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Cytoprotective and DNA Protective Activity of *Carica Papaya* Leaf Extracts

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ABSTRACT
Papaya (Carica papaya Linn) is commonly called as paw-paw and it belongs to the family Caricaceae. The properties of papaya fruit and other parts of the plant are also well known in traditional system of medicine. Papaya possess excellent medicinal properties for treatment of different ailments. These curative properties are based on the presence of phytochemical nutrients with antioxidant effect in different parts of the plant. It is considered as valuable nutraceutical fruit plant due to its biological activity and medicinal application.The present study was designed to determine the Cytoprotective and DNA protective activities of different fractions (Aqueous, Chloroform, Ethanol and Ethyl acetate extracts) of Carica papaya leaves. Cytoprotective capacity was assessed using erythrocytes, where ferrous sulphate was used to induce stress and the ability of the extracts to combat the induced stress was evaluated. The DNA protective potential against free radical-mediated oxidative stress was evaluated by a DNA damage inhibition assay involving agarose gel electrophoresis and UV spectrophotometric analysis. All the four fractions displayed significant cytoprotective effect on erythrocytes and prevented oxidative damage to DNA in presence of DNA damaging agent. Altogether, the results of our study lend pharmacological credence to the anti-cancerous and ethno medical use of this plant in traditional system of medicine and these resultscould be used to develop antimutagenic compounds for cancer therapy.
Keywords: *Carica papaya, Cytoprotection, DNA damage inhibition, ethyl acetate, aqueous, ethanol, chloroform fractions.*

I. Introduction

Carica papaya, which comes from Caricaceae family is one of many important cash crops which is believed to have its origin from Central America. The papaya plant is a large herb that grows rapidly, producing a soft wood and its height can be reached of more than twenty-five feet. The plant does not consume much time to grow and bears fruit within a year. The papaya tree needs a tropical climate, which is dry when cold and wet when warm, consequently, the best conditions for the tree to grow is at 25 °C.

Plants have the main advantage of still being the most effective and cheaper alternate source of drugs. In addition to the nutritional value of its fruit, the leaves of Carica papaya (CP) possess medicinal properties and are frequently used in traditional medicines. The leaves of papaya have been shown to contain many active components such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic-glucosides and glucosinolates that can increase the total antioxidant activity in blood and reduce lipid peroxidation level[1]. The alkaloids, flavonoids, saponins, tannins, and glycosides are related with anti-inflammatory activity. CP leaf extract was also found to have antibacterial effect[2], antitumor and immunomodulator activities[1]. The leaves also contain cardiac glycosides, anthraquinones, carpaine, pseudocarpaine, phenolic compounds[3,4].

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA[5]. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks. Oxidative stress also contributes to tissue injury following irradiation and hyperoxia, as well as in diabetes. Thus the importance of searching for alternative plant products against a number of diseases associated with ROS continues to be popular because of limited toxicity associated with it as against synthetic products.

The antioxidant, anti-inflammatory, anti-sickling, wound healing and immunomodulatory potentials of leaf extract of Carica papaya along with the potential to treat dengue, malaria and cancer has been previously reported[6]. In the continuous effort to evaluate potential medicinal values of the leaves of C. papaya, the present

research was conducted to investigate cytoprotective and DNA protective activities of ethanolic, ethyl acetate, chloroform and aqueous extracts of C. papaya.
The present study is designed to investigate the cytoprotective action of CP leaves extract against membrane damage in erythrocytes and DNA protective property against induced oxidative DNA damage by ferrous sulphate.

II. Materials And Methodology

Chemicals:
Agarose, Acetone, L-Ascorbic acid, Bromophenol blue, Chloroform, Disodium hydrogen orthrphosphate (Na_2HPO_4), Ethylenediamminetetraaceticacid (EDTA), Ethanol, Ethidium bromide (EtBr), Ethyl acetate, Ferrous sulphate heptahydrate ($FeSO_4.H_2O$), Potassium Chloride, Potassium dihydrogen orthophosphate (KH_2PO_4), Salmon milt Deoxyribonucleic Acid (DNA) Sodium Salt. (HiMedia), Sodium Chloride, Tris-HCl, Xylene cyanol.

Equipment:
Equipment used were Grinder, Weighing balance, Homogenizer, Rocker 300 Vacuum pump, Autoclave, REMI 2MLH Magnetic Stirrer, REMI R-8C Centrifuge, Water bath, Glass slides, OLYMPUS CKX41 Phase contrast microscope connected with INFINITY-1 Lumenera analyzer, Sterile lancets, Systronics UV-VIS Spectrophotometer 117, Agarose gel electrophoresis unit, Elico LI-120 pH meter, Alpha Innotech Imager Mini Gel documentation unit.

Plant source:
Carica papaya leaves were taken from a fruit bearing papaya plant at Mount Carmel College, Vasanth Nagar, Bangalore, Karnataka. India.Young and healthy leaves were used for the study.

Acetone powder preparation
Procured CP leaves were deveined, ground and depigmented using ice cold acetone. It was then filtered and dried in a vacuum pump. The resulting acetone powder was stored in an air tight plastic container and used further for the preparation of different fractions.

Preparation of 0.5% extracts using different solvents
50 mL of respective solvents- chloroform, distilled water, ethanol and ethyl acetate, were added to 250 mg of the acetone powder and each extract was placed on a magnetic stirrer at 120 rpm at low temperature for 3 hours. Each extract was then centrifuged at 3000 rpm for 10 min. Supernatant was collected. 0.5 % extracts of supernatant were prepared with water and used for further analysis.

In-vitro Erythrocyte Cytoprotection Assay
On a microscopic glass slide, a small drop of freshly drawn blood sample was taken. It was diluted 5 to 7 folds with PBS, mixed gently and viewed under high power of Phase contrast microscope. It was treated with $FeSO_4$ – a damaging agent and the effects noted. The cells were then subjected to co-treatment with ascorbic acid - a potent antioxidant and the subsequent effects recorded.
Each of the four extracts was then used, both before and after treatment with the oxidant and the cytoprotective action of each was noted. In the first set of experiment, RBCs are subjected to damage induced by ferrous sulphate and then co-treated with the extracts. In the next set of experiment, the erythrocytes were pre-treated with the extracts and then treated with the oxidant. The cytoprotective potential of each extract was assessed based on the time taken for the appearance of erythrocyte membrane damage in each case.

Detection of DNA protection by Carica papaya leaves using UV-Spectrophotometer
The level of Salmon milt DNA protection by Carica papaya leaves was compared to the standard antioxidant (Ascorbic acid) using UV Spectroscopy. For the standard reaction mixtures Salmon milt DNA (200µg/mL), the standard antioxidant Ascorbic acid (10mM) and standard oxidant ferrous sulphate (10mM) were taken. The absorbance was read at 260nm, TAE buffer was used as reference standard. Different reaction mixtures were prepared as follows and absorbance was noted down. DNA plus buffer, DNA plus ascorbic acid, DNA plus ferrous sulphate, DNA plus ascorbic acid plus ferrous sulphate and vice versa with 5 min intervals, DNA plus 1.0% samples, DNA plus samples before and after treatment of ferrous sulphate.

DNA damage inhibition assay using Agarose gel electrophoresis
The ability of ethanol, ethyl acetate, chloroform and aqueous extracts of C. papaya leaves to protect Salmon milt double stranded DNA from devastating effects of free radicals generated was assessed by the DNA damage

inhibition assay. The reaction mixtures contained 100µL DNA (10mg/mL), 50µL 10mM ferrous sulphate and 50µL 100mM ascorbic acid or 50µL of 0.5% extract. The DNA was processed and tested in two different treatments. In the first treatment (treatment 1) reaction mixture was prepared with DNA and $FeSO_4$ incubated for 5 minutes at 37 ^{0}C, and then addition of the extracts followed by another incubation for 5 minutes at 37 ^{0}C. In the second treatment (treatment 2), reaction mixture constituted DNA and the extracts incubated for 5 minutes at 37 ^{0}C, and then addition of $FeSO_4$ followed by another incubation for 5 minutes at 37 ^{0}C . 20µL of each reaction mixture were analyzed on 1% agarose gel (prepared by dissolving 0.5 g of agarose in 50 mL of 1X TAE Buffer) followed by ethidium bromide staining. Electrophoresis was carried out at 100V and 120A for 1 hour.

III. Results And Discussion

Cytoprotection of Carica papaya extracts against membrane damage in RBCs

The relative in vitro antioxidant and free radical scavenging activities of the extract and ascorbic acid were studied and it was observed that the extract possessed significant potential to overcome the induced toxicity when compared with ascorbic acid even at a low concentration of 0.05%.

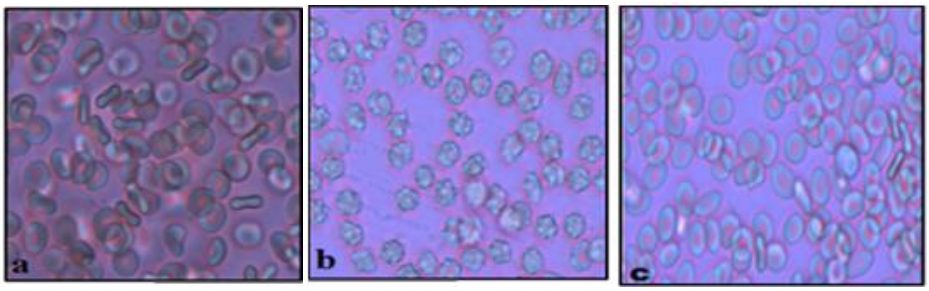

Fig 1: Treatment of RBCs with standard oxidant and antioxidant- (a) Human RBCs diluted with PBS as seen under 40X of phase contrast microscope. (b) Appearance of crenation on treatment with 0.5mM ferrous sulphate. (c) Restoration of crenation on treatment with 0.5mM ascorbic acid.

Ferrous sulphate induced crenation in the cell membrane of erythrocytes, causing the loss of its biconcave shape and thereby impairing its deformability. Red Blood Cells (RBCs) need to deform and squeeze through narrow capillaries. Decreased deformability is therefore one of the factors that can contribute to the elimination of aged or damaged RBCs from the circulation[7]. This process can also cause impaired oxygen delivery, which contributes to the pathology of a number of diseases. Damage to erythrocytes manifests in haemolysis which is associated with some haemoglobinopathies and deficiencies in a number of erythrocyte antioxidant systems[8]. Proteolysis is also activated by oxidative challenge of erythrocytes which leads to the alteration of the shape and function[9].

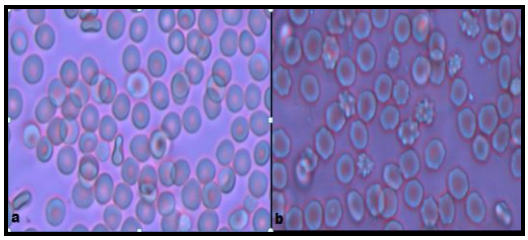

Fig 2: Treatment of RBCs with 0.05% extracts- (a) Co treatment of crenated cells with extracts. (b) Delayed appearance of crenation on addition of ferrous sulphate to cells pretreated with extracts.

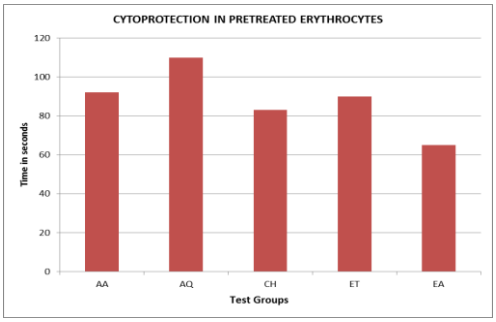

Fig 3: Pretreatment of RBCs with 0.5mM ascorbic acid and 0.05% extracts- AA: ascorbic acid, AQ: aqueous extract, CH: chloroform extract, ET: ethanol extract, EA: ethyl acetate extract

Treatment with ascorbic acid and the four extracts were seen to negate the cytotoxic effect of ferrous sulphate and restored the biconcave shape of RBCs. On pretreatment of RBCs with the extracts followed by treatment with the oxidant, it was observed that the extracts had the capacity to protect the cells from immediate morphological changes (crenations) induced by ferrous sulphate. The best protection was observed in cells pre-treated with aqueous extract followed by ethanol extract.

Erythrocytes are highly susceptible to attack by reactive oxygen species because of the high amount of polyunsaturated fatty acid content in their membranes and the metal catalyzed oxidation reactions because of haemoglobin. The oxidation of erythrocytes is a good model for the oxidation of bio membranes in general. Since C. papaya extracts reduce the ferrous sulphate-induced alterations in erythrocytes, they could also restore or reduce the morphological changes that accompany erythrocyte oxidation. The activity may be due to the presence of different phytochemicals which acted in synergy. From the above findings it can be said that these components responsible for cytoprotection are best extracted in aqueous system and least extracted with ethyl acetate.

DNA protection and repair by Carica papaya leaves using UV-Spectrophotometer.

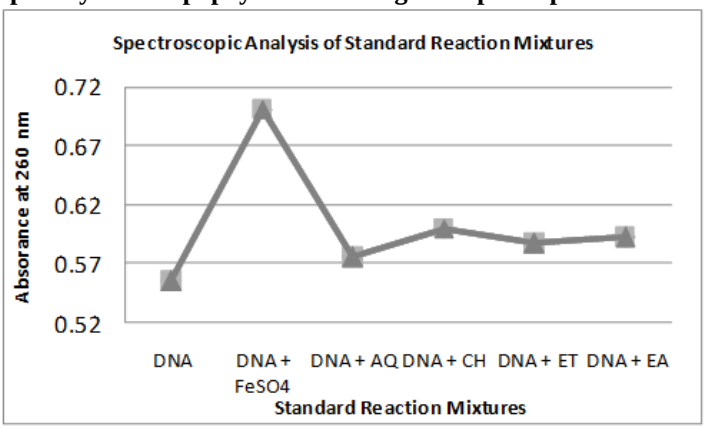

Fig 4: Treatment of DNA with 10mM $FeSO_4$ and 1.0% CP extracts- AQ: aqueous extract, CH: chloroform extract, ET: ethanol extract, EA: ethyl acetate extract

Free radicals can both initiate and exacerbate damage to cellular DNA in humans, and even partial damage to DNA can make a cell cancerous. In principle, the level of oxidative DNA damage in an organ or cell may be studied by measurement of strand breakage in extracted DNA using UV Spectrophotometer[10]. An optical density (absorbance) value higher than that of standard DNA implies either denaturation or strand breakage. DNA damage inhibition by the aqueous, chloroform, ethanolic and ethyl acetate extract of C. papaya leaves is shown in Fig. 4. It was seen that addition of oxidant to the standard DNA, resulted in increased absorbance at 260nm. On addition of extracts relatively low increase in the absorbance was noted, indicating that the extracts did not have DNA damaging activity.

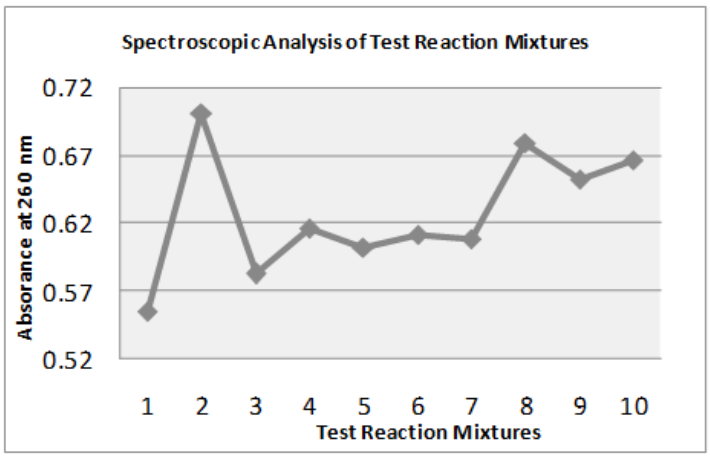

Fig 5: Spectroscopic analysis of DNA protection by CP extracts- **(**1) DNA, (2) DNA+ Oxidant, (3) DNA+ aqueous extract+$FeSO_4$, (4) DNA+ chloroform extract+ $FeSO_4$, (5) DNA+ ethanol + $FeSO_4$, (6) DNA+ ethyl acetate + $FeSO_4$, (7) DNA+ $FeSO_4$,+ aqueous extract (8) DNA+ $FeSO_4$ + chloroform extract (9) DNA+ $FeSO_4$ + ethanol extract (10) DNA+ $FeSO_4$ + ethyl acetate extract

In figure 5, 1 is the control, 2 is the effect of ferrous sulphate on DNA, while 3 to 6 are the results of treatment 1 (pretreatment with extracts followed by treatment with the oxidant) and 7 to 10 represent treatment 2 (pretreatment with oxidant followed by treatment with extracts). Treatment 1 is an assay of DNA protection provided by the extracts and treatment 2 was performed to evaluate their DNA repair potential. It was seen that the extracts exhibited significant DNA protective activity in treatment 1 and moderate to low DNA repair in treatment 2. The results however indicate that aqueous extract of CP showed highest DNA protective and repair potential in both the treatments followed by the ethanol extract as seen by their O.D values. Conversely, it was observed that chloroform extract displayed the least DNA protective and repair function in both the cases.This assay was based on the ability of extracts to protect the salmon (Oncerhynchusketa) testes DNA against oxidative damage. Salmon milt DNA has a molecular mass of 1.3 x 10^6 Da (~2,000 bp) and is double stranded[11]. Oxidative damage to DNA causes denaturation of the double strands or fragmentation of strands, both of which give a higher OD value at the same concentration as that of dsDNA. Thus an elevation in OD values above that of the standard DNA is a direct measure of DNA damage, and the reduction in these values on treatment with low concentration (1%) of extracts is indicative of the DNA protection and repair potential of the CP extracts.

DNA damage inhibition using Agarose gel electrophoresis

Figure 6 corresponds to the assay of DNA protective potential of aqueous and chloroform extracts of CP while figure 7 corresponds to that of ethanol and ethyl acetate. In both the figures Lane 9 & 13 represent the results of treatment 1 (pretreatment with extracts followed by treatment with the oxidant) and lane 10 and 14 represent treatment 2 (pretreatment with oxidant followed by treatment with extracts).

Fig 6: DNA protective activity of aqueous and chloroform extract seen by agarose gel electrophoresis:

Lane 1 and 2: Control (Buffer + DNA) ; Lane 3: positive control (DNA + ascorbic acid) ; Lane 4: Negative control (DNA + $FeSO_4$). Line 5: DNA + ascorbic acid + $FeSO_4$; Lane 6: DNA + $FeSO_4$ + ascorbic acid ; Lane 8: DNA + aqueous extract ; Lane 9: DNA + aqueous extract + $FeSO_4$; Lane 10: DNA+ $FeSO_4$,+ aqueous extract ; Lane12: DNA + chloroform extract ; Lane 13: DNA + chloroform extract + $FeSO_4$; Lane 14: DNA+ $FeSO_4$ + chloroform extract.

Fig 7: DNA protective activity of ethanol and ethyl acetate extract seen by agarose gel electrophoresis:

Lane 1 and 2: Control (Buffer + DNA) ; Lane 3: positive control (DNA + ascorbic acid) ; Lane 4: Negative control (DNA + $FeSO_4$). Line 5: DNA + ascorbic acid + $FeSO_4$; Lane 6: DNA + $FeSO_4$ + ascorbic acid ; Lane 8: DNA + ethanol extract ; Lane 9: DNA + ethanol extract + $FeSO_4$; Lane 10: DNA+ $FeSO_4$,+ ethanol extract ; Lane12: DNA + ethyl acetate extract ; Lane 13: DNA + ethyl acetate extract + $FeSO_4$; Lane 14: DNA+ $FeSO_4$ + ethyl acetate extract.

Treatment 1 is an assay of DNA protection provided by the extracts and treatment 2 was performed to evaluate their DNA repair potential. Oxidative stress can lead to several types of DNA damage, including oxidized bases and single- and double-strand breaks[12,13].DNA molecules are separated by size within an agarose gel in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight[14,15]. Gel

electrophoresis shows the negatively supercoiled native form as the main band, while nicked DNA (open circular form) and the relaxed closed circular form appears as minor bands. In the above gel pictures two patterns are visible- the lighter one corresponds to relaxed DNA following oxidation-dependent single and double strand breaks, while the darker more compact one corresponds to the intact DNA in its supercoiled form. Control sample (lane 1and 2) shows DNA almost only in the supercoiled form, whereas in lane 4, a maximum conversion of form I (supercoiled form) to form II (relaxed form) can be observed following addition of ferrous sulphate. In lane 9, 10, 13 and 14 of both the gels DNA was co-incubated with different CP extracts. A potent protective effect toward DNA damage was evident as shown in the figures after addition of 0.5% CP extracts. The order of DNA damage inhibition potential was found to be: aqueous>ethanol>ethyl acetate>chloroform. These findings are in accordance with those of UV spectrophotometric analysis.

IV. Conclusion

The importance of searching for alternative plant products against a number of diseases associated with ROS continues to be popular because of limited toxicity associated with it as against synthetic products. This is corroborated by the less frequent side effects of these plants and plant-derived products when compared to many synthetics.The present research was conducted to investigate the cytoprotective and DNA damage inhibitory activities of ethanolic, ethyl acetate, chloroform and aqueous extracts of C. papaya.

The oxidation of erythrocytes is a good model for the oxidation of bio membranes in general. From the in-vitro Erythrocyte Cytoprotection Assay it was observed that Co-treatment of erythrocytes with ferrous sulphate and extracts showed that CP leaves extract had the ability to restore the crenated RBCs to their native biconcave form, while pretreatment with extracts followed by treatment with oxidant showed delayed damage. These findings thus demonstrate CP leaves extracts possess significant cytoprotective property.

From the results of UV spectrophotometric analysis it was observed that pretreatment with extracts followed by treatment with the oxidant and pretreatment with oxidant followed by treatment with extracts had significant DNA protective and repair activity. These findings were further confirmed with agarose gel electrophoresis whose results were in accordance with those of UV spectrophotometric analysis. The order of DNA damage inhibition potential in both cases was found to be: aqueous>ethanol>ethyl acetate>chloroform.

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