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Research Article

ANTICANCER COMPOUND ISOLATED FROM THE LEAVES OF *TRIDAX PROCUMBENS* AGAINST HUMAN LUNG CANCER CELL A-549

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ABSTRACT

Tridax procumbens is a semi prostate annual or short lived perennial herb. The wide spread nature and contribution of the plant in medicine has been identified. The phytochemicals in dried leaves of *T. procumbens* has been investigated. *T. procumbens* compounds were tested for cytotoxicity against human lung cancer by MTT assay. The compounf of Rf value 0.66 showed 90% reduced cell viability. NMR, MS and IR spectra revealed the compound as Lupeol. The anticancer potential of the Lupeol against human lung cancer has been evaluated by colonogenic survival determination, cell cycle control, Cell based assay for inhibition of COX-2 activity and DNA fragmentation analysis, an amount of 320 µg/ml concentration of Lupeol compound exhibited potential anticancer property.

Keywords: *Tridax procumbens*; Lupeol; Anticancer potential

INTRODUCTION

Cancer is a disease in which disorder occurs in the normal processes of cell division, which are controlled by the genetic material (DNA) of the cell. Viruses, chemical carcinogens, chromosomal rearrangement, tumor suppressor genes, or spontaneous transformation have been implicated in the causes of cancer. According to the[1] Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and via the environment. At least 35% of all cancers worldwide are caused by an incorrect diet, and in the case of colon cancer, diet may account for 80% of the cases. When one adds alcohol and cigarettes to their diet, the percentage may increase to 60%. Genetic predisposition to cancer lends itself to ~20% of cancer cases, thus leaving the majority of cancers being associated with a host of environmental carcinogens. Plants have been demonstrated to be a very viable source of clinically relevant anticancer compounds. However, ethnopharmacological information has been poorly utilized in the past in the search for new principles against cancer. In many ethnomedical systems, reports of specific antitumor uses of plants are rarely found, mainly because cancer is a disease that involves a complex set of signs and symptoms[2]. Since, many of the plant products and their derivatives are approved for cancer control the development of new drugs to play an important role in cancer control is greatly desired[3].

Tridax procumbens is a semi-prostrate annual or short-lived perennial herb. Leaves are membranous, scaberulous above, glabrate beneath, auricled at base, irregularly toothed. Flower heads have long stalk, Yellow hard, rounded, 2.4-3.9 cm across, often 2-5 clustered together in the axils of leaves or terminal. Petals are about 2 cm long, tubular, yellow in colour. Anther tails are fimbriate. Achenes are curved, compressed *ca*. 8 mm long, tip narrowed, with one rib on each face. In the Indian systems of medicine (*Ayurveda, Siddha,* and *Unani*) *Tridax procumbens* is used either as a single drug or in combination with other drugs. Traditionally, it is used for the treatment of bronchial catarrh, dysentery, malaria, stomachache, diarrhoea, high blood pressure and to check haemorrhage from cuts, bruises and wounds and to prevent falling of hair. The leaf extract has been extensively used in Indian traditional medicine as anticoagulant, anticancer, antifungal and insect repellent[4]. The basic fraction from the leaves of *T. procumbens* has been identified as alkaloids, carotenoids, flavonoids (catechins and flavones), saponins and tannins[5]. Dexamethasone luteolin, glucoluteolin, β-sitosterol quercetin, β-sitosterol-3-O- β -D-xylopyranoside and flavonoid procumbenetin have been isolated from leaf[6]. Several workers have reported on the different biological activities of *T. procumbens* in various *in vitro* and *in vivo* test models. Different extracts of this plant have been found to exhibit antibacterial, anti-inflammatory, hepatoprotective, anti-ulcer and anticancer, immunomodulatory[7, 8, 9]. The aim of the present study was to isolate anticancer compound from *T. procumbens* and determine its anticancer potential against human lung cancer.

MATERIALS AND METHODS

Collection of plant material

Leaves of *Tridax procumbens* were collected from Medicinal Plant Garden at Sri Sairam Siddha Medical College and Research Centre, West Tambaram, Chennai 600 044, a recognized institution of Government of Tamilnadu and the Department of AYUSH, Government of India.

Cell line and culture

Human lung cancer cells A549 (p53 wt), were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured following their instructions.

Phytochemical analysis

The aqueous extract was freshly prepared and divided into different test tubes and various chemical constituents were analysed according to methods described by Allen[10] and Harbone[11].

Extraction and isolation of anticancer compounds from *Tridax* procumbens

Leaf powder (1.500Kg) of *Tridax procumbens* which had green–brown colour, was extracted three times with 5 L of each of the following solvents: dichloromethane, methanol and water for 24 h for each extraction. The dried methanol extract (60 g) was partitioned between ethyl acetate and water. The ethyl acetate extract (20 g) was subjected to silica gel vacuum liquid chromatography using a gradient solvent system (CH_2Cl_2/ MeOH = 100/0–99:1–98:2–97:3–96:4–95:5–90:10–80:20– 50:50) to give 4 fraction.

Identification of Compound

NMR spectra were recorded on Varian AS 400, JEOL JNM AL-300 and Bruker AM-500 NMR spectrometers. HRESIMS data were obtained on an Agilent Series 1100 SL mass spectrometer. EIMS and FABMS were measured on AEI MS-50, VG-ZAB-HS and APEX II FTICR mass spectrometers. IR spectra were measured on a Nicolet Impact 410 spectrometer. UV spectra were obtained on a U-2000 UV–vis spectrophotometer. Column chromatography (CC) was performed using silica gel (Qing Dao Ocean Chemical Technology. Factory, 200–300 mesh). Thin layer chromatography was carried out on silica gel 60 plates (Qing Dao Ocean Chemical Technology Factory).

Cytotoxicity screening *Tridax procumbens* compound

The cytotoxicity of *T. procumbens* compounds were determined by Methyl tetrazolium (MTT) assay[12]. Cells ($2X10^3$/well) were plated in 100 µl of medium/well in 96-well plates. After overnight incubation, four different fraction of compound were added 5 wells for each concentration. After treatment with four compounds for 1, 2, 3, 4, and 5 days, 20 µl of 5 mg/ml MTT (pH 4.7) was added to each well and cultivated for another 4 h. The supernatant was removed and 100 µl DMSO was added per well. Samples were then shaken for 15 min. The absorbance at 570 nm was measured with microplate reader (Bio-Rad), using wells without cells as blanks. All experiments were performed in triplicate. The effect *T. procumbens* compounds on the proliferation of cancer cells was expressed as relatively cell viability, using the following formula[13].

$$\text{Percent viability} = \frac{\text{OD of drug treated sample}}{\text{OD of none treated sample}} \times 100$$

Clonogenic survival determination

Cells were seeded at specified numbers (300/well for U251 cell, 400/well for A549 (p53 wt) in 6-well plates. Colony-forming ability was tested following 12-day lupeol treatment at 80, 160, and 320 µg/ml, respectively. This was done by staining cells with 0.5% crystal violet in absolute ethanol and counting colonies with >50 cells under dissection microscope.

Cell cycle analysis by flow cytometry

Cell cycle was analyzed by flow cytometry (FCM) analysis[14]. A total of $1X10^7$ cells were harvested from control culture and cells treated with 80, 160 and 320 µM of lupeol for 48 h. Cells were washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h. The sample was then concentrated by removing ethanol and treated with 1% (v/v) Triton X-100 and 0.01% RNase for 10 min at 37 ºC. Cellular DNA was stained with 0.05% propidium iodide for 20 min at 4 ºC in darkness. Cell cycle distribution and apoptotic cells were detected with FCM (Model FACSC420, USA). Data of more than $1X10^4$ cells was then analyzed with the Multi Cycle software package (Phoenix, USA). All data represents the results from three independent experiments.

Cell based assay for inhibition of cox-2 activity

Human lung cancer cells A549 (p53 wt) were cultured in 75 cm^2 culture flask in RPMI-1640 medium supplemented with 10 % bovine calf serum and 60 mg/l amikacin, at 37 ºC in an atmosphere of 95 % humidity and 5 % CO_2. For the assay, cells were seeded in the wells of 96-well plates (50,000 cells/well) and incubated at 37 ºC for 24 h. Confluent cells were treated with 250 µM aspirin for 30 min to completely inactivate COX-1 activity. After washing thoroughly with medium, cells were incubated with 5 µg/mL LPS for 16 h to induce the production of COX-2. Induced cells were washed thoroughly with medium to remove LPS completely and treated with different concentrations lupeol compound for 2 hours. Arachidonic acid (300 µM,) was added and the cells were further incubated for 30 minutes. Supernatants were removed and the amount of PGE2 released in the medium was determined using PGE2 Enzyme Immunoassay kit (Cayman Chem. Co. USA). COX-2 activity was estimated by the conversion of exogenous arachidonic acid to PGE2 and is expressed as % of the vehicle control (DMSO, 0.5 %). NS-398, a specific inhibitor of COX-2, was included as positive control in each assay[15, 16].

Analysis of DNA fragmentation

The cells were treated with or without 320 µM concentration of Lupeol for 16 h. Cells were collected and suspended in denaturing solution (10 mM Tris–HCl, ph 8.0, 100 mM NaCL, 25 mM EDTA, pH 8.0 and 100 µg/ml of Proteinase K) and incubated at 50 °C for 12–18 h. Total DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and then solubilized in TE buffer (10 mM Tris–HCl and 100 µM EDTA, pH 8.0). Ten micrograms of the collected DNA was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and then visualized under UV illumination.

Statistical analysis.

The statistical significance of difference between control and Lupeol-treated Cell line was determined by one-way ANOVA followed by Tukey test for multiple comparisons. Dunnett's t tests (2-sided) were employed, as needed, and result was considered significant at $P < 0.05$.

RESULT

Phytochemical screening of Aqueous leaf extract of *Tridax procumbens*

The phytochemical screening of the *Tridax procumbens* studied presently showed the presence of alkaloids, flavonoids, glycosides and terpenoids (Table-1).

Cytotoxicity screening of *Tridax procumbens* compounds on human lung cancer cells- A549

Tridax procumbens Compounds 1-4 were tested for their in vitro anticancer activity using the MTT assay. One cell lines representing one type of human malignancies (Human lung cancer cells A-549) was cultured with test compounds at concentrations in the range of 80, 160 and 320 µM for 72 h, and cell viability was determined. Out of the 4 compound Rf value 0.66 showed 90% cytotoxic potential against Human lung cancer cells A549.

Identification and analysis of anticancer compound from *Tridax procumbens*

The *Tridax procumbens* compound with Rf value 0.66 under IR spectrum of intensely broad band at 331, 3320 and 3320 cm^{-1}, showed presence of OH stretching and so they were assumed to be a triterpenoid. The melting point of Rf value 0.66 was 213^0C; and the UV λ_{max} value of *Tridax procumbens* compound was 350 nm. The ^{1}H NMR spectrum showed the presence of multiplets tertiary methyl's at δ 0.77, 0.79, 0.84, 0.97, 0.98 1.04 and 1.69, appeared as singlets except the signal appeared at δ 1.69 which showed allylic coupling (*J*=1.3Hz). A pair of multiplets at δ 4.56 and 4.69 was indicating of terminal isopropenyl moiety. This indicated that 16 belongs to the lupine of triterpenoids.

^{13}C NMR spectrum which showed seven methyl groups at [*δc*: 28.0 (C-23), 19.3 (C-30), 18.0 (C-28), 16.1 (C-25), 15.9 (C-26), 15.4. (C-24), 14.5 (C-27)], an exomethylene group at [*δc*: 150.8 (C-20), 109.3 (C-29)] and a secondary hydroxyl bearing carbon at [*δc*: 78.9 (C-3)], in addition to ten methylene, five methine and five quaternary carbons. The shielding of C-23 methyl of *Tridax procumbens* compound could be due to the influence of the adjacent C-3 hydroxyl group and further confirmed the indentity of *Tridax procumbens* compound as lupeol. The molecular formula was established by HR-EIMS at *M/Z* 426.3855 ($C_{30}H_{50}O$). Besides the molecular ion peak at *M/Z* 436, the EI mass spectrum also showed other fragment ion peaks at *m/z* (rel. int.), 426 [M^+] (2), 411 [M^+-CH_3] (3), 408 [M^+-H_2O] (3), 218 (5), 207 (6), 189 (58), 163 (80), 135 (57), 107 (68), 105 (55), 79 (54), 41 (100). *M/Z* 411 (M-CH_3), 218 (M-$C_{14}H_{28}$), 207(M-$C_{16}H_{27}$), which are diagnostic for pentacyclic triterpenes with an isopropenyl moiety.

Effect of lupeol on human lung cancer cells A549 for inhibition of colony formation

The inhibition of colony formation of lupeol was determined. The compound suppressed the colony formation at 320 µM concentration. Lupeol at 320 µM suppressed the A-549 cell numbers to118 ± 2.0, whereas untreated A-549 cells colony number were 384 ± 2.1 (Fig-1).

The Colony Number of lupeol treated cells were compared with that of the control, the single (*) indicates a very significant difference from the Control (P < 0.05), one way ANOVA Dunnett C Test. Results are mean values ± standard deviation of independent experiments performed in triplicate.

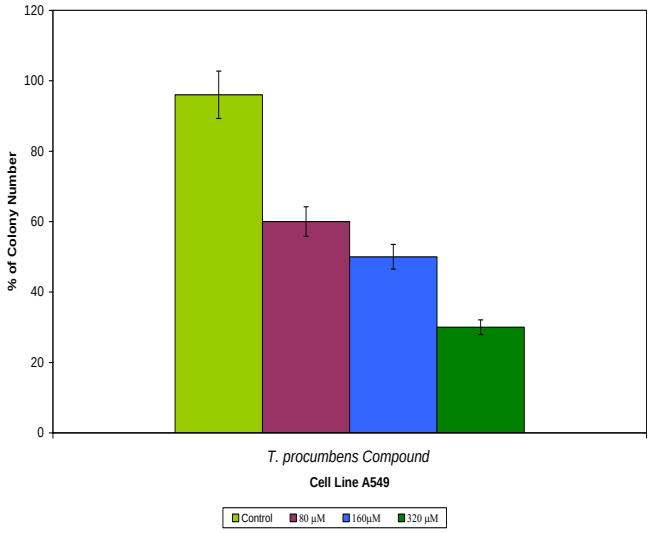

Fig 1 : Colony Formation inhibition of lupeol on human lung cancer cells A-549

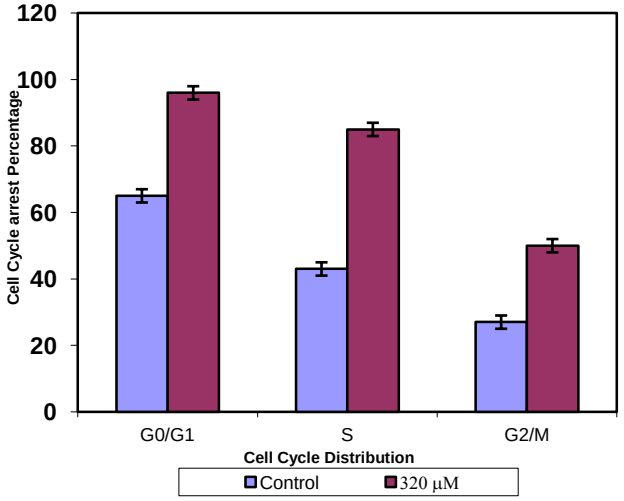

Fig-2. Cell Cycle Analysis of lupeol on cell cycle control of Human Lung Cancer cell A-549

Effect lupeol on cell cycle control of human lung cancer cell A-549

To investigate whether the lupeol affected cell cycle regulation, flow cytometry was performed. Incubation of lupeol with human lungs cancer cell A-549 for 24 h significantly reduced the DNA content, making them appear in the sub-G0/G1 or A0 region indicative of apoptosis, with consequent loss of cells in the G1 phase. 90 ± 2.0.1 of cells were in sub G0/G1 phase, with 65 ± 1.6 cells in the same phase in the respective control (Fig-2). After treatment with 320 µM lupeol for 48 h, the apoptotic cancer cells increased significantly (P < 0.05). Specifically, the cell population in G0/ G1 phase increased (P < 0.05), those in S and G2/M phases decreased (P < 0.05), suggesting a growth arrest in the G0/G1 phase of the cell cycle.

Cell Cycle Analysis of lupeol treated cells were compared with that of the control, the single (*) indicates a very significant difference from the Control (P < 0.05), one way ANOVA Dunnett C Test. Results are mean values ± standard deviation of independent experiments performed in triplicate.

Effect of lupeol compound on inhibition of cyclooxygenase activity of human lung cancer cell A-549

The investigation on the cyclooxygenase enzyme activity in the lupeol treated human lung cancer cells A-549 resulted in the maximum inhibition of the enzyme activity at 320µM concentration. About 67± 5.03%, 66± 2.08%, 78±3.6% and 81 ± 5.7% of enzyme activity was observed in the treated cells (Fig-3).

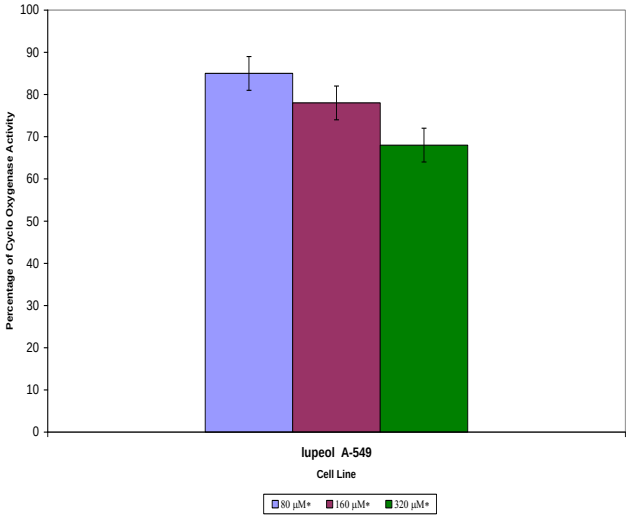

Fig-3 :Cyclooxygenase enzyme activity of lupeol compound treated A-549 Cells

Cyclooxygenase enzyme activity of lupeol treated A-549 Cells treated cells compared with that of control, the single (*) indicates a very significant difference from the Control (P < 0.05), one way ANOVA Dunnett C Test. Results are mean values ± standard deviation of independent experiments performed in triplicate.

Effect of lupeol on Human lung cancer cell A-549 DNA fragmentation.

Degradation of genomic DNA, due to activation of endogenous endonucleases, is one of the early events of apoptosis, for this reason, lupeol was tested for its ability to induce apoptosis in whole cells by DNA fragmentation. Human lung cancer cell A549 (p53 wt) (inoculated at 10^5 cells/ dish) were exposed for different periods of time to lupeol at IC_{50} concentration. Human lung cancer cell A549 (p53 wt) treated with lupeol for 24 h or more showed a marked DNA fragmentation pattern not observed in untreated cells (Fig-4). Although a typical DNA ladder due to the release of oligonucleosome associated DNA fragments was not observed, after 48 h of incubation with lupeol a clear disintegration of genomic DNA into high molecular weight fragments was obtained.

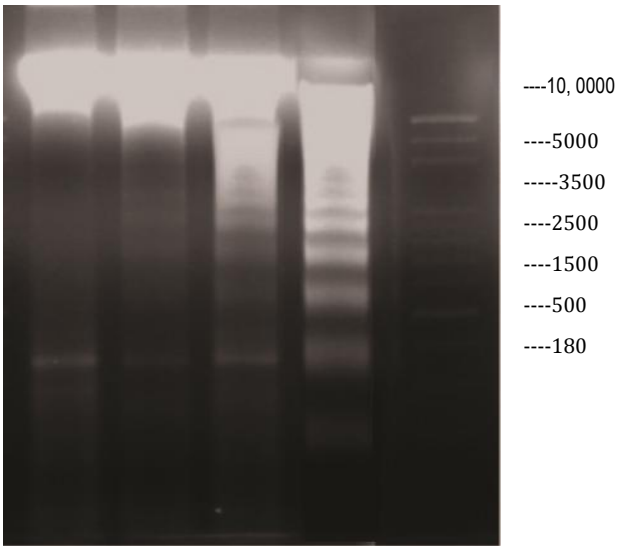

Fig 4: Effect of lupeol on human lung cancer cell A-549 on DNA fragmentation.

DISCUSSION

Medicinal plants are the important source in the discovery of novel anticancer compounds since there is limited number of anticancer drug available in modern medicine. The identification of the new drug is important in the lung cancer treatment so that survival rate of the lung cancer patient will be effectively increased. *T. procumbens* are common weed distributed all over the world. Tribal and traditional healers of India use *T. procumbens* for the treatment of inflammation bronchial disease and chronic wound. The plant is used as anticeptic and is recommended for external application for wound washing[4]. The family Compositae is the major source of terpenoid compound. They are widely used in modern medicine for the inflammation, cancer, bacterial and fungal diseases.

The present study on the phytochemical screeing was in accordance to the previous studies on the phytochemical screening in *Tridax procumbens* which revealed the presence of alkaloids, carotenoids, flavonoids (catechins and flavones), saponins and tannins[7]. Cytotoxicity screening results of this study demonstrate that the terpene and phenolic substances had the ability to reduce the viability of lung cancer cells, probably through induction of apoptosis. The result of the present study on cytotoxicity are similar to 160 µM of curcumin decreased the cell viability to 95% in human lung cancer cell[17]. Also, the results are consistent with the work of Mahavir et al[18], whose data showed that Noscapine at 30-50 µM concentration enhanced the cytotoxicity of Cisin-H460 and A-549 NSCLC cell sinasynergistic to strongly synergistic manner respectively. Generally, secondary metabolites of medicinal plants strongly inhibit growth of cancer cell line[19]. In the effective search for anticancer agents, the consumption of fruits, vegetables and medicinal plants was known to reduce cancer risk, which was also scientifically proved[20]. Thus medicinal plants constitute the main source of new drugs and health care products[21]. The compound of *Tridax procumbens* was identified as Lupeol. The data for Lupeol was in close agreement with those reported literature for lupeol in previous study[22, 23, 24].

The inhibition of colony formation of lupeol correlated with previous studies. Mohammad et al[25] recorded even after 21 days, the colony formation was significantly decreased in Lupeol-treated cells as compared with control that exhibited numerous colonies. Result from previous studies had shown similar effect of inhibition of colony formation of isochaihulactone of *Bupleurum scorzonerifolium* on human lung cancer cells[26].

Apoptosis induction was additionally measured by in situ EB/AO staining for apoptotic cells[27] and flow cytometry (FCM) analysis[28]. Cells treated by the drug undergo inhibited spindle formation resulting in mitotic arrest and cell death[29]. Flow cytometric analysis of lupeol on cell cycle matched alike with the results of cavacrol treated cells which showed increase of sub G0/G1 phase apoptic peak of cell cycle and decrease of cells at S phase in concentration dependent manner indicating induction of apoptosis and inhibition of DNA synthesis in S phase[30]. The reduction of DNA content and amount of increase of cells in G1 phase coincide with the previous report on the increase in distribution of number of cells on sub G0/G1 phase of Thiadiazole based compound treated in human non small lung cancer cell A-549 cells[31] was in similar to the present study.

Lupeol compound strongly inhibited cyclooxygenase enzyme and was in accordance to cyclooxygenase inhibitory effect of Neolignans[32]. The cyclooxygenase inhibitory effect was nontoxic to mammalian cells as was observed in pervious report[33]. Alfred et al[33] studied apoptosis in A549 cells by using nonaerosolized nimesulide with or without doxorubicin. The nimesulide at concentrations equal to or below its IC_{50} value enhanced the apoptotic response of doxorubicin against A549 cells by caspase-3 activity and DNA degradation assays. Caspase-3 activation is an early event in the apoptosis cascade, where its activation triggers PARP cleavage, which is parallel to apoptosis detection by DNA fragmentation and TUNEL assays[35].

Table 1: Phytochemical screening of aqueous leaf extract of *Tridax procumbens*

Sl.No.	Phytochemical Constituents		Observation	Aqueous extract of *Tridax procumbens*
1	Alkaloids	Dragendorff's test Mayers test	Orange / red precipitate Yellowish precipitation	+
2.	Flavonoids Alkalai Reagent		Intense yellow color	+
3.	Glycosides Bornbager's test		Pink color (Ammonia layers)	+
4.	Tannin $FeCl_3$ test		Green of blue black coloration	--
5.	Saponins Frothing test		Foam	--
6.	Terpenoids Nollers test		Purple color to Red	+

– = Negative (absent) **+ = Positive (present)**

Table-2 : Cytotoxicity screening of *Tridax procumbens* compounds 1-4 on Human lung cancer cells A-549

Tridax procumbens Compounds Rf Value	Human lung cancer cells A-549 Cell viability %		
	80 µM	160 µM	*320 µM
0.44			
0.66			
0.92			
0.98			

Inhibition <20% in green; 20-90% in Orange; >90% in Pink *Highest concentration.

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