

***In vitro* Antioxidant and Cytotoxic Effects of Methanol Extracts of *Vitex negundo*, *Lantana camara*, *Bauhinia variegata* and *Bauhinia racemosa* on Human Cancer Cell lines**

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Badgujar, et al.: *In vitro* Antioxidant Properties and Cytotoxic Effect of Selected Medicinal Plant Extracts

In current study, the phytochemical, antioxidant and cytotoxic effects of methanol extracts of *Vitex negundo*, *Lantana camara*, *Bauhinia variegata* and *Bauhinia racemosa* were reported. Methanol extract of medicinal plants (*V. negundo*, *L. camara*, *B. variegata* and *B. racemosa*) were extracted using different extraction methods and then tested for their antioxidant activity by 2,2-diphenylpicrylhydrazyl, superoxide scavenging method, chelation of metal ions and cytotoxic potency using MTT assay on human cancer cell lines. In different extraction methods, highest yield was obtained with hot extraction method. Total phenolic and flavonoid contents were found higher in methanol extract of *V. negundo* having 173.3 ± 1.54 mg gallic acid equivalent/g DW and 24.41 ± 1.5 mg quercetin equivalent/g DW contents, respectively. Leaves of *V. negundo* have maximum IC_{50} value of 73 ± 2.76 μ g/ml and 350 ± 1.23 μ g/ml in 2,2-diphenylpicrylhydrazyl and iron chelating activity compared to all other methanol extracts. *B. racemosa* leaves have higher superoxide scavenging effect with IC_{50} value of 223 ± 1.23 μ g/ml. *V. negundo* and *L. camara* leaves showed pronounced cytotoxic effect against HELA and KB human cancer cell lines with LD_{50} value of 222 ± 3.35 and 188.69 ± 1.4 μ g/ml, respectively. The present study shows that *V. negundo* and *L. camara* may be a probable source of natural antioxidant and as an anticancer drugs.

Key words: Cancer, superoxide scavenging activity, metal chelating activity, MTT assay, DPPH assay

Cancer is largely an uncontrolled cell proliferative disease in humans and currently there is a significant scientific and commercial interest in discovering new antitumor drugs from natural sources^[1]. The possibility of using natural products as anticancer agents was acknowledged in 1950s by the U.S. National Cancer Institute (NCI), which was involved in the discovery of new natural products as anticancer agents^[2]. This is the second most common disease after cardiac disorders leading to high rates of mortality in the world^[3]. It is believed that in the upcoming years the number of cancer patients would increase in the emerging and underdeveloped countries up to 70% to become an issue of serious concern. In India, lung, breast, colon, rectum, stomach and liver cancers are frequently detected^[4-6].

Plants have been used as medicines from thousands of years and people rely on plant products for their

primary health^[7]. These medicines initially were taken in the form of crude drugs such as powders, teas, poultices, tinctures and other natural remedies^[7,8]. Secondary metabolites present in plants among which phenolic compounds form the groups of secondary phytochemicals, which occur in high amounts and been found to be responsible for antiinflammatory, antiallergic and anticancer effects^[9,10]. Use of herbal drug as an alternate form in health care is increasing and screening of plants for bioactive compounds became an essential source of cancer-related drugs^[11].

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Hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (HO^\bullet) are some spontaneous oxygen species that are natural by-products of human metabolism. They are hazardous in high amount and interfere with proteins, enzymes, RNA and DNA, leading to tissue injury and degenerative diseases^[12,13]. Mammalian body has certain protection mechanisms to combat and reduce oxidative injury. Eating of food products rich in antioxidant phytonutrients especially flavonoids and other polyphenolic compound is beneficial for human health^[14,15]. Crude plant extracts with potentially useful biological properties have been used for further detailed pharmacological investigations through *in vitro* screening methods. The medicinal plants selected for the present investigation, which included leaves of *Vitex negundo*, *Lantana camara*, *Bauhinia variegata* and *Bauhinia racemosa* have been used in the traditional system because of their possible health endorsing and pharmacological attributes. The present study was carried out to study antioxidant and cytotoxic activity of crude methanol extracts.

MATERIALS AND METHODS

Selected plant materials were collected from Pune in August 2013 under close supervision and all plant materials were authenticated in the Botany department, Pune.

Preparation of extracts and stock solutions:

The selected plants were washed, shade-dried and chopped in to fine pieces. Extractions were performed using cold maceration and Soxhlet methods. Soxhlet extractions of all selected plant powders were carried out using methanol and all methanol extracts were concentrated in a rotary evaporator at 45° and desiccated under low pressure. The plant powders were extracted thrice with methanol to obtain 3 different extracts, which were found to have no major variation in yield and content of phytoconstituents.

Using cold maceration method, the extracts of all plants were prepared in methanol at room temperature. Dried plant powder of 10 g was mixed with 100 ml solvent in 250 ml flasks and kept on shaker for 24 h and then it was allowed to stand for 4 h to settle down the plant materials. Thereafter, supernatant was filtered and collected. The solvent was evaporated at 45° in rota evaporator (Heildolph, Germany). Yield was calculated for both extraction methods by Eqn. 1: extraction yield (%) = (weight of the dried extract)/(weight of original plant sample) $\times 100$. In airtight bottles dried extracts

were stored at -20° for further studies. Dimethyl sulfoxide (DMSO) was used to dissolve each extracts and sterilized using 0.22 μm syringe filters (Axiva, Scichem Biotech) for further use.

Total phenolic content:

Total phenolic content was determined using a method reported by Savitree *et al.*^[16] with certain modifications. Standard curve of gallic acid was plotted by mixing Folin-Ciocalteu reagent with methanol solution of gallic acid ($r^2=0.914$). About 0.5 ml of plant extracts dissolved in respective solvent was mixed with 0.1 ml Folin-Ciocalteu reagents (0.5 N) and the solution was kept for 15 min prior to the addition of 2.5 ml of saturated sodium carbonate. Absorbance was measured at 760 nm after 30 min of incubation. Gallic acid equivalents (GAE) mg/g of the dry extract was used to measure total phenolic content in the extract.

Determination of total flavonoid content:

For determination of total flavonoid content, a method described by of Pourmorad *et al.* was used with minor changes^[17]. Briefly, 1 ml of plant fraction and quercetin standard solution was mixed with 0.5 ml of potassium acetate (120 mM), followed by addition of 0.5 ml of aluminium chloride (1.2%) solution. The mixture was kept for an half an hour. At 415 nm the absorbance was measured instantly against prepared blank. Quercetin equivalents (QE) mg/g of the dry extract was used to measure total flavonoids content. A calibration curve was plotted using quercetin ($r^2= 0.955$).

2,2-diphenylpicrylhydrazyl (DPPH) activity:

Scavenging activity on DPPH free radicals by the extract was assessed according to the method reported by Blois^[18] with slight modifications. Briefly, 1.0 ml solution of the extract at different concentrations in ethanol was mixed with 1.0 ml of 0.2 mM DPPH in ethanol. The mixture was kept at room temperature for 30 min and the absorbance was measured at 517 nm. Ascorbic acid was used as the standard to compare the result. The DPPH activity of the sample extract was presented as IC_{50} value and also represented that the concentration of sample essential in reduction of DPPH radicals by 50%. DPPH radical scavenging activity was calculated using the following Eqn., percent scavenging = $((A_c - A_s)/A_c) \times 100$, where, A_c is the absorption of control, A_s is the absorption of tested extract.

Superoxide radicals scavenging assay:

The superoxide radical scavenging activity was measured by the reduction of nitro blue tetrazolium (NBT) according to a previously reported method with slight modification^[19]. Superoxide radical formed the non-enzymatic PMS/NADH system by the oxidation of NBT to purple formazan^[20]. Superoxide radical formed in 3 ml reaction mixture containing sodium phosphate buffer (20 mM pH 7.8) NADH (73 mM), NBT (50 mM), PMS (15 mM) and different concentrations of sample solution were mixed. The solution mixture was kept at 25° for 5 min. The formation of blue colour formazan was followed by observing the raise in absorbance at 560 nm against an appropriate blank. Reaction mixture without plant sample served as blank.

Metal chelating activity:

The metal ion chelating potential of plant extracts was measured by a standard method with slight modification^[21]. Briefly, in 1 ml of FeSO₄ solution (12.5 µM) was mixed with plant extracts of different concentration and ferrozine (75 µM) was added in this mixture to start the reaction. The mixture was kept for 20 min at room temperature and then the absorbance was determined at 562 nm. Citric acid was used as standard to compare the result. Following equation used to calculate metal chelating activity was calculated using following Eqn., chelating effect (%) = $(A_c - A_s) / A_c \times 100$, where, A_c is the absorbance of control and A_s is the absorbance of sample.

Antiproliferative assay:

Hela (cervical cancer) and KB (oral cancer) cell lines were procured from National Centre for Cell Science (NCCS) Pune, used to study antiproliferative activity. Cells were grown in DMEM-F₁₂ (Sigma, Germany), having 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin solution. Cells were incubated in CO₂ incubator containing 5% CO₂ at 37° temperature.

Cytotoxic activity:

In MTT assay, cytotoxicity of methanol extracts of

all selected plant extracts were determined on Hela (cervical cancer) and KB (oral cancer) cells depended on the reduction of MTT dye (Sigma, Germany) by calculating the quantity of insoluble formazan produced in live cells. Briefly, in 96-well plate the cells were inoculated in 100 µl at number varying from 10 000-20 000 cells/well based on cell growth characteristics. Then the 96-well plates were kept at 37° and 5% CO₂ humidified incubator for 24 h before addition of plant extracts. Furthermore, methanol extracts dissolved in 2% DMSO were added into a 96-well plate at different concentration (50, 100, 200, 400, 500 µg/ml) by diluting it in medium. After 24 h, in each well 20 µl of MTT reagent (5 mg/ml) was added. After 4 h of incubation 100 µl DMSO was added, which was followed by 1 h of incubation at 37° to dissolve formazan crystals. Using an ELISA plate reader (Promega, USA) absorbance was taken at 570 nm and background wavelength at 750 nm. Following equation was used to calculate the percentage of cell viability: cell viability (%) = $(OD \text{ sample (mean)}) / (OD \text{ control (mean)}) \times 100$, where, OD is the optical density; OD of sample: mean absorbance of treated cells and OD of control: mean absorbance of control cells. The dose-response graph was plotted and from the graph (LD₅₀) value was calculated^[22,23]. Concentration that required inhibiting 50% of cell growth was used as a stricture for cytotoxicity.

RESULTS AND DISCUSSION

In the present study, methanol extracts of four selected plants were evaluated for their antioxidant and anticancer activities using DPPH, superoxide scavenging, metal chelating activity and MTT assay. The antioxidant and anticancer activity of medicinal plants were not similar due to the different array of bioactive components present in the plants that might be responsible for their activity. Table 1 showed the yield of crude methanol extract of different plants by hot and cold extraction method. Table 1 also summarized that maximum yield was obtained by hot extraction method compare to cold extraction method in all plants. The maximum yield was obtained in *L. camara* followed by *V. negundo*, *B. racemosa*

TABLE 1: EXTRACTION YIELD (%) OF DIFFERENT PLANTS THROUGH HOT AND COLD EXTRACTION METHODS

Plants name	Parts	Solvent	Hot extraction yield (%)	Cold extraction yield (%)
<i>Vitex negundo</i>	Leaves	Methanol	23.7%	11.2%
<i>Lantana camara</i>	Leaves	Methanol	45.8%	8%
<i>Bauhinia variegata</i>	Leaves	Methanol	18%	16%
<i>Bauhinia racemosa</i>	Leaves	Methanol	18.4%	12.3%

and *B. variegata*. Phenolic compounds are commonly found in both edible and non-edible plants and plant parts. Flavonoids represented a largely well-known group of polyphenolic plant secondary metabolites. These compounds were reported to play a significant role in many activities including free radical scavenging properties^[24]. Total phenolic and flavonoid contents in methanol extracts of different plants were exhibited in Table 2. Table 2 also summarized that methanol extract of *V. negundo* leaves have the maximum total phenolic (173.3±1.54 mg GAE/g dry extract) and flavonoid content (24.41±1.54 mg QE/g dry extract). Phenols and flavonoids are very important constituents of plants and play a significant role in scavenging activity. These components contain hydroxyl group, which do not directly take part in plants antioxidant activity^[25]. In protection against oxidative stress, phenolic compounds like flavonoids play important roles. Due to functional hydroxyl groups present in these groups, they showed antioxidant activity by chelating metal ions or scavenging free radicals^[26,27]. Metals ion chelation could be essential in the avoidance of free ion generation, which harms target biomolecules^[28].

DPPH free radical scavenging activity was carried out with the methanol extracts of all selected plants and the results were presented in fig. 1. Different plants for

free radicals of DPPH showed significant scavenging activities mentioned in Table 3. The DPPH scavenging activity was increased as concentration was increased. These results provide a comparison of the antioxidant activity directly with ascorbic acid. *V. negundo* exhibited the highest scavenging activity (IC_{50} : 73±2.76 µg/ml) followed by *L. camara*, *B. variegata* and *B. racemosa*. The influence of antioxidants on scavenging of DPPH radical was supposed to be owed to their hydrogen-donating capacity. In the presence of oxygen radicals formed ($ROO\cdot$, $HO\cdot$, $O_2^{\cdot-}$) are extremely reactive species vary in their lifespan and organic properties.

Superoxide ($O_2^{\cdot-}$) radical is known to be very dangerous to cellular components as a precursor of the extra reactive oxygen species, cause damage to tissue and because of that various diseases occur. *B. racemosa* leaves (223±3.21 µg/ml) possessed the highest superoxide activity while *L. camara* showed the lowest superoxide radical scavenging activity (487±3.12 µg/ml). Results are presented in fig. 2 and Table 3.

The IC_{50} values for metal chelating ability of the *V. negundo*, *L. camara*, *B. variegata* and *B. racemosa* are 350±1.23, 355±2.32, 430±2.44 and 512±3.34 µg/ml, respectively, while that of EDTA was 288±3.23 µg/ml as shown in Table 3. Fe^{2+}

TABLE 2: THE TOTAL PHENOLICS AND FLAVONOIDS CONTENT IN DIFFERENT METHANOL EXTRACTS

Plants	Total phenolic content (mg GAE/g dry extract)	Total flavonoids content (mg QE/g dry extract)
<i>Vitex negundo</i>	173.3±1.54	24.41±1.54
<i>Lantana camara</i>	144.7±1.34	12.44±2.85
<i>Bauhinia variegata</i>	112.0±2.43	20.73±1.43
<i>Bauhinia racemosa</i>	61.5±1.29	21.59±1.61

Total phenolic content analysed as GAE mg/g of dry weight of sample; values are the mean±SD of triplicates analysis, Total flavonoid content analysed as QE mg/g of dry weight of sample; values are the mean±SD of triplicates analysis, SD is standard deviation, GAE is gallic acid equivalents, QE is quercetin equivalents

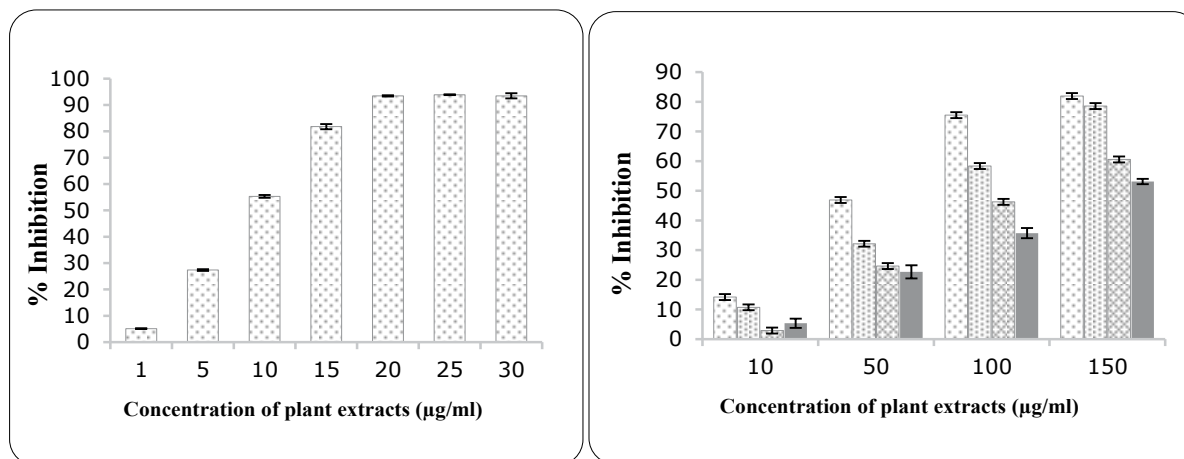


Fig. 1: DPPH scavenging assay

(A) DPPH scavenging assay of standard ascorbic acid, ascorbic acid. (B) DPPH scavenging assay of crude methanol extracts of selected four plants, *V. negundo*; *L. camara*; *B. variegata*; *B. racemosa*

ferrozine complex formation was inhibited by leaf extracts of all four plants as indicated by the formation of colour decrease in dose-dependent manner (fig. 3). A main mechanism behind antioxidant action is reduction of the Fe^{2+} ion, which indicate electron-donating activity of extract. Ferricyanide complex is converted to ferrous form in the presence of antioxidants in the extracts. Oxidative stress mediated because formation of ROS via Fenton chemistry, which may lead to

human diseases. Free radicals are generated due to metal ions leads to lipid peroxidation and DNA damage^[29]. These harmful effects of metal ions may be stuck by metal deactivation and chelation. A Ferrozine form complex with Fe^{2+} , but the complex formation is not occurs in the presence of metal chelating agents. Many reports on metal chelation by other plant extracts also authenticate these findings^[29,30]. Previous studies reported that oxidized form of metal ion stabilized

TABLE 3: IC₅₀ VALUE OF DIFFERENT PLANTS FOR VARIOUS ANTIOXIDANT SYSTEMS

Plants	DPPH radical scavenging assay activity [IC ₅₀ (μg/ml)]	Super oxide radical scavenging assay [IC ₅₀ (μg/ml)]	Iron chelating activity assay [IC ₅₀ (μg/ml)]
<i>Vitex negundo</i>	73±2.76	465±1.02	350±1.23
<i>Lantana camara</i>	89.24±2.21	487±3.12	355±2.32
<i>Bauhinia variegata</i>	117±3.33	485±2.72	430±2.44
<i>Bauhinia racemosa</i>	139±2.66	223±1.23	512±3.34
Standard	Ascorbic acid 11.40±0.33	Gallic acid 93.115±1.23	EDTA 288±3.23

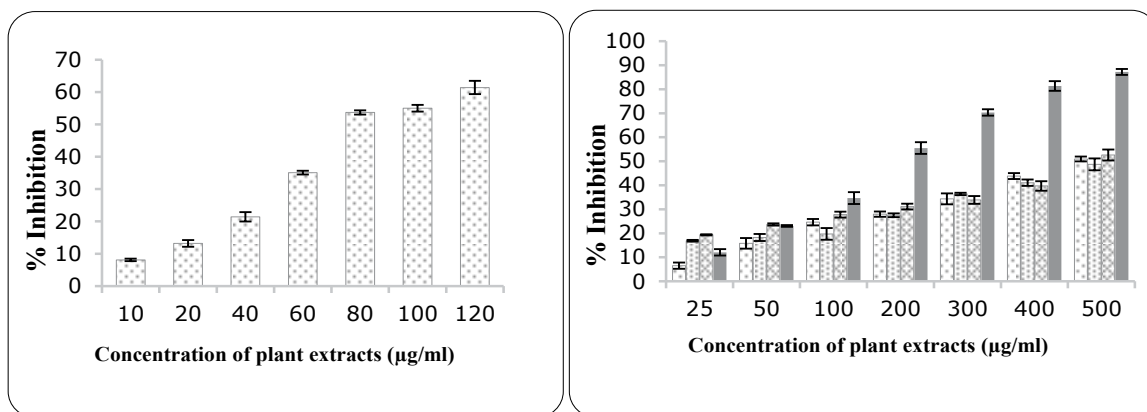


Fig. 2: Superoxide scavenging activity

Superoxide scavenging activity of extracts was measured at different concentrations (50-500 μg/ml), and absorbance was recorded at 560 nm. The results are expressed as mean±SEM of three replicates. (A) Superoxide scavenging activity of standard gallic acid, (B) Superoxide scavenging activity of crude methanol extracts of selected four plants, *V. negundo*; *L. camara*; *B. variegata*; *B. racemosa*

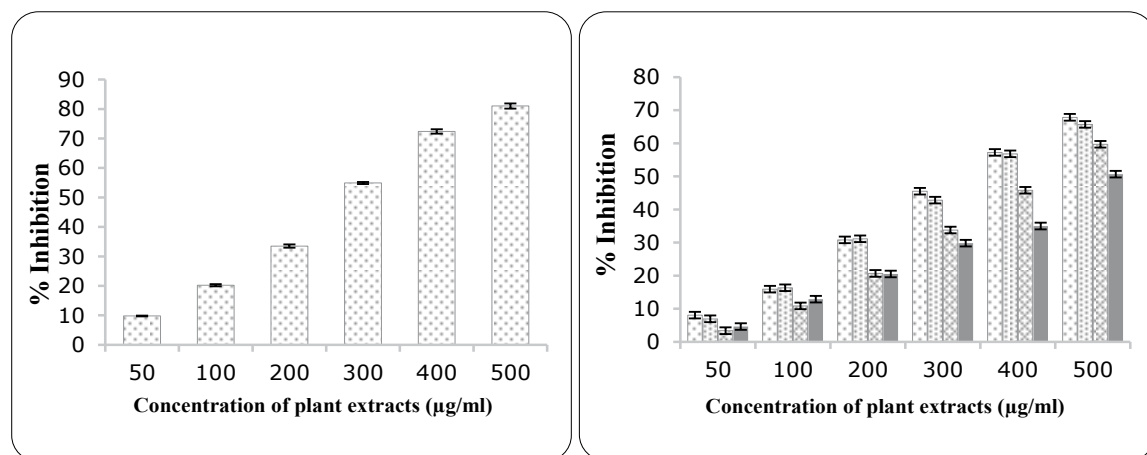


Fig. 3: Metal ion chelating ability

Chelating activity of extracts was measured at different concentrations (50-500 μg/ml), and absorbance was recorded at 562 nm. The results are expressed as mean±SEM of three replicates. (A) Metal chelating activity of gallic acid, (B) metal chelating activity of crude methanol extracts of selected four plants, *V. negundo*; *L. camara*; *B. variegata*; *B. racemosa*

by chelating agents because they reduce the redox potential therefore they were considered as efficient as antioxidants^[31].

The crude methanol extracts of four plants were tested for cytotoxicity against Hela (cervical cancer cell line) and KB cells (oral cancer cell line). Results showed that crude methanol extracts inhibited the viability of Hela and KB cells with their LD₅₀ values, where shown in Table 4. Crude methanol extracts were tested at concentration range (500, 400, 200, 100 and 50 µg/ml). As shown in figs. 4 and 5, these extracts show significant concentration-dependent inhibition on propagation and viability of the Hela cells and KB cells. The results exhibited that cytotoxic activity of *V. negundo* extract on Hela cells was more potent than all other extracts, similarly *L. camara* was more effective on KB cells. LD₅₀ values for all plants in both cell lines were summarized in Table 4. Many anticancer drugs derived from plants were useful clinically include vinblastine irinotecan, topotecan and paclitaxel^[31,32]. Several biological processes and biological interactions occur between flavonoids, phenolic compounds or polyphenols, with enzymes, proteins that make them toxic to the cell or inhibit the growth of cells^[33]. Several reports indicated that the cytotoxicity and anticancer properties of natural plants were due to the existence of flavonoid compound and it play a significant function in chemoprevention through showing its effects on transduction of signal

and angiogenesis of cells^[34,35]. In 1998, Bravo reported that polyphenolic compounds, including flavonoids are important for cancer prevention^[35]. It was reported in previous study that methanol extracts from *V. negundo* had high concentrations of polyphenolic compounds^[36]. This could have contributed to the cytotoxicity of the cells to methanol *V. negundo* and *L. camara* extract.

The current study showed that the presence of phytochemicals in selected plant leaf extracts has effective cytotoxic activity against cervical and oral cancer cells. The free radical scavenging ability probably is one of the mechanisms by which herbal medicines exhibit higher antioxidant activity. In addition crude methanol extracts have potential to fight against oxidative damage because of its DPPH activity, superoxide scavenging activity and iron binding ability. Knowing the exact compounds responsible for the plant's anticancer activity will be useful in finding new anticancer agents. Lastly, *in vivo* and clinical evaluations need to be performed for the successful commercialization of this plant to benefit both the food and pharmaceutical industries.

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TABLE 4: LD₅₀ VALUE OF DIFFERENT PLANTS ON HELA AND KB CANCEROUS CELL LINES

Plants	LD ₅₀ value on HELA cells (µg/ml)	LD ₅₀ value on KB cells (µg/ml)
<i>Vitex negundo</i>	222±3.35 µg/ml	247±2.33 µg/ml
<i>Lantana camara</i>	229±4.23 µg/ml	188.6±1.4 µg/ml
<i>Bauhinia variegata</i>	371.8±3.12 µg/ml	299.23±3.55 µg/ml
<i>Bauhinia racemosa</i>	442.61±2.1 µg/ml	334.65±2.34 µg/ml

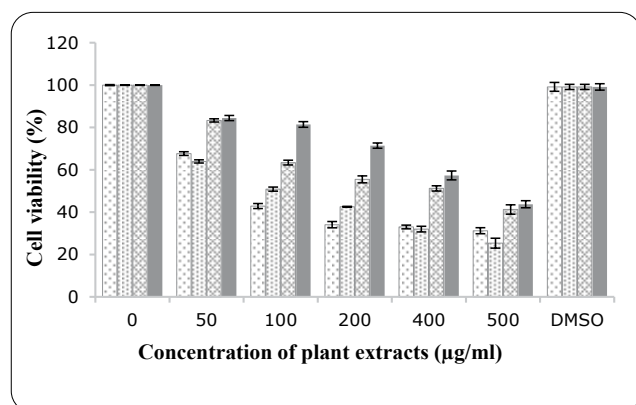


Fig. 4: Cytotoxic effect of the four methanol extracts against HeLa cell line using MTT assay
 ▨ *V. negundo*; ▩ *L. camara*; ▤ *B. variegata*; ▥ *B. racemosa*

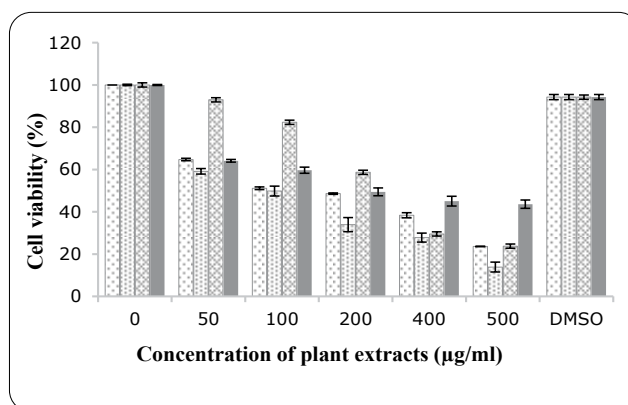


Fig. 5: Cytotoxic effect of the four methanol extracts against KB (Oral cancer) cell line using MTT assay
 ▨ *V. negundo*; ▩ *L. camara*; ▤ *B. variegata*; ▥ *B. racemosa*

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Conflict of interest:

All authors declare no conflict of interests.

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