Original article

Synergistic hepatoprotective potential of ethanolic extract of *Solanum xanthocarpum* and *Juniperus communis* against paracetamol and azithromycin induced liver injury in rats

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A B S T R A C T

Previously explored combination therapies mostly involved the use of bioactive molecules. It is believed that herbal compounds containing multiple plant products have synergistic hepatoprotective effects and could enhance the desired actions. To investigate the combination of ethanolic fruits extract of *Solanum xanthocarpum* (SX) and *Juniperus communis* (JC) against Paracetamol (PCM) and Azithromycin (AZM) induced liver toxicity in rats. Liver toxicity was induced by combine oral administration of PCM (250 mg/kg) and AZM (200 mg/kg) for 7 days in Wistar rats. Fruit extract of SX (200 and 400 mg/kg) and JC (200 and 400 mg/kg) were administered daily for 14 days. The hepatoprotective activity was assessed using liver functional test, oxidative parameters and histopathological examination. The results demonstrated that combine administration of AZM and PCM significantly produced liver toxicity by increasing the serum level of hepatic enzymes and oxidative parameters in liver of rats. Histopathological examination also indicated that AZM and PCM produced liver damage in rats. Chronic treatment of SX and JC extract significantly and dose-dependently attenuated the liver toxicity by normalizing the biochemical factors and no gross histopathological changes were observed in liver of rats. Furthermore, combine administration of lower dose of SX and JC significantly potentiated their hepatoprotective effect which was significant as compared to their effect per se. The results clearly indicated that SX and JC extract has hepatoprotective potential against AZM and PCM induced liver toxicity due to their synergistic antioxidant properties.

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1. Introduction

Liver diseases are one of most serious and common disease in worldwide but, despite tremendous advanced in modern medicine, their prevention and treatment options still remain limited. The role of oxidative stress and inflammation is well noted in the pathogenesis of hepatic diseases. Liver is vital organ in the body which plays an important role in drug elimination and detoxification. Liver diseases are characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma and are associated with high morbidity and mortality. The management of liver disease is still a challenge to modern system of medicine. The herbal drugs are widely used in the treatment of hepatic disorders. The plant extract from the Herbal plant are now in great demand in the developing world for the treatment of hepatic disorders. The plant extract from the Herbal plant are now in great demand in the developing world for primary health. It is considered to be inexpensive and safe to recommend for the treatment of liver disorder.

The hepatotoxicity can be produced by alcohol, chemical and xenobiotics. Paracetamol induced toxicity in animal is one of the most commonly experimental model to evaluate the hepatoprotective activity. At therapeutic doses paracetamol is considered a safe for liver. However, when given at overdoses, it is the leading cause of liver, kidney, and other organ damages in both humans and animals. It is rapidly metabolized in the liver by...
conjugation with glucuronic acid (40–67%) and sulfates (20–46%) and also metabolized by cytochrome P450 isoenzymes to the extremely toxic substance N-acetyl-p benzoquinoneimine (NAPQI). It depletes hepatic glutathione stores in hepatic cells. This condition leads to the formation of reactive oxygen and nitrogen species, and initiates lipid peroxidation that eventually results in damage, necrosis or apoptosis of the liver cells.

*Solanum xanthocarpum* Schrad. & Wendel. (Solanaceae) commonly known as Yellow Berried Nightshade (kantakari), found as weed throughout India. The fruits are well known for their anthelmintic, laxative, anti-inflammatory, diuretic, urinary stone and aphrodisiac activities. Plant is reported to have steroid saponin as glycoalkaloids, flavonoids, sterols and phenolic tannins. It is has been reported to have anti-inflammatory, anti-nociceptive, spasmolytic, antioxidant, hepatoprotective and diuretic activities.

*Juniperus communis* Linn. (Cupressaceae) is a coniferous shrub. It is widely distributed across the Himalayas from Kumaon at an altitude of 1700–4200 m. Plant has been reported as a traditional cure for chest troubles such as bronchitis and for tuberculosis. The *J. communis* is reported to have anti-inflammatory, anti-pyretic, analgesic and antimicrobial activities. However there is no single report yet demonstrated on interaction studies among *S. xanthocarpum* and *J. communis* for potent hepatoprotective potential against additive/synergistic effect of paracetamol and azithromycin induced liver toxicity. It has been well reported that *S. xanthocarpum* was found to be hepatoprotective by modulating only liver enzymes, which was not sufficient to provide enough protection from free radical injury. Moreover, *J. communis* was demonstrated for anti-oxidant potential against different animal models. Therefore, combining both the individual action for counteracting the liver pathogenesis would be novel challenge in liver diseases. Therefore, the present study was designed to investigate the synergistic effect of the combination of ethanolic fruits extract of *S. xanthocarpum* and *J. communis* against paracetamol and azithromycin induced liver toxicity in rats.

2. Material and methods

2.1. Chemicals and reagents

The plant extracts of *S. xanthocarpum* and *Juniperus communis* were procured from the CSIR-Institute of Himalayan Bioresource Technology (IHBT), Himachal Pradesh, India. Silymarin, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Ethylendiaminetetraacetic acid (EDTA), were purchased from Sigma Aldrich, co., Mumbai. The diagnostic kits for Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and Total bilirubin (TB) were purchased from Calkine and coral private Ltd. All other reagents or chemicals used were of highest commercial analytical grade obtained from CDH and Sigma-Aldrich, USA.

2.2. In-vivo model of paracetamol and azithromycin induced hepatotoxicity in rats

2.2.1. Experimental animals

Wistar albino rats of either sex weighing between 180 and 220 g were selected in the present study vide IAEC approval no ISF/CPCSEA/IAEC/2013/121. The experiments were conducted according to the ethical norms approved by Institutional Animal Ethics Committee (IAEC) guide lines for animal care and were adhered to as recommended by CPCSEA guidelines for the use and care of experimental animals. Animals were housed in environmentally controlled (25 ± 2 °C, 12 h light & dark cycle) small cages, with free access to standard laboratory chow diet and water *ad-libitum*.

2.2.2. Experimental protocol and procedure

The animals were divided into six groups (n = 6). The SX extract (200 and 400 mg/kg), JC extract (200 and 400 mg/kg), and standard drug silymarin (50 mg/kg) were administered orally for 14 days. Liver toxicity was induced by continuous administration of PCM (250 g/kg, p.o.) and AZM (200 mg/kg, p.o.) for 7 days from 7th day to 14th day after 2 h after the test and standard drug administration.

The experimental groups can be summarized as follows:

- **Group 1**: Normal control (Rats received 0.5% CMC for 7 days)
- **Group 2**: Toxicant control (Rats administered with 0.5% CMC + toxicant PCM (250 g/kg) and AZM (200 mg/kg) for 7 days)
- **Group 3**: Silymarin (Rats treated with silymarin suspension (50 mg/kg; p.o.) for 14 days + toxicant PCM (250 g/kg) and AZM (200 mg/kg) for 7 days)
- **Group 4 and 5**: SX (Rats treated with SXE extract (200 and 400 mg/kg; p.o.) for 14 days + toxicant PCM (250 g/kg) and AZM (200 mg/kg) for 7 days)
- **Group 6 and 7**: JC (Rats treated with JCE extract (200 and 400 mg/kg; p.o.) for 14 days + toxicant PCM (250 g/kg) and AZM (200 mg/kg) for 7 days)
- **Group 8**: SX + JC (Rats treated with SXE and JCE extract (200 mg/kg; p.o. each) for 14 days + toxicant PCM (250 g/kg) and AZM (200 mg/kg) for 7 days)

On day 14, animals were anaesthetized by ketamine, blood was collected, allowed to clot, and serum was separated for assessment of enzyme activity. The rats were then sacrificed by bleeding; the livers were carefully dissected then removed and rinsed with ice-cold isotonic saline then kept on ice and the liver was separated and weighed. A 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × g for 15 min and aliquots of the supernatants were separated and used for tissue biochemical estimation. Some part of the liver tissue was immediately transferred into 10% formalin for histopathological investigation.

2.3. Estimation of serum biochemical parameters

Biochemical parameters like serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and Total bilirubin (TB) were estimated using commercial enzymatic biochemical diagnostic kits according to manufacturer’s instructions.

2.4. Estimation of tissue biochemical parameters

2.4.1. Measurement of lipid per oxidation

The extent of lipid per oxidation in the liver was determined quantitatively by performing the method as described by Ohkawa. The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using Schimadzu spectrophotometer (Japan). The values were calculated using the molar extinction co-efficient of chromophore (1,56 × 105 M⁻¹ cm⁻¹) and expressed as percentage of control.

2.4.2. Estimation of nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid). Equal
volumes of the supernatant and the Greiss reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using Schimadzu spectrophotometer (Japan). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve and expressed as percentage of control.

2.4.6. Histopathological studies

Samples were then centrifuged at 12,000 g for 15 min at 4 °C. The supernatant obtained was centrifuged at 2,700 rpm for 1 h at 48 °C. The samples were then centrifuged at 1200 g for 15 min at 4 °C. To 1 ml of the supernatant obtained, 2.7 ml of phosphate buffer (0.1 mmol/l, pH 8) and 0.2 ml of 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB) was added. The yellow color formed was measured at 412 nm using Schimadzu spectrophotometer (Japan). Results were calculated using the molar extinction coefficient of the chromophore ((1.36 × 10⁻⁴ mol/l)⁻¹ cm⁻¹) and expressed as percentage of control.

2.4.3. Estimation of reduced glutathione levels

Reduced glutathione was estimated according to the method described by Ellman.²⁷ 1 ml supernatant was precipitated with 1 ml of 4% sulphosalicylic acid and cold digested for 1 h at 48 °C. The samples were then centrifuged at 1200 g for 15 min at 4 °C. To 1 ml of the supernatant obtained, 2.7 ml of phosphate buffer (0.1 mmol/l, pH 8) and 0.2 ml of 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB) was added. The yellow color formed was measured at 412 nm using Schimadzu spectrophotometer (Japan). Results were calculated using the molar extinction coefficient of the chromophore ((1.36 × 10⁻⁴ mol/l)⁻¹ cm⁻¹) and expressed as percentage of control.

2.4.4. Catalase estimation

Briefly, the assay mixture consisted of 12.5 mM H₂O₂ in phosphate buffer (50 mM of pH 7.0) and 0.05 ml of supernatant from the tissue homogenate (10%) and the change in absorbance was recorded at 240 nm. The results were expressed as mM of H₂O₂ decomposed per milligram of protein/min.²⁸

2.4.5. Protein estimation

The protein content was estimated by the biuret method using bovine serum albumin as a standard.

2.4.6. Histopathological studies

Liver tissues were fixed in 10% formalin for at least 24 h, embedded in paraffin, and cut into 5 μm-thick sections using a rotary microtome. The sections were stained with Hematoxylin–Eosin dye and observed under a microscope (IX51, Olympus, Japan) to observe histopathological changes in the liver.

2.5. Statistical analysis

All experiments were done in triplicate and results were reported as mean ± S.E.M. (n = 6). The data were analyzed by one-way ANOVA, and statistically significant effects were further analyzed by means comparison using Tukey’s multiple comparison analysis. The p < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of ethanolic extract of S. xanthocarpum and Juniperus communis fruits extract on liver enzymes against AZM and PCM induced liver toxicity in rats

Oral combined administration of AZM and PCM significantly increased the levels of liver enzymes (SGPT, SGOT and ALP) as compared to the sham control group treated with ethanolic extract of SX (200 and 400 mg/kg) and JC (200 and 400 mg/kg) treatments significantly attenuated the level of the lipid peroxidation and nitrite concentration of PCM treated rats dose dependently. Further, low dose combination of SX and JC extract significantly potentiated their anti-oxidant effect as compared to their effect per se respectively (Figs. 1 and 2).

3.2. Effect of S. xanthocarpum and Juniperus communis on bilirubin, protein and albumin levels against AZM and PCM induced toxicity in rats

Chronic administration of PCM significantly impaired in the activity of Bilirubin, Protein and Albumin as compared to the sham control treated group. Extract of SX (200 and 400 mg/kg) and JC (200 and 400 mg/kg) treatments significantly restored bilirubin, protein and albumin in PCM treated animals. Additionally, combination treatment of sub therapeutic dose of Extract of SX and JC significantly potentiated the hepatoprotective effect which was significant to their individual effects, suggesting hepatoprotective potential (Table 2).

3.3. Effect of S. xanthocarpum and Juniperus communis on oxidative stress parameters in AZM and PCM treated rats

Chronic treatment with PCM (3 g/kg, p.o.) for 7 days showed significant increase oxidative damage indicating increase of lipid peroxidation and nitrite concentration in the liver of the rat as compared to control group. However, co-administration of extract of SX (200 and 400 mg/kg) and JC extract (200 and 400 mg/kg) alone significantly attenuated the increased level of the lipid peroxidation and nitrite concentration of PCM treated rats dose dependently. Further, low dose combination of SX and JC extract significantly potentiated their anti-oxidant effect as compare to their effect per se respectively (Figs. 3 and 4).

3.4. Effect of ethanolic extract of S. xanthocarpum and Juniperus communis on catalase and reduced glutathione against AZM and PCM induced toxicity in rats

Administration of Paracetamol significantly reduced GSH and catalase enzyme activity in the liver as compared with the sham control treated group respectively (Fig. 3). Extract of SX (200 and 400 mg/kg) and JC (200 and 400 mg/kg) treatments significantly restored endogenous antioxidant enzymes (reduced GSH and catalase) activities as compared with diseased treated animals. Furthermore, low dose combination of SX with JC significantly potentiated their protective effect (antioxidant like effect) as compared to their individual effect produced at higher doses (Figs. 3 and 4).

3.5. Histopathological observations

The different groups of rats were studied for cellular architecture of the liver tissue by histopathological analysis which is presented in Fig. 5(A–H). The photomicrograph of the liver of rat showed normal architecture of hepatic cells with clear cytoplasm and slightly dilated central veins, normal kupffer cells and all cells had normal large nuclei (Fig. 5A). The liver tissue showed distorted architecture with broad area of necrosis in AZM and PCM control group (Fig. 5B). The silymarin group-3 also showed less inflammation and no necrosis in liver cells (Fig. 5C). The animal group 4–8 pretreatment with the SX and JC extracts (100 and 200 mg/kg) and combination of both extract (200 mg/kg; p.o. each), showed the more of normal architecture of the liver tissue with minimum inflammation (Fig. 5D–H). The induction of hepatotoxicity by AZM along with PCM and hepatoprotective effect of SX and JC extract is also supported by histological observations.

4. Discussion

In the present study, we demonstrated that SX and JC play an important role in drugs induced liver damage. In the assessment of
liver injury, firstly in AZM and PCM induced hepatotoxicity levels of ALT and AST in serum should be determined. These enzymes are very sensitive markers employed in the diagnosis of liver diseases.30

Nonsteroidal anti-inflammatory drugs and antibiotics are considered the most common medications associated with drug-induced liver injury mainly through an idiosyncratic form of hepatotoxicity. PCM-induced liver hepatotoxicity as an experimental model of drug-induced acute hepatic necrosis is well established. PCM induced hepatocellular injury and death involves its conversion to a toxic highly reactive metabolite, N-acetyl-p-benzoquinonemine (NAPQI). Generally, PCM is primarily metabolized via cytochrome P-450 to form the highly electrophilic NAPQI which is metabolized to a mercapturic acid which is excreted through the urine.31

The mode of action of paracetamol on the liver is by covalent binding of its toxic metabolite, n-acetyl-p-benzoquinonemine to the sulfhydryl group of protein resulting in cell necrosis and lipid peroxidation.32 Due to liver injury caused byparacetamol overdose, the transport function of the hepatocytes gets disturbed resulting in the leakage of the plasma membrane,33 thus causing an increase in serum enzyme levels.

Recently, Antibiotic-associated hepatotoxicity is another challenge for liver disease. Azithromycin has been linked to two forms of hepatotoxicity. The first is an acute, transient and asymptomatic elevation in serum aminotransferases which occurs in 1–2% of patients treated for short periods and a somewhat higher proportion of patients are given azithromycin for long-term.
The present study showed that combination of AZM and PCM caused significant increase of liver enzymes like SGPT, SGOT, ALP, bilirubin and albumin in serum of rats which is attributed to the liver dysfunction. These results are in consistent with previous reports for drug induced liver toxicity.34 Though the complete mechanism action of PCM induced liver injury is well demonstrated, however, AZM associated toxicity are still unclear. AZM was reported to cause cholestatic as an adverse effect. However, it is also well known that cholestasis with hepatitis is a common feature of macrolide hepatotoxicity.35,36 Macrolides is known to induce its own biotransformation by enhancing microsomal enzymes in the liver with high affinity to AZM. Azithromycin has a long half-life and it has been demonstrated that the protein concentration of AZM is high in liver due to its long half life.37 Our results also revealed that combine administration of AZM and PCM for 7 days significantly increased the oxidative damage indicating the increased level of MDA and nitrite concentration and decreased the activity of endogenous anti-oxidant like reduced glutathione and catalase in liver homogenate of rats. Many common diseases are caused by marked oxidative stress in cellular proteins. Oxidative stress is another possible marker of liver disease. Liver injury is involved to production of ROS which can cause abstraction of a hydrogen atom from an unsaturated lipid and initiating lipid peroxidation. Peroxidation of lipids results in considerable changes in membrane structure and cause DNA damage, cytotoxicity and cell death.38 MDA was a biomarker to measure the level of oxidative stress in organisms.39 In the present study, MDA level has markedly increased in toxicant group in comparison with the normal control group. This condition is closely related to paracetamol-induced lipid peroxidation and damage to plasma membrane because of oxidative stress. Endogenous antioxidants play a key role in protecting liver against different toxicant.40 GSH is an endogenous antioxidant enzyme which protecting against liver injury. It plays a key role in detoxification of the acetaminophen metabolite, NAPQI. Moreover it scavenges reactive oxygen species.41 The reduction in SOD and CAT activity in drug exposed animals may be due to the overproduction of superoxide radical anions.42 Liver histopathology slides show vacuolated cytoplasm with pyknotic nuclei and evidence of hepatocellular damage in AZM and PCM treated rats. However, pretreatment with SX extract (200 and 400 mg/kg) groups significantly decreased MDA level.
Administration of SX extract at concentrations of 200 and 400 mg/kg for 7 days resulted in a significant (p < 0.05) reduction of paracetamol induced elevation of serum enzyme markers, comparable to the effect of silymarin as the positive control. Silymarin is a known hepatoprotective compound. It is reported to have a protective effect on plasma membrane of hepatocytes.43

The liver enzyme marker such as SGOT (serum glutamic oxaloacetic transaminase) and SGPT (serum glutamic pyruvic transaminase) have still remained the standard for the assessment of liver toxicity, and have been used as biomarkers of choice for decades.44 Tests of liver function may therefore prove useful in assessing especially the toxic effects of drugs on the liver. These parameters for the determination of SGOT and SGPT. The liver cell necrosis leads to a significant rise of these enzymes in the blood serum. Hence, SOD and CAT enzyme activity in liver was significantly lower in the toxicant group. However, the SOD and CAT enzyme activity in doses (200 and 400 mg/kg) of the pretreated groups significantly increased when compared with the toxicant group.

In the present study, the SX extract appears to be efficient in reducing the AZM and PCM induced injury as observed from a significant reduction of AZM and PCM induced elevated serum enzyme levels. It was also noted that the histopathological cell injury was improved in rat liver treated with SX extract. This implies that concomitant administration of SX extract prevented hepato necrotic changes. Hepatoprotective effect of SX extract was further confirmed by histopathological studies of the liver, which basically supported the results from the serum parameter. Histopathological studies of the liver showed fatty changes, swelling and necrosis with loss of hepatocytes in paracetamol treated rats. SX treated groups showed regeneration of hepatocytes, normalization of fatty changes and necrosis of the liver. The maximum protection against hepatic damage was achieved with the SX extract at a dose of 400 mg/kg. The histopathological investigation of the liver of rats treated with SX extract showed a more or less normal architecture of the liver having reverted to a large extent, the hepatic lesions produced by paracetamol, almost comparable to the normal control groups.

5. Conclusion

In conclusion, the present study has demonstrated that the SX extract has shown the potent hepatoprotective activity as compared to JC extract against paracetamol-induced hepatotoxicity in rats and on the basis phychochemical data the plant has reported to have the phenolic compound and flavonoid. The hepatoprotective potential may be due to their anti-oxidant potential.

Conflict of interest

Authors declare that there is no conflict of interest throughout the study period or in future.

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