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IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF SELECTED MEDICINAL PLANTS AGAINST CANCER CELL LINES

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ABSTRACT

Natural compounds have a long history of being used as anticancer and antioxidant agents. This work aimed to find out about the phytochemical contents, antioxidant activity, and *in vitro* cytotoxicity of *B. variegate*, *L. camara, C. papaya,* and *V. negundo* against HeLa and KB cell lines. The plant leaves were dried, powdered, and extracted by cold percolation with methanol for 24h. Total flavonoid content and total phenolic content were analyzed by phytochemical screening. All extracts assessed for antioxidant activity were examined against ABTS and the DPPH radical. Using a cytotoxicity assay, they tested the crude extracts against HeLa and KB cell lines. *V. negundo* had the highest TFC, measuring 58.19±2.04 mg of quercetin equivalent, and *L. camara* had the highest TPC, measuring 191.7±2.17 mg of gallic acid equivalent. The extracts exhibited an IC_{50} value of 53.13±2.24 µg/ml of the *C. papaya* methanolic extract compared to the IC_{50} value of 19.65±0.88 µg/ml, as shown by the Ascorbic acid. *L.camara* leaves have a higher superoxide scavenging effect with an IC_{50} value of 39.5±1.15 µg/ml compared to the IC_{50} value of 13.21±2.13 µg/ml, as shown by the BHT. The *B. variegate* methanolic extract exhibited effective cytotoxicity with an LD_{50} of 78.91 µg/ml for HeLa cells. *C. Papaya* methanolic extract exhibited effective cytotoxicity

with an LD_{50} of 97.96 µg/ml for KB cells. With the data on the total flavonoid, phenolic, and cytotoxic activity of *B. variegate* and *C. papaya* leaves extracts, these species could be evaluated as possible sources of anticancer compounds.

Keywords: Medicinal plants, Total phenolic and flavonoids, Antioxidant activity, Cytotoxic activity on HeLa and KB cell lines

INTRODUCTION

Cancer is the leading cause of death worldwide, providing a significant public health issue that has motivated a search for anticancer agents, particularly those derived from natural resources, which has become a primary scientific research focus **[1]**. Cancer's characteristics are transformation, apoptotic dysregulation, multiplication, invasion, tumorigenesis, and metastatic spread **[2]**. Cancer treatment has become a serious difficulty because no single medication is effective for all types of cancer. The medical industry has seen significant progress in curing diseases, particularly those that are life-threatening, such as diabetes, cancer, and cardiovascular disease **[3]**.

Natural products are essential in the origination of novel pharmacological molecules **[4]**. Traditional remedy has been explored and researched to develop a cancer-fighting therapy **[5]**. Plants have several phytochemicals that can act as natural antioxidants, such as flavonoids, tannins, phenolic diterpene, and polyphenolic acids, which can have antioxidant properties with potential health advantages **[6]**. Bioactive compounds in plant extracts have a wide range of targets and modes of action **[4]**. Plant-based phytochemicals can change tumor cytogenesis by inhibiting or modifying epigenetic processes **[7]**. Researchers are increasingly consulting traditional medicine for fresh insights into how to create better drugs **[8]**. These plant-derived chemicals have clinical importance and could be developed into cancer-fighting medications in the upcoming time **[5]**. According to recent synthetic chemistry studies, natural molecules have an excellent potential to give better aspects of therapy and prevention of many diseases **[9]**. Traditional medicine has been found to have anticancer properties in human cancer cell lines, with some promising results **[10]**.

Oxidants are everywhere in biological systems and can harm membranes, proteins, and nucleic acids **[4]**. Oxidative stress is defined as the presence of oxidants and ROS **[11]**. Several promoters enhance cell proliferation, recruit inflammatory cells, and increase the ROS level, which causes oxidative DNA damage and reduces DNA repair **[12]**. ROS exposure to

biological components causes lipid and protein oxidation and changes in signal transduction pathways that increase cancer risk **[13]**. Antioxidants are essential in determining the medicinal impact of plants and making them effective anticancer drugs **[5]**. Extracts from numerous plants, like their bark, fruits, seeds, leaves, and roots, must have been widely researched for their antioxidant activities **[14]**. Plants with antioxidant-rich bioactive chemicals are particularly effective at preventing cancer by inducing apoptosis **[15]**. Antioxidants are obtained from natural or artificial sources such as quenching singlet oxygen, radical chain breakers, and chelating pro-oxidative metal ions.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods are quick procedures that use both hydrogen and single electron transfer. They are also less expensive and easier to develop in the lab **[1]**. The DPPH assay is a straightforward method for determining the proportion of antioxidants in any source. Only metabolically active cells reduce MTT, which helps to check cell viability **[5]**.

As an effect, we concentrated on establishing a link between total phenolic-flavonoid content and antioxidant activity with cytotoxic activity on human cancer cell lines using methanolic extract (ME) from *B. variegate*, *L. camara*, *C. papaya*, and *V. negundo*.

MATERIALS AND METHODS

Plant materials

B. variegate, *L. camara*, *C. papaya*, and *V. negundo* were the four medicinal plants chosen for the study.

Preparation of plant extracts

The plant materials were mechanically ground into powder after being sun-dried. The 10 gm of powdered plant material was used for extraction. All the plant extracts were prepared using the cold percolation method with 250 ml solvents **[6]**. The plant extracts were filtered, and the solvent was dried at 45˚C in a rotary evaporator and further dried overnight at very low pressure. The dried plant extracts were kept in airtight vials for subsequent research and stored at -20 °C. Stock solutions of the plant extracts were prepared at 1mg of all extracts in 1 ml DMSO. The extract solution was later sterilized using 0.22µm Syringe filters and used as a stock solution for subsequent assays. To acquire a range of required concentrations of plant extracts, the stock was serially diluted with a cell culture medium. All solvents worked in this research are analytical grade.

Determination of phytochemicals

Determination of total phenols

The TPC was determined using the Folin-Ciocalteau method according to Perera MG *et al*. Briefly, an aliquot of 0.2ml diluted

extract solution was mixed with 1.0 ml Folin-Ciocalteau reagent and allowed to react at 30ºC for 5 minutes in the dark condition. After that, 1 ml of saturated Na_2CO_3 solution was added to the reaction mixture and left to stand for 2 hours. A blue complex detected at 760 nm by visible-light spectrometry is created when polyphenols react with the Folin-Ciocalteu reagent in the plant extract. Gallic acid was used to generate a standard curve, and the final results were expressed as mg Gallic acid equivalents (GAE) (mg/g of extracted substance) **[13]**.

Determination of total flavonoids

A spectrophotometric technique utilizing aluminum chloride was applied to check the flavonoid content according to Paudel, M. R. *et al*. 1 ml of methanol was mixed with 4 mg/ml quercetin solution to make a standard sample. The mixture was prepared with 0.5 ml of plant extract (1 mg/ml stock concentration in methanol), 0.1 ml of 10% W/V solution of aluminum chloride, 0.1 ml of 1 Molar potassium acetate solution, 1.5 ml methanol, and then the total volume was makeup to 5 ml by adding distilled water. After 30 minutes of RT incubation, the absorbance of both reaction solutions was measured at 415 nm with a UV-visible light spectrophotometer. A standard curve for quercetin was used to compare the total flavonoid content, and the overall flavonoid content was demonstrated in micrograms of quercetin equivalent **[15]**.

Antioxidant activity

DPPH free radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used to determine the plant extract's scavenging capacity. When DPPH, a stable free radical, accepts an electron or hydrogen radical, it transforms into a stable diamagnetic molecule. The antioxidant activity of a compound is frequently assessed using the DPPH radical as a substrate. When DPPH absorbs an electron from an antioxidant molecule, it becomes decolored and may be quantitatively assessed by changes in absorbance. The concentration and intensity of the antioxidants are correlated with the degree of color change. In 100% ethanol, a stock solution of DPPH was produced at a 10 mg/ml concentration. The positive control was ascorbic acid. After 30 minutes of incubation at 37°C in the dark condition, the O.D (optical density) was determined at 517 nm. The half-maximal antioxidant concentration was calculated using the obtained dosage response curves (IC_{50}) **[6]**.

$$\% \text{ DPPH scavenging activity} = \frac{\text{Control O.D} - \text{Sample O.D}}{\text{Control O.D}} \times 100$$

ABTS decolorization assay

According to Herrera Calderon O *et al*., the free radical decolorization assay uses

ABTS. The pre-formed radical monocation of ABTS was produced by combining a 7.45 mM potassium persulfate solution with an ABTS solution. The mixer was left at RT in the dark for 15 hours. Separately, the plant extract dissolves in methanol to give a 1 mg/ml concentration. At 734 nm, the absorbance was measured. To test the free radical scavenging activity, the BHT (Butylated hydroxytoluene) equal antioxidant capacity, defined as mMol of BHT per gram of material, was utilized **[1]**. In each test, appropriate solvent blanks were used.

% ABTS scavenging activity= Control O.D − Sample O.D × 100

Control O.D

Cytotoxicity assay

Cell culture medium and cell lines

The National Centre for Cell Science (NCCS) in Pune provided the human cervical cancer cell line (HeLa) and the oral cancer cell line (KB). The Dulbecco's Modified Eagle Medium (DMEM) contains 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 mg/ml of streptomycin used to maintain the cancer cell lines. At 37ºC, the cells were cultivated in an incubator with 5% CO_2. MTT assay was used to assess the *in vitro* response to the extract using a growth inhibition assay. The cell culture process has been carried out under sterile circumstances inside a Class II biosafety cabinet.

Determination of cell viability by MTT assay

The cytotoxic potential was evaluated using the MTT assay **[16]**. Cytotoxic activity toward the HeLa and KB cell lines. The conventional procedure for hemocytometer cell counting was used after the Trypsinization of the HeLa and KB cell lines. 100 µl of fresh DMEM media with 10% FBS was used to seed cells in each well of 96-well microtiter plates coated with poly L-lysine. After that, cells were cultured for an overnight period at 37ºC and a 5% CO_2 overnight period to allow for attachment. INFINITE M NANO TECAN measured absorbance at a wavelength of 570 nm. For screening, the cells with 70-80 % confluency were treated with different doses of plant extracts. The media was replaced with a fresh supplemented medium containing the most active extracts (100 µl) and was tested for cytotoxicity at 50-1000 µg/ml concentrations in sequence to derive a dose-response curve. The cells were then cultured for 24 hours at 37ºC. Next to 24 hr of the treatment, the media was aspirated, and 20 µl of MTT solution, prepared at 5mg/ml in phosphate buffer saline, was added to every well and was incubated at 37ºC and 5% CO_2 for 4 hours. After incubation, 150 µl DMSO was added to every well and then incubated for 1 hr at

37ºC to conclude the dark blue formazan crystals resulting from MTT reduction. Triplicates of the test were run. The negative control and blank samples were present on each plate.

By differentiating the optical density (O.D) of the samples from a negative control, the cell viability (%) was calculated. The subsequent formula was applied to compute the cell viability percentage:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of control sample - Absorbance of treated sample}}{\text{Absorbance of control sample}} \times 100$$

O.D. stands for optical density. The cytotoxicity was determined using a concentration that was 50% of cell viability. The concentration that inhibited cell growth by 50% (LD_{50}) was identified using a dose-response curve.

RESULTS AND DISCUSSION

The yield of plant extraction

The yield of crude methanolic extract from medicinal plants using the cold extraction method is summarized in Table 1. The formula shown below was used to calculate the yield of various extracts. *L. camara* acquired the most significant yield, whereas *C. papaya* acquired the lowest yield.

$$\text{The yield of plant extracts (\%)} = \frac{\text{weight of the freeze-dried extract}}{\text{Weight of the original sample}} \times 100$$

Total phenolics and total flavonoid content

Phytoconstituents, including flavonoids and phenols, are essential for herbal plants' antioxidant action. In particular, phenolics and flavonoids, which are largely found in plants, have a range of biological activity, such as anti-neovascularization, antioxidant, anticarcinogenic, and anti-proliferation **[17]**.

The Folin-Ciocalteu assay is a colorimetric assay based on the electron transfer between the reagent and any phenolic component present. Flavonoids and phenols are substances that are beneficial to health **[1]**. **Table 2** displays the wide range of phenolic contents found in the plant extracts. Gallic acid standard curve (y = 0.008-0.263; R^2 = 0.9816) was used to calculate phenolic content. According to the results, *L. camara* methanolic extracts had the highest total phenolic content (191.7–2.17 g GAE/mg), and the *V. negundo* extract had the second-highest level of TPC. As per the study by Kumar *et al*., the phenolic content in the methanolic extracts of *L. camara* leaves was 232.99±15.97 mg GAE/g dry extract, which was higher than our obtained results **[18]**.

Flavonoid is secondary plant metabolites that play a role in preventing oxidative stress-related diseases in humans **[16]**. The total flavonoid content of plant extracts is

shown in **Table 2**. The linear regression equation of the calibration curve [y = 0.0388 - 0.327 (R2 = 0.9708)] derived from varied quercetin concentrations was used to calculate the total flavonoid content of each extract. Extracts of mg quercetin equivalent/g were used to determine the results **(Table 2)**. *V. negundo* methanolic extracts showed a very high total flavonoid content (58.19±2.04 µg of QE/mg), and the second-highest amount of TFC was found in the *B. variegate* extract. As per the study by Koirala *et al*., the flavonoid content in the methanolic extracts of *V. negundo* leaves was 123.78 mg QE/g dry extract, which was higher than our obtained results **[19]**.

According to common assumption, there is a direct relationship between total phenol content and antioxidant activity, with plants with higher phenolic content thought to exhibit better antioxidant activity **[4]**. It can be suggested that a plant's phenolic content may be a reliable predictor of its antioxidant ability. The important biomolecules (such as DNA, lipids, and proteins) involved in many diseases are protected from oxidative damage by phenolic compounds, which act as antioxidants **[9]**. Flavonoids are a subclass of phenolic compounds; therefore, it was expected that lower flavonoid content values than phenolic compounds **[20]**. The above results indicate that flavonoids and phenolics are important to the antioxidant and cytotoxic activity. Due to their pro-oxidant properties, phenolics and flavonoids are well recognized to have cytotoxic effects and induce apoptosis in cancer cell lines.

Antioxidant activity

Cancer and cardiovascular disease are chronic diseases that the antioxidant property helps to prevent. Natural antioxidants differ in terms of their structures, reactivity, types, and modes of action **[12]**. The antioxidant activity of the methanolic extracts was compared using the DPPH and ABTS assay. Thus, the free radical scavenging potentials of methanolic extracts at various concentrations were investigated, and the IC_{50} was calculated. Where the scavenging activity is dose-dependent, i.e., the scavenging ability increases as the concentration of plant extract increases. Table 3 shows the IC_{50} values calculated to evaluate the antioxidant activity further. The greater the free radical scavenging activity, the lower the IC_{50} value. The methanolic *C. papaya* extract (53.13±2.24µg/ml) had the highest inhibitory activity, whereas the *V. negundo* extract (296.80±1.98µg/ml) had the lowest. However, ascorbic acid has a more substantial radical scavenging capacity than the extracts, with an IC_{50} of 19.65±0.88 µg/ml. As per the study by Vuong *et al*., the DPPH scavenging activity in methanol

extract of *C. papaya* leaves was 96.44±4.58 µg/ml, which was lower than our obtained results **[21]**. The flavonoid concentration increased DPPH activity. Further isolation and characterization could be decided based on the of the findings.

A constant generation of the $ABTS^+$ radical mono cation is indicated by the chemistry of $ABTS^+$. When added, potassium persulfate transforms into its radical cation and absorbs light at 734 nm **[22]**. **Table 3** shows that ABTS radical scavenging activity indicated that the extracts inhibit the radicals depending on the concentration. Methanolic *L. camara* extract of leaves (39.5±1.15µg/ml) had stronger antioxidant activity than the other plant extracts but was lower than the standard, BHT (13.21±2.13µg/ml). As per the study by Kumar *et al*., the ABTS scavenging activity in methanol extract of *L. camara* leaves was 52.84±1.82 µg/ml, which was lower than our obtained results **[18]**. Some phenolic compounds could affect the strong ability to scavenge ABTS radicals **[16]**. Previous studies showed an excellent linear correlation between phenolic contents and antioxidant capacity.

Cytotoxicity assay

The MTT assay is a reliable, efficient, and sensitive colorimetric technique. This test depends on the mitochondrial enzyme succinate dehydrogenase, which turns tetrazolium ions into the colored product. The quantity of surviving cells is directly related to the formazan product produced because only metabolically active cells can split tetrazolium salts **[11]**. The study investigated the anticancer potential of *B. variegate, L. camara, V. negundo,* and *C. papaya* extracts on HeLa and KB human cancer cell lines. The extracts were used to screen the cells at 50-1000 µg/ml concentrations. The selectivity of the cytotoxic activity of the four tested extracts was determined by comparing the cytotoxic activity (LD_{50}) of each plant extract against each cancer cell line. Table 4 shows the extract's % growth inhibition of HeLa and KB cells. **Figures 3 and 4** show the results of the plant extracts cytotoxicity against the HeLa and KB cell lines, respectively. The cytotoxic properties of the plant extracts were often dose-dependent. The *B. variegate* extract showed the strongest cytotoxic effects against the HeLa cell line (LD_{50} = 78.91 g/ml), and the *C. papaya* extract showed the strongest cytotoxic effects against the KB cell line (LD_{50} = 97.96 g/ml). The extract of Nendran peel had an LD_{50} value of 120.6 g/ml against the MCF-7 cell line, according to a study by Durgadevi *et al*., **[6]**. Cytotoxic substances can trigger apoptosis or necrosis by activating various biochemical pathways, resulting in cell death **[9]**. More information on how these extracts affect

cytotoxic and apoptotic activity at the molecular level is still essential.

Table 1: Cold extraction yield of methanolic extract from plant leaves

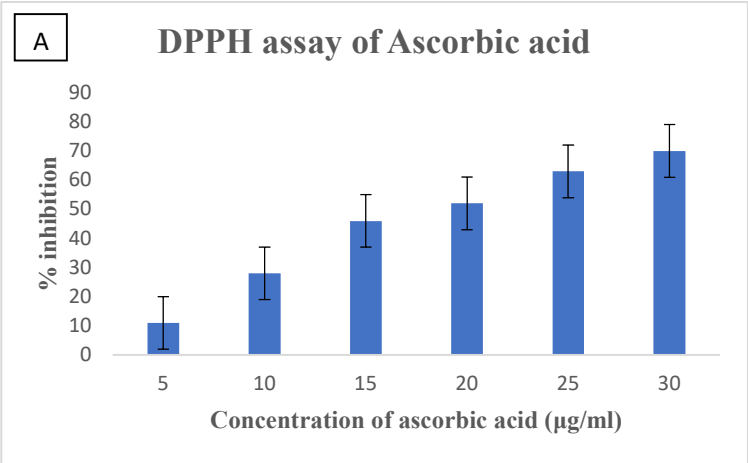
S. No.	Plant name	Yield (%)
1	*B. variegate*	10.5 %
2	*L. camara*	22.3 %
3	*V. negundo*	12.6%
4	*C. papaya*	9 %

Table 2: The total amount of phenolics and flavonoids in various methanolic extracts

S. No.	Plants name	Total phenolic content (mg gallic acid/gm dry extract)	Total flavonoid content (mg quercetin/gm dry extract)
1	*B. variegate*	78.10±1.86	35.71±3.54
2	*L. camara*	191.7±2.17	21.8±1.73
3	*V. negundo*	123.43±3.16	58.19±2.04
4	*C. papaya*	54.67±2.28	29.02±1.20

Table 3: Effect of plant methanolic extracts on antioxidant activity

Sr. no	Plants name	DPPH radicals IC_{50} Value(µg/ml)	ABTS radicals IC_{50} Value(µg/ml)
1	*B. variegate*	138.17±1.62	73.3±0.63
2	*L. camara*	197±2.63	39.5±1.15
3	*V. negundo*	296.80±1.98	95.06±3.43
4	*C. papaya*	53.13±2.24	48.78±2.33
5	Standard	19.65±0.88	13.21±2.13



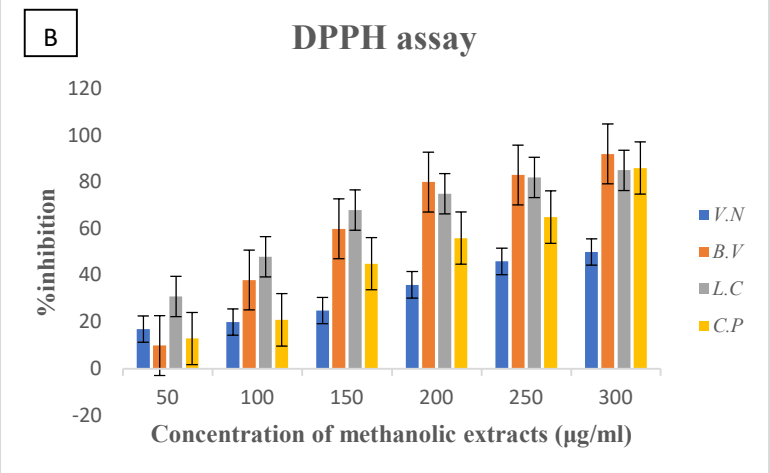
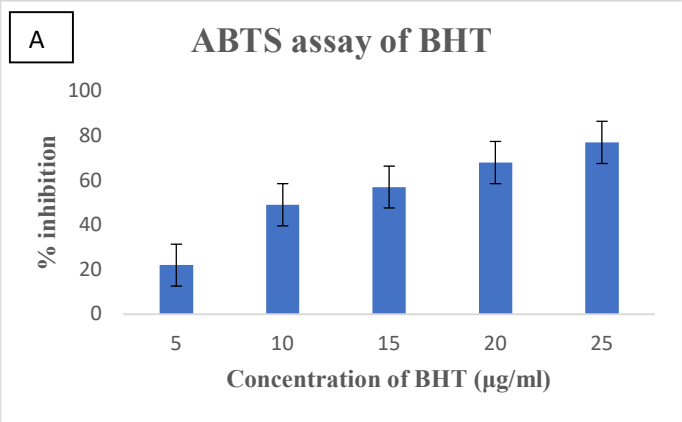


Figure 1: (A) DPPH scavenging activity of standard (Ascorbic acid). (B) Methanolic extracts of the plants *V. negundo* (V. N.), *B. variegate* (B. V.), *L. camara* (L. C.), and *C. papaya* (C.P) showed DPPH scavenging activity. All the values are presented as mean±SD of three replicates



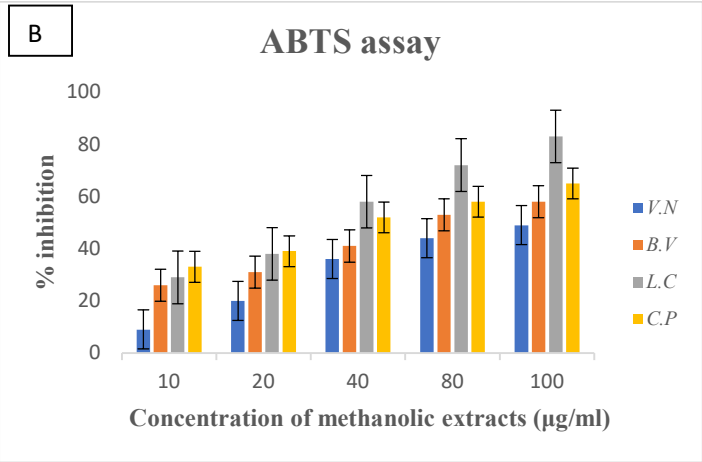


Figure 2: (A) ABTS activity of standard (BHT). (B) Methanolic extract of *V. negundo* (V. N.), *B. variegate* (B. V.), *L. camara* (L. C.), and *C. papaya* (C.P) showed ABTS scavenging activity. All the values are presented as mean±SD of three replicates

Table 4: LD_{50} value of different plant extracts on HeLa and KB cancer cell lines

Sr. no	Plants name	LD_{50} value on HeLa cells (µg/ml)	LD_{50} value on KB cells (µg/ml)
1	*B. variegate*	78.91	120.0
2	*L. camara*	121.8	195.9
3	*V. negundo*	105.7	137.5
4	*C. papaya*	139.4	97.96

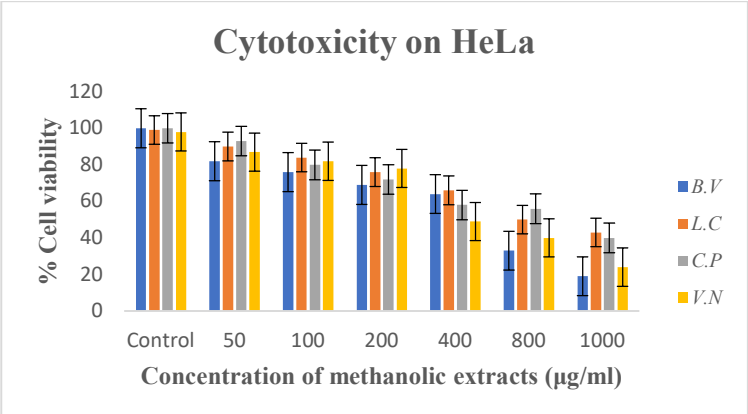


Figure 3: Cytotoxicity from methanolic extracts of the *B. variegate* (B. V.), *L. camara* (L. C.), *C. papaya* (C. P), and *V. negundo* (V. N.), against HeLa cells. Various concentrations (50-1000 µg/ml) of methanolic extracts of plants were treated for 24 hours. Triplicates of each experiment were performed.

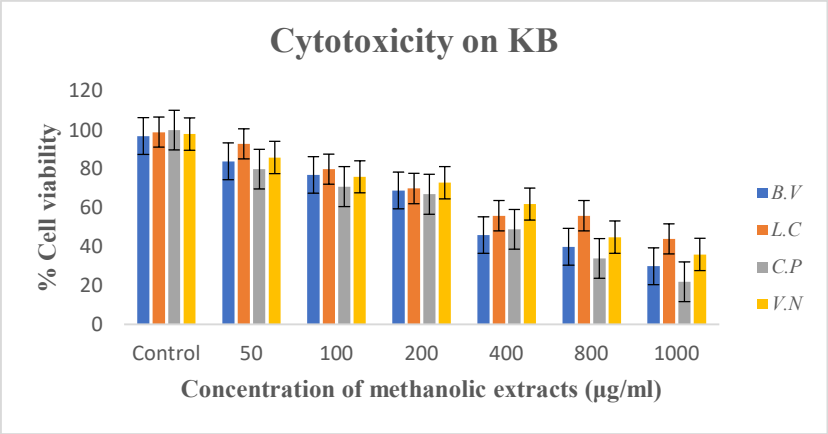


Figure 4: Cytotoxicity from methanolic extracts of the *B. variegate* (B. V.), *L. camara* (L. C.), *C. papaya* (C. P), and *V. negundo* (V. N.), against KB cells. Various concentrations (50-1000 µg/ml) of methanolic extracts of plants were treated for 24 hours. Triplicates of each experiment were performed

The findings of this study showed that *B. variegate* and *C. papaya* have various biological activities and can act as effective sources of antioxidants and anticancer agents. Additionally, all extracts demonstrated significant anticancer properties in the examined cell lines. The plant extract's ability to prevent the growth of cancer cells could not entirely due to their polyphenolic content. The presence of saponins, triterpenes, flavonoids, and tannins is believed to contribute partially by their antiproliferative activity through their antioxidant activity. High antioxidant concentrations have been shown to induce apoptosis and cytotoxic activity in tumor cell lines, indicating that the cytotoxic effects of many plant extracts may be related to their antioxidant capacity. Cytotoxic substances can trigger apoptosis or necrosis by activating various biochemical pathways, resulting in cell death.

CONCLUSION

Overall, this study suggests that *B. variegate*, *L. camara*, *V. negundo*, and *C. papaya* extracts cytotoxic and antioxidant properties may help prevent or delay the progression of many oxidative stress-related diseases, including cancer. The plants' antioxidant and increased flavonoid and phenolic contents may be responsible for the biological activities seen in the study. Compared to other evaluated extracts, the methanolic extract of *C. papaya* and *L. camara* leaves exhibits a higher level of antioxidant activity. Therefore, these extracts might be seen as novel sources of free radicals. Specifically, it was found that the extracts of *B. variegate* and *C. papaya* had high levels of cytotoxicity against the HeLa and the KB cell lines, respectively. The other extracts

presented low cytotoxicity. Nevertheless, the investigation revealed new information regarding the potential of *B. variegate* and *C. papaya* as anticancer drugs. As this research is preliminary, we advise additional research on these plants to assess their potential in other cancer cell lines. Additionally, *in vivo* studies are required to verify the antioxidant potential of this species further. We conclude that plants such as *B. variegate, L. camara, V. negundo,* and *C. papaya* are the most potential plants for usage as natural antioxidants and anticancer compounds for human health.

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