Hepatoprotective activity and phenolic profile of *Zanthoxylum alatum* Roxb. fruit extract

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Abstract: *Zanthoxylum alatum* Roxb. (Rutaceae) is a medicinal plant, which abundantly grows in the hilly areas of Pakistan. In the present study, the hepatoprotective activity of the *Z. alatum* whole fruit was evaluated. The hepatoprotective effect of the aqueous extracts was studied on mice liver damage using a single dose of paracetamol (640 mg/kg) orally by monitoring biochemical parameters. Pre-treatment of mice with *Z. alatum* (100 mg/kg) aqueous extract orally for four days prevented the paracetamol induced rise in serum transaminases (ALT and AST), restored the altered levels of antioxidant enzyme (catalase), thiobarbituric acid reactive substances (TBARS), non-protein thiol and ascorbic acid to near normal levels. The major phenolic acids, some flavonoid aglycone and glycosides were identified in fruit by high performance liquid chromatography. Ellagic acid (24.57±0.01mg/g), chlorogenic acid (10.65±0.01mg/g), gallic acid (9.15±0.02mg/g), chrysin (16.81±0.03), quercetin (16.81±0.03mg/g) and epicatechin (10.93±0.01) were prédominant in infusion of *Z. alatum*. The result substianted the hepatoprotective activity of *Z. alatum* which may be associated with its potential use in liver disease.

Keywords: *Zanthoxylum alatum*, lipid peroxidation, Pro-oxidants, hepatoprotective activity, HPLC analysis.

INTRODUCTION

Liver is a vital organ present in vertebrates and other animals, which performs detoxification of the exoge-nous xenobiotic, drugs, viral infection and chronic alcoholism. The liver is involved in wide range of physiological functions, which includes the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (Ward *et al.,* 1999). Problems due to liver diseases are worldwide and are associated with significant morbidity and mortality. Herbal drugs play an important role in the management of different liver disorders most of which fasten the natural healing processes of the liver. Several medicinal plants and their formulations are used for liver disorders in traditional system of medicine from a prolonged period of time (Subramoniam *et al.,* 1998).

The genus *Zanthoxylum* L. comprises more than 200 trees, shrubs and lianes (Mabberley 1997) and is included in the tribe Zanthoxyleae of the Rutaceae (Waterman 1975). Species of *Zanthoxylum* are distributed in pan-tropical regions. Different species of *Zanthoxylum L.* are reported from India (Anonymous 1970) and Pakistan (Majid *et al.,* 2004). Among these *Zanthoxylum alatum* Roxb. is a medicinal shrub locally called "Timber" and found in moist and hilly areas of Pakistan (Majid *et al.,* 2004). The powdered seed of *Z. alatum* has excellent spice value and are used in different conditions as aromatic tonic, in stomachic ache, for fever, in dyspepsia and cholera etc. (Rout *et al*., 2007). The fruits, branches and thorns are considered to be carminative and stomachic are used as a remedy for toothache (Chopra *et al.,* 1986). In Pakistan the dry fruit is mixed with salt, *Mentha avensis* and *Carum capticum* for blanks, dyspepsia and headache (Majid *et al.,* 2004). The fruit contains 1.5% essential oil (Chopra *et al.,* 1986) and is used to purify water (Gupta 1945). The essential oil of fruits of *Z. alatum* contains geraniol which shows antifungal activity against *Colletotrichum falcatum* and *Ceratocystis paradoxa* fungal pathogens of sugar cane and was more potent compared to the commercial synthetic fungicides (Rao and Singh 1994). The phytochemical analysis of *Zanthoxylum alatum* oil showed the presence of 33% monoterpene hydrocarbons. The main constituents are 1,8-cineole (15.7%), linalol (18.8%) and undecan-2-one (17.0%) (Peter *et al.,* 1999).

The antioxidant activity of other species of *Zanthoxylum* such as *Zanthoxlum piperatum,* which is a Japenese pepper is well reported (Yamazaki *et al.,* 2007). Moreover, *Zanthoxylum armatum,* which is synonym to *Zanthoxylum alatum* has shown hepatoprotective activity (Lalitsingh *et al.,* 2010). Our earlier studies have shown the antioxidant and free radical scavenging activities of *Z. alatum* fruit (Batool *et al.,* 2010). This tempted us to study the *in vivo* antioxidant activity and hepatoprotective effect of the plant against paracetamol induced liver damage in albino mice.

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MATERIALS AND METHODS

Preparation of plant extract

The fruits of the *Z. alatum* were collected from District Rawalakot Azad Kashmir Pakistan, identified by a botanist. The whole fruit (with seeds) was dried in an oven at temperature 40-50^{o}C, powdered in a blender and was extracted in boiling water (250ml) for 30 minutes with constant stirring, and then filtered using what man filter paper No. 1. The obtained residues was further extracted twice and then concentrated in a rotary evaporator at low temperature. Filtrates were dried in an oven at 50°C to a powder and their percentage yield was 11.2%. The stock solution was serially diluted to the desired concentration of plant.

Test animals

All animal studies were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Ethical Council of University Federal de Santa (UFSM 10069). For *in vivo* studies male albino mice (three to four months old) having weight of 35-40g were used. The animals were housed in separate cages and provided with food *ad libitum* in a room with controlled temperature (22°C±3) and 12h light/ dark cycle.

In vivo hepatoprotective activity

Animals were divided into four groups, which comprise five mice in each group. Group I (control) received only saline. Group II (Plant group) was given aqueous extract at a dose of 100mg/kg body weight of animals for 4 days. Group III (Paracetamol-control) was given a daily dose of saline and on the fourth day a single dose of paracetamol (640mg/kg body weight) orally. Group IV (Plant extract +Paracetamol) was given a daily dose of plant (100 mg/kg body weight) for four days and then treated with paracetamol (640 mg/kg body weight). The dose of plant (100 mg/kg) is based on our previous experiments (Sabir and Rocha 2008). The treatments were administered orally with the help of a gavage. All the mice were sacrificed 24 hours after paracetamol administration under light ether anesthesia and blood was collected in sterile tubes. Livers were quickly removed, placed on ice and homogenized in 7 volumes of NaCl (0.9%). The homogenates were centrifuged at 4,000x g for 10 minutes to yield a low-speed supernatant fraction that was used for the biochemical analysis.

Enzyme assays

The blood was clotted and serum was obtained by centrifugation at 1,500xg for 10 minutes. The activities of enzymes, serum alanine amniotransferase (ALT) and aspartate aminotransferase (AST) were determined using assay kits as described by supplier (Lab Test, MG, Brazil) specifications.

Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 as described by Aebi (1983). Lipid per oxidation in terms of MDA content was measured following the thiobarbituric acid method described by Ohkawa *et al.* (1979). Ascorbic acid was measured by the method of Natelson (1963). In liver homogenate, Non-protein thiol (NPSH) was estimated by the method of Jollow *et al.* (1974).

Identification and quantification of phenolic compounds

Twenty-microliter of *Zanthoxylum alatum* fruit extract was injected using C_{18} column (4.6 mm x250 mm) packed with 5µm diameter. The mobile phase was composed of solvent A (water with 2% formic acid) and solvent B (80% of acetonitrile and 20% of solvent A). The programmer began with gradient elution with 95% A (0-5 min); and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively following the method described by Silva *et al*. (2014) with slight modifications. The flow rate was 0.7mL/min, and the runs were integrated at 270nm for gallic and ellagic acids, 280 nm for catechin and epicatechin, 237 nm for caffeic, chlorogenic and coumaric acids, 313 nm for chysin, and 356nm for quercetin and rutin. Scanning was performed from 200 to 600nm. Phenolic compounds were identified by comparing retention times and UV-VIS spectra with those of pure standards. The sample and mobile phase were filtered through 0.45µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.025-0.250mg/ml for chrysin, catechin, epicatechin, quercetin and rutin and 0.030-0.200mg/ml for gallic, caffeic, coumaric, ellagic and chlorogenic acids. Calibration curve for gallic acid: Y= 12479x +1260.8 (r=0.9997); chlorogenic acid: Y=11983x +1263.4 (r=0.9999); caffeic acid: Y=12875x + 1365.1 (r= 0.9999); coumaric acid: Y=13765x+1185.7 (r=0.9998); ellagic acid: Y=11809x+1163.8 (r=0.9995); catechin: Y=12681x+1382.5 (r=0.9998); epicatechin: Y=12643x+ 1275.3 (r=0.9999); rutin: Y=11976x+1185.6 (r=0.9997); quercetin: Y=13074x+1196.5 (r=0.9994) and chrysin: Y= 13569x+1349.1 (r=0.9999). All chromatography operations were carried out at ambient temperature and in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Silva *et al.*, (2014). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

STATISTICAL ANALYSIS

The results were represented as mean± standard deviation. The data were statistically subjected to one-way ANOVA and means of different group were compared by Duncan's multiple range test (DMRT).

RESULTS

In vivo assays

Treatment with a single oral dose of paracetamol (640 mg/kg) body weight of mice induced a marked elevation (p<0.05) in ALT, AST and TBARS, whereas CAT activity was significantly decreased (p<0.05) indicating liver damage. Similarly administration of paracetamol resulted in significant decrease (p<0.05) in non-protein thiol and ascorbic acid level. However, the pretreatment with the aqueous extract of *Z. alatum* (100mg/kg) orally for four days caused a significant reduction (p<0.05) of ALT, AST and TBARS and recovered the decreased levels of tissue CAT, which supports the hepatoprotective activity of the extract. The aqueous extract brought back the altered levels of non-protein thiol and vitamin C compared to the normal. Results are reported in figs. 1-6.

HPLC analysis

HPLC fingerprinting of *Zanthoxylum alatum* fruit extract revealed the presence of the gallic acid (t_R=9.81 min; peak 1), catechin (t_R=16.25 min; peak 2), chlorogenic acid (t_R=19.83 min; peak 3), caffeic acid (t_R=23.79 min; peak 4), ellagic acid (t_R=26.13min; peak 5), epicatechin (t_R=28.05 min; peak 6), coumaric acid (t_R=30.64 min; peak 7), rutin (t_R=35.97 min; peak 8), quercetin (t_R= 47.61 min; peak 9) and chrysin (t_R=51.02 min; peak 10) (fig. 7 and table 1).

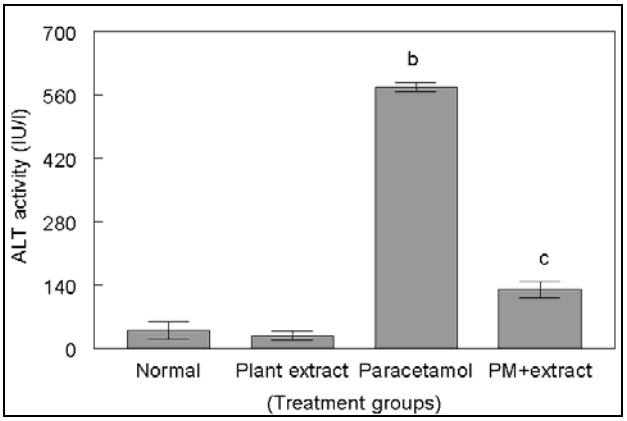

Fig. 1: Hepatoprotective action of *Z. alatum* in mice: Serum enzyme activity of ALT among different treatment groups. Values are mean ± SD (n=5), [b]p<0.05 versus normal control group; [c]p<0.05 versus paracetamol control group.

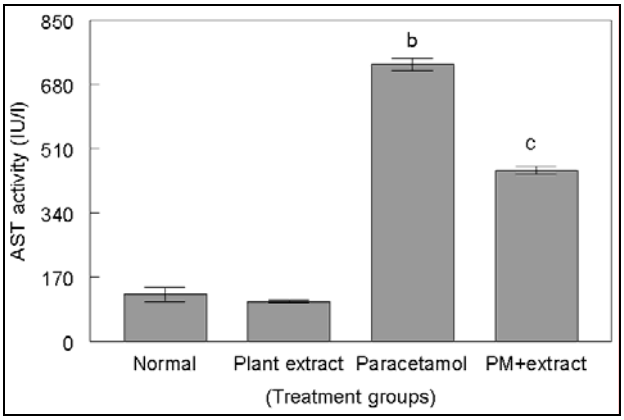

Fig. 2: Hepatoprotective action of *Z. alatum* in mice: Serum enzyme activity of AST among different treatment groups. Values are mean ± SD (n=5), [b]p<0.05 versus normal control group; [c]p<0.05 versus paracetamol control group.

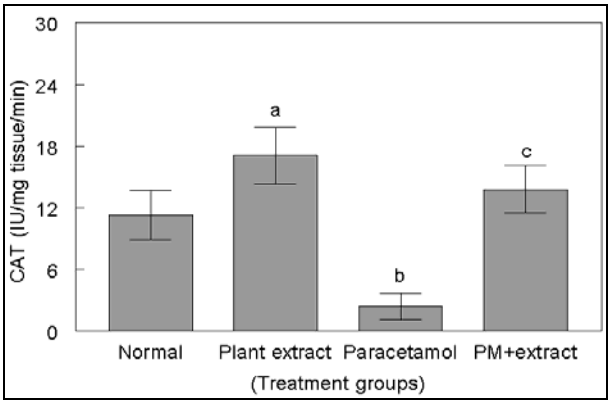

Fig. 3: Hepatoprotective action of *Z. alatum* in mice: Catalase activity among different treatment groups. Values are mean ± SD (n=5), [a]p<0.05 versus normal control group; [b]p<0.05; [c]p<0.05 versus paracetamol control group.

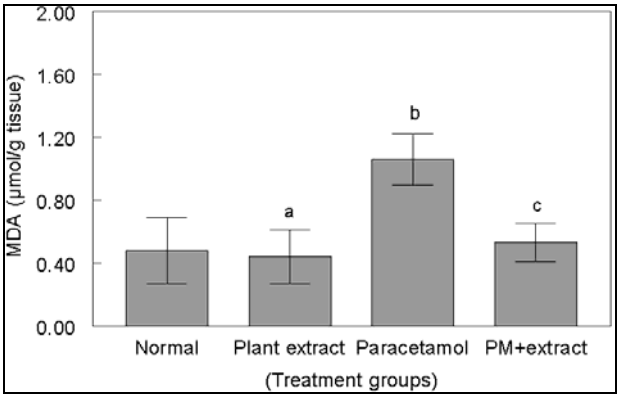

Fig. 4: Hepatoprotective action of *Z. alatum* in mice: lipid peroxide content (TBARS) among different treatment groups. Values are mean ± SD (n=5), [a]p<0.05 versus normal control group; [b]p<0.05 versus normal control group; [c]p<0.05 versus paracetamol control group.

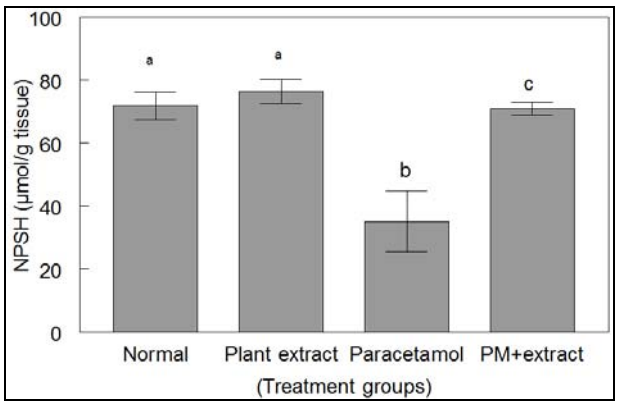

Fig. 5: Hepatoprotective action of *Z. alatum* in mice: Non protein thiol (NPSH) among different treatment groups. Values are mean ± SD (n=5), [a]$p<0.05$ versus normal control group; [b]$p<0.05$ versus normal control group; [c]$p<0.05$ versus paracetamol control group.

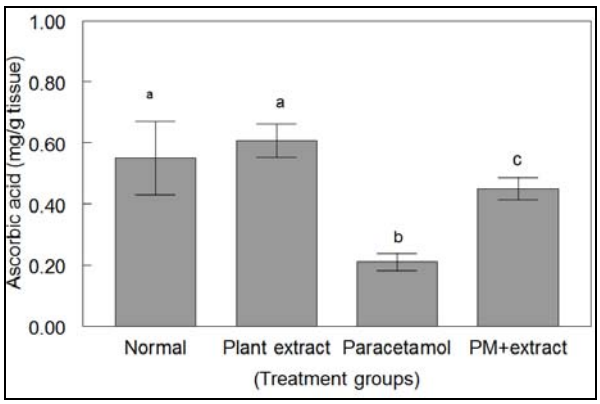

Fig. 6: Hepatoprotective action of *Z. alatum* in mice: Ascorbic acid in liver tissue among different treatment groups. Values are mean ± SD (n=5), [a]$p<0.05$ versus normal control group; [b]$p<0.05$ versus normal control group; [c]$p<0.05$ versus paracetamol control group.

DISCUSSION

In paracetamol-induced hepatotoxicity, reduction of hepatic glutathione is (Gupta *et al.,* 2006; Moron *et al.,* 1979) common; ALT and AST enzymes are present in high concentration in liver. For screening of hepatoprotective agents, paracetamol induced hepato-toxicity is a reliable method. Paracetamol is normally eliminated mainly as sulfate and glucoronide. Only 5% of the paracetamol is converted into N-acetyl-p-benzoquineimine. However, when paracetamol is administered in toxic dose the sulfation and glucoronidation routes are saturated and hence, higher percentage of paracetamol are oxidized to highly reactive N-acetyl-p-benzoquineimine (NAPQI) by enzymes of cytochrome-450. NAPQI reduces to semiquinone radicals which can covalently binds to macromolecules of cellular membrane and increases the lipid per oxidation, which results in oxidative stress (Dahlin *et al.,* 1984). In this study, we have demonstrated for the first time that *Zanthoxylum alatum* aqueous extract has an important hepatoprotective effect in mice against paracetamol. Paracetamol is safe in therapeutic doses, but can result in fatal hepatic necrosis in experimental animals and humans (Amar and Schiff 2007; Frank *et al.,* 1997) and is used as an experimental hepatotoxic agent. A clear sign of hepatic injury is the release of enzymes from cells into the plasma due to the disturbance caused in the transport functions of hepatocytes (Zimmerman and Seeff 1970). The hepatocellular damage can be estimated by measuring the level of serum enzymes. The toxic dose of paracetamol caused hepatotoxicity in mice, which was seen by a substantial increase in the quantity of serum hepatic enzymes (ALT and AST). Pretreatment with *Z. alatum* extract (100 mg/kg p.o) have protective effect on liver and significantly reduced ($p<0.05$) the levels of ALT and AST, which protects the injurious effect of paracetamol. The level of TBARS was also significantly increased ($p<0.05$) in liver, which was decreased in the group treated with plant extract, which indicates that the extract is effective in reducing the lipid peroxidation *in vivo*. The increase in TBARS level in liver induced by paracetamol suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism. A massive decrease in lipid peroxidation in liver tissue of plant extract treated groups indicates that extract possess antioxidative properties (Parmer *et al*., 2009). When the paracetamol was given at a dose (640 mg/kg, p.o) there was significant decrease ($p<0.05$) in CAT enzyme activity and restored in the group treated with the plant. In fact, treatment with extract alone caused a significant increase ($p<0.05$) in CAT activity, which justifies in part its protective effect on liver. A number of studies have shown that paracetamol toxicity results in sharp decrease in glutathione (Gerber *et al.,* 1977) and ascorbic (Dahlin *et al.,* 1984). The results have shown that treatment with *Z. alatum* restored the levels of NPSH and ascorbic acid that were reduced by paracetamol intoxication. The treatment with extract alone increased the amount of ascorbic acid in liver, which reinforces its hepatoprotective action.

The high content of phenolics and flavonoids (Janbaz *et al.,* 2002) has contributed to the hepatoprotective activity of *Z. alatum*. Ellagic acid (24.57±0.01mg/g), chlorogenic acid (10.65±0.01mg/g), gallic acid (9.15±0.02mg/g), chrysin (16.81±0.03), quercetin (16.81±0.03mg/g) and epicatechin (10.93±0.01) were detected as major compounds in infusion of *Z. alatum*. The observed antioxidant activity and protecting ability of *Z. alatum* against liver damage is due to the phenolic acids (ellagic acid, gallic acid, chlorogenic acid, caffeic acid and coumaric acid) and flavonoids (chrysin, catechin, epicatechin, rutin and quercetin). The exogenous antioxidants from *Z. alatum* extracts may act directly or indirectly with the internal antioxidant system for synergetic effects to protect several diseases linked to free

Table 1: Phenolics and flavonoids composition of *Zanthoxylum alatum* extract.

Compounds	Z. alatum seeds extract		LOD	LOQ
	mg/g	%	µg/mL	µg/mL
Gallic acid	9.15±0.02a	0.91	0.015	0.049
Catechin	2.43±0.01b	0.24	0.009	0.030
Chlorogenic acid	10.65±0.01a	1.06	0.024	0.081
Caffeic acid	5.08±0.02c	0.50	0.013	0.042
Ellagic acid	24.57±0.01d	2.45	0.027	0.089
Epicatechin	10.93±0.01a	1.09	0.018	0.060
Coumaric acid	2.17±0.02 b	0.21	0.007	0.023
Rutin	3.35±0.01 e	0.33	0.031	0.102
Quercetin	16.81±0.03 f	1.68	0.014	0.047
Chrysin	15.07±0.02 g	1.50	0.010	0.033

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p<0.05$.

radicals such as heart diseases, neurodisorders and other stress related disorders.

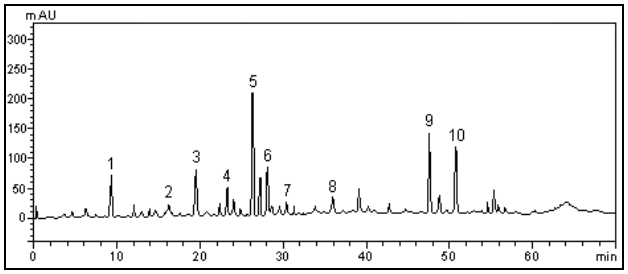

Fig. 1: Representative high performance liquid chromatography profile of *Zanthoxylum alatum* fruit extract, detection UV was at 325nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), coumaric acid (peak 7) rutin (peak 8), quercetin (peak 9) and chrysin (peak 10).

In conclusions, the aqueous extracts of *Z. alatum* fruit exhibited protective effect against paracetamol-induced hepatotoxicity and may be effectively utilized in liver disease. The investigation results that *Z. alatum* can be used not only as accessible source of natural antioxidants but also as an ingredient of the functional food and justifies its use in natural medicine.

ACKNOWLEDGEMENTS

SM Sabir is the recipient of TWAS-CNPq fellowship. JBTR is the recipient of CNPq research fellowship.

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