RESEARCH ARTICLE

Essential Oil of *Tridax procumbens L* Induces Apoptosis and Suppresses Angiogenesis and Lung Metastasis of the B16F-10 Cell Line in C57BL/6 Mice

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Abstract

Background: To determine the effect of essential oil obtained from a traditionally used medicinal plant *Tridax procumbens L*, on lung metastasis developed by B16F-10 melanoma cells in C57BL/6 mice. Materials and Methods: Parameters studied were toxicity, lung tumor nodule count, histopathological features, tumor directed capillary vessel formation, apoptosis and expression levels of P^{53} and caspase-3 proteins. Results: *In vitro* the MTT assay showed cytotoxicity was found to be high as 70.2% of cancer cell death within 24hrs for 50μg. *In vivo* oil treatment significantly inhibited tumor nodule formation by 71.7% when compared with untreated mice. Formation of tumor directed new blood vessels was also found to be inhibited to about 39.5%. TUNEL assays also demonstrated a significant increase in the number of apoptotic positive cells after the treatment. P^{53} and caspase-3 expression was also found to be greater in the essential oil treated group than the normal and cancer group. Conclusions: The present investigation showed significant effects of the essential oil of *Tridax procumbens L* in preventing lung metastasis by B16F-10 cell line in C57BL/6 mice. Its specific preventive effect on tumor directed angiogenesis and inducing effect on apoptosis warrant further studies at the molecular level to validate the significance of *Tridax procumbens L* for anticancer therapy.

Keywords: *Tridax procumbens L* - MTT assay - anti-metastatic - anti-angiogenic - P^{53} assay - caspase-3

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Introduction

Cancer is one of the main reasons for the cause of largest mortality worldwide. Many number of people die of lung cancer than colon, breast and prostate cancers (Zhi et al., 2007). Among cancer deaths, lung cancer is the one having top most mortality rate of 6 million per year Worldwide (Wenfeng et al., 2011). Among lung cancer types non-small-cell lung cancer accounts for 80% of deaths (Yang et al., 2008). In spite of having several chemotherapeutic and immunomodulating agents for treating cancer, there is still search for an ideal treatment that has minimal side effects and cost-effective. Most of the anticancer drugs available in the market like toxol and vinca alkaloids (vincristine, vinblastine) are obtained from medicinal plants (Shan et al., 1999). Evidences are there that these medicinal plant based drugs are believed to suppress the transformative, hyper proliferative and inflammatory processes. These activities may ultimately suppress the final steps of carcinogenesis such as angiogenesis and metastasis and also induce apoptosis (Bharat et al., 2006). According to cancer experts, metastasis is a serious aspect and is the common cause of cancer deaths. Most of the treatment for cancer involves the control of invasion and metastasis of tumors. Generally, angiogenesis is essential for the normal reproduction, development and organ repair. On the other hand, angiogenesis is also important in a variety of tumor processes for tumor growth and metastasis. These two extreme activities are regulated by a variety of distinct pro-angiogenic and anti-angiogenic molecules. Several anti-angiogenic agents such as Angiostain, endostain and thrombspondins (endogenous proteins) have been proved both *in vitro* and *in vivo* studies for their anti-angiogenic activity and some are in clinical trials (Pan et al., 2005). Many anticancer drugs exert their cytotoxicity through DNA damage and induction of apoptosis. P^{53} gene is an important protein in regulating DNA repair and triggering apoptosis after cellular DNA is injured (Porter et al., 1999). P^{53} act through many mechanisms for anticancer function, and plays a role in initiating apoptosis, genomic stability, and inhibition of angiogenesis. P^{53} mutations are common in lung cancer and range from 33% in adenocarcinomas to 70% in small cell lung cancers (Oren et al., 1994). On the other hand, Caspase-3 (CASP-3) is also a primary effector CASP that executes programmed cell death (apoptosis) where it is responsible for chromatin condensation and DNA fragmentation. Caspase occupy a pivotal position in the final common pathway of apoptosis like P^{53} does (Mraz et al., 2009). But the fact is that tumor cells are resistant to apoptosis. Hence, selective killing of tumor cells by promoting apoptosis factors is a promising and efficient way for treating cancer. Hence, the development of new

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anti-cancer drugs to promote apoptosis and suppress tumor directed angiogenesis is in need to overcome these problems.

The Ethno pharmacological and traditional use of plants often results in the discovery of new biologically active molecules (Alisi et al., 2008). Plants have a long history of use in the treatment of cancer (Mohammad et al., 2006). However, many researchers claim that the efficacy of such treatments should be viewed with some skepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Cragg et al., 1994). Hence, any discovery of anticancer agents (Plant based) must be related to novel molecular targets through specific mechanisms but less toxic to normal cells. Terpenes are a wide-spread group of natural compounds with considerable practical significance. Almost all terpenes have some biological activities in animals and play a meaningful role in human medicine. Hence, there is a growing interest in natural terpenoids in their scientific aspects of extraction and structural analysis. Terpenes are well reported for bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anti-cancer, spermicidal and anti-allergic activities (Pezzuto et al., 1997). The most volatile components of terpenes are α β-pinene and they are the dominant odorous compounds emitted by most of the medicinal plants. The effects of α-pinene vary depending on the composition of monoterpenes and sesquiterpenes. It is reported that the β-pinene generally accompany α-pinene in low quantities in the volatile extracts, essential oleoresins and oils of plants. Some specific studies have shown that the β-pinene, along with α-pinene and other terpenes are cytotoxic on cancer cells (Setzer et al., 1999). The α- and β-pinenes were strongly reported for its cytotoxic activity on several cell lines like breast cancer and leukemic cell lines (Zhou et al., 2004). When these pinenes are the major constituents of an essential oil, they warrant the anti-inflammatory and analgesic activities (Erazo et al., 2006). Our study plant *Tridax procumbens L* is an annual herb, native to tropical America and rapidly spread in many parts of tropical, subtropical and mild temperate regions Worldwide. The plants extract has been reported by earlier studies that it is used for lowering blood pressure (Ikewuchi et al., 2010), blood glucose level (Bhagwat et al., 2008) and also its hepatoprotective activity in rats (Vilwanthan et al., 2005). All these *in vivo* studies and traditional use did not reveal any deleterious effect in rats and higher animals including man (Satish et al., 2012). Moreover, different solvent extracts from this plant as well as essential oil have been reported for many significant pharmacological properties such as, anti-microbial (Mahato et al., 2005), anti-inflammatory (Nia et al., 2003), anti-oxidant (Habila et al., 2010), wound healing (Raina et al., 2008) and anti-cancer (Prostate Cancer) activities (Vishnu et al., 2011). The leaf extract of *Tridax procumbens L* is being used widely as traditional medicine for healing open wounds due to is greater anti-inflammatory effect. As the essential oil of *Tridax procumbens L* has revealed to have α-pinene, β-pinene l-phellandrene and Sabinene as their major bioactive compounds as identified by GC-MS by us, we intend to study its preventive/chemotherapeutic effect on experimentally induced lung cancer development (Manjamalai et al., 2010). In the present study, we have extracted the essential oil from *Tridax procumbens L* to evaluate its anti-cancer activity by assessing the apoptosis induction level suppressing effect on tumor directed angiogenesis and preventive/chemotherapeutic effect on lung metastasis in experimentally induced lung cancer in C57BL/6 mice using B16F-10 cell line.

Materials and Methods

Collection and authentication of plants

Fresh leaves of the selected plant *Tridax procumbens L* having medicinal value was collected from Western Ghats of siruvani hills of Coimbatore, India. The plant material was taxonomically identified and authenticated by the Botanical Survey of India and the voucher specimen (**No.BSI/SC/5/23/09-10/TECH.1449**) is retained in our laboratory for future reference.

Extraction of essential oil

Extraction of essential oil from the study plant was done by hydro distillation method using clevenger-type apparatus for 3 hours (Ahmed et al., 2011). Plant material (leaves) was immersed directly in a round bottom flask filled with water. This was then brought to boil. Vapours were condensed on a cold surface using condenser attached to it. Essential oil gets separated based on the difference in density and immiscibility, is then collected and dried over anhydrous sodium sulphate and stored in a vial at low temperature until analysis.

GC-MS analysis

GC-MS analysis was performed in Indian Institute of Spices Research (IISR)-Calicut-Kerala-[PMT/IISR/28(13)09], using CARBOWAX capillary column and helium as carrier gas to identify the major compounds present in essential oil (Philip et al., 2001). Briefly, 0.2 μl of essential oil was injected in to the column of 1 μl/min at 250^{o}C and the oven temperature was programmed as 60^{o}C for 15 minutes, and then gradually increased to 280^{o}C for 3 minutes. The identification was based on comparison of their mass spectra and retention indices.

Experimental animals

C57BL/6 (20-25g) of male sex mice (6 animals/group/cage) were purchased from National Institute of Nutrition (Hyderabad, India). The animals were housed in ventilated plastic cages and maintained at 12 hour light/12 hour dark cycle with free access to food and water. All the experiments involving animals were performed according to the standard protocol and guidelines after getting proper approval from Institutional Animal Ethical Committee.

In vivo acute drug toxicity study

Overnight-fasted C57BL/6 mice of male sex weighing 20-25 g were divided into 6 groups of 6 animals each. Each group of animals was given different doses of drug (essential oil) such as 50, 100, 200, 500, 1,000 and 2,000 μg via i.p. The acute toxicologic effect was observed for 72 hours in terms of mortality (Fernando et al., 2010).

Cell line

The B16F-10 melanoma cell line was purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium buffered with 2 g/L of HEPES and sodium bicarbonate, supplemented with dextrose, penicillin, streptomycin and 10% of fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37ºC. When needed for experiments (or during routine passaging steps), the cells were harvested with trypsin: EDTA (0.05: 0.03 [W/V] solution, and then washed in phosphate buffered saline (PBS, pH 7.4).

In vitro cytotoxicity study by MTT assay

This assay is extensively used for measuring cell survival and proliferation. It depends on the cell type, cellular metabolism and incubation time with MTT (Mosmann et al., 1983). This method is based on the capacity of mitochondrial enzyme succinate dehydrogenase of viable cells to reduce the yellow soluble tetrazolium salt MTT [3-(4, 5-dimethyl-thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide] into a purple blue insoluble formazan precipitate which is quantified spectrophotometrically after dissolving in DMSO. There is a direct proportionality between the formazan produced and the number of viable cells. Different concentrations of essential oil such as 5, 10, 25 and 50 μg/ml of media were treated with the cell line (1×10^6) and 20 µl of MTT (5 g/ml) reagent. The test tubes were incubated for 4hrs at 37°C. Later 1 ml of DMSO was added to solubilize the formazan crystals and the absorbance was taken at 570 nm. The percent specific cytotoxicity was calculated as follows and expressed as percentage of viable cells, %Cell viability=[(O.D of control-O.D of test compound)/(O.D. of control)]x100.

In vivo anti-cancer study using B16F-10 cell line

The effect of essential oil on suppressing the lung metastasis was studied using male C57BL/6 mice which were injected through tail vein with 1×10^6/0.1 ml B16F-10 mouse melanoma cells having high metastasis activity from small inoculums (Sheeja et al., 2010). Animals were divided into 4 groups of 6 mice in each group. Group 1 normal mice were left as such without any treatment. Group 2 cancer controls in which cancer cells were injected to develop cancer in mice and were left without any treatment. Group 3 vehicle controls injected with cancer cells and were treated with 0.1 ml of 50% of ethanol. Group 4 essential oil treated group injected with cancer cells and were treated with 0.1 ml of 50 μg of essential oil of *Tridax procumbens L*. The brief experimental design is given below (6mice/group for 21 days); Group 1: Normal mice. Group 2: Cell line alone. Group 3: Cell line+vehicle treated. Group 4: Cell line+essential oil of treated

Body weight and lung weight in the experimental animals

The body weight and lung weight were noted on the initial and final (22nd) day in all the experimental groups. The values are expressed in mean of all the six mice in a group.

Tumor nodule count

On the 22nd day after scarification their lungs were excised, weighed, and placed in phosphate-buffered saline (PBS, pH 7.4). The number of lung tumor nodules on the surface of the lung was counted and the percentage inhibition of lung tumor nodule formation was calculated (Korangath et al., 2010).

Haematological changes

Blood was collected from all mice by cardiac puncture after scarification on 22nd day and the blood parameters such as hemoglobin (Hb) and white blood cells (WBC) were observed in all the groups (Mahesh et al., 2007).

Histopathological analysis of lung

Lung tissues (Tumor nodules) were dissected out after scarification of mice, fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax for histological studies. From the blocks, 4 µm sections were then stained with Hematoxylin and Eosin (H and E), mounted in DPX and examined under a microscope for histopathological changes of lung cancer (Gopal et al., 2010).

Study of apoptosis by TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed with in-situ apoptosis detection kit (Promega Corporation, 2800 Woods Hollow Road, Madison, USA) following the manufacturer's instructions (Wong et al., 2011). Briefly, the deparaffinized tissue section in xylene was washed in 100% ethanol for 5 minutes. Then the samples were rehydrated by sequentially graded ethanol washes (100%, 95%, 85%, 70%, and 50%) for 3 minutes each at room temperature. Again washed the samples by immersing the slides in 0.85% NaCl for 5 minutes and in PBS for 5 minutes. Fixed the tissue sections by immersing the slides in 4% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature followed by washing in PBS for 5 minutes. Added 100µl of the 20 µg/ml proteinase K to each slide to cover the tissue section and incubated for 8-10 minutes. Proteinase K helps permeabilize tissues and cells to the staining reagents in subsequent steps. After washing with PBS fixed the tissue sections by immersing the slides in 4% methanol-free formaldehyde solution in PBS for 5 minutes at room temperature and again washed with PBS. Then the sections were incubated in equilibration buffer for 10 minutes and added 100µl of rTdT reaction mix (Equilibration buffer 98µl+biotinylated Nucleotide mix 1µl + rTdT 1µl) for all slides. Finally, after the incubation, 100 µl of Streptavidin HRP solution (1:500 in PBS) was added 100 µl to each slide and incubated for 30 minutes. Then added 100 µl of DAB solution to each slide and kept until a light brown background was developed. After mounting the slides with DPX the stained cells were examined at 40x magnification by using a light microscope (Olympus BH-2).

Cell death was quantitated by counting 200 cells in five to seven separate fields of view per slide and noting the percentage of apoptotic cells based on morphological appearance.

Immunodetection of P^{53} and Caspase-3 in lung tissue

The level of expression of P^{53} and Caspase 3 was checked by immunohistochemistry using immunofluorescence (Gopal et al., 2003). Briefly, frozen tissue sections (16 µm) of tumor sample and normal were fixed with acetone for 20 minutes followed by permeabilization with 0.5% (v/v) Triton×100 in PBS for 10 minutes at room temperature. After blocking with 5% horse serum in PBS for 1 hour, the sections were incubated with primary antibody [mouse monoclonal antibodies to P^{53} and Caspase 3 (Santa Cruz Biotechnology, USA] for 1 hour and then incubated with FITC conjugated secondary antibody for 1hour at room temperature. To reduce auto fluorescence, the sections were treated with CuSO4 (10 mM) in ammonium acetate buffer (50 mM CH_3COONH_4, pH 5.5) for 30 minutes. The sections were counterstained with DAPI-(4', 6-diamidino-2-phenylindole) for 5 minutes and mounted in vector shield (Vector laboratories). The normal and tumor sections treated as above, but without primary antibody, served as negative control. After mounting the slides, the section were viewed for immunofluorescence under a confocal laser scanning immunofluorescence microscopy (CLSM) using a Zeiss LSM 510 META confocal microscope. Image analysis was done using LSM510 META software (Carl Zeiss) and images were assembled using adobe Photoshop 7.0.

Tumor directed angiogenesis by assay of capillary vessel formation

C57BL/6 mice were divided into three groups and each group contains six mice. In brief, B16F-10 cells ($1x10^6$) were injected intradermally on the shaven ventral skin of each mouse (Xiyun et al., 2003). Drug (50 µg/dose) was given through IP for 10 days after cell line injection in which group 1 receives no treatment left as mice injected with cancer cells alone, group 2 was injected with cancer cells and receives vehicle 0.1 ml of 50% ethanol and group 3 the drug treatment group was injected with cancer cells and receives 0.1 ml of 50 µg of essential oil of *Tridax procumbens L* and mice were sacrificed on the eleventh day. The skin from the ventral side was dissected out, washed with phosphate buffered saline and the number of tumor directed capillaries was counted using a dissection microscope. The percentage of inhibition of capillaries formation was also calculated using the formula as follows, %inhibition=Tumor directed capillaries of cancer control-Tumor directed capillaries of treated mice/Tumor capillaries of cancer control×100.

Statistical Analysis

Data was statistically analyzed using one-way ANOVA as primary test followed by Dunnett's test one way ANOVA, using Graph pad InStat3.0 software (Guruvayoorappan et al., 2007). All the results were expressed as mean±S.D of 6 animals in each group and considered significant when P≤0.05, P≤0.01 and P≤0.001.

Results

Phytochemicals identified by GC-MS analysis

In the GC-MS analysis of the essential oil of *Tridax procumbens L* we found that the oil contains 14 compounds namely α-pinene, 1, 3, 6-octatriene, Camphene, β-pinene, Sabinene, Phellandrene, L-limonene, β-ocimene, Trans-beta-ocimene, Trans-Caryophyllene, Gama-elemene, Spathulenol, Torreyol and Aromadendrene. Out of these compounds α-pinene, β-pinene, Phellandrene and Sabinene were found to be the major compounds with 96% similarity with Wiley and NBS library.

In vivo acute drug toxicity and In vitro cytotoxicity

The acute drug toxicity study has shown that even the higher dosage of 2,000 µg did not show any observable toxic effects in C57BL/6 mice. However, an optimal dose of 50 µg was used for all our *in vivo* studies which would be much lower than the IC_{50} value. As shown in Figure 1, *in vitro* Cytotoxicity study has shown that the 50 µg of the essential oil of Tridax procumbens exhibited 70.23% of cancer cell (B16 F-10) death within 24 hrs.

Body and lung weight of experimental groups

We have observed a significant difference in the body and lung weight of mice among the groups as shown in Figure 2. The initial mean body weight of tumor induced mice was 21.38±0.18 and on the final 22nd day it was found to be decreased (19.81±0.19) whereas, in essential oil treated group it was found to be increased on final day (21.38±0.18) from initial day (20.85±0.21). The relative lung weight of the normal mice was found to be (2.31±0.02) and it was found to be increased in the tumor induced group (3.22±0.06) whereas, in the treated group it was found to be significantly decreased (2.56±0.05).

Tumor nodule count

Table 1 shows the percentage inhibition of tumor nodule formation in which a high % of inhibition (71.67%) was found in the mice treated with the essential oil of *Tridax procumbens L* when compared with cancer induced

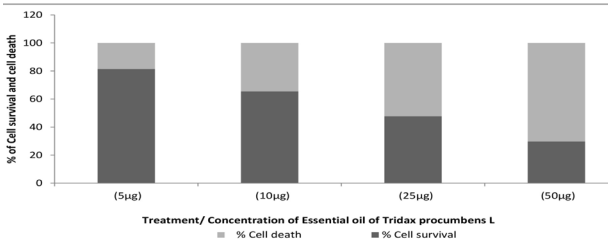

Figure 1. *In vitro* Cytotoxicity Activity of Essential Oil Towards B16F-10 Cell Line by MTT Assay

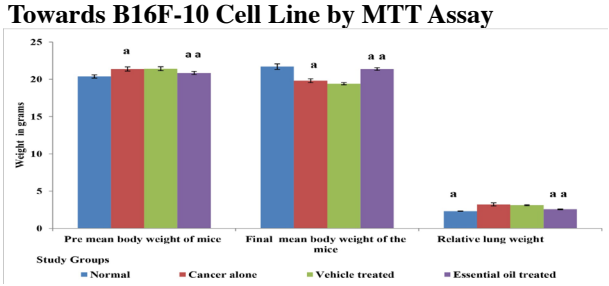

Figure 2. Effect of essential oil of *Tridax procumbens L* on body and relative lung weight of experimental groups. Data are expressed as mean±S.D. Normal Vs Cancer alone [a-P≤0.001]. Normal Vs Essential oil treated [a-P≤0.001]. Cancer alone Vs Essential oil treated [a-P≤0.001]

group. The number of tumor nodules found in cancer group is 157.66±2.81 and that of in the treated group is 44.66±4.63.

Hematological parameters

Figure 3A and 3B shows the level of WBC on the 22nd day. It was found to be (7325±17.60) in the normal mice and (7148±17.65) in the essential oil treated groups. On the other hand WBC was found to be high (7443±10.23) in the tumor control mice. Similarly, on the 22nd day, the hemoglobin level also was found to be increased (13.43±0.17) in the essential oil treated group when compared with tumor control groups (11.25±0.21).

Histopathological changes

The Hematoxylin and Eosin stained sections of lung tissues are shown in Figure 4. The lung from healthy normal mice shows normal architecture of lungs with bronchioles, alveoli and interstitium (Figure 4A). The lungs of cancer control animals showed massive tumor cell proliferation around the bronchioles and infiltration of metastatic colonies of melanoma in the interstitium of the lung. Increased fibrosis reduces alveolar space, which leads to reduction in vital capacity of the lung. Multiple area of necrosis with infiltration of neutrophils appeared in between and within lobules. Neoplastic tubular epithelial cells metastasized into Lungs. Entire alveolar region of lungs were occupied by multiple lobes of tubular neoplastic cells (Figure 4B). Simultaneous administration of essential oil (Figure 4C) showed significant reduction in tumor mass, metastatic foci and regeneration of alveolar passage with ciliated columnar epithelial cells. Luminal epithelial-like morphology with well differentiated secretory glands was seen.

Level of apoptosis by TUNEL assay

We have observed an increase in the number of apoptotic cells with mean apoptotic nuclei of 32.66±3.77 in treated mice which is significantly higher when compared with that of in the normal mice 5.33±1.51 and cancer control 13±1.26 respectively. The data is shown in Figure 5 and the apoptotic nuclei staining are shown in Figure 6.

Immunoflourescence for P53 and caspase 3 expressions

Examination of the expression of P53 and Caspase 3 was performed using confocal microscope. Lower expressions of P53 and Caspase 3 were found in the neoplastic epithelial cells of all the sections of cancer group when compared with the normal lung tissue. Expressions of P53 and Caspase 3 in the essential oil treated group were found to be increased when compared with the cancer control. Not much immunofluorescence was observed in the normal mice for both P53 and Caspase 3 proteins as shown in Figure 7 and Figure 8.

Level of tumor directed capillary vessel formation

Table 2 shows the level of capillary vessel formation

Table 1. Effect of Essential Oil of *Tridax procumbens L* in the Inhibition of Lung Tumor Nodule Formation

Group	No. of tumor nodules/lung	% Inhibition of tumor nodules
1	—	—
2	157.66/±2.81	—
3	156.66/±1.03	—
4	44.66[a]/±4.63	71.67

*Data are expressed as mean±S.D. Group 2Vs Group 4 [a-P≤0.001]

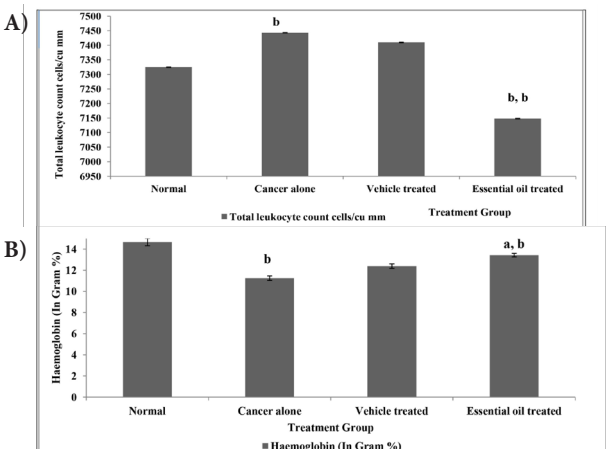

Figure 3. A) Effect of Essential Oil of *Tridax procumbens L*, on Total Leukocyte Count (WBC). Data are expressed as mean±S.D. Normal Vs Cancer alone [b-P≤0.001]. Normal Vs Essential oil treated [b-P≤0.001]. Cancer alone Vs Essential oil treated [b-P≤0.001]. **B) Effect of Essential Oil of *Tridax procumbens L*, on Haemoglobin Level.** Data are expressed as mean±S.D. Normal Vs Cancer alone [b-P≤0.001]. Normal Vs Essential oil treated [a-P≤0.05]. Cancer alone Vs Essential oil treated [b-P≤0.001]

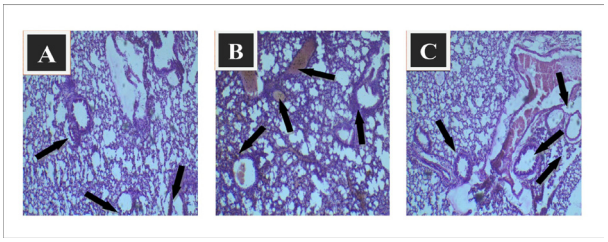

Figure 4. Histopathological Analysis of Lung. A) Normal mice-[Shows normal architecture of lungs with bronchioles, alveoli and interstitium]. B) Lung of mice injected with B16F-10 cell line (Cancer alone)-[Shows massive abnormal cell growth around the bronchioles and infiltration of melanoma in the interstitium of the lung, coarse granules of black melanin pigment are seen both inside the macrophages as well as outside them]. C) Lung of treated group shows significant reduction in tumor mass and regeneration of alveolar passage with ciliated columnar epithelial cells and reduced metastatic foci in lungs showing luminal epithelial-like morphology with well differentiated secretory glands are seen and reduction in the coarse granules of black melanin pigment are also seen

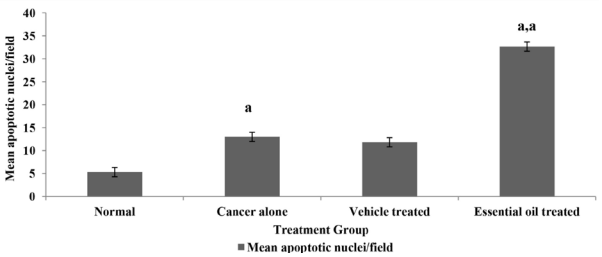

Figure 5. Levels of Apoptosis by TUNEL Assay. Data are expressed as mean±S.D. Normal Vs Cancer alone [a-P≤0.001]. Normal Vs Essential oil treated [a-P≤0.001]. Cancer alone Vs Essential oil treated [a-P≤0.001]

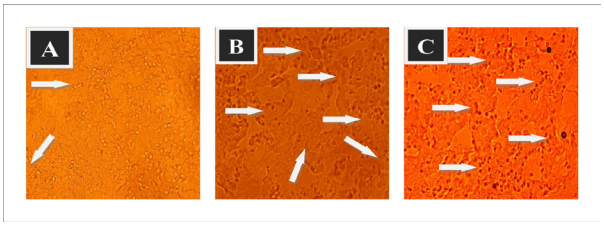

Figure 6. Level of Apoptosis by TUNEL Assay. A) Normal lung section showing the minimal number of apoptotic cells which exist in normal growth cycle. B) Lung section of mice injected with B16F-10 cell line (cancer alone) showing a moderated number of apoptotic cells due to enhanced proliferation. C) Lung section of treated mice with the essential oil Tridax procumbens. L. Shows enhanced apoptotic cells when compared with cancer alone case

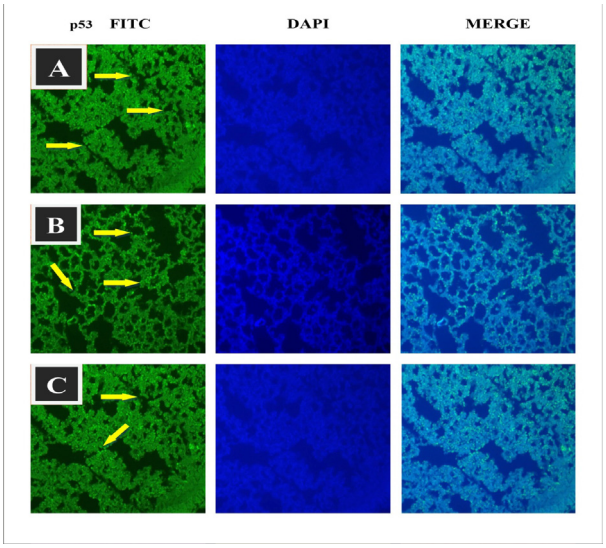

Figure 7. Immunodetection of P^{53} Expression. Intensity of green fluorescence indicates the expression level of P^{53} at 100x. A) Normal mice lung with normal expression of P^{53}. B) Cancer mice lung with lower expression of P^{53}. C) Treated mice lung showing enhanced expression of P^{53} compared to cancer control. [Magnification-100x] FITC, DAPI, MERGE [FITC+DAPI]

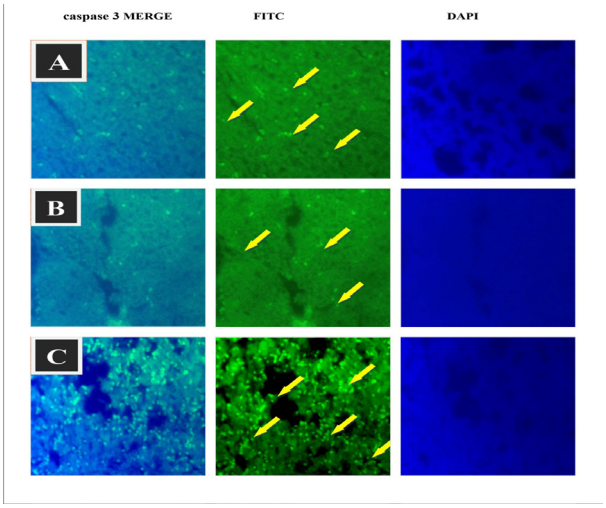

Figure 8. Immunodetection of Caspase 3 Expression. Intensity of green fluorescence indicates the expression level of Caspase 3 at 100x. A) Normal mice lung with normal expression of Caspase 3. B) Cancer mice lung with lower expression of Caspase 3. C) Lung histology of treated mice showing enhanced expression of Caspase 3 compared to cancer control. [Magnification-100x] FITC, DAPI, MERGE [FITC+DAPI]

Table 2. Effect of Essential oil of *Tridax procumbens L* on Tumor Directed Capillary Vessel Formation

Group	No. of tumor directed capillaries/cm^2	% of inhibition
1	21.48/±1.39	—
2	20.49/±1.37	—
3	13/±2.19[a]	39.47

*Data are expressed as mean±S.D. Group 2Vs Group 4 [a-P≤0.001]

in C57BL/6 mice upon induction with B16 F-10 cell line. The result shows that the essential of *Tridax procumbens L* exhibit a significant and high percentage of inhibition in formation of tumor directed new blood vessels accounting for 39.47%.

Discussion

The essential oil of *Tridax procumbens L* was found to have 14 compounds and out of which four compounds namely α-pinene (C10H16) β-pinene (C10H16) phellandrene (C10H16) and Sabinene (C10H16) were found to be the major compounds. All the major compounds identified belong to the monoterpene family and hence the significant activity exhibited by this plant's essential oil may be due to these major compounds. The biological activities of each compound have been reported decades back by different researches.

It has been proven earlier that the α and β pinene have significant biological properties like anti-cancer effect when they were used in breast cancer and *in vitro* studies showed that they are cytotoxic to human cancer cells but not on the healthy cells like the red blood cells. On the other hand, researchers have reported on the possible synergistic effect of these molecules with other monoterpenes, sesquiterpenes like Caryophyllene (Mercier et al., 2008). All these studies have proven the potential use of terpenes or a mixture of terpenes as the inducers of apoptosis in cancer cells. A number of medicinal values have been reported for *Tridax procumbens L* both by leaf extract as well as by essential oil.

In the present study, we have evaluated the anti-metastatic activity on lung cancer development of the essential oil of *Tridax procumbens L*. From the *in vivo* drug toxicity study it is clear that the drug even in its highest dosage did not show any lethal effect/abnormality on C57BL/6 mice, and we have taken 50 μg as the minimal dose for the anti-cancer studies. The cell line which we have used for the study (B16F-10 melanoma cells) is highly metastatic and form tumor cell colonies in the lungs when administered through tail vein. The *in vitro* cell cytotoxicity assay (MTT Assay) has shown a dose dependent cytotoxic effect for the essential oil of the plant towards B16F-10 cells. The essential oil of Tridax procumbens showed a high cytotoxicity of cancer cell death within 24 hrs for 50 μg which shows the potency of essential oil on killing B16F-10 cells *in vitro*.

The *in vivo* studies for chemotherapeutic effect of essential oil were carried out by involving tumor nodule counting (anti-metastatic study), apoptosis study (TUNEL assay), Assay of tumor directed capillary vessel formation (anti-angiogenesis assay) and histopathological analysis. Modern therapies focus on the anti-angiogenic therapy as a

highly recognized strategy for cancer treatment (Eichhorn et al., 2007).

Metastasis is one of the three hallmarks of cancer. Metastatic tumors are very common in the late stages of cancer (Chiang et al., 2008). The most common places for the metastases to occur are the lungs, liver, brain and the bones (Ait et al., 2007). A recent study showed that the essential oil of black seeds inhibits the metastasis in mouse models (Zhou et al., 2004). and moreover, cancer fighting essential oils effectively kill cancer cells while being non-toxic to normal cells (non neoplastic cells). Some of the most effective oils studied included sandalwood essential oil which inhibited growth by up to 90% of several different types of cancer cells (cervical, breast, skin and prostate) even at very small concentrations while having little or no harmful effect on normal cells. In our study, the cell line used was B16F-10 melanoma metastatic cell line, having a high metastatic potency to host in the lungs. The present study showed a significant inhibition of tumor nodule formation in the lungs by essential oil of *Tridax procumbens L* with an inhibition of 71.67%. This may because of the major bioactive compounds present in the essential oil such as monoterpenes like α and β pinene which have been reported to inhibit the formation of new blood vessel and thereby arresting the metastasis (Guy et al., 2012). Metastasis is associated with loss of body weight and changes in haematological parameters (Richard et al., 2007). In our study we have observed a decrease in the body weight, increase in WBC and decrease in haemoglobin in cancer group, whereas they were almost normalized like normal group in essential oil treated group.

Apoptosis and necrosis are two typical types of cell death and has been recognized earlier as a key feature of normal animal development and it is a regulated progress that is under the control of several signaling pathways, such as caspase and mitochondrial pathways (Folkman et al., 1987). Research article stated that accumulating evidence has indicated that the dysregulation of apoptosis contributes to carcinogenesis. Therefore, it has been suggested that the chemotherapeutic agents should have the ability to enhance apoptosis. Apoptosis is characterized by several biochemical criteria such as changes in mitochondrial membrane permeability, caspase signaling activation, internucleosomal DNA cleavage, and the release of intermembrane mitochondrial proteins (Takada et al., 2005). Although the molecular mechanisms underlying this phenomenon have been exclusively studied for a long time and it has become apparent that it that may be regulated by interactions with other cells (Konig et al., 1997). Caspase is an important apoptosis signaling molecule trigger a cascade of molecular interaction leading to apoptosis. Studies have shown that the extrinsic activation triggers the hallmark Caspase cascade characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role (Devi et al., 1996). The P^{53} is a mutated gene in all human tumors to about its half and is a transcription factor whose activity gives rise to a variety of cellular outcomes, most commonly cell cycle arrest and apoptosis, eliminating cancer-prone cells. Usually, P^{53} protein is present within a cell in minute amounts. Furthermore, the tumor-suppressor protein P^{53} protects against cancer by regulating the cellular response to DNA damage, apoptosis, and oncogene activation (Chendil et al., 2004). P^{53} has many mechanisms for anticancer function, and plays a role in apoptosis, genomic stability and inhibition of angiogenesis. TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades (Ramirez-Tortosa et al., 2001).

In the present study, hence we employed TUNEL assay for the evaluation of effect of the essential oil on apoptotic activity. Evaluation of the expression of P^{53} and Caspase -3 was also done by immunohistochemistry using FITC-labeled monoclonal antibodies. The result showed a higher level of positive apoptotic nuclei in the *Tridax procumbens L* essential oil treated mice when compared with the cancer mice and this may be because of the activity of the major compounds present in the essential oil. Correspondingly, expression of P^{53} and caspase was found to be high in the treated cases than the normal and it clearly indicates that the essential has induced apoptosis. Many anti-cancer drugs currently on the market were developed from plants. For instance, compounds in frankincense prevented formation of mouse melanoma tumors and decreased invasion and metastasis of advanced tumors (Klein et al., 2007). Lemongrass essential oil was reported to induce apoptosis in human leukemia cells and in several human cancer cell lines such as human colon, neuroblastoma, and sarcoma cancer cell lines (Glucksmann et al., 1951). Finally all the above study results and our study result for essential oil of *Tridax procumbens L* warrant for further studies at the molecular level for detecting the detailed mechanism of the drug in preventing cancer.

Tumor induces blood vessel growth (angiogenesis) by secreting various growth factors (e.g., VEGF) (Raff et al., 1992). Angiogenesis research is a cutting-edge field in cancer research and recently the first FDA-approved therapy targeting angiogenesis in cancer came to the market in the United States. The therapy is through a monoclonal antibody (AVASTIN) directed against an isoform of VEGF against colorectal cancer (Wyllie et al., 1980). Plants with medicinal properties and other culinary herbs with anti-angiogenic and little toxicity properties have gained importance in the last decade. Non-toxic anti-angiogenic phytochemicals are useful in combating cancer by preventing the formation of new blood vessels to support the tumor growth. Plants and plant-derived compounds find very much importance in various diseases including cancer. Extensive research over the last 50 years has indicated that Curcumin, Withanolide, Boswellic acid, Zerumbone and Resveratrol are plant derived major compounds from essential oil which both prevent and treat cancer by inhibiting angiogenesis and thereby preventing metastasis (Thornberry et al., 1998). Various other studies also have demonstrated that the extracts, natural pigments, essential oils, flowers, fruits exert anti-carcinogenic and anti-proliferative effects (Kitanaka et al., 1999). Our study has shown that the essential oil of *Tridax procumbens L* has a significant anti-angiogenic effect in B16F-10 injected tumor model with 39.47% inhibition on formation of tumor directed blood vessels.

In conclusion, based on the results obtained from our study and other related studies it can be concluded that the synergistic effects of essential oil of *Tridax procumbens L* on chemoprevention of lung cancer development in B16F-10 injected mice makes them potentially valuable drug for cancer treatment. Essential oils have a long history of use in traditional medicine, and most have been scientifically proven to be safe for use.

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References

Ahmed A, Lidia AG, Hajer El Jani, et al (2011). Antioxidant and antitumor activities of artemisia campestris and thymelaea hirsuta from Southern Tunisia. *Food Chem Toxicol*, **49**, 342-7.

Ait Mbarek L, Ait Mouse HN, Elabbadi N, et al (2007). Anti-tumor properties of black seed (Nigella sativa L) extracts. *Brazilian J Med Biol Res*, **40**, 839-47.

Alisi CS, Emejulu AA, Alisi PNC, et al (2008). Decreased cardiovascular risk and resistance to hyperlipemia-induced hepatic damage in rats by aqueous extract of Urtica dioica. *Afr J Biochem Res*, **2**, 102-6.

Bhagwat DA, Killedar SG, Adnaik SG, et al (2008). Antidiabetic activity of leaf extract of *Tridax procumbens L. Int J Green Pharm*, **2**, 126-8.

Bharat B, Aggarwal, Haruyo I, et al (2006). From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. *Expert Opin Ther Targets*, **10**, 87-118.

Chendil D, Ranga RS, Meigooni, D, et al (2004). Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. *Oncogene*, **23**, 1599-607.

Chiang AC, Massague J (2008). Molecular basis of metastasis. *N Engl J Med*, **359**, 2814-23.

Cragg GM, Boyd MR (1994). Ethno botany and the search for new drugs. *Ciba Foundation Symposium*, **185**, 178-96.

Devi PU (1996). Withania somnifera Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. *Indian J Exp Biol*, **34**, 927-32.

Eichhorn ME, Kleespies A, Angele MK, et al (2007). Angiogenesis in cancer: molecular mechanisms, clinical impact. *Langenbecks Archives of Sur*, **392**, 371-9.

Erazo S, Delporte C, Negrete R, et al (2006). Constituents and biological activities of Schinus polygamus. *J Ethnopharmacol*, **107**, 395-400.

Fernando SF, Guimaraes, Lucas FA, et al (2010). *In vitro* and *in vivo* anticancer properties of a Calcarea carbonica derivative complex (M8) treatment in a murine melanoma model. *BMC Cancer*, **10**, 1-14.

Folkman J, Klagsbrun M (1987). Angiogenic factors. *Sci*, **235**, 442-7.

Glucksmann A (1951). Cell deaths in normal vertebrate ontogeny. *Biol Rev Camb Philos Soc*, **26**, 59-86.

Gopal K, Kishor KR, Nandini R, et al (2010). High fat diet containing cholesterol induce aortic aneurysm through recruitment and proliferation of circulating agranulocytes in apoE knockout mice model. *J Thromb Thrombolysis*, **30**, 154-63.

Gopal K, Nagarajan P, Raj TA, et al (2011). Effect of dietary b carotene on cerebral aneurysm and subarachnoid hemorrhage in the brain apo E2/2 mice. *J Thromb Thrombolysis*, **32**, 343-55.

Guruvayoorappan C, Girija K (2007). Immunomodulatory and antitumor activity of biophytum sensitivum extract. *Asian Pac J Cancer Prev*, **8**, 27-32.

Guy P, Kamatou, Vermaak, et al (2012). Eugenol-from the remote maluku islands to the international market place: a review of a remarkable and versatile molecule. *Molecules*, **17**, 6953-81.

Habila JD, Bello IA, Dzikwi AA, et al (2010). Total phenolics and antioxidant activity of *Tridax procumbens L. Afr J Pharm Pharmacol*, **4**, 123-6.

Ikewuchi CJ, Ikewuchi CC, Onwuka CF, et al (2010). Effect of aqueous extract of *Tridax Procumbens L*inn on plasma electrolytes of salt-loaded rats. *Pak J Nutr*, **9**, 103-5.

Kitanaka C, Kuchino Y (1996). Caspase-independent programmed cell death with necrotic morphology. *Cell Death Diff*, **6**, 508-15.

Klein CA (2007). Cancer-the metastasis cascade. *Sci*, **321**, 1785-7.

Konig A, Schwartz GK, Mohammad RM, et al (1997). The novel cyclin-dependent kinase inhibitor flavopiridol downregulates Bcl-2 and induces growth arrest and apoptosis in chronic B-cell leukemia lines. *Blood*, **90**, 4307-12.

Korangath C, Preethi (2010). Inhibition of Metastasis of B16F-10 Melanoma Cells in C57BL/6 Mice by an Extract of Calendula Officinalis L Flowers. *Asian Pac J Cancer Prev*, **11**, 1773-9.

Mahato RB, Chaudhary RP (2005). Ethnomedicinal study and antibacterial activities of selected plants of Palpa district. *Nepal Sci World*, **3**, 26-31.

Mahesh Kumar MJ, Ponvijay KS, Nandhini, et al (2007). A mouse model for Luminal epithelial like ER positive subtype of human breast cancer. *BMC Cancer*, **7**, 1-12.

Manjamalai A, Sardar SS, Guruvayoorappan C, et al (2010). Analysis of phytochemical constituents and anti-microbial activity of some medicinal plants in Tamil Nadu, India. *Global J Biotech and Biochem*, **5**, 120-8.

Mercier B, Prost J, Prost M, et al (2008). Antioxidant activity of bol d'air jacquier breathing sessions in wistar rats-first studies. *Int J Occup Environ Hlth Res*, **21**, 31-46.

Mohammad Shoeb (2006). Anticancer agents from medicinal plants. *Bangladesh J Pharmacol*, **1**, 35-41.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunolo Methods*, **65**, 55-63.

Mraz M, Malinova K, Kotaskova J, et al (2009). MiR-34a, miR-29c and miR-17-5p are down regulated in CLL patients with TP53 abnormalities. *Leukemia*, **23**, 1159-63.

Nia R, Paper DH, Essien EE, et al (2003). Investigation into *in vitro* radical scavenging and *in vivo* anti-inflammatory potential of *Tridax procumbens L. Niger J Physiol Sci*, **18**, 39-43.

Oren M (1994). Relationship of P^{53} to the control of apoptotic cell death. *Seminars in Cancer Biology*, **5**, 221-7.

Pan Y, Song QL, Lin YH, et al (2005). GLB prevents tumor metastasis of Lewis lung carcinoma by inhibiting tumor adhesion actions. *Acta Pharm Sin*, **26**, 881-6.

Pezzuto JM (1997). Plant-derived anticancer agents. *Biochem Pharmacol*, **53**, 121-33.

Philip JM, Robert S, Charles C, et al (2001). Gas chromatographic technologies for the analysis of essential oils. *J Chromatogr*, **936**, 1-22.

Porter AG, Janicke RU (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death & Differ*, **6**, 99-104.

Raff MC (1992). Social controls on cell survival and cell death. *Nature*, **356**, 397- 400.

Raina R, Prawez S (2008). Medicinal plants and their role in wound healing. *Vetscan*, **3**, 221-4.

Ramirez-Tortosa C, Andersen OM (2001). Anthocyanin rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biol Med*, **31**, 1033-7.

Richard AD, Georgina SB, Yamina Hamma-Kourbali, et al (2007). Identification of candidate angiogenic inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based proteomic screens: disruption of vascular endothelial growth factor (VEGF)/heparin affin regulatory peptide (pleiotrophin) and VEGF/connective tissue growth factor angiogenic inhibitory complexes by MMP-2 proteolysis. *Mol and Cell Biol*, **27**, 8454-65.

Satish A, Bhalerao, Tushar SK, et al (2012). Phytochemical and pharmacological potential of *Tridax Procumbens L* inn. *Int J Adv Biol Res*, **2**, 392-5.

Setzer WN, Setzer MC, Moriarity DM, et al (1999). Biological activity of the essential oil of myrcianthes sp. nov. "Black fruit" from Monteverde, Costa Rica. *Planta Med*, **65**, 468-79.

Shan B, Medina JC, Santha E, et al (1999). Selective, covalent modification of beta-tubulin residue Cys-239 by T138067, an antitumor agent with *in vivo* efficacy against multidrug-resistant tumors. *Proceedings of the Natl Acad Sci*, **96**, 5686-91.

Sheeja K, Kuttan G (2010). Andrographis paniculata down regulates Proinflammatory Cytokine Production and Augments Cell Mediated Immune Response in Metastatic Tumor-Bearing Mice. *Asian Pac J Cancer Prev*, **11**, 723-9.

Takada Y, Murakami A, Aggarwal BB, et al (2005). Zerumbone abolishes NF- kappa B and IkappaBalpha kinase activation leading to suppression of anti-apoptotic and metastatic gene expression, up regulation of apoptosis and down regulation of invasion. *Oncogene*, **24**, 6957-69.

Thornberry NA, Lazebnik Y (1998). Caspases: enemies within. *Sci*, **281**, 1312-6.

Vilwanthan R, Shivshangari KS, Devaki T, et al (2005). Hepatoprotective activity of *Tridax procumbens L* against d-galactosamine/lipopolysaccharide-induced hepatitis in rats. *J Ethnopharmacol*, **101**, 55-60.

Vishnu priya P, Radhika K, Siva kumar R, et al (2011). Evaluation of Anti-cancer activity of Tridax procumbens flower extracts on PC3 Cell Lines. *Int J Advan Pharma Sci*, **2**, 28-30.

Wenfeng Huang, Kun Zou (2011). Cytotoxicity of a plant steroidal saponin on human lung cancer cells. *Asian Pac J Cancer Prev*, **12**, 513-7.

Wong Yau Hsiung, Habsah Abdul Kadir (2011). Leea indica Ethyl Acetate Fraction Induces Growth-Inhibitory Effect in Various Cancer Cell Lines and Apoptosis in Ca Ski Human Cervical Epidermoid Carcinoma Cells. *J Evid Based Complementary Altern Med*, **11**, 1-13.

Wyllie AH, Kerr JFR, Currie AR, et al (1980). Cell death: the significance of apoptosis. *Int Rev Cytol*, **68**, 251-306.

Xiyun Y, Yun L, Dongling Y, et al (2003). A novel anti-CD146 monoclonal antibody, AA98, inhibits angiogenesis and tumor growth. *Blood*, **102**, 184-91.

Yang Zhou, Wenyuan Gao, Kefeng Li (2008). Chinese herbal medicine in the treatment of lung cancer. *Asian J Trad Med*, **3**, 1-11.

Zhi Yao, Xu-Chun Che, Rong Lu, et al (2007). Inhibition by Tyroserleutide (Ysl) on the invasion and adhesion of the mouse melanoma cell. *Mol Med*, **13**, 14-21.

Zhou JY, Tang FD, Mao GG, et al (2004). Effect of alpha-pinene on nuclear translocation of NF-kappa B in THP-1 cells. *Acta Pharmacol Sin*, **25**, 480-1.

Zhou JY, Tang FD, Mao GG, et al (2005). Effect of α-pinene on nuclear translocation of NF-kappa B in THP-1 cells. *Acta Pharmacol Sin*, **25**, 480-4.