IJPSR (2019), Volume 10, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 April 2019; received in revised form, 25 July 2019; accepted, 21 October 2019; published 01 November 2019

CYTOTOXIC AND APOPTOGENIC EFFECTS OF A BIOACTIVE FRACTION ISOLATED FROM THE LEAVES OF A TRADITIONAL MEDICINAL PLANT *TRIDAX PROCUMBENS*

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Keywords:

Tridax procumbens, Synergism, Apoptosis, Mitochondria mediated, Selective killing

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ABSTRACT: Objective: To investigate the anti-proliferative effects of *T. procumbens* leaf extracts on the growth of MCF 7, Hep G2 and K562 cells. An attempt was made to partially purify the cytotoxic acetone extract using a bioassay-guided isolation procedure and the apoptosis-inducing activity of bioactive sub-fraction, F15 (fraction 15), was probed further against K562 cells. **Methods:** Cytotoxicity of extracts was evaluated by MTT assay. Hoechst 33258, acridine orange/ethidium bromide and AnnexinV- FITC staining was performed to detect apoptosis. Mitochondrial membrane potential and cell cycle distribution were measured by flow cytometry. DNA fragmentation, alkaline comet assays and Western blotting of key apoptosis-related proteins were also performed. **Results:** The F15 showed the highest antiproliferative activity against K562 cells leaving the normal peripheral blood lymphocyte unaffected. TLC of F15 displayed a total number of four bands that individually exhibited lower anticancer activities than that of F15, suggesting the possibility of synergism among them. F15 induced phosphatidylserine externalization, loss of mitochondrial membrane depolarization, DNA laddering, increased sub-G1 population and altered expression of caspase 3, -9 and PARP. **Conclusion:** Taken together, our results indicate that F15 triggers inhibition of tumor cell growth by inducing intrinsic apoptotic pathway in K562 cells.

INTRODUCTION: *Tridax procumbens* belonging to the family Asteraceae is a common medicinal herb used in Ayurvedic system of medicine for treating various ailments [1-19]. The anticancer potential of *Tridax procumbens* has rarely been reported [20-25]. The aim of the present study was to investigate the antiproliferative and apoptogenic effects of different organic solvent extracts prepared from leaves of *Tridax procumbens* on the growth of MCF7, Hep G2, and K562 cancer cell lines.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(11).5075-86

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(11).5075-86

MATERIALS AND METHODS:

Sample Collection: *Tridax procumbens* were collected locally from Calicut University campus on February 2015 and a voucher specimen (Voucher no. #6879) was deposited in Calicut University Herbarium.

Preparation of Leaf Extracts: The dried powdered (20 g) leaves were subjected to defatting by refluxing with petroleum ether (100 ml) at 60-80 °C for 12 h. This was followed by successive solvent extractions by a process of continuous Soxhlation. The extractions were done with individual solvents (100 ml) possessing increasing polarities such as chloroform, acetone, ethyl acetate and methanol. The crude extracts obtained were filtered through Whatman no.1 filter paper. Following evaporation of the solvents, the resultant residues were dissolved in DMSO to get a stock

solution of 20 mg/ml. Using the first two letters to denote the plant name and the third to denote the solvent used (chloroform, ethyl acetate, acetone and methanol), *T. procumbens* leaf extracts were denoted as TPC, TPE, TPA and TPM respectively.

Bioassay Guided Isolation of Anticancer Fractions from *T. procumbens*:

Column Chromatography: When tested for antiproliferative activity, TPA was found to be highly cytotoxic and was subjected to further purification. The schematic diagram of the extraction procedure is given in **Fig. 1**. TPA (4.0 g) was subjected to column chromatography using silica gel 60 (100-200 meshes) with an internal volume of 75% equilibrated with petroleum ether. Sequential elution was performed with 160 ml each of the solvents of increasing polarity *viz.* petroleum ether, dichloromethane, ethyl acetate, ethyl acetate: acetone (6:4), ethyl acetate: acetone (4:6), acetone and methanol. The semi-purified sub-fractions were collected and subjected to Thin Layer Chromatography (TLC) analysis [26]. The individual residues were then tested for anti-proliferative activity using MTT assay.

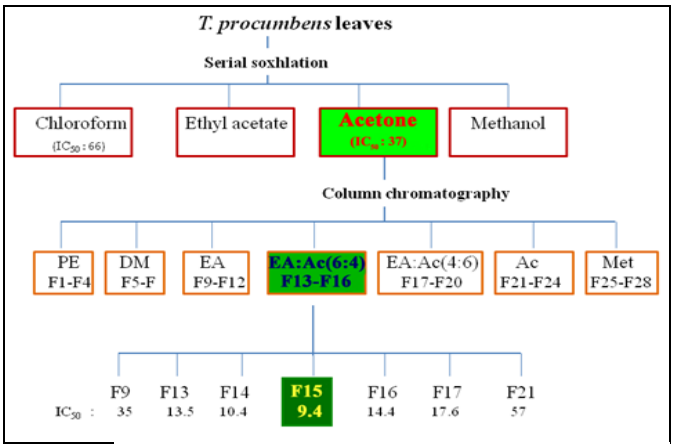

FIG. 1: SCHEMATIC REPRESENTATION OF THE BIOASSAY - GUIDED FRACTIONATION OF *T. PROCUMBENS* LEAVES. PE-Petroleum ether, DM-Dichloromethane, EA- Ethyl acetate, Ac- Acetone, Met-Methanol.

Preparative TLC: The fractions obtained by Column Chromatography were separated by TLC (silica gel G 60) using hexane: ethyl acetate (80:20) and chloroform: methanol (95:5) as the solvent systems. Based on the results of MTT assay, the sub-fractional residue - F15 - with the highest antiproliferative activity of 9.4 µg/ml, was identified. Following a preparative TLC, the sub-fractionated band component(s) was collected by scraping it off the TLC plates, which was then subjected to MTT procedure once again.

Anti-proliferative Activity:

Cells and Culture Conditions: The cytotoxicity was tested against three human cancer cell lines - chronic myelogenous leukemia K562 cells, breast cancer MCF 7 cells and hepatic carcinoma Hep G2 cells. K562 cells were cultured in RPMI-1640 medium while MCF 7 and Hep G2 cells were cultured in DMEM. Both media were supplemented with FBS (10% v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml) and maintained in an incubator at 37 °C in a humidified, 5% CO_2 atmosphere. The human peripheral blood lymphocytes (hPBL) cells were isolated from healthy donors to serve as the normal control cells for a strict comparison with the leukemic K562 cells. hPBL cells were separated from whole blood collected from healthy donors and cultured in HiKaryo XL^{TM} RPMI media according to the manufacturer's protocol (Hi-media Laboratories, Mumbai).

MTT Assay: The cytotoxicities of the different extracts were evaluated by the MTT assay. For this, cells were cultured in a 96 well microtitre plate with 2×10^4 K562 and 7000 each of MCF 7 and Hep G2 cells per well. The hPBL (10^5cells/ml) were separately added to 5 ml HiKaryo XL^{TM}RPMI medium and incubated at 37 °C for 48 h. To determine the IC_{50} concentration, cells were initially exposed to varying concentrations of the different leaf extracts - TPA, TPC, TPE, and TPM - for a period of 24, 48 and 72 h. MTT (500 µg/ml) was added to each well, and the cells were then incubated for 3 h at 37 °C. The medium was discarded, and an equal amount of DMSO (150 µl) was added to each well. After 10 min of shaking at room temperature to dissolve the formazan crystals, absorbance was measured at 570 nm on a plate reader (Multiscan EX, Thermo scientific, USA).

The hPBLs, taken as positive controls, were treated with concentrations - lower, equal to, and higher than the IC_{50} values obtained for the extracts. Untreated and DMSO-treated cells served as negative controls. The difference of absorbance between the treated and untreated control groups was used to determine cell viability [27].

Microscopic Evaluation: The control and F15-treated K562 cells were harvested and washed with ice-cold PBS and then visualized through a phase-contrast inverted microscope to note the morphological differences. Separate aliquots of control and F15-treated cells were subjected to staining with Hoechst 33258 as well as ethidium bromide/acridine orange to detect changes induced by apoptosis. The cells were then observed under a fluorescence microscope [28].

Cell Cycle Analysis: Flow cytometry of the control and F15-treated cell populations was carried out to check for cellular distribution in the different phases of cell cycle. For this, the cells were stained with PI and the percentage sub-population of cells in sub-G1, G0/G1, S, and G2/M phases were quantified using the BD FACS Diva software version 5 on a BD Biosciences FACS-SORP cytometer.

Analysis of Phosphatidylserine Externalization, Intracellular Ca^{2+} Concentration, and Mito-chondrial Membrane Potential: Externalization of phosphatidylserine (PS) in control and F15-treated cells (1×10^6) was evaluated by using annexin V/FITC apoptosis detection kit as per the manufacturer's instructions (APOAF- Annexin V-FITC detection kit, Sigma Aldrich, USA).

Alterations in levels of intracellular Ca^{2+} content were determined by employing a fluorescent dye, Fluo 3-AM. Control and F15- treated cells were washed twice with PBS following incubation with 5 μM Fluo 3-AM at 37 °C for 30 min. Mitochondrial membrane potential was determined by incubating cells with lipophilic cationic dye rhodamine 123 (10 μg/ml) for 30 min followed by a wash with PBS [29]. All assessments mentioned above were carried out by FACS analysis using BD FACS Diva software version 6.1.3 on a BD Biosciences FACS-ARIYA II cytometer.

Genotoxicity Evaluation by Comet Assay: The control and treated cells were subjected to comet assay essentially as described by Singh *et al.*, 1988 [30]. The slides were stained with ethidium bromide (10 μg/ml) for 30 min and observed under a fluorescence microscope.

DNA Fragmentation Assay: DNA isolated from control and treated cells were analyzed on 1% agarose gels to check for DNA laddering arising due to internucleosomal cleavage, which is considered to be a hallmark of apoptosis induction [31].

Western Blot Analysis: SDS PAGE and Western blotting were performed essentially as described in Ausubel *et al.*, 1992 [32] for the detection of apoptosis and cell cycle-related proteins. For this, the control and F15-treated cells were first washed with cold PBS and lysed in RIPA buffer containing protease / phosphatase inhibitors. The protein concentration was measured by the Bradford method. Equivalent amounts of proteins (50 μg) were electrophoresed on a 12.5 % SDS-PAGE gel and then transferred onto a positively-charged nylon membrane. Membranes were blocked for 1 h at room temperature in 3% BSA in TBS, and separately immunostained overnight with mouse/ rabbit derived primary antibodies (dilution 1:1000) at 4 °C against caspase -3, -9, PARP, and γH2AX. Antibody against β-actin was used as a loading control to detect housekeeping function.

The membranes were then washed in TBS and incubated for 1 h at room temperature with ALP-conjugated secondary antibody, which was either anti-mouse or anti-rabbit (1:2000). Following another TBS wash, the blots were exposed to BCIP/NBT solution to visualize the immunostained polypeptides.

Statistical Analysis: The data are expressed as the mean ± S.D. from three independent experiments. Results were analyzed for significance by one-way ANOVA using SPSS software version 16.0. Differences with $P<0.05$ were considered significant.

RESULTS:

Effect of *T. procumbens* Leaf Extracts in Human Cancer Cell Lines - K562, MCF 7 and HepG2: The antiproliferative activity of the extracts was determined by the standard MTT assay. The cytotoxic properties of different extracts of *T. procumbens* on K562, MCF 7 and Hep G2 cells were evaluated using MTT assay following exposure to the extracts for 24, 48 and 72 h. The IC_{50} values obtained in **Table 1** revealed that all extracts except acetone and chloroform showed negligible activity against the three cell types tested.

TABLE 1: IC_{50} VALUES OF *T. PROCUMBENS* EXTRACTS AGAINST DIFFERENT CELL LINES

Extract	24 h	48 h	72 h
		K562	
TPC	66 ± 5 µg/ml	58 ± 1 µg/ml	49 ± 4 µg/ml
TPA	37 ± 3 µg/ml	26 ± 6 µg/ml	16.3 µg/ml
TPE	>120 µg/ml	>120 µg/ml	>120 µg/ml
TPM	>120 µg/ml	>120 µg/ml	>120 µg/ml
		Hep G2	
TPC	>120 µg/ml	83±3 µg/ml	55±0.5 µg/ml
TPA	59±6 µg/ml	44±3 µg/ml	39±0.5 µg/ml
TPE	>120 µg/ml	>120 µg/ml	>120 µg/ml
TPM	>120 µg/ml	>120 µg/ml	>120 µg/ml
		MCF 7	
TPC	92±2.83 µg/ml	119.33±1.15 µg/ml	> 120 µg/ml
TPA	46±2 µg/ml	44±1 µg/ml	45.33±4.04 µg/ml
TPE	>120 µg/ml	>120 µg/ml	>120 µg/ml
TPM	>120 µg/ml	>120 µg/ml	>120 µg/ml

Values represent mean ± SD (n=3).

The highest anti-proliferative activity was exhibited by TPA with IC_{50} values ranging from 16 to 37 µg/ml for K562 cells following 24 to 72 h of exposure. TPA was observed to induce cell death in K562 and HepG2 in a concentration and time-dependent manner but not in MCF 7. The above results indicated that TPA possessed significant antiproliferative activity against K562 cells. Hence, this extract was subjected to further purification in an attempt towards the identification of bioactive sub-fractions.

Column Chromatography of TPA Fractions: The Column Chromatography of TPA yielded 28 fractions (F), in which only seven fractions (F9, F13 -17 and F21) were found to possess significant toxicity against K562 cells. Among the seven active fractions, F15 showed the highest anti-proliferative activity with an IC_{50} value of 9.42 ± 0.01 µg/ml. F9 (ethyl acetate fraction) and F21 (methanol fraction) showed higher IC_{50} values of 35 ± 4 and 57 ± 6 µg/ml respectively. F13-17 showed IC_{50} values ranging from 9.42 ± 0.01 to 17.6 ± 0.76 µg/ml. As F15 [E:A (6:4) fraction] showed the highest antiproliferative activity on K562 cells, it was subjected to further enrichment by preparative TLC.

Thin Layer Chromatography of Bioactive Sub-fractions of TPA: The thin layer chromatogram of F13-17 in solvent system chloroform: methanol (95:5, v/v) revealed 4 major bands with R_f values 0.2, 0.26, 0.39 and 0.42 respectively **Fig. 2**. Again, in hexane: ethyl acetate (80:20) solvent system, four bands in F9 and five in F21 were also detected. The R_f values of the separated bands of F9 were 0.675, 0.77, 0.87 and 0.961 while that of F21 was found to be 0.454, 0.571, 0.688, 0.779 and 0.883 respectively **Fig. 3**.

The four sub-fractionated bands obtained from a preparative TLC of F15 were collected separately for the assessment of IC_{50} concentration by MTT assay in K562 cells. The results clearly revealed that only one out of the four bands, with an R_f value of 0.39, harbored the bioactive constituent(s) apparently responsible for the highest anti-proliferative activity with an IC_{50} concentration of 16 ± 0.05 µg/ml **Table 2**, much higher than that of F15 (a mixture of four bands including band 3). In other words, the F15 mixture was found to be relatively more potent than the fractionated bands, highlighting the synergetic effect or combined action of the components of the sub-fraction.

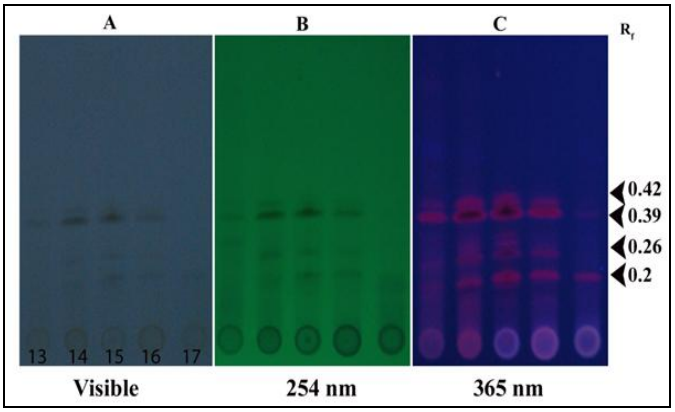

FIG. 2: TLC FINGERPRINT OF ACTIVE SUB-FRACTIONS (F13-17) OF TPA USING CHLOROFORM: METHANOL (95:5) AS SOLVENT SYSTEM VIEWED UNDER (A) VISIBLE LIGHT, (B) UV AT 254 nm AND (C) UV AT 365 nm. ARROWS INDICATE R_f VALUES OF FRACTIONATED BANDS

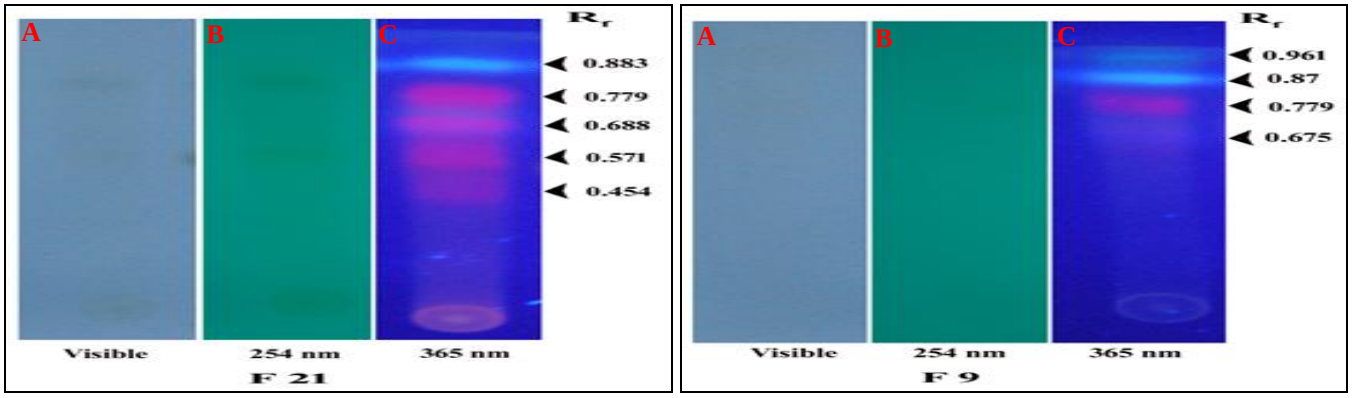

FIG. 3: TLC FINGERPRINT OF F9 AND F21 USING HEXANE: ETHYL ACETATE (80:20) AS SOLVENT SYSTEM VIEWED UNDER (A) VISIBLE LIGHT, (B) UV AT 254 nm AND (C) UV AT 365 nm. ARROWS INDICATE R_f VALUES OF FRACTIONATED BANDS

TABLE 2: R_f VALUES AND THE IC_{50} VALUES OF THE 4 INDIVIDUAL BANDS COLLECTED FROM F15

Band number	R_f value	IC_{50} value
1	0.2	62.3±0.26
2	0.26	66.2±3.36
3	0.39	16±0.05
4	0.42	-

F15 does not Affect Human Peripheral Blood Lymphocytes: The MTT assay performed with cultured hPBLs, treated with F15 at different concentrations (5, 10, 30 µg/ml), however, indicated no apparent differences between proliferation rates of treated and untreated lymphocytes **Fig. 4**, revealing the selective killing potential of F15.

Effect of F15 on Cell Morphology: Light microscopy of cells treated with F15 revealed distortions and disruptions in cell structure in addition to cell shrinkage and fragmentation. These morphological changes were observed to increase in a dose-dependent manner; the untreated and DMSO - treated control cells showed normal cell morphology with intact cell boundaries **Fig. 5**. The untreated and DMSO-treated cells, stained with Hoechst 33258, displayed intact and round uniformly stained nuclei, emitting relatively lesser bright blue fluorescence compared to that from F15 treated cells. Distinct F15-induced, concentration-dependent morphological changes like cell shrinkage, nuclear fragmentation, and condensation were observed which indicated induction of apoptosis **Fig. 6**. In acridine orange and ethidium bromide dual staining, the untreated control and DMSO-treated cells appeared green with intact nuclei and normal cell morphology. But F15-treated cells were stained orange to red and showed characteristics of apoptosis such as chromatin condensation, nuclear fragmentation, and alterations in the size and shape of cells **Fig. 7**.

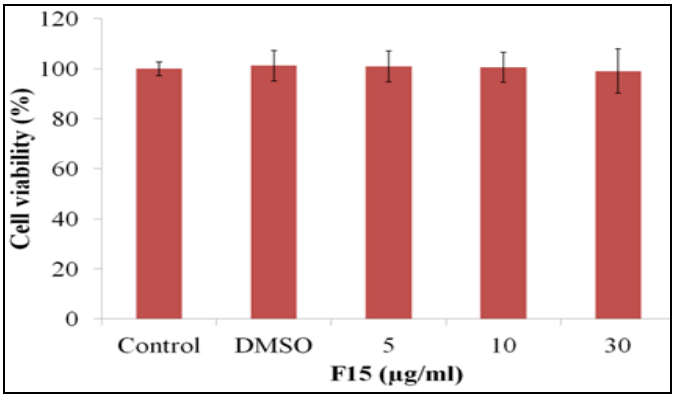

FIG. 4: EFFECT OF F15 ON HPBLS. CELL VIABILITY OF NORMAL HPBLS WAS ASSESSED BY MTT METHOD AFTER 24 h TREATMENT

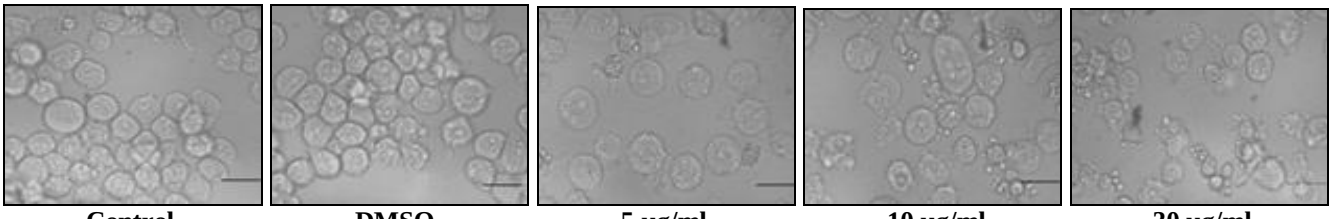

FIG. 5: LIGHT MICROSCOPY IMAGES OF K562 CELLS AFTER 24 h TREATMENT WITH DIFFERENT CONCENTRATIONS OF F15. Untreated control and vehicle (DMSO)-treated cells have been included in all figures. Scale bars represent 15.5 µm in all figures.

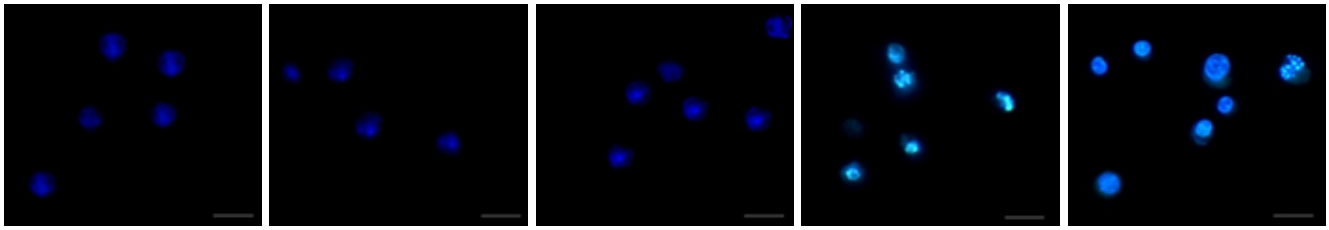

FIG. 6: FLUORESCENT MICROSCOPY IMAGES OF HOECHST 33258 STAINED K562 CELLS AFTER 24 h TREATMENT WITH F15

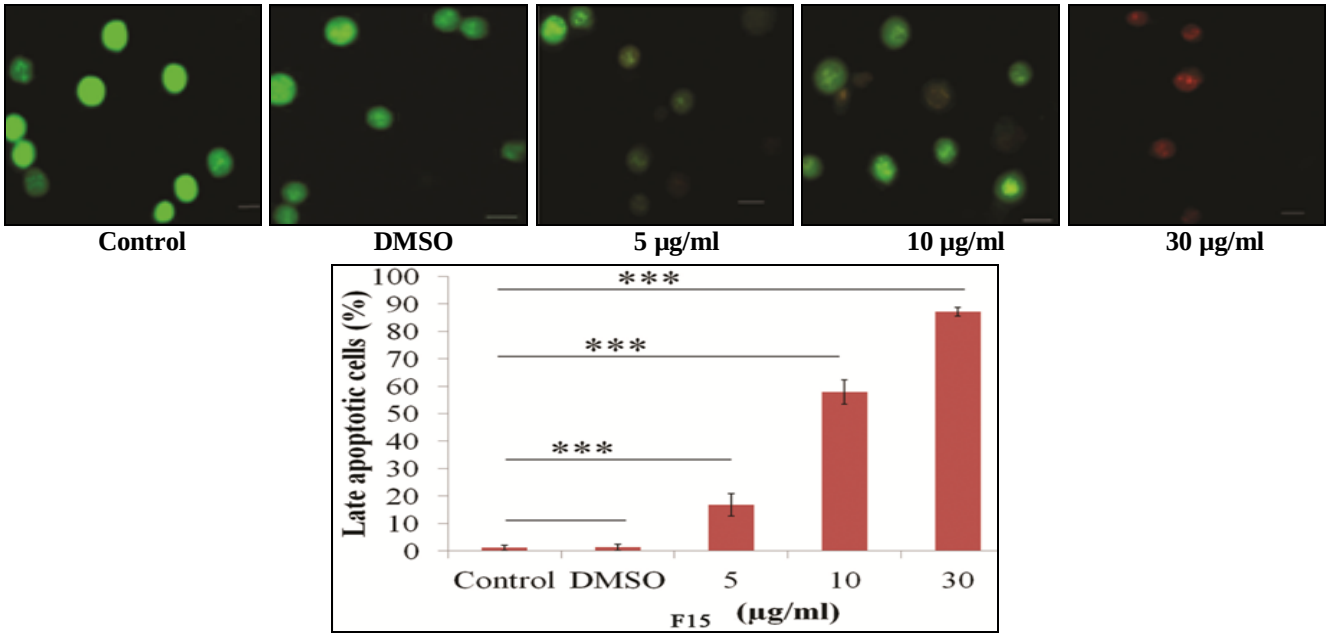

FIG. 7: EFFECT OF F15 ON K562 CELLS AFTER 24 h TREATMENT. (A) Fluorescence microscopy images of F15-treated cells stained with Acridine orange/Ethidium bromide. (B) Quantitative analysis of late apoptotic cells. ***p < 0.001.

Effect of F15 on Cell Cycle Distribution: In F15-treated cells, an alteration in the percentage of cells in each stage of the cell cycle - Sub G1, G0/G1, S, and G2/M- was observed in comparison to controls.

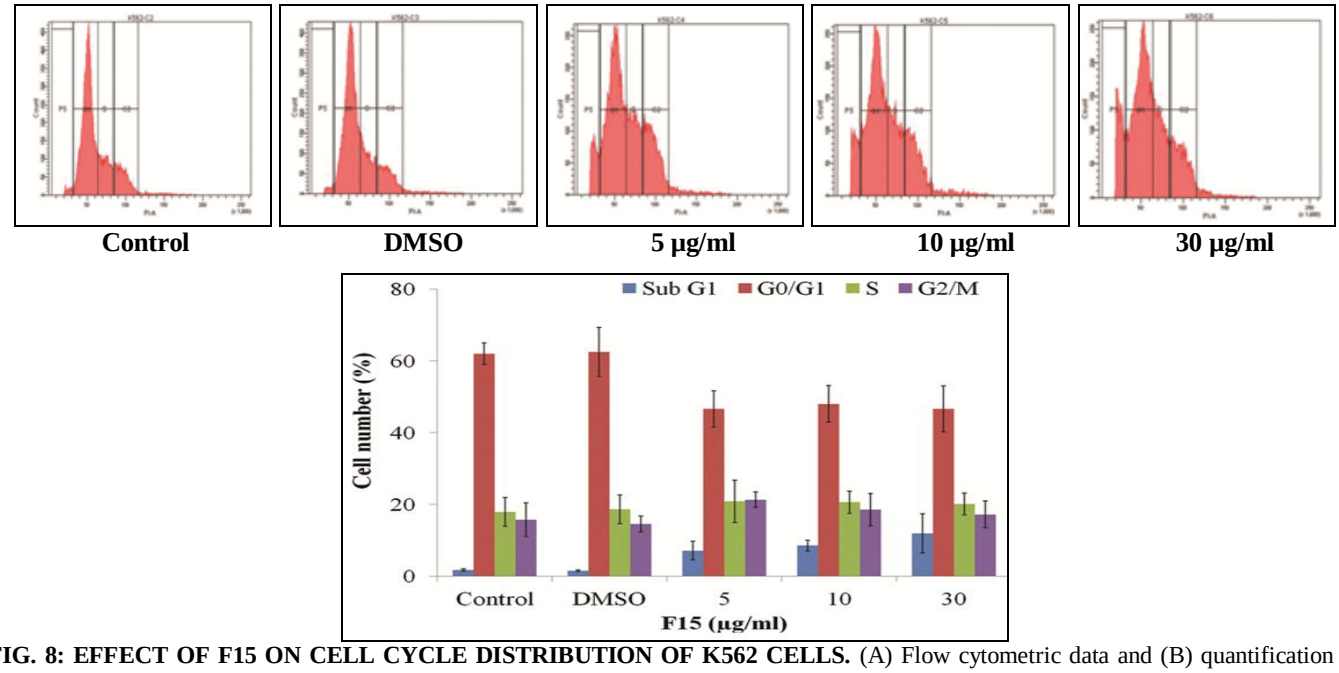

FIG. 8: EFFECT OF F15 ON CELL CYCLE DISTRIBUTION OF K562 CELLS. (A) Flow cytometric data and (B) quantification of cells in various cell cycle phases including sub G1. Fluorescence data from control and vehicle (DMSO)-treated cells have been included in all figures representing FACS analysis.

F15-treatment at 5 to 30 µg/ml led to an increasing, concentration - dependent, accumulation of apoptotic cells at sub- G1 phase from 7.4 to 12.42%, respectively, in comparison to controls. A concomitant decrease in G0/G1 (63.72 to 48.63%), a slight increase in S (18.3% to 20.98%) and G2/M sub-populations (16.14 to 17.95%) were also noted in **Fig. 8**.

Effect of F15 on Phosphatidyl Serine Externalization, Intracellular Ca^{2+} Release, Destabilization of Mitochondrial Membrane Potential: K562 cells treated with different concentrations (5, 10, and 30 µg/ml) of F15 for 24 h were stained with annexin V/FITC and PI. The percentage of apoptotic cells was found to increase in a dose-dependent manner with values of 4.6% at 5 µg/ml, 9.2% at 10 µg/ml, and 25.3% at 30 µg/ml compared to 4.8 % recorded in control cell cultures.

About 5.9% of the cell population treated with 30 µg/ml of F15 showed necrotic signs. Thus, the results clearly showed a considerable increase in apoptotic cell death in F15 treated cells compared to that in controls **Fig. 9**.

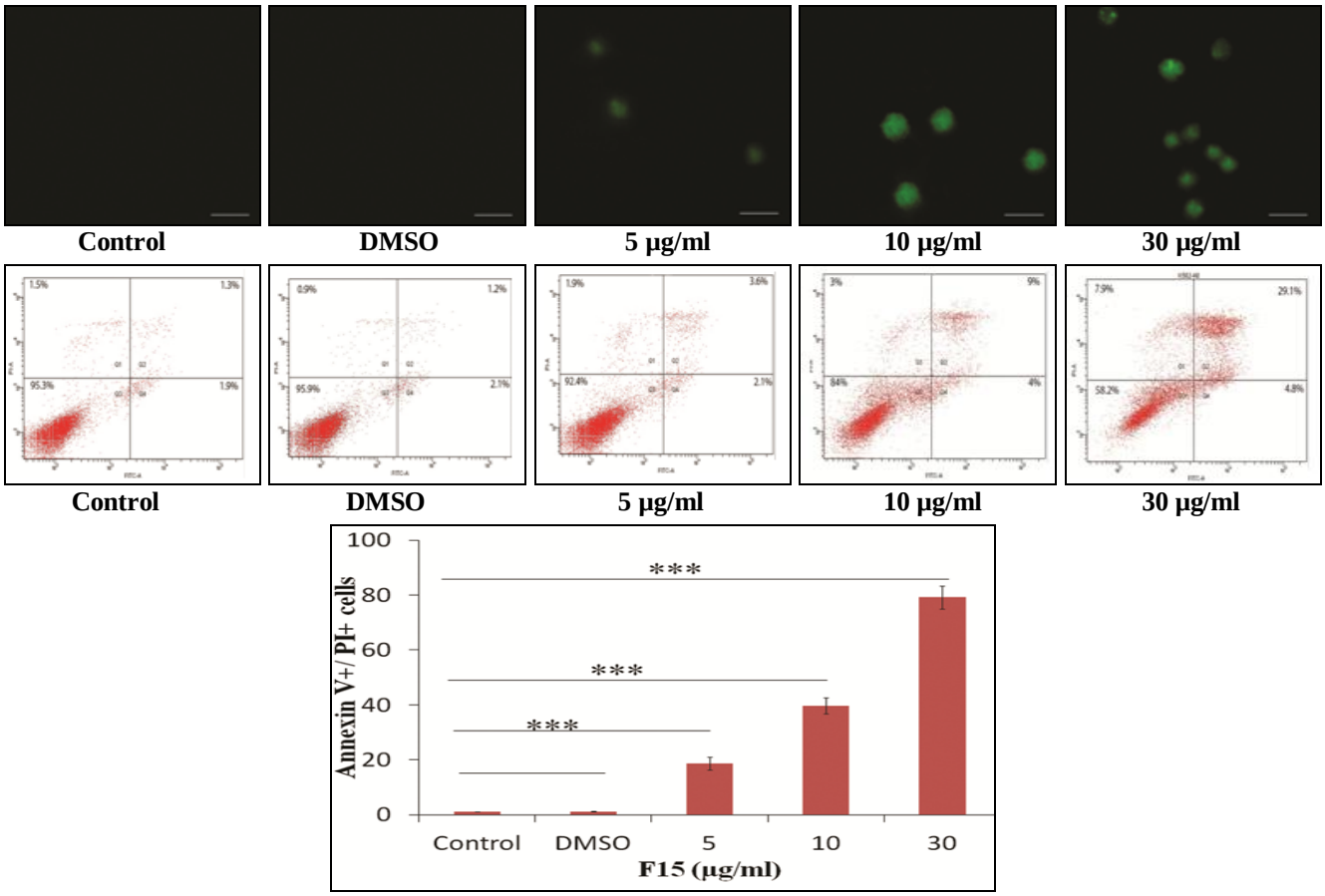

FIG. 9: APOPTOGENIC EFFECT OF F15 ON K562 CELLS AFTER 24 h TREATMENT. (A) Microscopy images, (B) flow cytometric analysis of annexin V-FITC / PI stained cells and (C) quantitative analysis of apoptotic cells ***p<0.001.

To investigate whether exposure to F15 induced release of Ca^{2+} in K562 cells, the Ca^{2+} sensitive dye Fluo 3-AM was used. The cells were found to display an increase in Fluo 3-AM fluorescence intensity following exposure to F15 in a concentration dependent manner compared to untreated, and DMSO treated controls **Fig. 10**.

To assess the involvement of mitochondria in F15 - induced cell death, measurements of mitochondrial membrane potential were carried out employing rhodamine-123 staining. The fluorescence of F15-treated cells was found to be increased in comparison to controls indicating loss of mitochondrial membrane potential in them **Fig. 11**.

Genotoxic Effect of F15: The control and vehicle-treated cells displayed compact DNA within the nucleus while comets were observed with increasing tail lengths in a concentration dependent manner in the range tested from 5-30 µg/ml, following 24 h of F15-treatment **Fig. 12**.

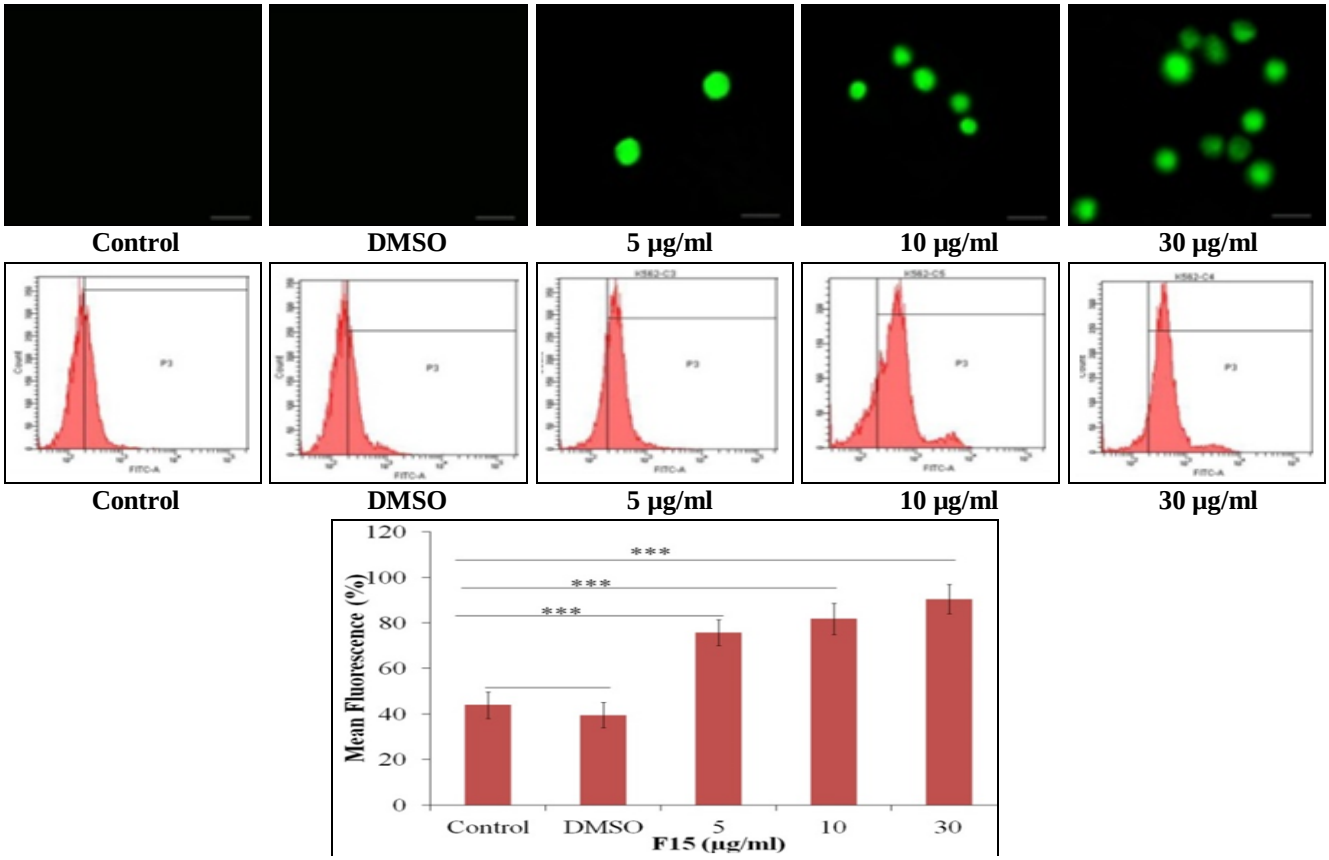

FIG. 10: MEASUREMENT OF INTRACELLULAR CALCIUM CONCENTRATION OF K562 CELLS USING INTRACELLULAR Ca^{2+} INDICATOR FLUO 3-AM, FOLLOWING 24 h OF F15 TREATMENT.(A) Microscopy images, (B) flow cytometric analysis and (C) mean fluorescence intensity of cells ***p<0.001.

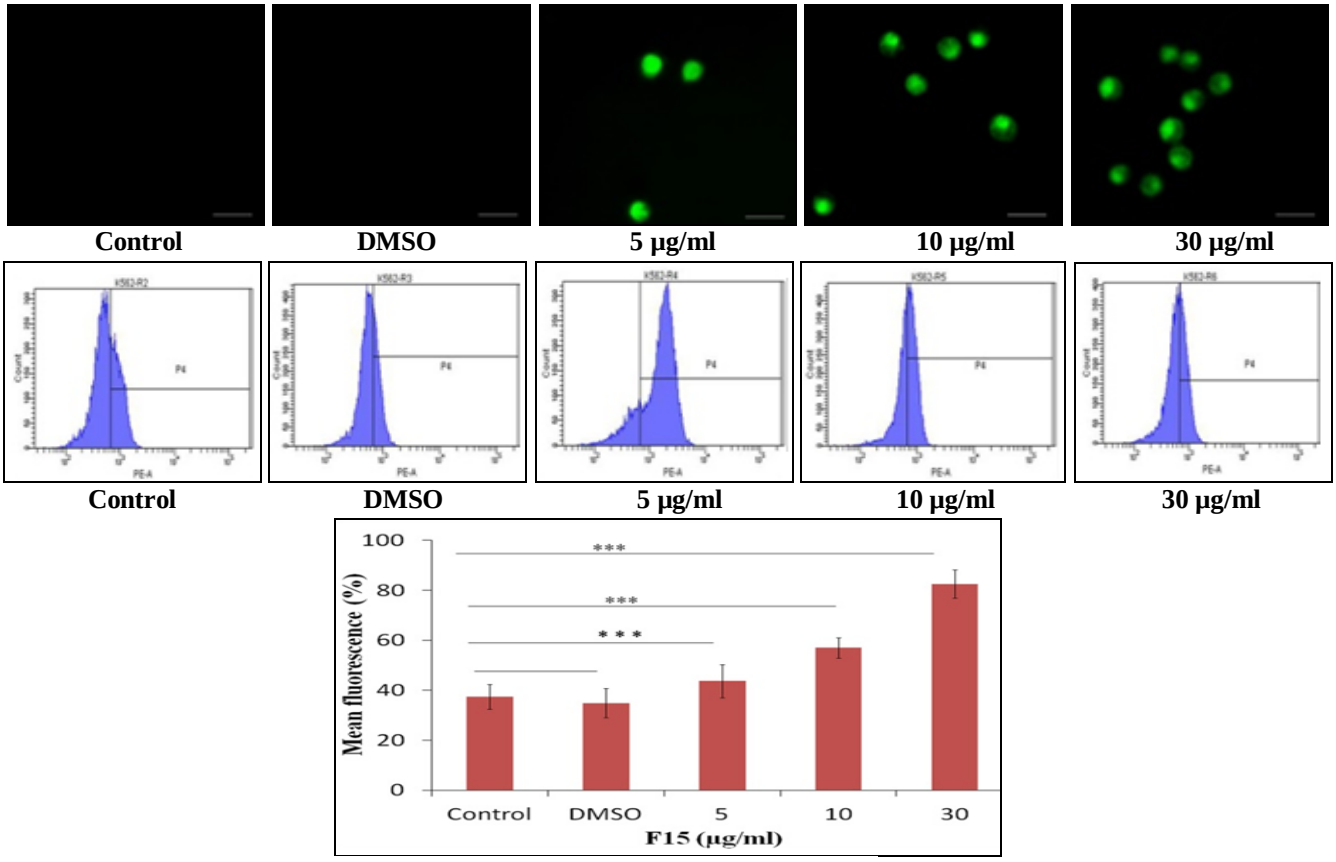

FIG. 11: MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL USING RHODAMINE -123 FOLLOWING 24 h OF F15 TREATMENT. (A) Microscopy images, (B) flow cytometric analysis, and (C) mean fluorescence intensity of K562 cells ***p < 0.001.

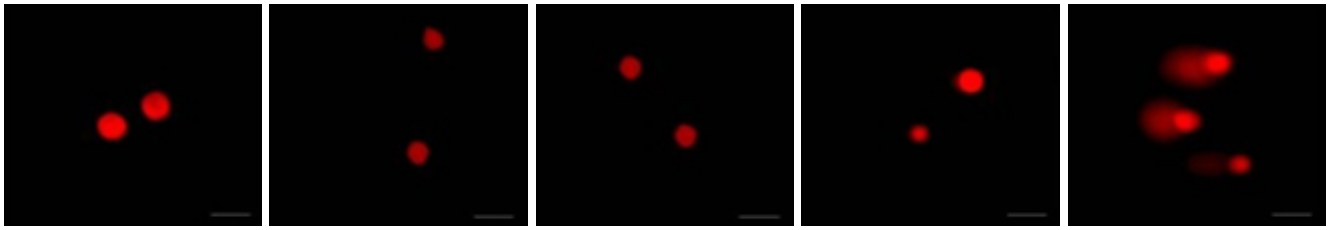

FIG. 12: DETECTION OF DNA DAMAGE BY ALKALINE COMET ASSAY IN K562 CELLS TREATED WITH F15 FOR 24 h

Induction of DNA Fragmentation by F15: DNA extracted and electrophoreses from F15-treated cells showed a distinct laddering pattern that was absent in the DNA obtained from control cells, thereby confirming induction of apoptosis in K562 cells **Fig. 13**.

F15 Affects the Expression of Proteins Associated with Apoptosis: In F15 treated cells, Western blot analysis revealed upregulated expression of damage-inducible histone variant H2AX, cleavage of apoptosis-related procaspase 9, -3 and poly (ADP-ribose) polymerase (PARP) **Fig. 14**.

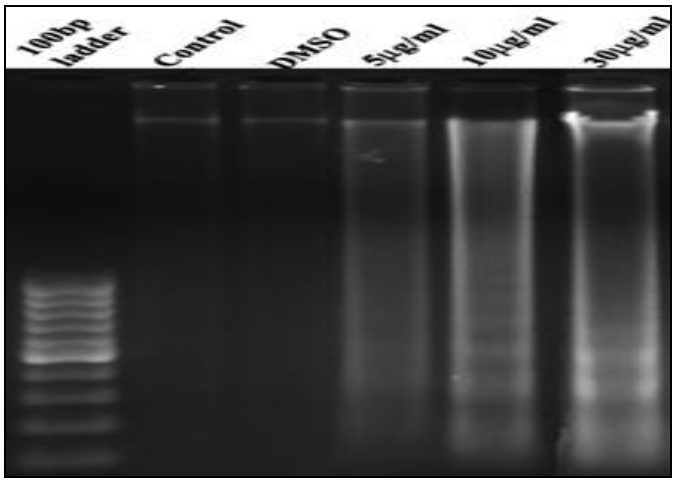

FIG. 13: DNA FRAGMENTATION ASSAY FOR DETECTION OF APOPTOSIS IN K562 CELLS. Lane 1-100bp DNA ladder, Lane 2-DNA from untreated cells, Lanes 3, 4, 5, 6 - DNA from cells treated with DMSO, 5, 10 and 30 µg/ml of F15 respectively.

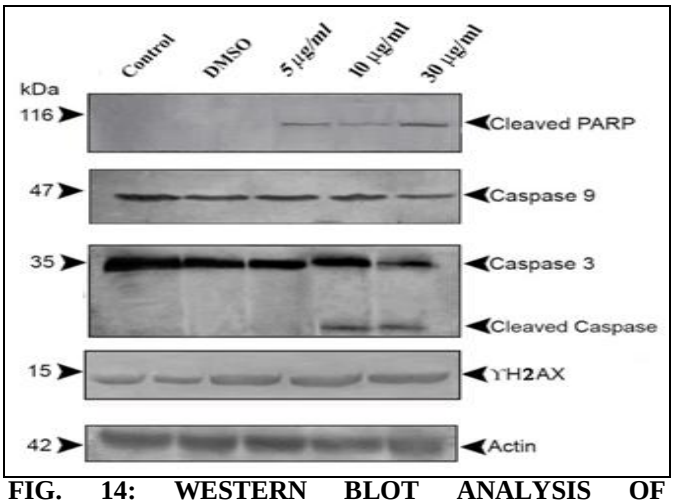

FIG. 14: WESTERN BLOT ANALYSIS OF APOPTOTIC PROTEINS IN K562 CELLS TREATED WITH 5, 10 AND 30 µg/ml OF F15. Expression of PARP, Caspase 9, Caspase 3, ϒ H2AX. All proteins were normalized against beta actin taken as the loading control.

DISCUSSION: The role of natural products as cancer chemotherapeutic agents, either in their unmodified (naturally occurring) or synthetically modified forms has been established in recent years [33, 34]. The medicinal plants mentioned in ancient texts of Ayurveda and other such medicinal systems represent a time-tested ancient wisdom which merits modern scientific exploration to guide the discovery of better leads in the field of pharmacognosy.

The present study revealed that TPA derived sub-fraction, F15, was cytotoxic towards K562 cells. The individual sub-fractions of F15, separated by preparative TLC, showed lower anticancer activity in comparison to F15, thereby revealing a synergistic effect of compounds present in two or more different bands present therein. Hence, further analysis of F15 was carried out using K562 cells. Many previous reports have in fact shown that crude plant extracts can be more active pharmacologically than their constituents [35, 36]. Enhanced anticancer activity of crude extracts might also arise from the potentiating of pharmacokinetics, wherein one ingredient enhances the therapeutic effect of another (active ingredient or drug) by modulating its pharmacokinetic properties such as absorption, distribution, metabolism, and/or excretion [37].

Cancer researchers have been on the lookout for anticancer drugs that can exploit the unique biochemical difference between normal and cancer cells to preferentially kill the cancer cells leaving the normal cells unaffected [38, 39]. Our results provided additional proof of F15's potential to

preferentially kill cancerous lymphocytic, K562 cells compared to normal human lymphocytes. To check whether the growth inhibitory activity of F15 was attributed to the apoptosis-inducing activity, acridine orange / ethydium bromide, Hoechst staining, and DNA fragmentation analysis were performed.

According to the results, morphological changes like membrane blabbing, cell shrinkage and increased DNA fragmentation were observed in K562 cells, indicating that cells underwent apoptosis in response to F15-treatment. Consistent with this observation, F15 also increased the cell population in the sub-G1 phase. To further confirm the role of F15 in promoting apoptosis annexin V/FITC-PI staining was performed. Our results showed a considerable increase in the early apoptotic cells in F15 treated cells in comparison to untreated control cells.

Our results also showed F15-treatment significantly increased the levels of intracellular calcium concentration. Calcium may activate Bax, which induces permeabilization of the mitochondrial membrane and release of mitochondrial pro-apoptotic factors, culminating in the activation of cell death effector caspases such as caspase-3 [40], which is also activated by F15.

We found that the apoptosis induced by F15 was accompanied by the activation of caspase-3 and caspase-9 with the cleavage of PARP, suggesting that F15-induced apoptosis involves a caspase-dependent mitochondrial pathway. Likewise, one of the earliest signs of DNA damage is the formation of γH2AX as a result of the occurrence of double-strand breaks. γH2AX formation in the chromatin precedes phosphatidylserine externalization on the plasma membrane of apoptotic cells [41, 42]. The results collectively indicated that F15 induced apoptosis in K562 cells.

CONCLUSION: The study revealed that *T. procumbens* was found to harbor phytoconstituents which preferentially affected the proliferative activity of leukemic cancer cells without having any deleterious effect on normal lymphocytes. Thus, *T. procumbens* holds the promising potential to serve either as a bio-resource or as a template for future anticancer-drug development.

ACKNOWLEDGEMENT: T. Lakshmipriya acknowledges the financial support from the University Grants Commission (Govt. of India) for Rajiv Gandhi National Fellowship. The authors thank Dr. Pradeep, Department of Botany, University of Calicut, for plant identification and Dr. Santhosh Kumar K., Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for allowing use of FACS machine and Mrs. Indu Ramachandran for technical help with FACS.

CONFLICT OF INTEREST: There are no conflicts of interest.

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How to cite this article:
Lakshmipriya T, Soumya T, Jayasree PR and Kumar PRM: Cytotoxic and apoptogenic effects of a bioactive fraction isolated from the leaves of a traditional medicinal plant *Tridax procumbens*. Int J Pharm Sci & Res 2019; 10(11): 5075-86. doi: 10.13040/IJPSR.0975-8232.10(11).5075-86.