

Cytotoxic effect of methanolic extracts and partially purified fractions of some medicinal plants used in traditional medication

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In this study, the cytotoxic activity of methanolic extracts different parts of seven plant species was checked on NRK-52E (Rat renal proximal tubular cells) using MTT assay. Based on their cytotoxic activities, methanol extract of *Vitex negundo* (*V. negundo*) was selected and their partition in hexane, chloroform, ethylacetate, butanol and water was done. Among all fractions, chloroform fraction was most active on NRK-52E cells as determined by MTT assay. In NRK-52E cells induction of apoptosis was checked by analyzing DNA fragmentation by agarose gel electrophoresis. To study the molecular mechanism of apoptosis, expression levels of different genes BCL-2, BCL-XL, SOD, TGF, Foxo and BAX were assessed using quantitative real-time PCR. Chloroform fraction of *Vitex negundo* (VnCE) was found to be highly antiproliferative and also showed DNA fragmentation in NRK-52E cells. VnCE showed up regulation of BCL-2, BCL-XL, SOD, Foxo and BAX genes and down regulation of TGF gene.

Keywords: Apoptosis, Cytotoxic activity, DNA fragmentation assay, MTT assay, Real-time PCR assay

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Introduction

One of the major causes of death worldwide is cancer and is characterized by the uncontrolled growth of abnormal cells leading to a constant increase in the number of cancer patients. Due to the insufficient ability of presently accessible cancer treatments the death rate among cancer patients remains high¹. Now there is a considerable scientific and marketable interest in the outcome of new anticancer drugs from natural sources. It is believed that the number of cancer patients in future will increase in the developing and underdeveloped countries, which may increase up to 70%; a serious issue for all of us. In exposure to some ionizing radiation, toxins and anticancer drugs physiological cell suicide program known as apoptosis gets activated which is an efficient and promising strategy to kill cancer cells². Drug-induced apoptosis is one of

the main approaches to treat cancer and many phytochemicals from plants have been stated to encourage apoptosis in many cancerous cell lines³⁻⁵. Intrinsic and extrinsic pathways are the main signalling pathways involved in the processes which meet in the mitochondria as they play an important role in apoptosis⁶. The targeted or combination therapies are a promising approach for cancer treatments⁷. The use of alternative medicine to eliminate the side effects and improve the effect of chemotherapy is prevalent in Western countries⁸. Medicinal plants contain a diversity of secondary metabolites, which can be used to inhibit or cure diseases, or to promote general health and well-being^{9,10}. A huge number of foodstuffs and dietary constituents have been assessed as potential chemopreventive agents and herbal preparations used in traditional medicine provide a largely unexplored source of novel drugs¹¹. Evidence shows that active compound present in plants work as a potent chemotherapeutic agent with less toxicity and few side effects. Plants have been used in the treatment of

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cancer since antiquity and over 60% of medicines used for the cancer treatments are derived from plant-derived sources¹². Around 80% of people in the rural area depend on plant products for their primary healthcare needs¹³. Anticancer plants are associated with induction of apoptosis, cell cycle arrest, inhibition of various signalling pathways and signal transducers^{14,15}. The modern pharmaceutical industry relies mainly on the variety of secondary metabolites in plants for finding new compounds with novel biological properties. In all drugs, 50% drugs in clinical use are obtained from natural foodstuff^{16,17}. Therefore, it is important to screen the crude extracts for their apoptosis-inducing potentials. In order to assess medicinal plants for their therapeutic potential and to select plants for future phytochemical study, different plant species with medicinal uses (*Vitex negundo*, *Lantana camara*, *Bauhania variegata*, *Bauhania racemosa*, *Bauhania purpurea*, *Argyrea nervosa* and *Butea monosperma*) were selected from literature and assessed for their *in vitro* cytotoxic activity on NRK-52E cells (Rat renal proximal tubular cell-line) through expression of different apoptotic genes in the present study.

Materials and Methods

Chemicals

All analytical grade chemicals were procured from Hi-Media and Merck. Standard drugs were procured from Sigma-Aldrich chemicals. For *in vitro* experiments, media like DMEM (Dulbecco's modified eagle's cell culture media) and fetal bovine serum (FBS) were procured from Gibco. Trypsin and 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were procured from Sigma Aldrich Company.

Plant materials

A fresh sample of selected medicinal plants *V. negundo*, *L. camara*, *B. variegata*, *B. racemosa*, *B. purpurea*, *A. nervosa* and *B. monosperma* were collected from Pune, in August 2013 under the supervision of a botanist and all material were submitted in organ form to Botany Department, Pune. Authentications of all plant materials were done using botanical parameter such as macroscopic, microscopic, histochemical and phytochemical evaluation by Dr. Subhash Sadhu Deokule, Head of Botany Department, Pune.

Cell line

In our study, NRK-52E cell line was procured from NCCS Pune, India and was used as an *in vitro* model. Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) Penicillin Streptomycin solution used as a culture medium. 10% (v/v) FBS obtained from Gibco (USA). The cells were maintained at 37 °C in a 5% CO₂ incubator. The medium was changed after every 2-3 days.

Preparation of the plant extracts

The selected plants were washed, shade dried and ground to get fine powder using a grinder mixer. Exactly 10 g of plant powder was soaked in 200 mL of methanol at room temperature for overnight and filtered by Whatman filter paper. In a rotary evaporator under reduced pressure (at 40-50 °C) filtrate obtained was concentrated to get ME (methanolic extract). At different time periods, the extraction process was repeated three times. In percentage yield, no major change was observed. Then yield was calculated for the extraction method. The dried extracts were stored at -20 °C in airtight bottles for further studies. Exactly 10 mg of each extract was dissolved in 1 mL dimethyl sulfoxide (DMSO) and were diluted with DMEM medium. Then, test solutions were sterilized using 0.22 µm Syringe filters (Axiva, Scichem biotech) and used as a stock solution for further experiments.

Total phenolic and flavonoid determination

Total phenolic contents in methanolic extracts from seven plants were determined by Folin-Ciocalteu method¹⁸. Total phenolic content (TPC) was expressed in terms of micrograms of gallic acid equivalents (GAE) per mg of dry extract. Total flavonoid contents (TFC) in methanolic extracts were measured as described previously¹⁹. Total flavonoid content of the different extracts was expressed in micrograms of quercetin equivalents (QE) per milligrams of extract.

Evaluation of extracts cytotoxicity

MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to check the cytotoxic activity. In 96-well plates 1X10⁴ cells/ well were seeded and treated with different concentrations (50–500 µg/mL) of methanolic extracts of selected medicinal plants for 24 hours. After 24 hours, the incubation medium was removed and 20 µL MTT, Sigma-Aldrich (5 mg/mL) was

added to each well. The plate was again kept for incubation for 4 hours at 37° C. To solubilise formazan products 150 µL of DMSO was added to each well. Reading was taken at 570 nm using ELISA plate reader (Promega, USA). The cell viability was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{O.D of control sample} - \text{O.D of treated sample}}{\text{O.D of control sample}} \times 100.$$

Partial Purification of screened plant

Based on the cytotoxic activity, VnME (*V. negundo*'s methanol extract) was selected and partitioned into fractions using separator funnel and solvent in increasing order of polarity. A powdered sample of *V. negundo* leaves (50 g) was extracted with methanol (300 mL X 6) for 24 hours at room temperature and concentrated under reduced pressure to get ME. The methanolic extract was suspended in water (500 mL) and extracted successively with hexane (250 mL X 3), chloroform (250 mL X 3), ethyl acetate (250 mL X 3), butanol (250 mL X 3) and water. The hexane extract obtained was pooled together, dried using anhydrous sodium sulphate. To obtain residue hexane extract (2.9 g) was concentrated under low pressure. Chloroform (1.9 g), ethyl acetate (2.4 g), butanol (1.1 g) and water (4.34 g) extracts were processed similarly. Stock solutions of different extracts were prepared by dissolving the extract in 1 mL DMSO and were diluted with DMEM medium. Then, test solutions were filtered through a 0.22 µm membrane filter (Axiva, Scichem biotech) and used as a stock solution for further experiments.

Evaluation of DNA fragmentation

In apoptosis, by activation of nuclear endonuclease DNA gets cleaved into multimers of 180-200 base pairs and can be visualized as a ladder by standard agarose gel electrophoresis. NRK-52E cells were seeded in a 6 wells plate and the plate was kept in CO₂ incubator. After 24 hours NRK-52E cells were treated with LD₅₀ concentration (µg/mL) of VnCE for 24 hours. Then, the cells were centrifuged at 1500 rpm for 10 minutes at 14 °C and pellet was resuspended in a lysis buffer for 10 seconds. (1% NP-40, 50 mM Tris-HCl, 10 mM NaCl, 20 mM EDTA, pH 7.5). The supernatant was collected and extraction is repeated with lysis buffer. The supernatant was brought to 1% SDS and treated with RNAase-A (5 µg/mL) at 56 °C for 2 hours followed by digestion with Proteinase K (2.5 µg/mL) at 37 °C for at least 2 hours.

After addition of half the volume of 10 M ammonium acetate, the DNA was precipitated with 2.5mL of ethanol. DNA samples were electrophoretically separated on 1.8% agarose gel containing ethidium bromide (0.4 µg/mL). Untreated cells were used as control. DNA extracted from cells treated with camptothecin was used as positive control.

Evaluation of apoptosis-regulatory genes by real-time assay

Expression levels of apoptosis-related genes, BCL-2, BCL-X1, SOD, TGF, Foxo and BAX were analyzed using real-time assay. LD₅₀ concentration of VnCE was used to treat cells for a period of 24 hours. RNA isolation was done by Quigen kit and the procedure was followed as per the manufacturer's kit. cDNA was synthesized using fermentas kit in order to further amplify the DNA segment for q-PCR and analyse the genes. The components of the kit after thawing were mixed, briefly centrifuged and stored on ice. RNA template, random hexamer and RNA (1 µg) were mixed. The mixture was placed at 65 °C for 3 minutes and snap cooled. The expression level of apoptotic related genes was determined by real-time q-PCR on thermocycler (Applied Biosystems using a real-time SYBR Green gene expression assay kit (Quigen). According to the manufacturer instruction, q-PCR was performed in a reaction volume of 15 µL. In brief, 7.5 µL master mix, 0.5 µL primer, 5 µL MiliQ water and 2 µL template cDNA were added to each well. The PCR plate was subjected to q-PCR. Thermal profile used was: initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. To study the fold change expression in untreated samples, comparative threshold (Ct) value was used to analyze q-PCR data. Expressions of the apoptotic genes were compared using beta-actin as an endogenous control gene. Sequences of the primers used to amplify BCL-2, BCL-X1, SOD, TGF, Foxo, BAX and endogenous reference beta-actin gene were mentioned in Table 1.

Statistical analysis

Data represented in this study were based on mean±standard deviation (SD) of identical experiments made in triplicates. Statistical significance was determined by analysis of variance (ANOVA). *P* value ≤0.05 was considered statistically significant. LD₅₀ value represented the concentration of the test samples that caused 50% inhibition.

Table 1 — Sequences of the primers used in Real-time PCR

Genes	Forward primer sequence (FP)	Reverse primer sequence (RP)
Beta-Actin	5'-GGGAAATCGTGCCTGACAT-3'	5'-CAGGAGGAGCAATGATCTT-3'
BCL-2	5'-CTGGTGGACAACATCGCTCTG-3'	5'-GGTCTGCTGACCTCACTTGTG-3'
BCL-XL	5'-AGGCTGGCGATGAGTTTCAA-3'	5'-TGAAACGCT CCTGGCCTTTC-3'
SOD	5'-GGGCTGGCTTGGCTTCA-3'	5'-AGCAGGCGGCAATCTGTAA-3'
TGF	5'-GCAACAACGCAATCTATGAC-3'	5'-CCTGTATTCCGTCTCCTT-3'
FOXO	5'-CAGCCAGGCACCTCATAACA-3'	5'-ATGTTGCTGCTCACTAACTCC-3'
BAX	5'-CCAGGACGCATCCACCAAGAAGC-3'	5'-TGCCACACGGAAGAAGACCTCTCG-3'

Results

Nowadays, significant scientific records are available on cancer cell death via induction of apoptosis using different herbal extracts. Currently used chemopreventive agents destroy both cancerous and normal cells triggering side effects. But, the usage of plant-derived agents precise to tumour cells could reduce the adverse effects making them ideal therapeutic candidates.

Extraction yield of different plants

Table 2 enumerates the yield of crude methanolic extract of different plants in cold extraction methods. The yield of various extracts was calculated by the formula given below. The maximum yield was obtained in *B. variegata* (17.1%) and minimum yield was obtained in *A. nervosa* leaves (4.3%).

Yield of plant extracts (%) = (weight of dried plant extract/weight of original plant sample) X100

Total phenolic and flavonoids contents

It is well known that phenolics and flavonoids are the important antioxidant substances that are obtained from most natural plants. Moreover, a few studies on the selected plants revealed that they are good dietary sources of antioxidants. So, we determined the total phenolic and flavonoid contents of the methanolic extracts of selected plants (Table 3). The highest concentration of TPC and TFC was found in methanolic extract of *V. negundo* (leaves) (217±1.5µg GAE/mg and 102±1.3 µg of QE/mg).

Cytotoxicity assay of methanolic extracts of different medicinal plants

The crude methanolic extracts of selected plants were checked for their toxicity against NRK-52E cells. Crude ME were tested under same conditions but different concentrations (500, 400, 300, 200, 100 and 50 µg/mL) for 24 hours. The crude methanolic extract inhibited the viability of NRK-52E cell with

Table 2 — Extraction yield (%) of different plants in cold extraction method

Plant name	Parts	Solvent	Extraction yield (%)
<i>Argyrea nervosa</i>	Leaves	Methanol	4.3
<i>Argyrea nervosa</i>	Root	Methanol	7.5
<i>Butea monosperma</i>	Leaves	Methanol	5.6
<i>Bauhania purpurea</i>	Leaves	Methanol	15.3
<i>Bauhania purpurea</i>	Bark	Methanol	15.3
<i>Bauhania racemosa</i>	Leaves	Methanol	16.8
<i>Bauhania Variegata</i>	Leaves	Methanol	17.1
<i>Lantana camara</i>	Leaves	Methanol	8.1
<i>Vitex negundo</i>	Leaves	Methanol	11.2

Note: The data are expressed as mean.

Table 3 — Total phenolic contents and total flavonoid contents of selected plants' methanolic extracts

Plant name	Total phenolic (mg of gallic acid per gram of dry extract)	Total flavonoid milligram of quercetin per gram dry extract
<i>Argyrea nervosa</i> (leaves)	112±2.9	19±3.2
<i>Argyrea nervosa</i> (Root)	167±4.3	12±5.2
<i>Butea monosperma</i> (leaves)	195±3.5	27±2.4
<i>Bauhania purpurea</i> (leaves)	143±2.8	23±4.9
<i>Bauhania purpurea</i> (Bark)	189±1.3	25±4.2
<i>Bauhania racemosa</i> (leaves)	190±1.9	97±2.8
<i>Bauhania Variegata</i> (leaves)	130±3.4	20±2.4
<i>Lantana camara</i> (leaves)	175±2.1	23±5.5
<i>Vitex negundo</i> (leaves)	217±1.5	102±1.3

Note: The data are expressed as mean ± SD (n = 3).

their LD₅₀ values as mentioned in Table 4. These extracts had significant concentration-dependent inhibition on viability and proliferation of the NRK-52E cancer cells as shown in Fig. 1. According to the results, the cytotoxic activity of VnME against NRK-52E cells was higher than other methanolic extracts.

Cytotoxicity assay of different fraction

Based on the preliminary cytotoxicity screening assay best result was obtained in VnME. So, partial purification of VnME in different solvent systems starting from hexane, chloroform, ethyl acetate, butanol

and water was done and each fraction was checked for its cytotoxic potency on NRK-52E cells. As shown in Fig. 2, chloroform extract showed more cytotoxic effect on NRK-52E cells. The LD₅₀ value of all fractions on NRK-52E cells was mentioned in Table 5.

Apoptosis induction

Disruption of DNA molecule into small fragments is a unique sign linked with apoptosis in which there is inhibition of DNA replication due to internucleosomal cleavage²⁰. DNA fragmentation was observed in the cells treated with chloroform extracts which confirmed the apoptotic effect (Fig. 3). But in the control cell, DNA fragmentation did not occur.

Expression level of apoptosis-related genes

To study the molecular mechanism behind apoptosis, induced by chloroform fraction in NRK-52E cells, the expression levels of apoptotic genes were studied. The relative quantification of BCL-2, BCL-X1, SOD, TGF, Foxo and BAX mRNA expression levels was performed by SYBR Green-based q-PCR using a Real-time 7500 Fast System (Applied Biosystem). NRK-52E cells

treated with LD₅₀ levels of VnCE for 24 hours showed increased expressions of most of the transcripts including BCL-2, BCL-X1, SOD, Foxo and BAX by several fold but decreased expression of TGF gene as compared to the levels in untreated control cells (Fig. 4). All together these data advocates that these were induced by chloroform fraction in a dose and time-dependent manner. Change in expression level of the above genes showed that the chloroform extract killed NRK-52E cells through apoptosis mechanism.

Table 5 — LD₅₀ value of *Vitex negundo* plant's different fraction on NRK-52E cells

Different fraction of <i>Vitex negundo</i> leaves	LD ₅₀ value on NRK-52E cells (µg/mL)
VnHE	219.3±0.8
VnCE	152.2±0.1
VnEAE	197.7±0.5
VnBE	260.7±0.4
VnWE	1123.6±1.1

Data presented as mean ±S.D (n=3), LD₅₀: Concentration of the extract causing 50% growth inhibition of the cells.

Table 4 — LD₅₀ value of methanolic extract of plants on NRK-52E cells

Plant name	LD ₅₀ value on NRK-52E cells (µg/mL)
<i>Argyrea nervosa</i>	290.1±1.4
<i>Argyrea nervosa</i>	493.6±1.3
<i>Butea monosperma</i>	361.9 ± 0.78
<i>Bauhania purpurea</i>	571.7±0.9
<i>Bauhania purpurea</i>	472.2±2.3
<i>Bauhania racemosa</i>	524.1±1.0
<i>Bauhania variegata</i>	531.8±1.1
<i>Lantana camara</i>	262.0±1.1
<i>Vitex negundo</i>	257.2±1.6

Data presented as mean ± S.D (n=3), LD₅₀: Concentration of the extract causing 50% growth inhibition of the cells.

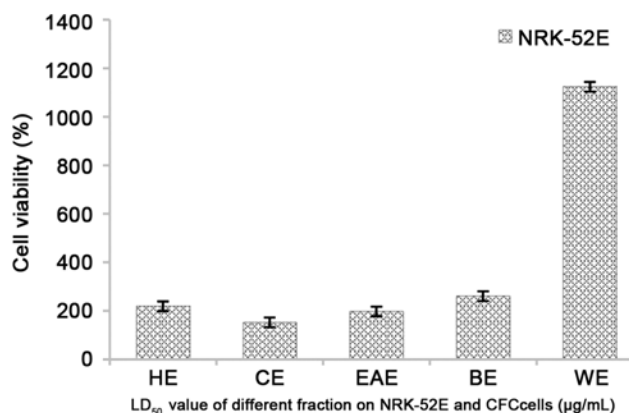


Fig. 2 — LD₅₀ value of *Vitex negundo* plant's different fractions against NRK-52E cells using MTT assay. Data presented as mean±S.D (n=3)

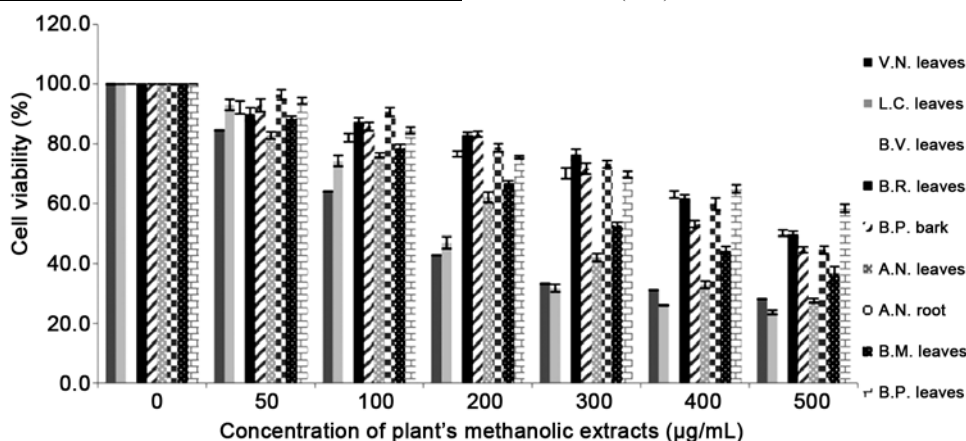


Fig. 1 — Cytotoxic effect of different plant extracts against NRK-52E cell line using MTT assay. Data presented as mean±S.D (n=3)

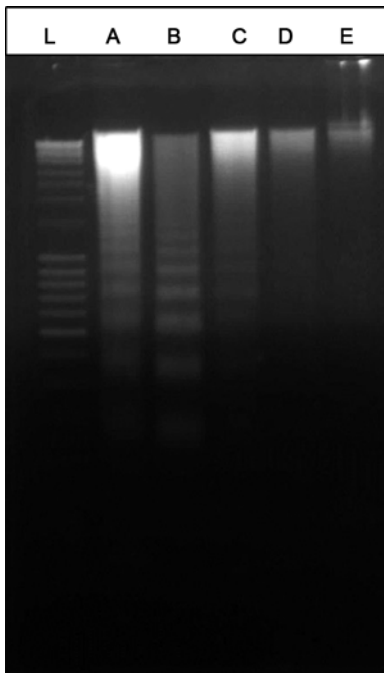


Fig. 3 — Agarose gel electrophoresis (1.8%) of the chromosomal DNA extracted of NRK-52E cells. L- DNA ladder, A- cells treated with camptothecin B and C- cells treated with chloroform fraction of *V. negundo* at LD₅₀ concentration for 24 hours D and E- Control cells treated with 0.5% DMSO, respectively.

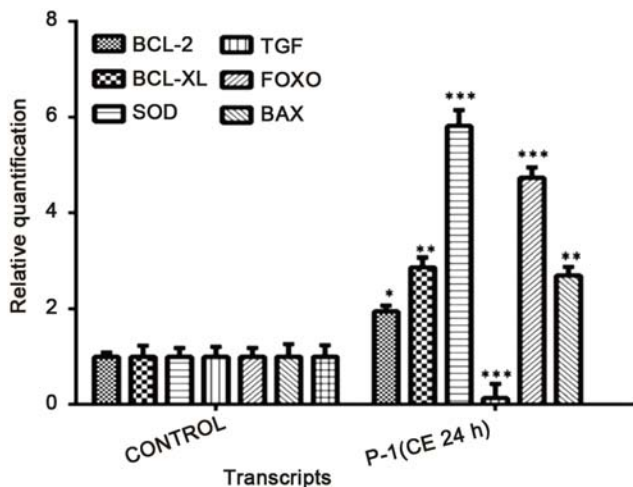


Fig. 4 — Expression of selected gene's mRNA levels in NRK-52E cells (Rat Renal proximal tubular cell, incubated with LD₅₀ value of VnCE for 24 hours incubation). Beat-actin was used as an internal reference gene. Data represented are means±SD of three independent experiments.

Discussion

Presently, there is an increasing curiosity to develop safe and more active therapeutic agents using phytochemicals for cancer treatment all over the world, owing to the adverse side effects of and resistance to

many anticancer drugs that have been established^{21,22}. Medicinal plants have been used widely in traditional medicine for cancer treatment. However, only a few medicinal plants have gained the interest of scientists to study them in therapeutic for neoplasm (cancer). Therefore, it is still a challenge to find effective clinical approaches for cancer treatment. Herbal plants remain the most common substitutes for cancer prevention and treatment throughout the world for a long time²³. To inhibit the growth of cancerous cell recently there has been an increasing interest in studying the effects of phytochemicals in combination with chemotherapy, radiotherapy or other therapies²⁴⁻²⁷, such as curcumin from *C. longa*, epicatechin gallate from tea and paclitaxel from Pacific yew. Because of its safety and nourishment, phytochemicals would be a promising strategy to provide complementary methods for cancer prevention and treatment. In this study, anti-proliferative activity from methanolic extracts of some selected plants was studied *in-vitro* using MTT assay. In MTT assay mitochondrial dehydrogenase enzyme converts yellow tetrazolium dye to a purple formazan dye in viable cells which are metabolically active²⁸. Result of MTT showed that methanolic extract of *V. negundo* possessed the highest cytotoxic effect on NRK-52E cells and significantly reduced purple formazan dye in a concentration-dependent manner (Fig. 1). Next, based on this result we examined the sensitivity of NRK-52E cells to the different fractions of *V. negundo* (*Vitex negundo*'s Hexane extract, chloroform extract, ethylacetate extract, butanol extract and water extract). Different fractions of *V. negundo* varied in their cytotoxic effect towards the NRK-52E cells and VnCE was found to be the most cytotoxic towards the NRK-52E cells which demonstrated the strongest growth inhibitory effect (lowest LD₅₀ value). The chloroform-soluble extract of the leaves of *V. negundo* isolated the flavone vitexicarpin also exhibits anticancer property²⁹. Therefore, to understand the mechanism of cell death responsible for growth inhibitory action VnCE was selected for further studies. Mode of action of many anticancer drugs depends on their capacity to induce apoptosis^{30,31}. In the intrinsic pathway DNA fragmentation marks the start of apoptosis, or programmed cell death, which is associated with the contribution of a specific caspase-activated enzyme. Activation of this enzyme by a variety of chemicals triggers cleavage of the whole DNA to produce small nucleosomal fragments of specific size of 180–200 bp

giving a ladder-like appearance. Cell death via apoptosis is used as a biochemical hallmark. The DNA fragmentation was used to examine the apoptosis effect of VnCE extract on NRK-52E cells. Apoptosis is a vital procedure in human growth, protection and tissue homeostasis³². It can be enhanced in cells through many mechanisms. This may be due to sequence communication between the genes, caspases and death receptors of extrinsic and intrinsic pathways³³. Apoptotic effect of sodium para-hydroxybenzoate tetrahydrate (SPHT) isolated from *V. negundo* leaves has been studied on colon cancer cell-lines³⁴. Ethanol extracts of leaves of *V. negundo* is reported to induce apoptosis in human breast cancer cell line (MCF-7)³⁵. Antiproliferative and apoptotic activity from ethylacetate extract of *V. negundo* has been studied in lung cancer cell line³⁶. Chloroform fraction increased the transcripts of almost all the genes namely, BCL-2, BCL-XL, SOD, Foxo and BAX by several fold. In mitochondrial pathway of apoptosis Bax and Bcl-2 are the two main molecules inter-dependently triggered by p53, leading to release of cytochrome c followed by apoptosis, which may be indirect activation of caspases. TGF- β gene regulation is specific to cell-type, such as dermal fibroblasts, rat intestinal epithelial cells, cervical cancer cells and corneal epithelial cells³⁷⁻⁴⁰. The expression level of TGF gene was decreased. Considering the dual role of TGF- β on transformed and normal cells some where it confers apoptosis and inhibition of growth to normal epithelial cells but helps in metastasis of cancer cells⁴¹⁻⁴⁴. Inhibition of SOD causes accumulation of cellular O₂⁻ and leads to free-radical-mediated injury to mitochondrial membranes, the release of cytochrome c from mitochondria and apoptosis of the cancer cells.

One of the main findings of this study is that *V. negundo* exhibits an antiproliferative effect on NRK-52E cells. Phenolic acids, proanthocyanins, anthocyanins and flavonols are important polyphenolic constituents in *V. negundo* sps. We also observed that the phenolic compound present in the extract might be responsible for the antiproliferative activities of *V. negundo* against cancerous cells.

Conclusion

In this study, it was found that chloroform extract of *V. negundo* was capable of inducing growth-suppressive and apoptotic effects in NRK-52E cells, suggesting that VnCE possess cytotoxic activity. Elaborate studies of the detailed mechanism underlying its apoptotic action

are necessary. Lastly, *in vivo* study needs to be done for the effective commercialization of this plant to profit pharmaceutical and the food industries.

Conflict of interest

The authors have declared that no conflict of interest exists.

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