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IN VITRO ANTIOXIDANT ACTIVITY OF *Carica papaya* SEEDS

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ABSTRACT

In the present study to investigate the *in vitro* antioxidant activity of *Carica papaya* seed through DPPH, total antioxidant assay, nitric oxide, superoxide scavenging, hydrogen peroxide, hydroxyl radical and iron reducing power activity. The results of the present study showed that *Cariac papaya* possesses strong antioxidant activity tested by various radical scavenging activities. The antioxidant potential of seeds of *Carica papaya* is directly proportional to concentrations (i.e. increase the concentration of *Carica papaya* is increase the scavenging activity). This work has gathered experimental evidence on the *Carica papaya* as natural antioxidant for its capacity to scavenge reactive oxygen/nitrogen species/free radicals and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the *Carica papaya* reported to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Thus, it can be concluded that *Carica papaya* can be used as an accessible plant antioxidants with consequent health benefits.

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INTRODUCTION

In recent years there is an upsurge in the areas related to newer developments in prevention of disease especially the role of free radicals in health and disease. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen (Tiwari, 2004). One can have too much of a good thing including oxygen, which is necessary for life, but in the form of free radical, it can cause harm. Oxygen is a dangerous friend. The by-products of its metabolism called free radicals are unstable, violently reactive, potentially destructive and short lived. Free radicals are the new "buzz word" in pathophysiology today. They have a special affinity for lipids, proteins and nucleic acids (DNA). Most molecules have all their electrons in pairs and are therefore not free radicals. Molecules are held together by pairs of electrons forming stable bonds, but breaking a bond forms highly reactive free radicals (Cheeseman and Slater, 1993). Therefore, the objectives of the present study to investigate the phytochemicals and *in vitro* antioxidant activity of *Carica papaya* seed through DPPH, total antioxidant assay, nitric oxide, superoxide scavenging, hydrogen peroxide, hydroxyl radical and iron reducing power activity.

MATERIAL AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), Ethylene Diamine Tetra Acetic acid (EDTA), Sodium nitroprusside (SNP), Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-Ascorbic acid were purchased from SISCO Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials

The fully mature seeds of *Carica papaya* were collected in January 2013 from pasupathi kovil, Thanjavur district, Tamil Nadu, India.

Preparation of ethanolic extract

The seeds of *Carica papaya* were first washed well and dust was removed from the seeds. Seeds were washed several times with distilled water to remove the traces of impurities from the seeds. The seeds were dried at room temperature and coarsely powdered. The powder was extracted with 50% ethanol for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in refrigerator for further study. The different concentrations (20µg/ml, 40µg/ml, 60µg/ml and 80µg/ml respectively) of plant extract were prepared and for the study.

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method of Shimada *et al.*, (1992). 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations (20µg/ml, 40µg/ml, 60µg/ml & 80µg/ml respectively). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 minutes. Then the absorbance was measured at 517nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity.

$$\text{Radical scavenging activity (\%)} = \frac{A_{\text{Sample}} - A_{\text{Control}}}{A_{\text{Control}}} \times 100$$

Where a control is the absorbance of the control (ascorbic acid) and A sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.*, (1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The total antioxidant capacity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where A_0 was the absorbance of the control (blank, without extract of *Carica papaya*) and A_1 was the absorbance in the presence of the *Carica papaya*.

Superoxide anion scavenging activity assay

The scavenging activity of the *Carica papaya* towards superoxide anion radicals was measured by the method of Liu *et al.* (1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3ml of Tris-HCl buffer (100mmol, pH 7.4) containing 0.75ml of NBT (300 µmole) solution, 0.75ml of NADH (936µmol) solution and 0.3ml of different concentrations of the *Carica papaya*. The reaction was initiated by adding 0.75ml of PMS (120µmol) to the mixture. After 5minutes of incubation at room temperature, the absorbance at 560nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where A_0 was the absorbance of the control (blank, without extract of *Carica papaya*) and A_1 was the absorbance in the presence of the *Carica papaya*.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2ml of 10mM sodium nitroprusside in 0.5ml phosphate buffer saline (pH 7.4) was mixed with 0.5ml of *Carica papaya* at various concentrations and the mixture incubated at 25°C for 150mintues. From the incubated mixture 0.5ml was taken out and added into 1.0ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5min. Finally,

1.0ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30min. The absorbance at 540nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$

Where A_0 was the absorbance of the control (blank, without extract of *Carica papaya* and A_1 was the absorbance in the presence of the *Carica papaya*.

Reducing power assay

The Fe^{3+} reducing power of the *Carica papaya* was determined by the method of Oyaizu (1986) with slight modifications. The *Carica papaya* (0.75ml) at various concentrations was mixed with 0.75ml of phosphate buffer (0.2mole, pH 6.6) and 0.75ml of potassium hexacyanoferrate $[K_3Fe(CN)_6]$ (1%, w/v), followed by incubating at 50°C in a water bath for 20min. The reaction was stopped by adding 0.75ml of trichloro acetic acid (TCA) solution (10%) and then centrifuged at 3000r/min for 10minutes. 1.5ml of the supernatant was mixed with 1.5ml of distilled water and 0.1ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10minutes. The absorbance at 700nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction (Yu *et al.*, 2004). Reaction mixture contained 60µl of 1mmole of $FeCl_2$, 90µl of 1mmole of 1, 10-phenanthroline, 2.4ml of 0.2mole of phosphate buffer (pH 7.8), 150µl of 0.17mole of H_2O_2, and 1.5ml of *Carica papaya* extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5min, the absorbance of the mixture at 560nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$

Where A0 was the absorbance of the control (blank, without extract of *Carica papaya* and A_1 was the absorbance in the presence of the *Carica papaya*.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration (Zhang, 2000). Aliquot of 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H_2SO_4 and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS_2O_3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as

% Inhibition = $(V_0 - V_1) / V0 \times 100$

Where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of the extract.

Tests were carried out in triplicate for 3–5 separate experiments. The free radical scavenging activity of *Carica papaya* was expressed in percentage (%).

RESULTS

In the present work design to investigate the *in vitro* antioxidant activity of *Carica papaya* seeds against various free radicals scavenging methods.

Superoxide anion radical scavenging activity

Ethanolic extract of the seeds of *Carica papaya* shows scavenging activity,20mg concentration produce 58.57%.40mg concentration shows 67.61% superoxide radical scavenging activity.60mg and 80mg concentration produces 73.80% and 82.71% respectively(Table I &Fig 3).

Hydrogen peroxide scavenging activity

Table I reverals 64.95% and 67.35% of hydrogen peroxide radical scavenging activity in 20mg and 40mg concentration of the seed of *Carica papaya*. whereas this activity in 60mg and 80mg is 70.91% and 78.60% respectively.

Nitric oxide radical scavenging activity

Nitric oxide scavenging activity in gradually increased from 20 to 60mg whereas nitric oxide scavenging activity in doubled in 80mg concentration. 20mg produces 38.1% activity. 40mg and 60mg produce 40.36% and 47.8% respectively. 90.63% activity was produce in 80% concentration of the seeds of papaya(Table I & Fig3).

DPPH Radical scavenging activity

20mg concentration of the seeds of *Carica papaya* produces 36.47% of DPPH radical scavenging activity whereas 40mg and 60mg shows 74.91% and 76.76% respectively 80mg concentrate exhibits maximum of 95.15% DPPH radical scavenging activity (Table I &Fig 3).

Hydroxyl radical scavenging activity

Carica papaya shows hydroxyl radical scavenging activity in a concentration dependent manner (Table II & Fig 4).

Table II reveals the hydroxyl scavenging activity in 20mg of the seeds of *Carica papaya* shows 82.64% hydroxyl radical scavenging activity and 40mg of this seeds exhibits of 91.06% hydroxyl radical scavenging activity.60mg and 80mg produced a significant OH radical scavenging activity of 93.7% and 98.3% respectively.

The ferrous ion chelating activity

Iron chelating activity in Carica papaya is concentration dependent.

20mg and 40mg concentration of the seeds exhibit 38.1% and 40.36% of iron chelating activity,60mg concentration of seed shows 47.8% activity, whereas 80mg concentration has the maximum iron chelating activity of 90.63% (Table II & Fig 4).

Iron reducing power activity

Table 3(Fig5) reveals depicts the reductive effect of *Carica papaya* similar to the antioxidant activity. The reducing power of *Carica papaya* increased with increasing dosage. All the doses showed significantly higher activities than the control.this indicater the greater reducing power of seeds of *Carica papaya*.

Table 1 - Radical scavenging activity of* Carica papaya *ethanolic extract at different concentration.

Concentration (mg)	Superoxide radical scavenging %	Hydrogen peroxide radical scavenging (%)	Nitric oxide radical scavenging %	DPPH radical scavenging (%)
20	58.57 ± 1.91	64.95 ± 2.61	38.1 ± 1.85	36.47 ± 4.36
40	67.61 ± 1.45	67.35 ± 1.14	40.36 ± 1.47	74.91 ± 2.74
60	73.80 ± 2.29	70.91 ± 3.00	47.8 ± 2.30	76.76 ± 2.53
80	82.71 ± 3.03	78.60 ± 1.62	90.63 ± 1.51	95.15 ± 1.02

Values are means ± SD.

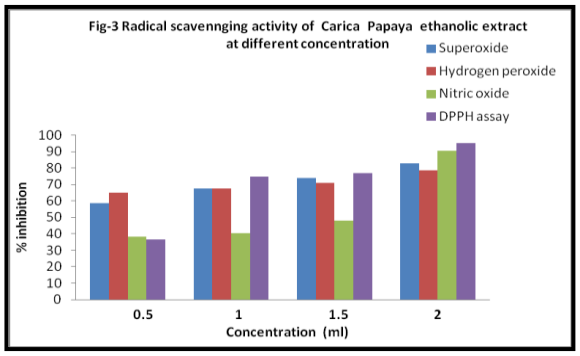


Table 2 - Radical scavenging activity of *Carica papaya* ethanolic extract at different concentration.

Concentration (mg)	Hydroxyl radical scavenging %	$Fe2^{+}$ chelating activity (%)
20	82.64 ± 1.51	38.1 ± 1.85
40	91.06 ± 2.60	40.36 ± 1.47
60	93.7 ± 3.09	47.8 ± 2.30
80	98.3 ± 2.08	90. 63 ± 1.51

Values are means ± SD.

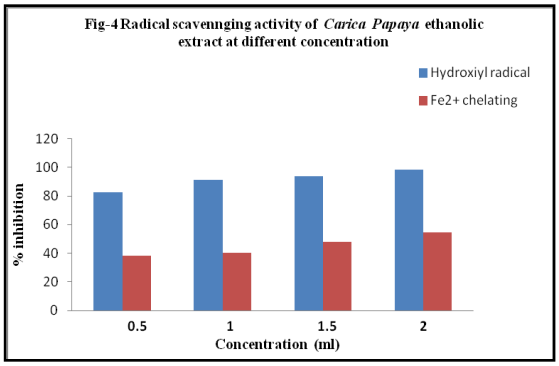
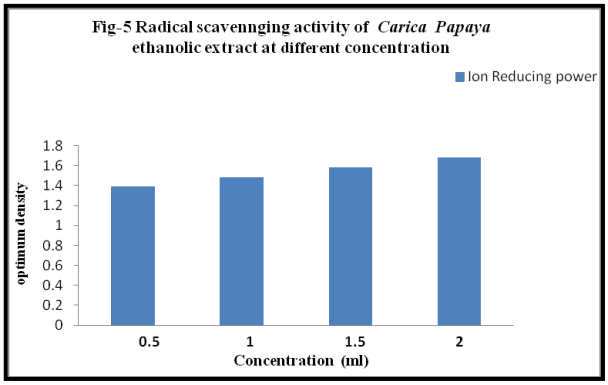


Table 3 - Reducing power activity of *Carica papaya* ethanolic extract at different concentration.

Concentration (mg)	Reducing power activity (O.D.)
20	1.39 ± 0.01
40	1.48 ± 0.02
60	1.58 ± 0.007
80	1.68 ± 0.01

Values are means ± SD.



DISCUSSION

Damages of biological systems caused by reactive oxygen species belonged to processes directly linked with development of cardiovascular and malignant diseases. Human organism possesses systems controlling oxidation processes posing a threat to structures and functions of cells. Three defense mechanisms has been developed, including: prevention of reactions of reactive oxygen species with biologically-significant compounds, breaking free-radical chain reactions and undesirable non-radical oxidation reactions, scavenging the products of free radicals reactions with biological substances and repair of damages (Bartosz, 1995). .Recently importance has been given for *in vitro* antioxidant study to understand the pharmacological role of medicinal plant. *In vitro* techniques have been used for detection of antioxidants, which are based on the ability of compounds to scavenge peroxy radicals (Velavan *et al.*, 2007).

In recent years, focus on plant research has increased all over the world and a large number of evidences have been collected to show the immense potential of medicinal plants used in traditional systems. Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential. Natural antioxidants are in high demand for application as nutraceuticals, bio-pharmaceuticals, as well as food additive because of consumer preference. A number of methods and variations have been developed and applied for the measurement of antioxidant capacity and efficacy (Harbone, 1973). The plants and its derivatives may considered as good sources of natural antioxidants for medicinal uses such as against cancer, diabetic mellitus, cardiovascular diseases, aging and other diseases related to radical mechanisms. Plant derived antioxidant therapy may be helpful for various free radical mediated diseases (Ighodaroro *et al.*, 2009).

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Korycka-Dahl and Richardson, 1978). The superoxide anion radical scavenging activity *Carica papaya* assayed by the PMS-NADH system**.** The superoxide scavenging activity of *Carica papaya* was increased markedly with the increase of concentrations. These results suggested that *Carica papaya* had superior superoxide radical scavenging effect.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Miller *et al*., 1993). Present study moderately inhibited nitric oxide in dose dependent manner.

DPPH free-radical scavenging activity Free radicals are harmful by-products generated during normal cellular metabolism, which could initiate oxidative damage to body (Halliwell and Gutteridge, 1999). Antioxidants are believed to play a significant role in the body's defense system against free radicals. Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs and cereals extracts (Hou *et al.*, 2005). The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.*, 2003).

The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The antioxidant activity of The APCP exhibited a significant dose dependent inhibition of DPPH activity.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Halliwell and Gutteridge, 1993). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. As concentration increases, hydrogen peroxide scavenging activity of *Carica papaya* in a concentration dependent manner.

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the presence of *Carica papaya*, indicating that have chelating activity (table 2 and fig 4). Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as

accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Hali well, 1991).

Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that form s bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion (Gordon, 1990). Thus, *Carica papaya* exhibits a marked capacity for iron binding, suggesting their ability as iron chelator proved to be an antioxidant.

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. Table 2(fig 4) showed the *Carica papaya* exhibited concentration dependent scavenging activities against hydroxyl radicals. A fenton reaction system. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups (Hagerman *et al*., 1998).

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the seed of *Carica papaya.* The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging (Diplock, 1997; Yildirim *et al*., 2000).

Antioxidant activity of the seeds of *Carica papaya* may be due to the presence of various phytochemicals.

SUMMARY

The increasing evidences suggesting that the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of antioxidants in the maintenance of human health and prevention and treatment of diseases. The natural antioxidants present in foods, fruits, beverages, spices, and supplements have received much attention from nutraceutical and cosmetic interest, while various synthetic antioxidants have also been prepared from pharmaceutical viewpoints. The capacity of these antioxidants has been assessed in numerous studies by different methods under different conditions.

Determination of the natural antioxidant compounds will help to develop new drug for antioxidant therapy. Keeping in view, the present study was to investigated the *in vitro* antioxidant activity of seeds of *Carica papaya* through the DPPH radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, nitricoxide scavenging, metal chelating and reducing power assay.

The results of the present study showed that *Cariac papaya* possesses strong antioxidant activity tested by various radical scavenging activities. The antioxidant potential of seeds of *Carica papaya* is directly proportional to concentrations (i.e. increase the concentration of *Carica papaya* is increase the scavenging activity). This work has gathered experimental evidence on the *Carica papaya* as natural antioxidant for its capacity to scavenge reactive oxygen/nitrogen species/free radicals and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the *Carica papaya* reported to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Thus, it can be concluded that *Carica papaya* can be used as an accessible plant antioxidants with consequent health benefits.

Overall, the *Carica papaya* seeds are a source of natural antioxidants that can be important in disease prevention and health preservation. However, the *in vivo* safety of *Carica papaya* seeds needs to be thoroughly investigated with which chemical compounds are responsible for *in vitro* antioxidant activity.

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