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

Priyadurairaj, Palagati Rohith Kumar Reddy, Palaniyandi Thiruvanavukkarasu, Rajeswary Hari*

Dr.M.G.R. Educational and Research Institute University, Chennai-95. Tamil Nadu, India

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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 doxrubicidin 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.

* Corresponding Author

Name: Rajeswary Hari
Phone: +91-9840774705
Email: rajihar@gmail.com

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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; Zunjar *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 wereevaporaised, 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 4^0C 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 25ºC 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 H_2O_2 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 H_2O_2 were added, and its absorbance was measured at 230 nm after10 min. Ascorbic acid was used as the standard. The inhibition of H_2O_2 by the extracts was calculated as follows. Hydrogen peroxide radical scavenging activity (%) = $[(A_0 - A_1/A_0) \times 100]$.

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 cm^2 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% CO_2 and kept at 37ºC (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 ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator for 24 hours and used for carrying out the antiproliferative 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. Doxrubicin 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 strained 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 doxrubicin 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 (diluted1:10, v/v) and 2 ml of Na_2CO_3 (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 100mg 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 for15 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; Ethanolic extract exhibited better anticancer activity than 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.

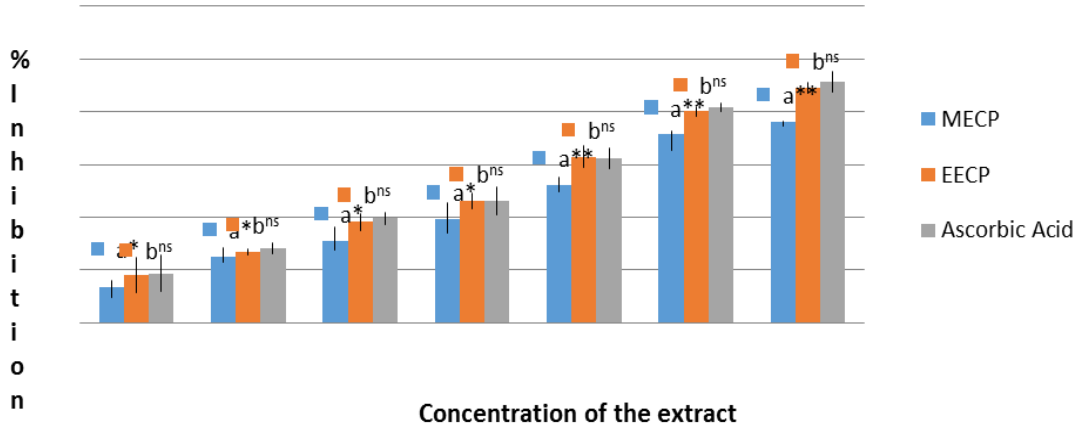

Figure 1: Superoxide scavenging activity of different plant extracts
Superoxide scavenging activity of MECP, EECP and Ascorbic acid. The value represents the mean ± SEM (n = 3). Comparison between a-MECP vs Ascorbic acid and b-EECP vs Ascorbic acid.*p<0.05, * *p<0.01, NS–Not Significant

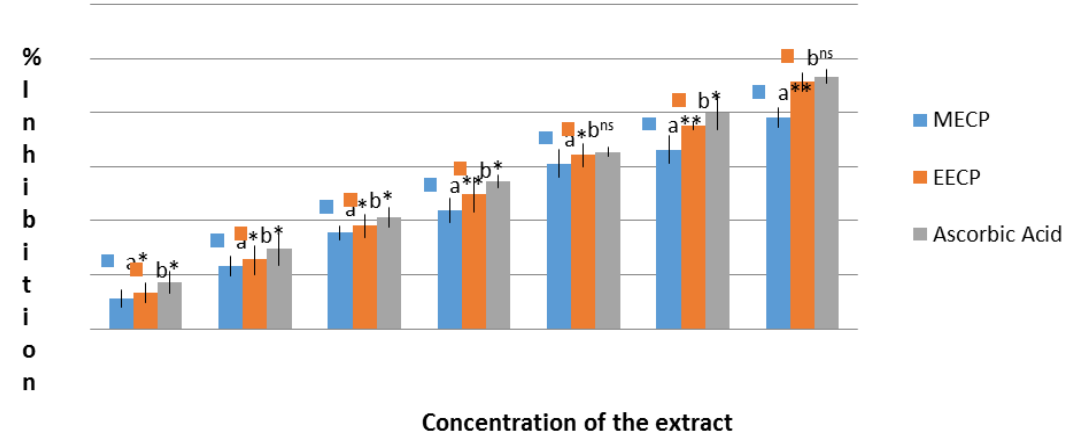

Figure 2: Nitric oxide scavenging activity of different plant extracts
Scavenging activity of Nitric oxide of both MECP, EECP and Ascorbic acid. Each value represents the mean ± SEM (n = 3). The comparison between a-MECP vs Ascorbic acid and b-EECP vs Ascorbic acid. * *p<0.01, *p<0.05, NS–Not Significant

DISCUSSION

The human cell system when utilizing oxygen for its metabolism produces a certain amount of Reactive oxygen species (ROS) such as O_2^-, H_2O_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

Table 1: Hydrogen peroxide scavenging activity of different plant extracts

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

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

Concentration (µg/ml)	Cell death in control (%)	Cell death by MECP (%)	Cell death by EECP (%)	Cell death by doxorubicin(%)
5	97.51±0.35	37.4±0.71 a***	43.23±0.30b**	63.26±1.25
10	97.34±0.12	41.4±0.66 a**	50.6±0.47 b**	72.81±0.69
25	97.30±0.09	52.01±0.42 a**	62.02±0.35 b**	76.52±0.70
50	97.42±0.10	63.39±0.40 a**	79.17±0.23 b^{ns}	80.89±0.10
75	97.52±0.19	70.7±0.34 a**	83.05±0.33 b^{ns}	85.03±0.08
100	97.52±0.19	79.6±0.38 a**	86.91±0.14 b^{ns}	89.90±0.09

Typhan 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

Extracts	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
EECP	48.46±4.30	63.30±5.02
MECP	32.67±6.60	47.17±2.30

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 is may be due 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 O_3, which ultimately produces hydroxyl radicals through Fenton reactions (Halliwell *et al.,* 1991). According to (Mahakunakorn *et al.,* 2005) the H_2O_2 exerts its injurious effect by altering intracellular Ca^{2+} homeostasis, inducing DNA damage and increasing intracellular ATP by easily crossing the cell membrane. Our plant extracts scavenged H_2O_2 since the removal of H_2O_2 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 Igwe 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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