Original Article

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Antioxidant and hepatoprotective activity of an ethanol extract of *Syzygium jambos* (L.) leaves

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ABSTRACT: Herbal medicines have traditionally been used worldwide for the prevention and treatment of liver disease with fewer adverse effects. The leaves of the *Syzygium jambos* (SJL) plant were chosen and studied for their antioxidant activity *in vitro* and hepatoprotective activity *in vivo*. The antioxidant activity of the ethanol extract was examined *in vitro* using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, reducing capacity, total phenol, total flavonoid content, and total antioxidant capacity. The extract had significant dose-dependent antioxidant activity in all *in vitro* experiments. IC_{50} values of SJL and ascorbic acid (standard) were found to be 14.10 and 4.87 µg/mL, respectively, according to a DPPH radical scavenging assay. Hepatoprotective activity of the plant extract was evaluated in a rat model of carbon tetrachloride (CCl_4)-induced liver damage. CCl_4 significantly altered serum marker enzymes, total bilirubin, total protein, and liver weight. The extract caused these values to return to normal in rats with CCl_4-induced liver damage that were given SJL. This indicated the hepatoprotective potential of SJL and was comparable to use of the standard drug silymarin. Thus, the present study revealed that SJL may have antioxidant and hepatoprotective activity.

Keywords: *Syzygium jambos*, antioxidant, hepato-protective activity

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by normal physiologic processes and fulfill important functions in the body at minute or moderate concentrations. ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host's defense system. The importance of ROS production by the immune system is clearly evident in patients with granulomatous disease. These patients have a defective membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system that precludes them from producing the superoxide anion radical (O_2^-), thereby resulting in multiple and persistent infections (*1,2*). Oxygen radicals have crucial action such as signal transduction, gene transcription, and regulation of synthesis of cyclic guanosine monophosphate (cGMP) in cells (*3,4*). Nitric oxide (NO) is a common signaling molecule and participates in virtually every one of the body's cellular and organ functions (*5*). Optimum amounts of NO produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (*5*). In addition, NO produced by neurons serves as a neurotransmitter (*6*).

However, the generation of even slightly larger amounts of these essential compounds during metabolism or in response to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods, and physical stress can cause massive physiologic problems by oxidation of bio-molecules (protein, amino acids, lipid, and DNA). Free radicals presumably play a major role in all pathologies. Free radicals are believed to be responsible for more than one hundred conditions like cancer, diabetes, atherosclerosis, arthritis, neuropathy, nephropathy, retinopathy, aging, compromised immunity, and cardiovascular diseases (*7-10*).

The liver has a central role in transforming and clearing chemicals and is closely related to the gastrointestinal tract, which makes it susceptible to drug toxicity, xenobiotics, and oxidative stress. Dysfunction of this organ results in impairment of energy metabolism and intracellular oxidant stress with excessive formation of ROS. CYP2E1 is a cytochrome P450 isoenzyme produced by the liver that also facilitates oxidative stress and cell injury (*11,12*). Although Kupffer cells and recruited neutrophils in the liver are part of the host-defense system, these inflammatory cells initiate additional liver injury under certain circumstances, such as when excess free radicals are present (*13-16*).

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Although the body has mechanisms to defend against the damaging properties of ROS (*17,18*), its capacity to control ROS can be overwhelmed, causing irreversible oxidative damage and various conditions that threaten the liver and other parts of the body. Several synthetic and semi-synthetic chemicals are often used to protect the liver from the detrimental effects of free radicals but they are not sufficiently effective and cause adverse reactions. Modern medicine includes many important bioactive molecules with antioxidant and hepatoprotective properties that were derived from plants. Curcumin and lycopene are used as antioxidants and are respectively obtained from the plants *Erythroxylum coca* and *Lycopersicon esculentum*. *Andrographis paniculata* and *Silybum marianum* are two important plant sources of andrographolide and silymarin, respectively, that are widely used as hepatoprotective agents (*19*). Hence, recent research has hastened to identify notable hepatoprotective agents from plant products that will reduce the harmful effects of and problems associated with free radicals while causing minimal adverse reactions. As part of ongoing research, the current study examined the ethanol extract of *Syzygium jambos* leaves for its use as a hepatoprotective agent to reduce the damage caused by ROS and RNS.

Syzygium jambos (L.), commonly known as rose apple, belongs to the family Myrtaceae and may merely be a shrub but is generally a tree found all over parts of Bangladesh, Pakistan, and India (*20,21*). It has been used in traditional medicine to treat various ailments. The fruit is regarded as a tonic for the brain and liver. The seeds are used to curb diarrhea, dysentery, and catarrh and help treat diabetes. A decoction of the leaves is applied to sore eyes, it serves as a diuretic and expectorant, and it is used to treat rheumatism. The bark contains 7-12.4% tannin. The leaf extract of *Syzygium jambos* reportedly has antinociceptive and antimicrobial activity (*22,23*). No reports have described the plant's hepatoprotective activity. Thus, the present study sought to investigate the leaves of the *S. jambos* (SJL) for its hepatoprotective activity using a rat model of CCl_4-induced liver damage and different *in vitro* antioxidation experiments.

2. Materials and Methods

2.1. *Chemicals*

1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), 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.

2.2. *Preparation of extract*

An ethanolic extract of leaves was used in the present study. Mature leaves were collected in July 2009 from Rajshahi, Bangladesh. The leaves were dried in the shade and pulverized in a mechanical grinder. The powder was extracted with ethanol. The extracted solution was filtered using a clean cloth and then filter paper. The extract was concentrated first in a rotary vacuum evaporator and then in a water bath. The extracted residue was weighed and the percent yield of leaves of *S. jambos* was 9.21% w/w. The extract was then frozen prior to examination of its potential antioxidant and hepatoprotective properties.

2.3. *DPPH radical scavenging activity assay*

The free radical scavenging capacity of the extract was determined using the stable free radical DPPH (*24*). The leaf extract was mixed with 95% ethanol to prepare a stock solution (5 mg/mL). DPPH solution (0.004%, w/v) was prepared in 95% ethanol. A freshly prepared DPPH solution (0.004%, w/v) was placed in test tubes and SJL was added followed by serial dilution (1 μg to 500 μg) in every test tube so that the final volume was 3 mL. After 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DR UV-visible spectrophotometer, USA). Ascorbic acid was used as a reference standard and dissolved in distilled water to prepare a stock solution with the same concentration (5 mg/mL). A control sample of the same volume was prepared without any extract and reference ascorbic acid. A solution of 95% ethanol served as a blank. The % scavenging of the DPPH free radical was measured 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 experiments in triplicate and expressed as the % of mean inhibition ± standard deviation. IC_{50} values were obtained by Probit analysis (*25*). The IC_{50} value is the concentration of the sample required to inhibit 50% of the radical.

2.4. *Total antioxidant capacity assay*

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method in accordance with the procedure described by Prieto *et al.* (*26*). The assay is based on the reduction of Mo(VI) to Mo(V) by the extract/sample and subsequent formation of a green phosphate/Mo(V) complex at an acidic 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). Test tubes containing the reaction solution were incubated at 95°C for 90 min. The absorbance of the solution was then measured against a

blank at 695 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer) after the solution cooled to room temperature. Ethanol (0.3 mL) in the place of extract/sample served as the blank. Antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

2.5. *Reducing capacity assay*

Reducing power of the extract was evaluated using the Oyaizu method (*27*). Different concentrations of SJL extract (125, 250, 500, and 1,000 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, 2.5 mL of 10% trichloroacetic acid solution was added to each test tube and the mixture was centrifuged at 3,000 rpm for 10 min. Five mL 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 served as the reference standard. Phosphate buffer (pH 6.6) served as the blank solution.

2.6. *Determination of total phenol content*

Total phenol content in the extract was determined with Folin-Ciocalteu reagent. Extract (200 μg/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 brought to 10 mL using distilled water. The mixture was allowed to stand for 2 h. The absorbance at 765 nm was then determined. The total phenol content in SJL was then determined as the mg of gallic acid equivalent using equations that were obtained from a standard gallic acid graph (*28*).

2.7. *Determination of total flavonoid content*

The total flavonoid content was determined using a method previously described by Kumaran and Karunakaran (*29*). One 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 for quercetin (12.5-100 μg/mL) and was expressed as mg of quercetin equivalent (QE/g of extract).

2.8. *Hepatoprotective activity*

The hepatoprotective activity of SJL extract was determined using a rat model of carbon tetrachloride-induced hepatotoxicity. After seven days of acclimatization, rats were divided into four groups consisting of three rats each. Treatment lasted for 8 days. Group I served as the normal control and received only normal saline (1 mL/kg, *i.p.*) for eight days. Group II served as the toxic control and was administered carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Group III served as the positive control and was administered silymarin (25 mg/kg/day, *p.o.*) along with carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Group IV was administered SJL (300 mg/kg, *p.o.*) daily and carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Twenty-four h after the last dose, blood was taken from the retro-orbital plexus under sodium phenobarbital anesthesia and rats were dissected to remove the liver. Before blood was collected, the syringe was ringed with heparin to prevent hemolysis/clotting. The blood samples were then centrifuged at 2,500 rpm at 37°C to separate serum and were used to estimate the biochemical markers of liver damage, *i.e.* SGOT, SGPT (*30,31*), ALP (*32*), bilirubin (*33*), and total protein levels (*34*).

2.9. *Statistical analysis*

Linear regression analysis was used to calculate IC_{50} values wherever needed. All results are expressed as average ± SEM. Data were statistically evaluated in InStat software using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *DPPH radical scavenging activity*

A DPPH assay is one of the most widely used methods of screening for the antioxidant activity of plant extracts. DPPH is a stable, nitrogen-centered free radical that produces a violet color in ethanol solution. When DPPH encounters proton donors such as antioxidants, it is reduced to a yellow product, diphenyl picryl hydrazine, and then absorbance decreases. The antioxidant activity of an ethanol extract of SJL was evaluated by measuring its scavenging capacity for the DPPH free radical; this capacity was expressed using the IC_{50} value. A lower IC_{50} value indicates an extract with greater scavenging activity. The extract had dose-dependent activity, *i.e.* DPPH scavenging activity increased proportionate to the increase in concentration of the extract. Results are shown in Figure 1. The IC_{50} value of the extract was 14.10 μg/mL while that for reference ascorbic acid was 4.87 μg/mL. These results indicate that the extract had definite free radical scavenging activity in comparison to ascorbic acid.

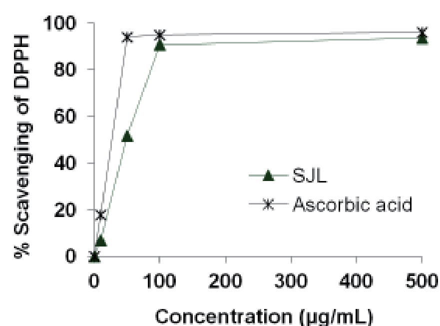

Figure 1. DPPH radical scavenging activity of the ethanolic extract of *S. jambos* leaves. Values are the average of experiments in triplicate and are expressed as mean ± standard deviation.

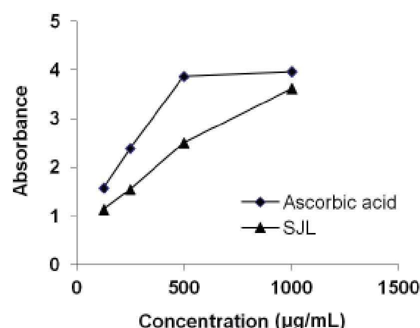

Figure 2. Reducing power of *S. jambos* leaves and ascorbic acid.

Table 1. Effect of ethanol extract of *S. jambos* leaves and silymarin on serum biochemical parameters in CCl_4-induced liver damage in rats

Treatment group	Serum biochemical parameters					Liver weight (g)
	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 ± 0.18	5.96 ± 0.18
CCl_4	74.3 ± 0.51	85.4 ± 0.39	68.12 ± 0.12	5.68 ± 0.027	7.67 ± 0.064	9.57 ± 0.31
SJL	41.7 ± 0.60	66.9 ± 0.34	49.29 ± 0.09	4.59 ± 0.019	8.12 ± 0.06	7.33 ± 0.27
Silymarin	26.8 ± 0.33	46.0 ± 0.34	24.47 ± 0.13	2.33 ± 0.036	11.08 ± 0.08	6.98 ± 0.42

Values are mean ± STD and each group contained three rats. Drug treatment lasted for 8 days. $^{\#}$ $p < 0.001$ for the CCl_4-treated group compared to the normal control group; * $p < 0.05$ for experimental groups compared to the control group. Significance was tested using one-way ANOVA followed by a post hoc Dunnett's test.

3.2. *Total antioxidant capacity*

The total antioxidant capacity of SJL was determined using a calibration curve for ascorbic acid ($y = 0.0043x + 0.1503$) and was expressed as the ascorbic acid equivalent (AE). Total antioxidant capacity was 335.70 ± 65.77 mg AE/gm of extract.

3.3. *Reducing capacity*

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*35*). Reducing properties are generally associated with the presence of reductones, which have been shown to have antioxidant action by breaking the free radical chain reaction by donating a hydrogen atom (*36,37*). 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 SJL was investigated using Fe^{3+}-Fe^{2+} transformation. The presence of reductones causes the reduction of 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 extract had significant reducing activity compared to ascorbic acid and this activity increased proportionate to the increase in concentration of the extract, as shown in Figure 2. An increase in the reaction mixture's absorbance indicates an increase in the reducing capacity of the sample.

3.4. *Total phenol content and flavonoid content*

The Folin-Ciocalteu reagent was used to estimate total phenols present in the extract and this value was expressed as gallic acid equivalents (GAE). The total phenolic content of the sample, calculated on the basis of the standard curve for gallic acid, was found to be 161.78 ± 11.78 mg GAE/gm of SJL extract. The total flavonoid content of SJL was 1,033.37 ± 62.05 mg of quercetin equivalent per gram of extract.

3.5. *Hepatoprotective activity*

The hepatoprotective activity of the crude ethanol extract at a dose of 300 mg/kg body weight in rats with carbon tetrachloride-induced damage is shown in Table 1. For comparison, the table also shows the untreated group (control), carbon tetrachloride-treated group (induction control), and the group treated with the drug (silymarin). The control group (I) had serum SGPT of 22.1 ± 0.33 U/L, SGOT of 38.8 ± 0.39 U/L, ALP of 19.26 ± 0.04 mg/dL, total bilirubin of 1.06 ±

0.016 mg/dL, total protein of 12.14 ± 0.18 KA, and liver weight of 5.96 ± 0.18 g. In the group with CCl_4-induced liver damage (II), serum SGPT increased to 74.3 ± 0.51 U/L, SGOT increased to 85.4 ± 0.39 U/L, ALP increased to 68.12 ± 0.12 mg/dL, total bilirubin increased to 5.68 ± 0.027 mg/dL, total protein increased to 7.67 ± 0.064 KA, and liver weight increased to 9.57 ± 0.31 g. Administration of SJL extract in rats with CCl_4-induced liver damage resulted in gradual normalization of SGPT, SGOT, ALP, total bilirubin, total protein, and liver weight ($p < 0.001$ compared to the CCl_4-treated group).

4. Discussion

There is growing evidence that the altered production and spatiotemporal distribution of ROS/RNS causes deleterious oxidative/nitrosative stress (*38*). This condition leads to the interaction of ROS/RNS and bio-molecules (protein, amino acids, lipid, and DNA) and interferes with the expression of a number of genes and signal transduction pathways. Thus, ROS/RNS play a key role in damage to cell structures as well as various diseases and aging (*39*). The liver is one of the most important organs in the body. It plays a vital role in regulating, synthesizing, storing, secreting, transforming, and breaking down many different substances in the body. The liver plays a central role in transforming and clearing chemicals and hence it is most susceptible to the free radicals from these agents (*40*). The body has several mechanisms to counteract oxidative/nitrosative stress with antioxidants, either naturally generating them in situ (endogenous antioxidants) or obtaining them externally through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to help prevent disease.

Results of the current study indicated that the ethanol extract of SJL has significant antioxidant capacity, obvious reducing capacity, and definite DPPH radical scavenging activity. These pharmacological effects of the extract may at least in part be due to phenols and flavonoids components that were found in the SJL ethanol extract. Phenols are ubiquitous secondary metabolites in plants and have a wide range of therapeutic uses because of their antioxidant, antimutagenic, anticarcinogenic, and free radical scavenging activity (*41*). 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 (*42*). Flavonoids are a group of polyphenolic compounds with known properties, which include free radical scavenging and inhibition of hydrolytic and oxidative enzymes (*43*). Furthermore, these compounds have a strong affinity for iron ions (which are known to catalyze many processes and lead to the appearance of free radicals), so their antiperoxidative activity could also be attributed to a concomitant ability to chelate iron. Therefore, the phenol and flavonoid components in the SJL ethanol extract may have contributed directly to antioxidant action noted in this study.

In most developing countries, there is a high incidence of viral hepatitis. Identification of an efficient hepatoprotective drug derived from natural sources is an urgent necessity. The changes associated with CCl_4-induced liver damage are similar to those of acute viral hepatitis (*44*). CCl_4 is therefore a useful tool for inducing hepatic damage in experimental animals. The hepatotoxicity of CCl_4 is the result of its reductive dehalogenation. It is catalyzed by cytochromic P450 to produce the highly reactive metabolite trichloromethyl ($CCl_3\cdot$) free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical ($CCl_3OO\cdot$). These free radicals bind covalently to cellular proteins or lipids or extract a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and consequently leading to liver damage. A substantial increase in the level of serum marker enzymes (SGOT, SGPT, and ALP) and total bilirubin was noted in the CCl_4 control group. The return of elevated levels of serum enzymes to near normal values in groups treated with the leaf extract or standard silymarin is an indication of the stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl_4. A decrease in the level of total proteins in rats with CCl_4-induced liver damage is attributed to damage primarily in the endoplasmic reticulum. This results in a loss of P450 and subsequent decrease in protein synthesis. The rise in protein levels in treated groups suggests the stabilization of the endoplasmic reticulum and subsequent protein synthesis. In addition, CCl_4 led to a significant increase in liver weight because it blocks the secretion of hepatic triglycerides in plasma (*45*). Silymarin and the extract were found to prevent an increase in liver weight in rats. These results suggest that the ethanol extract of SJL offers hepatoprotection by reducing damage or by preserving normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin such as CCl_4. This finding indicates that SJL has protective action *in vivo*.

Results of the present study indicate that an ethanol extract of SJL has potential antioxidant activity *in vitro* and hepatoprotective activity *in vivo.* However, further studies are needed to examine underlying mechanisms of antioxidant activity *in vitro* and hepatoprotective activity *in vivo*. Studies also need to isolate the active compound(s) responsible for this pharmacological activity.

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