Semecarpus anacardium Linn. leaf extract exhibits activities against breast cancer and prolongs the survival of tumor bearing mice

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ABSTRACT

Background: *Semecarpus anacardium* Linn. is a commonly used Ayurvedic medicinal plant. Their different parts including nuts, have various medicinal properties to treat clinical ailments such as vitiligo, inflamation, microbial infection, geriatric problem, baldness and neuro-related problems.

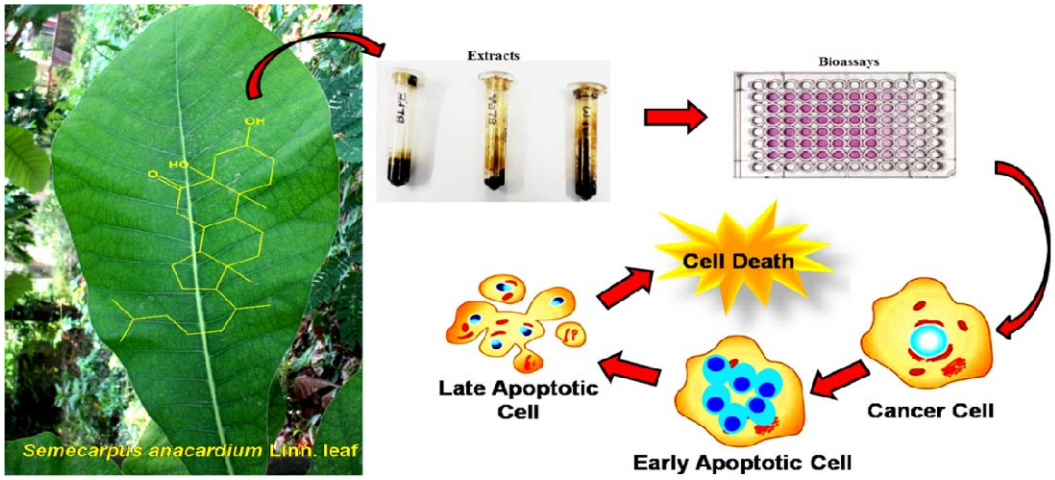
Aim of the study: The main objective of this study was an evaluation of the anticancer activity of the leaves of *Semecarpus anacardium* Linn and phytochemical screening.

Materials and methods: The phytochemical screening was carried out through GC-MS analysis. Cytotoxicity was examined using MTT assay whereas mode of cell death evaluated by fluorescence microscopy. Finally, antitumor activity was determined in EAC cell-induced tumor-bearing mice.

Results: The ethyl acetate extract induced cytotoxicity in cancer cells in a dose dependent manner (IC_{50}: 0.57 µg/ml in MCF-7 cells) in different cancer cell lines. However, normal cells were relatively insensitive to the extract. Fluorescence microscopy results, confirmed that the extract induced apoptosis in cancer cells effectively. The extract induce cell cycle arrest at the G1 phase and suppressed cancer cell migration. An oral administration of the extract suppressed the tumor growth in the mice model bearing Ehrlich ascites carcinoma cells. Whereas, the ethyl acetate extract was also found to prolong the survival of tumor-bearing mice.

Conclusion: Overall, these observations suggest the anticancer activities of the ethyl acetate extract of *S. anacardium* leaves. The study opens a new window to examine the phytochemical constituents from the leaves as a source for the anticancer compounds.

Keywords: *Semecarpus anacardium*; *Ayurveda*; Breast cancer; Apoptosis; Cell migration; Phytochemical.



1. INTRODUCTION

Breast cancer is the most common cancer in women and a major health issue globally. There was 2.09 million new cases diagnosed in 2018 and 0.63 million mortality with a projection of 3.06 million new cases and 0.99 million death in 2040 (Lam F, Ervik M, Ferlay J, Soerjomataram I, Mery L, 2018). Chemotherapy always remains the pharmacological approach for cancer treatment. This method has a several drawback due to involvement of large amount of drug. (Arya et al., 2015). These drugs are often cytotoxic and induce cancer cell death in solid tumors through various route like cell cycle arrest, DNA synthesis inhibition, necrotic cell death, or by inducing apoptosis. Whereas, these chemotherapeutic drugs draw have the capability to damage the vital organs and normal proliferating cells such as heart, liver, kidneys, hair follicles, stem cells, etc (Vijayalakshmi et al., 2000, 1997). So, in this concern new efficient and non toxic chemotherapeutic agents always concern to overcome this problem.

Natural products have been used to treat many clinical ailments since antiquity (Kim et al., 2018).Their presence and effect also concern in different traditional system of medicine across the world. Ayurveda, one of the traditional system of medicine, specially popular in Indian subcontinent.(Joshi et al., 2010). The a large number of phytoconstituents are isolated from Ayurvedic medicinal plants which are promising lead molecules such as alkaloids from *Rauwolfia serpentina*, *Holarrhena antidysenterica*, *Piper longum*, *Withania somnifera*, *Curcuma longa* etc. for the treatment of hypertension, Amoebiasis, cough, stress and cancer, respectively (Patwardhan and Vaidya, 2010). Several compounds isolated from *Azadirachta indica* and Bharangin from *Pigmacopremna herbacea* shows potent anticancer property with regulating numerous signal pathways (Awasthee et al., 2018; Gupta et al., 2017). Many isolated compounds are in clinical trial for cancer prevention such as curcumin, flavopiridol, genistein, capsaicin, berberine, boswellic acid, caffeic acid, resveratrol, etc (Pandey et al., 2017).

Semecarpus anacardium Linn. (Family: *Anacardiaceae*) is used for treatment of inflammation, vitiligo, neural problem, microbial infection, geriatric problem, diabetes etc in Ayurveda. (P Mathivadhani et al., 2007a). Plant fruit part has been used in numerous Ayurvedic formulations since a time ago (Semalty et al., 2010). Recent studies have explored its various properties such as antioxidant (Ramprasath et al., 2005), antimicrobial (Purushothaman et al., 2017), hypoglycemic (Arul et al., 2004), anti-inflammatory (Khan et al., 2013), anticancerous (Joice P. Joseph et al., 2013), antiatherogenic (Semalty et al., 2010), etc. This study also advocate to the different properties of its nuts like antioxidant and cytotoxicity in cancer cells of breast, liver, lung, cervical and blood (Selvam et al., 2004; Semalty et al., 2010; Sharma et al., 1995). It also has antitumor activity in tumor bearing animal model. The nut contains bhilawanols, cardol, anacardic acid, semecarpol and anacardol (Adhami et al., 2012; Nair et al., 2009). Its two contents, bhilawanol and anacardic acids are immunogenic and their exposure causes irritation, blisters, contact dermatitis and toxicity (Llanchezhian et al., 2012).

The obtained results show that the ethyl acetate extract of *S. anacardium* Linn. leaves induces apoptosis and arrests the cell cycle progression in G1 phase. Whereas, their oral administration of the extract shows inhibition of tumor growth, delays the tumor associated death and enhances

the survival in tumor bearing mice significantly. The results show that the potency of extract for being used as raw material for future anticancer drug discovery to treat breast cancer. Therefore, this study explores the anticancer property of leaves for the first time with less toxicity and more efficacy as compare to the nuts.

2. METHODS

Collection of plant materials and phytochemical analysis

The leaves of *Semecarpus anacardium* Linn. were collected in the spring season of 2017 from Banaras Hindu University, Varanasi, India and identified by the Department of Dravyaguna (Ayurvedic Pharmacognosy & Pharmacology), Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (Accession Number: **DG/18/171**).

Firstly, the plant materials (1 kg of leaves) were dried at room temperature for two weeks and ground into a coarse powder through electrical domestic grinder. The powder was macerated up to 24 h with continuous stirring in 5 litres of petroleum ether, ethyl acetate and methanol three times respectively. The supernatant was recovered by filtration through Whatman filter paper of 11µm pore size. Further, each filtrate was finally dried by rotary vacuum evaporator in reduced pressure (Singh et al., 2019a). The compounds of ethyl acetate extract of *S. anacardium* leaves (SLE) were assessed by Gas chromatography–mass spectrometry (GC-MS), the JEOL GCMATE II GC-MS at SAIF, IIT Madras using standard procedure (Usharani and Vasudevan, 2017). The data were matched with the standards (Fitriani et al., 2015; Ibms et al., 2015).

Cell Culture

Human breast adenocarcinoma (MCF-7) cells, human triple negative breast carcinoma (MDA-MB-231) cells, human colon adenocarcinoma (HCT-15) cells, mouse insulinoma (MIN-6) cells, mice undifferentiated breast carcinoma (Ehrlich-Lettre ascites carcinoma, EAC) cells and mice fibroblast (NCTC clone 929, L929) cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in DMEM with high-glucose, sodium bicarbonate, L-glutamine and sodium pyruvate which supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotic (10000 unit/mL of penicillin G, 10 mg/mL of streptomycin sulfate and 25 µg/mL amphotericin B in 0.9% normal saline) solution at 37 °C in a humidified environment with 5% CO_2 gas. About 70-80% confluent cells at log phase were harvested by trypsinization (1X Trypsin-EDTA Solution, Himedia) (Singh et al., 2019a). MCF-7 cells were used throughout the study while EAC and L929 cells were used in cytotoxicity assay.

Cytotoxicity assay

MTT assay was performed for evaluation of the cytotoxicity of the leaves extracts in which tetrazolium rings of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide is cleaved by mitochondrial enzyme succinate dehydrogenase of live cells. The yellowish MTT solution is turned into water-insoluble violet colored formazan crystals depending on the number of viable cells. This assay is also sensitive, reliable, quantitative and colorimetric method to measure cell

viability. The 100 µl of cell solution in fresh media at a cell density of 1 X 10^4 cells/ml was seeded in each well of 96-well plate and incubated overnight. Then, the old culture media was replaced with 200 µl fresh media and treated with the extracts by performing serial dilution which was further incubated for 48 hours. On completion of treatment duration, the treated culture media was replaced with 100 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution at a concentration of 0.5 mg/ml incomplete culture media, i.e., the culture media without fetal bovine serum was kept for 4 hours at 37 °C. The treated cells were centrifuged at 750 rpm for 5 minutes (Remi R8C) and the supernatant was replaced with 100 µl DMSO to solubilize water-insoluble purple colored-formazan crystal and absorbance was measured at 570 nm using microtitre plate reader (Biorad, India) (Singh et al., 2019a). The percentage of cell viability was calculated as Percentage of cell viability = (OD of the treated cells /OD of the untreated cells as control) × 100, then IC_{50} was calculated.

Analysis of Cell morphology

The MCF-7 cells were seeded into 96 wells plate and then old media were replaced with fresh media after 24 h, the extract was administred and further washed with 10 mM PBS (pH 7.4) aftr incubation of 24 h and observed under the inverted light microscope (Dewinter, India) at 40X objective lense (Singh et al., 2018, 2019a). Further, Acridine orange (AO) and Ethidium bromide (EtBr) double staining was used for detection of the mode of cell death. Acridine orange is a cell permeable dye which binds with the nucleic acid to emit green fluorescence while ethidium bromide can enter the cell membrane and binds to the nucleic acid to emit red fluorescence. MCF-7 cells were seeded into 96 wells plate (1X10^4 cells per well) for 24 h at 37 °C with fresh culture medium and the extract to test the drug, Paclitaxel was used as control positive group while 10 mM PBS was taken as control negative. Then the culture media were removed and cells were washed with 10 mM PBS (pH 7.4) and incubated for further 30 minutes in 100 µg/ml AO and 100 µg/ml EtBr in dark. It was washed with 10 mM PBS (pH 7.4) and finally cells were examined underthe fluorescence inverted microscope (Dewinter, India) using 40× objective lense (Singh et al., 2018, 2019a).

Analysis of nuclear morphology

Nuclear Morphology was studied by DAPI (4′-6-diamidino-2-phenylindole) stain (Genetix, India) which forms fluorescent complexes with natural double-stranded DNA (Singh et al., 2019b). Human breast cancer (MCF-7) cells were seeded at a density of 1 X 104 cells per well and incubated for 24 hours. Then, the treatments were given as per previous experiment followed by washing of cells with PBS and stained with DAPI solution. After 30 minute incubation, the cells were again washed with 10 mM PBS (pH 7.4) and cells were studied by fluorescence inverted microscope using the blue filter (Singh et al., 2019a).

Cell cycle analysis

The cell cycle analysis was perfomed with slight modifiedification of previously described method (Awasthee et al., 2018). In brief, the cells were washed with PBS at the end of the

treatment time, then stained with 70% chilled methanol, treated with RNaseA and stained with PI. The population of cells in different phases of the cell cycle was determined by FACScan and the analysis was performed by Cell Quest software (Becton Dickinson).

Scratch wound healing assay

MCF-7 cells were grown upto 80% confluency and trypsinized for seeding into 6-well plate (1×10^5 cells/ml) at 37 ˚C for 24 h in DMEM medium. Further, media were replaced with fetal bovine serum free media further 24 h and scratch wound was created in the middle of the well with a sterile 20 µl pipette tip and washed with 10 mM PBS thrice. 1 ml of complete medium was given to the wound containing cells and extract was administrated in test group while medium in control group. The cells were examined at an interval of 24 h, 48 h and 72 h. Images were analyzed for cellular migration by inverted microscope (Dewinter, India) using 10X objective lense (Singh et al., 2019a).

Animal model and Ethical statement

For *in vivo* studies, Swiss albino mice of either sex weighing 18–22 grams of about 8–10 weeks old were procured from the central animal facility, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India with approval of the institutional ethical committee (**Dean/2016/CAEC/337**). The animals were kept in polypropylene cages and provided standard pellet diet (Agro Corporation Pvt. Ltd., Bengaluru, India) and water *ad libitum.* The mice were accommodated under controlled temperature (27 ± 2 ˚C) and humidity with a 12 hours light and dark cycle by following CPCSEA guidelines for experimental study.

Antitumor evaluation

To evaluate antitumor activity, a total of 18 tumor-bearing mice and 6 normal mice were divided into four groups. Mice of group I, group II and group III were administered 20 mg/kg body weight Paclitaxel, 100 mg/kg bodyweight BLEA and equal volume of water (as BLEA suspended in it) respectively, while group IV was normal mice without tumor. All mice of group I and Group II were given respective drugs regularly throughout the experimental period of 28 days. The mice of group IV were given no treatment to served as control negative group. Tumor size was measured using vernier calipers on alternative days for tumor animals and tumor volume was calculated using the formula $V = 0.5 \times a \times b2$, where ‘a' and ‘b' indicates the major and minor diameter, respectively. At the end of 28th day of experimental period, animals from each group were sacrificed to collect kidney, liver, thigh and spleen were collected from Paclitaxel, BLEA treated, tumor and normal animals and were evaluated for morphological changes using hematoxylin-eosin stained histological slides (Srivastava et al., 2014a).

Animal survival study

The effect of BLEA on life span of the tumor-bearing mice was assessed. For this study, total 12 mice have received EAC cells in their left thigh. They were further divided into two groups (6 mice in each). Group I was served as tumor control and group II received oral administration of BLEA 100 mg/kg body weight daily till the survival of last animal, starting after 7 days of tumor (EAC) cell injection.

The life span of BLEA treated mice was calculated and compared with untreated tumor-bearing animals. The life span of experimental animals was monitored and calculated by using the following formula $[(T - C)/C] \times 100$, where 'T' indicates the number of days the treated animals survived and 'C' indicates that number of days that tumor animals survived (Arya et al., 2015).

Histology

The tissues were collected from control, BLEA treated animals, and further processed for histological evaluations (Thomas et al., 2016). The excised tissues were fixed in 4% neutral saline formalin, dehydrated in alcohol and embedded in paraffin wax. The section (5 μm) of tissues was cut using Microtome (Leica Biosystems, Germany) and then stained with haematoxylin and eosin. Images of all tissue sections were captured using light microscope (Dwinter).

In silico studies

ADME Screening: The parameters of pharmacokinetics such as absorption, distribution, metabolism and elimination (ADME) play an important role in drug discovery and development (Daina et al., 2017). The ADME properties of compounds were analyzed using SwissADME (http://www.swissadme.ch) and the data were recorded to analyze the possibility of identified compounds to be the drug candidates (Baell et al., 2013).

Molecular docking studies: All identified phytochemicals were used as ligands and their structures were drawn using Chembiodraw Ultra 14.0 (Cambridge Soft) and saved as pdb file. Crystal structures of receptor proteins CDK2 (protein ID: 1W0X) was downloaded from Protein Data Bank (https://www.rcsb.org/structure/2B53), then water molecules and ligand were removed. Patchdock (Tel Aviv University, Israel) was used for molecular docking studies using CDK2 receptor protein and ligands considering settings as clustering RMSD 4.0 and complex type default (Ronchi et al., 2017). The resultant complex of receptor-ligand with solution number, docking score, area and six-dimensional transformation space was recorded. The docking solutions obtained in Patchdock were further refined with FireDock (Tel Aviv University, Israel) and top 10 solutions among 1000 rescored solution were downloaded with global energy, atomic contact energy, attractive Van der Waals and considered for further analysis. Each complex of ligand-receptor obtained through FireDock was ranked based on their minimum global energy and Chimera 1.12 used for surface visualization of the complex. Discovery Studio 2017 R2 Client was used for analysis of interaction of the receptor-ligand complex.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0. The analysis of variance was performed on three independent cytotoxicity results by one way ANOVA analysis using tukey as post test. The results were considered significant at p value <0.05. The data were expressed as mean±SD.

3. RESULTS

the plant leaf extract was selected for this study , as it showeddue to higher cytotoxicity in cancer (MCF-7) cells and less toxicity for normal (L929) cells (selectivity 7.84) among all four extracts i.e. root, bark, leaf and fruit (supplement Fig 1).

Phytochemical constituents of active extract

The ethyl acetate extract of *S. anacardium* leaves was analyzed by GC-MS and seventeen major compounds (table 1, supplement figure 2) were identified. Among these compounds, (E)-octadec-9-enoic acid had highest peak area (11.30%), followed by palmitic acid (9.89%), (E)-3,7,11,15-tetramethylhexadec-2-en-1-ol (8.77%), methyl3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoate (8.06%), and other phytochemicals were listed in the table 1. Compound 1 and 11 were alkaloids, compound 13, 14, 15 were steroids, compound 2 was terpenoids, compound 15, 16 were phenolic compound, compound 17 was flavonoid and remaining were aliphatic compounds.

The extracts induce cytotoxicity in cancer cells.

There is six cell lines MCF7, MDA-MB-231, HCT-15, MIN6, EAC and L929 have been used to evaluate the anticancer activity of leaf extracts in organic solvent, ethyl acetate and which was coded as BLEA . The above cell lines were treated with different concentration of the extracts, ranging from lowest (0) to highest (200 μg/ml), to identify the significant cytotoxicity in dose-depended manner as evaluated by MTT assay (Fig. 1).

BLEA induced cytotoxicity among all cancer cell lines significantly (supplementary table 1). MCF-7 and EAC cells exhibited the highest sensitivity with an IC_{50} of 0.57 and 1.71 μg/ml respectively. HCT-15, MIN-6 and HepG2 cells showed least sensitivity with an IC_{50} of 6.66, 15.77 and 44.93 μg/ml respectively towards the extracts (fig. 1).Whereas, BLEA showed very low cytotoxicity in normal L929 cells (IC_{50} value 29.62 μg/ml), indicate that the extracts were safe for normal cells. Based on the selectivity indices of the extracts in different cell lines, extract BLEA was selected against MCF-7 cells for further studies (supplement table 1). Significantly, BLEA show very less cytotoxicity in normal cells compare to paclitaxal (supplement fig. 3), whereas BLEA was very effective in cancer cells.

BLEA alters cell morphology

After the treatment of cells with BLEA, it is necessary to know, how the cells behave with extract. For this purpose, BLEA treated MVF-7 cells were examined through light microscope and obtained results showed the cytoplasmic vacuolization in the treated cells. There was no cytoplasmic vacuolization in untreated cells. This result shows the drug have ability to induce stress in cancer cells. The details are shown in figures (fig. 2A-C).

BLEA induces apoptosis

BLEA induced cytotoxicity through altering cellular morphology and intracellular vacuolizations. After that it is neccessary to know the mode of cell death. Firstly, the cells were stained with DAPI staining and further examined under fluorescence microscopy. The obtained results revealed a significant alteration in the morphology of nucleus in treated group of cells, such as chromatin condensation, nuclear fragmentation and shrunken nuclear morphology(fig. D-F). Whereas, the untreated cells showed intact nuclear morphology. This result also supports apoptosis as a mode of cell death. Along with the above obtained results, the cells were treated with BLEA at same concentration and apoptosis assay was performed using Ethidium bromide-Acridine orange double staining medoth. MCF-7 cells were treated with BLEA at 0.57 μg/ml concentrations (equivalence IC_{50}) for 48□h, then stained with Ethidium bromide and Acridine orange which showed that the death induced in MCF-7 cells by BLEA was apoptosis, as the nuclear fragmentation, nuclear contraction, cytoplasmic membrane blebbing, entry of ethidium bromide into the cells, leading orange to red color fluorescence in cells were observed (fig. 2G-I).

BLEA induces cell cycle arrest

BLEA treated cells were died through apoptosis, so, it is necessary to know the effect of BLEA on cell cycle alteration. BLEA found to modestly increase the population of cells at the G1 phase. Furthermore, the sub G1 population of cells was also modestly increased at the higher concentration of BLEA (500 ng/ml). Conversely, the percentage of cells in the S and G2/M phase decreased with an increase of BLEA concentration (fig 2J-L). Overall, these observations suggest that BLEA induces cell cycle arrest at the sub-G1 and G1 phases.

BLEA inhibits cellular migration

Cellular migration is one of the important characteristics of cancer cells and enables them to metastasis. The scratched wound was created and treated with BLEA extract, subsequently, the cells were examined on 1st, 3rd and 5th day for the healing of wound and observed that BLEA decreases the cellular migration significantly, resultant from the delay in wound healing process. (fig. 3). The migration of cancer cells was ceased by the extract.

BLEA safe for oral administration in mice

The toxicity of BLEA for oral administration was evaluated by acute oral toxicity with the standard protocol (test number 425 of OECD) in mice. The BLEA did not show any sign of toxicity even at the high dose in mice. The tissues of vital organs were further analyzed for any

cellular architectural alteration due to toxicity which did also not show any sign of toxicity. Interestingly, there was no body weight loss or any toxicological symptoms recorded in the animal treated with BLEA, which indicates that BLEA is non-toxic to the animals for oral administration (supplementary table 2 and supplement fig. 4).

BLEA inhibits of tumor progression in mice

The anticancer potency of BLEA was evaluated using EAC (Ehrlich ascites carcinoma) cells induced mice tumor model. Based on a pilot study (Supplementary fig. 5), 100 mg/kg b.wt. dose of BLEA was selected for evaluation of anti-tumor activity. The treatment of tumor-bearing mice was started from 8th day of EAC cells injection, after that the tumors became measurable, with a daily dose of 100 mg/kg b.wt of BLEA with feeding needle till 28th day. The volume of tumors was measured on alternative days. The oral administration of BLEA resulted in a significantly ($p \leq 0.05$) suppress the tumor progression, whereas the tumor volume was continuously increasing in the control group of tumor-bearing mice (fig. 4A).

The mean volume of the tumors were observed on the day 8th, 18th and 28th and found 58.67, 388.98 and 780.44 mm^3 respectively in the untreated tumor-bearing control group of mice, whereas BLEA treatment reduced the tumor volume and found 58.67, 224.00 and 298.67 mm^3 respectively as compare to the control (fig. 4B-D). The histological examination of tissues from thigh, liver and spleen from tumor-bearing control group of mice showed alterations in cellular morphology compared to the treated and normal mice (fig. 4F-H). There were dead cells seen in tumor tissue of treated mice. Therefore, the study suggests that BLEA has potency to reduce tumor progression significantly in mice models.

BLEA delays tumor associated death and enhances the survival of tumor bearing mice

Importantly, this study showed that the tumor associated death in mice was delayed. The first death in BLEA treated mice was recorded on the day 49th whereas the first death in control tumor bearing mice was on the day 22nd. It was observed that about 50% survival in tumor bearing mice treated with BLEA was increased as compare to the control group of tumor bearing mice. The control untreated tumor bearing mice survived only up to 73 days after EAC injection, whereas the BLEA treated tumor bearing mice were survived more than 110 days. These results showed the anticancer potential of BLEA which caused tumor growth regression as well as increased survival with the delay in tumor associated death in tumor bearing mice model. Further, the histological examination of dissected tumor tissues was done using haematoxylin-eosin double staining to explore the mechanism lying behind the tumor growth in tumor bearing mice models. The study showed a significant decrease in number of EAC cells in the tissue of thigh tumor of the mice received BLEA orally in comparison of untreated controls tumor bearing mice (fig. 4E).

Compounds of BLEA inhibit CDK2

All 17 compounds of BLEA extract were found to have zero violation of Lipinski rule, which was suggested the possibility of the compound to be drug candidate with good bioavailability (data not shown). Compound 14 was bound to CDK2 with lowest Gibbs free energy, forming hydrogen bond with amino acid residues of CDK2 side chain such as Ile10, Thr14, Val18, Leu134, Ala144 and Asp145, while other closely interacting residues were Gly11, Glu12, Gly13, Tyr15, Lys33, His84, Gln85, Asp86, Lys89, Asp127, Lys129, Gln131 and Asn132 with a pocket area of 760.10 $Å^2$. The binding affinity of compound 13 was highest after compound 14, followed by compound 12 and 10 with -45.27, -41.94 and -40.75 kcal/mol respectively. Compound 13 was found to interact with amino acid residues Ile10, Tyr15, Val18, Lys89, Leu134 and Ala144 through hydrogen bond directly, while closely interacting residues were Gly11, Glu12, Gly13, Thr14, Lys33, Gln85, Asp86, Asp127, Lys129, Gln131, Asn132 and Asp145 with a pocket area of 693.30 $Å^2$. Similarly, other compounds were found to have inhibitory action against CDK2 protein with different affinities and pocket areas, as showed in the table 2 and figure 5.

Overall, obtained results confirmed that BLEA induced cytotoxicity by inducing apoptosis, inhibited tumor growth, delayed the tumor associated death leading to significant increase in the survival of tumor bearing mice model. Therefore, this study suggests that BLEA could be served as a prime source for development of chemotherapeutic agent for human cancer prevention.

4. DISCUSSION

The development of chemoresistance among cancer cells is major obstacle in its treatment, leading to high mortality rate in cancer patients. The drug inactivation, drug target alteration and drug efflux are influencing the chemoresistance development for existing target specific single molecule drug (Arya et al., 2015). Therefore, plant extract or fractions which are mixture of multi-targeted compounds, having antitumor activity are potent tools to overcome the drug resistance. Traditionally used medicinal plants serve as the prime source for development of chemotherapeutic drugs with minimal side effect. They also have a capacity to reduce the time and cost to develop potential chemotherapeutic agents.

Semecarpus anacardium Linn. is well known medicinal plant, its fruit is described in Ayurveda for preparation of several polyherbal formulations to treat clinical ailments such as inflammation, neurological problem, geriatric problem, vitiligo, cardiac troubles, enlargement of spleen, hemorrhoids, rheumatism, diabetes, baldness, etc including cancer (P Mathivadhani et al., 2007b). Whereas, *S. anacardium* nut extract has potency to regulate the key enzymes involved in carbohydrate metabolism which is elevated in cancer cells during tumor growth progression to enhance the synthesis of nucleic acid and lipids (Sujatha and Sachdanandam, 2002) and cellular respiration (Arathi and Sachdanandam, 2003). It also maintains normal physiological level of cyto-protective Glutathione enzyme (Mathivadhani et al., 2006), modulates tumor hypoxia associated factors (Panneerselvam Mathivadhani et al., 2007a) and extracellular matrix associated enzymes (P Mathivadhani et al., 2007b), leading to inhibition of tumor progression and metastasis in DMBA induced tumor bearing rats. Although, the milk of nut has reported to

have anticancer property in hepatocellular carcinoma in wistar rats (Joice P Joseph et al., 2013). The nut extract is cytotoxic and induced cell death through apoptosis in breast cancer (T47D) cells by arresting the treated cells in G2/M phase (Panneerselvam Mathivadhani et al., 2007b). The compounds isolated from its nuts, show cytotoxicity with an IC50 value range from 0.10 – 5.9 µg/ml among various cancer cell lines, 3-O-methyl quercetin and kaemeferol, show cyto-protective property against free radical induced cellular damage, the isolated compounds also shows synergistic effect with conventional drugs such as doxorobocin and induce apoptosis via arresting cells in G2/M phase of cell cycle in cancer cells (Kumar et al., 2016; Nair et al., 2009).

In this study, anticancer properties of *S. anacardium* leaves was investigated, which is an alternative source of phytochemicals and could be used for therapeutic purposes. The medicinal application of leaves remain unknown till the date. Here, the leaf extracts in three different organic solvents were prepared and evaluated first time for anticancer activity using various cell lines and animal tumor model. The ethyl acetate extract was found more potent cytotoxic against different cancer cell lines and induces apoptosis in MCF-7 cells. However, it was found fairly safe for normal (L929) cells. Moreover, the treatment of MCF-7 cells with BLEA exhibited cell cycle arrested in G1 phase and apoptotic cell death. The in vivo study using mice as animal model also exhibited that BLEA is safe for oral administration and potent for tumor growth inhibition. The extract delays the tumor related death and enhance the survival of tumor bearing mice.

The IC_{50} values of the leaves extracts shows different range for all three extracts, i.e. the range 1.33-8.65 µg/ml for petroleum ether, 0.57 – 15.77 µg/ml for ethyl acetate and 0.97 – 43.66 µg/ml for methanol in various cancer cells respectively which is slightly more than the compounds isolated from the nuts (Nair et al., 2009). Moreover, the IC_{50} values of all three extracts of nuts are 32.73, 29.62 and 17.45 µg/ml for petroleum ether, ethyl acetate and methanol respectively which are also higher than the previous studies (Nair et al., 2009). The selectivity index is an important tool in bioassay guided fractionation for selection of the best extract/fraction which indicate cytotoxicity to cancer cells and safety for normal cells (Mfotie Njoya et al., 2017). The selectivity index is range from 0.40 to 52.17 for all three extract in different cancer cells. Among these, the selectivity index of ethyl acetate extract of the leaf is higher in human breast carcinoma (MCF-7) cells, resulting the selection of BLEA and MCF-7 cells for further studies.

The discovery and development of anticancer chemotherapeutic agents focuses on mode of cell death induced and their effect on cell cycle (Hanahan and Weinberg, 2011). As apoptotic cell death is highly organized and programmed process. The drugs with apoptosis inducing property and inhibiting the tumor progression in cancer, are considered as novel anticancer chemotherapeutic drugs (Singh et al., 2016). There are several cytoplasmic vacuoles induced after treatment with BLEA in breast cancer (MCF-7) cells which indicating the induction of apoptosis like cell death (Mallick et al., 2015; Vijayarathna et al., 2017). Double staing with AO-EtBr, followed by fluorescence microscopy for detection mode of cell death and differentiation

of untreated cells, early apoptotic cells, late apoptotic cells and necrotic cells are indicating BLEA induced apoptosis in MCF-7 cells. As Acridine orange permeable to the unbroken plasma membrane and has ability to bind DNA, resulting green fluorescence, whereas Ethidium bromide enters inside the cells only broken plasma membrane, binds the nucleic acid ladder and emits red fluorescence. The fluorescing color green, yellowish orange, orange and red represent normal, early apoptotic, apoptotic and late apoptotic/necrotic cell death respectively (Ramya et al., 2018). The altered morphology was seen in the cells treated with BLEA and stained with DAPI stain, which further confirmed the apoptosis mode of cell death induction (Ramya et al., 2018). The cell cycle is series of events, regulated by various molecular signals at different checkpoints, resulting synthesis of DNA and division of cell into daughter cells. BLEA alters the cell cycle and arrests the MCF-7 cells at G1 phase. Interestingly, the nut extract has reported for arresting the cells at G2/M phase (Panneerselvam Mathivadhani et al., 2007b). The above findings suggest that the molecular mode of action of nut extract and leaf extract are different in cancer cells (Krishnan et al., 2017). The scratched wound assay is an important tool for study of cell migration as well as metastasis. Numerous phytochemicals and herbal extract have been reported for inhibition of cellular migration which may play an important role in prevention of metastasis in cancer patients (Gao et al., 2018; Jiang et al., 2017; Liang et al., 2015). The leaf extract, BLEA has also showed inhibition of cellular migration of MCF-7 cells in this study.

The progression of cell cycle through G1 phase depends on CDK2-Cyclin E complex (Schulze-Gahmen et al., 1995). The genotoxic stress induces cell cycle arrest with the help of CDK inhibitors such as P^{21}, P^{27}, P^{57}, etc. The CDK inhibitors bind to CDK, as a result the function of CDK-Cyclin complex is ceased. Similarly, the binding of P^{21} to CDK2 causes cells arrest in G1 phase of cell cycle (Schulze-Gahmen et al., 1995). Therefore, CDK2 inhibitor phytochemicals may be implemented as an efficient G1 phase arrest in treatment of cancer cells. Numerous phytochemicals are reported to have CDK2 inhibitory activity such as Artocarpin, Curcumin, Piperine, Urosolic Acid, etc (Lapenna and Giordano, 2009). All identified compounds were showed binding affinity to the target protein (CDK2), whereas compound 10, 12, 13, 14 were showed strong binding affinity to the active site of CDK2 protein. The compounds of the potent extract BLEA, which have higher affinity to the target protein CDK2, also showed strong hydrogen bonding with the conserved amino acid residues such as Val18 and Leu134 as shown in table X and figure Y. The molecular docking studies shows that the compounds of BLEA are capable to bind to the active site of CDK2, inhibit the binding of CDK2 to cyclin E, resulting arrest of cell cycle progression in G1 phase. The interaction of compounds with CDK2 shows possible cause of cell cycle arrest in G1 phase of cancer cells.

The different extracts of *Semecarpus anacardium* nuts are well known for their toxicity in human and animals (Llanchezhian et al., 2012; Patwardhan et al., 1988; Sundaram et al., 2018). The purification processes are described in Ayurveda to reduce its toxicity of nuts before clinical use (Maurya et al., 2015). However, in this study it has found that the ethyl acetate extract of leaf is safe for oral administration. There was no any morphological, physiological or behavioral sign of

toxicity sign as well as death occurred in mice even received high dose (5000 mg/kg b. wt.) of the extract (BLEA). Interestingly, haematoxylin and eosin stained histological examination of stomach, liver, kidney and lung tissues did not show any alteration in cellular architecture in treated group of animals.

The study has further extended to evaluate *in vivo* anti-tumor activity in EAC cells induced mice tumor model. The EAC cells are mouse derived ascetic carcinoma cells which easily accepted in mice body with high transplantable efficacy, rapid growth and confirmed malignancy. The subcutaneous injection of EAC cells, results the development of solid tumor model in mice, which is selected in this study (Ezzat et al., 2018; Mishra et al., 2018). BLEA has evaluated for anti-tumor potency using EAC induced allograft tumor mice model, which showed significant inhibition of tumor growth progression, delayed the tumor associated death and increase the survival of tumor bearing treated mice. These results are similar to the previously reported results of other herbal extracts such as extracts of *Achras sapota*, *Vernonia condensate*, *Eclipta alba*, etc (Arya et al., 2015; Srivastava et al., 2014b; Thomas et al., 2016). Moreover, the examination of histological slide of tumor tissue stained with hematoxylin and eosin showed the death of tumor cells, induced by BLEA (Srivastava et al., 2014b).

5. Conclusion

The study explored the possibility of leaf extract as a prime source for anticancer phytochemicals with minimal toxicity, as it induces cytotoxicity in various cancer cell lines via cell cycle arrest, leading to apoptosis and safe for normal cells. Moreover, BLEA inhibited the tumor growth progression, delayed the death and enhanced survival significantly in tumor bearing mice model. Therefore, BLEA is potential extract for anticancer chemotherapy development. However, additional advanced studies are necessary for further development and elucidation of molecular pathways involved to develop a new drug.

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Author Contributions

RKS, AKS and SKS designed the study; RKS performed the experiments; SSV and SCG helped in cellcycle analysis; RKS, AR, RT, VS, AKS and SKS contributed to the formatting, writing and critical reviewing of the manuscript. All authors have read and approved the manuscript for publication.

Conflict of interest

All authors have declared for no conflicts of interest.

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SUPPLEMENTARY DATA

Supplementary methods

Evaluation of the oral acute toxicity

The evaluation of oral acute toxicity was carried out as per the Organization for Economical Cooperation and Development (OECD test 425) described procedure. Total 18 mice of either sex were divided into three groups (3 male & 3 female in each group) randomly *viz.* experimental groups 1 & 2 and control group 3 respectively. A day before an experiment, animals were kept on fasting. Next day, the ethyl acetate extract was administered orally using a 16G oral round ball tip feeding curved needles to the mice of the experimental groups. A single dose of 2000 mg/kg and 5000 mg/kg body weight were administrated among the mice of experimental group 1 and 2 respectively, while distilled water was administrated to the mice of control group. All mice were observed individually after first 30 min dosing, special attention was given during the first 4 h and followed up from 24 h to 14th day of the dosing to collect signs and symptoms of toxicity (Amelo et al., 2014; Sreeja et al., 2018). The attention was paid to investigate the potential occurrence of tremors, convulsions, salivation, diarrhea, lethargy and drowsiness. Live weight of the animals was monitored on days 0, 7 and 14 of the dosing as one of the toxicity parameters. At the end of the experiment, the animals were sacrificed by cervical dislocation of anaesthetised animals and the organs *viz.* brain, stomach, liver, spleen, lungs, kidneys, muscles, esophagus and small intestine were collected to evaluate histopathological changes.

Tumor model development

EAC cell induced tumor model was chosen for this study, as it is of mouse origin and can be easily transplanted in immunocompetent mouse. The EAC cells were harvested from 75 cm^2 cell culture flask with 70-80% cell confluence by trypsinization and followed by centrifugation at 750 rpm (Remi R8C) for 5 min. The harvested cells were diluted in 10 mM phosphate buffered saline (1X PBS) at a density of 5×10^6 cells, and 200 µl of viable cell solution were injected into the peritoneal cavity of each recipient mouse of 21±1 g body weight and allowed to multiply. The EAC cells were withdrawn from peritoneal cavity of the donor mice after 8–10 days of inoculation and diluted in 1X PBS and centrifuged at 750 rpm (Remi R8C) for 5 minutes and the cell pellet was resuspended in sterile 10 mM PBS which was injected (100 µl at 1×10^7 cells/ml) in left thigh of female mice for developing solid tumor (Bhattacharjee et al., 2017; Mishra et al., 2018).

Pilot study for effective dose determination

After 4 days of EAC inoculation, a pilot study was set up for determination of effective dose in EAC induced tumor-bearing mice which were divided into four groups of 3 mice in each. The

tumor-bearing mice were administered 200, 100 and 50 mg/kg body weight of ethyl extract of test drug in group 1, 2 and 3 respectively while100 µl distilled water was given to the mice of group 4 named as control once daily for 14 days (Srivastava et al., 2014b) along with normal diet and water *ad libitum*. At the end of 14th days, the mice were sacrificed and tumors were dissected out for their volume calculation. Tumor size was measured by Vernier calipers using the formula $V = 0.5 \times a \times b^2$, where 'a' and 'b' indicates the major and minor diameter, respectively.

Supplement table 1

Selective index of different extracts against cancer cell lines.

Selective Index	**MCF-7**	**MDA-231**	**HCT-15**	**MIN6**	**EAC**	**L929**
BLPE	14.92	5.07	4.69	24.61	3.79	1.00
BLEA	**52.17**	2.36	4.45	1.88	17.37	1.00
BLM	17.96	3.13	1.11	0.40	1.36	1.00

Supplement table 2

Observational study for BLEA oral toxicity in mice (OECD test 425).

Observation	0 h		0.5 h		1 h		2 h		4 h		24 h		14th day	
	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2
Skin & fur	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Tremors	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	Y	Y	N	Y	N	N	N	N	N	N
Coma	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mortality	N	N	N	N	N	N	N	N	N	N	N	N	N	N

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FIGURES

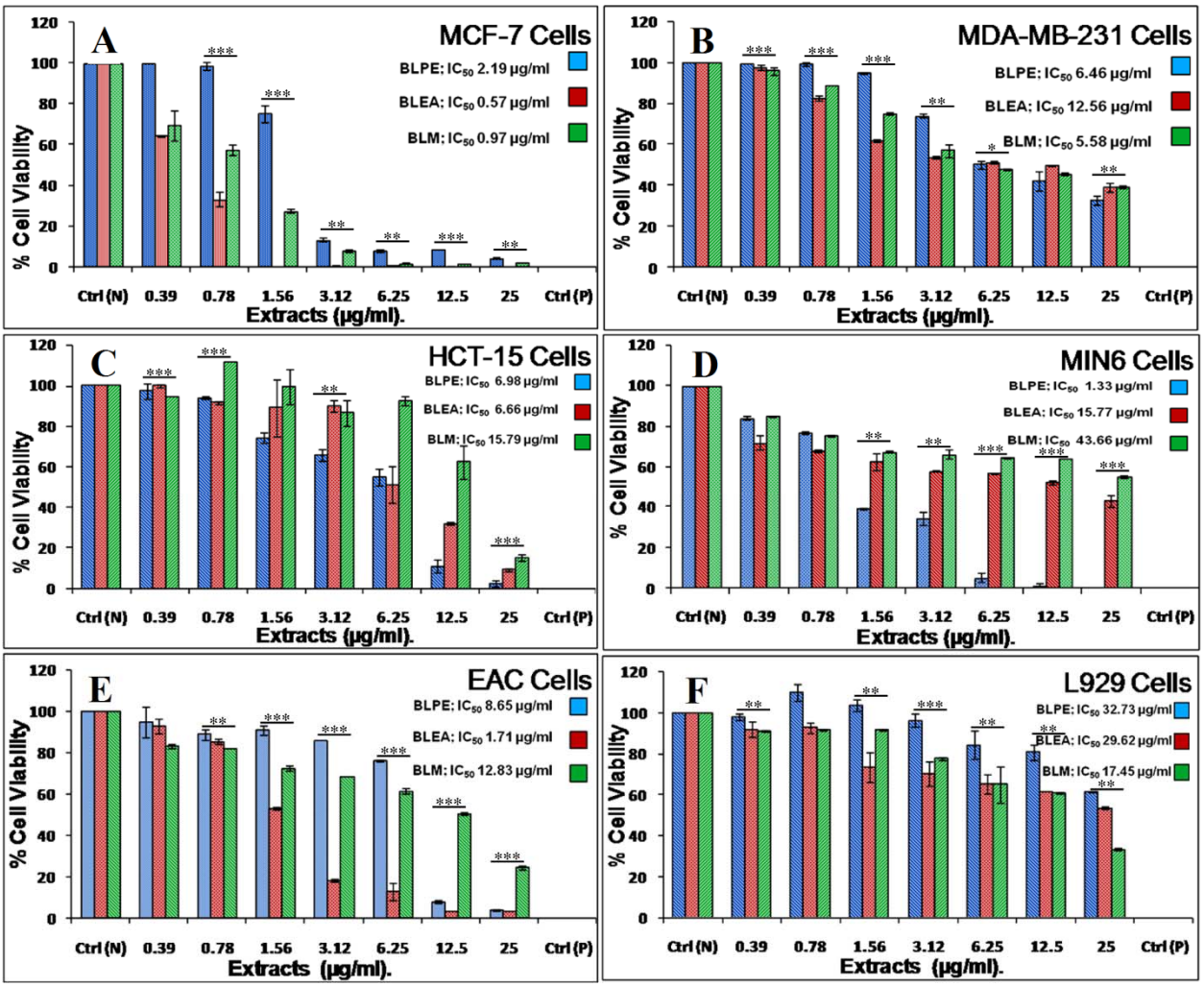


Figure 1: Evaluation of cytotoxicity of different extracts on different cell lines. Different cell lines (A) Human breast cancer cells, MCF-7, (B) Human triple negative breast cancer cells, MDA-MB-231, (C) Human colorectal carcinoma cells, HCT-15, (D) Mouse insulinoma cells, MIN6, (E) Mouse ascetic carcinoma cells, EAC and (F) Mouse fibroblast cells, L929 treated with BLPE, BLEA & BLM respectively and cytotoxicity was evaluated by MTT assay after 48 hours of treatment.

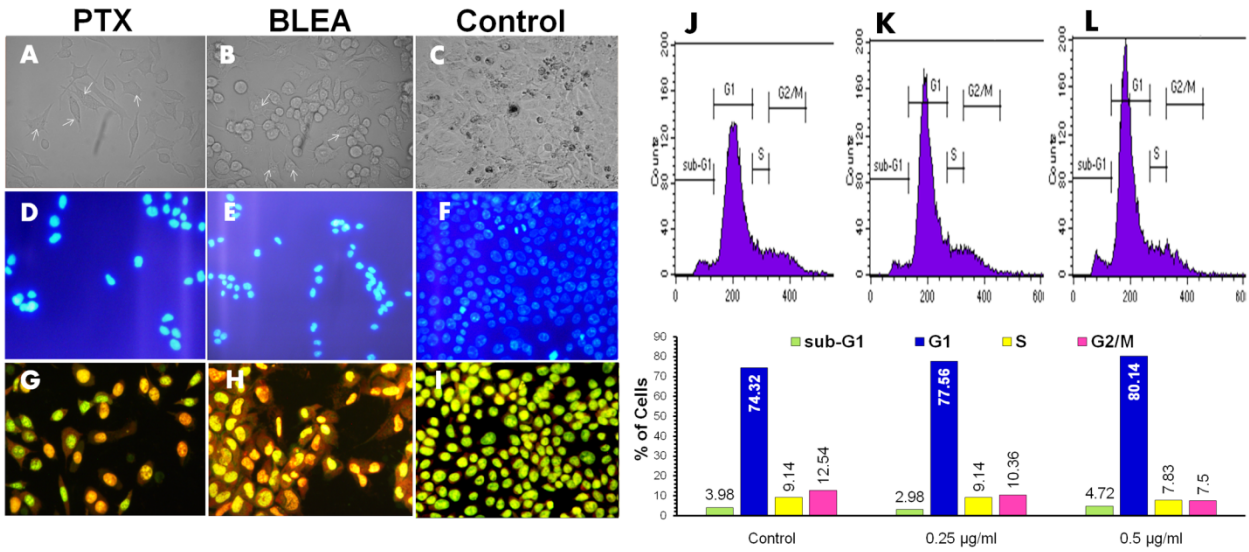


Figure 2: Effect of BLEA on cell morphology, death and cell cycle in MCF-7 cells. ***Light microscopy***: Cellular vacuolization in cells treated with (A) Paclitaxel (2.13 ng/ml), (B) BLEA (0.57 µg/ml) and (C) control. ***Fluorescence microscopy***: evaluation of nuclear morphology using DAPI staining in cells treated with (D) Paclitaxel (2.13 ng/ml), (E) BLEA (0.57 µg/ml) and (F) control, and evaluation of cell death using Ethidium bromide-Acridine orange double staining in cells treated with (G) Paclitaxel (2.13 ng/ml), (H) BLEA (0.57 µg/ml) and (I) control. ***FACS analysis***: The treated cells showed arrest of cell cycle in G1 phase dependent on concentration (J) control cells, (K) 0.10 µg/ml and (L) 0.20 µg/ml.

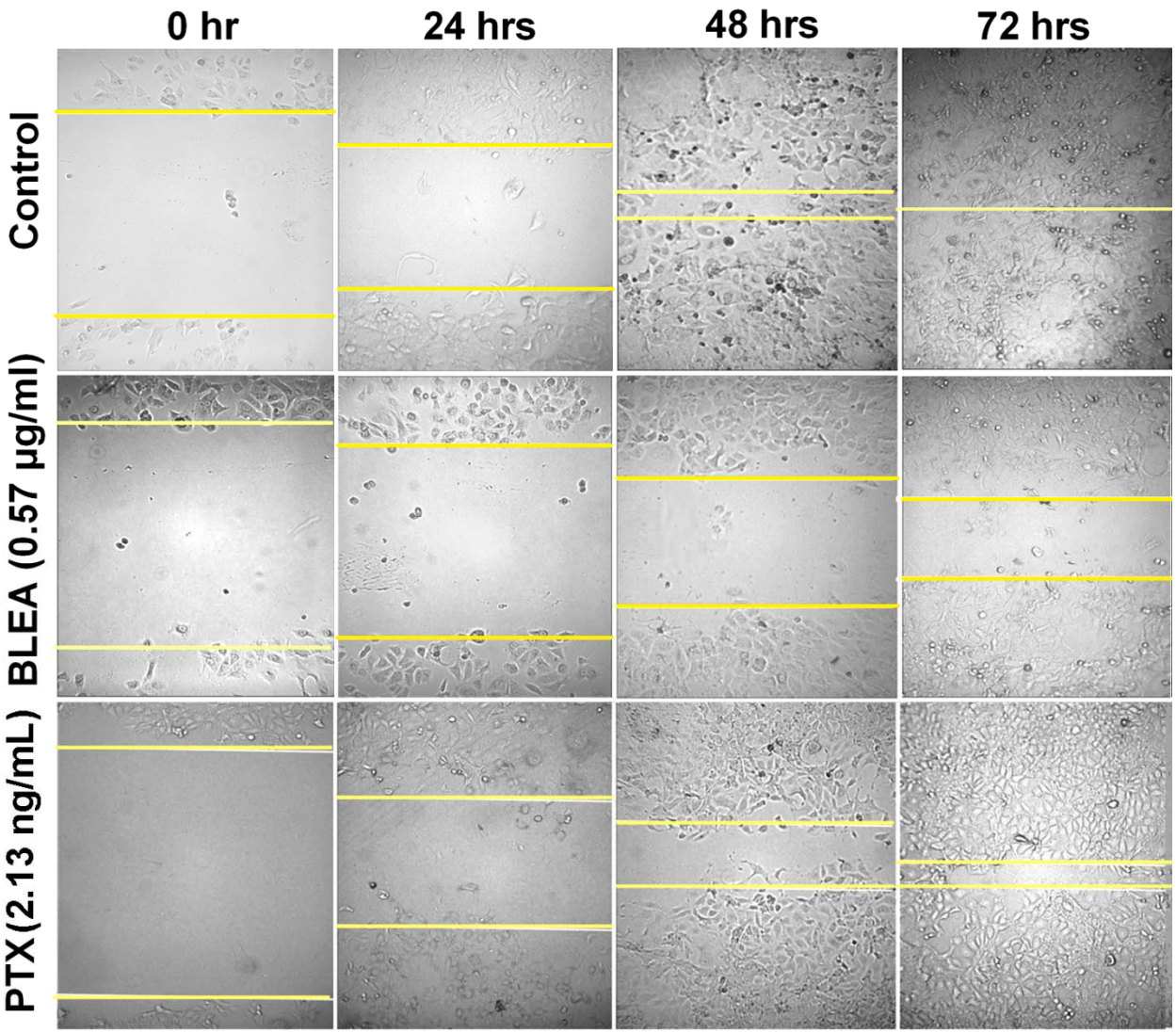


Figure 3: BLEA inhibited the cellular migration in MCF-7 cells. The wound healing assay was used to examine the effect of BLEA on wound closure and cellular migration rate.

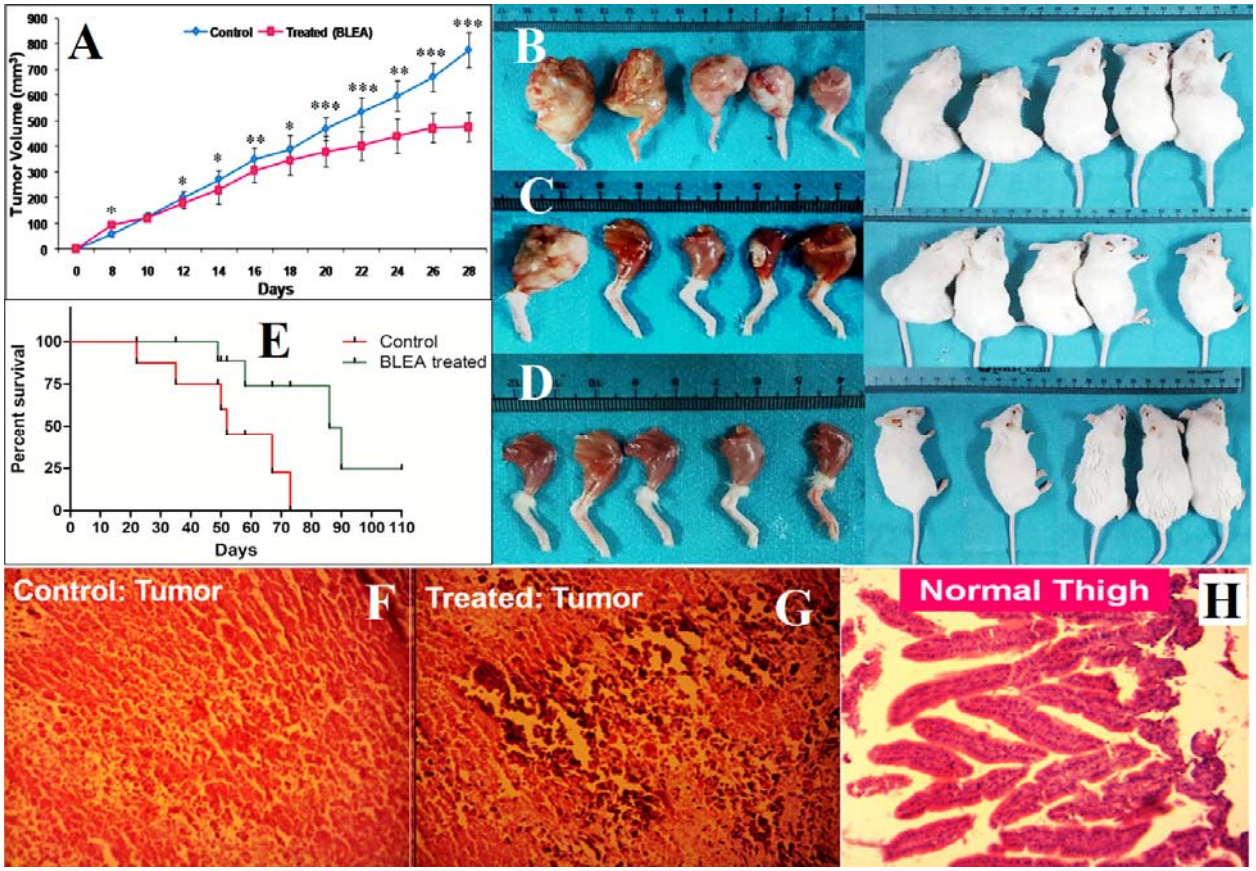


Figure 4: Effect of BLEA on tumor progression, survival and cellular architecture of tumor in tumor bearing mice. Mouse origin EAC cells induced solid tumor was developed in left thigh of mice and 100 mg/kg b. wt. dose of BLEA was administered orally after eight days of EAC injection throughout the experimental period. (A) Effect of BLEA on tumor progression, (B) tumor of control group, (C) tumor of treated group, (D) thigh of normal mice, (E) survival curves (Kaplan–Meier) of treated and untreated tumor bearing mice, (F) section of untreated HE stained tumor, (G) section of treated HE stained tumor and (H) normal thigh section.

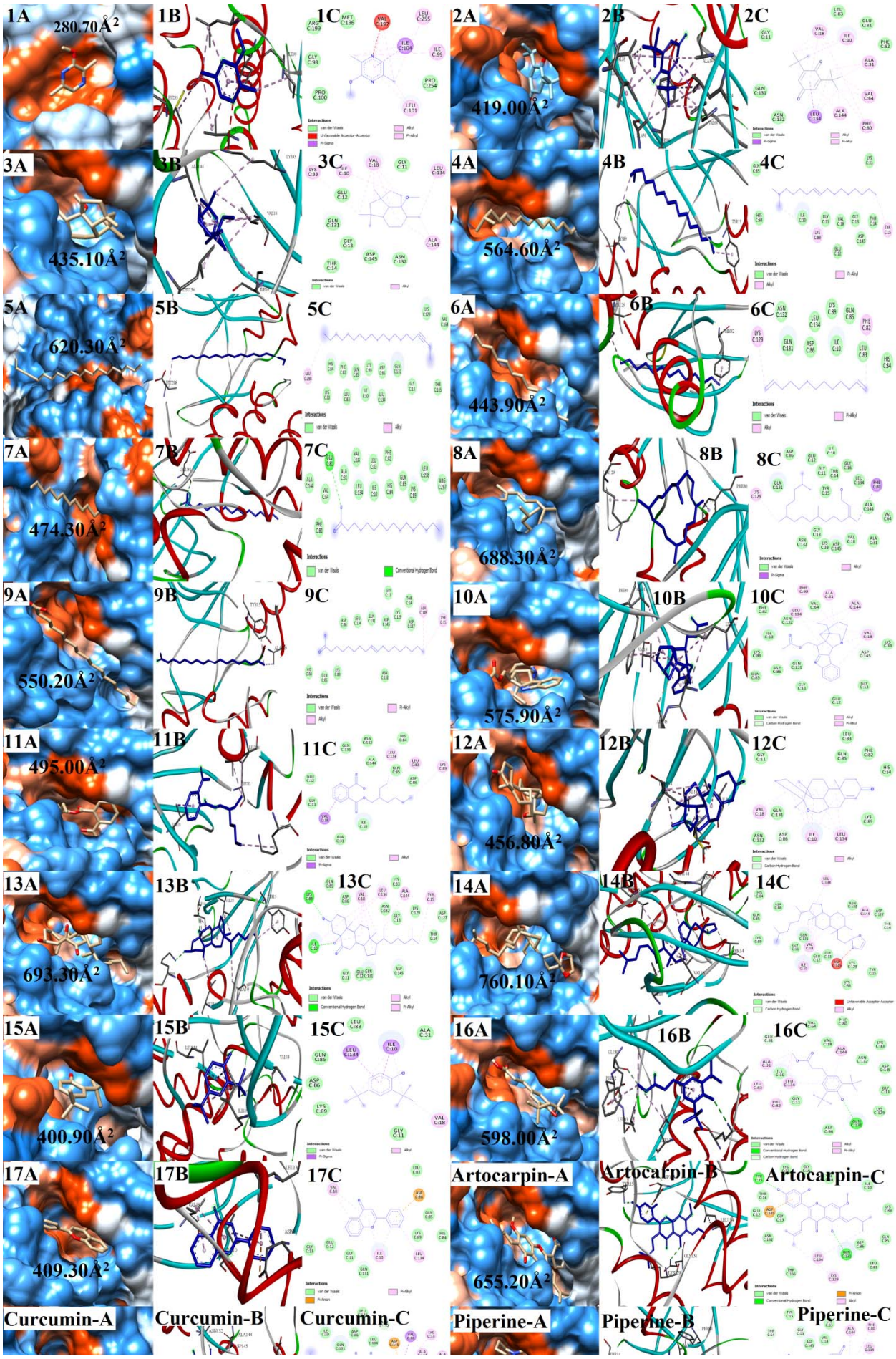
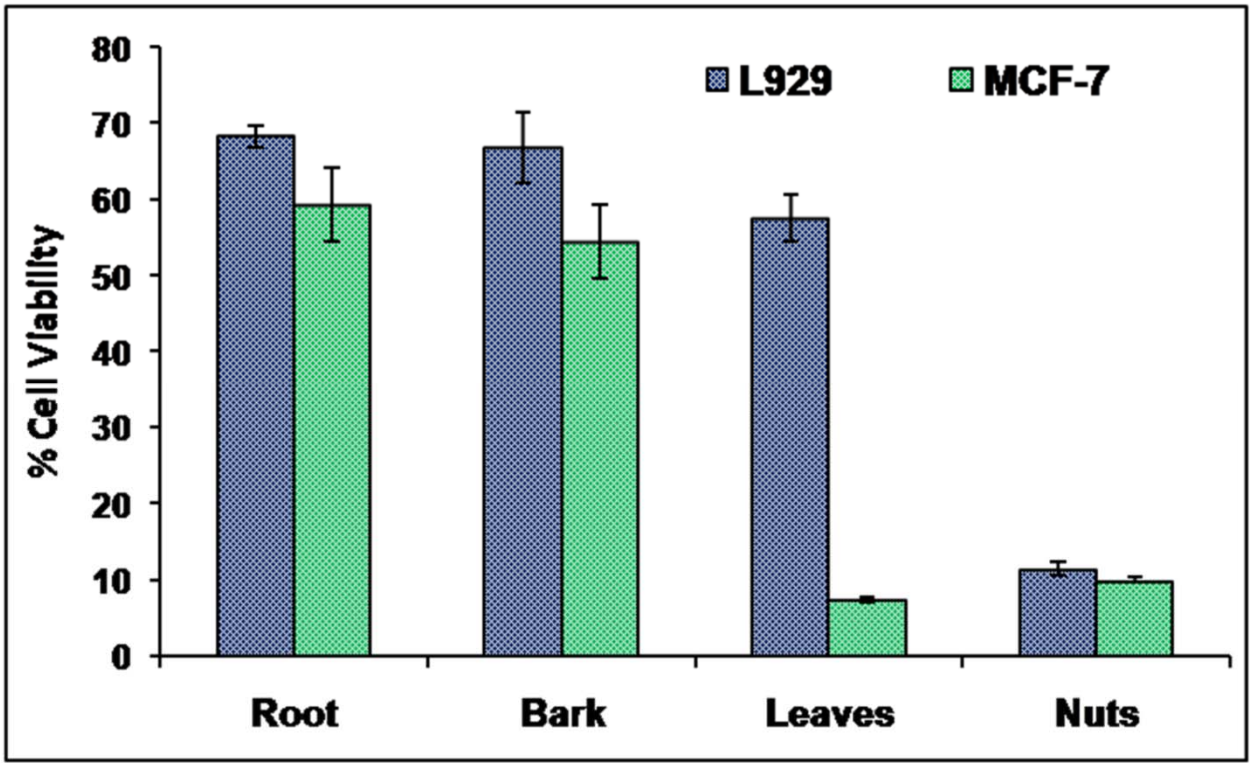
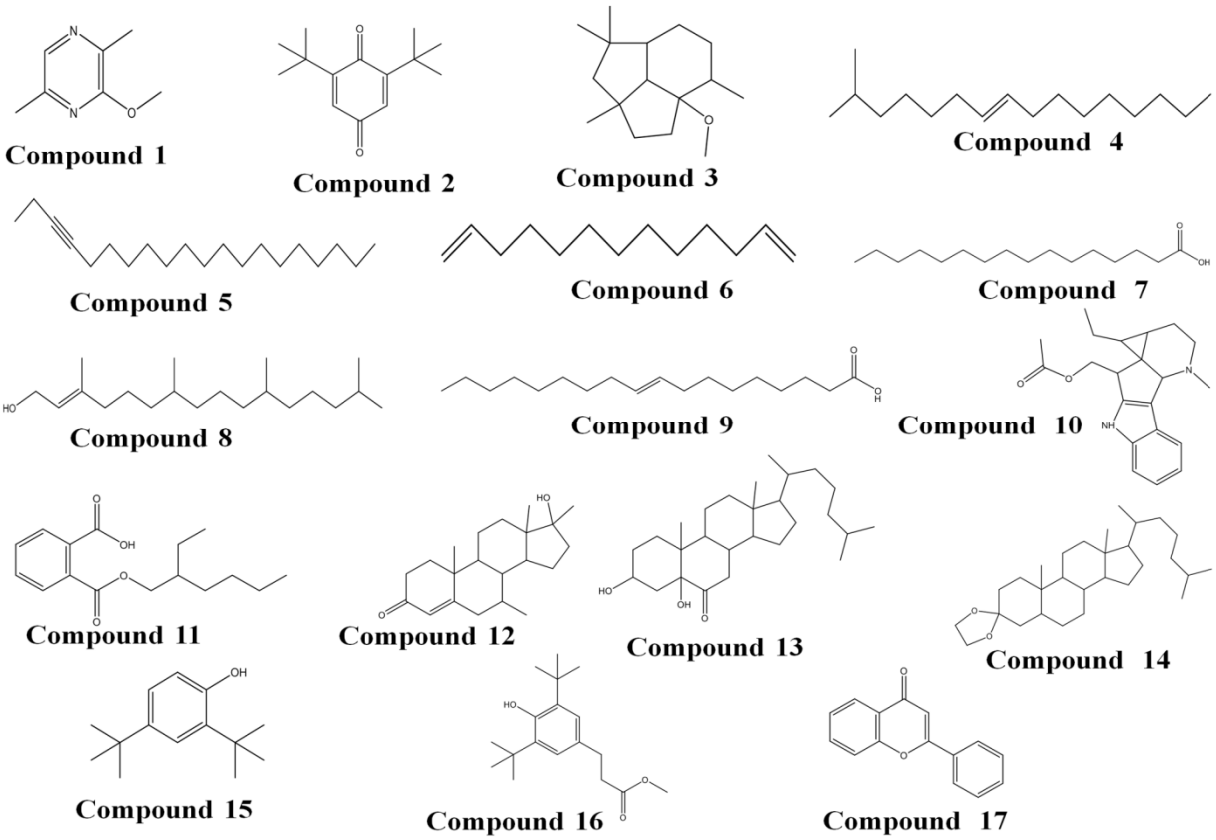

1A
280.70Å²
1B
1C
2A
419.00Å²
2B
2C
3A
435.10Å²
3B
3C
4A
564.60Å²
4B
4C
5A
620.30Å²
5B
5C
6A
443.90Å²
6B
6C
7A
474.30Å²
7B
7C
8A
688.30Å²
8B
8C
9A
550.20Å²
9B
9C
10A
575.90Å²
10B
10C
11A
495.00Å²
11B
11C
12A
456.80Å²
12B
12C
13A
693.30Å²
13B
13C
14A
760.10Å²
14B
14C
15A
400.90Å²
15B
15C
16A
598.00Å²
16B
16C
17A
409.30Å²
17B
17C
Artocarpin-A
Artocarpin-B
Artocarpin-C
655.20Å²
Curcumin-A
Curcumin-B
Curcumin-C
Piperine-A
Piperine-B
Piperine-C

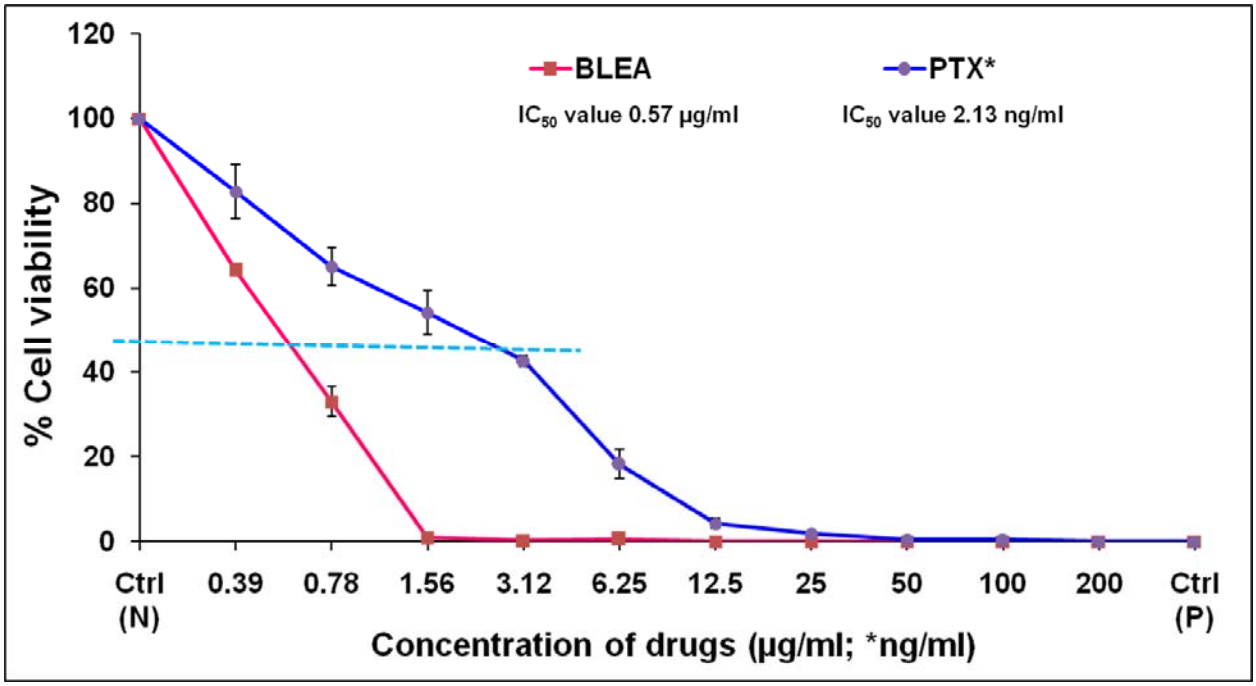
Figure 5: Visualization of the interaction of receptor protein, CDK2 with ligands molecules in which A represents 3D surface view of binding site of compounds, B represents 3D spatial interactions and C represents 2D interaction of the compounds respectively. **1-17** represents the compounds.



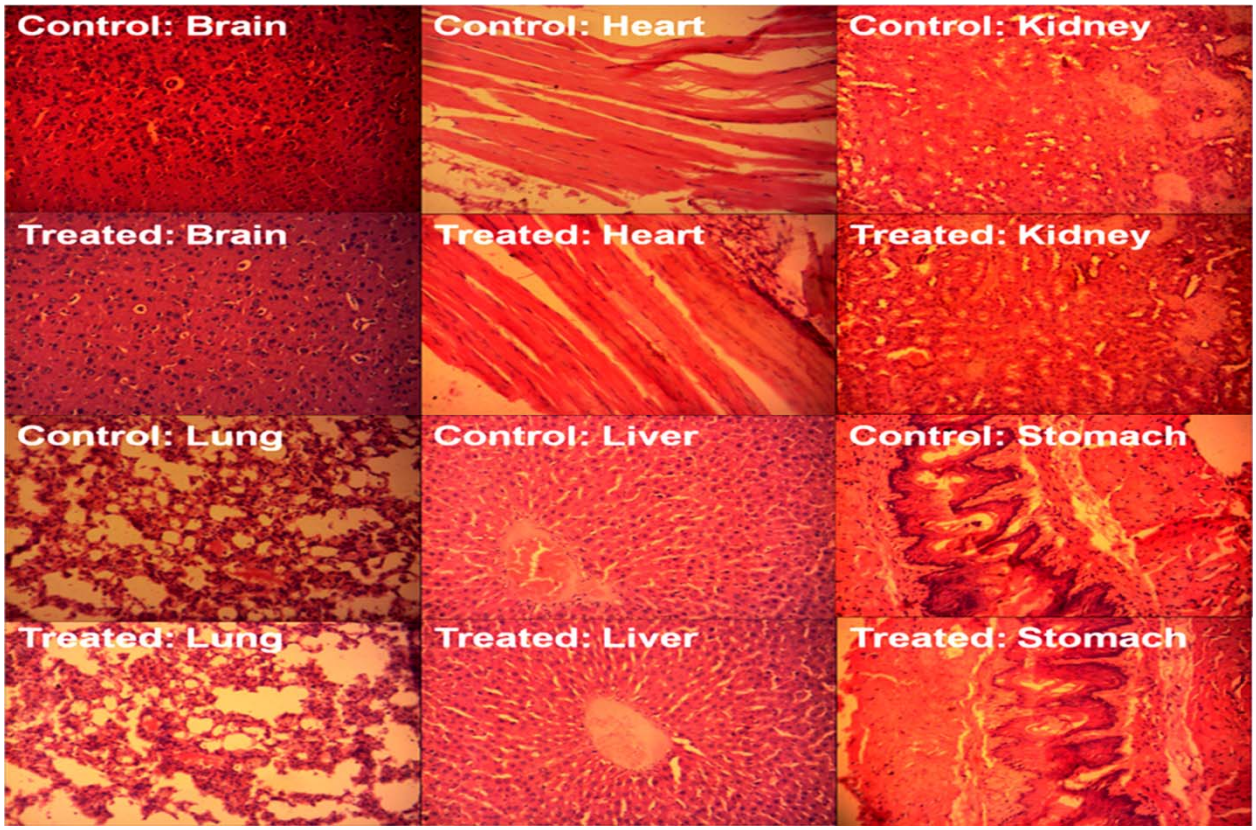
Supplement figure 1: the cytotoxicity of root, bark, leave and fruits in cancer cells (MCF-7) and normal cells (L929).



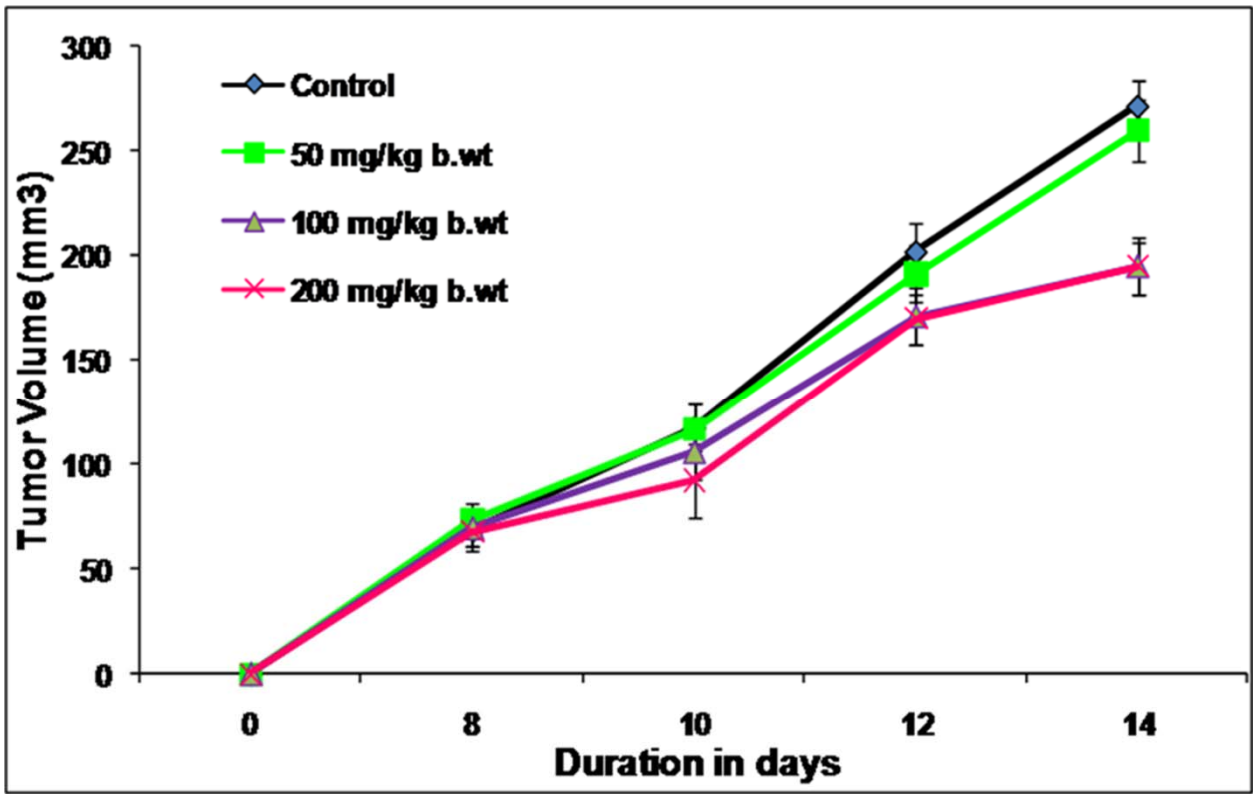
Supplement figure 2: structure of the identified compounds.



Supplement figure 3: the cytotoxicity of PTX and BLEA in MCF-7 cells.



Supplement figure 4: Histological evaluation of toxicity of BLEA in different vital organs. HE staining was done to visualize the cellular architecture of different tissues of control and treated groups.



Supplement figure 5: Effective dose determination of BLEA using different doses (50, 100 and 200 mg/kg b. wt.) in comparison of control (pilot study).

TABLES

Table 1

The names and numbers of the compounds identified in BLEA by GC-MS spectral analysis.

Compounds identified by GC-MS analysis	Mol. Wt.
Compound 1: 3-methoxy-2,5-dimethylpyrazine	138.17
Compound 2: 2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione	220.31
Compound 3: 4a-methoxy-1,1,2a,5-tetramethyldecahydro-1H-cyclopenta[cd]indene	206.32
Compound 4: (E)-2-methylhexadec-7-ene	222.24
Compound 5: icos-3-yne	236.39
Compound 6: trideca-1,12-diene	238.45
Compound 7: Palmitic acid	278.52
Compound 8: (E)-3,7,11,15-tetramethylhexadec-2-en-1-ol (**Phytol**)	180.33
Compound 9: (E)-octadec-9-enoic acid	292.41
Compound 10: (1-ethyl-4-methyl-1,1a,2,3,4,4a,9,10-octahydrocyclopropa[3',4']pyrido[2',3':3,4]cyclopenta[1,2-b]indol-10-yl)methyl acetate	256.42
Compound 11: 2-(((2-ethylhexyl)oxy)carbonyl)benzoic acid	296.53
Compound 12: 17-hydroxy-7,10,13,17-tetramethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one (**Calusterone**)	282.46
Compound 13: 3,5-dihydroxy-10,13-dimethyl-17-(6-methylheptan-2-yl)hexadecahydro-6H-cyclopenta[a]phenanthren-6-one	338.44
Compound 14: 10,13-dimethyl-17-(6-methylheptan-2-yl)hexadecahydrospiro[cyclopenta[a]phenanthrene-3,2'-[1,3]dioxolane	278.34
Compound 15: 2,4-di-tert-butylphenol	316.48
Compound 16: methyl3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanoate	418.65

Compound 17: 2-phenyl-4H-chromen-4-one (**Flavone**)	430.71

Table 2

Different parameters of interaction, affinity and amino acid residues of CDK2 interacted with the ligand (compounds).

Compound Name	Atomic Contact Energy	Attractive VdW	ΔG (Kcal/mol)	Residues directly interacted with ligand through H-bonds	Residues closely interacted with ligand through other interactions
1	-8.77	-9.46	-25.67	Ile99, Leu101, Ile104, Val197, Leu255	Gly98, Pro100, Met196, Arg199, Pro254
2	-9.22	-12.16	-30.83	Ile10, Val18, Ala31, Val64,Phe80, Leu134, Ala144	Gly11, Glu81, Phe82, Leu83, Gln131, Asn132
3	-7.17	-12.65	-29.67	Ile10, Val18, Lys33, Leu134, Ala144	Gly11, Glu12, Gly13, Thr14, Gln131, Asn132, Asp145
4	-5.85	-15.2	-30.48	Tyr15, Lys89	Ile10, Gly11, Glu12, Gly13, Thr14, Val18, Lys33, His84, Gln85, Asp145
5	-6.33	-16.43	-30.46	Leu298	Ile10, Gly13, Lys20, Phe82, Leu83, His84, Gln85, Asp86, Lys89, Lys129, Gln131, Leu134, Val164, Thr165
6	-5.08	-11.51	-23.94	Phe82, Lys129	Ile10, Phe82, Leu83, His84, Gln85, Asp86, Lys89, Gln131, Asn132
7	-9.28	-16.74	-31.21	Glu81	Ile10, Val18, Ala31, Val64, Phe80, Phe82, Leu83, His84, Gln85, Lys89, Leu134, Ala144, Arg297, Leu298
8	-6.97	-20.57	-36.73	Phe80, Lys129	Ile10, Gly11, Glu12, Gly13, Thr14, Tyr15, Gly16, Val18, Ala31, Lys33, Val64, Asp86, Gln131, Asn132, Leu134, Ala144, Asp145
9	-3.4	-15.66	-28.00	Tyr15, Ala149	Gly13, Thr14, His84, Gln85,

					Asp86, Lys89, Asp127, Lys129, Gln131, Asn132, Leu134, Asp145,
10	-10.46	-17.3	-40.75	Val18, Ala31, Phe80,Leu134, Ala144	Ile10Gly11, Glu12, Gly13, Lys33, Val64, Phe82, Gln85, Asp86, Lys89, Gln131, Asn132, Asp145,
11	-10.37	-15.72	-36.09	Val18, Leu83, Lys89, Leu134	Ile10, Gly11, Glu12, Ala31, His84, Gln85, Asp86, Gln131, Asn132, Ala144,
12	-10.31	-17.69	-41.94	Ile10, Val18, Leu134	Gly11, Phe82, Leu83, His84, Gln85, Asp86, Lys89, Gln131, Asn132
13	-9.81	-22.92	-45.27	Ile10, Tyr15, Val18, Lys89, Leu134, Ala144	Gly11, Glu12, Gly13, Thr14, Lys33, Gln85, Asp86, Asp127, Lys129, Gln131, Asn132, Asp145
14	-8.08	-25.53	-48.27	Ile10, Thr14, Val18, Leu134, Ala144, Asp145	Gly11, Glu12, Gly13, Tyr15, Lys33, His84, Gln85, Asp86, Lys89, Asp127, Lys129, Gln131, Asn132
15	-7.67	-11.65	-28.76	Ile10, Val18, Leu134	Gly11, Ala31, Leu83,Gln85, Asp86, Lys89
16	-8.27	-15.14	-31.51	Ala31, Glu81, Phe82, Leu83, Gln131, Leu134, Ala144	Ile10, Gly11, Gly13, Val18, Lys33, Val64, Phe80, Asp86, Lys129, Asn132, Asp145
17	-6.89	-13.09	-29.85	Ile10, Val18, Asp86, Leu134	Gly11, Glu12, Gly13, Leu83, His84, Gln85, Lys89, Gln131
Artocapin	-7.46	-25.52	-48.97	Tyr15, Lys129, Gln131, Leu134, Asp145	Ile10, Glu12, Gly13, Thr14, Gly16, Val18, Lys33, Leu83, Gln85, Asp86, Lys89, Asn132, Thr165

Curcumin	-8.6	-20.73	-40.68	Val18, Ala31, Lys33, Phe80, Glu81, Asn132, Ala144, Asp145	Ile10, Gly11, Glu12, Val64, Asp86, Gln131, Leu133, Leu134
Piperine	-6.11	-18.23	-34.88	Gly13, Thr14, Ala31, Val64, Phe80, Asn132, Leu134, Ala144	Ile10, Tyr15, Val18, Lys33, Glu81, Phe82, Leu83, Asp127, Lys129, Gln131, Asp145
Urosolic acid	-13.07	-24.56	-49.37	Ile10, Val18, Ala31, Lys129, Asn132, Leu134, Ala144, Asp145	Gly11, Glu12, Gly13, Lys33, Val64, Phe80, Glu81, Phe82, Leu83, Asp86, Gln131