 MHz spectrometer using tetramethylsilane (TMS, δ scale, 0.00 ppm) as internal standard. The chloroform (δ-$CDCl_3$) and dimethyl sulfoxide (DMSO-δ6) as the solvents, and their chemical shifts were stated in ppm (δ). The NMR data were processed using MestreNova 12.0 software (Mestrelab Research, Spain). Coupling constants (J) are given in Hertz (Hz). Splitting of peaks are abbreviated as s for singlet, d for doublet, t for triplet, q for quartet, bs for broad singlet, and m for multiplet. Electrospray mass analysis was done on API 3000 Triple Quad LC–MS (Applied Biosystem, USA) mass spectrometer after dissolving samples in methanol or acetonitrile. IR spectra were recorded on a Perkin Elmer Frontier apparatus (ν, cm^{-1}) (PerkinElmer Inc., Waltham, MA, USA).

C. Experimental methods

Synthesis of BS-$(CH_2)_3$-Br

β-sitosterol (1 g, 2.4 mmol) was dissolved in anhydrous THF (100 mL) in a two-necked flask equipped with a magnetic stirrer and rubber septum under inert argon atmosphere. TEA (0.632 g, 6.24 mmol) was then added to the reaction vessel, which was cooled to − 15 °C using an ice–salt mixture. A mixture of 4-bromobutyryl chloride (0.586 g, 3.16 mmol) and THF (20 mL) was added dropwise to the reaction mixture for 20 min. The whole mixture was stirred at room temperature for 48 h, diluted with DCM (100 mL), and washed with 1 N HCl solution (2 × 50 mL). After the organic layers were combined and dried with Na_2SO_4, the solvent was removed by a rotary evaporator and the compound was dried under reduced pressure at ambient temperature until a constant weight was obtained.

Yield: 1.132 g, 86%. m.p: 56–60 °C. FTIR (ATR, cm^{-1}): 2950 and 2870 (C-H), 1720 (C=O), 1472 (C-H), 1236 ((C=O)-O), and 1044 (C-O-C). ^1H NMR (500 MHz, CDCl3, δ, ppm): 5.38 (s, H-6, 1H), 4.63 (m, H-3, 1H), 3.47 (t, J= 11 Hz, O(C=O)$CH_2CH_2CH_2$Br, 2H), 2.48 (t, O(C=O)$CH_2CH_2CH_2$Br, 2H,), 2.17 (m, O(C=O)$CH_2CH_2CH_2$Br, 2H), 1.02 (s, H-18, 3H), 0.92 (d, J= 5.6 Hz, H-21, 3H), 0.85 (d, J= 8 Hz, H-26, 3H), 0.84 (d, J=7.2 Hz, H-29, 3H), 0.68 (s, H-18, 3H). Elemental analysis: Calcd for $C_{33}H_{55}O_2Br$, C, 70.31; H, 9.83; O, 5.68; Br, 14.18. Found 70.18; H, 9.86; O, 5.66; Br, 14.3. HRMS (m/z): $(M+H)^+$, Calcd for $C_{33}H_{55}O_2Br$, 562.33635, found 562.33510.

Synthesis of BS-$(CH_2)_3$-N_3

BS-$(CH_2)_3$-Br (1 g, 1.82 mmol) and NaN_3 (0.35 g, 5.42 mmol) were dissolved in anhydrous DMF (20 mL) under inert argon atmosphere and stirred for 24 h at 90 °C. This mixture was cooled to room temperature, transferred into water (300 mL), extracted with diethyl ether (2 × 50 mL), and the organic phases were collected and dried with Na_2SO_4. Consequently, the solvent was removed by a rotary evaporator, and the compound was dried under reduced pressure at ambient temperature until a constant weight was obtained.

Yield: 0.82 g (88%). m.p: 51–53 °C; FTIR (ATR, cm^{-1}): 2945 and 2850 (C-H), 2100 (N_3), 1720 (C=O), 1465 (C-H), 1256 ((C=O)-O), and 1045 (C-O-C). ^1H NMR (500 MHz, $CDCl_3$, δ, ppm): 5.38 (s, 1H, H-6), 4.62 (m, 1H, H-3), 3.35 (t, J= 10.6 Hz, 2H, O(C=O)$CH_2CH_2CH_2N_3$), 2.38 (t, J= 11 Hz, 2H, O(C=O)$CH_2CH_2CH_2N_3$), 1.90 (m, 2 H, O(C=O)$CH_2CH_2CH_2N_3$), 1.01 (s, 3H, H-18), 0.90 (d, J= 5.6 Hz, 3H, H-21), 0.86 (d, J= 8 Hz, 3H, H-26), 0.86 (d, J=7.2 Hz, 3H, H-29), 0.67 (s, 3H, H-18). Elemental analysis: Calcd for $C_{33}H_{55}O_2N_3$ C, 75.38; H, 10.54;

O, 6.09; N, 7.99. Found 75.13; H, 10.45; O, 6.01; N, 8.41. HRMS (m/z): $(M+H)^+$, Calcd for $C_{33}H_{55}O_2N_3$ 525.42945, found 525.43612.

Synthesis of curcumin mono alkyne

Curcumin (5 g (purity is around 65%), 8.82 mmol) was reacted with propargyl bromide (0.783 mL 80 wt. % in toluene, 8.82 mmol) in the presence of K_2CO_3 (1.21 g, 8.82 mmol) with 70 mL acetone under N_2 atmosphere for 48 h at room temperature. The reaction solvent was removed under reduced pressure, the reaction mixture was extracted with CH_2Cl_2 and the combined organic phases were washed with water and brine, dried and concentrated. The resulting residue was purified by column chromatography (silica gel, 0.5% ~ 1% acetone in CH_2Cl_2) to give the desired product as an orange solid.

Yield: 42%. FTIR (ATR, cm^{-1}): 3290, 3260, 2970, 2920, 1620, 1578, 1410, 1250. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.62–7.58 (d, 2H), 7.15–6.98 (m, 6H), 6.52–6.49 (d, 2H), 5.93 (d, 1H), 5.80 (s, 1H), 4.81 (d, 2H), 3.92 (s, 6H), 2.54 (s, 1H). Elemental analysis: Calcd for $C_{24}H_{22}O_6$ C, 70.92, H, 5.46, O, 23.62. Found C, 70.94, H, 5.43, O, 24.63. HRMS (m/z): for $C_{24}H_{22}O_6$ $[M+H]^+$calcd. 407.1484, found 407.1857.

Synthesis of BS-CUR conjugate

BS-$(CH_2)_3$-N_3 (0.511 g, 1 mmol) and CMA (0.487 g, 1.2 mmol) were dissolved in degassed DMF (5 mL) under argon atmosphere. PMDETA (0.554 g, 3.2 mmol) was then added, and the solution was gently purged with argon for 5 min. Then, copper (I) bromide (0.459 g, 3.2 mmol) was added to the reaction mixture, and again degassed by purging with argon for 5 min. After stirring the reaction mixture at room temperature for 48 h, azido-functional Merrifield resin (0.150 g) was added [17]. This suspension was purged with argon for 5 min and stirred for additional 72 h at ambient temperature. The insoluble resin was removed by gravity filtration through a paper filter (Schleicher & Schuell, blue ribbon, 2 μm) and washed with DCM. Then, the solution was diluted to 100 mL with DCM and washed with brine (50 mL) and water (50 mL). After the collected organic phases were combined and dried with Na_2SO_4, the solvent was removed by a rotary evaporator and product was obtained as an orange solid.

Yield: 0.8 g (86%). m.p: 112–116 °C. FTIR (ATR, cm^{-1}): 3350-3290 (O-H), 2940 and 2868 (C-H), 1730 (C=O), 1655 (C=C), 1460 (C-H), 1250 ((C=O)-O), and 1040 (C-O-C). ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm): 8.18 (s, 1H, -C=C*H*-N_3 triazole ring), 7.71-6.66 (m, $H_{curcumin}$), 5.95-5.88 (d, 2H), 5.34 (s, 1H, H-6), 4.64-4.62 (m, 5H, H-3, O(C=O)CH_2CH_2*CH_2*N_3, and O-C*H_2*-triazole, respectively), 2.46 (m, 2H, O(C=O)*CH_2*$CH_2CH_2N_3$ and m, 2 H, O(C=O)CH_2*CH_2*CH_2N_3), 1.00 (s, 3H, H-18), 0.92 (d, J= 5.6 Hz, 3H, H-21), 0.86 (d, J= 8 Hz, 3H, H-26), 0.86 (d, J=7.2 Hz, 3H, H-29), 0.67 (s, 3H, H-18). ^{13}C NMR (500 MHz, $CDCl_3$, δ, ppm): 195.19 (2C), 173.98, 151.80, 150.38, 149.33, 148.11, 147.36 (2C), 141.20, 140.82, 129.66, 127.80, 127.13, 126.82, 123.72, 123.30, 122.19, 121.85, 115.67, 114.78, 111.77, 111.12, 75.20, 60.84, 60.06, 56.51 (2C), 56.46, 56.21,56.24, 51.18, 45.26, 43.60, 40.25 (2C), 39.78 (2C), 37.22, 36.64, 32.55 (2C), 31.84, 31.59, 31.09, 31.03, 30.49, 28.79, 24.87, 21.43, 19.89 (2C), 19.55, 19.43, 18.16, 18.30, 12.66. Elemental analysis: Calcd for $C_{57}H_{77}O_8N_3$ C, 73.44; H, 8.33; O, 13.73; N, 4.51. Found 73.61; H, 8.42; O, 12.98; N, 4.99. HRMS (m/z): $(M+H)^+$, Calcd for $C_{57}H_{77}O_8N_3$ 931.5711, found 932.5622.

D. In vitro cell viability assays in cell cultures

Cell culture

L929 healthy mouse fibroblast and breast adenocarcinoma (MDA-MB-231) cell lines (American Type Culture Collection (ATTC) Manassas, VA, USA) were used in the present study. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin–streptomycin were obtained from PAN BIOTECH.The cells were cultured in DMEM supplemented with 10% FBS, 10 000 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin at 37 ºC in a humidified environment with 5% CO_2. The culture medium was replenished over alternating days, and the cells were sub-cultured by detaching with trypsin (0.25%)/ethylene diamine tetra acetic acid (EDTA) (0.02%) in phosphate buffer solution. The elution test method was used to obtain an extract from the samples. 0.5 g mL^{-1} of samples were placed in DMEM with 5% FBS and 100 mg mL^{-1} of penicillin–streptomycin in the incubator with 5% CO_2 and at 37 ºC for 24 h so that any soluble factor could leach into DMEM. After elution, the eluents were sterilized using a 0.22 mm syringe; various concentrations of target compounds were prepared using fresh DMEM for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, Biofroxx, Germany) assay. β-sitosterol and curcumin were used as a positive control and tested in the same manner.

Cytotoxic activity studies (MTT assay)

L929 and MDA-MB-231 cells were plated in flat-bottom 96-well plates at a seeding density 10^5 cells per mL. After seeding, the cells were allowed to attach in an incubator at 37 ºC in an environment with 5% CO_2 for 24 h. After attachment, the culture media were removed and replaced with 100 mL of fresh medium containing the test compounds with a concentration gradient of compound ranging from 0.5 µM to 100 µM. Cells with extract-free media were used to determine relative cell survival. The cells were treated with DMSO (Biofroxx, Germany) as a positive control. After the addition of extract concentrations, the cells were incubated for 24 h to observe the cytotoxic and proliferative effects. Finally, a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to determine the cytotoxicity and proliferation. 10 mL of MTT solution (5 mg of MTT mL^{-1} in phosphate buffer saline (PBS) (15 mM, pH 7.4, Gibco, Paisley, UK) was added to each well and incubated for 3 h at 37 ºC so that formazan crystals were formed in the cells. Then, 100 mL of DMSO was added to each well to dissolve the formazan crystals. After 30 min, the optical density (OD) at 570 nm was measured using an Elisa Reader (Thermo scientific Multiskan GO). The mean OD570 value of the negative control group without extract was standardized as 100% alive, and the OD570 values of the test samples were compared to this value. The half-maximal inhibitory concentration (IC_{50}) was determined by nonlinear regression analysis using the Boltzmann sigmoidal function from Origin 9 (OriginLab, Northampton, USA). Selectivity index (SI) values, indicating selectivity for tested cell lines, were calculated from the ratio of IC_{50} values of the compounds obtained for normal vs. cancer cells. An SI score >3 represented good selectivity. SI was calculated as a ratio of IC_{50} for a normal cell line to IC_{50} value for the respective cancerous cell line using the following equation:

$$SI = \frac{IC_{50} \text{ for normal cell line}}{IC_{50} \text{for cancerous cell line}}$$

E. Statistical analysis

All statistical analyses were performed by the analysis of variance (ANOVA) at the 5% probability level. ANOVA was used to identify the statistical significance between different groups. All measurements were done for at least three replicates. In all studies, $P \leq 0.05$ was considered to be of statistical significance. The results were each presented as mean ± standard error.

F. Abbreviation List

American Type Culture Collection	ATTC
Analysis of variance	ANOVA
Coupling constants	*J*
Dulbecco's Modified Eagle's Medium	DMEM
Ethylene diamine tetra acetic acid	EDTA
Fetal bovine serum	FBS
Fourier-transform infrared spectroscopy	FTIR
Half-maximal inhibitory concentration	IC_{50}
Hertz	Hz
High resolution mass spectrometry	HRMS
Nuclear magnetic resonance	NMR
Optical density	OD
Phosphate buffer solution	PBS
Ppm	δ
Selectivity index	SI
Thin layer chromatography	TLC
Tetramethylsilane	TMS
Wavelength	λ

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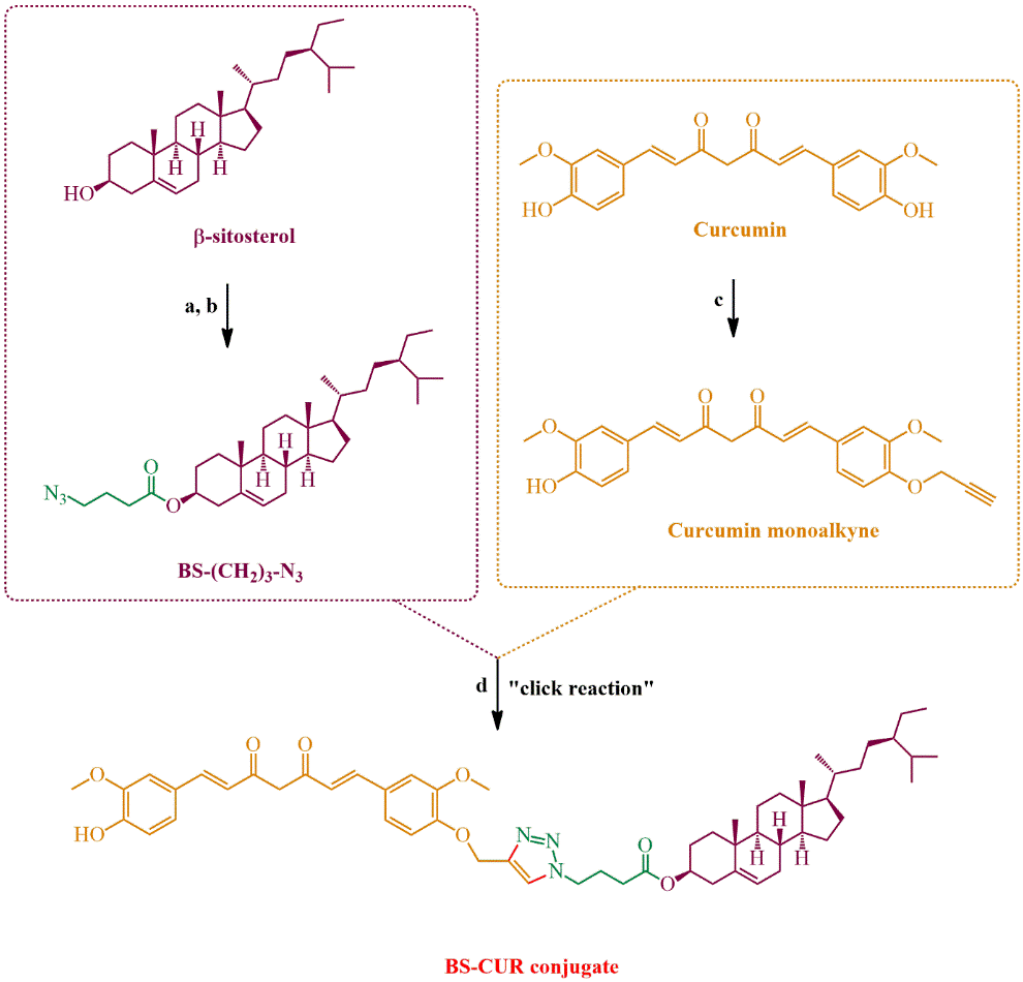
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III. RESULTS AND DISCUSSION

A. Chemistry

The synthesis of the β-sitosterol-curcumin (BS-CUR) conjugate was successfully accomplished using a strategy similar to the one described previously [18]. The general synthetic method for the preparation of BS-CUR is outlined in Scheme 1. The crucial consideration in designing the BS-CUR conjugate is to determine that these two molecules retain their biological activity after chemical bonding. It was reported that the functionalization of the 3-OH of sterols does not affect their binding affinities to cells. On the other hand, it was announced that one of the phenolic oxygens and the C-4 position of curcumin was used to bind the sterol does not change the effect of curcumin in that study [19]. We selected easily available steroid β-sitosterol as a starting compound, which was converted initially to the bromide-functionalized derivative (BS-$(CH_2)_3$-Br) in high yield (86%) and purity. BS-$(CH_2)_3$-Br was obtained via reacting the 3-OH group of BS with 4-bromobutrylchloride in the presence of a sufficient amount of TEA in the THF solution. This compound was then reacted with sodium azide to yield an azide-functionalized derivative (BS-$(CH_2)_3$-N_3). It served as the intermediate for the preparation of the BS-CUR conjugate. On the other hand, for the alkyne derivative, we prepared the curcumin mono alkyne which was then coupled to the BS-$(CH_2)_3$-N_3 using the "click reaction" to give BS-CUR conjugate. The copper catalyzed "click reaction" was used between the alkyne and azide group for its simplicity and high selectivity. The isolated yield for the BS-CUR conjugate was 86%. The structure of BS-CUR was fully characterized by FTIR, elemental analysis, ^{1}H NMR techniques, and mass spectrometry. The results are in good agreement with the expected structures.



Scheme 1. Synthesis of BS-CUR conjugate. Reagents and conditions: (a) 4-bromobutrylchloride, THF, TEA; (b) NaN_3, DMF; (c) Propargyl bromide, K_2CO_3, acetone; (d) PMDETA, CuBr.

The FTIR spectrum of BS-CUR conjugate and the intermediate derivatives was shown in Fig.1. The spectrum of BS was also included for comparison. The disappearance of the absorption peak at 3500 cm^{-1} (Fig. 1a) after the reaction BS with 4-bromobutryl chloride, confirmed the conversion of the hydroxyl group into the bromide group (Fig.1b). The strong and sharp absorption peak at 1720 cm^{-1} is attributed to the carbonyl group in the BS-$(CH_2)_3$-Br. After reaction with NaN_3, the absorption peak at 2100 cm^{-1} belonging to the azide group was seen in Fig.1c. The absence of the azide signal at 2100 cm^{-1} indicated that the click reaction occurred successfully. As seen in Fig.1d, the peaks at 1650, 1732, and 3355-3295 cm^{-1} in the FTIR spectrum of BS-CUR indicated the presence of the C=C double bond, the ester bond, and the OH group vibrations, respectively. Moreover, the absorption peaks at 2935-2870 cm^{-1} corresponded to the aliphatic C–H stretching band of the compounds.

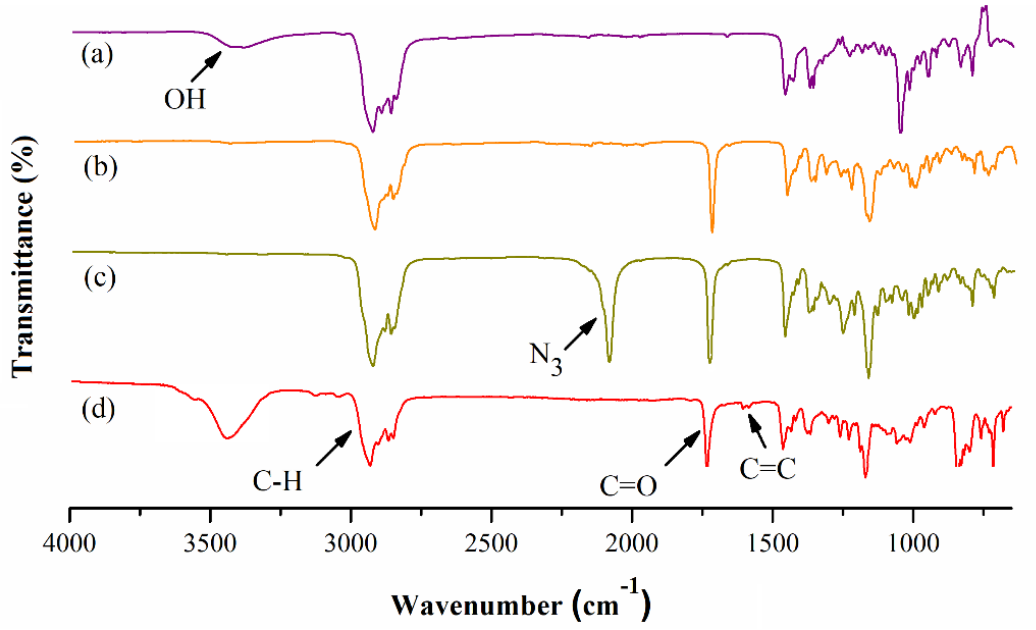


Figure 1. FTIR spectrum of (a) β-sitosterol (b) BS-$(CH_2)_3$-Br (c) BS-$(CH_2)_3$-N_3 (d) BS-CUR conjugate.

The structure of the target BS-CUR conjugate was confirmed by ^{1}H NMR analysis based on chemical shifts (Fig. S1, S2, S3, and S4). BS-CUR conjugate showed the signals at lower fields in the range of 7.71–6.66 ppm corresponding to the aromatic protons of curcumin moiety, and the singlet signal at 8.18 ppm assigned to CH proton in the triazole ring as shown in Fig. 2, which confirmed the successful conjugation of curcumin and BS. The distinctive signal for the olefinic proton in the part of steroidal BS was located as a singlet at 5.34 ppm. The signal of methine proton attached to the hydroxyl group was observed at about 4.6 ppm. The CH protons close to the triazole ring were junction with the methine proton and seen between 4.64 and 4.62 ppm. The multi signals that appeared in their ^{1}H NMR spectra at higher fields were attributed to the other protons of the BS unit. In addition, steroidal BS backbone showed the signals for methyl groups at 1.02 (H-19, 3H), 0.92 (H-28 and H-29, 6H), 0.86 (H-26 and H-21, 3H), and 0.67 (H-18, 3H). In the ^{13}C NMR spectrum (Fig.S5), 57 signals were found, the signals of carbonyl carbons at curcumin moiety at δ_c 195.07, the signals of the double bonds C-5 and C-6 in the β-sitosterol moiety at δ_c 140.85 and 122.13, and the signal of C-3 in the β-sitosterol moiety at δ_c 75.21 were observed. The conjugation of BS with curcumin was also supported by mass spectral data. It revealed a molecular peak $[M+H]^+$, m/z= 932.5662 was consistent with the calculated value of the molecular weight.

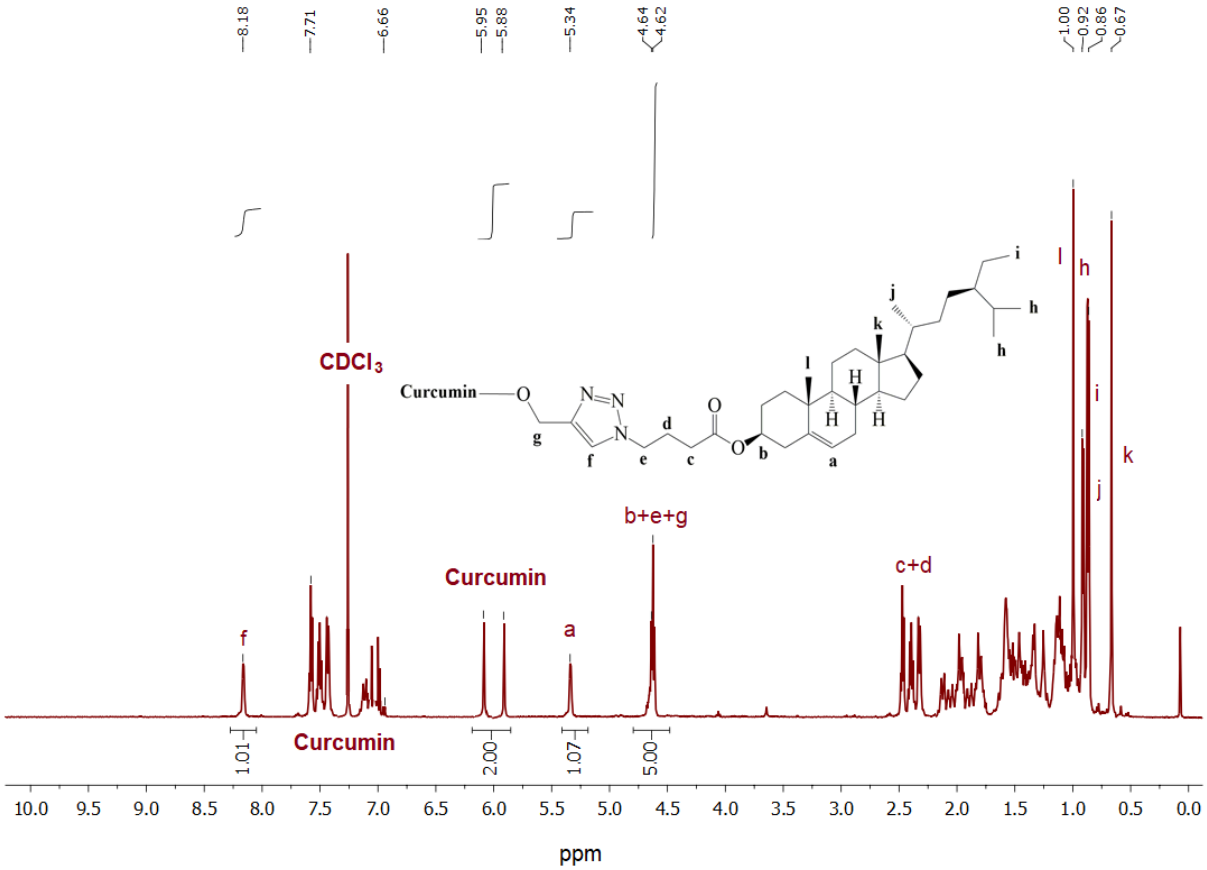


Figure 2.[1]H NMR spectrum of BS-CUR conjugate.

The UV-vis absorbance and fluorescence properties of curcumin mono alkyne and BS-CUR were demonstrated in Fig. 3. BS-CUR conjugate showed the absorption bands at 426 and 443 nm (π–π* transition) similar to that of curcumin mono alkyne, which indicates the successful conjugation [20]. The solution of curcumin mono alkyne exhibited a bright yellow color due to the π–π* transition of the chromophore. The solution of BS-CUR conjugate showed a blue shift after conjugation which were recorded by irradiating with a 365-nm UV lamp. It is related to the conjugating with BS increases the energy level of the π* orbital. Hence the visual demonstration of BS-CUR gave a color towards the blue. The fluorescence emission spectrum of BS-CUR conjugate exhibited the hypsochromic shift (to lower wavelength) by ∼15 nm attributed to the conjugation between curcumin mono alkyne and BS [21, 22]. The conjugate also showed a similar peak reflecting that the optical properties of curcumin mono alkyne were retained after binding.

B. Biological assays

In vitro evaluation of cytotoxic activity

The evaluation of the *in vitro* cytotoxicity of curcumin, β-sitosterol, and BS-CUR conjugate was carried out by cell viability assay for 24 h against human breast cancer cell line (MDA-MB-231) and healthy mouse fibroblast cell lines (L929). The cell viabilities were assessed versus increasing concentrations for tested compounds. The cell viability rate for healthy mouse fibroblast L929 cell line was similar to the control group, indicating that the studied compounds were non-toxic within the test range (0–50 µg/mL) (Fig. 4A). The cell viability in the positive control group was considered as 100%, and the percentage values for BS-CUR conjugate were calculated as 112.6%, 116.8 %, 120.2 %, and 122.5 % at the 5, 10, 25, and 50 µg/mL concentrations, respectively. For the MDA-MB-231 cells, statistical significant differences were observed for experimental groups, compared with control group ($p < 0.05$) (Fig.4B). Percentages of cell viability significantly lower than control group for all experimental groups at the same concentrations. The cell viability percentages for BS-CUR conjugate were 72.4 %, 42.02 %, 25.3 %, and 16.1 % at the 5, 10, 25, and 50 µg/mL concentrations, respectively.

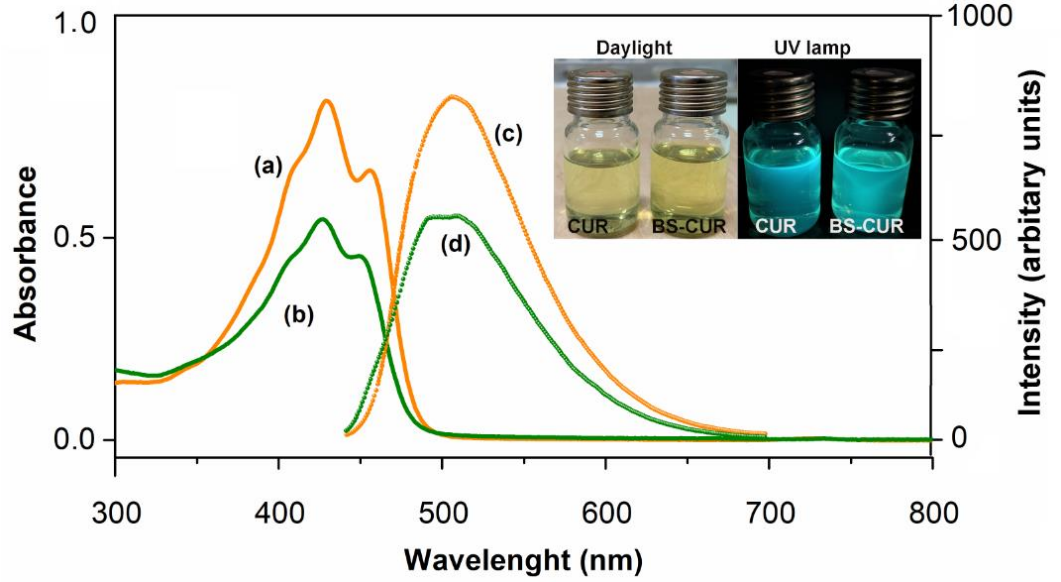


Figure 3. Absorption and Emission spectra of CUR mono alkyne ($1x10^{-3}$ mol L^{-1}) (a and c) and BS-CUR ($1x10^{-3}$ mol L^{-1}) conjugate (b and d) in DMF.

The chemopreventive impacts of curcumin have been credited to different natural properties, including neutralization of cancer-causing free radicals and anti-angiogenesis activity, which restricts the blood supply to quickly developing threatening cells [23]. β-sitosterol, on the other hand, can influence distinctive levels of tumor improvement, such as their inhibitory effects on the creation, development, and acceptance of cancerous cells, as well as restraint of tumor cell attack and metastasis [24]. The half-maximal inhibitory concentration (IC_{50}) values were 8.41 ± 0.51 µg/mL, 5.98 ± 0.31 µg/mL, and 4.47 ± 0.14 µg/mL for curcumin, β-sitosterol, and BS-CUR conjugate, respectively. According to the criteria of the National Cancer Institute, natural extracts with an IC_{50} value of ≤20 µg/mL are considered highly cytotoxic [25, 26]. As indicated by the IC_{50} values in Table 1, the BS-CUR conjugate showed a significantly increased toxic effect in comparison with the bare compounds, β-sitosterol and curcumin. Hence, the combination of CUR and BS has a synergistic cytotoxic effect and strong *in vitro* cytotoxic activity against MDA-MB-231 cells with IC_{50} values ≤10 µg/mL. On the other hand, the IC_{50} value of BS-CUR conjugate was compared with cisplatin, one of the most commonly used chemotherapeutic agents for breast cancer treatment. IC_{50} values of cisplatin against the MDA-MB-231 cells were previously determined as 9.03 and 13.85 µg/mL at 24 h, respectively [27, 28]. The BS-CUR conjugate showed a good IC_{50} value compared to that of the free cisplatin. To determine the real therapeutic potential of novel anticancer drug candidates, it is necessary to check their selectivity towards normal cells. The SI, which indicates the safety level of a compound toward normal breast cells, indicated that BS-CUR has high cytotoxic activity against MDA-MB-231 (SI=48.31) but was less harmful to normal L929. Higher values of SI indicate greater anticancer specificity, and the compounds displaying an SI above 3.0 are considered highly selective agents [29]. Commonly used anticancer drugs, such as cisplatin or doxorubicin have a big disadvantage of being toxic to both cancer and normal cells.

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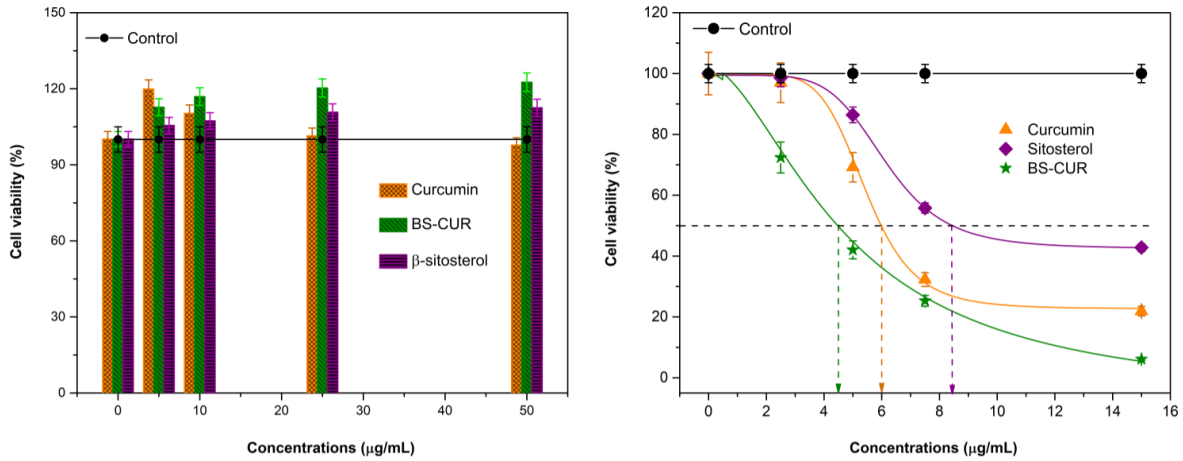


Figure 4. Cell viability of L929 cells (A) and MDA-MB-231 (B) cells treated with curcumin, β-sitosterol, and BS-CUR conjugate (n=3, error bars represent standard deviation (P <0.05)).

Table 1. SI and IC_{50} values of compounds on the L929 andthe MDA-MB-231.

Compounds	(IC_{50}, μg/mL)		
	MDA-MB-231	**L929**	**SI**
Curcumin	8.41 ± 0.51	126.05 ± 1.1	15.00
β-sitosterol	5.98 ± 0.31	197.07 ± 0.5	32.95
BS-CUR	4.47 ± 0.14	215.97 ± 0.9	48.31
Cisplatin[a]	9.03[b,] [27]	18.06 [30]	2.00
	13.85[b,] [28]		1.30

[a] Reference drug
[b] The IC_{50} value of cisplatin was referred from already published work.

Structure activity relationship

The analysis of structure-activity relationships demonstrates, to begin with, that the cytotoxicity of the tested compounds appears to be connected to the incorporation of the heterocyclic rings into the steroid moiety as comparing the cytotoxicity results in Table 1. The following structure-activity relationships (SARs) were drawn: (a) Curcumin has two (or three) functional sides: aromatic rings joined by means of olefin bonds to a β-diketone. The olefin double bonds, while recognized to be important for activity, are usually considered to be a linker between the two key basic components and have not been widely modified. Instead, synthetic endeavors have basically been coordinated at variations of the aromatic rings and their substituents [31]. (b) β-sitosterol has three functional sides for inhibitory effects. A double bond on C-5, the ethyl group on C-24, and absence of double bond on C-22 position of β-sitosterol [32]. In a structure-activity study on the effects of different groups in phytosterols on the inhibition of the relative absorption of cholesterol, the following conclusions were reached for β-sitosterol and stigmasterol, which have similar structures: the presence of C-5 double bond and C-24 ethyl groups of β-sitosterol makes it more effective than stigmasterol [33]. Elmegeed et al. reported previously the inhibitory activity of heterocyclic steroids and curcumin derivatives on breast cancer cells. The results suggested that the hybridization of curcumin and steroid derivatives formed promising anticancer agents. The different functional groups on the steroid backbone increased the inhibitory effect of derivatives with compare to the bare compounds [34]. The synthesis study of steroid curcumin derivatives as anti-Alzheimer's disease candidates resulted that the functional groups such as carbonyl and enolic groups, methoxy and phenolic hydroxyl groups, and the phenyl rings on the curcumin moiety and the presence of steroid moiety showed anti-Alzheimer's disease properties [35].

IV. CONCLUSIONS

In the present study, β-sitosterol and curcumin conjugate was designed and synthesized. The "click chemistry" approach was used in the conjugation of compounds containing alkyne and azide-end groups. The chemical structure of the conjugate was assessed with FTIR, HNMR, HRMS, and UV/fluorescence spectroscopy techniques. The results showed the two natural compounds were successfully conjugated in good yields. *In vitro* cytotoxicity studies showed that the conjugate was safe and biocompatibility with healthy fibroblast cell lines. A biological evaluation of this conjugate to the proliferation of the estrogen-negative breast cancer cell line, MDA-MB-231, was also performed. The conjugate showed the lowest IC_{50} values against the MDA-MB-231 cell line compared to the BS and CUR. It can be suggested that the BS-CUR conjugate may be used as a potential therapeutic compound for cancer treatment *in vitro* and *in vivo*.

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Supplementary Information for

Design, Synthesis, and Biological Evaluation of Curcumin-β-sitosterol Conjugate a Potential Candidate for Breast Cancer Therapy

Meme Kanseri Tedavisi için Potansiyel Bir Aday Olan Kurkumin-β-Sitosterol Konjugatının Tasarımı, Sentezi ve Biyolojik Değerlendirmesi

Sevinç İlkar Erdağı[1*]

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^{1}H and ^{13}C NMR spectra of compounds

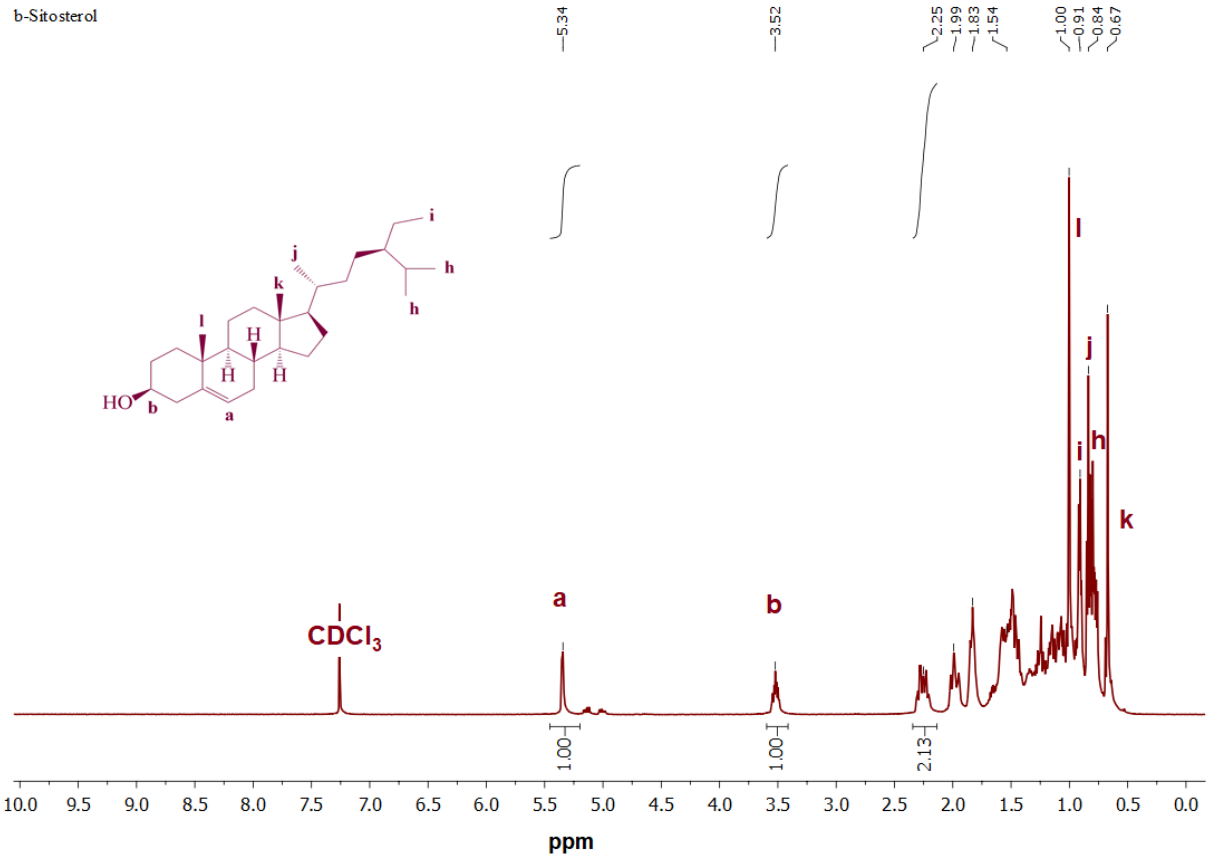


Figure S1. ^{1}H NMR spectrum of β-sitosterol.

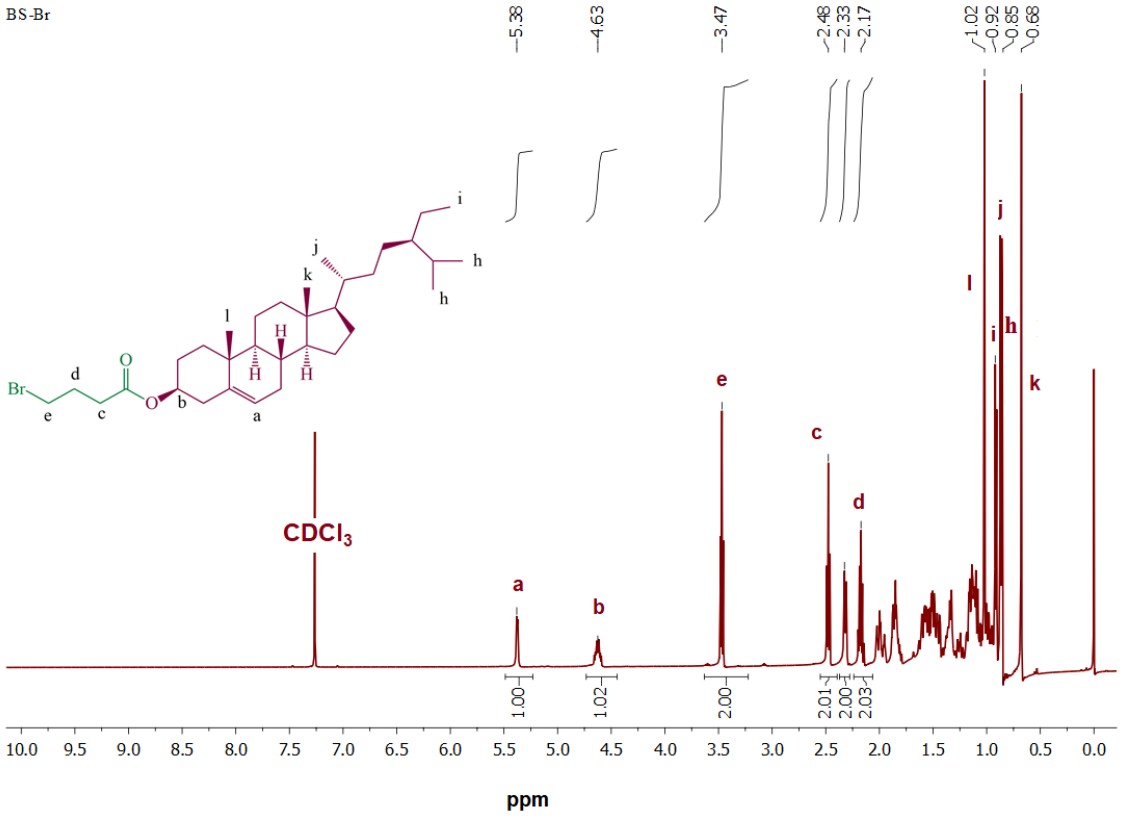


Figure S2. ^{1}H NMR spectrum of BS-$(CH_2)_3$-Br.

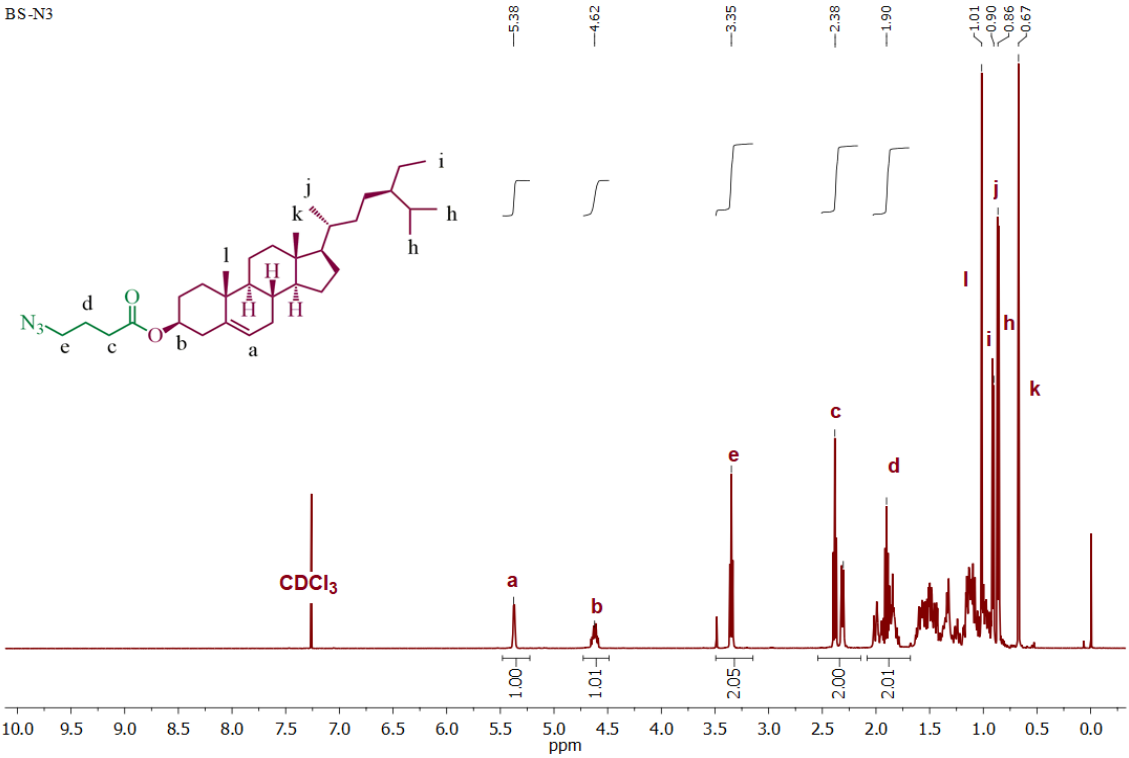


Figure S3. ^{1}H NMR spectrum of BS-$(CH_2)_3$-N_3.

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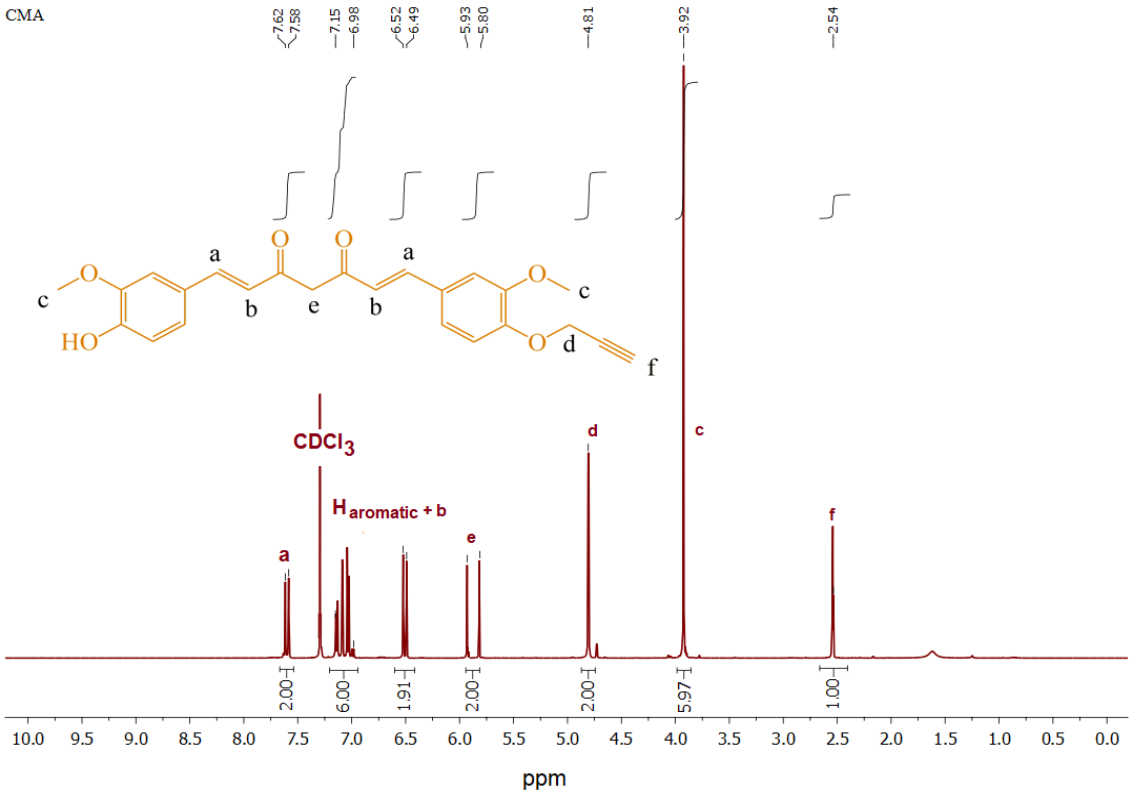


Figure S4. ^{1}H NMR spectrum of curcumin mono alkyne

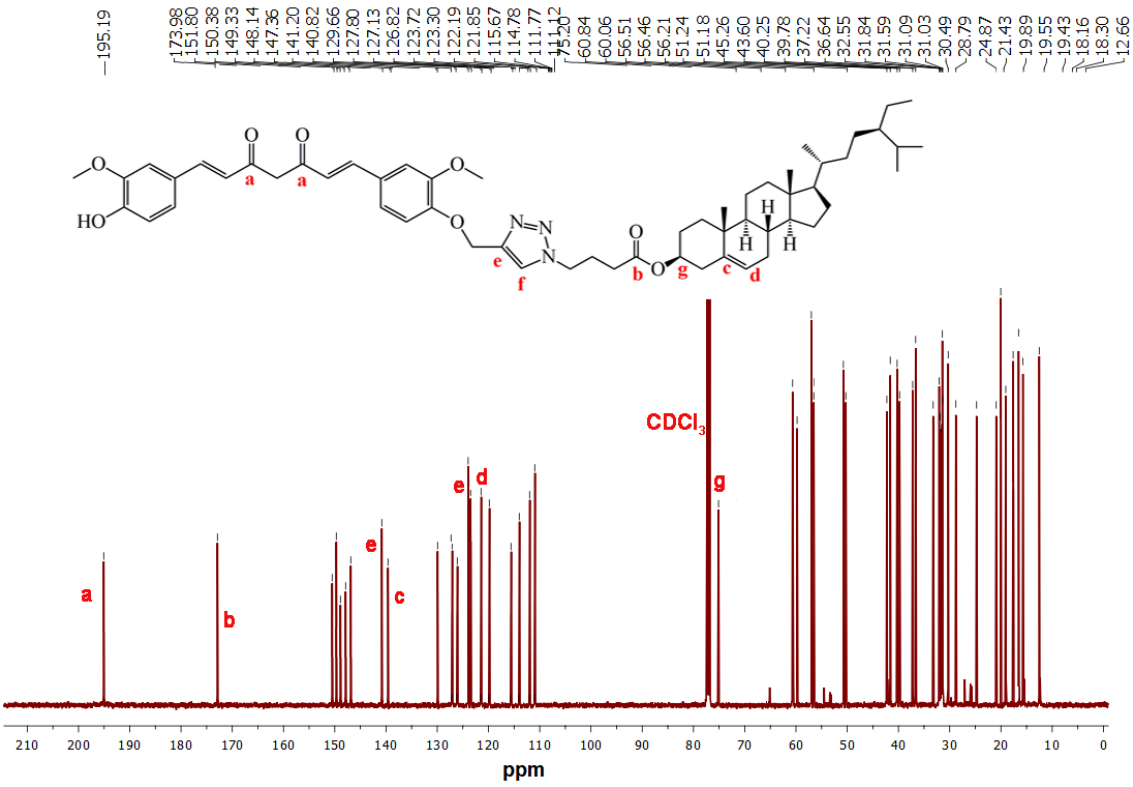


Figure S5. ^{13}C NMR spectrum of BS-CUR