Antimicrobial, Antioxidant and Anticancerous Studies on Dodonaea Viscosa Leaf Extracts Against Human Breast Cancer Cell Line (MCF-7)

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Abstract

Cancer is a group of disease that can be invading the body tissues, due to normal growth especially Breast Cancer is the most dangerous disease to women. The main objective of this work finding a Pharmacological activities of herbal plants Dodonaea viscosa against Cancer. The study investigated with methanolic and chloroform extraction from leaves of D. viscosa for presence of Phytochemical compounds. Results revealed that chloroform leaf extract have major phytocompounds than methanolic leaf extract. Further chloroform leaf extract showed antimicrobial activity with Minimum Inhibitory Concentration (>32µg/ml in E. coli and 32µg/ml in Staphylococcus aureus). The higher antioxidant activity (DPPH) observed in leaf extract of D. viscosa at the concentration of 100µL. The Cytotoxicity activity of leaf extract D. viscosa using MCF Cell Line by MTT assay and Trypan Blue Assay further evidenced the inhibitory action on Cancer cell line by the leaf extract. This study validated the medicinal potential of the D. viscosa against MCF-7 Cell line.

Keywords: Dodonaea viscosa, anticancer activity, MTT assay, Trypan blue assay, Breast cancer cell line.

INTRODUCTION

Cancer is a group of disease that can be invade the body tissues, due to uncontrollable or abnormal cell growth. Nowadays, Cancer is mostly affecting the human beings and there is no complete treatment for preventing the cancer¹. Breast cancer is the most dangerous disease to women because of mutational changes in breast tissue². The symptoms of breast cancer are lumps in the breast, change in breast shape, dimpling of the skin, fluid coming from the nipple, red scaly patch of skin³. Alternative and Modern healing therapies approaching again Breast Cancer. The novel invention can be needing to cure the cancer and for preventing them. There are 122 bioactive compounds identified from 94 species of plants and it can be produced drugs against Cancer and 80% of traditional medicine can be identical and tested from herbal plants. The traditional plants have lower toxicity. The screening of the plant compound and plant-based drug delivery to patient against anticancer activity⁴.
Nowadays research focusing on Herbal method with screening and structural identification of novel plant and natural products such as herbs, fruits, spices, teas, and vegetables. These natural products have been studied and proved massive chemotherapeutic effect inhibit the development of Cancer in Laboratory animal models. The wide range of specific constituent of Secondary metabolites present in medicinal plants much more focused by Pharmaceutical importance for a source of medicinal source. Mainly focused on herbal plants than other plants because it is having less toxicity, but the recovery time from disease is slow in process. Researching of herbal plants with combat of antibiotic resistant Microorganisms to developing a therapeutic use against Cancer. Analyzing the presence of phytochemicals in plants to exhibit potential compounds such as Flavonoids, Terpenoids, Alkaloids, Carotenoids. With the help of the compounds, it scavenging the free radicals also preventing the Reactive Oxygen Species (ROS) causing the cell damage. The Epidemiological studies suggested antioxidant supplements reducing the breast cancer effect or breast cancer-related mortality. Mostly Chemotherapy is widely used against Cancer, but it will cause lot of side effects rather than Herbal medicine. The Reduction of (4,5- dimethythiazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT) by the succinate dehydrogenase system of mitochondria in metabolically active cells yields a water insoluble purple formazan product.

*Dodonaea viscosa* is an evergreen shrub and has many medicinal properties for using traditional purpose. A lot of Pharmacological studied in *Dodonaea viscosa* such as Anti-inflammatory, Antioxidant and hypolipidemic effect. Stem or leaf infusion used for sore throats and Rheumatism, Root infusion for colds, leaves used for fever and itching, seeds for Malaria and used as an Antispasmodic agent. The leaves and roots also used for toothaches and headaches. A complex mixture of species contains di-and tri-triterpene, Saponins, flavonoids and eighteen flavonoids include glycosides of Quercetin studied through leaf extract. The present study was carried out to investigate phytoconstituents and evaluating the antimicrobial, antioxidant and Cytotoxicity effect of *D.viscosa* leaf extract against Breast cancer cell line (MCF-7). To analyze the Phyto constituents and identify the qualitative compound using GCMS. To screen the leaf extract against MCF-7 Cell line antimicrobial activity by using Minimum Inhibitory Concentration method, antioxidant activity by using DPPH radical scavenging activity, anticancerous activity by using invitro methods such as MTT assay, Trypan blue assay.

MATERIALS AND METHODS

Plant Collection and Authentication

The leaves of *Dodonaea viscosa* were collected from Mudumalai Hills Region, Coimbatore district of Tamil Nadu, India. The plant was identified and authenticated by Dr. P. Jayaraman, Botanist, Plant Anatomical Research Centre, Chennai-45, and a voucher specimen number PARC/2017/3552.

Preparation of Plant Material

The collected leaves of *Dodonaea viscosa* was washed with distilled water and air dried in a room temperature (shade) without contact in a direct sunlight to avoid the chemical losses in plant. After that, the dried leaves were grounded into fine powder using mortar and pestle or electric blender. The leaf powder was weighed and stored in an airtight container or zip-lock cover for future study.

Preparation of Plant Extracts

Solvent Extraction

The dried leaves of *Dodonaea viscosa* were crudely powdered and subjected to extraction by a Soxhlet extractor. The extraction process carried with 250ml of different solvents and 20gm of powdered plant material.
material was uniformly packed into a thimble. The extraction was done with different solvents such as chloroform and methanol. The process becomes continue till changing of extract to colorless continues for 24 hours in Siphon tube. After that, the extract was taken in a beaker and kept on hot plate and heated at 30-40°C. Dried extract was kept in refrigerator at 4°C for their future use in phytochemical analysis19.

### Phytochemical Analysis

Phytochemical screening was performed to assess the qualitative chemical composition of *D. viscosa* leaf extract for identification of the major secondary metabolites like alkaloids, flavonoids, phenolic compounds, saponins, tannins and terpenoids. The phytochemical analyses were carried out using standard procedures20,21.

### GC-MS Analysis

The GC-MS of chloroform extract was performed for phytochemical investigation. Thermo GC-TRACE ultra-version 6.0. Experimental conditions of GC-MS system were as follows: TR 5-MS capillary standard non-polar column, dimension: 30 Mts, ID: 0.25 mm, Film thickness: 0.25μm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature program (oven temperature) was 40°C raised to 250°C at 5°C/min and injection volume was 2 μl. Samples dissolved in chloroform were run fully at a range of 50-650 m/z and the results were compared by using Wiley Spectral library search program22.

### Antimicrobial Activity

Anti-microbial activity against *Escherichia coli* and *Staphylococcus aureus* and concentration ranges from 64 mg/l - 0.125 mg/ml were used. Minimum inhibitory concentrations (MICs) of antimicrobial agents dissolved with the help of Dilution methods23. Antimicrobial assay was performed in 11 well, sterile, flat bottom microtiter plates, based on broth microdilution assay, which is an automated colorimetric method, uses the absorbance of cultures in a microtiter plate. Each well of microtiter plates was filled with 100 μl of nutrient broth (0.5 McFarland standard). Plant extract is dissolved in respective solvent and 150 μl of the stock is taken, serially diluted in tubes containing DMSO. Different concentration of leaf extracts added as follows 64μg/ml, 32μg/ml, 16μg/ml, 8μg/ml, 4μg/ml, 2μg/ml, 1μg/ml, 0.5μg/ml, 0.25μg/ml and 0.125μg/ml. 100 μl of inoculums were added from well 1 to well 11, positive control containing 100 μl of broth and 100 μl of inoculum, negative control containing 200 μl of broth only. For bacteria, microtiter plates were incubated at 35±2°C for 24 h. The sufficient growth can be attained and take the absorbance or turbidity value. After the incubation period the plates were read at 535 nm using ELISA reader (ELX 800 MS, Biotek Instruments, Inc. USA). The amount of growth in each well is compared with positive growth control and the MIC recorded. The lowest concentration of the agent that inhibits growth of the microorganism such as *Escherichia coli* and *Staphylococcus aureus* i.e., equal to or greater than 50% of the growth. MIC of the drug against the organism *E. coli* and *S.aureus* can be determined by either based on the absorbance or by visual turbidity24.

### Antioxidant Activity

**1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Assay**

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma– Aldrich, Ascorbic acid and methanol were used. In order to determine the radical scavenging ability, the method reported by Mensor et al. (2001)25, was used. Briefly, an alcohol solution of DPPH (50 μl, 2mg/ml) was added to 3mg/ml samples containing different concentrations of extracts (10 μL, 20 μL, 30 μL, 40 μL, 50 μL, 60 μL, 70 μL, 80 μL, 90 μL,100 μL) originating from leaves of the *D. viscosa*. The samples were first kept in a
dark place at room temperature for 30 mins and their absorbance was read at 517 nm using distilled water as blank in a spectrophotometer. The Inhibition (I) was determined using the following formula: 

\[
\% \text{Inhibition} = \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \times 100;
\]

Where, A\text{Control}: tube containing only methanol; A\text{Test}: tube containing extract. Test samples contained 1850 µl methanol + 100 µl of plant extract + 50µL of DPPH solution; control sample contained 50µL of DPPH solution + 1950µL methanol; standard sample contain 1850 µl methanol + 100 µl of Ascorbic acid +50µL of DPPH solution.26

Cell Line

The human breast adenocarcinoma (MCF-7) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 µU/mL penicillin, and 100 µg/mL streptomycin. Cell cultures were incubated in a humidified atmosphere of 5% CO₂ in air and 37°C, and upon reaching 80% confluence, were passaged with a solution of 0.25% trypsin-EDTA. Exponentially growing cells were used for all the experiments.27

Invitro Cytotoxicity Assays

MTT Assay

The leaf extract of invitro Cytotoxicity activity determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using MCF cell line.28 The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) in a 5% CO₂ at 37°C. Cells were seeded at 5000 cells/well in 96-well plates and both were incubated for 48 hours. The different concentrations of the sample incubated for 24 hours. After the medium are removed it was washed with the phosphate saline solution (PBS). Then the sample was placed in a new medium containing 50µl of MTT solution(5mg/ml), to each well incubated for 4h. After the incubation MTT-containing medium was removed and the formazan crystals were dissolved with 50µl of DMSO29. The viable cells were determined by the absorbance at 570nm using a microplate reader (Spectra Max Plus) and percentage of cell viability was calculated using the following formula:

\[
\text{Viability} \% = \frac{[A_{570} \text{ (treated cells)} - \text{(background)}]}{[A_{570} \text{ (untreated cells)} - \text{background}]}) \times 100.
\]

The concentration that gave a 50% reduction in the number of living cells (IC₅₀) was estimated.30

Test of Cell Viability

Trypan Blue Assay

The dye trypan blue exclusion test is used to determine the number of viable cells present in a cell suspension. Centrifuge an aliquot of MCF-7 cell suspension being tested for viability 5 min at 100x g and discard supernatant. The size of the aliquot depends on the approximate number of cells present. The aliquot should contain a convenient number of cells to count in a Hematocytometer when suspended in 1ml PBS and then diluted again by mixing with 0.4% trypan blue (e.g., 5x10⁵ cells/ml). Mix 1 part of 0.4% trypan blue and 1-part cell suspension (dilution of cells). Allow mixture to incubate 3 min at room temperature. Cells should be counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts. Apply a drop of the trypan blue/cell mixture to a hematocytometer place the hematocytometer on the stage of a binocular microscope and focus on the cells. Count the unstained (viable) and stained (nonviable) cells separately in the hematocytometer and to obtain the total number of viable cells per ml of aliquot.31

Viability can be calculated with the formula:
% Viability = Total number of living cells / Total number of cells \times 100 \textsuperscript{32}

RESULTS

Preparation of Plant Extracts

Leaf powder was extracted with Methanol and Chloroform by using Soxhlet apparatus. The plant extract was subjected to condensation process at 40°C to yield crude extract.

After Extractions

The plant sample can be extracted, and the solvent can be evaporated, and it stored in a refrigerator.

Phytochemical Analysis

Freshly prepared leaf organic extracts were tested for the presence of phytochemical constituents using reported methods\textsuperscript{20} and the results are given in Table-1.
Terpenoids constitute the largest class of natural products and are a rich reservoir for drug discovery. Although the research and development of anti-cancer drugs derived from natural products have led to the identification of a variety of terpenoids that inhibit cancer cell proliferation and metastasis via various mechanisms. Terpenoids is present in chloroform plant extract and absent in methanol plant extract. Antioxidant and Anticancer activity proceeded with Chloroform leaf extract.

**GC-MS Analysis**

GC-MS analysis of the chloroform extract of *Dodonaea viscosa* showed six peaks which indicating the presence of five phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library, the five phytocompounds were characterized and identified. The various phytochemicals which contribute to the medicinal activities of the plant were shown. The mass spectra of all the phytochemicals identified in the chloroform leaf extract of *Dodonaea viscosa*. Of the five compounds identified, the most prevailing compounds were Phthalic acid, di(2-propylpentyl) ester, Dibutyl phthalate (25%), plasticizing compound (20%). Among the compounds, four compounds were reported to have antimicrobial activity and antioxidant activity was reported Quinoline, 1,2-dihydro-2,2,4-trimethyl-1-, cytotoxicity activity in 1-Dodecanol.

<table>
<thead>
<tr>
<th>Test</th>
<th>Methanol</th>
<th>Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for Phenol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Test for Terpenoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Test for Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Test for Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Test for Alkaloids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Test for Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Test for Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Present, -Absent
Antimicrobial Activity

*Dodonaea viscosa* is an effective antimicrobial agent against microorganisms. The absorbance values of the wells as follows: *E. coli* and *S. aureus* in Table 3.

---

**Fig 3. GC-MS chromatogram of *Dodonaea viscosa* chloroform extract**

**Table 2. Phytocomponents identified in the chloroform extract of *D. viscosa* by GC-MS**

<table>
<thead>
<tr>
<th>S.No</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>MW</th>
<th>Peak Area %</th>
<th>Nature of the compound</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9.872</td>
<td>Quinoline, 1,2- dihydro 2,2,4 trimethyl-</td>
<td>C_{12}H_{13}N</td>
<td>173.259</td>
<td>5.74</td>
<td>Acetone compound</td>
<td>Antioxidant activity</td>
</tr>
<tr>
<td>2.</td>
<td>10.367</td>
<td>1-Dodecanol</td>
<td>C_{12}H_{26}O</td>
<td>186.339</td>
<td>1.24</td>
<td>Alcoholic compound</td>
<td>Cytotoxicity activity</td>
</tr>
<tr>
<td>3.</td>
<td>21.183</td>
<td>Dibutyl phthalate</td>
<td>C_{32}H_{40}O_{4}</td>
<td>278.35</td>
<td>24.29</td>
<td>Plasticizer compound</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td>4.</td>
<td>24.212</td>
<td>Phthalic acid, 6-ethyl- 3-octyl butyl ester</td>
<td>C_{22}H_{38}O_{4}</td>
<td>362.51</td>
<td>4.25</td>
<td>Plasticizer compound</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td>5.</td>
<td>31.982</td>
<td>Oxirane octanoic acid, 3-octyl-, methyl ester</td>
<td>C_{13}H_{22}O_{5}</td>
<td>312.49</td>
<td>2.88</td>
<td>Fatty acid compound</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td>6.</td>
<td>35.576</td>
<td>Phthalic acid, di (2-propyl pentyl) ester</td>
<td>C_{24}H_{44}O_{4}</td>
<td>390.55</td>
<td>50.7</td>
<td>Plasticizer compound</td>
<td>Antimicrobial activity</td>
</tr>
</tbody>
</table>

The biological activity of the compound can refer with help of article^{34}.
Based on absorbance

The absorbance of the sample in the microtiter plate was read at 595nm after the specific incubation period in Table 4

Based on turbidity

The lowest concentration at which there is visible reduction in the turbidity of the organism growth in the wells of the microtiter plate is considered as the MIC of the drug against that specific organism in Table 4.

### Table 3. The absorbance value of *E. coli* and *S.aureus*

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th><em>E. coli</em></th>
<th><em>S.aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.176</td>
<td>0.049</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.386</td>
<td>0.195</td>
</tr>
<tr>
<td>32µg/ml</td>
<td>0.208</td>
<td>0.084</td>
</tr>
<tr>
<td>16µg/ml</td>
<td>0.227</td>
<td>0.097</td>
</tr>
<tr>
<td>8µg/ml</td>
<td>0.236</td>
<td>0.098</td>
</tr>
<tr>
<td>4µg/ml</td>
<td>0.232</td>
<td>0.119</td>
</tr>
<tr>
<td>2µg/ml</td>
<td>0.238</td>
<td>0.108</td>
</tr>
<tr>
<td>1µg/ml</td>
<td>0.267</td>
<td>0.130</td>
</tr>
<tr>
<td>0.5µg/ml</td>
<td>0.288</td>
<td>0.135</td>
</tr>
<tr>
<td>0.25µg/ml</td>
<td>0.350</td>
<td>0.164</td>
</tr>
<tr>
<td>0.125µg/ml</td>
<td>0.325</td>
<td>0.165</td>
</tr>
<tr>
<td>0.0625µg/ml</td>
<td>0.367</td>
<td>0.186</td>
</tr>
</tbody>
</table>

The chloroform plant extract was taken absorbance value in 595 nm.

### Antioxidant Activity-DPPH Assay

Free radicals and reactive oxygen species (ROS) contribute to forming many diseases such as Arthritis, Cirrhosis, Cancer, Alzheimer’s disease, and aging\(^26\). At a concentration of 100 µL (1000 µg), the
scavenging activity of the Chloroform extract of Dodonaea viscosa leaves reached to 66.53%, while other different concentration showing averagely reduced the free radicals illustrated variation in figure 4.

![Graph of Concentration of extract vs % Scavenging](image)

Fig 4. Percentage of Scavenging radicals

**Cytotoxic Activity of Terpenoid Rich Chloroform Leaf Extract in Dodonaea Viscosa**

Dodonaea viscosa is a terpenoid rich leaf extract used in Anticancer studies for demonstrated strong cytotoxicity towards MCF-7 Cell line used MTT assay. Cytotoxicity = (IC_{50} value is 8.571).

![Graph of Response vs Log dose](image)

Fig 5. Anticancer activity of Terpenoid rich *Dodonaea viscosa* leaf extract in MCF-7 Cells
The significant reduction in cell viability among the MCF-7 cells in concentration variation manner and the values were determined from best-fit analysis. Comparison of cellular concentration of control and the concentrations such as 6.25µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml in (figure 6.a-e).

**Fig 6.a. Control**

**Fig 6.b. 6.25µg/ml**

**Fig 6.c. 25µg/ml**

**Fig 6.d. 50µg/ml**

**Fig 6.e. 100µg/ml**

**Fig 6. Comparison of cellular concentration of control and the concentrations such as 6.25µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml**

**Trypan Blue Assay**

Trypan blue assay determined the number of viable cells present in an MCF-7 cell suspension with the help of Hemocytometer.
DISCUSSION

Natural plants have many medicinal properties used in worldwide for more than thousands of years. Many research experiments showed *D. viscosa* have antimicrobial, antiviral, anti-malarial, anti-ulcer, antispasmodic, antifertility, antidiarrheal properties and wound healing effect. Cancer is one of the leading life-threatening disease to treat and prevention is quite difficult. Control and management therapy of Cancer is important and goal for every researcher doing a lot of experiment with natural products for complete cure and permanent solution to inhibit the cancer cell. In this research studied used natural plant leaf product activity against Anticancerous effect to Breast carcinoma cell. Preliminary phytochemical screening revealed the presence of Phenols, Alkaloids, Saponins, Glycosides, Terpenoids in methanol and Chloroform extract of *D. viscosa*. The Main Phytoconstituents Terpenoids and Phenols present in leaves part like antiulcer, antidiabetic and anti-inflammatory activities. The MIC value of antimicrobial activity concentration in *E. coli* is >32µg/ml and *S. aureus* is 32µg/ml. Typical phenolic acids and Flavonoids possess antioxidant activity. In the present study antioxidant was found which can be attributed to the presence of Alkaloids, Flavonoids. So, it concluded Chloroform extracts have higher antioxidant activity of these extracts. The relationship between Phenols and antioxidant activity of plant species because it is scavenging ability of their Hydroxyl groups. The Phenolic Compound donating the electrons scavenging the Hydrogen Peroxide and it is neutralizing the water and it mainly due to redox properties in adsorbing and neutralizing Super oxide Anion, Hydroxyl Peroxy radical. The result of DPPH inhibition by leaf extract of *D. viscosa* showed that maximum scavenging activity with concentration of 100 µL (1000 µg) at 66.53%.The result of Cytotoxic activity on human MCF-7 cell lines was determined according to the dose values of plant extract to reduce in the cell lines to 50%(IC<sub>50</sub>). The results showed that Chloroform extract of *D. viscosa* has strong cytotoxic activity as its IC<sub>50</sub> is value is 8.571.Trypan blue assay was used for evaluation of viability of MCF-7 cells determined the anticancer activity of chloroform extracts of *D. viscosa*.

CONCLUSION

The current study showed that Chloroform extract of *Dodonaea viscosa* leaves have antioxidant and anticancer activities. This Leaf extract result observed had high terpenoid content against Breast
carcinoma MCF cell lines. Based on the experiments *Dodonaea viscosa* have strong secondary metabolites which add Scientific evidence to conduct further studies, investigate the lead compounds in plant and evaluate the anticancer potential on in vivo animal models and forward an attempt to carry out trails on human beings.

**ACKNOWLEDGMENTS**

There are no financial Conflicts of Interest to disclose.

**REFERENCES**