Effect of three fatty acids from the leaf extract of *Tiliacora triandra* on P-glycoprotein function in multidrug-resistant A549RT-eto cell line

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ABSTRACT

Background: Cancer cells have the ability to develop resistance to chemotherapy drugs, which then leads to a reduced effectiveness and success of the treatment. Multidrug resistance (MDR) involves the resistance in the same cell/tissue to a diverse range of drugs of different structures. One of the characteristics of MDR is an overexpression of P-glycoprotein (P-gp), which causes the efflux of the accumulated drug out of the cell. The MDR human non-small cell lung carcinoma cell line with a high P-gp expression level (A549RT-eto) was used to investigate the bioactive compounds capable of reversing the etoposide resistance in this cell line. **Materials and Methods:** The leaves of *Tiliacora triandra* were sequentially extracted with hexane, dichloromethane, methanol and water. Only the hexane extract reduced the etoposide resistance of the A549RT-eto cell line, and was further fractionated by column chromatography using the TLC-pattern and the restoration of etoposide sensitivity as the selection criteria. **Results:** The obtained active fraction (F22) was found by nuclear magnetic resonance and gas chromatography-mass spectroscopy analyses to be comprised of a 49.5:19.6:30.9 (w/w/w) mixture of hexadecanoic: octadecanoic acid: (Z)-6-octadecenoic acids. This stoichiometric mixture was recreated using pure fatty acids (MSFA) and gave a similar sensitization to etoposide and enhanced the relative rate of rhodamine-123 accumulation to a similar extent as F22, supporting the action via reducing P-gp activity. In contrast, the fatty acids alone did not show this effect. **Conclusion:** This is the first report of the biological activity from the leaves of *T. triandra* as a potential source of a novel chemosensitizer.

Key words: Fatty acids, multidrug resistance, P-glycoprotein, *Tiliacora triandra*

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INTRODUCTION

Multidrug resistance (MDR) in cancer cells involves the resistance to a diverse range of currently used cytotoxic drugs with different structures in the same cells/tissue. One of the characteristics of MDR is the overexpression of P-glycoprotein (P-gp), a 170 kDa plasma membrane protein encoded by the *mdr1* (multidrug resistance 1) gene in humans.[1,2] The function of P-gp is an energy-dependent membrane efflux pump that transports a broad spectrum of anticancer drugs, including doxorubicin, vinblastine, vincristine, etoposide and palitaxel, across the plasma membrane out of the cell.[3] Since P-gp can pump the administered drugs out of the cancer cells and so diminish the efficacy of chemotherapy, the inhibition of its activity may reverse this characteristic. Recently, many natural compounds capable of reversing cellular MDR and thereby restoring the sensitivity to anticancer agents have been extensively explored and investigated.[4-6] The inhibition of the P-gp-mediated drug efflux by co-administration of natural compounds would be expected to result in a higher uptake of the chemotherapy drugs and so consequentially a potential reduction in the required systemic or targeted drug dose with reduced adverse effects.[7] These compounds, known as MDR-reversing agents, chemosensitizers or modulators, have been found across a broad spectrum of chemical structures.[3,8] In addition, hydrophobic agents, both synthetic (verapamil, phenothiazines,

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reserpine, trifluoperazine or cyclosporine) and natural (fatty acid-PEG-fatty acid diester,[9] capsanthin (carotenoid), zeaxanthin (carotenoid) and chrysin (flavonoid)),[10] and bisbibenzyl derivatives have been reported to reverse MDR in cancer cells by inhibition of P-gp.[11]

Tiliacora triandra, an angiospermic plant that is native to mainland Southeast Asia, is naturally abundant in Thailand.[12] The roots from this plant contain alkaloid compounds, especially the bisbenzylisoquinoline alkaloids tiliacorinine, tiliacorine and nortiliacorinine,[13] and have been widely used in folk medicine as an antipyretic agent for all kinds of fever and in the preparation of antimalarial drugs.[14] Many biological activities, for example, cytotoxic activity against the growth of cholangiocarcinoma cell lines,[15] antimycobacterial activity against the MDR strains of *Mycobacterium tuberculosis*[13] and antimalarial activity, have been reported.[16] In addition, *T. triandra* leaves have been shown to be a natural source of antioxidants, containing high levels of beta-carotene, condensed tannins, triterpenes, flavonoids and saponins, as well as minerals, such as calcium and iron.[17] The antioxidant and antimutagenic activities from these compounds further suggest their potential in cancer prevention.[18] It was, therefore, the objective of this work to isolate and identify the extract obtained from the leaves of *T. triandra* that posses a modulating effect on P-gp and so reverse the MDR in the non-small cell lung carcinoma cell line (A549RT-eto).

MATERIALS AND METHODS

Plant materials and extraction of *T. triandra*

Fresh leaves of *T. triandra* were collected during April and June, 2012 from Na Mom, Songkhla Province, Thailand. Plant authentication was performed by Ms. Parinyanoot Klinratana and voucher specimens (A 013629) have been deposited in the Kasin Suvatabandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. Fresh leaves of *T. triandra* were dried at 45°C and ground into powder. Briefly, 1 kg of the dried sample powder was sequentially extracted with 1000 mL each of hexane, dichloromethane (CH_2Cl_2), ethanol and water as previously described.[19] The residues were dried overnight at room temperature (RT) and later extracted with 300 mL of water at 60°C for 8 h with shaking. The respective solvent from the obtained hexane, CH_2Cl_2 and ethanol extracts was removed by rotary evaporation while the water was removed by freeze drying. The dried samples were then stored at -20°C until use.

Isolation and identification of the compounds

Quick column chromatography. A sintered glass 500-mL column was tightly packed with silica gel 60 G using a vacuum pump. The crude extract (~72 g) was solvated in 10 mL of CH_2Cl_2, mixed with sufficient silica gel 60 (pore size 60 Å, 70-230 mesh) to form a paste, left to dry and then loaded onto the packed column followed by a piece of filter paper (110 mm in Ø) and a cotton plug. The column was then eluted with a discontinuous five stage gradient formed from 1:1, 2.3:1 and 1:0 (v/v) CH_2Cl_2: Hexane followed by 1:19 and 1:9 (v/v) methanol (MeOH): CH_2Cl_2 to yield a total of 70 fractions. Fractions containing similar components, as determined by their one-dimensional thin layer chromatography (1D-TLC) pattern (see below) were pooled and evaporated to dryness. The obtained combined fractions were then solvated and screened for their cytotoxic activity against the A549 and A549RT-eto cell lines and their ability to restore cytotoxic sensitivity to etoposide in the A549RT-eto cell line (see below).

Gel filtration chromatography. Sephadex LH-20 gel (100 g, GE Healthcare Bio-sciences AB) saturated in 1:9 (v/v) MeOH: CH_2Cl_2 was loaded into a ~350-mL column (2.5 × 50 cm). The fractions with potent activities in reversing the etoposide resistance in the A549RT-eto cell line were then combined and loaded onto the column (1.54 g/loading), and then eluted with excess 1:9 (v/v) MeOH: CH_2Cl_2 to ensure no compounds were retained. The column was then washed with MeOH. The fractions were screened by 1D-TLC, pooled and subsequently screened for their cytotoxic activity, and the ability to restore cytotoxic sensitivity to etoposide in the A549RT-eto cell line, as described above.

Flash column chromatography. A silica gel (50 g, 60-230 mesh) column in hexane was prepared. Fractions which showed a good ability to restore etoposide sensitivity in the A549RT-eto cell line without being cytotoxic themselves were dissolved (190 mg) in the appropriate solvent, mixed with silica gel (1 g, 60-230 mesh) and left at RT until dry. The prepared extract was then transferred to the column and eluted with hexane: CH_2Cl_2. Fractions were then screened by 1D-TLC, pooled and subjected to screening of their cytotoxic activity and the ability to restore cytotoxic sensitivity to etoposide in the A549RT-eto cell line as described above.

Thin layer chromatography (1D-TLC). TLC was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer). The plate was cut to 5 × 5 cm, loaded with sample by a capillary tube and developed in TLC-developing tank. When the mobile phase reached the solvent front, the TLC plate was removed and left at RT to dry. The resolved compounds were visualized under ultraviolet light or by 5% (v/v) anisaldehyde reagent.

Chemical structure analysis by spectroscopy

Nuclear magnetic resonance (NMR). To analyze the apparently pure component or partially purified fraction, 5-10 mg

of each sample was dissolved in 500 μL of deuterated chloroform ($CDCl_3$) and transferred into an NMR tube. The NMR spectrum was recorded on a Varian Mercury$^+$ 400 NMR spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C nuclei in order to gain information on the functional groups. The chemical shift in δ (ppm) was assigned with reference to the signals of the $CDCl_3$ residue.

Gas chromatography mass spectroscopy (GC-MS). Free fatty acids were prepared as methyl esters prior to being analyzed by GC-MS. Briefly, one drop of 18 M H_2SO_4 was added to each fraction (~5 mg) dissolved in anhydrous MeOH (1 mL), refluxed at 60°C for 60 min and cooled down to RT. The obtained solution was concentrated by adding with equal volume of H_2O and extracted twice with diethyl ether ($(C_2H_5)_2O$). The organic layer was concentrated by evaporation and subjected to GC-MS analysis, performed commercially at The Central Instrument Facility, Faculty of Science, Mahidol University, Thailand. The GC-MS was performed on an Agilent technology 6890N-GC system (Agilent technologies, USA) coupled to MSD-5973N equipment operating in the electrospray ionization mode at 70 eV, with a HP-INNOWAX column (29.8 m × 0.25 mm × 0.25 μm). The temperature was held at 50°C for 2 min, then ramped to 200°C at 10°C/min and held for 2 min, ramped to 210°C at 5°C/min and held for 5 min and finally ramped to 22°C at 5°C/min and held for 17 min (total run time of 45 min). The injector temperature was fixed at 250°C. The flow rate of the carrier gas (helium) was 1 mL/min and a 1:5 split ratio was used. Identification of each individual constituent of the compound was achieved by comparing the retention times with those of authentic compounds as well as the spectral data obtained from the Wiley Database.

Cell and cell culture

The non-small cell lung adenocarcinoma cell line, A549, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the MDR derivative, A549RT-eto, was developed and kindly provided by Dr. Yodsoi Kanintronkul.[20] The major mechanism of MDR in the A549RT-eto cells involves the overexpression of the *mdr1* gene which encodes for the P-gp drug efflux transporter. These cell lines were cultured in complete media (CM; RPMI with 10% (v/v) fetal bovine serum, containing 100 units/mL penicillin, 100 μg/mL streptomycin and 25 μg/mL of amphotericin B) at 37°C with 5% (v/v) CO_2.

Measurement of the cytotoxicity and ability to restore etoposide sensitivity in the A549RT-eto cell line

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[19,21] was performed to determine the cytotoxicity to the A549 and A549RT-eto cell lines of each extract or fraction, as well as to determine the sensitivity of each cell line to etoposide and the ability of each extract or fraction to modulate this. Briefly, cell suspensions of A549 or A549RT-eto cells at 5×10^4 cells/mL in CM were seeded at 100 μL into each well of a 96 well microtiter plate (5×10^3 cells/well) and incubated at 37°C with 5% (v/v) CO_2. After 24 h, either crude extracts (two-fold serial dilutions from 100 to 6.25 μg/mL), or fractions in DMSO dissolved in CM was then added and further incubated for 72 hours. Thereafter, the medium was removed and replaced with fresh CM containing 5 mg/mL MTT and incubated at 37°C for 2 hours. This media was then removed and 100 μL DMSO was added and aspirated to lyse the cell membranes and solubilize the formazan crystals prior to measuring the absorbance at 550 nm (A_{550}) on a Biochrom Asys UVM 340 Microplate Reader (Holliston, MA, USA). The surrogate cell survival, as the relative number of viable cells, assuming the same mitochondrial activity per viable cell, was then calculated by the mean A_{550} of the treated cells (A_T) and the mean A_{550} of control cells (A_C) with Eq. (1),

$$\% \text{ Cell survival} = (A_T/A_C) \times 100 \quad \text{Eq. (1)}$$

Etoposide was used as the specific positive control for the A549RT-eto-resistant cells. The concentration which caused a half maximal inhibition of cell proliferation (IC_{50}), as determined by the MTT assay, was obtained from a semi-log plot of the concentration of etoposide (or the test fraction) against the percentage cell survival.

The ability of each fraction to reverse the etoposide resistance in the A549RT-eto cell line was evaluated using the MTT assay in the presence of different concentrations of etoposide in 96-well plates, as described previously.[19] The highest concentration of each extract that displayed >80% cell survival of the cell lines after exposure for 72 h was deemed to be non-cytotoxic and used in the chemosensitivity test (loss of resistance to etoposide cytotoxicity in the A549RT-eto cell line). A549RT-eto cells were treated with a mixture of the extract at the deduced maximal non-cytotoxic concentration and a two-fold serial dilution of etoposide (100-6.25 μg/mL) for 72 hours. After treatment, the relative viable cell number (surrogate % cell survival) was evaluated by the MTT assay, performed as above. The ability of the test fraction to restore etoposide sensitivity (reduced resistance) in the A549RT-eto cell line was interpreted as the reversal fold (RF) index, obtained from Eq. (2),

$$RF = (IC_{50} \text{ of etoposide})/(IC_{50} \text{ of etoposide with the test extract}) \quad \text{Eq. (2)}$$

Verapamil at 10 μM, a non-cytotoxic dose that reverses the etoposide resistance in the A549RT-eto cell line[20] was used as a positive control.

Rhodamine-123 accumulation and efflux assays

This assay was performed as previously described[22] with some modification. The A549RT-eto cells were seeded into 96-well microtiter plates and incubated as described above. After 24 h, the non-cytotoxic concentration of each extract or fraction was added to the wells in fresh CM and further incubated for 72 hours. Next, Rh-123 was added to 20 μM final and the cells were incubated at 37°C and 5% (v/v) CO_2 for 90 min and washed with ice-cold phosphate buffer saline pH 7.4 (PBS). The Rh-123 that had accumulated in the cells was released and solubilized by the addition of 200 μL 0.2 M NaOH/0.5% (v/v) Triton X-100 and then quantitatively determined using a spectrofluorometer (PerkinElmer Victor^3; PerkinElmer Life and Analytical) at an excitation wavelength of 485 nm and measuring the emission wavelength at 535 nm (E_{535}). The relative rate of accumulation (RR_A) of Rh-123 was calculated from Eq. (3);

$$RR_A = (TC\text{-}BC)/(CC\text{-}BC) \quad \text{Eq. (3)}$$

where BC is the E_{535} of the blank control with no cells, CC is the E_{535} of the non-treated cells with the test compound and TC is the E_{535} of the treated cells with the test compound. Verapamil (10 μM) was used as the positive control.

RESULTS

Cytotoxic effect of etoposide and the crude extracts of *T. triandra*, as evaluated by the MTT assay

The cytotoxic effects of etoposide on the sensitive (A549) and MDR (A549RT-eto) lung cancer cells were determined by the MTT assay to measure the surrogate cell survival as the relative number of viable cells. Dose-response curves were obtained after 72 h of exposure of A549 and A549RT-eto cells with etoposide at two-fold serial dilutions from 100 to 6.25 μg/mL [Figure S1, Supplementary information (SI)]. As expected, the A549RT-eto cells were more resistant to etoposide than the A549 cells with a 37-fold higher etoposide IC_{50} value for the A549RT-eto cells than the A549 cells [Table 1].

After sequential extraction of *T. triandra* leaves with hexane, CH_2Cl_2, ethanol and water, the four crude extracts obtained were then evaluated for their cytotoxicity against the A549 and A549RT-eto cells [Figure 1]. While the hexane (F1) and water (F4) extracts were essentially non-cytotoxic (IC_{50} > 100 μg/mL), the CH_2Cl_2 (F2) and ethanol (F3) extracts were found to be cytotoxic to both the A549 and (especially the) A549RT-eto cells with IC_{50} values of between 22.0 and 73.0 μg/mL [Table 1]. The F2 extract displayed the highest cytotoxicity to both cell lines. Accordingly, the F1 and F4 extracts from *T. triandra* leaves were subsequently selected to evaluate their ability to restore etoposide sensitivity in the A549RT-eto cell line.

Table 1: IC_{50} of *T. triandra* extraction A549 and A549RT-eto cells

Sample	IC_{50} (μg/mL) in the indicated cell line	
	A549	A549RT-eto
F1 (Hexane)	>100	>100
F2 (CH_2Cl_2)	22.0±2.0	48.5±10.0
F3 (Ethanol)	67.3±13.7	73.0±4.7
F4 (Water)	>100	>100
Etoposide (control)	0.95±0.02	35.0±9.5

The four crude extracts from the leaves of *T. triandra* were evaluated at different concentrations for their cytotoxic effect on A549 and A549RT-eto cells after a 72 h exposure using the MTT assay, and compared with that for etoposide as a positive control. The obtained IC_{50} values are shown as the mean±1 SD and are derived from triplicate repeats. Means with a different lowercase letter are significantly different (P<0.05; Duncan's MMT). IC_{50} : a half maximal inhibition of cell proliferation

Effect of *T. triandra* leaf extracts on MDR A549RT-eto cells to etoposide (MDR reversing activity)

Etoposide, a chemotherapeutic drug that is cytotoxic to A549 cells but not the MDR A549RT-eto derivative cell line, was selected to determine the ability of non-cytotoxic concentrations of F1 and F4 extracts to reverse the MDR phenotype of the A549RT-eto cells in comparison with that for verapamil (10 μM) as a positive control. The obtained IC_{50} values of etoposide, and etoposide in the presence of 10 μM verapamil or a non-cytotoxic concentration of the F1 and F4 (125 μg/mL) were used to calculate the RF indices using Eq. (2). Only the F1 extract could significantly reverse the etoposide resistance in the A549RT-eto cells (i.e. RF > 1) [Table 2], and so F1 was selected for further fractionation to isolate and identify the relevant bioactive compound (s).

Isolation and identification of MDR-reversing bioactive compounds in the F1 extract of *T. triandra* leaf

The bioactive compounds from the F1 extract were isolated by the procedure outlined schematically in Figure 1, with each fraction being evaluated for its cytotoxicity and then the ability to restore etoposide sensitivity to the A549RT-eto cells (MDR-reversing activity). Separation of the F1 extract by silica gel G60 column chromatography (see methods) yielded eight fractions (F5-F12), of which only one fraction (F7) reduced the etoposide resistance in the A549RT-eto cell line. Fractionation of F7 by Sephadex LH20 column chromatography yielded seven fractions (F13-F19), but the

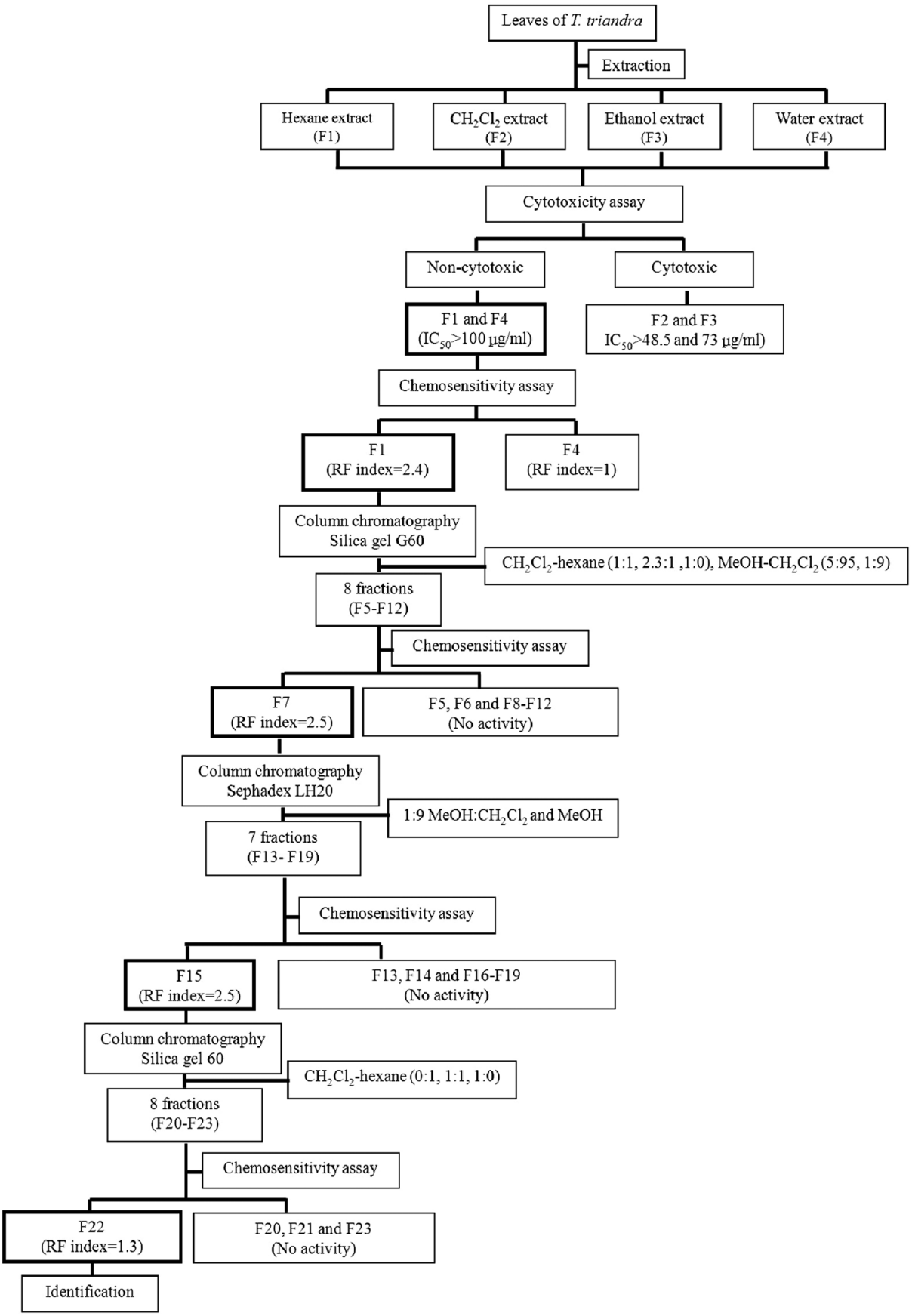

Figure 1: Schematic summary of the solvent extraction and chromatographic separation of bioactive compounds from the leaves of *Tiliacora triandra*

Table 2: Reversing fold (RF) index of the F1 and F22 bioactive fractions from *T. triandra* extracts, and the three fatty acids and their composite mixture (M3FA), on the etoposide sensitivity of A549RT-eto cells

Sample	Reversing fold index
F1 (125 μg/mL)	2.40±0.26
F22 (125 μg/mL)	1.30±0.13
M3FA (125 μg/mL; Net fatty acid of 464.2 μM)	1.24±0.11
Hexadecanoic acid (61.9 μg/mL; 241 μM)*	1.50±0.17
Octadecanoic acid (24.5 μg/mL; 85.9 μM)*	0.95±0.10
(Z)-6-octadecenoic acid (38.6 μg/mL; 136.8 μM)*	1.16±0.04
Verapamil (4.91 μg/mL; 10 μM)	2.67±0.09

The RF index of the F1 and F22 fractions from *T. triandra*, and the three pure fatty acids alone or as a composite mixture (M3FA) that mimics the composition of F22 were evaluated by the MTT-based chemosensitivity assay on A549RT-eto cells in the presence of various concentrations of etoposide for 72 h. Verapamil was used as a positive control. Data are shown as the mean±1 SD and are derived from triplicate repeats. Means with a different lowercase letter are significantly different (P<0.05; Duncan's MMT). *represents the concentrations of the three individual fatty acids represent their concentration in the M3FA and F22 samples. RF: Reversing fold

absence of cytotoxicity and the ability to restore etoposide sensitivity to the A549RT-eto cell line was only found in one of these, F15. Finally, F15 was further purified by silica gel 60 flash column chromatography to yield four fractions (F20-F23). From the qualitative 1D-TLC a single spot was obtained from F22 which could reverse the etoposide resistance in A549RT-eto cells with an RF value of 1.3 [Table 2].

Structure analysis of F22

F22 was preliminarily analyzed by ^{1}H and ^{13}C-NMR spectroscopy. F22 showed ^{1}H NMR signals of olefinic protons at δ 5.35 (m), in addition to characteristic signals of fatty acids at δ 2.34 (t, J = 7.5 Hz), 2.02 (m), 1.63 (m), 1.25 (brs) and 0.88 (t, J = 6.8 Hz) [Figure S2 (A), SI). The ^{13}C NMR spectrum also supported the presence of fatty acid by the signals resonated at δ 130.2 and 129.9 for unsaturation and at δ 179.1 for the carbonyl ester [Figure S2 (B), SI). It was, therefore, concluded that F22 contained free fatty acids. In fact, free fatty acids usually occur as a mixture of two or more components.

The fatty acid components in F22 were analyzed by GC-MS following their derivatization as methyl esters. Three prominent peaks with retention times (t_R) of 18.44, 21.32 and 21.66 min and a relative abundance of 49.53, 19.56 and 30.91% (w/w), respectively, were evident [Figure S3, SI). The peak at t_R 18.44 min was identified as hexadecanoic acid from the molecular ion peak of its methyl ester at m/z 270.06 and by co-injection with an authentic sample. By means of similar methods, the peaks at t_R 21.32 and 21.66 min were identified as the methyl esters of octadecanoic acid (m/z 298.29) and (Z)-6-octadecenoic acid (m/z 296.27), respectively.

Chemosensitivity assay

The composition of F22, a 49.53:19.56:30.91 (w/w/w) mixture of hexadecanoic acid: octadecanoic acid: (Z)-6-octadecenoic acid, was recreated using pure hexadecanoic acid (Cat. No. SI-P0500, Sigma, USA), octadecanoic acid (Cat. No. SI-S4751, Sigma) and (Z)-6-octadecenoic acid (Cat. No. SI-O1008, Sigma). The mixture (M3FA), as well as the free fatty acids on their own, was then screened for their ability to restore the etoposide sensitivity in the A549RT-eto cell line in comparison to F22 and verapamil (10 μM). The M3FA at 125 μg/mL had a clear ability to restore etoposide sensitivity to the A549RT-eto cell line and showed a broadly similar RF value (1.24) to that for F22 at the same concentration (RF = 1.30), although this was just over two-fold lower than that for the verapamil control [Table 2]. Moreover, hexadecanoic acid on its own at a lower concentration (the same as that of its concentration in F22 or the M3FA mixture) had an even higher (1.2-fold) RF value, whilst octadecanoic and (Z)-6-octadecenoic acids on their own showed lower RF values [Table 2].

Rhodamine-123 accumulation and efflux assays

The major cause of MDR in cancer cell lines is the over-expression of P-gp. To study the effect of F22 or M3FA on P-gp function, the activity of P-gp was assessed by measuring the intracellular retention of Rh-123 fluorescence in the A549RT-eto cells. F22 increased the intracellular Rh-123 accumulation with an RR_A value of 1.58 [Figure 2], which may reflect an interference with P-gp function. Furthermore, the RR_A of Rh-123 with F22 was very close to that for the positive control verapamil (RR_A = 1.57), and not that much higher (1.16-fold) than that for the M3FA mixture [Figure 2]. However, on their own the three fatty acids did not increase the RR_A of Rh-123, with RR_A values from 0.78 to 0.93, the lowest of which was hexadecanoic acid [Figure 2], in contrast to its high RF value.

DISCUSSION

A number of naturally derived agents serving as inhibitors, modulators or chemosensitizers have been identified and found to be capable of reversing the MDR of anticancer drugs *in vitro*.[4] Overexpression of P-gp is a well-known cause of MDR in cancer cell lines, where P-gp functions as a reverse flux protein and results in the efflux of the anticancer drugs and consequentially protects the cancer cells from the drug's cytotoxic effects.[23,24] The recently developed A549RT-eto cell line has a high expression level of P-gp on the plasma membrane and has been shown to exert a P-gp-dependent MDR including resistance to etoposide.[20] Accordingly, the A549RT-eto cell line was used in this study to screen for bioactive compounds from

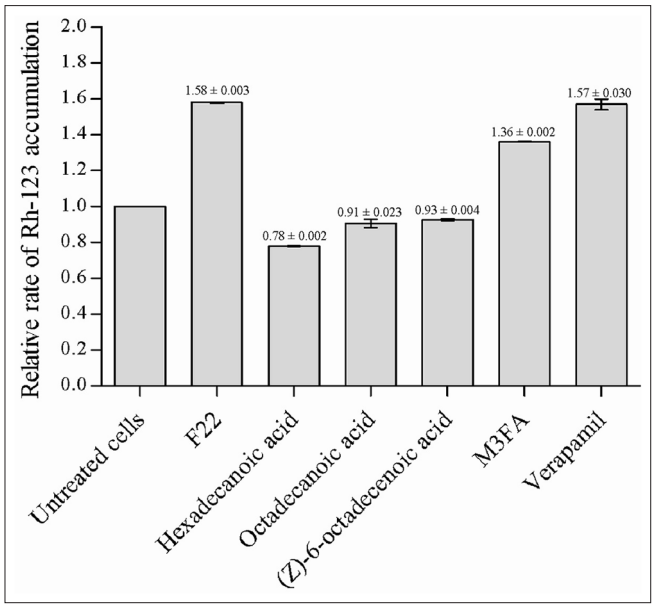

Figure 2: Effect of the bioactive fractions from *T. triandra* on Rh-123 accumulation in A549RT-eto cells. The relative rate of accumulation (RRA) of Rh-123 in A549RT-eto cells (F22 125 µg/mL, Hexadecanoic acid; 61.9 µg/mL; 241 µM, Octadecanoic acid (24.5 µg/mL; 85.9 µM, (Z)-6-octadecenoic acid 38.6 µg/mL; 136.8 µM, M3FA 125 µg/mL; Net fatty acid of 464.2 µM and Verapamil 4.91 µg/mL; 10 µM) was evaluated in terms of the intracellular Rh-123 level as measured by a fluorescence microplate reader at 535 nm. Data are shown as the mean ± 1 SD and are derived from triplicate repeats. Means with a different lowercase letter are significantly different ($P < 0.05$; Duncan's MMT)

T. triandra leaf extracts that could reverse the etoposide resistance through the (assumed) effect of inhibiting the P-gp drug efflux pump.

The sensitivity of A549RT-eto cancer cells to etoposide was partially restored in the presence of the hexane extract (F1) of *T. triandra*. Following further fractionation, one fraction (F22) was obtained with this ability. Structural analyses revealed the composition to be a 49.53:19.56:30.91 (w/w/w) mixture of hexadecanoic: octadecanoic: (Z)-6-octadecenoic acids, and when stochiometrically reconstituted from pure fatty acids the mixture (M3FA) at the same non-cytotoxic concentration (125 µg/mL) as F22 gave a similar RF index (1.24 vs. 1.30) and RR_A of Rh-123 (1.58 vs. 1.36) as F22. The mixture of the three fatty acids was important as alone at the same individual concentrations none could enhance the RR_A of Rh-123 and only hexadecanoic acid showed a RF value of more than 1.0. Although hexadecanoic acid has not previously been reported to increase the cytotoxicity of chemotherapy drugs, ceramide, with a similar structure to hexadecanoic acid, has been reported to increase the etoposide-induced apoptosis in leukemia MOLT-4 cells[25] and to enhance the doxorubicin-mediated apoptosis of leukemia cells.[26,27] In addition, other compounds with similar structures, such as various EFA's (γ-linolenic acid, eicosapentanoic acid, arachidonic acid and docosahexanoic acid), have also been reported to enhance the efficacy of chemotherapy drugs in cancer cell lines and to increase the sensitivity of the drug-resistant KB-ChR-8-5 human cervical carcinoma cells to the cytotoxic action of vincristine *in vitro*.[28] To support that the efficacy of these fatty acids on the etoposide sensitivity was via alteration of the P-gp activity in the A549RT-eto cells, the RR_A of Rh-123 was evaluated for F22 and M3FA in comparison to verapamil as a positive control. The RR_A of Rh-123 was increased in the presence of F22 or M3FA, supporting (but not confirming) that together the three fatty acids in F22 could reverse the etoposide resistance in the A549RT-eto cell line by modulating the P-gp function. Even though each of the three pure fatty acids alone showed a low efficiency of Rh-123 accumulation (RR_A of less than 1), M3FA can accumulate Rh-123 in the cell with a RR_A value of 1.36 which was fairly close to that of F22. Overall, the results suggested that the presence of all three fatty acids together might exert an effect on P-gp function, resulting in an increased sensitivity to etoposide in the A549RT-eto cell line.

Although this study demonstrates the ability of a mixture of three fatty acids (hexadecanoic acid, octadecanoic acid and (Z)-6-octadecenoic acid) from the hexane extract of *T. triandra* leaves to enhance the cytotoxicity of etoposide against A549RT-eto cells when together but not alone, the importance of the composition of the mixture remains unknown, or the presence of other minor components in the F22 hexane extract of *T. triandra* leaves. Further work at the molecular level will be investigated for more comprehensive understanding.

SUPPORTING INFORMATION

The dose response curves for etoposide on the A549 and A549RT-eto cell lines are available in Figure S1, whilst the ^{1}H-NMR, ^{13}C-NMR and GC-MS spectra of F22 are available as Figures S2-3, respectively, in the Supporting Information.

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