



In vitro anticancer efficiency of *vitex negundo* extracts

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Abstract

Background. *Vitex negundo* is a medicinal plant rich in bioactive phytochemicals, particularly phenolic and flavonoid compounds, which have been associated with antioxidant and anticancer activities. Natural plant-derived compounds have gained increasing attention as potential alternatives for cancer therapy due to their lower toxicity and diverse pharmacological properties. **Aim.** To evaluate the antioxidant and anticancer activities of aqueous and alcoholic leaf extracts of *Vitex negundo* through phytochemical analysis, antioxidant assessment, and cytotoxic evaluation against the human breast cancer cell line (MCF-7). **Methods.** Leaves of *Vitex negundo* were extracted using aqueous and 80% methanolic solvents by Soxhlet extraction. Total flavonoid and phenolic contents were determined using aluminum chloride and Folin–Ciocalteu methods, respectively. Antioxidant activity was evaluated using the phosphomolybdenum assay at concentrations of 50, 100, and 150 µg/mL. Cytotoxic activity against MCF-7 breast cancer cells and normal human dermal fibroblast (HdFn) cells was assessed by the MTT assay using extract concentrations ranging from 12.5 to 400 µg/mL. **Results.** The alcoholic extract exhibited significantly higher flavonoid (512.36 ± 2.82 µg/mL) and phenolic contents (261.83 ± 0.41 µg/mL) than the aqueous extract (287.59 ± 2.61 and 232.94 ± 0.45 µg/mL, respectively). Antioxidant activity was also greater in the alcoholic extract, reaching 17.84 ± 0.75 at 150 µg/mL compared with 4.88 ± 0.73 for the aqueous extract. Both extracts showed dose-dependent cytotoxicity against MCF-7 cells. The alcoholic extract demonstrated stronger anticancer activity with an IC₅₀ value of 56.2 µg/mL, whereas the aqueous extract exhibited an IC₅₀ of 102.0 µg/mL. Both extracts showed comparatively lower toxicity toward normal HdFn cells. **Conclusion.** *Vitex negundo* leaf extracts possess considerable antioxidant and anticancer activities, with the alcoholic extract exhibiting superior biological efficacy due to its higher phenolic and flavonoid contents. These findings suggest that *Vitex negundo* represents a promising natural source of bioactive compounds for the development of potential therapeutic agents against breast cancer.

Keywords: *Vitex negundo*, Anticancer, antioxidant, Breast cancer, MCF-7.

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Introduction

Plants that contain in one or more of their parts substances that can be used medically are considered medicinal plants (1). Humans have used medicinal plants to treat human diseases, considering them a primary source of therapeutic agents since ancient times (2). Because medicinal plants

are less expensive and readily available, they have been widely used by non-industrial societies (3). *Vitex negundo* (*V. negundo*), Its common name is the five-leaved chaste tree, back to family Verbenaceae, This shrub has white branches, thin gray bark, and a slender

trunk. The leaves are 3 to 5 leaflets (4). *V. negundo* originally from India and the Philippines and is found as a rare species in the United Kingdom. in Europe, Asia and the Antilles it was cultivated. The plant abundg it in humid conditions, so it is found in along riverbanks and wide areas and dry lands (5). Antioxidants are molecules that can remove and eliminate free radicals before they cause harmful effects on the body. They work to break down harmful substances (free radicals) and convert them into more stable compounds. Antioxidants are of two types: enzymatic antioxidants and non-enzymatic antioxidants (6). The plant contains polyphenolic compounds as well glycosidic iridoids, terpenoids and alkaloids. Multiple systems have been used in the laboratory to prove and verify the antioxidant power it contains because polyphenolic compounds have high antioxidant potential (7). Cancer is an uncontrolled growth and spread of cells. It is a disease with multiple factors and also targets the immune system (8).

Materials and Methods

Identification and Collection of *Vitex negundo*

The leaves of this plant were taken from the markets of Baghdad called Al-Razi Herbs Store, were obtained in 2024/7/21, and the plant was classified by Dr. Ibrahim Saleh Al-jubouri, the College of Pharmacy in Al-Mustansiriya University.

Preparation of the aqueous and alcoholic extract

After drying, the leaves of the plant were pulverized, and 150 g of sample was loaded in thimble and extracted in 250 ml of

distilled water for 4hours for aqueous extract, and methanol 80% for alcoholic extract using a Soxhlet apparatus. Then extracted successively by hot soxhlet extraction method. The extract solution was placed in the dishes at 37°C and incubated in the incubator (9). The dried plant extract was stored inside sterilized tube in refrigerator.

Determination of total flavonoid

A-Solutions

-Methyl Alcohol 50%

By mixing 45 ml of distilled water to 5 ml of absolute methanol, the solution was prepared to reach the required concentration.

-Sodium Nitrate solution 5%

The solution had been made by dissolving 5 g of NaNO_2 in 95 ml of D.W. to reach the required concentration.

-Sodium Hydroxide solution 10%

The solution was prepared by mixing 10 g of NaOH in 90 ml of D.W. to reach the required concentration.

-Aluminium Chloride solution 10%

By dissolving 10 g of AlCl_3 in 90 ml of D.W. The solution was prepared to reach the required concentration.

B-procedure

After preparing the solutions at the required concentrations, the total content of flavonoid was measured chemically in the aqueous and alcoholic extract of the plant based on the rutin(flavonoid standard) aluminium chloride colorimetric method (10).

1- plant extract 3.2 mg dissolved in 5 ml of "50% methanol".

- 2- Then 5% Sodium Nitrate solution (w/s) 1 ml added .
- 3- After 6 minutes, 1ml of Aluminum Chloride solution 10% (w/v) was poured to the mixture, then it was left for five minutes before adding 10 ml of Sodium Hydroxide solution 10% (w/v).
- 4- The mixture was quantum sufficit to 50 ml with distilled water and mixed.
- 5- The mixture was left for a quarter of an hour, then the absorbance was measured at a wavelength of 450 nm of flavonoids in the plant using a spectrophotometer.
- 6- The same procedure was applied for rutin at different concentrations (20, 40, 60, 80 µg/ml) as shown in figure (1) using curve fitting question.

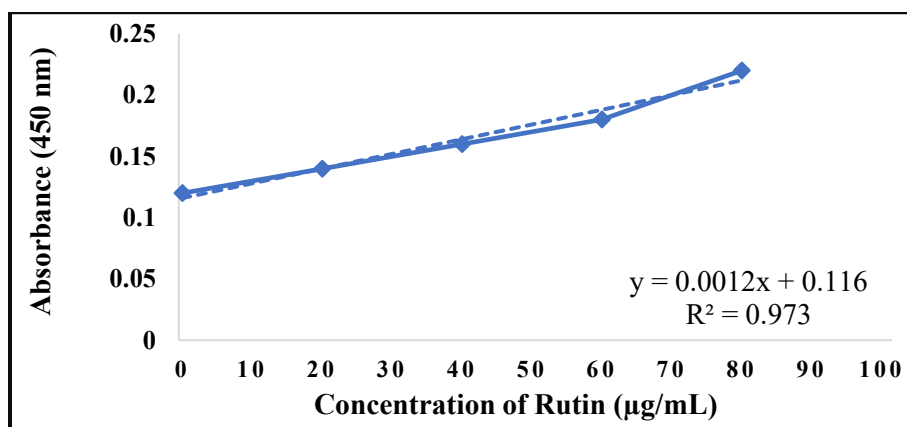


Figure (1): Standard curve to determine Rutin Concentrations.

Determination of total phenol contents

The total phenomenon of the extracts was measured using Folin-Ciocalteu method, 2.5 ml of the reagent (folin-ciocalten) were taken and mixed with 1 ml of each extract at a rate of 10% (w/v). 5 minutes later, Then, 2 ml of the Na₂CO₃ (75%) substance were taken and added to the previous mixture and incubated at a

temperature of 50°C for 10 min, stirring the mixture from time to time. Then the mixture was cooled to use a spectrophotometer to measure its absorbance at a wavelength of 765 nm opposite blank (11).

The same procedure was applied for Gallic acid at different concentrations (0.12, 0.25, 0.5, 1 mg/ml) as shown in Figure (2) using curve fitting question.

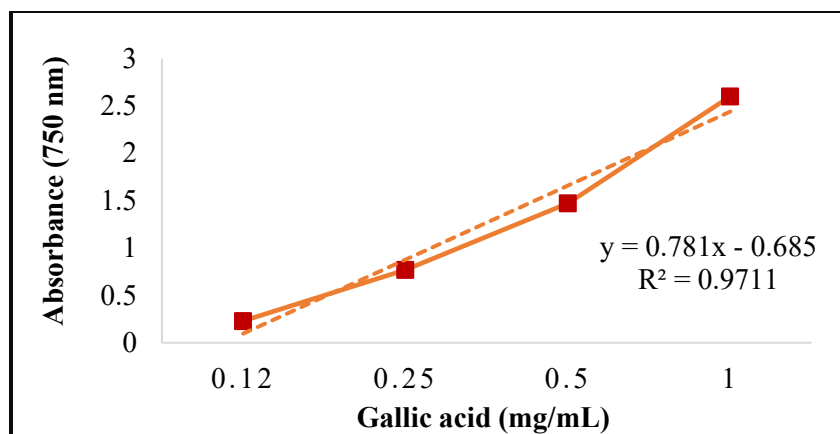


Figure (2): Standard curve to determine gallic acid concentrations

Phosphomolybdate antioxidant assay

Using the phosphomolybdenum method, the antioxidant capacity of the extracts was measured. 1 ml of the reagent consisting of (4 mM of ammonium molybdate, 28 mM of sodium phosphate, and 0.6 M of sulfuric acid) was taken and added to 0.1 ml of the extracts, where it was incubated for an hour and a half in a water bath at a temperature of 95°C after the tubes containing the mixture were covered with silver paper and then cooled at room temperature to measure the absorbance of the solution at a wavelength of 765nm. The high absorbance values indicated that the high antioxidant activity of the extracts was confirmed by using ascorbic acid as a standard (12).

Cytotoxicity MTT{3 - (4,5 dimethylthiazol -2- yl) - 2,5 - diphenyltetra zolium bromide} assay

The principle is one of the colorimetric assays used to estimate cytotoxicity of the cells by measuring the metabolic activity, and is commonly applied to estimate the inhibitory or lethal ability of extracts and compounds against cancer cells. The test depends on the ability of oxidoreductase

enzymes and the cellular ability based on the capacity of the NADPH Dehydrogenase enzyme to reduce the self-yellow MTT dye in water to the insoluble purple formazan compound, as the higher the percentage of the formed formazan compound, the more effective it is, which means an increase in the percentage of live cells (13).

A-Kit Contents

- A- vials 10 x 1ml: as a solution
- B- Solubilization solution: bottle 2 x 50 ml solution.

B-Assay procedure

This examination used MCF-7 from the “university of Malaxa / Collage of Medicine / Department of Pharmacology / center of investigation of New Therapies in Malaysia”.

To evaluate the cytotoxicity of aqueous and alcoholic extracts on MCF-7, The following steps were performed (14).

- The cells were planted in density (1×10^4 to 1×10^6 cells/ml) into 96 pits in a microtiter plate and 100 microliters of MCF-7 cells were added to them, repeating for each concentration in addition to the control.

- At 37°C and 5% CO₂, the plate was incubated for only one day, after which the culture medium was removed.
- Then, to each treated hole in the plate, 100 µL of the aqueous extract of the plant (25, 50, 100, 200, and 400 µg/ml) of different concentrations were added at 37°C for 48 hours.
- Three replicates were prepared from each concentration. After treatment with the compounds, 10 µL of MTT added at 37 °C and incubated for 4 hours.
- 100 ml of the solubilization solution was added and then incubated for five min.
- The results were read at 575 nm to check the absorbance by ELISA reader, and The growth inhibition percentage was

calculated for all extract concentrations using the equation.

$$\text{Viability (\%)} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

Results

Determination of total flavonoid content

The total flavonoid content of both the aqueous and alcoholic extracts of *V. negundo* was measured by spectrophotometer as rutin equivalent, the aqueous extract total content is 287.59 ± 2.61 µg/ml of flavonoids, while in alcoholic extract is 512.36 ± 2.82 µg/ml of flavonoids. These results indicate that the flavonoid in the alcoholic extract is higher than in the aqueous extract. As shown in Figure (3).

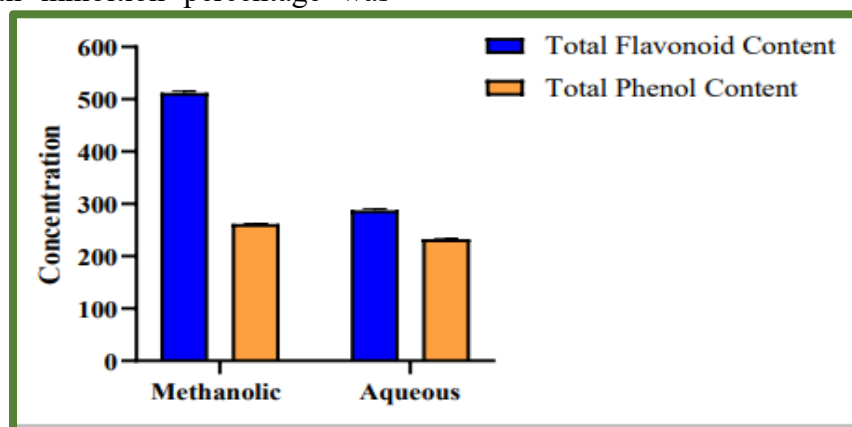


Figure (3): total flavonoid and phenol content in aqueous and alcoholic extracts of *Vitex negundo*.

Determination of total phenol content

The total phenol content was revealed using a spectrophotometer for the aqueous and alcoholic extracts of the *V. negundo*. The

amount of phenol in the aqueous extract was 232.94 ± 0.45 µg/ml, while the amount phenolic in the alcoholic extract was 261.83 ± 0.41 µg/ml. As shown in Figure (4).

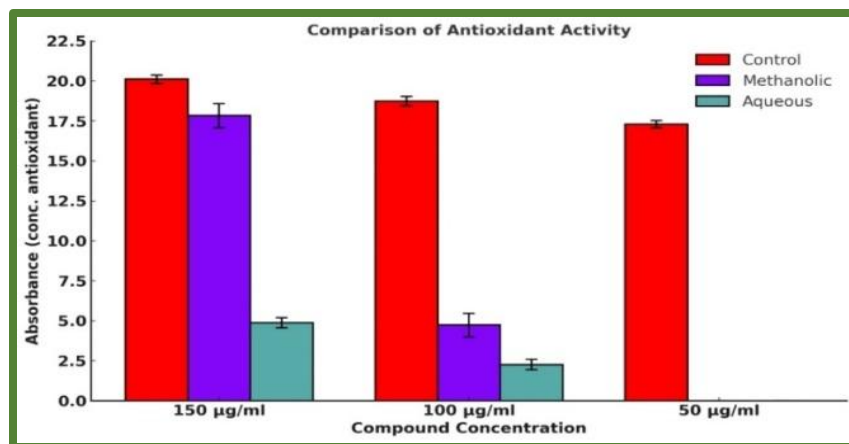


Figure (4): Total phenolic and flavonoid content in extracts of *Vitex negundo*.

Antioxidant activity

In measuring antioxidants, both the aqueous extract and the alcoholic extract of the *V. negundo* plant were tested using three different concentrations: 150 µL/ml, 100 µL/ml, and 50 uL/ml for each of extracts.

The results showed that 150 µL/ml is more efficient as antioxidant which is 17.84 ± 0.75 for alcoholic extract while it 4.88 ± 0.73 for aqueous extract which indicate that the alcoholic extract is more effective as antioxidant (Table 1 and Figure 5).

Table (1): Total antioxidant of extracts of *Vitex negundo*.

Conc. (µg/ml)	Alcoholic extract Mean±S.D	Aqueous extract Mean±S.D	Ascorbic acid Mean±SD
150	17.84±0.75 ^C	4.88±0.73 ^C	20.12±0.25 ^B
100	4.73±0.31 ^B	2.27±0.32 ^B	16.71±0.26 ^A
50	0.00±0.00 ^A	0.00±0.00 ^A	15.30±0.21 ^A

Different letters: Significant difference ($P \leq 0.05$) between means of columns.

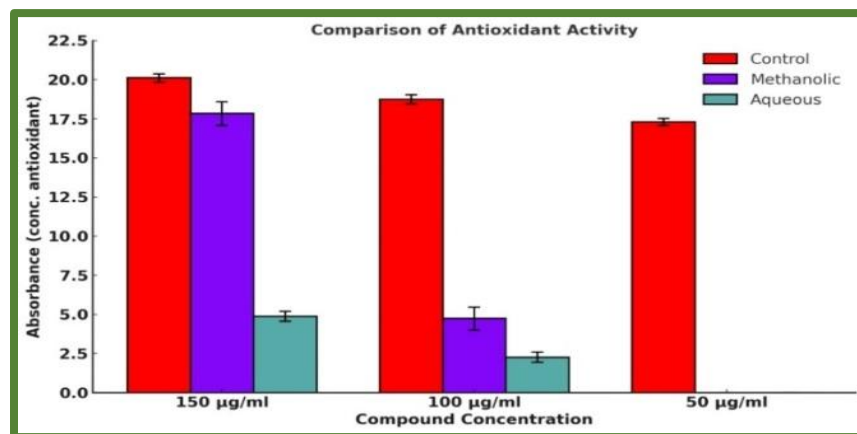


Figure (5): Total antioxidant of extracts of *Vitex negundo*.

Cytotoxicity MTT assay

A- Aqueous extracts

To know the extent of the cytotoxic effect of these extracts, this assay was used on MCF7, which is a breast cancer cell line. In this test, the rate of the extracts' ability to inhibit and keep alive the previously mentioned cancer cells was calculated, using different concentrations of the extracts (12.5-400 µg/ml). The toxic effect of the aqueous extract on MCF-7 was calculated at concentrations ranging from 12.5 µg/ml to 400 µg/ml as shown in the Table (2). The

maximum decrease was at a concentration of 400 µg/ml (79.38±0.37) while the effect on normal cells (88.15±1.03 µg/ml). This means that the aqueous extract inhibited 20.62% of cancer cells, while the highest viability of MCF-7 cells was reached at a concentration of 12.5 µg/ml (95.17 ± 0.33) while the effect on normal cells (95.52 ± 0.59) that means that the extract inhibited 4.83% of cancer cells. The aqueous extract showed significant cytotoxic activity with an IC₅₀ value of 102.0 µg/ml. While IC₅₀ was obtained from the effect of 157.2 µg/ml the extract on HdFn normal cell line (Figure 6).

Table (2): The cytotoxicity activity of *Vitex negundo* aqueous extract in the HdFn and MCF-7 cell lines

Conc. (µg/ml)	Viable cell count HdFn (Mean±SD)	Viable cell count MCF-7 (Mean±SD)
400	88.15±1.03 ^B	79.38±0.37 ^B
200	89.38±0.15 ^B	83.06±1.33 ^C
100	92.52±0.06 ^A	85.83±0.56 ^C
50	93.35±0.16 ^A	90.46±0.19 ^B
25	94.39±0.41 ^A	93.17±0.41 ^A
12.5	95.52±0.59 ^A	95.17±0.33 ^A

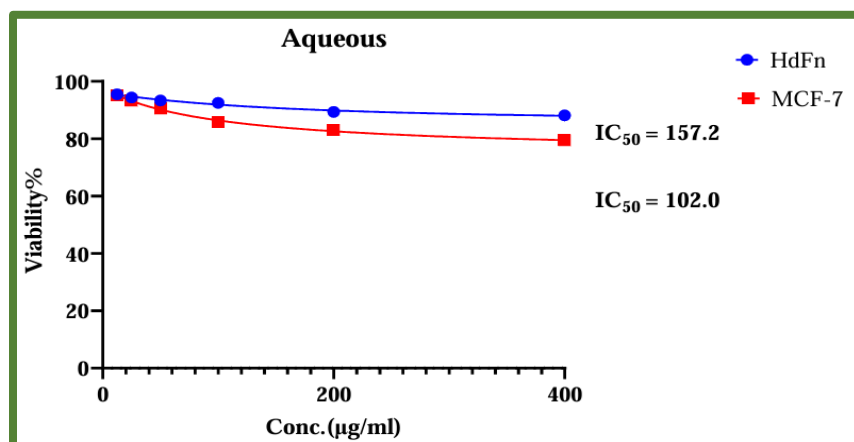


Figure (6): the cytotoxicity activity of *Vitex negundo* aqueous extract in the HdFn and MCF-7 cell lines using MTT assay.

B-Alcoholic extract

Different concentrations of the plant alcoholic extract were also used, ranging from 12.5 - 400 µg/ml. The alcoholic extract of the plant leaves had a greater effect on MCF-7, The results showed a significant

decrease in cell viability when the concentration increased. The lowest viability of cells was reached at a concentration of 400 µg/ml (40.01±1.51), While the highest viability was reached at a concentration of 12.5 µg/ml (94.73±2.23) Table (3). The

viability effectiveness of the plant alcoholic extract on the HdFn normal human cell line was ($87.15 \pm 0.41 \mu\text{g/ml}$) in a concentration of $400 \mu\text{g/ml}$ while ($95.79 \pm 0.70 \mu\text{g/ml}$) in a concentration of $12.5 \mu\text{g/ml}$ in this alcoholic

extract. The alcoholic extract showed significant cytotoxic activity with an IC_{50} value of $56.2 \mu\text{g/ml}$. While IC_{50} was obtained from the effect of $279.4 \mu\text{g/ml}$ the extract on HdFn normal cell line (Figure 7).

Table (3): The cytotoxicity activity of *Vitex negundo* methanol extract in the HdFn and MCF-7 cell lines

Conc. $\mu\text{g/ml}$	Viable cell count of HdFn (Mean \pm SD)	Viable cell count of MCF -7 (Mean \pm SD)
400	87.15 ± 0.41^C	40.01 ± 1.51^E
200	90.30 ± 0.22^B	45.24 ± 1.01^E
100	93.13 ± 0.63^A	55.96 ± 1.51^D
50	94.63 ± 0.48^A	71.62 ± 1.53^C
25	94.83 ± 0.43^A	86.05 ± 1.61^B
12.5	95.79 ± 0.70^A	94.73 ± 2.23^A

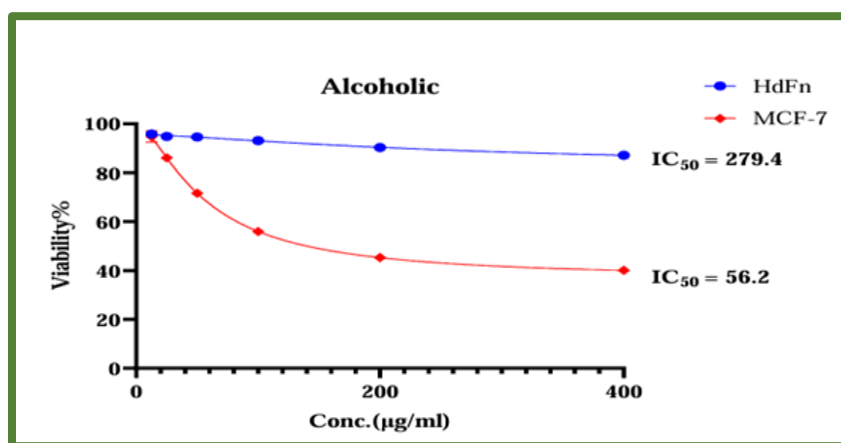


Figure (7): cytotoxicity activity of *V. negundo* methanol extract in the HdFn and MCF-7 cell lines.

Discussion

Determination of Total Flavonoid Content

The higher flavonoid content observed in the alcoholic extract compared with the aqueous extract can be attributed to the greater extraction efficiency of methanol for moderately polar phytochemicals. Methanolic solvents are well known to dissolve a wider range of flavonoid compounds than water, resulting in improved recovery of these secondary metabolites. Similar findings were reported

by (15), who demonstrated that methanolic extracts contained higher concentrations of flavonoids and exhibited stronger antioxidant properties than aqueous extracts.

Flavonoids are recognized as one of the major classes of natural antioxidants because of their ability to donate hydrogen atoms or electrons, chelate transition metals, and scavenge reactive oxygen species, thereby protecting biological molecules from oxidative damage (16). Their biological activities extend beyond antioxidant capacity and include anti-

inflammatory, antimicrobial, cardioprotective, neuroprotective, and anticancer effects. These compounds also contribute to food preservation by delaying lipid oxidation and maintaining food quality, explaining their increasing application in pharmaceutical and food industries (17). Likewise, plant-derived phenolic constituents have recently attracted attention as natural preservatives in food processing due to their antioxidant efficacy (18). Previous studies have demonstrated that *Vitex negundo* is particularly rich in flavonoids and flavonols, which largely account for its antioxidant potential (19). Similar observations were reported for *Plantago lanceolata*, in which flavonoids were identified as the principal bioactive constituents responsible for antioxidant activity (20).

Determination of Total Phenolic Content

The higher phenolic content detected in the alcoholic extract is consistent with the higher solubility of phenolic compounds in organic solvents. Phenolic compounds are among the most abundant secondary metabolites in medicinal plants and represent an important source of natural antioxidants because of their ability to neutralize free radicals and interrupt oxidative chain reactions (21). These compounds also contribute to the pharmacological properties of medicinal plants through anti-inflammatory, antimicrobial, and anticancer mechanisms. Therefore, the greater phenolic concentration in the alcoholic extract likely contributed to its superior biological activities observed in the present study.

Antioxidant Activity

The stronger antioxidant activity exhibited by the alcoholic extract is closely associated with its higher concentrations of flavonoids and phenolic compounds. Numerous studies have established a positive correlation between total phenolic content and antioxidant capacity because phenolic compounds readily donate electrons or hydrogen atoms to stabilize reactive oxygen species (22). Consequently, extracts enriched with these compounds generally exhibit greater antioxidant efficiency. Ascorbic acid remains one of the most potent natural antioxidants and is widely used as a reference standard because of its excellent free radical scavenging and reducing abilities (23). The findings of the present study therefore support the hypothesis that the antioxidant activity of *V. negundo* primarily depends on its phytochemical composition.

Cytotoxic Activity of the Aqueous Extract

The cytotoxic activity of the aqueous extract against MCF-7 cells indicates the presence of biologically active constituents capable of suppressing breast cancer cell proliferation. Although the aqueous extract exhibited moderate activity, its inhibitory effect increased progressively with increasing concentration, suggesting a dose-dependent response. Similar concentration-dependent cytotoxic effects have been reported for aqueous extracts of medicinal plants, including *Plantago lanceolata*, against cancer cell lines (24).

The observed cytotoxicity may be attributed to flavonoids and phenolic compounds, which are known to induce apoptosis, arrest the cell cycle, increase intracellular oxidative stress in tumor cells,

and modulate signaling pathways involved in cancer progression. Plant-derived phytochemicals have also been reported to reduce oxidative damage associated with various pathological conditions and may counteract cytotoxic effects induced by environmental stressors (25). These mechanisms collectively explain the inhibitory activity of the aqueous extract against MCF-7 cells.

Cytotoxic Activity of the Alcoholic Extract

The alcoholic extract demonstrated markedly stronger cytotoxic activity than the aqueous extract, indicating that methanol extracted a greater concentration of anticancer phytochemicals. This enhanced activity is consistent with the higher flavonoid and phenolic contents detected in the alcoholic extract. Breast cancer remains one of the leading causes of cancer-related mortality among women worldwide, emphasizing the importance of identifying effective plant-derived therapeutic agents (26).

MCF-7 cells are among the most widely used human breast cancer cell lines for evaluating the cytotoxic potential of natural products because they provide a reliable experimental model for screening anticancer compounds. The concentration-dependent reduction in cell viability observed in the present study agrees with previous reports demonstrating that increasing extract concentration enhances the inhibition of breast cancer cells (27). Previous investigations have also confirmed that *V. negundo* possesses significant anticancer activity owing to its antioxidant constituents and its ability to induce

genotoxic and apoptotic effects in MCF-7 cells (28).

The anticancer activity of *V. negundo* is likely mediated by the synergistic action of its secondary metabolites, including flavonoids, phenolics, alkaloids, glycosides, tannins, sterols, lignans, and saponins. These compounds interfere with multiple cellular signaling pathways involved in proliferation, apoptosis, angiogenesis, and oxidative stress, ultimately suppressing tumor growth (29). Similar biological activities have been reported for numerous medicinal plants rich in secondary metabolites, which exhibit antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer properties (30). Likewise, (31) demonstrated that medicinal plants containing high levels of flavonoids and terpenoids possess considerable therapeutic potential. Furthermore, citrus-derived polyphenols and flavonoids have been shown to exert substantial antioxidant and health-promoting effects, supporting the role of phenolic phytochemicals as major contributors to the biological activities observed in medicinal plants (32).

Conclusion

Vitex negundo extracts is effective as antioxidant and It has an effect on prevent the growth of breast cancer cells MCF-7, especially the alcoholic extract because it is more effective than the aqueous extract, as indicated by the results. Natural materials can be considered for use instead of chemotherapy in fighting cancer, in addition to their antioxidant potential.

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