Free radicals scavenging and antiproliferative activity of ethanolic and methanolic extracts of *Carica papaya* leaves in PA-1 cell line

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**ABSTRACT**

A comparative antioxidant and anticancer potential of methanolic and ethanolic extracts of leaves of *Carica papaya* were investigated. Both the plant extracts were prepared separately, and their anti-oxidant scavenging potential was investigated for the scavenging of superoxide, nitric oxide and hydrogen peroxide radicals using standard protocols. The anticancer property of these plant extracts was evaluated in PA-1 cell lines using tryptan blue assay. The total phenols and flavonoids contents responsible for the above said activities were estimated quantitatively. Among the two extracts, the ethanolic extract scavenged the free radicals to a greater extent when compared to the methanolic extract. Similarly, in the tryptan blue exclusion assay, the percentage of cell death was more for ethanolic extract. At high concentrations such as 50, 75 and 100 μg concentrations, the ethanolic extract showed higher cell death which was comparable to the positive drug doxorubicin used in the present study. Both the extracts possessed considerable quantities of phenols and flavonoids. It may be concluded that the presence phytochemicals along with the phenols and flavonoids may be the reason for the antioxidant and anticancer activity observed in the present study.

**INTRODUCTION**

Cancer is a multistep disease involving the physical, environmental, metabolic, chemical, and genetic factors, which is considered as a major public health burden in developed and developing countries (Ferlay *et al.*, 2010). Though there several clinical therapies including immune modulation, chemotherapy, surgery and radiation are available to treat cancer, they have limited success as evidenced by the high morbidity and mortality rates emphasizing the need for the new cancer management (Dai *et al.*, 2010). Reactive oxygen species (ROS)/Reactive nitrogen species (RNS) comes under the category of free radicals are contributing significantly in the pathogenesis of diseases such as diabetes, the ageing process, heart diseases, and cancer (Willcox *et al.*, 2004; Sireesha *et al.*, 2015). They are responsible for the damage of the nucleotide and deoxyribose sugar backbone which ultimately leads to the severe genetic mutation of the important genes (Klaunig JE, Kamendulis 2004). Due to these genetic changes initiation, promotion and advanced stages of cancer are activated. Phytotherapy has also significantly contributed to the treatment of cancer, and over the last half-century, secondary metabolites derived from plants have been successfully used for the treatment of cancer. (Willcox *et al.*, 2004).

*Carica papaya* belongs to the *Caricaceae* family is a herbaceous fruiting perennial tree-like plant is found throughout India and America (Pierson *et al.*, 2012). The leaves possessed many medical applications and used for treating amoebic dysentery, controlling fever, gastric digestion problems,
(Canini et al., 2007; Starley et al., 1999; Zunjjar et al., 2011). They are used as an anti-inflammatory agent and good burn healers (Owoyele et al., 2008) and reduces cardiovascular disease risk (Runnie et al., 2004). The serve as an anti-tumour activity and immune-adjuvant for vaccine therapy (Otsuki et al., 2010).

They are also used for reducing cardiovascular disease risk (Runnie et al., 2004). The present study was aimed to evaluate, the antioxidant activity and proliferative effect of the ethanolic (EECP) and methanolic (MECP) extracts of leaves of Carica papaya.

MATERIALS AND METHODS

Chemicals: The chemicals such as Catechin, Gallic acid, Ascorbic acid, Folin–Ciocalteu’s reagent, Sodium nitroprusside, Vitamin E, Potassium thiocyanate and Ferric chloride were obtained from SD Fine Chemicals Ltd., India. The chemicals and reagent are used for other purpose were of analytical grade.

Collection of Plant Material: The leaves of Carica papaya were collected from Chennai and were authenticated by Dr Jayakumari Prof and Head of the Department of Pharmacognosy, Vels college of pharmacy Chennai, India. The herbarium file of the same Centre contains the voucher specimen.

Preparation of methanolic and Ethanolic crude Extracts Carica papaya

The leaves of Carica papaya are entirely washed in tap water, dried and powdered. The ethanolic (EECP) and methanolic (MECP) extracts of leaves of Carica papaya were prepared by Soxhlet extraction method. The extracts werevaporized, and all the solvent had been removed. The yield of the EECP and MECP were calculated, and it was found to be 7-9% and 4-6% w/w respectively. The crude extract was kept and stored in 4°C until further use.

Antioxidant activity determination

Superoxide scavenging activity: The scavenging activity of Superoxide radical was performed by (Robok et al., 1988) method. These radicals are formed in nicotinamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) system and measured by the reduction in nitro blue tetrazolium (NBT). In brief 1 ml of 0.1mM NADH solution and different concentrations of the plant extracts were added to 1 ml of 50mM PMS solution and incubated at 25°C for 5 min and the absorbance was measured against the blank solution. The L-Ascorbic acid was used as the positive control. The inhibition percentage of superoxide radical = [(A0 –A1/A0) x100], Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

Nitric oxide scavenging activity: The nitric oxide scavenging ability of the plant extracts were measured by (Marcocci et al., 1944) method. The different concentrations of the EECP and MECP extracts were mixed with sodium nitroprusside, incubated for 150min at 25°C and 0.5ml of Griess reagent was added. The chromophore formed and the absorbance of was read at 546nm. Vitamin E was used as a standard.

Hydrogen Peroxide Scavenging Activity: The H2O2 scavenging ability of the plant extracts was performed by (Ruch et al., 1989) method. Aliquots of the plant extracts were dissolved in 3.4 ml of phosphate buffered saline (PBS), and 0.6ml of a 2mM solution of H2O2 were added, and its absorbance was measured at 230 nm after 10 min. Ascorbic acid was used as the standard. The inhibition of H2O2 by the extracts was calculated as follows. Hydrogen peroxide radical scavenging activity (%) = [([A0 –A1/A0] x100]

Procurement and maintenance of cancer cell lines: The PA-1 (Human ovarian cancer) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in DMEM (Dulbecco’s Modified Eagle Medium). The cell lines were cultured in 25 cm² tissue culture flask supplemented with DMEM 10% FBS, sodium bicarbonate, and an antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml). They were humidified with 5% CO2 and kept at 37°C (NBS Eppendorf, Germany). And two days old confluent monolayer of cells were trypsinized, and the cells were suspended in 10% growth medium, 100µl cell suspension (5x10⁴ cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO2 incubator for 24 hours and used for carrying out the anti-proliferative study of the plant extract.

Treatment of cells with EECP and MECP extracts

The 24 hrs incubated 96 well seeded PA-1 cell were treated with the different concentrations of EECP, and MECP extracts were prepared in 5% DMSO. In short, after 24 hours the growth medium was diluted and different concentrations freshly prepared each plant extracts in 5% DMSO was added to the cells and incubated for 72 hrs to analyse the potency of the plant extracts. Doxorubicin was used as a positive drug in this study, and negative Control was PA-1 cell lines without any treatment.
**Cell Viability Assay**

The cell viability tumour cells as a result of drug treatment were estimated by trypan blue dye in which the cells that stained with the dye were non-viable and those did not stained are non-viable. And 0.1 ml of freshly prepared PA-1 cell lines were treated with the different concentrations of the EECP and MECP extracts in DMSO and the last volume of 1ml were incubated for 3 hours at 37°C. Cells without any treatment was considered as negative control and cells treated with doxorubicin was used as a positive control. At the final stage of the incubation period, the cells were placed on the Neubauer counting chamber, the cells were stained with trypan blue dye, and the number of viable and non-viable cells were counted under the microscope (Haldar et al., 2009).% viable cells = [1.00 - (Number of blue cells + Number of total cells)] × 100. Cell count = (No. of cells × dilution factor)/(area × thickness of liquid film).

**Determination of Total Phenolic content**

The Total phenolic content of the extracts was done by Slinkard and Singleton method (Slinkard et al., 1977). In which 0.5 ml of aqueous solution containing 100 mg of the sample was treated with 2.5 ml FCR (diluted 1:10, v/v) and 2 ml of Na2CO3 (7.5%, v/v) solution. After incubation at 30°C for 90 min, the absorbance was at 765 nm. Results were used as gallic acid are equivalents as (mg gallic acid/100g dried extracts).

**Determination of Total Flavonoid content**

The total flavonoid content was followed by the colourimetric method (Zhishen et al., 1999). The sample containing 2ml of an aqueous solution containing 100 mg of the extracts were mixed with 0.15 ml of aluminium chloride solution, 0.15 ml of sodium nitrite solution, was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) and water was added to make the volume to 5ml. This complete mixture was incubated for 15 min and the absorbance determined at 510 nm against the water blank. Results were expressed as catechin equivalents (mg catechin/100 g dried extract).

**Statistical Analysis**

The experimental data were statistically analysed with Statistica/Mac software (Prism, USA). One-way ANOVA was used for Data analysis followed by Dunnet’s “t” test. They were expressed as mean ± SEM of three parallel measurements. P values of <0.05 were considered as significant.

**RESULTS**

**Antioxidant activity determination**

**Superoxide scavenging activity:** Figure -1 shows the nitric oxide scavenging action of the EECP, MECP and the positive control Ascorbic acid. Concentration-Dependent scavenging of nitric oxide was observed for all these three extracts. However, the ethanolic extract of the Carica papaya showed an enhanced scavenging activity when compared to the methanolic extract. At concentrations of 800and 1000 µgms, the EECP exhibited almost the same nitric oxide inhibition as that of the positive control ascorbic acid used in this study

**Nitric oxide scavenging activity**

The nitric oxide scavenging activity of the EECP, MECP in Figure -2 shows the positive control as Ascorbic acid. The nitric oxide concentration dependent scavenging activity was observed for all these three extracts. However, the ethanolic extract of the Carica papaya showed an enhanced scavenging activity when compared to the methanolic extract. At concentrations of 800and 1000 µgms, the EECP exhibited almost the same nitric oxide inhibition as that of the positive control ascorbic acid used in this study

**Hydrogen Peroxide Scavenging Activity**

In the present investigation higher inhibitory activity of hydrogen peroxide radicals was observed for EECP extract than the MECP extract. Ascorbic acid was used as positive control in the present study, the lower concentration (100µgms) the radical scavenging activity of EECP extract was compared. Table -3 explains the hydrogen peroxide Scavenging activity of the MECP, EECP and their positive control ascorbic acid

**Cell Viability assay:** The anticancer activity of the Carica Papaya extracts was assessed by trypan blue assay and were summarized in table- 4. A significant percentage of cell death was observed due to the plant extract treatment in PA-1 cell lines when compared to untreated control cells; Ethanol extract exhibited better anticancer activity that the methanolic extract of Carica papaya which is observed in terms of percentage cell death observed in the present study. At 50, 75, 100 µgms concentrations the anticancer activity was as similar to that of the positive drug doxorubicin.

**Total Phenolic and Flavonoid contents:** The total phenolic and flavonoid contents of MECP and EECP extracts were shown in Table-6, which were determined and expressed in terms of catechin equivalents and gallic acid. The antioxidant activity and antiproliferative of these plant extract may be probably due to these secondary metabolites.
DISCUSSION

The human cell system when utilizing oxygen for its metabolism produces a certain amount of Reactive oxygen species (ROS) such as $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and hydroxyl radicals (OH) (Ukeda et al., 2001). ROS readily affects the various biomolecular of the cell such as proteins, carbohydrates, fatty acids and DNA by oxidizing them leading to cell degeneration and death (Remacle et al., ). Our human body by its nature has several defense mechanisms to combat this oxidative stress. Many serious diseases such as cancer, gout, Alzheimer’s disease arteriosclerosis, may occur when there is an excess production of these free radicals beyond the body’s capacity to scavenge them. The natural antioxidants present in vegetables and fruits contain much importance as they protect the human body from the oxidative stress and diseases. (Ali et al., 2008). These holistic plant derived herbal medicines can be exploited for research to obtain a successful plant-derived drug for the treatment of cancer since the modern synthetic cancer treatment procedures are very expensive and also produces severe side effects.

In the present study investigation, the antioxidant potential of the methanolic and ethanolic extracts of *Carica papaya* was studied in forms of the scavenging of superoxide, hydrogen peroxide radicals and nitric oxide. The ethanolic extract was more effective in scavenging these radicals than the methanolic extracts. The Superoxide is considered as the reduced form of oxygen by one electron is a dangerous molecule since it acts as a precursor for other free radicals such as hydrogen peroxide and hydroxyl. These molecules have the ability to damage the biological macromolecules and initiate the tissue damage. In the present study, the plant extracts considerably reduced the superoxide radicals. (OUIgwe et al., 2015) Have reported six major chemical constituents in the isopropanol extracts of *Carica papaya* leaf extract. These compounds either individually or cumulatively might be responsible for the

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**Figure 1:** Superoxide scavenging activity of different plant extracts
Superoxide scavenging activity of MECP, EECP and Ascorbic acid. The value represents the mean ± SEM (n = 3). Comparison between a-MECP vs Ascorbic acid and b-EECP vs Ascorbic acid.*p<0.05, *p<0.01, NS–Not Significant

**Figure 2:** Nitric oxide scavenging activity of different plant extracts
Scavenging activity of Nitric oxide of both MECP, EECP and Ascorbic acid. Each value represents the mean ± SEM (n = 3). The comparison between a-MECP vs Ascorbic acid and b-EECP vs Ascorbic acid. *p<0.01, *p<0.05, NS–Not Significant
Table 1: Hydrogen peroxide scavenging activity of different plant extracts

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Inhibitory activity of MECP (%)</th>
<th>Inhibitory activity of EECP (%)</th>
<th>Inhibitory activity of Ascorbic acid (%)</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>12.19±0.54a**</td>
<td>20.72±4.60 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>21.30±0.72</td>
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<tr>
<td>200</td>
<td>14.82±1.28a**</td>
<td>23.35 ± 4.63 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>24.75 ± 0.77</td>
</tr>
<tr>
<td>400</td>
<td>20.88 ± 1.31a**</td>
<td>27.28 ± 3.43 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>28.52 ± 2.53</td>
</tr>
<tr>
<td>500</td>
<td>25.46 ± 3.32a**</td>
<td>35.00 ± 2.79 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>36.36 ± 0.70</td>
</tr>
<tr>
<td>600</td>
<td>45.47 ± 6.56a**</td>
<td>66.46 ± 2.07 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>66.72 ± 0.88</td>
</tr>
<tr>
<td>800</td>
<td>68.19 ± 2.83a**</td>
<td>79.63 ± 4.02 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>81.75 ± 0.56</td>
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<tr>
<td>1000</td>
<td>75.23 ± 4.57a**</td>
<td>87.25 ± 3.97 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>88.15 ± 4.01</td>
</tr>
</tbody>
</table>

Hydrogen peroxide scavenging activity of MECP, EECP and Ascorbic acid. Values represent the mean ± SEM (n = 3). Comparison between a-MECP vs Ascorbic acid and b-EECP vs Ascorbic acid NS—Not Significant * p<0.01, *p<0.05.

Table 2: Trypan blue assay of different plant extracts

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Cell death in control (%)</th>
<th>Cell death by MECP (%)</th>
<th>Cell death by EECP (%)</th>
<th>Cell death by doxorubicin(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>97.51±0.35</td>
<td>37.4±0.71 a&lt;sup&gt;***&lt;/sup&gt;</td>
<td>43.23±0.30 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>63.26±1.25</td>
</tr>
<tr>
<td>10</td>
<td>97.34±0.12</td>
<td>41.4±0.66 a&lt;sup&gt;**&lt;/sup&gt;</td>
<td>50.6±0.47 b&lt;sup&gt;**&lt;/sup&gt;</td>
<td>72.81±0.69</td>
</tr>
<tr>
<td>25</td>
<td>97.30±0.09</td>
<td>52.0±0.42 a&lt;sup&gt;**&lt;/sup&gt;</td>
<td>62.0±0.35 b&lt;sup&gt;**&lt;/sup&gt;</td>
<td>76.52±0.70</td>
</tr>
<tr>
<td>50</td>
<td>97.42±0.10</td>
<td>63.9±0.40 a&lt;sup&gt;**&lt;/sup&gt;</td>
<td>79.17±0.23 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>80.89±0.10</td>
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<tr>
<td>75</td>
<td>97.52±0.19</td>
<td>70.7±0.34 a&lt;sup&gt;**&lt;/sup&gt;</td>
<td>83.05±0.33 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>85.03±0.08</td>
</tr>
<tr>
<td>100</td>
<td>97.52±0.19</td>
<td>79.6±0.38 a&lt;sup&gt;**&lt;/sup&gt;</td>
<td>86.91±0.14 b&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>89.90±0.09</td>
</tr>
</tbody>
</table>

Trypan blue exclusion assay of control, MECP, EECP and doxorubicin. Different value represents the mean ± SEM (n = 3) and the comparison between a-MECP vs Doxorubicin and b-EECP vs Doxorubicin, a.*,p<0.05, * *p<0.01, NS—Not Significant.

Table 3: Total Flavonoid contents and Phenolic of MECP and EECP Extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Total phenolic content (mg/g)</th>
<th>Total flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EECP</td>
<td>48.46±4.30</td>
<td>63.30±5.02</td>
</tr>
<tr>
<td>MECP</td>
<td>32.67±6.60</td>
<td>47.17±2.30</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD (n = 3); Total flavonoid content was expressed as mg catechin equivalent/g dried extract; Total phenolic present was expressed as mg gallic acid equivalents/g dried extract.

They are scavenging effect of the superoxide seen in the present study.

The reactive oxygen species was very reactive and reactive nitrogen species or Nitric oxide are produced and according to Hemnani and Parihar these nitrogen species damage the DNA, oligonucleosomal fragments, destroy proteins by nitration of tyrosine residues and causes lipid peroxidation. In the present investigation, considerable scavenging of the nitric oxide was observed by both the plant extracts. It is an accepted fact that most of our Indian plants contain significant phyto pharmacological components which have the ability to protect our health. Moreover, a significant amount of flavonoids and phenols were present in both the extracts. It is an accepted fact that phenols and flavonoids contribute sufficiently towards the antioxidant activity. The extent of nitric oxide scavenging activity may be due by these phenols and flavonoids.

The Hydrogen peroxide produced in the biological systems by itself is not very reactive by can act as a transition metal ions and an oxidising agent in the presence of O₃ which ultimately produces hydroxyl radicals through Fenton reactions (Halliwell et al., 1991). According to (Mahakunakorn et al., 2005) the H₂O₂ exerts its injurious effect by altering intracellular Ca²⁺ homeostasis, inducing DNA damage and increasing intracellular ATP by easily crossing the cell membrane. Our plant extracts scavenged H₂O₂ since the removal of H₂O₂ it is more important for defending the antioxidant mechanism.

The anticancer activity of the Carica Papaya extracts was assessed by trypan blue assay using PA-1 cell lines. At higher concentrations, the plant extracts activity was comparable with the positive drug doxorubicin. (Ayoola et al., 2010) have reported the presence of bioactive compounds such as saponins, cardiac glycoside alkaloids and alkaloids. According to (Okwu et al., 2004) saponins are responsible for the bitter taste of the leaf and they are cytotoxic. Already Ou lgwe have reported the antimicrobial activity and (Robinson et al., 1985) have reported the anti-malarial activity of the leaf extract and both of them have said that the alkaloids present in the leaf extract are re-
sponsible for the activity. The same alkaloid may be expected to play a vital role in the cytotoxic activity of the PA-1 cell lines

CONCLUSION

It can be concluded based on the obtained results the *Carica papaya* leaf extract may be a good source of drug for ovarian cancer as well as a successful antioxidant. However further detailed investigations are important to prove the tentative action of the mechanism of its cytotoxic activity. Owing to the presence of alkaloids and saponins which can be developed into an anticancer drug for treating various types of cancers. Added to that with the cardiac help glycosides, the oxidative stress-related conditions which are common in cancer chemotherapy will also be taken care of. With these possibilities, the plant drug can be developed into a new antioxidant and anticancer drug after proper pharmacological evaluation and chemical characterisation.

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