Anticarcinogenic effects of *Cinnamomum verum* on HL60 leukemia cell lines

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ABSTRACT

**Background:** Phyto medicine are focused more by recent researchers for the treatment of different types of chronic diseases including cancer, because of its radical scavenging effect, antioxidant capacity and anticarcinogenic potentiality. The present study was aimed to assess to the presence of bioactive compounds and its anticarcinogenic activity of *Cinnamomum verum* (*C.verum*) bark. **Methods:** The quantitative and qualitative phytochemical analysis was carried out with the ethanol and methanol extracts *C.verum*. GCMS analysis, of methanolic extract was done. The radical scavenging activity and the antioxidant potentials were assessed by thio barbituric assay, metal chelating activity, superoxide anion radical scavenging activity, phospho molybdenum assay. Further studies were progressed with *in vitro* cell cytotoxicity assay, DNA fragmentation assay and cell morphology assay with ethanolic extract of *C.verum* using HL 60 leukemia cell lines.

**Results and Discussion:** The quantitative phytochemical analysis showed the presence of tannin, saponin, alkaloids, flavonoids, polyphenols and steroids. The total phenolic and flavanoid contents were estimated as 176.7mg/g and 153.2mg/g. From GCMS results the bioactive phytochemicals and their structural characterizations were identified. The radical scavenging activity of ethanol and methanol extracts of *C.verum* showed better scavenging activity against free radical generation. In *in vitro* antioxidant assay the results showed both the extracts ethanol and methanol of *C.verum* have significant antioxidant potentiality. The results of cell cytotoxicity assay show good inhibitory effect. DNA fragmentation analysis confirms the characteristic nuclear fragmentation in the late stage of apoptosis. The morphological identification of DNA shows cellular shrinkage, membrane blebbing, and typical nuclear fragmentation. **Conclusion:** The present study proves that *C.verum* bark is a good source of bioactive compounds and exhibit the anticarcinogenic effect through various mechanism.

KEYWORDS:*Cinnamomum verum*, Leukemia, Cytotoxicity assay, Apoptosis, HL60 cell lines

1. INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Many treatment options for cancer exist, with the primary ones including surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care. Which treatments are used depends on the type, location, and grade of the cancer as well as the person’s health and wishes. The treatment intent may be curative or not curative.1-2

Despite significant progress in prevention, diagnosis and development during the last 25 years, cancer still represents the second cause of mortality in developed countries, after cardiovascular diseases. Discovering new drugs that are more active, more selective, and less toxic, limiting deleterious side effects and tumour multidrug resistance will obviously be a challenge for the 21st century. The isolation of potent anticancer molecules from the natural environment has generated interest in many groups to purify original compounds, understand their biological activity and also identify the pharmacological targets of molecules, previously known for their ecological function. Therefore the concept of delaying or preventing these problems remains a viable and attainable goal for the future.3

Natural products consist of a wide variety of biologically active phytochemicals including phenolics, flavanoids, carotenoids, alkaloids and nitrogen containing as well as organo sulphur compounds, which have been shown to suppress early and late stages of carcinogenesis.4 Naturally occurring bioactive extracts or single compounds are believed to benefit human health and this has spawned an important and dynamic new area of research resulting in substantial advances in nutritional knowledge. There is also growing awareness that dietary source and form of food may affect overall health. Suitably, the role of food as an agent for improving health has been
recognized initiating the development of new classes of food known as functional foods⁴.

*Cinnamomum verum* (*C. verum*) has the following potential pharmacological properties- Antibacterial efficacy, Antioxidant ability, Larvicidal activity, Pesticidal activity. It is blood alterative, stimulant and analgesic. Mouth refresher and gives strength to gums. Hence, a piece of its bark should be chewed to stop vomiting and nausea. A swab made in its oil is held between teeth for dental caries. A thin paste should be applied to the skin in skin diseases. Application of oil cleanses and heals a tubercular ulcer⁵.

Today, the benefits and effectiveness of herbs are backed by scientific data. Because medicinal plants have no doubt remained the major sources of traditional medicine worldwide. The active principles of plant derived products have been isolated and characterized and their mechanisms of action are understood. This study attempts made to determine the phytochemical analysis, antioxidant, and anti-cancerous potentials of *C. verum*.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Sigma Chemicals / reagents and RPMI media were used for all experiments. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) SRL, India. All the chemicals used were extra pure and of analytical grade and cell culture tested.

2.2 Collection of plant

The plant *C. verum* was collected from the Indian Medical Practitioner’s Co-operative Pharmacy and Store Limited, Thiruvanmaiyur, Chennai – 600041, Tamilnadu, India. The dried plant sample was powdered and used for the preparation of extracts and further analysis.

2.3 Preparation of extract

2.3.1 Ethanol extract

5g of fine dry powder of *C. verum* is dissolved in 50ml of 70% ethanol. The procedure is repeated for 4 times under room temperature (28 ± 3°C) and then filtered using whatman No.4 filter paper. The pH of the extract is maintained between 2-3 using 4M HCl. To the volume of extract, equal volume of Ethyl acetate is added and separated in separation funnel. To this anhydrous sodium sulfate is added to dry. The dried extract is then weighed.

2.3.2 Methanol extract

To 100ml of 50% methanol, 2 drops of 1.2M HCl and 10 ml of 1% Ascorbic acid is added. To this 5g of fine dry powder of *C. verum* is added and kept under 80°C for 2 hours, filtered and dried. The dried extract is then weighed.

2.4. Phytochemical Analysis

2.4.1 Qualitative phytochemical analysis

The ethanolic and methanolic extracts of *C. verum* is analysed for the presence of phytochemical constituents such as tannins, saponins, flavonoids, proteins, alkaloids, steroids, quinones, terpenoids, cardio glycosides according standard methods.

2.4.2 Quantitative phytochemical analysis

2.4.2.1 Total Phenolics

The percentage of total phenol content of the methanolic extract was estimated by the Folinciocalteu method. The extract and different dilutions of standard gallic acid were mixed separately with 1 ml of Folinciocalteu reagent and a solution of 7% sodium carbonate was also added. The mixtures were incubated at room temperature for one and half hours. The total phenolic content for the extract was determined by colorimetry at 750nm. A standard curve for gallic acid in methanol was prepared using different concentrations (100 - 700 µg/ml). The total phenolic content was expressed in terms of gallic acid equivalents.

2.4.2.1 Total flavonoids

The amount of total flavonoids in the extract was determined using aluminum chloride colorimetric method. The extract was dissolved in methanol (1mg / ml) and with 0.1 ml of 1M potassium acetate, 1.5 ml of methanol, 0.1ml of 10% aluminum chloride and 2.8 ml of distilled water. This was maintained at room temperature for about 30mins. The absorbance was measured at 415nm. Standard quercitin was prepared and a calibration curve for the standard quercitin was obtained by taking 12.5ml, 25ml, 50ml, 75ml and 100ml in methanol. A plot of absorbance versus concentration was plotted and the total flavonoid contents were calculated as quercitin equivalent.

2.5 GC-MS analysis

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30mm×0.25mm ID×1µm Mdf, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; Helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 2µl was employed (split ratio of 10:1);Injector temperature 250°C; Ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2min.), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9min. isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5seconds and fragments from 45 to 450Da. Total GC running time was 26min.
2.6 In vitro Antioxidant Activities

2.6.1 Estimation of Radical Scavenging Activity (RSA):
The RSA activity of different extracts was determined using DPPH assay according to Nenadis and groups\(^7\). The decrease of the absorption at 517 nm of the DPPH solution after addition of the antioxidant was measured in a cuvette containing 2960 µl of 0.1 mM ethanolic DPPH solution was mixed with 40 µl of 50 – 250 µg/ml of *C. verum* extract of both ethanol and methanol. Blank containing 0.1 mM ethanolic DPPH solution without *C. verum* extract and vortexed thoroughly, the setup was left at dark at room temperature. The absorption was monitored after 20 mins. Ascorbic acid (AA) and Butylated hydroxytoluene (BHT) were used as references. \(\alpha\)-tocopherol was used as a standard. The ability to scavenge DPPH radical was calculated by the following equation.

\[
\% \text{ of DPPH radical scavenging activity (RSA)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

Where,

- \(A_{\text{control}}\) is the absorbance of DPPH radical + ethanol
- \(A_{\text{sample}}\) is the absorbance of DPPH radical + extract.

Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions. The IC\(_{50}\) (concentration providing 50% inhibition) was calculated graphically using a calibration curve vs percentage of inhibition.

2.6.2 In vitro antioxidant activity

2.6.2.1 Thiobarbituric acid (TBA) method:
2ml of 20% trichloroacetic acid and 2 ml of 0.67% 2-thiobarbituric acid was added to 1ml of both sample solution. The mixture was placed in a boiling water bath and, after cooling, was centrifuged at 3000 rpm for 20 minutes. Absorbance of supernatant was measured at 552 nm. \(\alpha\)-tocopherol was used as a standard for 20 mins. Absorbance of supernatant was measured at 552 nm. Alkaline boiling water bath and, after cooling, was centrifuged at 3000 rpm for 20 minutes. Absorbance of supernatant was measured at 552 nm. \(\alpha\)-tocopherol was used as a standard. The ability to scavenge DPPH radical was calculated by the following equation.

\[
\% \text{ of DPPH radical scavenging activity (RSA)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

2.6.2.2 Superoxide anion radical scavenging assay:
1ml of NBT (Nitro blue tetrazolium) solution (156 µM NBT in 100 mM phosphate buffer, pH 8) mixed with 1ml of NADH (Nicotinamide adenine dinucleotide) solution (468 µM in 100 mM phosphate buffer, pH 8). Mixed with 0.1 ml of the sample solution (10 mg/ml). The reaction was started by adding 100 µl of PMS (Phenazine metho sulfate) solution (60 µM PMS in 10 mM, phosphate buffer, pH 8). The mixture was incubated at 25°C for 5 minutes. A control was performed with reagent mixture but without the sample. Absorbance was measured spectrophotometrically at 560 nm. \(\alpha\)-tocopherol was used as a standard.

2.6.2.3 Metal chelating activity:
The chelating of ferrous ions by *C. verum* extract of ethanol and methanol was estimated. The reaction was initiated by the addition of 5 mmol/l ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard\(^8\).

2.6.2.4 Phosphomolybdenum assay:
The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto and groups\(^11\). An aliquot of 100 µl of both the sample solution was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 nM sodium phosphate and 4 mM ammonium molybdate) in a 4 ml vial. The vials were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. Ascorbic acid was used as a standard.

2.6.2.5 Catalase
The reaction mixture contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml 1:10 diluted tissue homogenate and 0.5 ml of 2 M hydrogen peroxide. The reaction was stopped by the addition of 2.0 ml of dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The tubes were heated in a boiling water bath for 10 mins. After cooling the contents, the optical density was measured at 590 nm. The activity was expressed as units/mg protein. One unit of enzyme activity was defined as the amount of enzyme required to decrease the absorbance by 0.5 units at 590 nm\(^12,13\).

2.6.2.6 Superoxide dismutase
Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of nitro tetrazolium (NBT) reduction and the extent can be assayed spectrophotometrically. The SOD activity was set up with an incubation medium in a final volume 3.0 ml containing equal volume of 50 mM potassium phosphate buffer pH 7.8, 45 µM methionine, 5.3 mM riboflavin, 84 µM NBT and 20 µM potassium cyanide and 0.5 ml of tissue homogenate. The tubes were placed in an aluminium foil lined box maintained at 25°C and equipped with 15 W fluorescent lamp and the reduced NBT was measured spectrophotometrically at 600 nm after 10 minutes exposure to light. The maximum reduction was evaluated in the absence of enzyme. The activity was expressed in units/mg protein. One unit of enzyme activity was defined as the amount of enzyme that gives 50 percent inhibition of the extent of NBT reduction\(^12,13\).
2.6.2.7 Glutathione peroxidase
Glutathione peroxidase activity was carried out with a reaction containing 0.4ml of sodium phosphate buffer (0.4M, pH 7.0); 0.1ml of 10mM sodium azide, 0.2ml of 4mM reduced glutathione, 0.5ml of tissue homogenate, 0.5ml of 2.5mM hydrogen peroxide and the final volume was made up to 2.0ml. The tubes were incubated at 37°C for 3 minutes. The reaction was terminated by the addition of 0.5ml of 10% trichloro acetic acid. To determine the residual glutathione the supernatant was removed by centrifugation. To this 3.0ml of 0.3M disodium hydrogen phosphate and 1.0ml of Dithio nitro benzoic acid (DTNB) were added. The color developed was read at 412nm. The GPx activity was calculated and expressed as units/mg protein. Unit of GPx activity was defined as the μg of glutathione utilized/mg protein\(^\text{15, 13}\).

2.6.2.8 Reduced glutathione
The reduced glutathione was measured by its reaction with DTNB, for the GSH determination in the tissues (0.5ml) by precipitating the proteins by adding 0.12ml of 25% trichloro acetic acid, the precipitated proteins were centrifuged at 1000Xg for 10 minutes. The supernatant was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8) and 0.2ml of freshly prepared DTNB (0.6mM) was added to the tubes and the intensity of the yellow color formed was read at 412nm in a spectrophotometer after 10 minutes. The results were expressed as nmols/g tissue\(^\text{16}\).

2.9 In vitro cytotoxicity assay

2.9.1 Cell line culture
HL60 cells obtained from NCCS (National Centre for Cell Science, Pune) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with penicillin/streptomycin/amphotericin (100U/ml), L-glutamine (200mM), obtained from Himedia, Mumbai, India and 10% fetal bovine serum, obtained from Invitrogen, USA. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO\(_2\). Cells were allowed to grow to confluence over 24 h before use.

2.9.2 MTT Assay
Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. Briefly, HL60 cells were seeded at a density of 5x10\(^3\) cells/well in 96-well plates for 24h, in 200μl of RPMI with 10% FBS. Then, RPMI medium containing various concentrations (50–250μg/ml) of test compound was added and incubated for 48h. After treatment, cells were incubated with MTT (20μl, 5mg/ml) at 37°C for 4h and then with DMSO at room temperature for 1h. The plates were read at 570nm with background wavelength as 630nm in an ELISA plate reader. Data represented the mean values for three independent experiments\(^\text{17}\).

\[
\text{Cell viability} = \frac{\text{Mean OD} - \text{Control OD}}{\text{Mean OD}} \times 100
\]

2.9.3 DNA fragmentation Assay
The isolation of fragmented DNA was carried out according to the previous procedure. Cells were seeded in a cell culture flask and treated with 50, 100, 200, 250μg/ml of test compound for 36h. After harvesting, the cells were washed with PBS and then centrifuged. The cell pellets were then treated with lysis buffer (1% NP-40, 120 mM EDTA and 50 mM Tris HCl (pH 7.5)) and then centrifuged. The supernatant obtained was incubated overnight with proteinase K (50 μg/ml) and then with RNase (50 μg/ml) for 1 h at 37°C. After extraction with phenol:chloroform: isooamyl (25:24:1), the DNA was separated in 1.8% agarose gel at 60 V for 2–3 h and the pattern of DNA fragmentation was viewed under UV trans-illuminator after staining with ethidium bromide\(^\text{18}\).

2.9.4. Observation of DNA Morphology
The HL60 cells were washed with PBS 1 x 10\(^6\) cells/ml were treated with 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, and 250 μg/ml of the C. verum ethanolic extract for 3 h, 6 h, 16 h, and 20 h at 37°C and 5% CO\(_2\). The cells were then fixed with absolute ethanol at -20°C for 15 minutes. After fixation, cells were washed and stained with 1μg/ml propidium iodide (PI) at 37°C for 15 minutes. The cells were washed again and 10 μl cell suspension was taken on a slide. Fluorescent images were scanned using fluorescence microscope and the images were captured by a digital camera. Similarly, to investigate apoptosis or necrosis, acridine orange (AO) and ethidium bromide (EB) staining method was performed. Acridine orange permeates all the cells and makes the nuclei appear green. Ethidium bromide is only taken up by dead cells when cytoplasmic membrane integrity is lost and the nucleus stains yellowish orange. Therefore, live cells have a normal green nucleus; early apoptotic cells show bright green/yellowish nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange/red chromatin while the cells that have died from direct necrosis have a structurally normal deep orange nucleus.

\[
\text{Percentage} \% \text{ of Apoptotic cells} = \frac{\text{Total number of apoptotic cells}}{\text{Total number of cells counted}} \times 100
\]

2.10 Statistical Analysis
Data were represented as means ± standard deviation. Variance analysis was used for statistical comparisons of the results using two way ANOVA. Values P<0.05 were considered as statistically significant.
3. RESULTS AND DISCUSSION

3.1 Extraction of C. verum

5g of dry bark of plant C. verum was extracted with ethanol and methanol. The bark extract shows dark brown color. The quantity of the extracted residues were weighed and found to be 567mg from ethanol extract and 657mg for methanol extract.

3.2 Phytochemical Analysis of C. verum

3.2.1 Qualitative Phytochemical Analysis

The phytochemical results observed that ethanol extract contains polyphenol, tannin, saponin, flavanoids and steroids, where as methanol extract contains tannin, saponin, alkaloids and steroids in detectable amounts. No alkaloid, protein, quinones, terpenoids and cardio glycosides in the ethanol extract and flavanoids, protein, quinones, terpenoids and cardio glycosides found in the ethanol extract. The various phytochemical compounds present in plant and plant products are known to have beneficial importance in health science. C. verum is said to contain about 200 such compounds that contribute significantly to its medicinal application. The selected C. verum used for study was also found to be rich in Polyphenols, Tannins, Saponins, Flavonoids, Alkaloids and Steroids which was agreeable with the study of Mbaebia and groups 19.

3.2.2 Quantitative Phytochemical Analysis

In the present study the total phenolic content of C. verum ethanolic extract was found to be 176.7mg/g, the amount of flavonoids was found to 153.2mg/g. Plant polyphenols and flavonoids are a major group of compounds which have the following effects; cholericetic and diuretic functions, decreasing blood pressure, reducing the viscosity of the blood and stimulating intestinal peristalsis 20, as well as primary antioxdation or free radicals scavenging activities 21, 22.

C. verum phenolic and flavonoid compounds are becoming increasingly popular because of their potential role in contributing to human health. A wide range of phenolic constituents are present in C. verum and most of these found in the form of flavonoids and phenolic acid. Phenolic compounds such as BHT (Butylated Hydroxy Toluene) and gallate are known to be effective antioxidant. The result obtained in the present study was in close agreement with of Mbaebia and groups 19.

3.3 GC-MS Identification of Bioactive Compounds

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name and Molecular weight of the components of the test materials were ascertained in Table 1 and

Table 1. GC-MS Identification of Bioactive Compounds from C. verum

<table>
<thead>
<tr>
<th>S.No.</th>
<th>RT</th>
<th>Name of the Component</th>
<th>Molecular Formula</th>
<th>MW</th>
<th>Peak Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3.37</td>
<td>Butane, 1,1-diethoxy-3-methyl-</td>
<td>C₈H₁₄O₂</td>
<td>160</td>
<td>0.51</td>
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<td>2.</td>
<td>3.81</td>
<td>Hexanoic acid, ethyl ester</td>
<td>C₄H₈O₂</td>
<td>144</td>
<td>3.03</td>
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<td>3.</td>
<td>4.77</td>
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<td>C₃H₈O₂</td>
<td>176</td>
<td>1.3</td>
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<td>4.</td>
<td>6.01</td>
<td>2-Propan-1-ol, 3-phenyl-</td>
<td>C₉H₁₂O₂</td>
<td>134</td>
<td>0.57</td>
</tr>
<tr>
<td>5.</td>
<td>6.43</td>
<td>4,7-Methano-1H-indene-1,8-dione, 3a,4,7a-tetrahydro-</td>
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<td>160</td>
<td>0.22</td>
</tr>
<tr>
<td>6.</td>
<td>7.09</td>
<td>Benzenepropanol</td>
<td>C₁₂H₁₆O₂</td>
<td>136</td>
<td>0.39</td>
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<tr>
<td>7.</td>
<td>7.64</td>
<td>Cinnamaldehyde, (E)-</td>
<td>C₁₀H₁₄O₂</td>
<td>132</td>
<td>61.57</td>
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<td>2-Propan-1-ol, 3-phenyl- (Synonym: Cinnamyl alcohol)</td>
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<td>10</td>
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<td>1.58</td>
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<td>11</td>
<td>9.56</td>
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<td>146</td>
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<td>9.81</td>
<td>2-Propan-1-ol, 3-phenyl-, acetate</td>
<td>C₉H₁₂O₄</td>
<td>176</td>
<td>1.01</td>
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<tr>
<td>13</td>
<td>10.35</td>
<td>Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylthyl)-</td>
<td>C₁₄H₁₉O₉</td>
<td>204</td>
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<td>14</td>
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<td>C₁₀H₁₄O₂</td>
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<td>Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylthyl)-, (1S-cis)-</td>
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<td>18</td>
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<td>12-Methyl-E,E-2,13-octadecadien-1-ol</td>
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<td>280</td>
<td>0.10</td>
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<tr>
<td>19</td>
<td>14.45</td>
<td>n-Butyl-α-phenylpropionate</td>
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<td>206</td>
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<td>20</td>
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<td>1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester</td>
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<td>16.66</td>
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<td>23</td>
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<td>Oleic Acid</td>
<td>C₁₈H₃₄O₂</td>
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<td>0.09</td>
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<tr>
<td>24</td>
<td>18.78</td>
<td>E-11-Hexadecenoic acid, ethyl ester</td>
<td>C₁₈H₃₄O₂</td>
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<tr>
<td>25</td>
<td>19.28</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
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<td>280</td>
<td>0.88</td>
</tr>
<tr>
<td>26</td>
<td>19.36</td>
<td>Oleic Acid</td>
<td>C₁₈H₃₄O₂</td>
<td>282</td>
<td>2.25</td>
</tr>
</tbody>
</table>
3.4 Anti Carcinogenic effect of C. verum

3.4.1 Radical scavenging activity (RSA) of C. verum extracts

The radical scavenging effect of ethanol and methanol extracts from the bark of C. verum on DPPH radicals was increased with the increase in concentration. The results indicated that ethanol showed high activity than the methanol at the concentrations tested (Figure 2). The result obtained in DPPH assay with C. verum at a concentration of 20-200µg/ml was in accordance with Gurdipsingh and group\textsuperscript{25}.

3.4.2 In vitro Antioxidant activity

Assay of TBARS measures malondialdehyde present in the sample, as well as malondialdehyde generated from lipid hydroperoxides by the hydrolytic conditions of the reaction. Ethanolic extract shows significant decrease in TBARS compared with control indicates its scavenging activity against peroxidation (Figure 3). The ethanol extract of C. verum have strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than control (Figure 3). Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydroxyl peroxide serving as in this study was in close relevant with Sindhu Mathew and Emilia Abraham\textsuperscript{26}.

It is reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. The data present in this study indicated that ethanol extract has the more ability for iron binding and could reduce the generation of hydroxyl radicals (Figure 3). However, these transition metal ions could form chelates with the antioxidants, which result in suppression of hydroxyl radicals generation and inhibit ion of peroxidation process of biological molecules. The metal chelating activity of C. verum was in correlates with the previous studies by Sindhu Mathew and Emilia Abraham\textsuperscript{26}.
Figure 2. Radical scavenging activity of *C. verum* extract

Values are mean ± SD of triplicates

Figure 3. *In vitro* Antioxidant Activities of *C. verum* extract

The ethanol and methanol extracts were used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The result indicates that the ethanol extract has more powerful antioxidant in the reduction of phosphomolybdenum complex (Figure 3). Phosphomolybdenum reduction potential of extracts was explained by the fact that the transfer of electrons/hydrogen from antioxidants occurs at different redox potential in various assay system and the transfer also depends on the structure of the antioxidants\(^{27}\).

*C. verum* was capable of scavenging free radicals through their catalase enzyme in a concentration dependent manner. It is known that free radical cause auto-oxidation of unsaturated lipids in food\(^{28,29}\). Antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby
forming a stable end product, which does not initiate or propagate further oxidation of lipid \(^{30}\) (Figure 4).

The extract significantly scavenged the superoxide radicals in a concentration dependent manner. Superoxide is not only one of the strongest reactive oxygen species among the generated free radicals but also a precursor to other active free radicals, which is an important role in the oxidative damage in lipids, proteins, and DNA and thereby inducing tissue damage \(^{31, 32}\) (Figure 4).

The initiating free radicals are scavenged by the glutathione peroxidase by inhibiting the production of secondary, more damaging free radicals. The endogenous antioxidants will interfere with the chain propagation, as they are more easily oxidized than polyunsaturated fatty acids. Finally, lipid hydro peroxides already formed will be reduced by this enzyme \(^{33}\) (Figure 4).

The ethanol extract was capable of scavenging superoxide dismutase in a concentration dependent manner. The ethanol extract significantly scavenged the superoxide radicals than the methanol extract (Figure 4).
3.5 Cytotoxicity Assay
In the present study the cytotoxic effect of C. verum ethanol extract was tested with HL60 leukemia cell lines. Cell lines free from any kind of bacterial and fungal contaminations. Plate 1 indicates dead cells and their cellular uptake of the dye which appear as blue in colour. In MTT assay cell death and cell viability of leukemia cell line of anticancer activity was estimated. The results showed 84.1% cell viability in the concentration of 1mg/ml. The IC₅₀ of cell viability was observed at concentration of 127µg/ml of the ethanol extract. The values obtained were shown in Figure 5, Plate 1 and Figure 6.

The inhibitory concentration (IC₅₀) was determined to be 127µg/ml after two days (48 hrs) of treatment.

Figure 6. Percent inhibition of HL 60 cell lines by ethanolic extract of C. verum
The cellular uptake study analysis confirmed a dose dependent inhibition of cell lines noticed during the specific incubation period. On treatment with ethanolic extracts in cell lines showed the better cytotoxic effect. The higher susceptibility was noted when the cell lines were treated even minimal concentration of 50µg for HL60 cells. Results indicated the involvement of various molecular mechanisms in the generation of anticancer action of C. verum.

3.6 DNA fragmentation Assay
To explicate the apoptotic induction by the ethanolic extract in cancer cells, DNA fragmentation assay was done in HL60 cells. DNA fragmentation was analysed after treatment with the ethanolic extract at 3h and 16h. In case of 3h incubation less laddering pattern was obtained which might be due to the presence of early apoptotic cells with less nuclear fragmentation as shown in Plate 2 when compared to control, whereas it produced a typical ladder-like pattern at 16h incubation, shown in Plate 2, confirming characteristic nuclear fragmentation in the late stage of apoptosis, Therefore, the results conclude that, with short incubation time, the ethanolic extract brings about early apoptosis, but when incubated for longer duration, later stage of apoptotic cells with more nuclear fragmentation was observed.

The present data are also in accordance with the findings of Mishra and his groups reported a DNA ladder pattern with aqueous ethanol seed extract of Ziziphus mauritiana in HL60 cells in a concentration-dependent manner and a time-dependent study showed typical ladder pattern due to induction of apoptosis. The result obtained from our experiment finally confirms the cell death via apoptosis by the C. verum ethanolic extract. Thus, this illustrates that the anticancer effect of the ethanolic extract may act through the apoptotic signaling.
Plate 2. DNA Fragmentation assay of ethanolic extract of *C. verum*

**3.7 DNA Morphology Assay**

The apoptotic features like chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies can be seen under fluorescence microscope after staining of nuclei with DNA. HL 60 cells were treated with 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, and 250 µg/ml of the ethanolic extract of *C. verum*. Acridine orange/ethidium bromide (AO/EB) staining was used to observe the apoptotic and necrotic cell nuclear morphology after treatment with the ethanolic extract at different time and concentration (Plate 3). The cells showing green fluorescence with intact green nucleus represent the live cells in control and in treated cells. The morphology of cells was found to be transformed after the treatment, showing cellular shrinkage, membrane blebbing, and typical nuclear fragmentation. The result found that treatment for short duration (5 h) showed early apoptotic cells with more membrane blebbing and yellowish/orange condensed chromatin. But with gradual increase in incubation time (5 h, 10 h, and 15 h) few membrane blebbing and more condensed and fragmented chromatin were observed in most of the cells; at concentrations 200 µg/ml and 250 µg/ml, even presence of necrotic cell was observed with deep orange nucleus which might be due to toxicity with higher dose.

Plate 3. DNA Morphology assay of ethanolic extract of *C. verum*

In the present study, the apoptotic index measured from acridine orange ethidium bromide staining exhibits significant increase in apoptotic cell death after treatment with the ethanolic extract both in dose- and time-dependent manner compared to control which can be...
interpreted from the histogram (Figure 7). The frequency of apoptotic cells with 5h of incubation was 29.4% at 50 µg/ml and 58% at 250 µg/ml with respect to 8.6% in control. Similarly with 10h of incubation the frequency of apoptotic cell was increased to 36% at 50 µg/ml and 72% at 250 µg/ml with 11.8% in control. Further, with 15h samples result in increase of 56% to 68% cell death 50 µg/ml to 250 µg/ml, respectively, in comparison to 19% and 27% with their respective control. Our results illustrate that there is significant increase in apoptotic cell death at 5 h, 10 h and 15 h, compared to their respective control and it was also observe there is significant increase in apoptotic cells when compared between different times of incubation. Therefore, results from our present study indicate an increase in apoptotic cell population induced by the ethanolic extract in concentration as well as time-dependent manner.

Figure 7. Percentage of Apoptosis cell by the ethanolic extract of C.verum

Values are mean ± standard deviation of triplicate.“#” represents significant difference (P<0.005) from 5 h, 10 h, and 15 h versus 3 h whereas “a” represents significant difference (p<0.01 from 5 h to 15 h.

4. CONCLUSION
The ultimate goal of the present study is to reveal the biological chemopreventive mechanism of bioactive compounds in selected plant bark. This study confirms that the bark C.verum is rich in biologically active phytochemicals. These phytochemicals show various beneficial effects that are supported by a variety of literature. Hence human consumption of C.verum may contribute to the improvement in quality of health benefit, antioxidant defense, delaying the onset of oxidative stress mediated disease and this could have a significant advantage over the synthetic antioxidants in food. The results of the present study may be helpful for understanding the role played by naturally occurring phytochemicals acting as radical scavengers. The present data seemed to indicate that extraction of the compounds responsible for the anticancer activity was related to the phytochemicals present in the extracts of C.verum bark. The results confirm that the regular use of C.verum bark as spices is more effective than the usage of regular antibiotic course.

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