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RESEARCH ARTICLE

Evaluation of Extracts of Seeds of *Syzygium cumini* L. for Hepatoprotective Activity Using CCl_4-Induced Stressed Rats

M Islam, K Hussain*, A Latif, FK Hashmi, H Saeed, NI Bukhari, SS Hassan, MZ Danish and B Ahmad

University College of Pharmacy, University of the Punjab, Allama Iqbal Campus, Lahore-54000, Pakistan
*Corresponding author: hussain_761@yahoo.com

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The hepatoprotective effects of seeds of *Syzygium cumini* L. (Family: *Myrtaceae*), presumed to be effective in treating gastrointestinal diseases of animals, have not been investigated before, particularly in liver damage caused by infections, chemicals and xenobiotics. Therefore, this aimed at investigating the hepatoprotective effects of methanol extracts of plant seeds in chemically (CCl_4) induced stress rats. Adult male, *Sprague Dawley* rats (n=30) were randomly segregated into 5 equal groups i.e., group-I (control), group-II (silymarin treated; 1.0 mg/kg BW), group-III (extract of *Syzygium cumini* seeds treated; 250 mg/kg BW), group-IV (extract treated; 500 mg/kg) and group-V (CCl_4 treated; 1.5 mg/kg). Rats were treated with respective treatments for 14 consecutive days. At day 14, four hours after the last dose, an oral dose of CCl_4 (1.5 mg/kg, 1:1 in olive oil) was administered to all the groups, except animals in the control group. Subsequently, 24h later, blood samples and liver tissues were collected for biochemical analysis and histopathology, respectively. The values of liver function markers were found to be significantly ($P<0.05$) lower while serum protein level was significantly higher in control and treated groups as compared to that of the CCl_4 treated group. Histological examination of liver tissues also indicated that the extract of *Syzygium cumini* seeds in both the doses, and silymarin protected the liver from CCl_4-induced stress. It was concluded that extract of seed of *Syzygium cumini* has hepatoprotective activity.

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INTRODUCTION

The contaminated food and water, chemicals, drugs and xenobiotics are major sources of gastroenteritis (Slifko *et al.,* 2000). These agents are neutralized by liver biotransformation enzymes of both phase–I and –II reactions (Trisciani *et al.,* 2011). Compromised enzyme activity or excessive intake of toxins induce hepatic stress, leading to various liver diseases (Vrba and Modriansky, 2002; Fernandez-Checa and Kaplowitz, 2005). To protect the liver from stress induced injury with the aim to prevent disease progression; supplementation with antioxidant drug compounds could be helpful in reducing liver damaging events (Bansal *et al*., 2000; Bansal *et al*., 2005). Plant materials possessing astringent and antioxidant activities are used to treat gastroenteritis and hepatic stress (Baliga *et al*., 2011). *Syzygium cumini* L. (Family: *Myrtaceae)* is a famous plant, the leaves, bark and seeds of which are used by folklore to treat gastrointestinal and liver diseases of the grazing animals (Baliga *et al*., 2011). To provide scientific evidence to this folklore use, the present study aimed to investigate hepatoprotective activity of methanol extract of seeds of the plant.

The plant is well known in many countries due to its medicinal properties and fruit value. Traditionally, various parts of the plant were utilized to treat different ailments of humans and animals (Thomson, 2000; Khare, 2004). The bark has been used for the treatment of sore throat, bronchitis, asthma, thirst, biliousness, dysentery and ulcers (Ayyanar and Subash-babu, 2012). Leaf juice, alone or in combination with herbs, goat milk and honey is reported to be effective in treating diarrhea, diabetes and stomach-ache (Nikhat *et al.,* 2009; Nikhat *et al.,* 2008). The literature review indicated a number of reports regarding antioxidant activity of extracts, mainly; fruit,

peel, leaves and bark of the plant, using various models (Banerjee *et al.,* 2005; Veigas *et al.,* 2007; Ruan *et al.,* 2008; Eshwarappa *et al.,* 2014). The antioxidant activity of all parts of the plant was attributed to diverse types of phytochemical constituents reported previously (Banerjee and Narendhirakhanna, 2011). To treat gastrointestinal diseases of sheep and goat, Pakistani people purchase fresh leaves of the plant from animal fodder markets.

Several lines of evidences suggest that carbon tetrachloride (CCl_4) can cause hepatic damage owing to overproduction of trichloromethyl free radicals leading to membrane lipid peroxidation and eventually cell death (Alavian *et al.,* 2014; Afifi *et al.,* 2014; Sahreen *et al.,* 2014). Due to such reasons, this chemical agent is commonly employed to develop animal models to study hepatoprotective, cardioprotective and antioxidant drugs (Olatosin *et al*., 2014; Rao *et al.,* 2014). Using this model, extracts of leaves of *Syzygium cumini* have shown hepatoprotective activity (Moresco *et al.,* 2007; Cheong and Ranghoo-Sanmukhiya, 2013.). Extracts of peels of fully ripe berries have shown ameliorating effects using CCl_4-induced isolated rat hepatocytes (Veigas *et al.,* 2008). There was only one report wherein aqueous extract of seeds were investigated for liver protection (Behera *et al.,* 2014), but the stress was induced by diabetes. In this study, the activity was attributed to tannins and flavonoids of the extract. Our literature review indicated that methanol extract of seeds of the plant was not investigated for hepatoprotective activity using CCl_4 induced oxidative stress in rats.

The seeds are reported to contain jamboline, traces of pale yellow essential oil, chlorophyll, fat, resin, albumen, tannins (Jadhav *et al.,* 2009), phenolic compounds such as ellagic acid, gallic acid, caffeic and ferulic acids and their derivatives (Williamson, 2002) and flavonoids like rutin and quercetin (Sharma *et al*., 2008). Based on such constituents, seed extracts are expected to possess excellent astringent and antioxidant potential, which may be beneficial in relieving gastroenteritis and liver inflammation.

Several compounds can be used to induce hepatic stress in experimental animals to screen hepatoprotective agents. In the present study, CCl_4 was used to induce oxidative stress in rats because of its well characterized mechanism, causing structural membranes damage and centrilobular necrosis. In the present study, silymarin was used as a standard drug because of its proven activity, safety and well-established role in inhibiting lipid peroxidation, stimulating protein synthesis along with antioxidant, anti-inflammatory and anti-fibrotic activity (Saller *et al.,* 2001; Pradhan and Girish, 2006).

MATERIALS AND METHODS

The study was conducted as per approved protocol of the Animal Ethical Committee, University College of Pharmacy, University of the Punjab, Lahore, Pakistan.

Procurement of plant material and its extraction: In May, 2010, 28 kg fully ripe berries of the plant (*Syzygium cumini*) were purchased from the fruit market of Multan, Pakistan. The material was authenticated by a Taxonomist, Department of Botany, Government College University (GCU), Lahore, Pakistan. A voucher specimen was deposited in herbarium of the GCU (Bot. Herb # 853). The seeds were separated, washed with water and dried in an oven at 50°C. The material was then crushed using colloidal mill and dried under shade for 4 weeks. Finally, the material was kept in an oven at 60°C for three days and pulverized. For extraction purposes, 500g material was extracted sequentially using solvents such as petroleum ether, chloroform and methanol by soxhlet apparatus. The extracts were filtered and dried in *vacuo* at 40°C.

Study design and treatments: Thirty male *Sprague-Dawley* rats, weighing 250±15g, were randomly segregated into 5 experimental groups that were control group (group-I, untreated, uncompromised), silymarin treated group (group-II) and extract treated groups (group-III and -IV) and toxic group (group-V) treated like the control, except the last day of the study. Each of the groups was kept in individual cage and allowed to acclimatize for 7 days at Animal House, University College of Pharmacy, University of the Punjab, Lahore, Pakistan. Two solutions of the extracts, 6.25 and 12.50mg/mL, and one solution of silymarin (0.25mg/mL) were prepared in distilled water. All the groups were treated orally (daily) for 14 consecutive days. Group wise treatments were as follows; group-I received vehicle control, group-II received silymarin (1.0mg/kg), group-III received 250mg/kg of the extract, group-IV received 500mg/kg of the extract and group-V received (Vehicle). At day 14, 4h after usual dosing, an oral dose of CCl_4 (1.5 mg/kg, 1:1 in olive oil) was administered to all the groups (Group-II, III, IV and V), except the control. An oral dose (1.0mL) of the extract, silymarin and vehicle was administered daily, for 14 days, using feeding cannula as per aforementioned study design.

Sample collection and analysis: For sample collection, 24h after CCl_4 administration, blood samples and liver tissues were collected for the biochemical analysis and histopathology, respectively. Blood samples were collected from the tail vein, while a piece of liver from each animal (obtained under chloroform anesthesia) was preserved in 10% formalin for histopathology. Blood samples were subjected to centrifugation (2700 rpm for 10 min) to collect serum, which was analyzed for total protein, aspartate transaminase (AST), alkaline transaminase (ALT), alkaline phosphatase (ALP), and bilirubin total (BiT) using *ROCHE HITACHI 902 Chemistry Analyzer (*Roche Diagnostics). For histopathology, liver tissues were processed by routine method of dehydration and paraffin embedding techniques. Sections of 4-5 µm thickness were cut and stained with hematoxylin and eosin. Then the slides were examined for pathological changes under light microscope.

Statistical analysis: The data were submitted to one way ANOVA with Post Hoc multiple comparisons Bonferroni test, using SPSS 13.0 and $P \leq 0.05$ was considered statistically significant.

RESULTS

Methanol extract of seeds of the plant prepared sequentially was investigated at two dose levels, 250 and 500mg/kg, for the assessment of liver protection, provided

Table 1: Biochemical parameters of CCl_4 and extract of seeds of *Syzygium cumini* treated/untreated rats

Parameters	Groups				
	I	II	III	IV	V
AST (U/L)	87.50±4.23a	108.00±11.66b	151.67±8.16b	132.50±8.22b	176.67±14.03b
ALT (U/L)	46.17±1.17a	54.00±15.53b	91.34±9.46b	80.34±2.43b	115.00±15.49b
ALP (U/L)	13.67±1.03a	17.34±1.22b	26.34±3.73b	22.34±2.07b	32.17±3.25b
Total Proteins (g/dL)	08.57±0.21a	07.97±0.11b	07.02±0.25b	07.28±0.76b	05.68±0.50b
Total bilirubin (mg/dL)	00.28±0.07a	00.34±0.08b	0.60±0.09b	00.42±0.21b	00.85±0.05b

Mean±SD values bearing different alphabets in a row differ significantly. Extract=Methanol extract of seeds of *Syzygium cumini*. Group-I (untreated - control), Group-II (silymarin), Group-III (250 mg/kg extract), Group-IV (500 mg/kg extract) and Group-V (toxic). After 14 days, CCl_4 was administered to all the groups, except control.

against a single oral dose of CCl_4. The levels of liver function biomarkers such as AST, ALT, ALP, TP and BiT in control, treated and toxic groups of animals are presented in Table 1. The lower levels AST, ALT, ALP and BiT in group-I (untreated and uncompromised) as compared to all the groups, which were exposed to oxidative stress ($P<0.05$) indicated the validity of the model used in the present study.

These results show that mean levels of all the markers, between the groups and within the groups, were quite different ($P=0.000$). The mean levels of AST, ALT and ALP in all the treated groups were significantly lower than that of the toxic group ($P<0.05$), which indicated that the extract in both the doses and silymarin protected the liver from the CCl_4-induced oxidative stress. The TP contents of the extract and silymarin treated groups were higher than that of the toxic group ($P<0.05$). The level of BiT was significantly lower in treated groups and control as compared to the toxic group ($P<0.05$).

The comparison of two dose levels of the extract, 250 and 500mg/kg, indicated that the levels of AST, ALT and ALP were significantly lower in the group treated with the higher dose ($P<0.05$). However, preservation of TP in both the extract-treated groups was found to be analogous to each other. Excluding the similarity of TP contents in both the dose-treated groups, a dose 500mg/kg has demonstrated better liver protection activity than that of the lower dose. Microscopic examination of liver of control group showed normal hepatocytes, lobular liver cells and sinusoids. In this group, nuclei were seemed to be normal which were stained blue. On the other hand, the slides of toxic group (group-V) showed necrosis, apparent due to absence of nuclei and accumulation of fat globules. The silymarin-treated group (group-II) and methanol extract-treated groups (group-III and -IV) showed mild centrizonal inflammation along with normal hepatocytes. These results further verified the activity of the extract, indicated by the biochemical analysis.

DISCUSSION

Carbon tetrachloride (CCl_4) has been widely used to induce liver injury in rodents. However, patho-physiological consequences depend upon the time of exposure, for example; a single dose of CCl_4 can cause centrizonal necrosis while prolonged administration leads to liver fibrosis and cirrhosis (Manibusan *et al.,* 2007). CCl_4 generates trichloromethyl (•CCl3) and trichloromethyl peroxy (•CCl3O2) free radicals leading to a pathological situation characterized by compromised membrane integrity due lipid peroxidation that further initiates events that not only affect membrane proteins but also the DNA, thus further aggravating hepatic complications (Oluba *et al.,* 2010). Furthermore, the propagation of lipid oxidation increases the interaction of free radicals with phospholipids that destroy cellular structures (Chidambara *et al.,* 2002). This extent of hepatic damage caused by ensuing oxidative stress is characterized or evaluated by higher biochemical biomarkers such as AST, ALT, ALP and BiT in the blood (Sahreen *et al.,* 2014).

In the present study, administration of methanol extract of seeds of the plant in two doses lowered the level of biochemical markers, which were increased by free radicals of CCl_4. It is probable that the administration of extract for 14 days increased the antioxidant capacity of animal to scavenge the free radicals generated by CCl_4. The free radical scavenging activity of the plant, under investigation, has been attributed to the presence of flavonoids and related compounds (Moresco *et al.,* 2007; Abalea *et al.,* 1999). The plant also contained ellagic acid, a polyphenol having lipid peroxidation inhibition activity (Thresiamma *et al.,* 1996). Presumably, treatment with methanolic extracts can modulate liver cytochrome P-450 enzymes to enhance scavenging of hepatotoxic free radicals and by increasing antioxidant defense activities (Vishnu Kumar *et al.,* 2013).

Silymarin is a well-known hepatoprotective drug, commonly used as a reference drug in hepatoprotective studies of test compounds. Our data suggest that 500mg/kg of plant seed extract exhibits similar hepatoprotective activity as shown by silymarin - evidence by reduced AST, ALT TP and BiT. These results were further corroborated by liver histopathological examination. In terms of efficacy, another study reported the similar evidence that 500mg/kg of plant seed extract was comparable to the standard against hepatotoxicity employing streptozotocin induced diabetic rats (Behera *et al.,* 2014). Furthermore, liver protective activity of the extract was attributed to saponins, tannins and flavonoids.

Thus, the present study not only corroborate previous findings but also is the first report regarding the hepatoprotective activity of sequentially obtained methanol extract of dried plant seeds as evidenced by comparable hepatoprotective effects of per oral administration of 500mg/kg methanol extract of plant seeds to that of silymarin.

Conclusion: Based on the results of the present study, it is concluded that methanol extract of seeds of *Syzygium cumini*, obtained by sequential extraction using solvents in the order of increasing polarity, possesses promising hepatoprotective activity.

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