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IN VITRO AND *IN VIVO* ANTIOXIDANT POTENTIAL OF ETHANOLIC EXTRACT OF *SYZYGIUM JAMBOS* (L.) BARK

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ABSTRACT

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. A variety of free radical scavenging antioxidants exist within the body, many of them are derived from dietary sources like fruits, vegetables and teas. In this study the antioxidant activity of the ethanolic extract of *Syzygium jambos* bark (SJB) was investigated for antioxidant potential. The *in-vitro* antioxidant activities of the extract was evaluated by DPPH free radical scavenging assay, total antioxidant capacity, reducing power capacity, total phenol and total flavonoid content. The extract demonstrated significant dose dependent antioxidant activity. The extract of SJB was evaluated for *in vivo* efficacy by carbon tetrachloride (CCl_4) induced liver damage rats in hepatoprotective model. CCl_4 produced significant alteration of serum marker enzymes, total bilirubin, total protein and liver weight. Restoration of these values towards normal which is comparable to control group indicated hepatoprotective potential of the extract. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic and flavonoid compounds present in SJB. Due to its natural origin and potent free radical scavenging ability SJB could be used as a potential preventive intervention for free radical mediated diseases.

KEYWORDS: SJB; Ethanolic Extract; Antioxidant Activity.

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INTRODUCTION

Free radicals stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging and ischemia. The production of free radicals is inextricable linked to the inflammatory process. Free radicals prime the immune response, recruit inflammatory cells and are innately bactericidal[1].

Some of these free radicals play a positive role *in vivo* such as energy production, phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically important compounds[2]. However, free radicals are very detrimental in attacking lipids in cell membranes and also DNA, inducing oxidations that cause membrane damage such as membrane lipid peroxidation and a decrease in membrane fluidity and also cause DNA mutation leading to cancer[3]. Free radicals and oxidants activate nuclear factor-κB, a nuclear transcription factor, resulting in an upregulation of pro-inflammatory mediators such as interleukin-1, interleukin-8 and tumor necrosis factor-α[1]. This in turn stimulates the immune response; increases oxidant production and can lead to further tissue damage.

A potent scavenger of these free radical species may serve as a possible preventive intervention for free radical mediated diseases[4]. Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions[5, 6].

Syzygium jambos (L.) commonly known as Rose apple belongs to the family Myrtaceae. It has been used in the indigenous system of medicine for the treatment of various ailments. The fruit is regarded as a tonic for the brain and liver. An infusion of the fruit acts as a diuretic. A sweetened preparation of the flowers is believed to reduce fever. The seeds are employed against diarrhea, dysentery, catarrh and are beneficial to diabetics. The leaf decoction is applied to sore eyes, also serves as a

diuretic and expectorant and treatment for rheumatism. The bark has astringent, emetic and cathartic properties. The decoction is administered to relieve asthma, bronchitis and hoarseness. The root is an effective remedy for epilepsy[7].

The objective of the present study was to investigate the antioxidant activity of the SJB using different in vitro and in vivo carbon tetrachloride induced hepatoprotective models as well as determination of total phenolic and flavonoid content in order to evaluate a relationship between the antioxidant activity and the phytochemical constituents.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid), ferric chloride, Gallic acid and Quercetin were obtained from Sigma Chemical Co. USA. Ascorbic acid and Aluminium chloride were obtained from SD Fine Chem. Ltd., Biosar, India. Ammonium molybdate, Methanol, Sodium Phosphate, Concentrated H_2SO_4, Folin-ciocalteu reagent, Sodium carbonate, Potassium Acetate, Mono-Sodium phosphate, Bi-sodium phosphate, Potassium ferricyanide and Trichloro acetic acid were purchased from Merck, Germany.

Preparation of Extract

Ethanol extract of Bark was used in the present study. The matured leaves were collected in the month of July 2009 from Rajshahi, Bangladesh. The Bark were dried under shade and pulverized in a mechanical grinder. The powder was extracted with ethanol. The mixture was filtered and the filtrate was concentrated in Rotary vacuum evaporator to yield semisolid (8.75 % w/w). The extract was preserved in a refrigerator till further use.

Experimental Procedure

All the following experiments were repeated three times and the results averaged.

In Vitro Antioxidant Activity

DPPH radical scavenging activity

The free radical scavenging capacity of the extract was determined using stable free radical 1, 1-Diphenyl-2-picrylhydrazyl, (DPPH)[8]. SJB was mixed with 95% ethanol to prepare the stock solution (5 mg/ml). DPPH solution (0.004% w/v) was prepared in 95% ethanol. Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and SJB was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% ethanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation:

% Scavenging Activity = Absorbance of the control- Absorbance of the test sample/Absorbance of the control ×100

The inhibition curve was plotted for triplicate experiments and represented as % of mean inhibition ± standard deviation. IC50 values were obtained by Probit analysis[9].

Total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto *et al.*[10]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract/sample and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^0C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer) against blank after cooling to room temperature. Ethanol (0.3 ml) in the place of extract/sample is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Reducing power capacity

Reducing power of the extract was evaluated by Oyaizu method[11]. Different concentrations of SJB extract (125, 250, 500, 1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). The mixture was incubated at 50°C for 20 min. After incubation, 2.5mL of 10% trichlorocacetic acid solution was added to each tube and the mixture was centrifuged at 3000 rpm for 10 minutes. 5mL of the upper layer solution was mixed with 5 ml of distilled water and 1 ml of ferric chloride solution (0.1% w/v) and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid was taken as reference standard. Phosphate buffer (P^H 6.6) was used as blank solution.

Total Phenol Content

Total phenol content in the extract was determined with Folin-Ciocalteu reagent. Extract (200µg/ml) was mixed with 400 µl of the Folin-Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken

thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 hrs. Then the absorbance at 765 nm was determined. The concentration of total phenol content in SJB was then determined as mg of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph[12].

Total Flavonoid Content

The total flavonoid content was determined using a method previously described by Kumaran and Karunakaran[13]. 1 ml of plant extract in ethanol (200 µg/ml) was mixed with 1 ml aluminium trichloride in ethanol (20 mg/ ml) and a drop of acetic acid, and then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 ml of plant extract and a drop of acetic acid, and then diluted to 25 ml with ethanol. The total flavonoid content was determined using a standard curve of quercetin (12.5-100 µg/ml) and expressed as mg of quercetin equivalent (QE/gm of extract).

***In Vivo* Antioxidant Study**

Preparation of test sample

Stock solution was prepared by dissolving 0.36 gm of the extract of SJB in 4.8 ml of distilled water. 0.4 ml of the stock solution per 100 gm rat is given orally so that the dose would be 300 mg/kg body weight.

Animals

Male Wister albino rats (150 gm) were used for the present study. They were purchased from ICDDR, B and placed in plastic cages with mesh bottoms in a room temperature. Prior to the commencement of the experiment, all the rats were acclimatized to the new environmental condition for a period of one week. They were maintained with 12h light and dark cycles and fed on a standard pellet diet supplied from ICDDR, B and fresh drinking water *ad libitum*.

Experimental design

The hepatoprotective activity of SJB extract was determined by using carbon tetrachloride induced hepatotoxic rat model. After seven days of acclimatization, the rats were divided into four groups each comprising of three rats and treatment was done for 8 days.

Group I**:** Normal control (0.9% normal saline; 1 ml/kg i.p.)

Group II**:** CCl_4 control (CCl_4: liquid paraffin (1:2); 1ml/kg i.p.)

Group III**:** CCl_4 + SJB (300 mg/kg/day; p.o)

Group IV**:** CCl_4 + standard drug Silymarin (25 mg/kg/day; p.o)

Group II-IV **:** Received CCl_4 in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h.

After 24 hrs of the last dose, blood was withdrawn from retro-orbital plexus under sodium phenobarbital anesthesia and the rats were dissected to isolate liver. Before collecting of the blood, the syringe was ringed with heparin to prevent hemolysis/clotting. The blood samples were then centrifuged at 2500 rpm at 37°C to separate serum and were used for estimation of the biochemical markers of liver damage viz. SGOT, SGPT[14], ALP[15], Bilirubin[16] and Total Protein levels[17].

Statistical Analysis

Linear regression analysis was used to calculate IC_{50} values wherever needed. All the results are shown as average ± S.E.M. Data was statistically evaluated by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using instat software. $P< 0.05$ was considered as statistically significant.

RESULTS

***In Vitro* Antioxidant Activity**

DPPH radical scavenging activity

The bark extract demonstrated H-donor function and showed dose dependent free radical scavenging activity. The percentage inhibition is shown in **Fig. 1**. The extract possessed significant DPPH radical scavenging activity (IC_{50} 6.75 µg/ml) compared with standard ascorbic acid (IC_{50} 4.87 µg/ml).

Total antioxidant capacity

Total antioxidant capacity of the SJB was determined using a standard curve of ascorbic acid and value was expressed as ascorbic acid equivalent (AE) which is 554.88± 71.53 mg AE/gm of extract.

Reducing power capacity

The extract showed significant reducing power activities as compared to ascorbic acid and proportionally increased with the increasing concentration of the extracts which is shown in **Fig. 2**.

Total Phenol Content

It is employed routinely in studying phenolic antioxidants. Phenolic content of the sample was calculated on the basis of the standard curve for gallic acid. The result was 299.64±5.38 mg gallic acid equivalent /gm of SJB extract (**Fig. 3**).

Total Flavonoid Content

Flavonoids are a large class of benzo-pyrone derivatives, ubiquitous in plants and exhibit antioxidant activity. The flavonoid content of SJB is shown in **Fig. 3**. The total flavonoid contents of SJB was 782.86 ± 150.80 mg of quercetin per gm of extract.

***In Vivo* Antioxidant Study**

Biochemical parameters (SGOT, SGPT, ALP, total protein, total bilirubin) and liver weight are shown in **Table 1**. The level of SGPT, SGOT, ALP, total bilirubin, total protein and liver weight was restored towards the

normal value in SJB and silymarin treated carbon tetrachloride intoxicated rats was the index of their hepatoprotective effects.

DISCUSSION

Reactive oxygen species (ROS) including free radicals and non free radicals along with various forms of active oxygen are involved in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, aging, hepatic damage, neurodegenerative, cardiovascular complications etc. Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms.

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. When DPPH encounters proton donors such as antioxidants, it gets reduced to a yellow colored product, diphenylpicryl hydrazine and the absorbance decreases. Results indicated definite scavenging activity of the extract in comparison with ascorbic acid.

The antioxidative effect of the extract is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes[18]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides[19].

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[20]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain reaction donating a hydrogen atom[21]. They can react with free radicals to convert them into more stable products. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The reducing capacity of SJB was investigated by Fe^{3+}-Fe^{2+} transformation. Presence of reductones causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form. This Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The reducing power of SJB was found remarkable.

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities. These also decrease cardiovascular complications. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. SJB showed significantly higher inhibition percentage (stronger hydrogen-donating ability) positively correlated with total phenolic content. Therefore, phenolic components of SJB may contribute directly to antioxidant action in this study.

Flavonoids are a group of polyphenolic compounds with known properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes[22]. Furthermore, as these compounds present a strong affinity for iron ions (which are known to catalyze many processes leading to the appearance of free radicals), their antiperoxidative activity could also be ascribed to a concomitant capability of chelating iron. Therefore flavonoids exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral and anticancer activities.

In most of the developing countries, the incidence of viral hepatitis is more. So, the investigation for an efficient hepatoprotective drug from the natural resource is an urgent necessity. The changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis. CCl_4 is therefore a useful tool for the induction of hepatic damage in experimental animals. The ability of hepatoprotectivity of SJB to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects.

The hepatotxicity of CCl_4 is the result of its reductive dehalogenation catalysed by cytochromic P-450 to produce the highly reactive metabolite trichloromethyl ($CCl_3^{\cdot}$) free radical. This then readily interacts with molecular oxygen to form trichloromethyl peroxy radical ($CCl_3OO^{\cdot}$). These free radicals bind covalently to cellular proteins or lipids or abstract a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and consequently leading to liver damage. Furthermore, influx of extracellular Ca^{2+} into the cell is claimed to be an important step of loss of functional integrity of the membrane. It was found the substantial increase in the level of serum marker enzymes (SGOT, SGPT and ALP) and total bilirubin in CCl_4 control group (**Table 1**). Restoration of the elevayed level s of serum enzymes towards the near normal value in case of fruit extracts as well as standard Silymarin treated groups is the indication of stabilization of plasma membrane and repair of hepatic tissue damage that occurred by CCl_4 .The reduction of the level of total proteins in CCl_4 challenged animals (**Table 1**) is attributed to the damage produced localized in the endoplasmic reticulum which results in the loss of P-450 leading to its functional failure with a decrease in protein synthesis. The rise in protein levels in the treated groups suggests the stabilization of endoplasmic reticulum leading to protein

synthesis. The CCl_4 induced a significant increase in liver weight, which is due to blocking of secretion of hepatic triglycerides in plasma[23]. Silymarin and the extract prevented increase of liver weight in rats.

Hence, the present investigation suggests that SJB shows good antioxidant activity, reducing power, free radical scavenging activity and hepatic protection. Phytochemical screening of the crude SJB reveals the presence of phenolic compounds, flavonoids, saponins, alkaloids, tannins. Thus these in vitro and in vivo antioxidant potential of SJB may be due to the presence of these phytoconstituents.

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Table 1: Effect of ethanolic extract of *S. jambos* and Silymarin on serum biochemical parameters in CCl_4 induced liver damage in rats

Treated Group	Serum Biochemical Parameters					Liver weight (gm)
	SGPT (U/L)	SGOT (U/L)	ALP (KA)	Bilirubin (mg/dl)	Total protein (mg/dL)	
Normal control	22.1±0.33	38.8±0.39	19.26±0.04	1.06±0.016	12.14±0.181	5.96± 0.18
CCl4	74.3±0.51#	85.4±0.39#	68.12±0.122#	5.68±0.027#	7.67±0.064#	9.57±0.31#
SJB	38.2±0.51*	61.9±0.51*	43.99±0.049*	4.167±0.027*	9.123±0.09*	7.24±0.38*
Silymarin	26.8±0.33*	46±0.34*	24.47±0.125*	2.33±0.036*	11.08±0.082*	6.98±0.42*

Values are Mean ± S.E.M.; n=3 in each group. Drug treatment was done for 8 days.
#P<0.001 CCl4 treated group compared with normal group; *P<0.05 Experimental groups compared with control group; where the significance was performed by One way ANOVA followed by post hoc Dunnett's test.

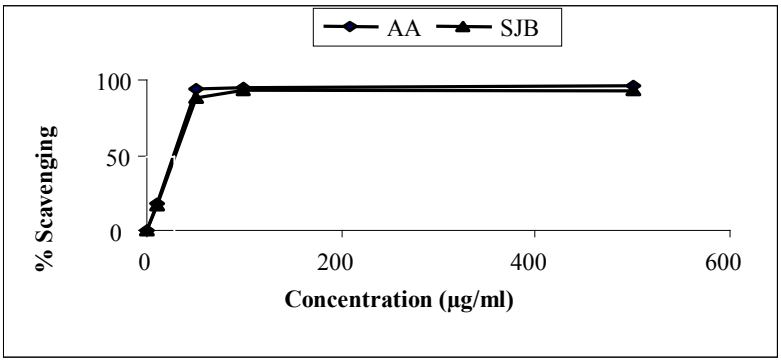

Fig. 1: DPPH radical scavenging activity of the ethanolic extract of *Syzygium jambos* bark

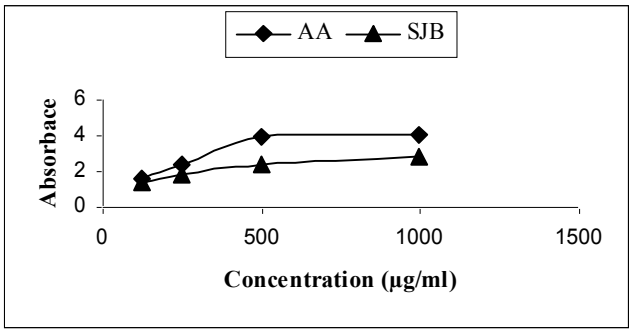

Fig. 2: Reducing power of SJB. Here, AA=Ascorbic acid

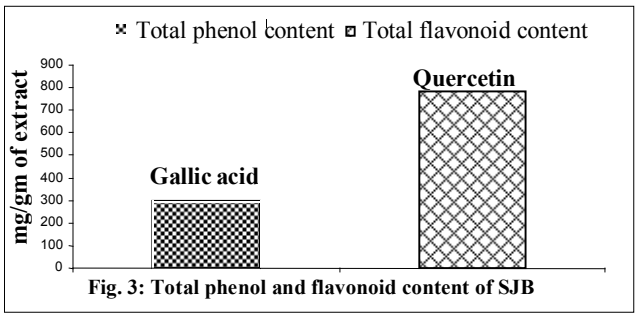

Fig. 3: Total phenol and flavonoid content of SJB

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