

## Original Article

# Thujone-Rich Fraction of *Thuja occidentalis* Demonstrates Major Anti-Cancer Potentials: Evidences from *In Vitro* Studies on A375 Cells

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Crude ethanolic extract of *Thuja occidentalis* (Fam: Cupressaceae) is used as homeopathic mother tincture (TOΦ) to treat various ailments, particularly moles and tumors, and also used in various other systems of traditional medicine. Anti-proliferative and apoptosis-inducing properties of TOΦ and the thujone-rich fraction (TRF) separated from it have been evaluated for their possible anti-cancer potentials in the malignant melanoma cell line A375. On initial trial by *S*-diphenyltetrazolium bromide assay, both TOΦ and TRF showed maximum cytotoxic effect on A375 cell line while the other three principal fractions separated by chromatography had negligible or no such effect, because of which only TRF was further characterized and subjected to certain other assays for determining its precise anti-proliferative and apoptotic potentials. TRF was reported to have a molecular formula of  $C_{10}H_{16}O$  with a molecular weight of 152. Exposure of TRF of *Thuja occidentalis* to A375 cells *in vitro* showed more cytotoxic, anti-proliferative and apoptotic effects as compared with TOΦ, but had minimal growth inhibitory responses when exposed to normal cells (peripheral blood mononuclear cell). Furthermore, both TOΦ and TRF also caused a significant decrease in cell viability, induced inter-nucleosomal DNA fragmentation, mitochondrial transmembrane potential collapse, increase in ROS generation, and release of cytochrome *c* and caspase-3 activation, all of which are closely related to the induction of apoptosis in A375 cells. Thus, TRF showed and matched all the anti-cancer responses of TOΦ and could be the main bio-active fraction. The use of TOΦ in traditional medicines against tumors has, therefore, a scientific basis.

## 1. Introduction

*Thuja occidentalis* (Fam: Cupressaceae), commonly known as Arbor vitae or white cedar, is an ornamental tree grown in Europe [1]. It is used as a medicinal plant in various forms of traditional medicines like folk medicine, homeopathy, and so forth. for treatment of bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea and rheumatism [2–5]. In homeopathy, the crude ethanolic extract of *T. occidentalis* is used as mother tincture (TOΦ). Thuja is also occasionally used for treating diseases of skin, blood, gastrointestinal tract, kidney, brain, warty excrescences, spongy tumors, and so forth. and claimed to have pronounced remedial effects. The extract has been reported to enhance the antibody response to sheep blood cells [6]. Protective effect of *T. occidentalis*

has also been reported against radiation-induced toxicity in mice [7]. Ameliorative effect of *T. occidentalis* has also been suggested in preventing congestive heart disease [8]. Therefore, it gained attention of pharmacologists to study the major constituents of the dried herbal substances of *T. occidentalis* [9–11]. Some 31 compounds were identified in *T. occidentalis* “globosa” and 27 in *T. occidentalis* “gracilis” [9]. Although some minor differences exist in the actual ingredients of several varieties of *Thuja*, generally the major components found in extracts of all varieties of *T. occidentalis* comprise essential oil (1.4–4%) as the principal constituent. Other constituents include coumarins (*p*-coumaric acid, Umbelliferone) flavonoids (Catechine, Gallocatechine, etc.), tannic acid, polysaccharides and proteins [12]. The essential oil of the fresh leaves (related to the monoterpene fraction) contains 65% thujone, 8% isothujone and fenchone each,

5% sabines and 2% a-pinene as the main monoterpenes. Among the constituents of the dried herbal substances, it is not known precisely as to which fraction(s) of the extract is(are) the most bioactive agent(s), particularly in respect of its (their) anti-tumor or anti-cancer activities. In homeopathic literature [13], TOΦ has been reported to be effective against various forms of skin diseases, particularly in the treatment of moles and papillomas. Homeopathic TOΦ has been reported earlier to have cytotoxic effect in Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC), and lung carcinoma L929 [14]. Dubey and Batra also reported hepato-protective activities [15] and antioxidant activity [16] of *T. occidentalis* in CCL<sub>4</sub>-treated liver damage in rats. However, to the best of our knowledge, anti-cancer potentials of TOΦ or any of its major fractions had not been tested earlier in skin cancer cell line A375 by utilizing some widely acceptable parameters of study, like S-diphenyltetrazolium bromide (MTT) assay, Trypan blue exclusion assay and lactate dehydrogenase (LDH) activity-based cytotoxicity assays, fluorescence microscopy, comet assay, DNA fragmentation assay, analysis of changes in morphological features of cells and in mitochondrial membrane potential, ROS production, flow cytometric analysis, immunofluorescence study and western blot analysis.

Thus, the hypotheses to be tested were:

- (i) whether TOΦ and all chromatographically separated fractions had potential anti-cancer effects in A375 cells;
- (ii) if any fraction could be demarcated as the most biologically active one;
- (iii) if, it is possible, to chemically characterize this fraction;
- (iv) whether the cytotoxic, anti-proliferative and apoptotic effects of the fraction, vis-à-vis TOΦ, could be compared with focus on its possible mechanism of action; and
- (v) if it is possible to assess the overall anti-cancer potentials of TOΦ in relation to its preferential cytotoxic effect over normal cells, by analyzing results of different relevant assays including the possible signaling pathways.

## 2. Methods

**2.1. Sources of the Homeopathic Drugs.** *Thuja occidentalis* Φ was procured from Dr Willmer Schwabe India Pvt. Ltd (a subsidiary of Schwabe International GmbH, Germany), Noida, India. The drug was prepared by following the procedure as laid down in the Pharmacopeia of India [17].

**2.2. Chromatographic Separation of the TOΦ.** The solvent was removed and the residue was dissolved in a minimum volume of ethanol and mixed with silica gel (60–120 mesh) and air dried. The mixture was then loaded on to a silicic acid column pre-equilibrated in petroleum ether. The column was then eluted sequentially with 100 mL volume of each of

(i) petroleum ether–ethyl acetate (9 : 1, v/v), (ii) petroleum ether–ethyl acetate (7 : 3, v/v); (iii) petroleum ether–ethyl acetate (3 : 7, v/v), and (iv) ethanol (90%v/v) [18]. A375 cells were treated with fractions for preliminary screening.

**2.3. Chemicals and Reagents.** Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraaceticacid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (USA). All organic solvents used were of HPLC grade. Propidium iodide (PI), acridine orange (AO), 4',6'-diamidino-2 phenyl indole (DAPI), 3-(4,5-dimethyl-thiazol-2-yl)-2, MTT and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**2.4. Cell Culture.** A375 human malignant melanoma cell line was collected from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM supplemented with 10% heated inactivated FBS and 1% antibiotic (PSN) and maintained at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Cells were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline and plated at required cell numbers and allowed to adhere for 24 h before treatment.

**2.5. MTT Assay.** The human melanoma A375 cells were dispensed in 96-well flat bottom microtiter plates at a density of 1 × 10<sup>4</sup> cells per well. After 24 h of incubation, they were treated with various concentrations of *T. occidentalis* Φ to determine the concentration of TOΦ at which the percentage of cell death was nearly 50%.

A375 cells were further treated with various concentrations of thujone-rich fraction (TRF) followed by 24 h of incubation. The control received no drug. After the incubation 10 μL of MTT solution (5 mg mL<sup>-1</sup>) was added to each well. The intracellular formazan crystals formed were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm [19] using an ELISA reader (Multiscan EX, Thermo Electron Corporation, USA). The relative percentage of viability was calculated as follows:

Relative percentage viability

$$= \left( \frac{\text{OD of drug-treated sample}}{\text{OD of control sample}} \right) \times 100. \quad (1)$$

**2.6. Trypan Blue Exclusion Assay (Cytotoxicity Assay).** For cytotoxicity assessment, trypan blue exclusion assay was performed [20]. After treatment with TRF (0–200 μg mL<sup>-1</sup>) for 24 h, A375 cells were stained with 0.4% trypan blue and 100 cells were counted at various fields in haemocytometer for each experiment. The level of cytotoxicity was calculated as per the following formula:

Relative percentage of cytotoxicity

$$= \left( \frac{\text{Total cells} - \text{Viable cells}}{\text{Total cells}} \right) \times 100. \quad (2)$$

**2.7. LDH Activity-Based Cytotoxicity Assay.** LDH activity was assessed using a standardized kinetic determination kit (Enzopak, Recon, India). LDH activity was measured in both floating dead cells and viable adherent cells. The floating cells were collected from culture medium by centrifugation (1000 g) at 4°C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp) [21]. The LDH released in the culture supernatant [designated as extracellular LDH (LDHe)] was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHi). The percentages of apoptotic and necrotic cell deaths were calculated as follows:

$$\text{Apoptosis\%} = \frac{\text{LDHp}}{(\text{LDHp} + \text{LDHi} + \text{LDHe})} \times 100, \quad (3)$$

$$\text{Necrosis\%} = \frac{\text{LDHe}}{(\text{LDHp} + \text{LDHi} + \text{LDHe})} \times 100.$$

**2.8. Observation of Morphological Changes.** Cells plated in six-well culture plates ( $2 \times 10^5$  cells per well) in DMEM supplemented with 10% FBS for 24 h were treated with or without TRF at a specified concentration. After 24 h, the cells were observed under inverted phase contrast microscope (Axiscop plus 2, Zeiss, Germany) and photographs were taken.

**2.9. Fluorescence Microscopy.** To determine the live apoptotic cells, cells treated for 24 h were stained separately with  $10 \mu\text{g mL}^{-1}$  of DAPI and with acridine orange:ethidium bromide (AO/EB) [22]. After 24 h, the control and treated cells were stained with acridine orange ( $50 \mu\text{g mL}^{-1}$ ) and ethidium bromide ( $50 \mu\text{g mL}^{-1}$ ) mixture. Then the cells were analyzed under fluorescence microscope (Axiscop plus 2, Zeiss, Germany) and representative photographs were taken for further quantitative analysis.

**2.10. Analysis of Changes in Mitochondrial Membrane Potential.** The changes in mitochondrial membrane potential of the treated cells were determined using a fluorescent probe, Rhodamine 123 [23]. Briefly,  $5 \mu\text{L}$  of Rhodamine 123 ( $1 \text{ mmol L}^{-1}$ ) were added to the cells ( $2 \times 10^5$ ). Then the cells were incubated for 15 min. Next, the cells were washed with PBS and observed under a fluorescence microscope (Axiscop plus 2, Zeiss) and photographs were taken.

The mitochondrial membrane potential changes were also determined by flow cytometer (FACS caliber, BD Bioscience) [24]. Briefly, cells after treatment were washed with ice-cold PBS before incubation with Rhodamine 123 ( $5 \text{ mmol L}^{-1}$ ) in darkness for 15 min at room temperature. The fluorescence emissions were analysed with a flow cytometer.

**2.11. Analysis of ROS Production.** Cells after treatment were suspended in growth medium and incubated with DCFDA (2'-7'-dichloro-fluorescein diacetate) ( $5 \mu\text{M}$ ) for 15 min. Cells were washed with PBS and analyzed under fluorescence microscope and photographs were taken.

To evaluate the intra-cellular ROS level, the cells were incubated with  $10 \mu\text{M}$  DCFDA for 30 min at room temperature. After washing the cells twice with PBS, the intensity of DCFDA fluorescence was determined by flow cytometer with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

**2.12. Flow Cytometric Analysis.** A375 cells were harvested and washed once in cold phosphate-buffer saline (PBS). Cell pellets were fixed in 3% paraformaldehyde and suspended in 1 mL of PI solution containing  $50 \text{ mg mL}^{-1}$  PI, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X-100. Cells were incubated at 4°C in the dark for 15 min and analyzed by a flow cytometer [25].

In order to detect the translocation of phosphatidylserine from inner cell membrane to outer cell membrane (a characteristic feature of cells undergoing apoptosis), cells were subjected to flow cytometric analysis after staining with Annexin V-FITC and PI [23].

**2.13. Comet Assay.** After treatment, the cells were trypsinized and washed in ice-cold PBS. The cell suspension was mixed with an equal amount of 1% low melting agarose kept at 37°C. Immediately after mixing,  $100 \mu\text{L}$  of the suspension was pipetted on to microscope slide pre-coated with normal melting agarose, then covered with cover slip and placed on a glass tray on ice. Then the slides were immersed in cold lysis solution ( $2.5 \text{ M NaCl}$ ,  $100 \text{ mM EDTA}$ ,  $10 \text{ mM Tris}$ , PH-10, with freshly added 1% Triton X-100 and 2% DMSO) followed by incubation at 4°C for at least 1 h. The electrophoresis in weak alkali ( $0.03 \text{ M NaOH}$ ,  $1 \text{ mM EDTA}$ , PH-12) at  $1 \text{ V cm}^{-1}$  and 30 mA for 15 min was preceded by a 20 min immersion of the slides in electrophoresis buffer to promote chromatin unwinding. After electrophoresis the slides were neutralized in  $0.05 \text{ M Tris}$  buffer. Then DNA was stained with ethidium bromide ( $50 \mu\text{g mL}^{-1}$ ) for 10 min, washed in distilled water and examined in a fluorescence microscope (Lyca, USA) [26].

**2.14. DNA Fragmentation Assay.** The cells were grown to 70% confluence and treated with various concentrations of TRF for 24 h. Following this treatment, the cells were washed twice with phosphate-buffered saline [ $10 \text{ mM Tris}$  (pH 7.5),  $150 \text{ mM NaCl}$ ,  $5 \text{ mM MgCl}_2$  and 0.5% Triton X-100], left on ice for 15 min, and pelleted by centrifugation (1000 rpm) at 4°C. The pellet was incubated with DNA lysis buffer [ $10 \text{ mM Tris}$  (pH 7.5),  $400 \text{ mM NaCl}$ ,  $1 \text{ mM EDTA}$  and 1% Triton X-100] for 30 min on ice and then centrifuged at 15 000 g at 4°C. The supernatant that was obtained was incubated overnight with RNase ( $0.2 \text{ mg mL}^{-1}$ ) at room temperature and then with proteinase K ( $0.1 \text{ mg mL}^{-1}$ ) for 2 h at 37°C. DNA was extracted using a phenol/chloroform mixture (1 : 1) and precipitated with 95% ethanol. The DNA precipitate was centrifuged at 15 000 g and 4°C for 15 min, and the pellet was air-dried and dissolved in  $20 \mu\text{L}$  of Tris-EDTA buffer [ $10 \text{ mM Tris-HCl}$  (pH 8.0) and  $1 \text{ mM EDTA}$ ]. The total amount of DNA was resolved over a 1.5% agarose gel, containing  $0.3 \text{ mg mL}^{-1}$

TABLE 1: One-way ANOVA for effect of different fractions of TOΦ on viability of A375 cells as measured by MTT assay.

Source of variation	Effect of different fractions of TOΦ on viability of A375 cells as measured by MTT assay		
	df	F	Sig.
Between groups	3	55 679.278	0.000*
Within groups	8		

\*  $P < .05$ .

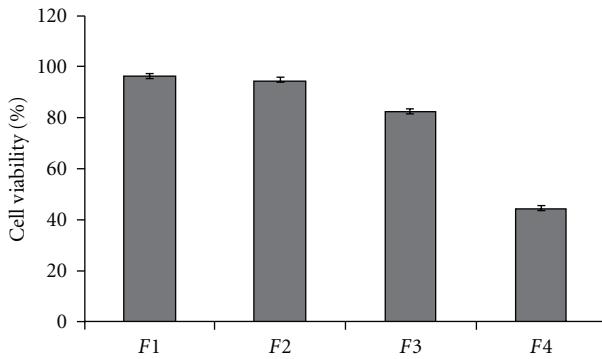
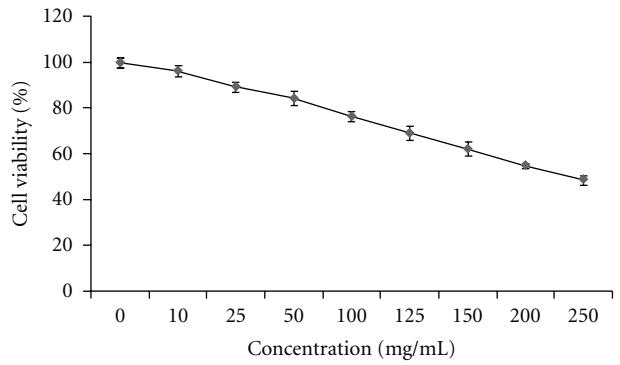


FIGURE 1: Effect of different fractions of TOΦ on viability of A375 cells as measured by MTT assay. Fraction 4 appeared to show maximum inhibitory effect against A375 cells while Fractions 1 and 2 had no effect and Fraction 3 had little effect on the viability of A375 cells. Results are expressed as mean  $\pm$  SEM of three independent experiments and analyzed using one-way ANOVA.  $P$ -value  $< .05$  was considered statistically significant.

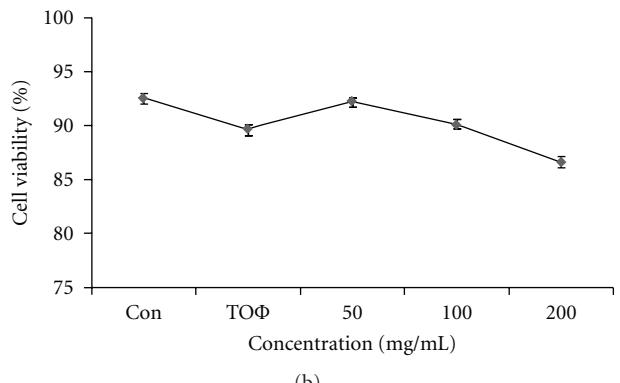
ethidium bromide in  $1\times$  TAE buffer. The bands were visualized under an UV transilluminator followed by digital photography.

**2.15. Immunofluorescence Study.** Cells were plated in six-well culture plates and allowed to adhere for 24 h before treatment. At the end of the treatments, cells were washed with PBS and fixed in 3% paraformaldehyde for 1 h. The cells were permeabilized with 0.2% CHAPS in PBS for 2 min and were blocked in 2% BSA with 0.2% Tween-20 for 30 min [27]. Then, the cells were incubated for overnight at  $4^{\circ}\text{C}$  with a 1 : 400 dilution of specific primary antibodies (anti-Bcl-2, anti-Bax, anti-cytochrome *c* and anti-caspase-3) and further incubated for 1 h with a 1 : 2000 dilution of goat anti-mouse IgG-FITC as a secondary antibody. Immunofluorescence was photographed.

**2.16. Preparation of Cell Lysates.** Lysates were prepared to examine the expression of Bax, Bcl2, cytochrome *c*, caspase-3. A375 cells were washed twice with PBS and then lysed in ice-cold lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton-X 100, 1 mM EDTA, 2 mM sodium orthovanadate, 100 mM Sodium fluoride, 10  $\mu\text{g mL}^{-1}$  leupeptin, and 1 mM PMSF, 10  $\mu\text{g mL}^{-1}$  aprotinin. After 60 min of incubation on ice, the cells were swelled and then centrifuged at 12 000 g



(a)



(b)

FIGURE 2: (a) Effect of different concentrations TRF on viability of A375 cells as measured by MTT assay. Histogram shows 50% of cell death at  $226.18 \mu\text{g mL}^{-1}$  of TOΦ. (b) Cytotoxicity of TRF on normal PBMC as determined by trypan blue staining assay. Cell cytotoxicity on PBMC was measured in the mentioned dose of TRF that showed minimal cytotoxic effects ( $\sim 14\%$ ) even at the highest dose. Results are expressed as mean  $\pm$  SEM of three independent experiments. Data are means  $\pm$  SD from three different measurements and were analyzed using one-way ANOVA.  $P < .05$  compared to the control group. Con = control.

for 20 min. The protein content of the supernatant was determined by Bradford assay with bovine serum albumin as standard and then was stored at  $-20^{\circ}\text{C}$  until analysis [28].

**2.17. Western Blot Analysis.** Both adherent and floating cells were collected, and then western blot analysis was carried out. Equal amounts of lysate protein were run on 12.5% SDS-PAGE and electrophoretically transferred to PVDF membrane [29]. After blocking, the blots were incubated with specific primary antibodies (anti-Bcl-2, anti-Bax, anti-cytochrome *c* and anti-caspase-3) overnight at  $4^{\circ}\text{C}$  and further incubated for 2 h with a 1 : 2000 dilution of goat anti-mouse IgG-ALP as a secondary antibody. Bound antibodies were developed by BCIP-NBT and quantification of proteins was done by densitometry using image analyzer (Gel Doc System; Ultra Lum, USA). The same membranes were also immunoblotted against  $\beta$ -actin (house keeping gene) for data normalization.

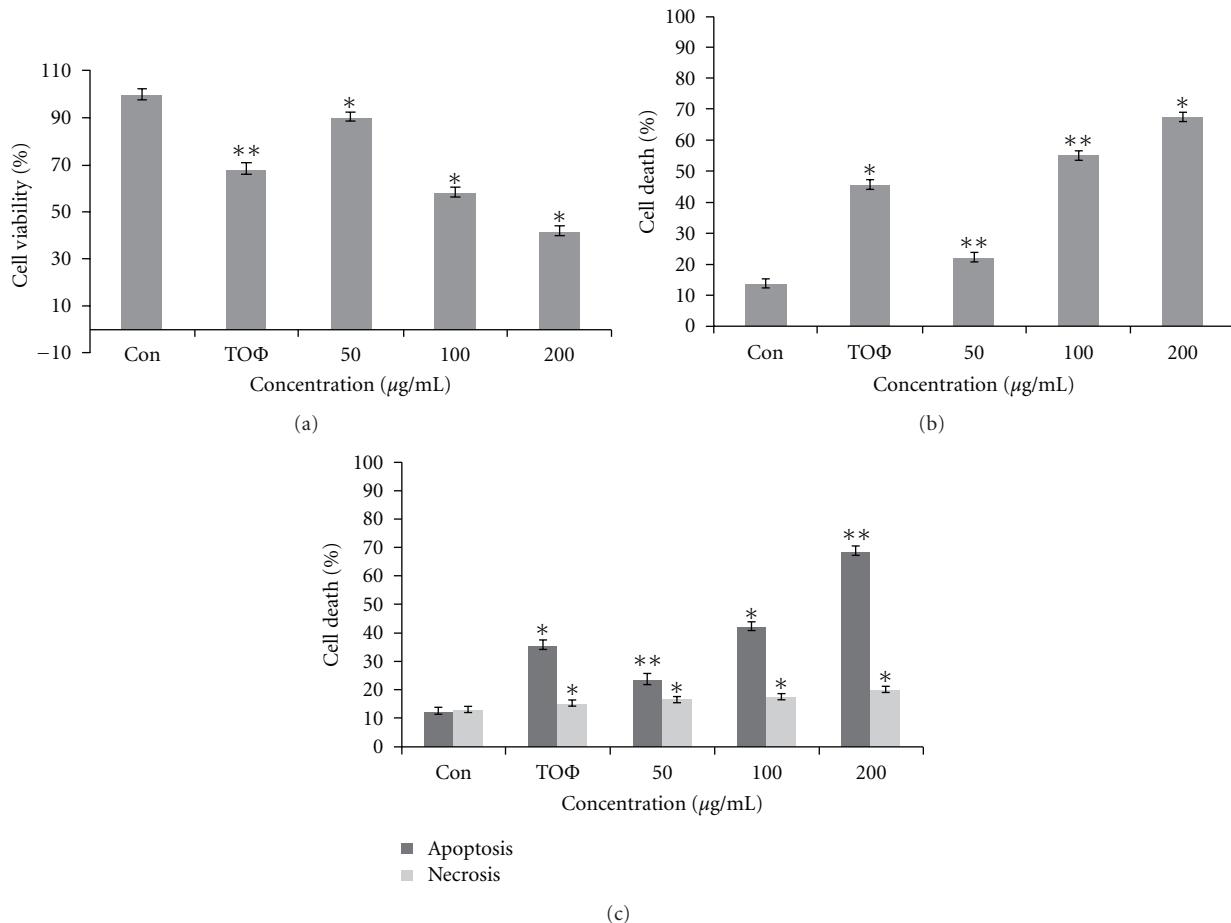


FIGURE 3: (a) Effect of *T. occidentalis* Φ and different concentrations of TRF on viability of A375 cells as measured by MTT assay. Treatment of A375 cells with 100 and 200  $\mu\text{g mL}^{-1}$  for 24 h resulted in ~42 and 59% cell deaths, respectively, where TOΦ had ~32% cell death. (b) Cytotoxic effect of TOΦ and different concentrations of TRF on A375 cells as determined by trypan blue exclusion method. An amount of 100 and 200  $\mu\text{g mL}^{-1}$  concentrations of TRF showed more cytotoxic effect compared with that of TOΦ for 24 h. (c) Cells treated with TOΦ and various doses of TRF (50, 100, 200  $\mu\text{g mL}^{-1}$ ) for 24 h were measured by LDH activity-based assay. The ratio of apoptotic cells increased from 23.59% at 50  $\mu\text{g mL}^{-1}$  to 68.83% at 200  $\mu\text{g mL}^{-1}$  for TRF, but that of necrotic cells was still negligible in the presence of 200  $\mu\text{g mL}^{-1}$  TRF. Data are each the mean  $\pm$  SD (bars) from three independent experiments. Data are means  $\pm$  SD from three different measurements and were analyzed using one-way ANOVA.  $P < .05$  compared to the control group (zero concentration). Con = control. \* =  $P < .05$ , \*\* =  $P < .01$ .

**2.18. Statistical Analysis.** Statistical analysis was performed by the Student's *t*-test for the significance of difference between the data of control and drug-treated cells. Data were obtained from representative experiments with triplicate and were expressed as mean  $\pm$  standard error (SE). Additionally, ANOVA was performed to identify if the differences were also significant among the means of different groups. The *P*-value  $< .05$  was considered to be significant (Tables 1–4).

### 3. Results

**3.1. Chromatographically Separated Fractions.** Four major fractions were obtained of which only Fraction 4 appeared to show strong inhibition of A375 cell viability, while the other fractions had no (Fractions 1 and 2) or little (Fraction 3) effect. For this, only Fraction 4 which was eluted with 90% (v/v) ethanol was selected for further detailed study.

Differences in cell viability between the Fractions 4 and 1, Fractions 2 or 3 were found to be statistically significant ( $P < .05$ ) (Figure 1).

**3.2. Viability of A375 Cells.** MTT assay was conducted on the A375 cell treated with different concentrations of TOΦ. The results revealed 50% of cell death at 226.18  $\mu\text{g mL}^{-1}$  of TOΦ (Figure 2(a)). The cell cytotoxicity on normal peripheral blood mononuclear cell (PBMC) was also measured in the mentioned dose of TRF that showed minimal cytotoxic effects (~14%) even at the highest dose (Figure 2(b)). Table 2 shows the results of the statistical analysis done by one way ANOVA.

MTT assay showed that TRF had significantly stronger inhibitory effects ( $P < .05$ ) on proliferation of A375 cells. Treatment of A375 cells with 100 and 200  $\mu\text{g mL}^{-1}$  for 24 h resulted in ~42 and 59% cell deaths, respectively

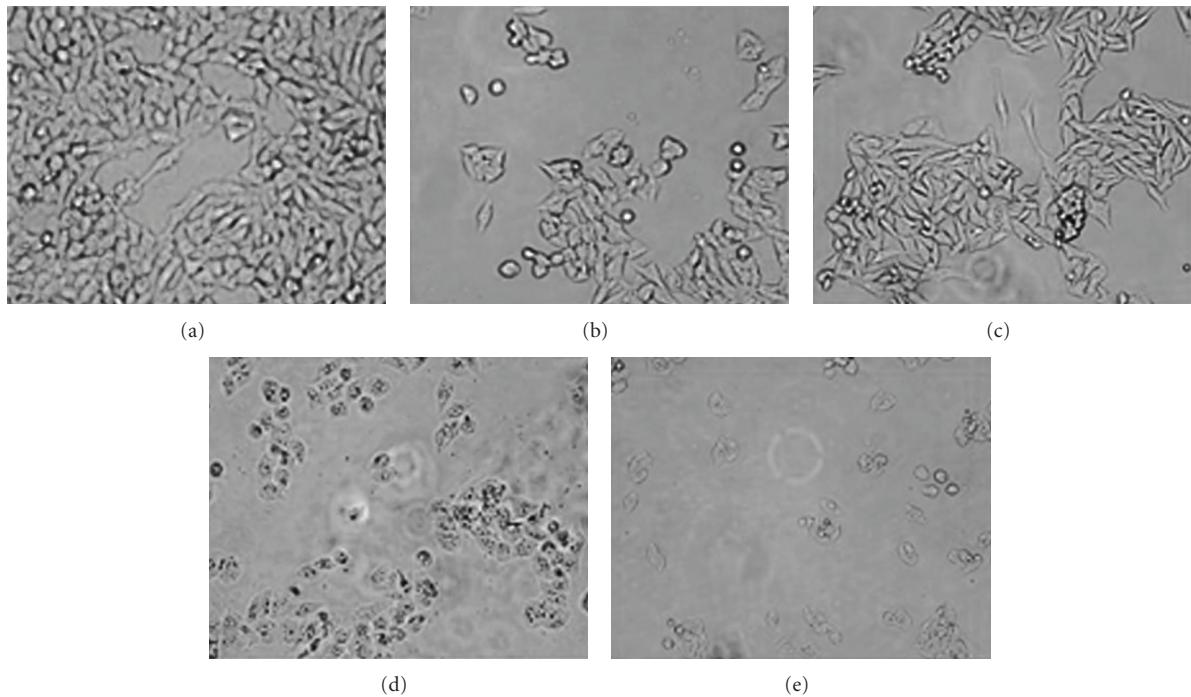


FIGURE 4: Morphology of control cells (a) and TOΦ-treated cells (b). Panels (c), (d), and (e) denotes morphology of cells treated with different concentrations of TRF ( $50, 100$ , and  $200 \mu\text{g mL}^{-1}$ ), respectively, for  $24$  h observed under a phase contrast microscope showing ( $10\times$  magnification). Cell rounding, cytoplasmic blebbing and detachment along with cell shrinkage were observed in treated cells.

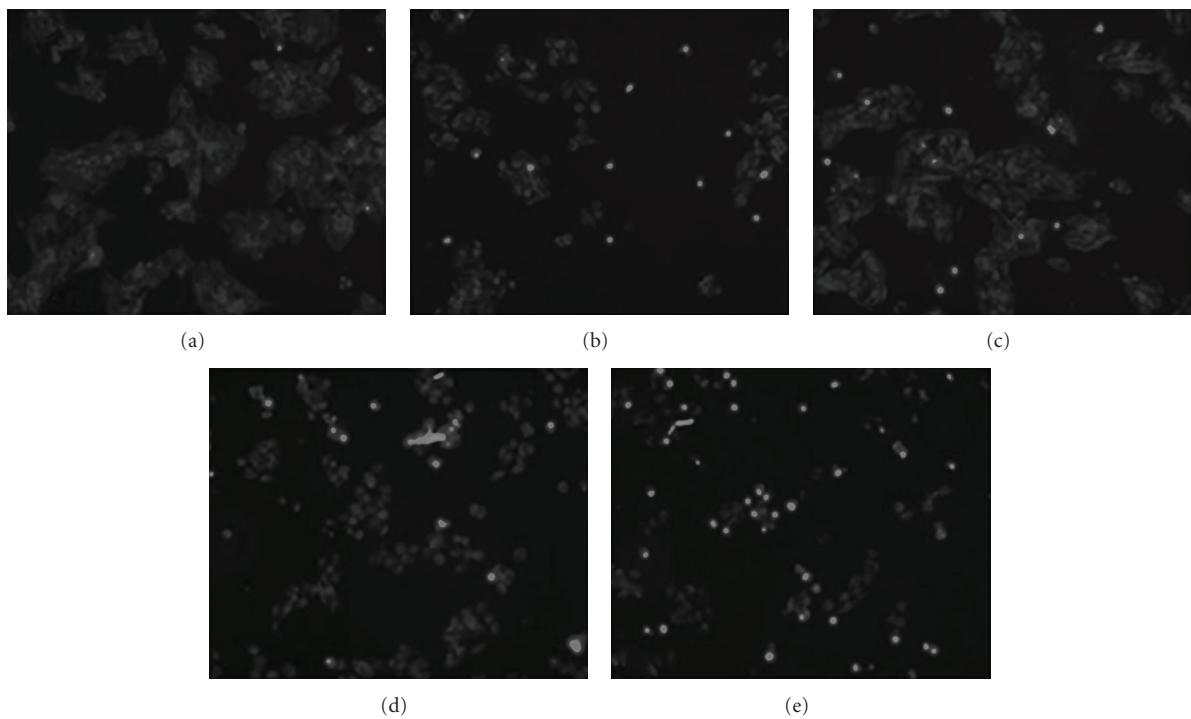


FIGURE 5: Cells were stained with DAPI ((a)= control, (b)= TOΦ, (c)=  $50 \mu\text{g mL}^{-1}$  (d)=  $100 \mu\text{g mL}^{-1}$ , (e)=  $200 \mu\text{g mL}^{-1}$ ) and viewed using fluorescence microscope ( $10\times$  magnification). Photographs demonstrate brightly stained apoptotic nuclei in TRF-treated cells. Note that the number of apoptotic nuclei was increased with the increasing concentrations of TRF where no or negligible number of apoptotic nuclei was observed in the control cells.

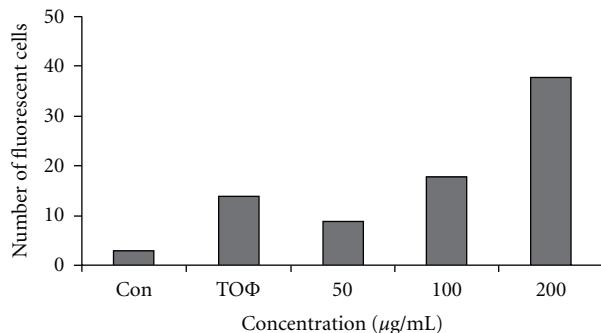


FIGURE 6: Quantitative data on DAPI staining of A375 cells treated with TOΦ and different concentrations of TRF represented by histogram. A positive dose-response was noted as number of fluorescent cells increases with the increase in concentration of TRF (50, 100, and 200  $\mu\text{g mL}^{-1}$ ). Con = control.

TABLE 2: One-way ANOVA for effect of different concentrations TRF on viability of A375 cells as measured by MTT assay and cytotoxicity of TRF on PBMC as determined by trypan blue staining assay.

Source of variation	Cytotoxic effect of different concentrations TPΦ on viability of A375 cells as measured by MTT assay			Cytotoxicity of TRF on PBMC as determined by trypan blue staining assay		
	df	F	Sig.	df	F	Sig.
Between groups	8	35 856.993	0.000*	3	389.971	0.000*
Within groups	18			8		

\*  $P < .05$ .

TABLE 3: One-way ANOVA for effect of *T. occidentalis* Φ and different concentrations of TRF on viability of A375 cells as measured by MTT assay, cytotoxic effect of TOΦ and different concentrations of TRF on A375 cells as determined by trypan blue exclusion method.

Source of variation	Cytotoxic effect of TOΦ and different concentrations of TRF on viability of A375 cells as measured by MTT assay			Cytotoxic effect of TOΦ and different concentrations of TRF on A375 cells as determined by trypan blue exclusion method		
	df	F	Sig.	df	F	Sig.
Between groups	3	81638.983	0.000*	3	68772.131	0.000*
Within groups	8			8		

\*  $P < .05$ .

(Figure 3(a)). The cell cytotoxicity in response to treatment in three concentrations has been shown in Figure 3(b). These results demonstrate that one of the causes of A375 cell death induced by TRF could be attributed to apoptosis. The results were statistically significant at various levels ( $P < .05$  through

TABLE 4: One-way ANOVA of LDH activity of TOΦ and various doses of TRF (50, 100, 200  $\mu\text{g mL}^{-1}$ ) on A375 cells for 24 h.

Source of variation	LDH activity-based assay of cells treated with TOΦ and various doses of TRF		
	df	F	Sig.
Apoptosis			
Between groups	3	58102.645	0.000*
Within groups	8		
Necrosis			
Between groups	3	327.647	0.000*
Within groups	8		

\*  $P < .05$ .

$P < .01$ ). The statistical data of ANOVA have been shown in Table 3.

To further characterize TRF-induced A375 cell death, the ratios of LDH release from viable cells, floating dead cells, and the culture medium were compared (Figure 3(c)). There was a significant ( $P < .05$  to  $P < .01$ ) increase in the ratio of apoptotic cells from 23.59% at 50  $\mu\text{g mL}^{-1}$  to 68.83% at 200  $\mu\text{g mL}^{-1}$  for TRF, but that of the necrotic cells was still negligible in the presence of 200  $\mu\text{g mL}^{-1}$  TRF. Table 4 summarizes the statistical findings of ANOVA performed to identify if the differences were significant among the means of different groups.

**3.3. Morphological Changes.** The morphological changes of A375 cells treated with TRF were also observed. Results revealed that morphological changes (specifically for chromatin condensation and cell shrinkage) were typical of apoptosis. These changes were apparent at 24 h, and became common in ~50% or more of the treated A375 cells after 24 h. Exposure to 100  $\mu\text{g mL}^{-1}$  for 24 h caused the majority of A375 cells to shrink, round up and detach from the culture dish. The effect was more pronounced in the cells treated with the highest dose of 200  $\mu\text{g mL}^{-1}$  (Figure 4).

**3.4. Fluorescence Microscopy.** The untreated A375 cells did not take positive staining with DAPI (Figure 5) and showed no cells with visible chromatin condensation. However, with different concentrations of treatment, cells with chromatin condensation appeared to increase in number along with the increase in dose (Figure 6).

The cells stained with AO/EB in untreated culture (Figure 7) showed intact chromatin without fragmentation; while there were nearly 39% cells with fragmented and condensed chromatin visible at 100  $\mu\text{g mL}^{-1}$  TRF-treated culture. In the 200  $\mu\text{g mL}^{-1}$  TRF-treated culture; nearly 50% cells were fragmented and condensed (Figure 8).

**3.5. Mitochondrial Membrane Potential.** As compared to the control (Figure 9(a)), A375 cells showed greenish stain in greater intensity. However, in the TOΦ and TRF-treated cells (Figures 9(b)–9(e)) reddish stain was more prevalent, indicating mitochondrial potential depolarization, and that

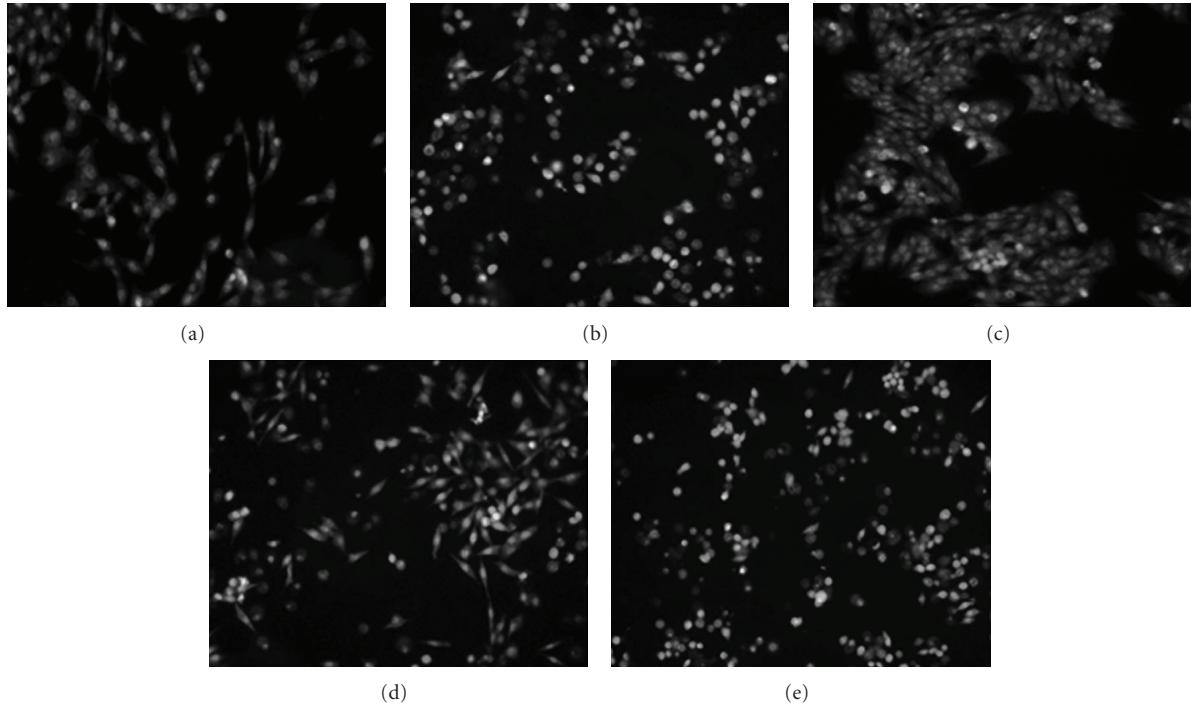


FIGURE 7: Cells were stained with acridine orange and ethidium bromide ((a) = control, (b) = TOΦ, (c)–(e) = TRF; (c) =  $50 \mu\text{g mL}^{-1}$ , (d) =  $100 \mu\text{g mL}^{-1}$ , (e) =  $200 \mu\text{g mL}^{-1}$ ) and viewed using fluorescence microscope ( $10\times$  magnification). Photographs demonstrate brightly stained apoptotic nuclei in TRF-treated cells where number of brightly stained cells was less in the control group, showing no or minimum apoptosis.

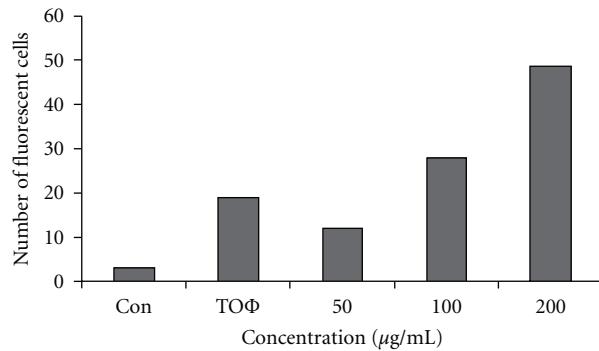


FIGURE 8: Quantitative data on acridine orange and ethidium bromide staining of A375 cells treated with TOΦ and different concentrations of TRF represented by histogram. Number of fluorescent cells increases with the increase in concentration of TRF ( $50, 100$ , and  $200 \mu\text{g mL}^{-1}$ ). Con = control.

was more pronounced in the higher dose (Figure 9(e)). Quantitative data obtained by flow cytometry (Figures 9(f)–9(j), resp.) would also support the mitochondrial membrane potential depolarization convincingly.

**3.6. Reactive Oxygen Species Generation.** In the TRF-untreated control (Figure 10(a)), the A375 cells did not take up much stain, showing less generation of reactive oxygen species (ROS). TOΦ showed intense staining as compared to the control (Figure 10(b)). In contrast, there were number of

cells that showed more intense staining with the increase in dose (Figures 10(c)–10(e)) signifying more amount of ROS generation.

The quantitative data generated by flow cytometry (Figures 10(f)–10(j)) would corroborate the same findings.

**3.7. Flowcytometric Analysis.** To further confirm whether the cause of A375 cell death induced by TRF was apoptosis, flow cytometric analysis was performed. Incubation of fixed and permeabilized cells with fluorochrome PI results in quantitative PI binding with total cellular DNA, and the fluorescence intensity of PI-labeled cells was proportional to DNA contents (Figure 11). Apoptotic nuclei with hypodiploid DNA correspond to the sub  $G_0/G_1$  peak. The maximal increase in the frequency of apoptotic cells was observed upon  $24\text{ h}$  treatment with  $200 \mu\text{g mL}^{-1}$  TRF (Figure 11(d)). These results demonstrate that the treatment with TRF at  $200 \mu\text{g mL}^{-1}$  induced A375 cell apoptosis maximally.

The percentages of apoptosis in TRF ( $200 \mu\text{g mL}^{-1}$ ) treated cells were found to be 34.69% as compared with a very low percentage of apoptotic cells (8.23%) in the untreated cell (Figure 12; Table 5).

**3.8. Comet Assay.** In comet assay, the nuclei were intact and round, without any fragmented DNA (Figure 13(a)) in the control. In the treated cultures (Figures 13(b)–13(e)), there was a linear increase in comet lengths (Figure 14) with the increase in concentrations from  $50, 100$  to  $200 \mu\text{g mL}^{-1}$ ,

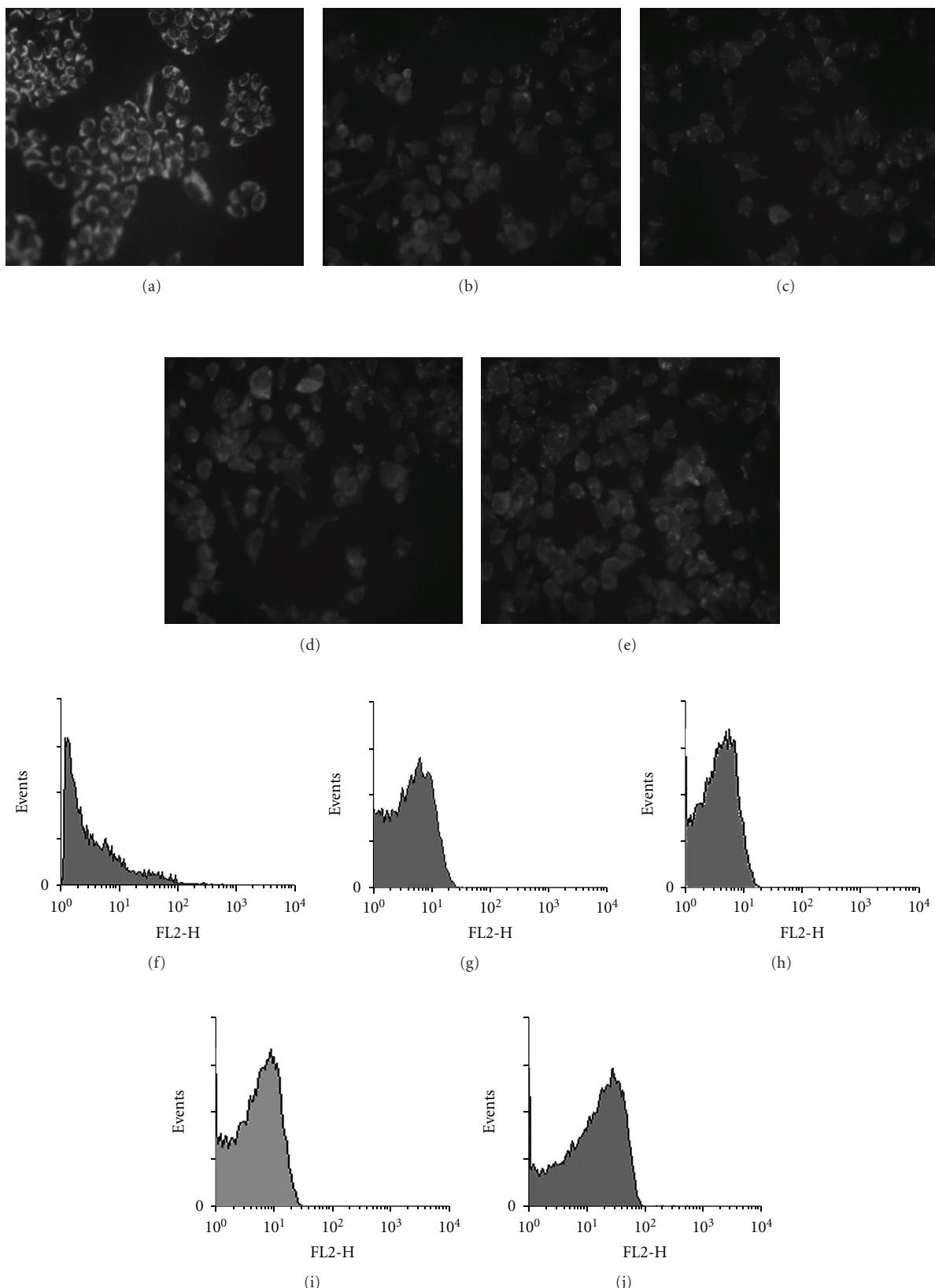


FIGURE 9: A375 cells ((a) = control) showed greenish stain with higher mitochondrial membrane potential. In the cells treated with TOΦ (b) and different concentrations of TRF cells ((c)–(e)) reddish stain depicts lower mitochondrial membrane potential. ((f)–(j)) shows the histogram of mitochondrial membrane potential depolarization measured by flow cytometry.

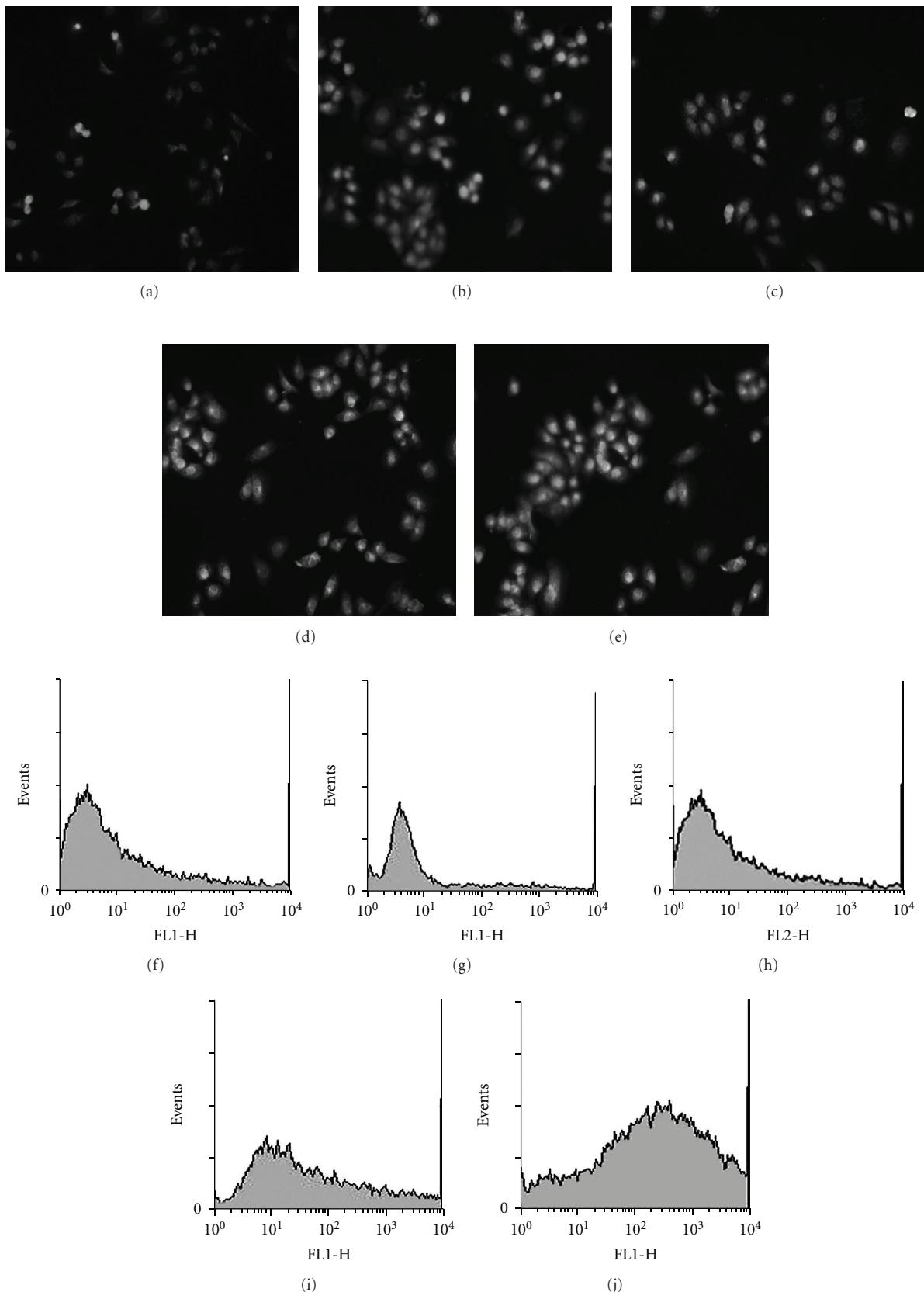
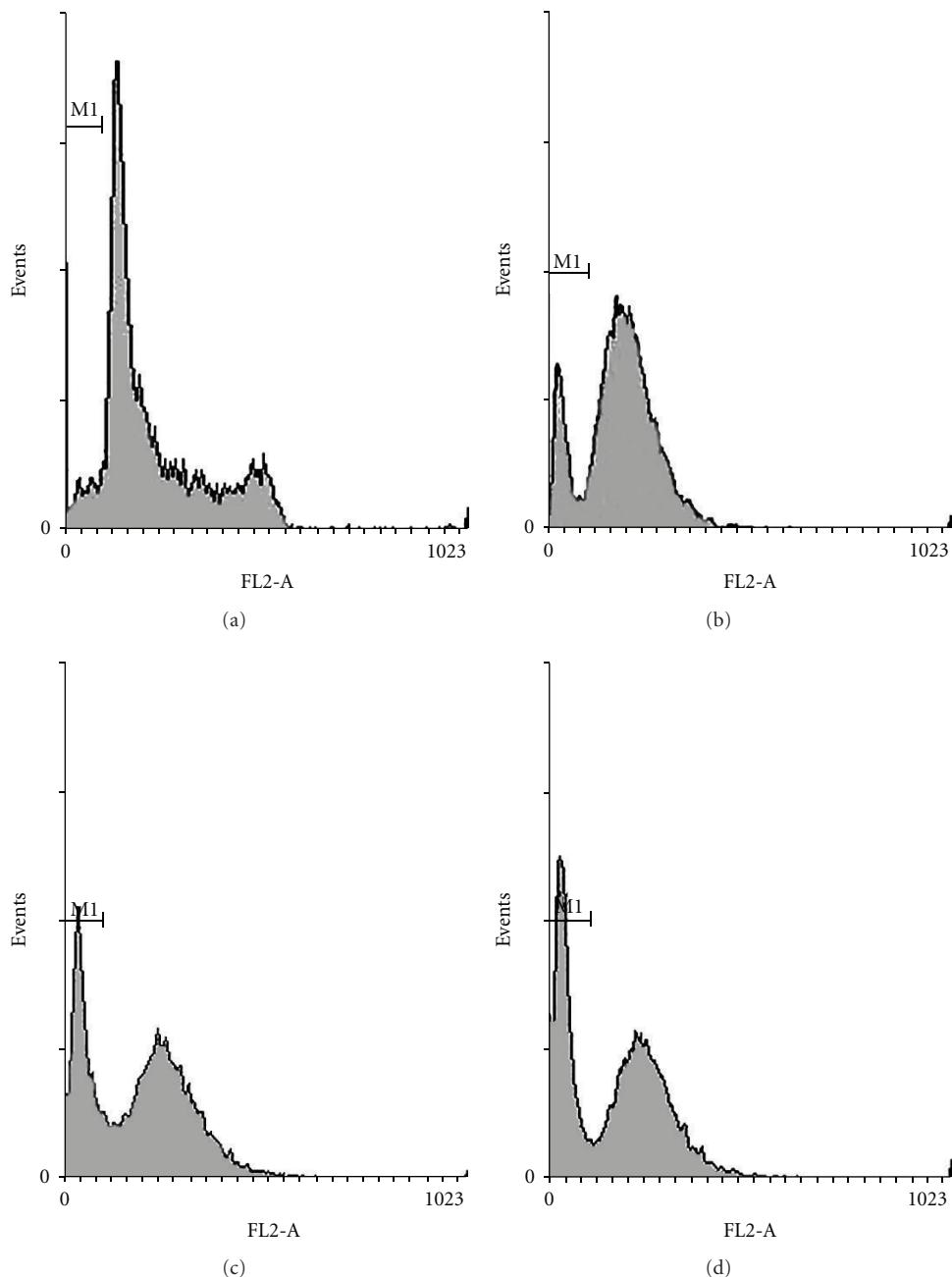


FIGURE 10: Induction of ROS produced in A375 cells (a)= control, (b)= TOΦ, (c)=  $50 \mu\text{g mL}^{-1}$ , (d)=  $100 \mu\text{g mL}^{-1}$ , (e)=  $200 \mu\text{g mL}^{-1}$ ) treated with TOΦ and different concentrations of TRF for 24 h. The generation of ROS was monitored by fluorescence microscopy. Lower panel ((f)–(j)) shows the histogram of intracellular ROS measured by flow cytometry.



**FIGURE 11:** Flow cytometric analysis of the cell cycle distribution of A375 cells with or without TRF for 24 h. Histogram shows TRF-induced A375 cell death by apoptosis. Number of apoptotic cells increased with the increase of dose. ((a)= Control, (b)= TOΦ, (c)= 100  $\mu$ g/mL TRF, (d)= 200  $\mu$ g/mL TRF).

showing signs of more DNA damage with extended comet tails.

**3.9. DNA Fragmentation Assay.** The DNA fragmentation was also tested by agarose gel electrophoresis. Figure 15 indicates a significant increase in inter-nucleosomal DNA fragmentation of A375 cells. When the DNA isolated from TRF-treated cells was subjected to agarose gel electrophoresis, a DNA

ladder characteristic of apoptotic DNA was observed in the cells treated with different concentrations.

**3.10. Immunofluorescence Study.** With the increase in dose of TRF treatment, there was an increase in fluorescence intensity of Bax, cytochrome *c* and caspase-3 and decrease in fluorescence of Bcl-2 (Figure 16) consistent with the immunoblot data (Figure 17).

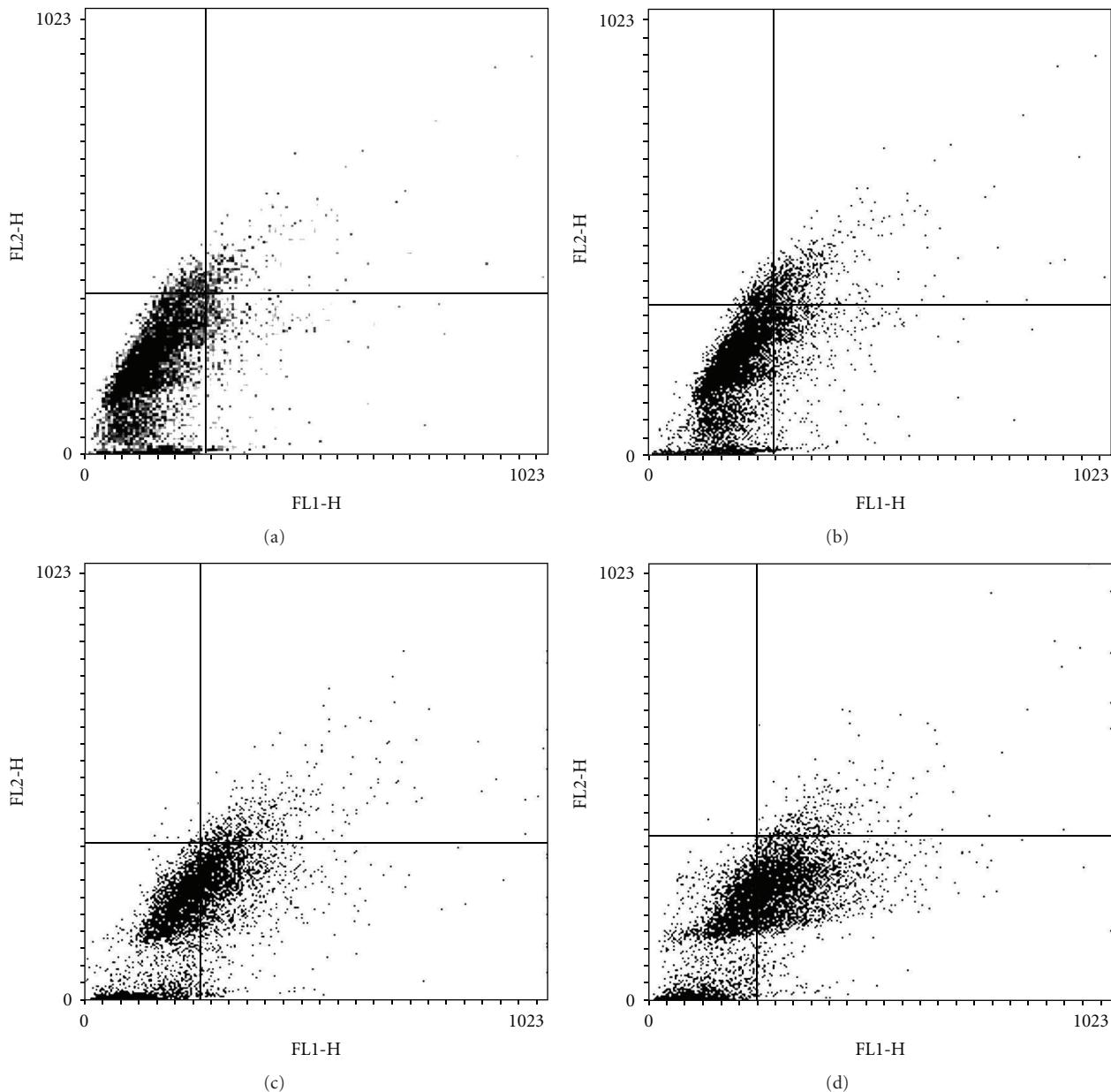


FIGURE 12: Flow cytometric analysis of TOΦ and TRF on A375 cells after staining with Annexin V-FITC and PI. Figure (d) represents more apoptotic cells at the lower right quadrant (early) at 200  $\mu\text{g}/\text{mL}$  dose. ((a)= Control, (b)= TOΦ, (c)= 100  $\mu\text{g}/\text{mL}$  TRF, (d) = 200  $\mu\text{g}/\text{mL}$  TRF).

TABLE 5: Quantitative evaluation of apoptosis through Annexin V-FITC staining method.

Quad	%Total			
	C	TOΦ	100	200
LL	81.46	75.13	72.85	61.77
LR	8.23	18.56	21.64	34.69

LL = lower left panel, LR = lower right panel.

**3.11. Immunoblot Analysis.** Along with the increase in dose of TRF, the expression levels of Bax, cytochrome *c* in cytosolic fraction and caspase-3 in the total cell lysate were

increased while the expression of Bcl-2 was downregulated (Figure 17).

#### 4. Discussion

Results of the present study would indicate that the homeopathic drug *T. occidentalis* Φ had four chromatographically separated fractions of which the thujone-rich fraction was found to be the most bioactive (anti-cancer, pro-apoptotic) component. Unfortunately, despite our best effort, the oily nature of this fraction did not allow us to purify it to 100%, for which we were able to perform only mass spectrometry of TRF. The mass spectrophotometric data obtained by us

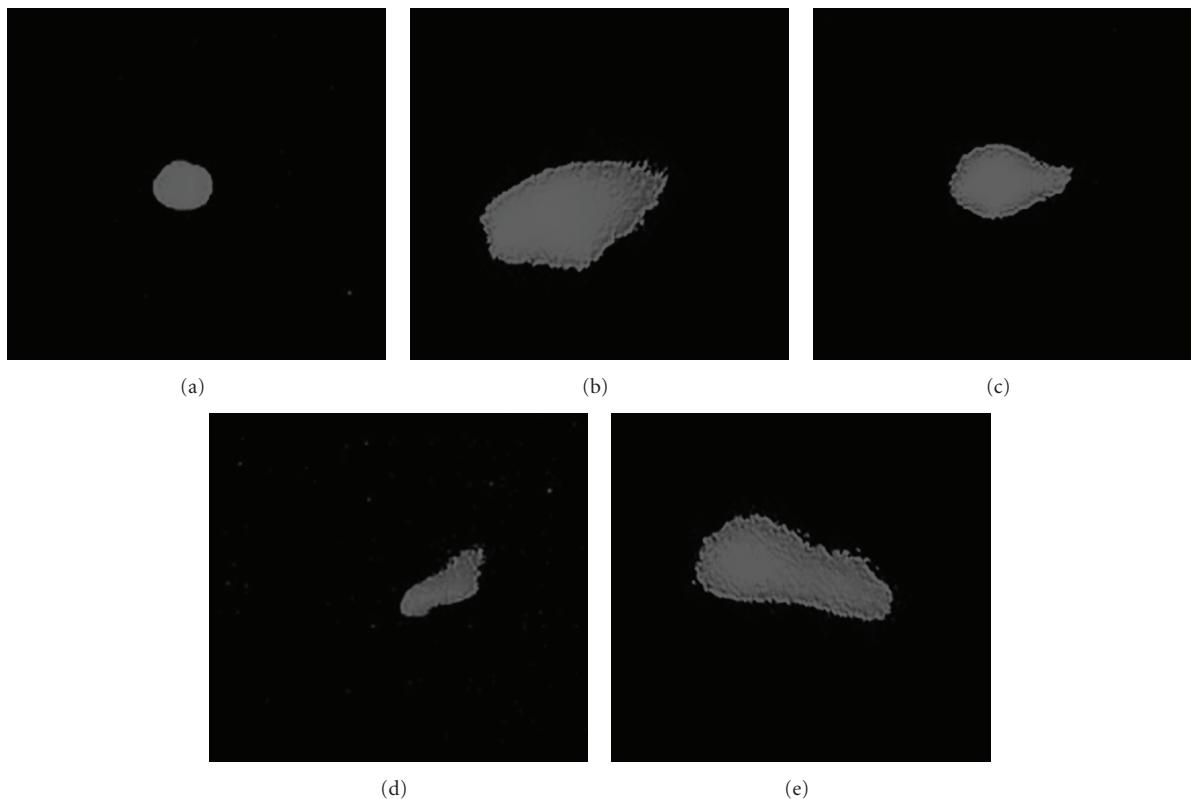


FIGURE 13: Photomicrographs show the control A375 cells (a) with round shaped nuclei and panels (b), (c), (d), and (e) show nuclei with comet tails when treated with TOΦ and different concentrations of TRF, respectively.

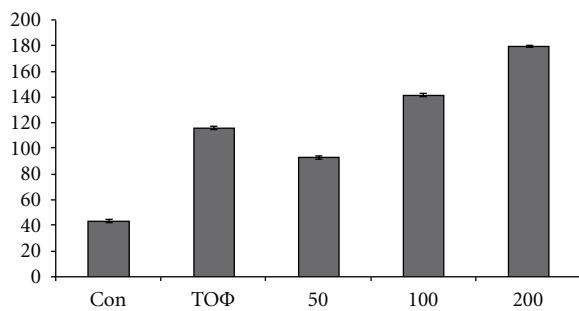


FIGURE 14: Histogram of the comet tail of A375 cells. Con=control, 2nd column = TOΦ, 3rd = 50 µg/mL, 4th = 100 µg/mL, 5th = 200 µg/mL.

supported the published data on the chemical structure of the C<sub>10</sub>H<sub>16</sub>O with a molecular weight 152 as reported by Tarcisia [30].

Anti-cancer activity of the alcoholic extract of *T. occidentalis* had earlier been reported by Sunila and Kuttan [14] from their *in vivo* studies in rats. In our present *in vitro* study involving several protocols, we confirm the pro-apoptotic and anti-cancer potential of *T. occidentalis* mother tincture. Additionally, it was revealed that the thujone-rich component was possibly the key bioactive compound showing its promising anti-cancer potentials in the skin cancer cell line A375, a fact which had not earlier been

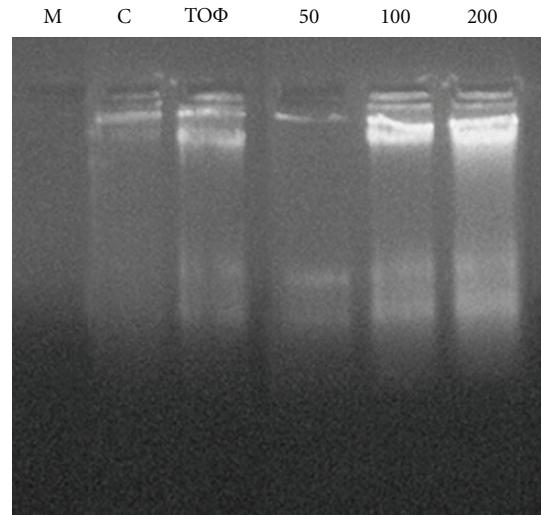


FIGURE 15: DNA fragmentation assay of A375 cells treated with different concentrations of TRF for 24 h. Lane (1) media, lane (2) control, lane (3) TOΦ, lane (4)  $50 \mu\text{g mL}^{-1}$ , lane (5)  $100 \mu\text{g mL}^{-1}$ , lane (6)  $200 \mu\text{g mL}^{-1}$ -treated TRF, respectively.

reported (Figure 18). Furthermore, the anti-cancer activity was mediated through activation of pro-apoptotic signalling via the activation of Bax, caspase-3 and cytochrome *c*.

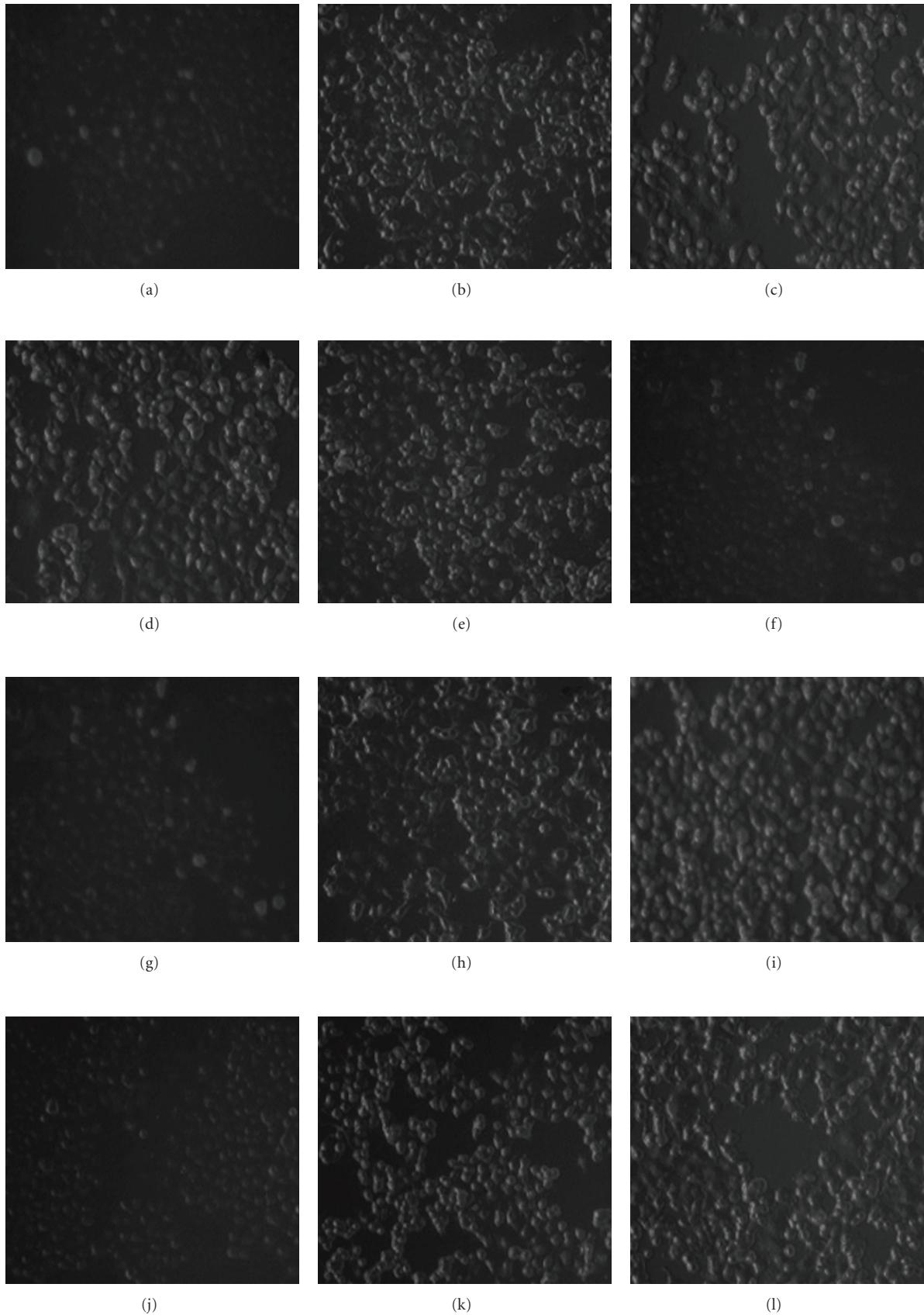


FIGURE 16: Immunofluorescence staining pictures of A375 cells following ((a)–(c)) Bax activation, ((d)–(f)) Bcl-2 deactivation, ((g)–(i)) cytochrome *c* activation and ((j)–(l)) caspase-3 activation.

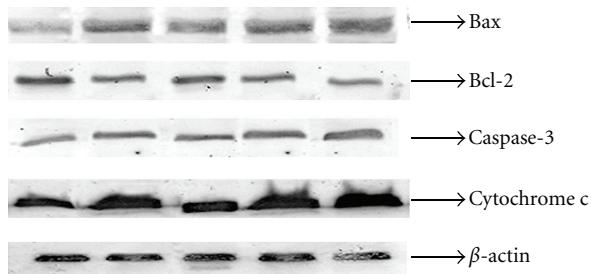


FIGURE 17: Western blot analysis of Bax, Bcl-2, caspase-3, cytochrome *c* and  $\beta$ -actin lane (1) Control; lane (2) TOΦ, lane (3)  $50 \mu\text{g mL}^{-1}$ , lane (4)  $100 \mu\text{g mL}^{-1}$ ; lane (5)  $200 \mu\text{g mL}^{-1}$ -treated TRF, respectively. The expressions of Bax, caspase-3 and cytochrome *c* were up-regulated with the increasing concentrations of TRF where Bcl-2 was down regulated.

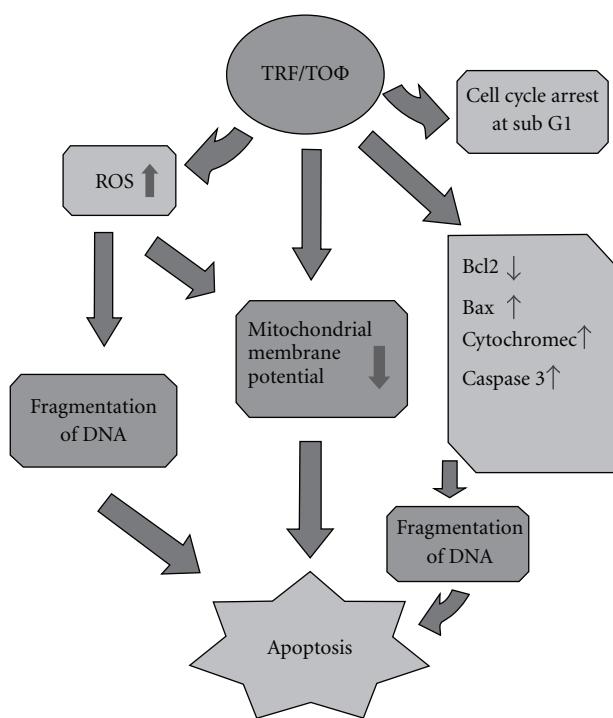


FIGURE 18: Simple schematic representation of effect of TRF and TOΦ on A375 cell line.

We determined the optimum dose through a range finding trial. We also studied the probable cytotoxic effects of both TOΦ and the TRF on a relative basis. The result revealed that the  $220.18 \mu\text{g mL}^{-1}$  of TOΦ could produce 50% cell death in A375 while that dose was relatively non-toxic to PBMC. Correspondingly, of the three doses of 50, 100,  $200 \mu\text{g mL}^{-1}$  of TRF used,  $200 \mu\text{g mL}^{-1}$  TRF showed the maximum effect surpassing the effect of treatment with the TOΦ as revealed from the result of the several protocols. Thus, the TRF appeared to be more potent anti-cancer agent when treated alone, a fact which may be of therapeutic importance in drug design and development of anti-cancer drug from natural sources.

In recent years, the use of complementary and alternative medicine (CAM) and other traditional medicines (TM) is increasingly becoming popular [31–37]. However, many people are hesitant to use such drugs either because they are not scientifically validated for their action or because the mechanism of their action is not properly known. Therefore, search should be on for finding out safe, affordable and efficient natural plant products that are experimentally proven to be effective and are relatively non-toxic in nature, because most of the orthodox anti-cancer drugs used in cancer therapy are toxic and have adverse side-effects. Thus, studies pinpointing confirmed efficacy of a particular fraction or compound among several found in crude extract are important for therapeutic purposes. Although the efficacy of TRF and TOΦ has presently been tested against an *in vitro* cancer cell line, it is very likely that the result can be extrapolated in animal or in human. However, to determine this, more experiments should be carried out on *in vivo* animal models, which will hopefully be taken up in the next phase of our program using mice as a model.

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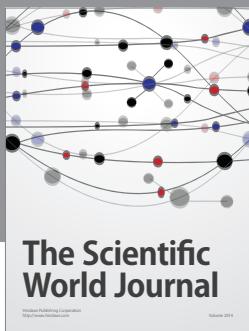
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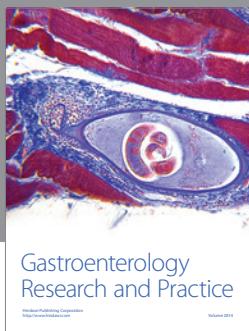
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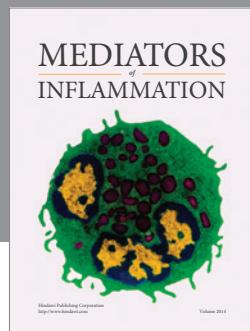
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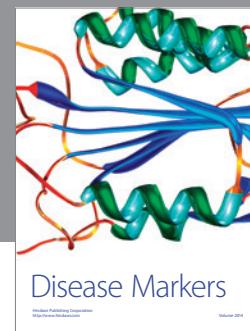
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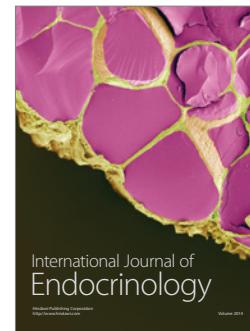
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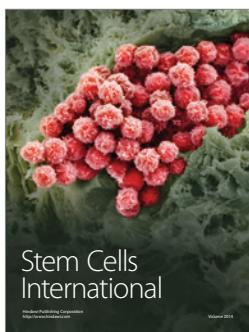
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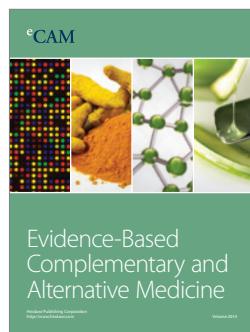
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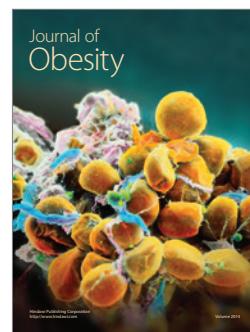
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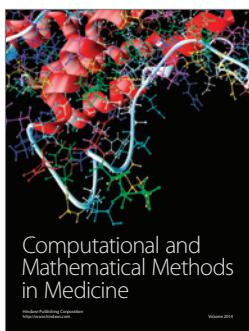
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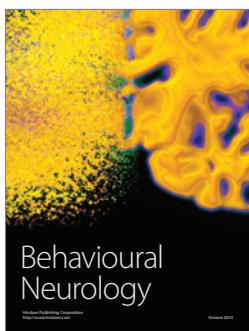
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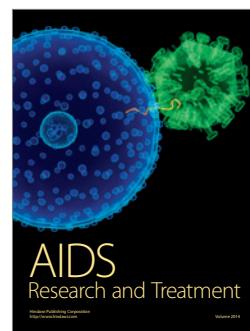
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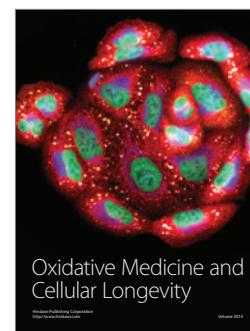
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