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ORIGINAL RESEARCH

Synthesis and evaluation of apoptotic induction of human cancer cells by ester derivatives of thujone

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Abstract

Thujone (**1**), thujol (**2**), and aromatic thujol esters (**3–9**) were evaluated for their ability to induce cell death in human cervical (HeLa), melanoma (A375), and colon (HCT-116) cancer cell lines, using etoposide as a positive control. The compounds showed dose-dependent activity at concentrations ranging from 50–400 µg/mL. Etoposide exhibited an IC_{50} value of 116 µg/mL in HeLa cells, and α-thujone, α/β-thujone (7:1), and thujol showed comparable activity with IC_{50} values of 191, 198, and 136 µg/mL, respectively. All seven ester derivatives were cytotoxic to HeLa and HCT-116 cells, while a subset was cytotoxic to A375 cells. In HeLa cells, t-cinnamate (**4**), t-isonicotinate (**5**), t-nicotinate (**6**), and t-furoate (**8**) were more potent than either α-thujone or α/β-thujone. Similarly, t-furoate (**8**) was more potent than thujone in A375 cells, and t-isonicotinate (**5**) and t-nicotinate (**6**) were more potent against HCT-116 cells. Based on cell morphology, PARP cleavage and an increase in the caspase-3/7 levels, the esters exert their cytotoxic effects by induction of apoptosis.

Keywords Thujone · Thujol esters · HeLa cells · HCT-116 cells · A375 cells · Anticancer · Cell death induction · Apoptosis

Introduction

Monoterpenes are produced by a number of plant species and are under investigation for human use and applications because of their varied biological properties. They are present in high quantities in a variety of plant essential oils, and many exert anticancer activity against human tumor cell lines with varying selectivity and mechanisms of action (Sobral et al. 2014). Limonene, carvacrol, thymol, and eugenol are known to have many pharmacological activities, and their activity against a range of human cancer cells is well-documented. Limonene and its naturally occurring metabolite, perillyl alcohol, have been demonstrated to induce both cell cycle arrest and apoptosis in colon cancer, non-small cell lung cancer and lung adenocarcinoma cell lines (Bardon et al. 2002; Elegbede et al. 2003; Yeruva et al. 2007; Mukhtar et al. 2018). Mechanistically, they do so by modulating the expression levels of p21, the universal CDK inhibitor, and key apoptotic proteins (Bardon et al. 2002; Elegbede et al. 2003; Yeruva et al. 2007). Carvacrol has shown antiproliferative effect against human breast, cervical, liver, and oral squamous cell carcinoma cell lines, with apoptosis as the primary mechanism of action (Özkan and Erdoğan 2011; Yin et al. 2012; Al-Fatlawi and Rahisuddin 2014; Dai et al. 2016). The isomer of carvacrol, thymol, has also demonstrated significant antiproliferative and apoptotic effects against breast, liver, and gastric cancer cells lines (Özkan and Erdoğan 2011; Kang et al. 2016; Jamali et al. 2018). Eugenol has been shown to induce apoptosis in leukemia, melanoma, breast, and colon cancer cells. In addition, eugenol has demonstrated the ability to reduce tumors in animal models (Jaganathan and Supriyanto 2012).

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Metabolites and synthetically modified derivatives of monoterpenes have also emerged as promising candidates for use as anticancer agents, and very often are more potent than the parent compounds. Perillic acid is a metabolite of both limonene and perillyl alcohol. It has a similar mechanism of action as both compounds but demonstrates greater potency. Similarly, various synthetic derivatives of perillyl alcohol, including esters and epoxides, showed enhanced anticancer activity relative to the parent compound (Mukhtar et al. 2018). Thiosemicarbazone derivatives of limonene showed moderate to excellent activity against a wide range of tumor cell lines (Vandresen et al. 2014). Azomethine linked hybrids containing carvacrol, thymol, and/or eugenol have shown comparable or enhanced activity relative to Doxorubicin in colon and pancreatic cancer cell lines (Rajput et al. 2017).

Thujone is a monoterpene that is naturally present in the essential oil extracted from *Thuja occidentalis* (white cedar) and several other plant species. Extracts of *Thuja occidentalis* have been reported to have several biological properties, including bactericidal, fungicidal, insecticidal, apoptotic, and anti-oncogenic (Naser et al. 2005; Alves et al. 2014). Thujone exists as two diastereomers, α-thujone and β-thujone, with α-thujone being primarily responsible for mediating the above-mentioned biological effects. The biological basis for the insecticidal activity of α-thujone is its ability to function as a neurotoxin by binding to and antagonizing the activity of GABA receptors in the brain (Hold et al. 2000). The antineoplastic effects of thujone or thujone-containing extracts have been examined in some cancer cell lines, including melanoma, cervical, and glioblastoma cells and is based on the ability to induce apoptosis and decrease angiogenesis (Biswas et al. 2011; Torres et al. 2016; Privitera et al. 2019). The cinnamate derivative of thujone is known, but to our knowledge there have been no reports on its potential as an anticancer agent. In this study, we examined the ability of seven aromatic ester derivatives of thujone to induce cell death of human cervical (HeLa), melanoma (A375), and colon (HCT-116) cancer cells, and determined their mechanism of action.

Materials and methods

Experimental

General experimental procedures

All solvents and reagents were ACS grade and were obtained from either Fisher Scientific or Aldrich Chemical Company. Solvents were dried according to standard procedures. Purification was performed by column chromatography using either a Teledyne Isco system with Rf-Gold prepacked silica gel columns (20–40 mm), or the traditional method using 230–400 mesh silica gel. NMR spectra were recorded on a JEOL ECZ 400S spectrometer (^{1}H: 400 MHz, ^{13}C: 100 MHz) using $CDCl_3$ as solvent and TMS as internal standard. FTIR data were acquired using a Thermo Scientific Nicolet iS50 FT-IR spectrometer, and UV data were obtained using a Varian Cary 4000 UV–Vis spectrophotometer. Optical rotations were measured using a Rudolph Research Analytical AUTOPOL III polarimeter. HRESIMS data were obtained on a Waters SYNAPT G2-S QTOFMS system, and EIMS data were measured on a ThermoFisher Scientific ISQ™ Series Single Quadrupole GC-MS system. The BioRad iMark Microplate absorbance reader was used for XTT assays to determine cell viability.

Sodium borohydride reduction of thujone (1) to thujol (2)

A solution of α/β-thujone, 7/1 (25.01 g) in absolute EtOH (200 mL) was cooled in an ice bath, and $NaBH_4$ (18.68 g) was added slowly over a 10 min period. The solution was stirred at room temperature for 3 h, followed by removal of the EtOH in vacuo. Saturated sodium chloride (200 mL) was added to the residue, and 10% aqueous HCl (15 mL) was added slowly. The resulting solution was extracted with CH_2Cl_2 (3 × 75 mL). The CH_2Cl_2 solution was dried (Na_2SO_4), filtered, and concentrated to afford thujol (**2**).

Thujol, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-ol (2)

Colorless oil (98%);$[\alpha]_D^{25}$ -3.4 (c 0.019, MeOH); IR (ATR) ν_{max} 3375 (O–H) cm^{-1};

2A$_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 3.95$ (1 H, H-3), 2.15 (1 H, H-4), 1.94/1.60 (2 H, H-2), 1.25 (1 H, H-7), 0.95 (3 H, H-8), 0.92 (3 H, H-10), 0.89 (3 H, H-9), 0.85 (1 H, H-5), 0.35/0.22 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 72.8 (CH, C-3), 37.6 (CH, C-4), 33.4 (CH, C-7), 31.3 (C, C-1), 33.3 (CH_2, C-2), 28.2 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 14.5 (CH_3, C-10), 14.4 (CH_2, C-6).

2A$_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 3.87$ (1 H, H-3), 2.05 (1 H, H-4), 1.92/1.52 (2 H, H-2), 1.28 (1 H, H-7), 0.92 (3 H, H-8), 0.91 (3 H, H-10), 0.89 (3 H, H-9), 0.80 (1 H, H-5), 0.41-0.38 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 80.3$ (CH, C-3), 45.3 CH, (C-4), 34.5 (C, C-1), 36.0 (CH_2, C-2), 33.0 (CH, C-7), 29.7 (CH, C-5), 20.2 (CH_3, C-9), 20.0 (CH_3, C-8), 20.3 (CH_3, C-10), 17.3 (CH_2, C-6); EIMS *m/z* 136 (32), 121 (82), 95 (100), 93 (84), 81 (52), 55 (61); HRESIMS *m/z* (pos): 155.1084 $C_{10}H_{19}O$ $[M + H]^+$ (calcd. 155.1066).

General procedure for the synthesis of thujol esters (3–9)

Acyl components: benzoyl chloride, phenacetyl chloride, cinnamoyl chloride, nicotinoyl chloride, isonicotinoyl

chloride, 2-furoyl chloride, and thiophene-2-carbonyl chloride.

A solution of the diastereomeric mixture of thujol (2.00 g, 13 mmol),* triethylamine (3.6 mL, 26 mmol), and 4-dimethylaminopyridine, DMAP (0.40 g, 3.3 mmol), in CH_2Cl_2 (20 mL) was cooled in an ice-bath. The acid chloride/acid anhydride (26 mmol) was added slowly, and the reaction mixture was allowed to warm to room temperature and stirred for 3–24 h, while monitoring the progress of the reaction by TLC. The dichloromethane solution was washed with saturated NaCl (30 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Silica gel column chromatography using ethyl acetate-–hexanes mixtures as eluents afforded the diastereomeric mixture of thujol esters as colorless or pale-yellow oils.

*1.5 g of thujol was used for some of the samples, and reagent ratios were adjusted accordingly.

Only the NMR data for the major diastereomers (thujol esters **$3A_1$/$3A_2$–$9A_1$/$9A_2$** are shown).

Thujol benzoate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl benzoate (3) Colorless oil (55%); $[\alpha]_D^{25}$ -10 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 273(2.78) nm; IR (ATR) ν_{max} 1712 (C=O), 1275 (C-O) cm^{-1};

$3A_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 8.04$ (2 H, H-3′/ H-7′), 7.53 (1 H, H-5′), 7.42 (2 H, H-4′/ H-6′), 4.97 (1 H, H-3), 2.50 (1 H, H-4), 2.12/1.86 (2 H, H-2), 1.29 (1 H, H-7), 0.99 (3 H, H-8), 0.97 (3 H, H-10), 0.92 (3 H, H-9), 0.85 (1 H, H-5), 0.46/0.32 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 166.2$ (C, C-1′), 132.9 (CH, C-5′), 129.6 (CH, C-4′, C-6′), 128.4 (CH, C-3′, C-7′), 130.6 (C, C-2′), 75.7 (CH, C-3), 36.1 (CH, C-4), 33.4 (CH, C-7), 31.1 (C, C-1), 30.3 (CH_2, C-2), 28.1 (CH, C-5), 20.3 (CH_3, C-9), 19.8 (CH_3, C-8), 15.5 (CH_3, C-10), 14.6 (CH_2, C-6);

$3A_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.99$ (2 H, H-3′/H-7′), 7.53 (1 H, H-5′), 7.42 (2 H, H-4′/ H-6′), 5.01 (1 H, H-3), 2.21 (1 H, H-4), 2.24/1.78 (2 H, H-2), 1.36 (1 H, H-7), 1.10-0.85 (10 H, H-5, H-8, H-9, H-10), 0.79/0.46 (2 H, H-6);

^{13}C NMR (CDCl3, 100 MHz): $\delta = 166.0$ (C, C-1′), 132.8 (CH, C-5′), 129.5 (CH, C-4′, C-6′), 128.4 (CH, C-3′, C-7′), 130.8 (C, C-2′), 82.7 (CH, C-3), 42.8 (CH, C-4), 34.5 (C, C-1), 33.2 (CH_2, C-2), 32.8 (CH, C-7), 29.6 (CH, C-5), 20.3 (CH_3, C-10), 20.0 (CH_3, C-9), 19.9 (CH_3, C-8), 16.1 (CH_2, C-6); EIMS *m/z* 136 (41), 121 (34), 105 (100), 93 (49), 77 (51); HRESIMS *m/z* (pos): 259.1710 $C_{17}H_{23}O_2$ $[M + H]^+$ (calcd. 259.1692).

Thujol cinnamate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl cinnamate (4) Colorless oil (43%); $[\alpha]_D^{25}$ -15 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 278(4.25) nm; IR (ATR) ν_{max} 1709 (C=O), 1168 (C–O) cm^{-1};

$4A_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.65$ (1 H, H-3′), 7.51 (2 H, H-5′/ H-9′), 7.37 (2 H, H-6′/H-7′/ H-8′), 6.42 (1 H, H-2′), 4.85 (1 H, H-3), 2.45 (1 H, H-4), 2.05/1.81 (2 H, H-2), 1.28 (1 H, H-7), 0.98 (3 H, H-8), 0.93 (3 H, H-10), 0.91 (3 H, H-9), 0.87 (1 H, H-5), 0.44/0.30 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 166.7$ (C, C-1′), 144.7 (CH, C-3′), 134.5 (C, C-4′), 130.3 (CH, C-7′), 128.9 (CH, C-5′, C-9′), 128.1 (CH, C-6′, C-8′), 118.3 (CH, C-2′), 75.3 (CH, C-3), 35.9 (CH, C-4), 33.2 (CH, C-7), 30.9 (C, C-1), 30.2 (CH_2, C-2), 28.1 (CH, C-5), 20.2 (CH_3, C-9), 19.8 (CH_3, C-8), 15.4 (CH_3, C-10), 14.5 (CH_2, C-6);

$4A_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.61$ (1 H, H-3′), 7.51 (2 H, H-5′/ H-9′), 7.37 (2 H, H-6′/H-7′/ H-8′), 6.37 (1 H, H-2′), 4.89 (1 H, H-3), 2.15 (1 H, H-4), 2.20/1.72 (2 H, H-2), 1.35 (1 H, H-7), 1.03 (3 H, H-8), 0.96 (3 H, H-10), 0.92 (3 H, H-9), 0.86 (1 H, H-5), 0.73/0.42 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 166.4$ (C, C-1′), 144.4 (CH, C-3′), 134.5 (C, C-4′), 130.3 (CH, C-7′), 128.9 (CH, C-5′, C-9′), 128.1 (CH, C-6′, C-8′), 118.9 (CH, C-2′), 82.2 (CH, C-3), 42.8 (CH, C-4), 34.5 (C, C-1), 33.4 (CH_2, C-2), 32.8 (CH, C-7), 29.6 (CH, C-5), 20.3 (CH_3, C-10), 20.0 (CH_3, C-9), 19.9 (CH_3, C-8), 16.0 (CH_2, C-6); EIMS *m/z* 136 (40), 131 (100), 121 (32), 103 (33), 93 (40), 77 (20); HRESIMS *m/z* (pos): 285.1874 $C_{19}H_{25}O_2$ $[M + H]^+$ (calcd. 285.1849).

Thujol isonicotinate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl isonicotinate (5) Colorless oil (43%); $[\alpha]_D^{25}$ -20 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 274(3.37) nm; IR (ATR) ν_{max} 1725 (C=O), 1279 (C–O) cm^{-1};

$5A_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 8.74$ (2 H, H-4′/ H-6′), 7.80 (2 H, H-3′/H-7′), 4.94 (1 H, H-3), 2.45 (1 H, H-4), 2.08/1.85 (2 H, H-2), 1.25 (1 H, H-7), 0.90 (3 H, H-8), 0.89 (3 H, H-10), 0.86 (3 H, H-9), 0.80 (1 H, H-5), 0.40/ 0.30 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 164.6$ (C, C-1′), 150.6 (CH, C-4′, C-6′), 137.6 (C, C-2′), 122.9 (CH, C-3′, C-7′), 76.7 (CH, C-3), 36.0 (CH, C-4), 33.1 (CH, C-7), 31.1 (C, C-1), 30.2 (CH_2, C-2), 27.9 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 15.4 (CH_3, C-10), 14.6 (CH_2, C-6);

$5A_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 8.74$ (2 H, H-4′/ H-6′), 7.80 (2 H, H-3′/H-7′), 4.98 (1 H, H-3), 2.18 (1 H, H-4), 2.16/1.74 (2 H, H-2), 1.32 (1 H, H-7), 1.10-0.80 (10 H, H-5, H-8, H-9, H-10), 0.66/0.45 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 164.4$ (C, C-1′), 150.6 (CH, C-4′, C-6′), 137.9 (C, C-2′), 122.8 (CH, C-3′, C-7′), 83.6 (CH, C-3), 42.8 (CH, C-4), 34.5 (C, C-1), 33.4 (CH_2, C-2), 32.6 (CH, C-7), 29.5 (CH, C-5), 20.3 (CH_3, C-10), 19.9 (CH_3, C-9), 19.8 (CH_3, C-8), 16.1 (CH_3, C-6); EIMS *m/z* 136 (61), 121 (74), 106 (78), 93 (100), 78 (51); HRESIMS *m/z* (pos): 260.1657 $C_{16}H_{22}NO_2$ $[M + H]^+$ (calcd. 260.1645).

Thujol nicotinate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl nicotinate (6) Colorless oil (57%); $[\alpha]_D^{25}$ -20 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 263(3.32) nm; IR (ATR) ν_{max} 1719 (C=O), 1282 (C–O) cm^{-1};

6A$_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 9.15$ (1 H, H-3′), 8.71 (1 H, H-5′), 8.20 (1 H, H-7′), 7.32 (1 H, H-6′), 4.94 (1 H, H-3), 2.46 (1 H, H-4), 2.06/1.84 (2 H, H-2), 1.13 (1 H, H-7), 0.89 (3 H, H-8), 0.90 (3 H, H-10), 0.89 (3 H, H-9), 0.80 (IH, H-5), 0.40/0.28 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 164.8$ (C, C-1′), 153.4 (CH, C-3′), 150.9 (CH, C-5′), 137.0 (CH, C-7′), 126.3 (C, C-2′), 123.3 (CH, C-6′), 76.3 (CH, C-3), 36.1 (CH, C-4), 33.1 (CH, C-7), 31.1 (C, C-1), 30.3 (CH_2, C-2), 27.9 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 15.4 (CH_3, C-10), 14.6 (CH_2, C-6);

6A$_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 9.12$ (1 H, H-3′), 8.71 (1 H, H-5′), 8.20 (1 H, H-7′), 7.32 (1 H, H-6′), 4.97 (1 H, H-3), 2.22 (1 H, H-4), 2.09/1.75 (2 H, H-2), 1.36 (1 H, H-7), 1.10-0.80 (10 H, H-5, H-8, H-9, H-10), 0.69/0.43 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 164.4$ (C, C-1′), 153.3 (CH, C-3′), 150.8 (CH, C-5′), 136.9 (CH, C-7′), 126.5 (C, C-2′), 123.3 (CH, C-6′), 83.2 (CH, C-3), 42.8 (CH, C-4), 34.5 (C, C-1), 33.4 (CH_2, C-2), 32.6 (CH, C-7), 29.5 (CH, C-5), 20.3 (CH_3, C-10), 19.9 (CH_3, C-9), 19.8 (CH_3, C-8), 16.1 (CH_2, C-6); EIMS *m/z* 136 (57), 121 (68), 106 (100), 93 (92), 78 (57); HRESIMS *m/z* (pos): 260.1662 $C_{16}H_{22}NO_2$ $[M + H]^+$ (calcd. 260.1645).

Thujol thiophenate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl thiophene-2-carboxylate (7) Pale yellow oil (60%); $[\alpha]_D^{25}$ -15 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 268(3.89) nm; IR (ATR) ν_{max} 1705 (C=O), 1259 (C–O) cm^{-1};

7A$_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.77$ (1 H, H-6′), 7.52 (1 H, H-4′), 7.08 (1 H, H-5′), 4.92 (1 H, H-3), 2.47 (1 H, H-4), 2.10/1.84 (2 H, H-2), 1.26 (1 H, H-7), 0.91 (3 H, H-8), 0.90 (3 H, H-10), 0.89 (3 H, H-9), 0.87 (1 H, H-5), 0.44/0.31 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 161.9 (C, C-1′), 134.2 (C, C-2′), 133.3 (CH, C-4′), 132.3 (CH, C-6′), 127.8 (CH, C-5′), 76.0 (CH, C-3), 36.2 (CH, C-4), 33.2 (CH, C-7), 31.1 (C, C-1), 30.3 (CH_2, C-2), 28.0 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 15.5 (CH_3, C-10), 14.6 (CH_2, C-6);

7A$_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.75$ (1 H, H-6′), 7.52 (1 H, H-4′), 7.08 (1 H, H-5′), 4.96 (1 H, H-3), 2.19 (1 H, H-4), 2.21/1.78 (2 H, H-2), 1.35 (1 H, H-7), 1.10-0.85 (10 H, H-5, H-8, H-9, H-10), 0.77/0.46 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 161.6$ (C, C-1′), 134.7 (C, C-2′), 133.1 (CH, C-4′), 132.2 (CH, C-6′), 127.8 (CH, C-5′), 82.9 (CH, C-3), 42.8 (CH, C-4), 34.4 (C, C-1), 33.2 (CH_2, C-2), 32.7 (CH, C-7), 29.5 (CH, C-5), 20.3 (CH_3, C-10), 20.0 (CH_3, C-9), 19.8 (CH_3, C-8), 16.0 (CH_2, C-6); EIMS *m/z* 136 (42), 121 (37), 110 (100), 93 (52); HRESIMS *m/z* (pos): 287.1076 $C_{15}H_{20}O_2NaS$ $[M + Na]^+$ (calcd. for; 287.1076).

Thujol furoate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl furan-2-carboxylate (8) Colorless oil (73%); $[\alpha]_D^{25}$ -15 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 252(3.52) nm; IR (ATR) ν_{max} 1713 (C=O), 1295 (C–O) cm^{-1};

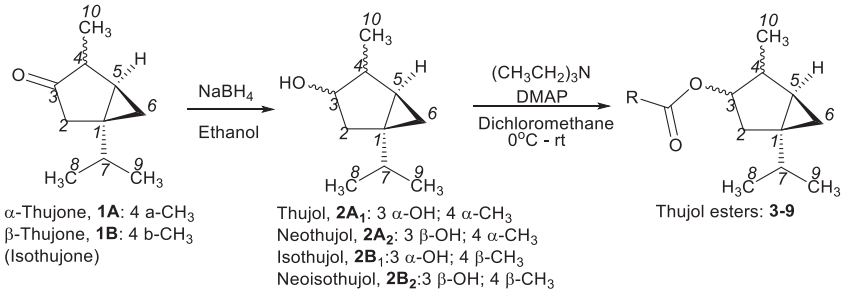
8A$_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.53$ (1 H, H-4′), 7.11 (1 H, H-6′), 6.44 (1 H, H-5′), 4.88 (1 H, H-3), 2.44 (1 H, H-4), 2.05/1.82 (2 H, H-2), 1.24 (1 H, H-7), 0.91 (3 H, H-8), 0.90 (3 H, H-10), 0.89 (3 H, H-9), 0.88 (1 H, H-5), 0.40/0.26 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 158.4 (C, C-1′), 144.9 (C, C-2′), 146.3 (CH, C-4′), 117.8 (CH, C-6′), 111.8 (CH, C-5′), 75.8 (CH, C-3), 36.0 (CH, C-4), 33.2 (CH, C-7), 30.9 (C, C-1), 30.2 (CH_2, C-2), 27.9 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 15.3 (CH_3, C-10), 14.5 (CH_2, C-6);

8A$_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.53$ (1 H, H-4′), 7.07 (1 H, H-6′), 6.44 (1 H, H-5′), 4.94 (1 H, H-3), 2.15 (1 H, H-4), 2.17/1.73 (2 H, H-2), 1.30 (1 H, H-7), 1.10-0.85 (9 H, H-8, H-9, H-10), 0.85 (1 H, H-5), 0.73/0.40 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 158.2$ (C, C-1′), 145.2 (C, C-2′), 146.2 (CH, C-4′), 117.5 (CH, C-6′), 111.7 (CH, C-5′), 82.6 (CH, C-3), 42.7 (CH, C-4), 34.3 (C, C-1), 33.3 (CH_2, C-2), 32.7 (CH, C-7), 29.4 (CH, C-5), 20.3 (CH_3, C-10), 19.9 (CH_3, C-9), 19.8 (CH_3, C-8), 15.9 (CH_2, C-6); EIMS *m/z* 136 (44), 121 (52), 95 (100), 93 (69); HRESIMS *m/z* (pos): 249.1512 $C_{15}H_{21}O_3$ $[M + H]^+$ (calcd. 249.1485).

Thujol phenylacetate, 1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl 2-phenylacetate (9) Pale yellow oil (62%); $[\alpha]_D^{25}$ -30 (c 0.0002, MeOH); UV (MeOH) λ_{max}(log ε) 258 (2.45) nm; IR (ATR) ν_{max} 1730 (C=O), 1156 (C–O) cm^{-1};

9A$_1$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.26$ (5 H, H-4′-H-8′), 4.72 (1 H, H-3), 3.58 (1 H, H-2′), 2.34 (1 H, H-4), 1.97/1.70 (2 H, H-2), 1.25 (1 H, H-7), 1.10-0.70 (10 H, H-5, H-8, H-9, H-10), 0.35/0.25 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 171.3$ (C, C-1′), 134.2 (C, C-3′), 129.4 (CH, C-4′, C-8′), 128.6 (CH, C-5′, C-7′), 127.1 (CH, C-6′), 75.7 (CH, C-3), 41.6 (CH_2, C-2′), 35.7 (CH, C-4), 33.4 (CH, C-7), 30.9 (C, C-1), 30.1 (CH_2, C-2), 28.0 (CH, C-5), 20.2 (CH_3, C-9), 19.7 (CH_3, C-8), 15.2 (CH_3, C-10), 14.4 (CH_2, C-6);

9A$_2$: ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.26$ (5 H, H-4′-H-8′), 4.75 (1 H, H-3), 3.53 (1 H, H-2′), 2.10 (1 H, H-4), 2.00/1.60 (2 H, H-2), 1.31 (1 H, H-7), 1.10-0.70 (5 H, H-5, H-8, H-9, H-10), 0.54/0.33 (2 H, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 171.1$ (C, C-1′), 134.2 (C, C-3′), 129.3 (CH, C-4′, C-8′), 128.6 (CH, C-5′, C-7′), 127.1 (CH, C-6′), 82.6 (CH, C-3), 42.7 (CH, C-4), 41.9 (CH_2, C-2′), 34.4 (C, C-1), 33.2 (CH_2, C-2), 32.7 (CH, C-7), 29.4 (CH, C-5), 20.3 (CH_3, C-10), 19.9 (CH_3, C-9), 19.8 (CH_3, C-8), 15.8 (CH_2, C-6); EIMS *m/z* 136 (64), 121 (52), 93 (64), 91 (100); HRESIMS *m/z* (pos): 295.1683 $C_{18}H_{24}O_2Na$ $[M + Na]^+$ (calcd. 295.1668).



R	Thujol ester 3 α-OCOR 4 α-CH_3	Neothujol ester 3 β-OCOR 4 α-CH_3	Isothujol ester 3 α-OCOR 4 β-CH_3	Neoisothujol ester 3 β-OCOR 4 β-CH_3
Benzoate	$3A_1$	$3A_2$	$3B_1$	$3B_2$
Cinnamate	$4A_1$	$4A_2$	$4B_1$	$4B_2$
Isonicotinate	$5A_1$	$5A_2$	$5B_1$	$5B_2$
Nicotinate	$6A_1$	$6A_2$	$6B_1$	$6B_2$
Thiophenate	$7A_1$	$7A_2$	$7B_1$	$7B_2$
Furoate	$8A_1$	$8A_2$	$8B_1$	$8B_2$
Phenylacetate	$9A_1$	$9A_2$	$9B_1$	$9B_2$

Fig. 1 Synthesis of ester derivatives of thujone

Cell culture materials

Human cervical cancer cells HeLa (CCL-2), A375 melanoma cells (CRL-1619), and HCT-116 colon cancer cells (CCL-247) were obtained from American Type Culture Collection. HeLa and A375 cells were cultured in Dulbecco's modified Eagles medium (DMEM) while HCT-116 cells were cultured in McCoy's medium. DMEM and McCoy's media were supplemented with 10% FBS, penicillin, and streptomycin and maintained in a humidified incubator at 5% CO_2 and 37 °C.

Cell viability assay

HeLa, A375 and HCT-116 cells were plated in 96-well plates at a density of 1×10^5 cells/mL, 2×10^5 cells/mL and 5×10^5 cells/mL, respectively. After 24 h of incubation, cells were treated with concentrations of test compounds ranging from 50 to 400 µg/mL. The control was treated with 10% DMSO. After incubation for 24 h in the presence of the compounds, 50 µL of XTT solution (0.3 mg/mL) was added to each well. Cells were incubated with XTT reagent for 4 h, and the absorbance at 495 nm was measured using an

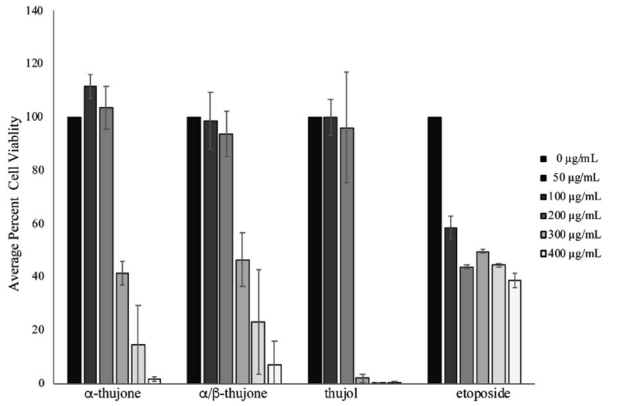


Fig. 2 Treatment of HeLa cells with thujone results in dose dependent increase in cytotoxicity. HeLa cells were treated with 50–400 μg/mL of the different compounds for 24 h. Data are average +/− SD of two independent experiments, each done in duplicate

ELISA plate reader. The average absorbance of the media was subtracted, and the percent of cell viability was calculated as follows:

$$Percent\ cell\ viability = \left(\frac{Absorbance\ of\ drug - treated\ cells}{Absorbance\ of\ control\ cells}\right) \times 100$$

IC_{50} values were determined using Graphpad Prism software.

Cell morphology

HeLa cells were plated in a 24-well plate, allowed to become 95% confluent and treated with 100 μg/mL of the different compounds for 24 h, following which images were captured using Nikon Eclipse TE300.

Western blotting

Cells were induced with either 1 μM of Staurosporine or 100 μg/mL of the ester derivatives of thujone and were harvested 3 h after drug addition in RIPA lysis buffer supplemented with protease inhibitors. The lysates were sonicated 2 × 30 sec and centrifuged at 14,000 rpm for 10 min at 4 °C. Proteins were resolved using 10% SDS-PAGE and transferred to nitrocellulose membrane. PARP protein was detected using Rabbit anti-PARP antibody (#9542 Cell Signaling Technology) at 1:1000 and anti-Rabbit-HRP (Jackson ImmunoResearch) at 1:5000. Z-VAD-FMK was added at a final concentration of 20 μM 1 h before the addition of the ester derivatives. The cells were harvested for western blotting 2 h after addition of the ester derivatives. For the hydrolysis experiments cells were treated with 250 μM of various compounds and harvested 4 h after drug addition in RIPA lysis buffer supplemented with protease inhibitors.

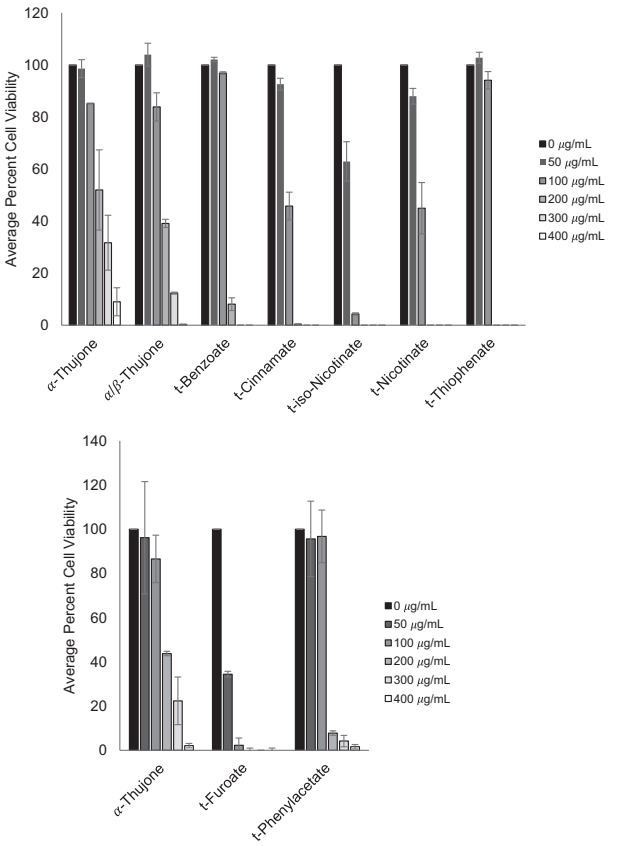


Fig. 3 Ester derivatives of thujone are more potent than α-thujone or α/β-thujone in inducing death of HeLa cells. HeLa cells were treated with 50–400 μg/mL of the different compounds for 24 h. Data are average +/− SD of two independent experiments, each done in duplicate

Activated caspase 3/7 detection

Cells were treated with either 1 μM of Staurosporine or 100 μg/mL of the ester derivatives of thujone for 3 h, following which they were stained with Cell Event Caspase 3/7 (Invitrogen #R37111) for 60 min according to the manufacturer's instructions. Cells were fixed with 3.7% formaldehyde for 15 min and imaged using Nikon confocal microscope.

Chemistry

A 7:1 mixture of α-(−)-thujone and β-(+)-thujone (isothujone) was reduced using sodium borohydride in absolute ethanol to obtain a mixture of four thujol diastereomers in near quantitative yield (Fig. 1). The NMR data indicated 49%, 31%, 14% and 6% of (−)-thujol (**$2A_1$**), (−)-neothujol (**$2A_2$**), (−)-isothujol (**$2B_1$**), and (+)-neoisothujol (**$2B_2$**), respectively. The structures were confirmed by comparison of the NMR data with literature values (Bergqvist and Norin 1963; Bohlman and Zeisberg 1975). The approximate ratio of 1.5:1 for thujol:neothujol

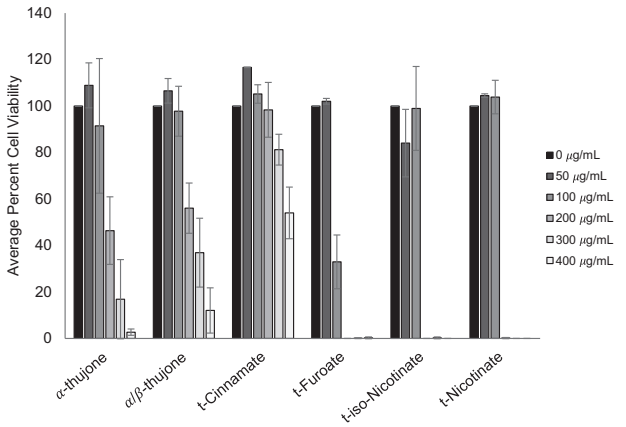


Fig. 4 A subset of thujone ester derivatives are cytotoxic to A375 cells. Cells were treated with 50–400 µg/mL of the different compounds for 24 h. Data are average +/− SD of two independent experiments, each done in duplicate

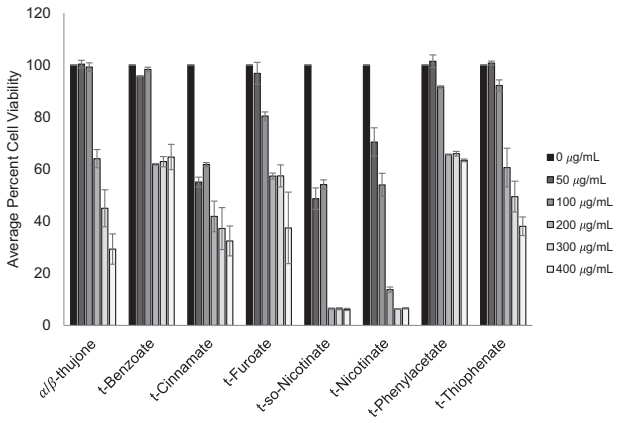


Fig. 5 Ester derivatives of thujone affect cell viability of HCT-116 colon cancer cells. HCT-116 cells were treated with 50–400 µg/mL of the different compounds for 24 h. Data are average +/− SD of two independent experiments, each done in duplicate

is in good agreement with the study by Banthorpe, which showed a similar ratio of products for the reduction of pure α-thujone (Banthorpe and Davies 1968). The ratio of products reported for the reduction of β-thujone in the same study is 1.2:1 neoisothujol:isothujol. However, in our study the amount of isothujol was greater than that of neoisothujol by factor of 2.3:1. The higher percentage of $\mathbf{2A_1}$ relative to $\mathbf{2A_2}$ can be rationalized based on the configuration of the isopropyl and methyl groups at positions C-1 and C-4. Since both groups are α-oriented, the hydride preferentially attacks the carbonyl group from the β-face of the molecule, resulting in the α-hydroxyl group at C-3[23]. Although β-thujone has the C-4 methyl and the C-6 methylene groups in the β-orientation, and only the C-1 isopropyl group in the α-orientation, based on our results there is still a preference for hydride attack from the β-face of the molecule. It can therefore be concluded that the isopropyl group has a greater impact on the stereochemical outcome of the sodium borohydride reduction of thujone than either the C-4 methyl or the C-6 methylene group.

Surprisingly, the approximate ratio of alcohol products derived from α-thujone to those from β-thujone is 4:1. This would indicate that there was slight epimerization of α-thujone to β-thujone under the reaction conditions. It has been reported by Hach that significant epimerization of pure α-thujone and pure β-thujone occur when the reduction is carried out in anhydrous solvents (Hach et al. 1971), but this was not observed by us.

The four thujol diastereomers were not readily separated by silica gel column chromatography, and the mixture was used without further purification. Seven thujol esters containing aromatic moieties (**3–9**) were prepared by reacting the mixture of thujols with benzoyl chloride, phenacetyl chloride, cinnamoyl chloride, nicotinoyl chloride, isonicotinoyl chloride, 2-furoyl chloride, and thiophene-2-carbonyl chloride as the acyl component. The esters were obtained in 43–73% yields after workup and purification, and the structures were confirmed by NMR, IR, and MS analyses.

Based on NMR integration, the ratios of the ester diastereomers were similar to those observed for the thujols. As with the mixture of thujols, we encountered difficulty trying to separate the diastereomeric mixture of esters by silica gel column chromatography. Attempts to separate the mixtures by reversed phase HPLC using an achiral column were similarly unsuccessful.

Results and discussion

Anticancer studies

Thujone has been shown to induce apoptosis of melanoma and glioblastoma cells (Biswas et al. 2011; Torres et al. 2016). In addition, an essential oil preparation containing thujone has been shown to decrease cell viability of HeLa cells (Privitera et al. 2019). However, thujone has been shown to have no effect on the proliferation of colon cancer cells (Zhou et al. 2016). In this study, we synthesized a series of ester derivatives of thujol and evaluated them for cytotoxic activity against human cervical (HeLa), melanoma (A375), and colon (HCT-116) cells, together with thujone, α/β-thujone, and thujol.

HeLa cells were treated with increasing concentrations of α-thujone, α/β-thujone (7:1), and thujol. As a positive control, we examined the ability of etoposide, a well-known chemotherapeutic drug, to induce cell death compared with α-thujone, α/β-thujone, and thujol. Treatment of HeLa cells with 50 µg/mL of etoposide resulted in reduction of cell viability to 59%, but treatment with either 50 or 100 µg/mL of α-thujone, α/β-thujone, and thujol did not have any effect

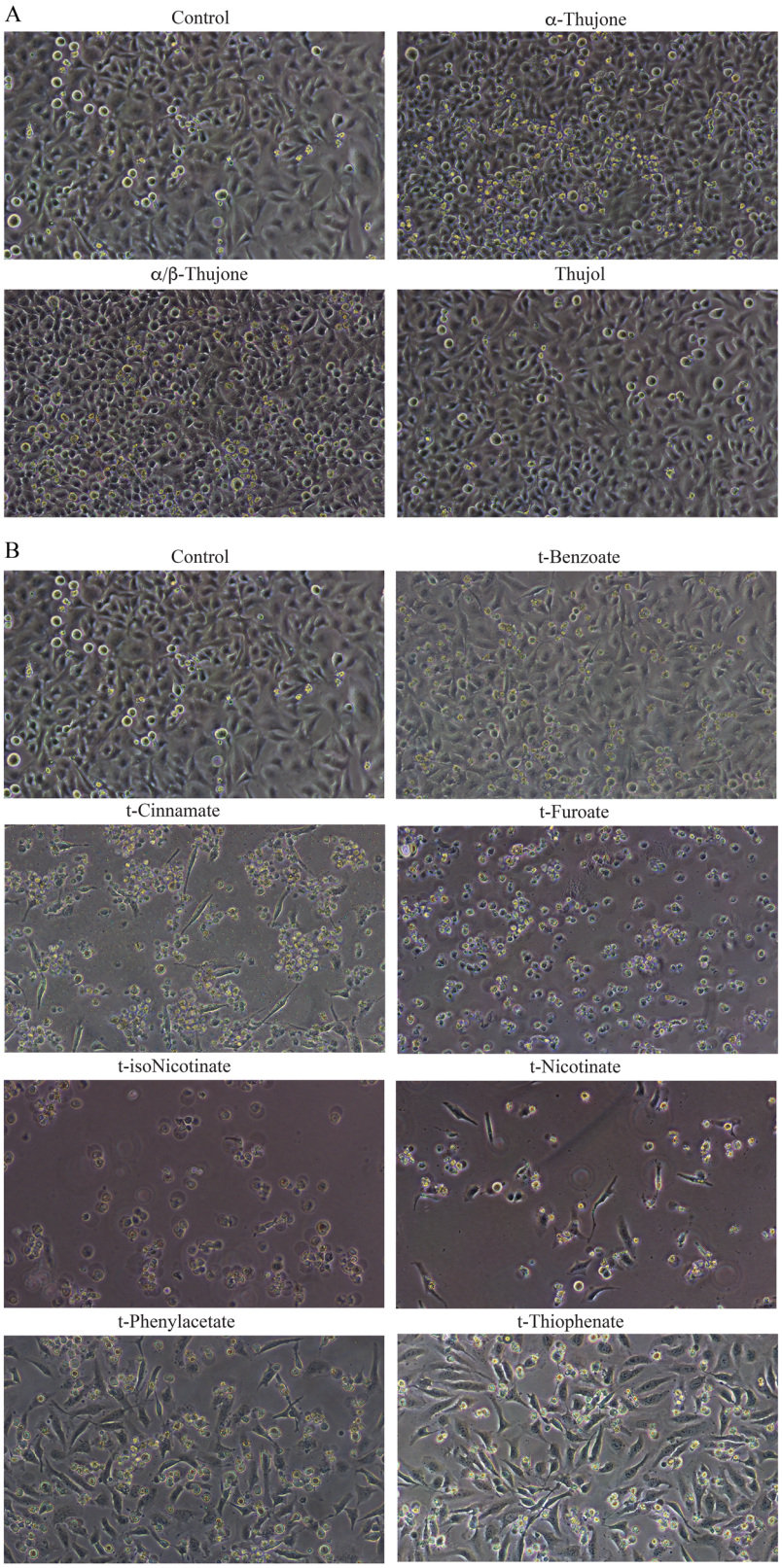


Fig. 6 HeLa cells exposed to ester derivatives exhibit apoptotic morphology. Morphology of HeLa cells treated with 100 µg/mL of α-thujone, α/β− thujone (**a**) or seven ester derivatives for 24 h (**b**)

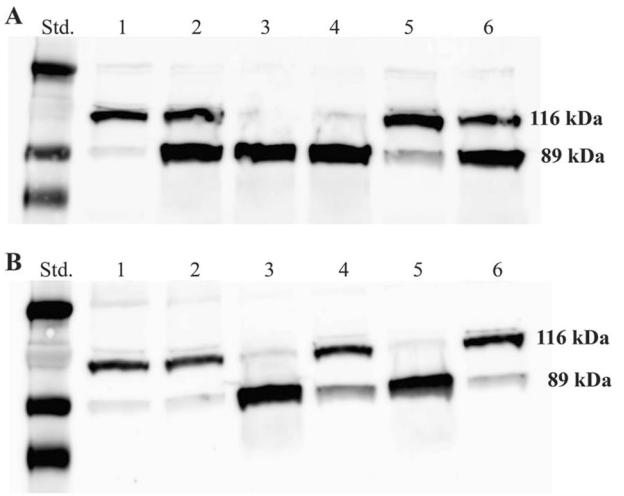


Fig. 7 PARP cleavage is detected in HeLa cells treated with ester derivatives of thujone. **a** HeLa cells were treated for 3 h with either 1 µM of Staurosporine (lane 2) or 100 µg/mL of t-furoate (lane 3), t-isonicotinate (lane 4), t-cinnamate (lane 5), and t-nicotinate (lane 6). SDS-PAGE and western blotting were performed to detect PARP in the lysates of control (lane 1) and treated cells. Full-length PARP is detected in control cells (lane 1) while the 89 kDa cleaved fragment is present in treated cells (lanes 2–6). Std. refers to protein standard. **b** PARP cleavage was examined in cells treated in the presence or absence of Z-VAD-FMK (20 µM), which was added an hour before addition of the apoptotic inducers. Addition of Z-VAD-FMK significantly inhibits the induction of PARP cleavage by t-furoate and t-isonicotinate. Std. refers to protein standard, Lane 1: control, Lane 2: Z-VAD-FMK only, Lane 3: t-furoate, Lane 4: t-furoate + Z-VAD-FMK, Lane 5: t-isonicotinate, Lane 6: t-isonicotinate + Z-VAD-FMK

on cell viability (Fig. 2). However, treatment of cells with 200 µg/mL of α-thujone, α/β-thujone, and etoposide resulted in comparable reduction in viability to 41%, 47% and 50%, respectively. Moreover, at higher concentrations, α-thujone, and α/β-thujone appear to be more effective than etoposide at inducing cell death, as treatment with 400 µg/mL of α-thujone, α/β-thujone, and etoposide, the highest concentration tested, reduced cell viability to 2%, 7%, and 39%, respectively (Fig. 2). Interestingly, while HeLa cells exposed to either α-thujone, α/β-thujone, or etoposide exhibited a dose-dependent decrease in cell viability, treatment with 200 µg/mL of thujol lead to a dramatic decrease in cell viability with only 2% of cells being viable. The IC_{50} values for α-thujone, α/β-thujone, and thujol were 191, 198, and 136 µg/mL, respectively, while etoposide exhibited an IC_{50} value of 116 µg/mL (197 µM). The IC_{50} values for etoposide vary depending on the cell line tested, but for HeLa cells it has been reported to be 209 µM (Xiao et al. 2014).

Interestingly, while treatment with α/β-thujone reduced the viability of human (U87MG) and rat (C6) glioblastoma cells, α-thujone was ineffective at inducing cell death (Torres et al. 2016). However, both α-thujone, and α/β-thujone were able to elicit the death of HeLa cells. Cell viability was reduced to 50% when treated with 300 µg/mL and 400 µg/mL concentrations of α/β-thujone (7:1), for U87MG and C6 cells (Torres et al. 2016), respectively, while 200 µg/mL of α-thujone, and α/β-thujone resulted in 41 and 47% of HeLa cells being viable. Therefore, lower concentrations of both α-thujone, and α/β-thujone are effective in inducing the death of HeLa cells when compared with their ability to induce death of glioblastoma cells.

We subsequently examined the ability of the ester derivatives of thujone (**3–9**) to induce HeLa cell death, and evaluated whether there were direct structure–activity correlations. Of the seven ester derivatives examined, t-isonicotinate (**5**) and t-furoate (**8**) are the most effective at inducing cell death, as treatment of HeLa cells with the lowest tested concentration of 50 µg/mL resulted in reduction of cell viability to 63% and 34%, respectively (Fig. 3). Increasing the dosage to 100 µg/mL further decreased cell viability to <5% for both of these derivatives. While treatment of HeLa cells with 50 µg/mL of t-cinnamate (**4**) and t-nicotinate (**6**) produced only a modest effect on cell viability, treatment with the next highest concentration of 100 µg/mL reduced cell viability to 46% and 45%, respectively. Further treatment with higher concentrations resulted in 100% cell death. No appreciable cell death was observed when HeLa cells were treated with 50 or 100 µg/mL of the remaining derivatives, t-benzoate (**3**), t-thiophenate (**7**), and t-phenylacetate (**9**). However, exposure of cells to the next highest concentration of 200 µg/mL reduced cell viability to 8%, 0%, and 8%, respectively (Fig. 3). Based on these results, it can be concluded that the structure of the thujone ester has an effect on the viability of HeLa cells.

Similar to HeLa cells, A375 cells treated with α-thujone and α/β-thujone exhibit a dose-dependent decrease in cell viability, with viability being reduced to 46% and 56%, when exposed to 200 µg/mL of the respective derivative (Fig. 4). This is comparable with what was observed previously, with viability decreasing to 58% and 41% when A375 cells were treated with 100 and 200 µg/mL concentrations of thujone rich fractions, respectively (Biswas et al. 2011). Of the seven ester derivatives, only t-cinnamate (**4**), t-isonicotinate (**5**), t-nicotinate (**6**), and t-furoate (**8**) had an effect on the viability of A375 cells, and their activity was lower than observed in HeLa cells at similar concentrations. The viability of A375 cells treated with 100 µg/mL of t-furoate decreased to 33%, with higher concentrations resulting in 100% cell death. While treatment of cells with either 50 or 100 µg/mL of t-isonicotinate and t-nicotinate had no effect, treatment with 200 µg/mL or higher concentrations resulted in 0% viability (Fig. 4). As

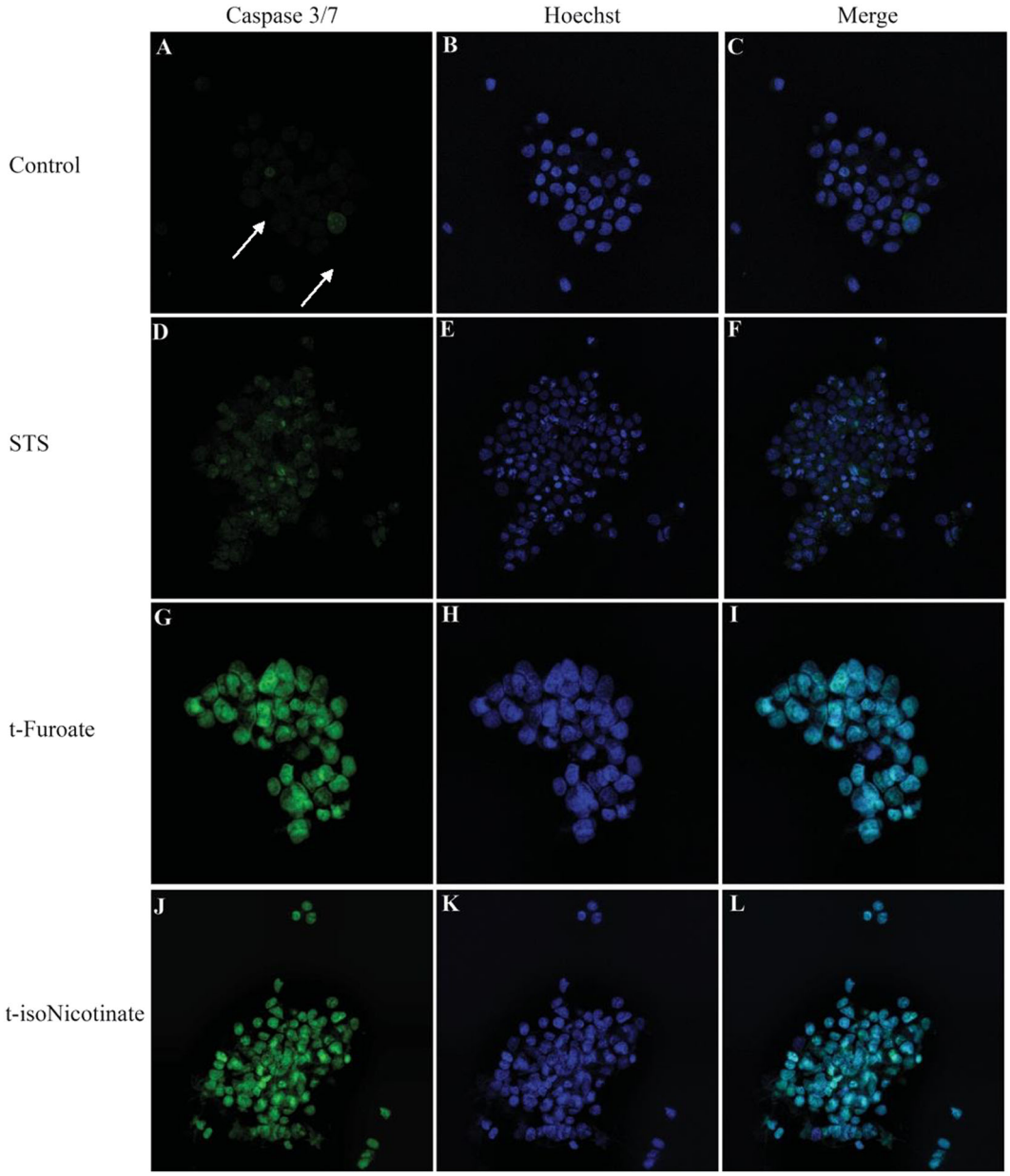


Fig. 8 The effector Caspase 3/7 is activated at high levels in t-furoate and t-isonicotinate treated cells. HeLa cells were treated with either 1 µM of Staurosporine (**d**–**f**), 100 µg/mL of t-furoate (G-H), or 100 µg/mL of t-isonicotinate (**j**–**l**) for 3 h and stained with Cell Event Caspase 3/7 Green to detect activated caspase 3/7 (**a**, **d**, **g**, **j**), Hoecsht to detect DNA (**b**, **e**, **h**, **k**) and merged (**c**, **f**, **i**, **l**). Arrows point to cells that show very low levels of activated caspase in the control

was observed for the parent compounds (α-thujone, and α/β-thujone), treatment with t-cinnamate also produced a dose-dependent cytotoxicity, but only treatment with the highest concentration of 400 µg/mL reduced the viability significantly to 54% (Fig. 4). The remaining derivatives had no appreciable effect on the viability of A375 cells. Interestingly, in contrast with HeLa cells, t-isonicotinate and t-nicotinate showed similar activity profile in A375 cells, with no effect in cell viability up to 100 µg/mL, but a dramatic reduction to <5% at 200 µg/mL.

Lastly, we examined whether the ester derivatives of thujone exhibited cytotoxicity against the HCT-116 colon cancer cell line. All seven ester derivatives affected cell viability of HCT-116 cells to varying degrees, with treatment of cells with 50 µg/mL of t-cinnamate (**4**), t-isonicotinate (**5**), and t-nicotinate (**6**) resulting in reduction of viability to 55%, 48% and 70%, respectively (Fig. 5). Neither 50 or 100 µg/mL of t-benzoate (**3**), t-phenylacetate (**7**), or t-thiophenate (**9**) has an appreciable effect on viability. However, while t-thiophenate (**9**) exhibited a dose

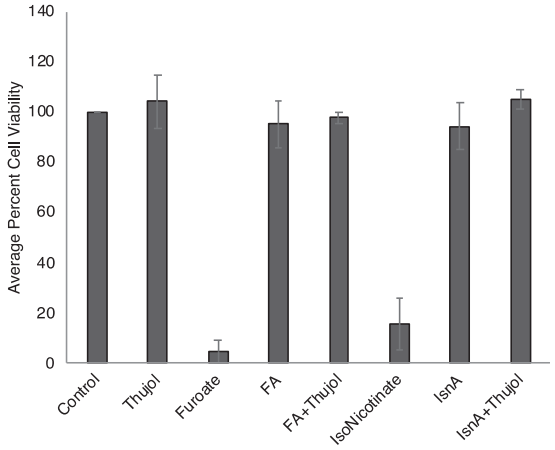


Fig. 9 The ester derivatives of thujone and not the hydrolysis products are responsible for the cytotoxic effects. HeLa cells were treated with 250 µM of thujol, t-furoate, t-isonicotinate, Furoic acid (FA), Isonicotinic acid (IsnA) for 24 h

dependent effect with increasing concentrations, treatment of cells with 200 µg/mL of t-benzoate (**3**), and t-phenylacetate resulted in viability being reduced to 61–65% with no further decrease in viability at higher concentrations. T-isonicotinate (**5**) and t-nicotinate (**6**) were the most cytotoxic to HCT-116 cells as viability decreased to 6% (Fig. 5).

Ester derivatives of thujone induce apoptosis

To determine whether the cytotoxic effects were as a result of apoptotic induction, we first examined the morphology of HeLa cells after exposure to 100 µg/mL of thujone, thujol, and the ester derivatives. Cells treated with α-thujone, α/β-thujone (7:1), thujol (Fig. 6a) were mostly normal which is consistent with the cell viability data. Cells treated with the ester derivatives of thujone (Fig. 6b) exhibited morphology that is consistent with the cells undergoing apoptosis. The most dramatic changes were observed when cells were treated with t-cinnamate (**4**), t-isonicotinate (**5**), t-nicotinate (**6**), and t-furoate (**8**), with a significant proportion of cells appearing rounded and detached from the substratum. This is consistent with the cell viability data, as these four derivatives were able to induce significant cytotoxicity when used at 50 or 100 µg/mL concentrations.

As the morphology of the treated cells is consistent with apoptotic cell death, we examined whether we could detect PARP cleavage when cells were treated with the four derivatives that exhibited the strongest effect on HeLa cell viability. PARP, a 116 kDa nuclear protein is involved in DNA repair and its proteolytic cleavage is a characteristic biochemical feature of Caspase mediated apoptotic cell death (Kaufmann et al. 1993). As a positive control, HeLa cells were treated with Staurosporine, a protein kinase inhibitor that has been shown to potently induce apoptosis of several human cancer cells (Tamaoki et al. 1986; Bruno et al. 1992). As expected, PARP cleavage was detected in HeLa cells treated with Staurosporine (Fig. 7a, lane 2). Similarly, treatment of HeLa cells with 100 µg/mL of t-cinnamate (**4**), t-isonicotinate (**5**), t-nicotinate (**6**), and t-furoate (**8**) resulted in PARP cleavage, which is consistent with caspase mediated apoptotic cell death (Fig. 7a, lanes 3–6). The role of caspases in mediating the death of HeLa cells treated with the ester derivatives was further supported by the ability of the pan caspase inhibitor Z-VAD-FMK to significantly reduce PARP cleavage in t-furoate and t-isonicotinate treated cells (Fig. 7b, lane 3 vs 4 and 5 vs 6). In addition, we examined whether we could detect the presence of activated effector caspase 3/7 in HeLa cells treated with t-furoate and t-isonicotinate. While very few control cells exhibited the presence of very low levels of activated caspase 3/7 (Fig. 8a), nearly 100% of t-furoate and t-isonicotinate treated cells exhibit very high levels of activated caspase 3/7 (Fig. 8g, j). The fluorescence signal detected in these cells is much brighter than seen in Staurosporine treated cells (Fig. 8d vs g/j), which is consistent with the degree of PARP cleavage. The ester derivatives of thujone therefore potently induce apoptosis of HeLa cells.

As the cytotoxic effects could potentially be mediated by the hydrolysis of ester derivatives, we compared the apoptosis inducing ability of t-isonicotinate (**5**) and t-furoate (**8**), with their corresponding acids alone and in combination with thujol. While treatment with 250 µM of either t-furoate or t-isonicotinate reduced cell viability to 4% and 15%, respectively, treatment with neither their corresponding acids alone nor in combination with thujol had a significant effect on viability (Fig. 9). To further confirm that the cytotoxic effects were not being mediated by the hydrolysis products of the ester derivatives, we examined the status of PARP cleavage (Fig. 10). PARP cleavage was observed only in cells treated with t-furoate (lane 3) and t-isonicotinate (lane 6) and not in cells treated with either of their corresponding acids alone (lanes 4 and 7), or in cells treated with thujol and the acids combined (lanes 5 and 8).

Conclusions

We have examined seven ester derivatives of thujone and compared their ability to induce the death of HeLa, A375, and HCT-116 cells with that of thujone and thujol. In HeLa cells, t-Cinnamate (**4**), t-isonicotinate (**5**), t-nicotinate (**6**), and t-furoate (**8**) are more potent than the parent compounds, α-thujone, α/β-thujone, and thujol in eliciting cell death at lower

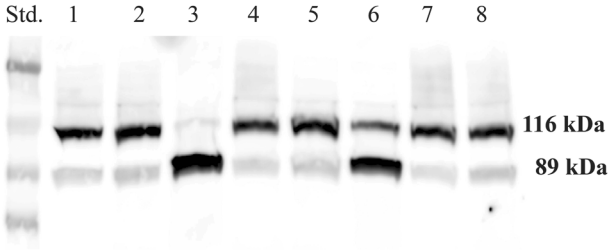


Fig. 10 PARP cleavage is detected only in cells treated with the ester derivatives. HeLa cells were treated with 250 μM of thujol (lane 2), t-furoate (lane 3), Furoic acid (FA: lane 4), FA + Thujol (lane 5), t-isonicotinate (lane 6), Isonicotinic acid (IsnA: lane 7), and IsnA + Thujol (lane 8) for 4 h. SDS-PAGE and western blotting were performed to detect PARP in the lysates of control and treated cells. Full-length PARP is detected in lanes 1, 2, 4, 5, 7, and 8 while the 89 kDa cleaved product is detected in lanes 3 and 5. Std. refers to protein standard

concentrations. The same four derivatives were also cytotoxic to A375 and HCT-116 cells, with t-furoate (**8**) being the most effective against A375 cells, while t-isonicotinate (**5**) and t-nicotinate (**6**) were the most effective in HCT-116 cells. We also demonstrate that the derivatives induce caspase-mediated apoptosis as evidenced by PARP cleavage and presence of high levels of activated effector caspases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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