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Studies on *in-vitro* antioxidant activities of *Carica papaya* aqueous leaf extract

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ABSTRACT

The free radical scavenging activity of the aqueous leaf extraction of *Carica papaya* was studied by using different anti oxidant models of screening.e.g lipid peroxide(rat brain and liver),1,1-diphenyle hydrazyl (DPPH) ,2,2-azinobis –(3-ethyle benzothiazoline -6-sulphonate) (ABTS) ,nitric oxide ,super oxide and hydroxyl radical model. The extract showed good dose dependent free radical scavenging activity in all the in vitro models. IC50 values were found to be 198,141,185,244, 323, 461 and 922 µg/ml respectively in DPPH, ABTS, nitric oxide, superoxide, hydroxyl ion, lipid peroxidation (liver and brain) inhibition assays. However the extract showed only moderate scavenging activity of hydroxyl radical and anti lipid peroxidation potential, which was performed using rat liver and brain homogenate. The results were observed in a concentration dependent manner. All the above in vitro studies clearly indicate that aqueous extract of *Carica papaya* has a significant antioxidant activity.

Key words: Antioxidant activity, free radicals, scavenging activity, lipid peroxidation.

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INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism [1]. The most common reactive oxygen species (ROS) include super oxide (0_2.) anion, hydrogen peroxide (H_20_2), peroxyl (ROO) radicals, and reactive hydroxyl (OH.) radicals. The nitrogen derived free radicals are nitric oxide (NO.) and Peroxynitrite anion (ONOO.). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome [2]. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers [3]. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic. etc. They were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties.

In Indian system of medicine *Carica papaya* is an important medicinal plant and its fruit juice, leaves and roots has been used in various ailments and as health tonic. Carica papaya Linn,(Caricaceae) (Eng:Paw or Papaya tree,Tamil:poppayi,Tel: Bappayi),is a well known ,short lived, fast –growing ,woody ,large herb ,cultivated in gardens through out India; indigenous to America [4]. The plant is traditionally used for the treatment of gastric ulcers, dental caries,to expel intestinal worms, as heart tonic, for severe jaundice, for inflammation, haemorrhoids etc. reports, indicates that the pharmacological activities of *C.papaya* include anti hypertensive, ulcer protective ,anti microbial and anti oxidant, anti-fertility, anti fungal, anthelmintic [5-10]. Carica papaya contains alkaloid (carpine and carposide), citric acid, Vit-C, Nicotinic acid, papain or papayotin, papaya oil. There fore, the objective of the present study was to investigate the in vitro anti oxidant activity of Carica papaya leaf extract through the free radical scavenging activity.

MATERIALS AND METHODS

All chemicals and solvents used were of analytical grade and were obtained from Ranbaxy Fine chemicals, Mumbai, India. 1,1-di phenyl ,2-picryl hydrazyl(DPPH) was obtained from Sigma Chemicals,USA. The other chemicals used were 2,2-azinobis –(3-ethylebenzothiazoline-6-sulphonate)(ABTS), Sodium nitro prusside,potassium chloride(KCl) ,ferrous sulphate($FeSO_4$), thio barbituric acid(TBA), tri chloro acetic acid(TCA),nitro blue Tetrazolium(NBT),ethylene diamine tetra acetic acid(EDTA), ascorbic acid,2-Deoxy-D-ribose,phenozine methosulphate(PMS). Nicotinamide Adenine Dinucleotide Hydrazine (NADH).

Plant material

The leaves of CP were collected during December and January 2007 from Berhampur, Ganjam district, Orissa.

Plant extract

The leaves were cut in to small pieces and shade dried at room temperature for 15 days, finely powdered and used for extraction. The powdered material was extracted with distilled water using soxhlet apparatus. The extract was concentrated *in vacuo* and kept in a vacuum dessicator for complete removal of solvent. The yield was 14% w/w with respect to dried powder. Preliminary qualitative analysis of the aqueous extract showed the

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presence of alkaloids, citric acid, maleic acid and Vit-C. The dried extract thus obtained was used directly for the assessment of antioxidant activity through various invitro methods.

Animals

Adult Wistar rats of either sex and of approximately the same age weighing about 200-250 g were used (Ghosh Enterprises, Kolkata). The rats fed with the standard animal pellets (Rayans bio technologies, Hyd) and water *ad libitum*.They were housed in polypropylene cages maintained under standard conditions (12:12 hr L: D cycle: 25±3^{0}C : 35-60% RH). The experimental protocol was subjected to the scrutinistion of the Institutional Animal Ethics Committee and was cleared by the same.

EXPERIMENTAL METHODS

In-vitro anti-oxidant study

Carica papaya aqueous extract was tested for its free radical scavenging activity using different in vitro models. All experiments were performed thrice and the results averaged. L-Ascorbic acid was used as standard in all experiments. The Institutional Animal Ethics Committee approved the use of animals for the lipid peroxidation assay. (Ethical committee Reg, no: 926/Ab/06/CPCSEA, approval no: 05)

Inhibition of lipid peroxidation in rat brain

Preparation of rat brain homogenate [11]: Randomly selected rats were fastened overnight. They were sacrificed by cervical dislocation, dissected and the whole brain except cerebellum was removed quickly. It was further processed to get 10% homogenate in 0.15 M KCl [12]. Using Teflon homogeniser. The homogenate was filtered to get a clear solution and used as a source of poly unsaturated fatty acids for the extent of lipid peroxidation.

In-vitro anti lipid peroxidation assay: The extract samples were individually added to the brain homogenate (0.5 ml). This mixture was incubated with 0.15 M KCL (100µ L) .Lipid peroxidation was initiated by adding 100 µ L of 15mM $FesO_4$ Solution.

The reaction mixture was incubated at 37^{o}C for 30 min. An equal volume of TBA: TCA (1:1,1ml) was added to the above solution followed by the addition of 1ml BHT. This final mixture was heated on a water bath for 20 min at 80^{o}C and cooled, centrifuged and absorbance read at 532nm [13]. Percentage inhibition was calculated using the following formula.

$$\text{Percentage inhibition} = \frac{\text{Average control O.D-Test sample O.D}}{\text{Average control O.D}} \times 100$$

(Control-blank, without extract, Test- in the presence of extract)

Inhibition of lipidperoxidation in rat liver

Rat liver homogenate was used as the source of poly unsaturated fatty acids for determining the extent of lipid per oxidation [13]. Liver was collected immediately after the sacrifice of the animals by cervical dislocation under ether anaesthesia. The liver was homogenized with 40mM Tris-HCl buffer (pH 7) and centrifuged at 3000rpm for 10 min to get a clear supernatant. Reaction mixture 4ml containing 0.5ml of supernatant, Carica papaya aqueous extract solution of different concentrations (25-1000µg/ml) and 100µL of each of 0.15 M KCl, 15 mM $FeSO_4$ and 6mM ascorbic acid was incubated at 37^o C for 1 hr. TCA (1 ml; 10%) was added to the mixture and

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the sample centrifuged at 3000 rpm for 20 min at 4° C to remove insoluble proteins. Supernatant was removed and 1ml TBA (08%) was added to this fraction followed by heating at 90° C for 20 min in water bath. After cooling the colored TBA-MDA complex was extracted with organic solvent (2ml butanol) and absorbance was measured at 532 nm. Percentage inhibition was calculated using the above formula

Determination of DPPH scavenging activity [14]

The free radical scavenging activity of the Carica papaya aqueous leaf extract and ascorbic acid(std) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH [15]. 0.1mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations.(25-1000µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated using the above formula.

Determination of ABTS scavenging activity [16]

ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12-16 hr before use. For the study, different concentrations (25-1000 µg/ml) of the extract(0.5ml) were added to 0.3ml of ABTS solution and the final volume was made up to 1ml. the absorbance was read at 745nm and the percentage of inhibition calculated by using the same formula as given above.

Determination of nitric oxide scavenging activity [17, 18]

Nitric oxide (NO) radicals were generated from the sodium nitro prusside solution at physiological pH. Sodium nitro prusside (1ml of 10mM) was mixed with 1ml *Carica papaya* extract of different concentrations (25-1000 µg/ml) in phosphate buffer (pH 7.4) .the mixture was incubated at 250C for 150min. to 1ml of the incubated solution,1ml of Greiss ' reagent (1% Sulphanilamide, 2% ortho phosphoric acid and 0.1% Naphthyle ethylene diamine di hydro chloride) was added . Absorbance was read at 546nm and percentage inhibition was calculated using the above formula.

Determination of Super oxide radical scavenging activity

Measurement of super oxide anion scavenging activity of CPAE based on the method described by Liu et al, 1997) with slight modifications [19]. Super oxide 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 Nitro Blue Tetrazolium (NBT). In this experiment the super oxide anion was generated in 3 ml of Tris-HCl buffer (16mM,pH 8.0) containing 1ml of NBT (50 µM) solution, 1.0 ml of NADH (78 µM) solution and different concentrations (25-1000µg/ml) of the extract in water. The reaction was initiated by adding 1.0 ml of PMS (10 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer against blank samples. Percentage inhibition was calculated using the above formula.

Determination of hydroxyl radical scavenging activity [20, 21, 14].

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the test compound i.e.; *Carica papaya* extract for hydroxyl radical generated from Fe^{3+}/ascobate/ EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose that eventually results in thiobarbituric acid reacting substances [TBARS] formation. This was measured at 532nm. In this experiment 1ml of reaction mixture containing 500 µl (0.5ml) of extract solution in different concentrations(25-1000µg/ml) , and 100 µl (0.1 ml) of each of 2-Deoxy-D-ribose(28 mM) ,EDTA (1 mM) ,$FeCl_3$ (0.2 mM) and Ascorbic acid(1 mM) were incubated at 37°C for 1 hr. The above reaction mixture [0.4ml] was treated with dodecyl sulphate [8.1%, 0.2ml], thiobarbituric acid [0.8%, 1.5ml] and acetic acid [20%, 1.5ml, and pH 3.5]. The total volume was then made upto 4ml by adding distilled water and kept

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in oil bath maintained at 95^0C for one hour. After cooling the absorbance was measured at 532nm [spectrophotometer Model Elico SL 159]. Percentage inhibition was calculated using the above formula.

Statistical analysis

Linear regression analysis was used to calculate the IC_{50} values.

RESULTS

Several concentrations, ranging from 25-1000µg/ml of the aqueous extract of Carica papaya were tested for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The maximum percentage inhibition in all the models viz; ABTS,DPPH,nitric oxide,superoxide, hydroxyl ion and lipid peroxidation in brain and liver were found to be 86.54,85.32,88,82.36,81.92,51.69 and 72.56 respectively at 1000 µg/ml concentration. On a comparative basis the extract showed better activity in quenching ABTS with the an IC_{50} value of 141 µg/ml, DPPH radicals with an IC50 value of 198 ,nitric oxide radicals with an IC_{50} value of 235 and super oxide radicals with an IC_{50} value of 238 µg/ml . However, the extract showed encouraging response in quenching hydroxyl ion radicals (IC_{50} value-323 µg/ml) and the activity was moderate in the remaining antioxidant models. Effect of aqueous extract of Cp on different antioxidant models (values are mean 3 replicates) has been shown in Table 1.

Table1; Effect of aqueous extract of Cp on different antioxidant models
(values are mean 3 replicates)
Inhibition (%)

Conc (µg/ml)	Lipid peroxidation (Brain)	Lipid peroxidation (Liver)	DPPH	ABTS	Nitric Oxide	Super Oxide	Hydroxyl ion
25	2.99±0.54	18.25±0.84	34.58±1.23	32.56±0.90	26.95±0.58	26.42±0.93	22.89±0.66
50	6.815±0.29	23.21±0.42	38.52±0.30	35.22±0.79	35.01±0.98	36.25±1.70	33.45±0.40
100	10.99±0.84	31.24±0.83	43.2±0.48	40.25±1.53	44.96±0.67	44.92±2.10	39.87±0.55
200	12.14±0.78	39.55±0.99	54.65±0.57	52.65±1.11	56.42±0.96	49.65±1.40	45.98±0.57
250	20.12±1.05	43.65±0.52	58.98±1.78	72.88±0.68	68.22±2.64	62.95±0.56	49.32±0.43
500	35.12±0.40	61.95±0.72	72.92±1.45	76.58±0.49	78.12±0.89	68.21±1.34	67.84±0.55
750	40.33±1.48	65.45±1.72	76.54±0.43	81.57±0.36	83.25±0.80	76.32±1.76	74.63±0.42
1000	51.69±0.58	72.56±0.36	85.32±1.26	86.56±0.36	86.21±2.63	82.36±1.16	81.92±0.68
IC_{50}	922	461	198	141	185	238	323

DISCUSSION

The increased demand for medicinal plants has created an ecological crisis for medicinal herbs growing in the wild raising alarm about their rate of extinction .The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties due to the presence of flavonoids, a class of natural polyphenols found in green plant cells [22]. Leaf senescence is a crucial developmental state in the life of plants. It is the time during which compounds synthesized by the plant during its growth phase are mobilized into younger tissues. Leaf senescence

also indicates the beginning of the harvest period [23] and has been shown to affect antioxidant activity [24]. Anti oxidant may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation etc. In lipid peroxidation Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals [25], which is accepted as an indicator of lipid peroxidation [26]. The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex [27] or through OH radicals by Fenton reaction [28] there by initiating a cascade of oxidative reactions. The results obtained in the present study may be attributed to several reasons viz; the inhibition of ferryl-perferryl complex formation, scavenging of OH or super oxide radical or by changing the ratio of Fe+3/Fe+2;reducing the rate of conversion of ferrous to ferric or by chelation of iron itself [29]. The moderate activity of the extract may probably be due to the rapid and extensive degradation of the antioxidant principles in an ex vivo state [30]. It is also known that the OH radical which initiates lipid peroxidation has a very short life time (10^{-9} at 37^0C) and is hence very difficult to investigate [31]. The reduction in free radical yield and the subsequent decrease in harm and damage to the cell membrane is due to decrease in MDA production. DPPH is a stable free radical having maximum absorption at 517 nm that accepts an electron or hydrogen atom to become a stable diamagnetic molecule [32]. We have studied the ability of the extract to neutralize the free radicals such as DPPH radicals. In the presence of the extract capable of donating an H atom its free radical nature is lost hence the reduction in DPPH radical was determined by the decrease in its absorbance at 517 nm. The decrease in DPPH absorption in the presence of varying concentrations of extract has been monitored. The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$ which has a characteristic long wavelength absorption spectrum [33]. The results obtained imply the activity of the extract either by inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidant towards $ABTS^+$ radicals have been reported earlier [34]. ABTS radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer; the reactions with ABTS radicals involve electron transfer process.

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins [35]. The effect of Carica papaya leaf extract on the inhibition of free radical mediated deoxy ribose damage was assessed by means of the iron (2) dependent DNA damage assay. The extract has the moderate activity against hydroxyl radicals. Super oxide 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 [36].The super oxide radical scavenging activity of the extract from Carica papaya leafs assayed by the PMS-NADH system. The super oxide scavenging activity of Carica papaya leaf extract was increased markedly with the increase of concentration. These results suggested that the extract had important super oxide scavenging activity. Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. In the present study the nitrite produced by the incubation of solution of sodium nitroprusside in standard phosphate buffer at 25^0C was reduced by the aqueous extract of Carica papaya. This may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide there [17] by inhibiting the generation of nitrite.

CONCLUSION

An aqueous extract of Carica papaya was examined for its in vitro antioxidant activity in different model systems. The antiradical activity could be correlated with the Polyphenolic components present in the extract. The results gained by these methods provide some important factors responsible for the antioxidant potential of Carica papaya leaf and offer evidence for the large number of in vivo beneficial effects of the leaf reported in the literature.

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