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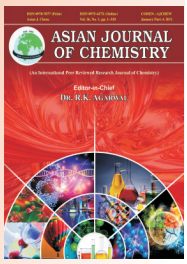

Characterization and Anticancer Potential of Newly Synthesized Propofol Conjugates

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The present study reports the synthesis, characterization and anticancer potential of novel ester conjugates of stearic acid (SA) and palmitic acid (PA). An ester conjugates were chemically designed by esterification of the terminal COOH group of the fatty acid to C^1-OH position of 2,6-diisopropylphenol (propofol). The structure of new ester conjugates *viz*; propofol stearate and propofol palmitate were characterized by UV, NMR (^1H, ^{13}C) and FAB mass spectroscopy. The anticancer efficacy of the conjugates was examined on HepG2, Lovo, HT1080, A549 and MDA-MB-231 cancer cell lines. Treatment with propofol stearate significantly inhibited the growth of MDA-MB-231 cancer cells whereas propofol palmitate exhibited cytotoxicity towards A549 cancer cells. However, both of the conjugates showed significant ($p < 0.05$) growth inhibition of all types of tested cancer cells in a dose-dependent manner. The preliminary results suggest that novel conjugates possess anticancer properties that reduce the proliferation of cancer cells *in vitro*.

Keywords: Saturated fatty acids, Propofol, Cytotoxicity, Cancer cell lines.

INTRODUCTION

Anticancer drugs despite showing progress in the treatment of malignant diseases are frequently associated with systemic toxicity and other side-effects[1,2]. Their efficient widespread distribution and rapid elimination are highly desirable through selecting and/or developing suitable substitutes. The common approach to overcome such obstacles and to enhance the drug efficacy is to use the anticancer drug conjugated with other substitute. Therefore, a molecule that shows suitable properties is chemically altered by coupling to another therapeutically active compound to obtain the best combination of properties informing the most effective anticancer drug[3,4]. A variety of anticancer drugs with dietary supplementations have been successfully evaluated because of their better treatment responses and less toxicity[5,6]. Among these chemically modified drugs are the esters of fatty acids, preferred as anticancer drug conjugates because of their lower toxicity, better specificity and potential bioactivities towards various therapeutic targets[7,8]. The synthetic phenolic ester conjugates of various unsaturated fatty acids have been reported to be far more efficacious than free fatty acids against various types of cancer cell lines [9-13]. Among the various categories of fatty acids, long chain saturated fatty acids (LCSFAs) are a class of lipids that contain 16-20 carbons and no double bonds along the carbon chain. The two main LCSFAs *i.e.*, stearic acid and palmitic acid, are two of the most common dietary fatty acids which play an important role in the cellular physiological processes. They are freely present in serum, with stearic acid and palmitic acid accounting for close to 13 and 28 % of the total fatty acids, respectively[14,15].

Dietary stearic acid (SA; C18) has been reported for its therapeutic potential against breast cancers. *in vitro*[16-18], *in vivo*[19,20] and epidemiological studies[21], all support dietary stearic acid to be associated with the inhibition of breast cancer. In our previous study, we also reported that an ester derivative of stearic acid inhibits the growth of human breast cancer cells *in vitro* and induce apoptosis[22]. Stearic acid is found in relatively high concentrations in foods like beef, chocolate and milk fats. Similarly, palmitic acid (PA; C16) is most commonly found in butter, cheese, milk and meat. Likewise stearic acid, palmitic acid is also known for its selective cytotoxicity towards cancer cells. DNA topoisomerase I is a prime molecular target of palmitic acid in tumor cells[23]. Studies have shown that treatment of human leukemic cells[23] or lung adenocarcinoma cell line A549[24] with palmitic acid results in decrease in cell viability. Its antitumor activity has also been reported in mice[23]. Therefore considering the anticancer role of palmitic acid, we prepared its ester derivative just like that of stearic acid. Since

our stearic acid conjugate showed anticancer efficacy towards breast cancer, in the present study we tested it and palmitic acid ester conjugate for anti-proliferative activities against other types of cultured human cancer cells. Both ester conjugates were synthesized by conjugating them with 2,6-diisopropylphenol (propofol). Propofol has been found to have a protective role against human cancer cells[25]. Besides being an intravenous anaesthetic agent in humans and animals[26], it is also a potent antioxidant. The studies have shown the efficacy of propofol in enhancing the antineoplastic activity of various polyunsaturated fatty acids in various human carcinoma cells[10,12] and in augmenting the apoptosis in carcinoma cells[27]. Recently, we have also shown that ester conjugates of fatty acid (saturated and unsaturated) with propofol inhibits proliferation, adhesion and migration of human cancer cells[13,22]. In recent years, more and more evidences indicate that propofol has the ability to influence the motility, proliferation and invasiveness of cancer cells *in vitro* and *in vivo* [25,28,29].

In the present study, in continuation of our previous work[22], herein we report the synthesis and characterization of ester conjugates of stearic acid and palmitic acid. The synthesized conjugates were characterized on the basis of UV, ^{1}H NMR and ^{13}C NMR and FAB-MS spectroscopy. As anticancer regimen the *in vitro* enhanced efficacies of novel anticancer agents was largely based on the testing of cytotoxic activity against a panel of human cancer cell lines.

EXPERIMENTAL

All the reagents used during the course of the experiment were purchased from Sigma (St Louis, MO, USA) whereas fetal calf serum was procured from Bio-Whittaker, Houston, TX, USA. Sterile filters of 0.22 µm size were purchased from Millipore, Billerica, MA, USA. Thin-layer chromatography (TLC) plates (60 Å, 0.2 mm thick) and silica gel (60-120 mesh) were purchased from Fisher Scientific, Loughborough, LE, UK.

Cell cultures: Five human cancer cell lines: HepG2, hepatocellular carcinoma; LOVO, colon malignant melanoma; A549, lung adenocarcinoma; HT1080, skin malenoma and MDA-MB-231, breast carcinoma were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell cultures were maintained in DMEM containing 5 % fetal bovine serum (Gibco, USA) and 1 % antibiotic-antimycotic solution (Gibco, USA). Cells were maintained at 5 % CO_2 in a humified chamber.

Synthesis: The protocol for synthesis of palmitic acid ester conjugate was the same as that used for the synthesis of stearic acid ester conjugate[22]. Firstly, the saturated fatty acid (stearic acid or palmitic acid) was dissolved in DCM (dichloromethane). The coupling reagent DCC (N,N-dicyclohexylcarbodiimide) was added to the reaction mixture and was stirred for 10 min. For esterification of propofol to the fatty acid, propofol and a catalyst DMAP (4-dimethylaminopyridine) was mixed in the reaction mixture. After stirring the mixture for overnight in the dark, it was filtered and concentrated under reduced pressure to yield the product. Progress of the synthesized product was viewed under UV lamp on Thin Layer Chromatographic (TLC) plates by using iodine vapours. The semi-solid mass was further purified by silica gel column chromatography using n-hexane and diethyl ether (1:1 v/v) as eluent.

Spectral analysis: Presence of propofol in the synthesized conjugate was assessed by UV spectroscopy on a UV Mini-1240 spectrophotometer, Shimadzu, Kyoto, Japan. Proton (^{1}H) and Carbon-13 (^{13}C) NMR spectra were taken from JOEL-NMR Eclipse spectrometer. Chemical shifts were recorded as delta values in parts per million (ppm) relative to TMS. The multiplicity of the signals was documented as: s, singlet; d, doublet; t, triplet; m, multiplet. $CDCl_3$ was used as solvent for NMR unless otherwise stated. The FAB-MS spectrum was recorded on a JEOL SX 102 Mass Spectrometer/Data System using Argon/Xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature. M-Nitrobenzoyl alcohol (NBA) was used as the matrix. All the analysis was performed in triplicate.

2,6-Diisopropylphenyl stearate (propofol stearate): Yield 66 %; ^{1}H NMR ($CDCl_3$) δ : 0.88 (t, 3H, *J* = 6.68, 7.04 Hz), 1.19 (d, 1H, *J* = 6.88 Hz) 1.31-1.18 (m, 38H), 1.40 (m, 1H), 1.60 (m, 1H), 2.30 (t, 1H, *J* = 7.44, 7.68, Hz), 3.19-3.12 (m, 2H), 3.66 (s, 2H), 6.89 (t, 1H, *J* = 7.60, 7.68 Hz), 7.05 (d, 2H, *J* = 7.64 Hz); ^{13}C NMR ($CDCl_3$), δ : 14.15, 22.73, 22.77, 25.0, 25.10, 27.13, 27.54, 29.19, 29.30, 29.40, 29.49, 29.56, 29.63, 29.69, 29.73, 30.94, 31.96, 34.16, 34.24, 51.48, 120.62, 123.44, 123.88, 126.42, 133.67, 140.34, 145.64, 149.97, 172.46; MS: (*M/z* = 445, 417,100 %).

2,6-Diisopropylphenyl palmitate (propofol palmitate): Yield 60 %; ^{1}H NMR ($CDCl_3$) δ : 0.88 (t, 3H, *J* = 6.64, 7.04 Hz), 1.19-1.43 (m, 36H), 1.72-1.78 (m, 2H), 2.56 (t, 2H, *J* = 7.48, 7.56 Hz), 2.86-2.90, (m, 1H), 2.97-3.00 (m, 2H), 6.89 (d,1H, *J* = 8.24 Hz), 7.04 (dd, 1H, *J* = 2.2 Hz), 7.13 (d, 1H, *J* = 2.12 Hz,); ^{13}C NMR ($CDCl_3$), δ : 14.15, 22.72, 23.00, 24.12, 25.10, 27.49, 29.22, 29.31, 29.39, 29.52, 29.63, 29.69, 29.73, 31.95, 33.87, 34.45, 121.91, 124.37, 124.65, 139.56, 146.04, 146.51, 172.68; MS: (*m/z* = 373 (417-C_3H_7), 163, 100 %).

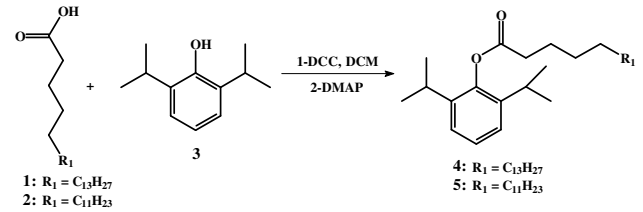
Cytotoxicity testing: MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazoliumbromide) reduction assay was used to assess the cytotoxic ability of the conjugates[12]. Cytotoxicity was examined on five human cancer cell lines; HepG2, Lovo, HT1080, A549 and MDA-MB-231. The cancer cells at a concentration of 5×10^5 cells/200 mL/well were seeded into 96-well culture plates in a specific medium containing 5 % FCS and grown for 24 h. After 24 h, various concentrations (0-30 µM) of conjugates or control were used to treat the cell. Ethanol as vehicle control and doxorubicin as positive control was also used to treat the cells using the same culture conditions. Triplicate wells of a 96-well culture plate were prepared for each concentration. Following 94 h incubation and replenishing the medium, 5 mg/mL MTT reagent (in PBS) was added to each well and incubated for 2-3 h at 37 ºC. After incubation, from each well the supernatant was aspirated and 100 µL of DMSO was added. The absorbance was measured at 620 nm and concentrations of conjugates that inhibit 50 % of cell growth (IC_{50}) were recorded.

Statistical analysis: Results are expressed as the means ± SD in triplicates for each treatment. Individual treatments were tested against the control by using Student's t-tests. Significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Cancer is a prominent cause of death in developing countries and the second leading cause of deaths in western countries after heart disease[30]. The problem contributing to cancer development is associated with various risk factors. Some studies have implicated that fatty acids and high intake of dietary fat is associated with appearance of cancer at certain sites[31,32]. However, specific saturated, monounsaturated, or polyunsaturated fatty acids are reported not to affect cancer risk[33]. The therapeutic effects of dietary fatty acids on tumor developments and metastasis are supported by studies using cultured cells and animal models[34-36]. Among dietary fatty acids, saturated fatty acids are known to inhibit breast cancer cell proliferation[35]. The present study is based on the synthesis and anticancer potential of ester conjugates derived from LCFAs (stearic acid or palmitic acid) and propofol.

A terminal carboxylic group of stearic acid and palmitic acid were directly coupled with the C1-hydroxy group of propofol with the help of DCC in the presence of a catalytic DMAP to produce the ester conjugates in quantitative yields (**Scheme-I**). The conjugates, propofol stearate and propofol palmitate were obtained as colourless viscous oil after the purification on silica gel column with *n*-hexane/ethyl acetate (1:1) as eluent. The conjugate, propofol stearate was obtained with a moderately high yield of 66 % and Rf value of 0.68. Propofol palmitate, the second conjugate, was obtained with a yield of 60 % and R_f value of 0.66 (**Scheme-I**).



Scheme-I: Schematic representation of chemical synthesis of propofol stearate and propofol palmitate. Both the ester conjugates were synthesized by esterification of the terminal carboxylic moiety of fatty acid to the hydroxyl moiety of propofol

The chemical formation of the conjugates was confirmed with the help of spectroscopic data. The UV spectrum was used to establish the formation of an ester conjugate. There was distinct new peak of propofol stearate at 270 nm and of propofol palmitate at 265 nm. The presence of single absorption peak of conjugates in the ultraviolet range (Fig. 1) means that the esterification reaction was carried out successfully resulting in the formation of novel conjugate.

The signals associated with the formation of novel propofol-LCSFA conjugates were recorded in their ^{1}H NMR and ^{13}C NMR spectra. Assignments of the signals were based on the chemical shift and intensity pattern of specific spectrum. In ^{1}H NMR spectrum, the absence of signal of phenolic proton as well as hydroxyl proton of carboxylic group at δ 4.99 and 10.5-13.5 ppm confirm the synthesis of propofol stearate and propofol palmitate, respectively. The methyl protons of isopropyl chain of the ring of propofol derivative were observed as a doublet and multiplet of propofol stearate and propofol palmitate at 1.19 ppm and 1.72-1.78, respectively. The -$COCH_2$- protons resonated in the range of 3.19-3.12 ppm

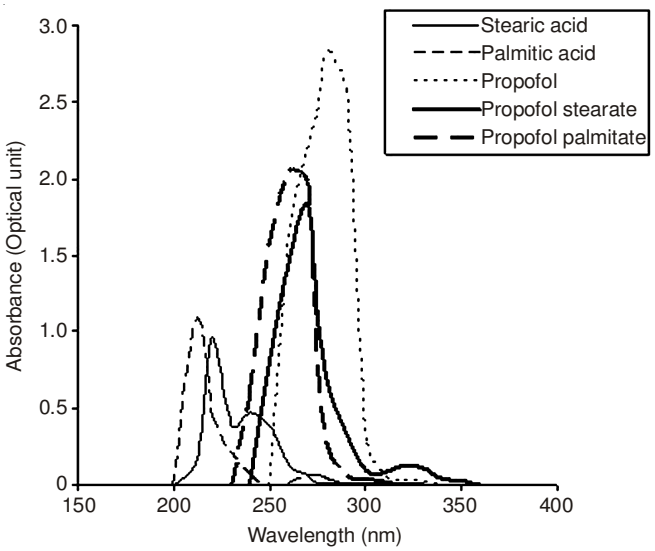


Fig. 1. UV absorbance spectra of novel ester conjugates. The UV spectroscopy establishes the formation of propofol stearate and propofol palmitate. Reference peaks of parent compounds are also shown

and triplet at 2.56 ppm, respectively, while Ar-CH < were resonating as a multiplet at 3.19-3.12 and 2.97-3 ppm, respectively. The aromatic protons appeared in the usual range of 6.89-7.13 ppm for propofol stearate and propofol palmitate, respectively.

The assignments of the ^{13}C signal for >C=O group of ester was quite specific and was observed at 172.46 and 172.68 ppm for propofol stearate and propofol palmitate, respectively. The terminal methyl group appeared at 14.15 ppm. A few other significant carbon signals were recorded at 34.24 and 34.45 ($COCH_2$) ppm, respectively.

The high-resolution FAB-MS spectral analysis further confirmed the structures of both the conjugates (Fig. 2). The FAB-MS of propofol stearate showed molecular ion peak at *m/z*: 445 $(M)^+$ followed by fragment ion peaks at 91, 163, 205, 239, 333 and *m/z*: 375. The base peak at *m/z*: 417 clearly indicate that phenyl derivative incorporated with fatty acid and terminal cleavage in the ethyl linkage. There is another peak at *m/z*: 163 representing loss of terminal methyl from base peak of propofol. Moreover, the peak at *m/z*: 163 again cleaved and gave peak at *m/z*: 135 due to loss of CO from phenyl moiety. The spectrum of the conjugate follow McLafferty and subsequently loss of methyl give characteristic peak at *m/z*: 205. The important fragments and their abundance are arranged systematically: 445 $(M)^+$, 417, 415, 403, 375, 333, 267, 239, 191, 178, 177, 163, 135, 121 and *m/z*: 91 (Fig. 3).

The FAB-MS of propofol palmitate showed molecular ion peak at 337 (*m/z*-C_3H_7) followed by fragment ion peaks at *m/z* = 81, 91, 107, 119, 121, 135, 161, 163, 177, 178, 205 and *m/z* = 219 (Fig. 4). The base peak at *m/z* = 163 clearly indicates that phenyl derivative incorporate with fatty acid and represents a cleavage in ester linkage. Moreover, the peak at *m/z* = 163 again cleaved and gave peak at *m/z* = 135 due to the loss of CO from phenyl moiety (Fig. 5). The spectrum of propofol palmitate also follow McLafferty and loss of methyl group give characteristic peak at *m/z* = 205. Another peak at *m/z* = 177 follow McLafferty ion indicating the cleavage of ester linkage and presence of propofol moiety. Other peaks at diffe-

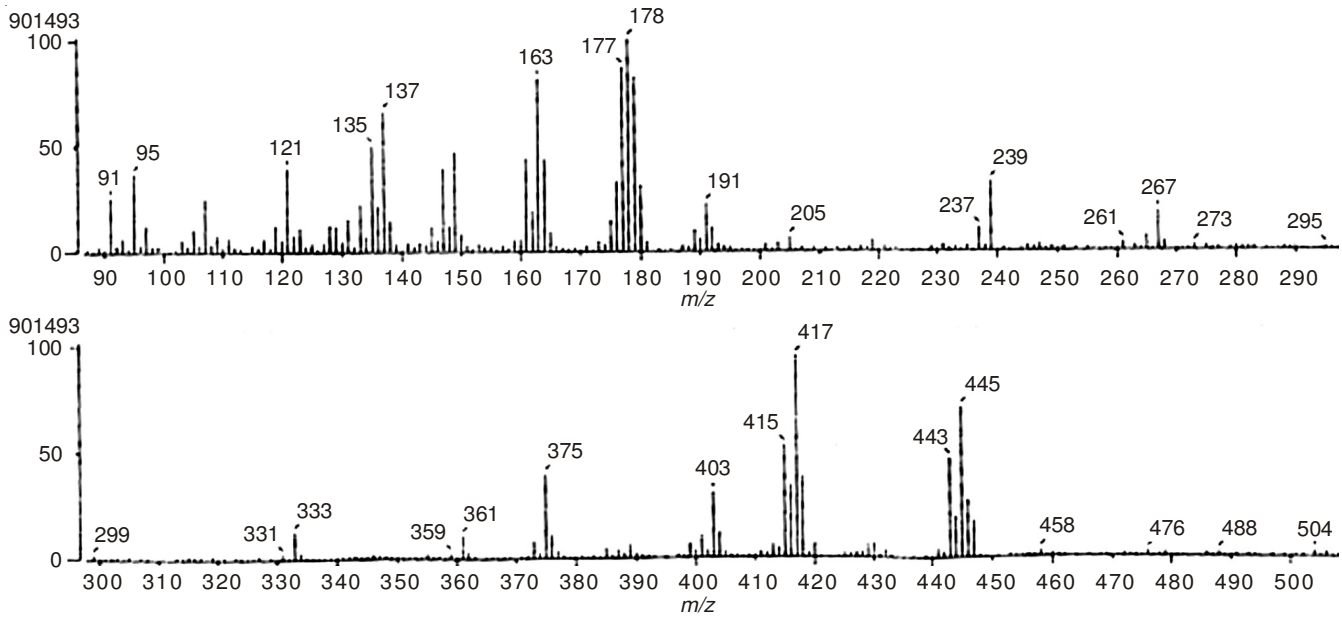


Fig. 2. FAB-MS spectrum of an ester conjugate, propofol stearate

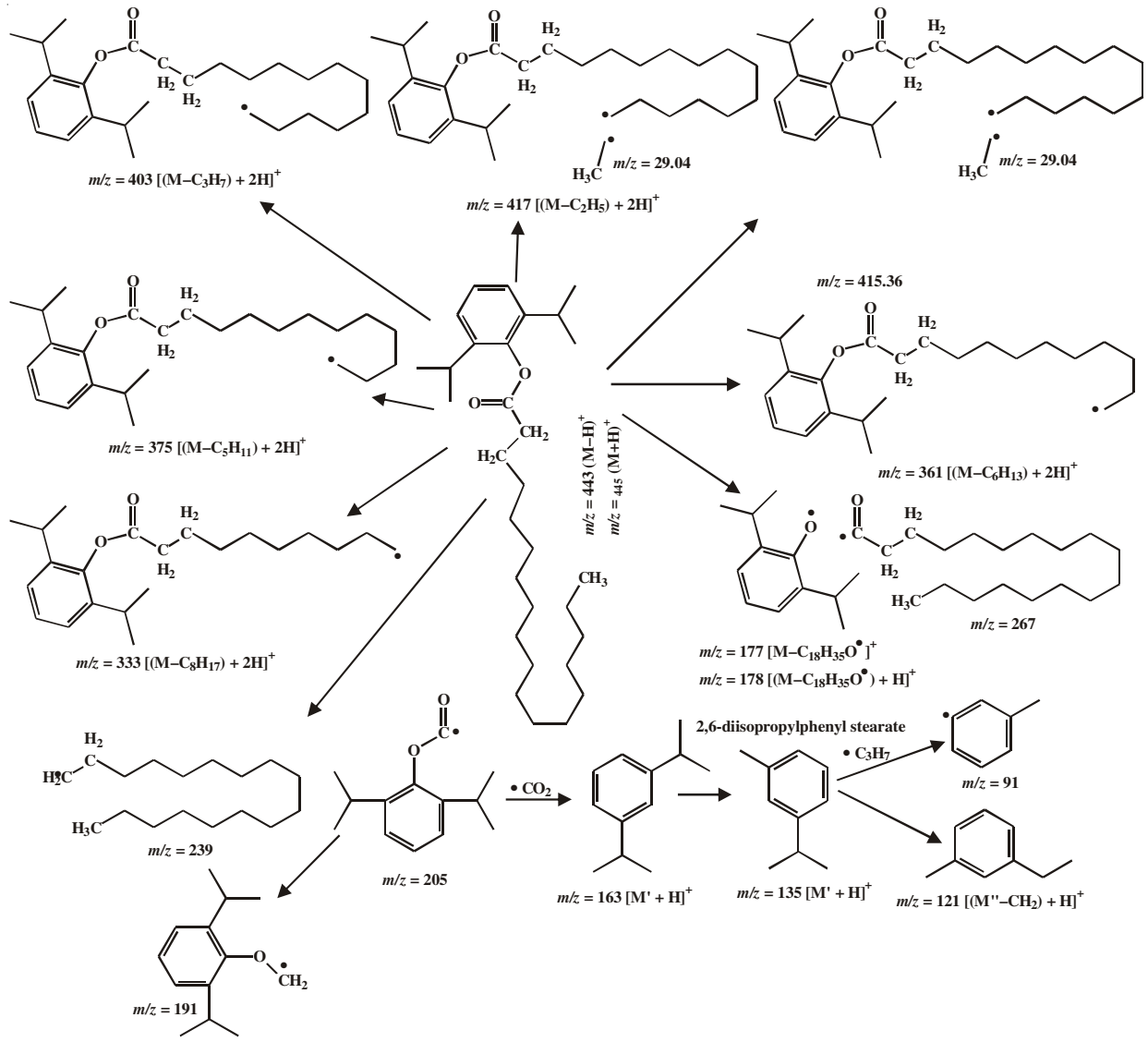


Fig. 3. Mass fragmentation pattern of propofol stearate. The important molecular ion peak and generated fragment peaks are represented diagrammatically

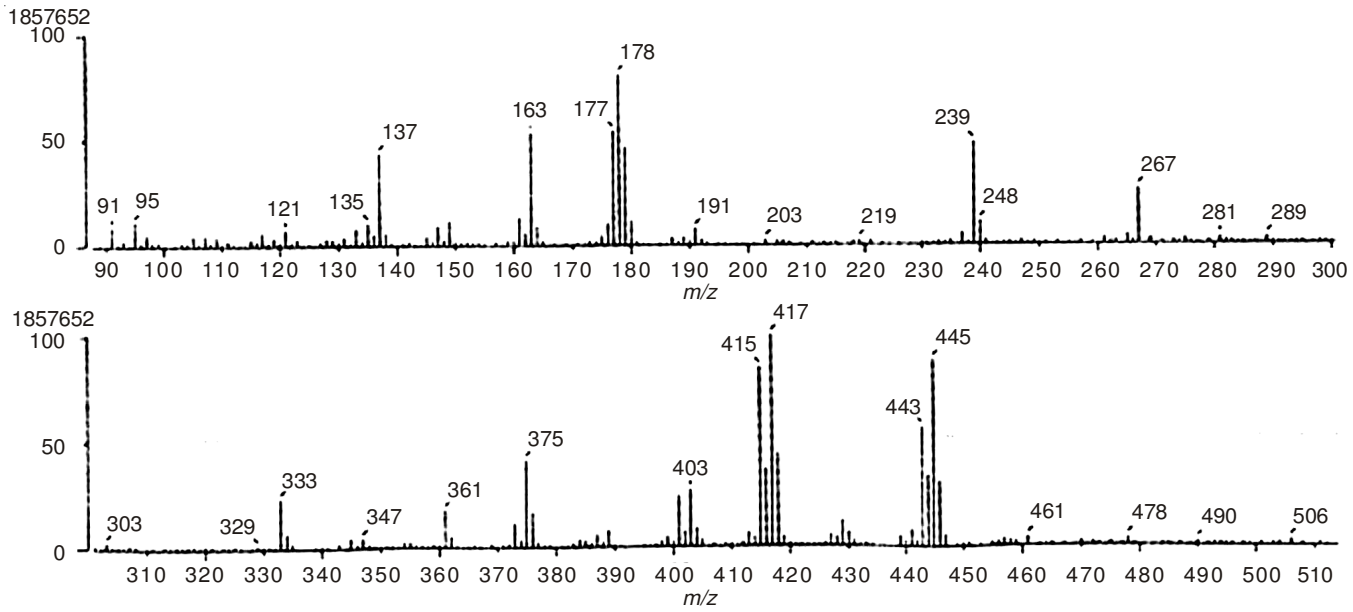


Fig. 4. FAB-MS spectrum of an ester conjugate, propofol palmitate

rent *m/z* show long chain fatty acid fragmentation patterns as mentioned in the scientific literature.

The spectral observations show that coupling reaction between LCFA (stearic acid or palmitic acid) and propofol is quite straightforward in nature. The absence of signal of hydroxyl group in ^{1}H NMR spectra confirms the synthesis of a new product. Moreover, shift in signals in ^{13}C NMR indicates that the ester bond was formed at C-19 hydroxyl position of propofol.

The conjugates were tested for their cytotoxic effect on the growth of cancer cells. Therefore, both ester conjugates were assayed *in vitro* against cultured HepG2, MDA-MB-231, LOVO, HT1080 and A549 cells. The IC_{50} values were determined using MTT assays, in which panel of cancer cells were exposed to 0-30 µM propofol stearate or 0-30 µM propofol palmitate. As shown in Fig. 6, treatment with propofol stearate and propofol palmitate significantly inhibited the growth of the cancer cells in a concentration dependent manner ($p < 0.05$). The sensitivities of cultured cells to each conjugate are shown in Table-1 in terms of IC_{50} values. Propofol stearate was more active conjugate with IC_{50} values ranging from 10.1 µM for MDA-MB-231 to 18.2 µM for $HepG_2$ cells. IC_{50} values of propofol palmitate ranged from 11.9 µM for A549 to 19.5 µM for HepG2 cells. The cell toxicity observed with conjugates against cancer cells was significantly higher ($p < 0.05$) than the parent fatty acid (stearic acid or palmitic acid) and propofol. The results showed that all the tested cancer cells were significantly sensitized to both conjugates and the ester conjugates were able to effectively cause cancer cell death at tested concentrations ($p < 0.05$). This implies that these conjugates are stable in media. Harvey *et al.*[11] has reported that the presence of two propyl groups around the ester linkages provide the stability to the conjugate. If there is no protective group around the ester linkage the conjugate is quickly cleaved off and loses its effectiveness. The results of present investigations also indicate that the presence of two methyl groups in 2,6-propofol around the ester bond provide structural hindrance that may be responsible for such activity.

The effects of propofol stearate on the breast cancer MDA-MB-231 cell line are consistent with our earlier published data[22]. In the case of MDA-MB-231 cells the effect of propofol stearate was more pronounced (10.1 µM IC_{50}) than that found for propofol palmitate (15.7 µM IC_{50}). Similarly, the effect of propofol palmitate was more pronounced (11.9 µM IC_{50}) on A549 cells than that found for propofol stearate (16.1 µM IC_{50}). The growth inhibition of HepG2 cells by both conjugates proved least potency (18.2 and 19.5 µM IC_{50} for propofol stearate and propofol palmitate, respectively) as compared with LOVO, A549, HT1080 and MDA-MB-231. Both conjugates were equally potent showing moderate growth inhibition of LOVO and HT1080 cancer cells. However, such significant differences in the anti-proliferative activity between the synthesized ester conjugates and the parent controls, indicate a specific character of the former, probably arising from the introduction of the 2,6-propofol moiety. It is anticipated that propofol as a partly lipophilic agent will facilitate the intake

TABLE-1
CYTOTOXICITY OF ESTER CONJUGATES ON CULTURED HUMAN CANCER CELLS. THE CYTOTOXIC EFFECT OF PROPOFOL STEARATE AND PROPOFOL PALMITATE WAS TESTED AT CONCENTRATION 0-30 µM. IC_{50} VALUES SHOW THE CONCENTRATION AT WHICH 50% GROWTH INHIBITION OF HEPG2, LOVO, A549, HT1080 and MDA-MB-231 CANCER CELLS OCCUR. VALUES ARE MEANS OF THREE OBSERVATIONS

Conjugates	Cell lines (IC_{50}, µM)				
	HepG2	LOVO	A549	HT	MDA-MB-231
Propofol stearate	18.2	17.4	16.1	13.7	10.1
Propofol palmitate	19.5	14.6	11.9	12.8	15.7

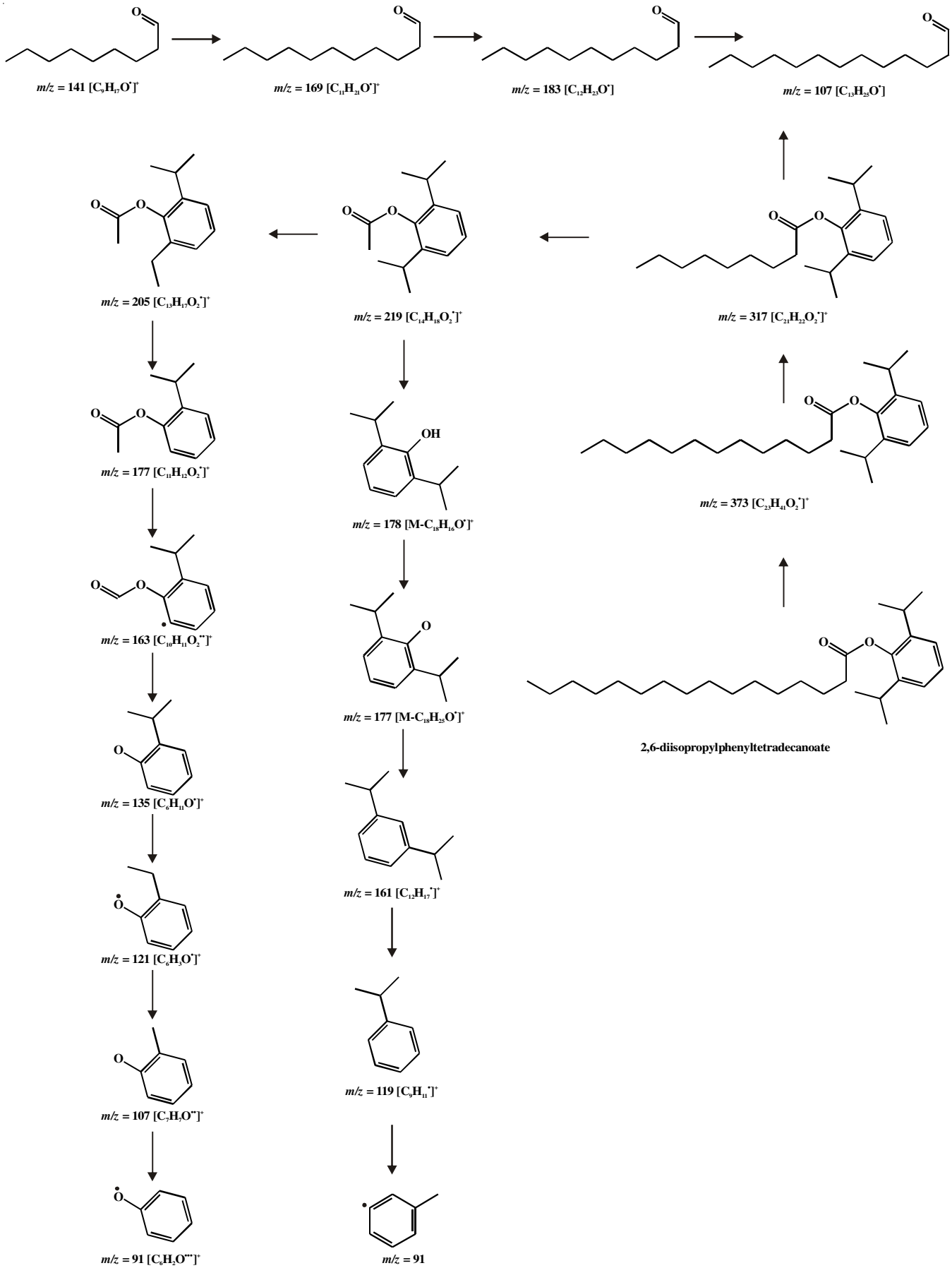


Fig. 5. Mass fragmentation pattern of propofol palmitate. The important molecular ion peak and generated fragment peaks are represented diagrammatically

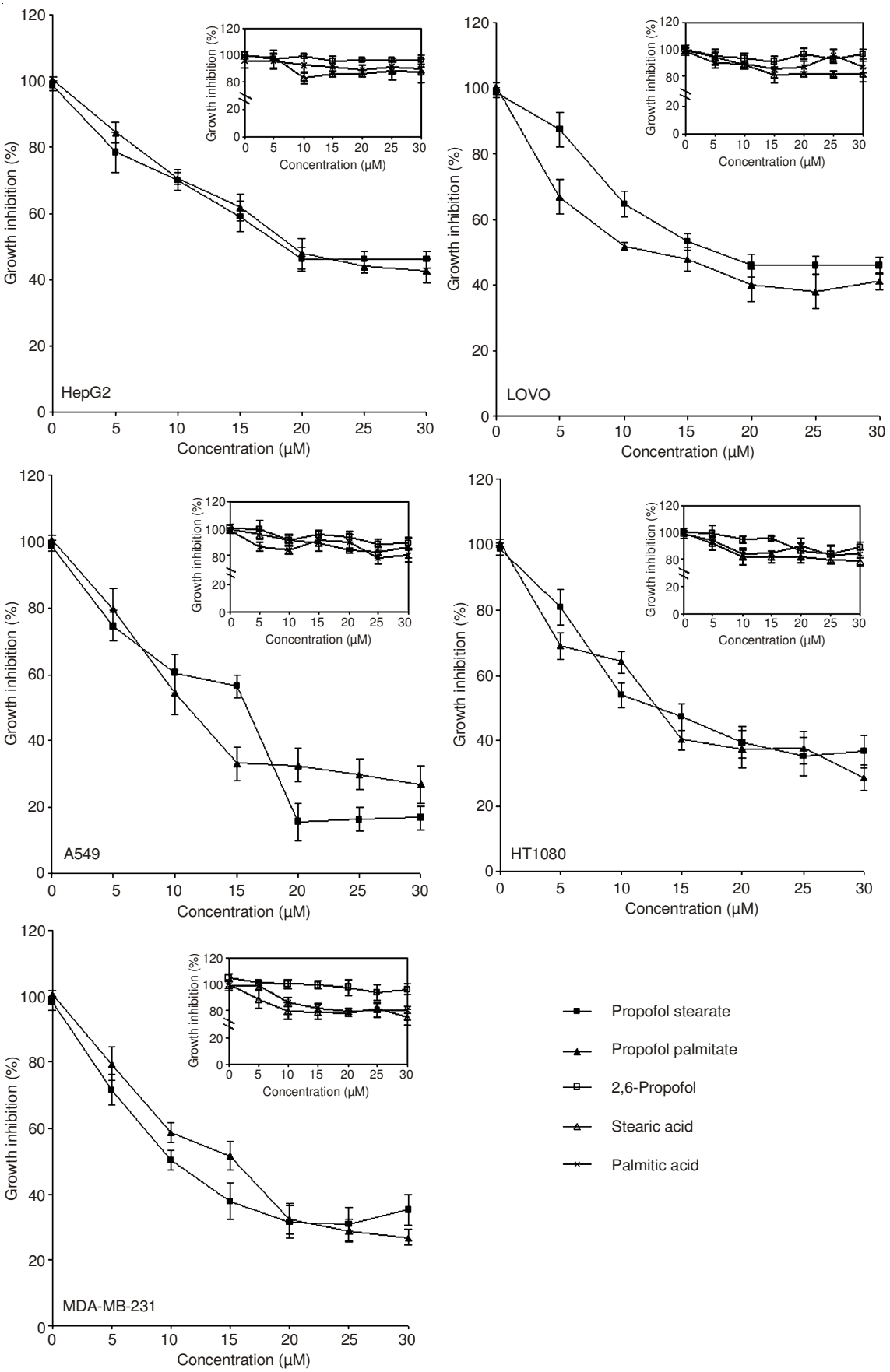


Fig. 6. MTT assay showing effects of novel ester conjugates on cultured human cancer cells. Growth inhibitory effects of propofol stearate and propofol palmitate on HepG2, LOVO, A549, HT1080 and MDA-MB-231 cancer cell lines. Effects of parent compounds are shown in inset of respective graph. Results are expressed as the mean ± SD of three experiments. Significant differences ($p < 0.05$) are indicated with an asterisk

of the chemotherapeutic fatty acid and strong hydrophobic nature of fatty acid will help in rapidly trans-locating against the plasma membrane.

Conclusion

The present study demonstrates the efficient and high-yielding synthetic method for the preparation of saturated fatty acid conjugates. The conjugates were positively identified and characterized using ^{1}H and ^{13}C NMR and FAB-MS spectrum. Both conjugates *viz*, propofol stearate and propofol palmitate exhibits potent anti-proliferative effects on various cultured cancer cells that are associated with decreased growth inhibition. The exceptional efficacy of the conjugates against cancer cells provides bright prospects for their applications in cancer chemotherapy. Further investigation will be required to determine whether these effects *in vitro* can be seen *in vivo* as well.

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