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Hepatoprotective activity of *Trianthema portulacastrum* L. against lipopolysaccharide/D-galactosamine induced hepatotoxicity in mice

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D-Galactosamine (D-GalN) is a well-established hepatotoxic agent, whereas lipopolysaccharide (LPS) is a bacterial endotoxin; and both in a combination induces the liver damage, which is similar to a human hepatic diseases. In this study, we have evaluated the hepatoprotective activity of the Giant or Black Pigweed, *Trianthema portulacastrum* L. against D-galactosamine in the presence of lipopolysaccharide (LPS/D-GalN) induced hepatotoxicity using Swiss albino mice. LPS/D-GalN treatment elevated the liver marker enzymes. The combined action of LPS/D-GalN decreased the production of antioxidants such as reduced glutathione (GSH), catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST) and increase in the malondialdehyde (MDA) and nitric oxide (NO) formation. Pre-treatment with *T. portulacastrum* (TP) extract showed dose dependently attenuated liver marker enzymes, increased GSH content, upregulated enzymatic activities of antioxidants in liver, decreased MDA and NO formation. The results also revealed that LPS/D-GalN-treatment increased the inflammation as observed through neutrophil infiltrations and increased the formation of sinusoids as evidenced from the histopathological studies of liver tissue and the TP extract pre-treatment mitigated the neutrophil infiltration and sinusoids formation dose dependently.

Keywords: Antioxidants, Black Pigweed, Giant Pigweed, Hepatoprotective, Inflammation, Jejunum, Liver

The liver is a vital organ that is frequently exposed to toxic substances, agents from pollution and drugs, which are responsible for hepatotoxicity[1]. D-Galactosamine (D-GalN) is a well-established hepatotoxic agent. It is responsible for the necrosis of hepatic cells[2]. On the other hand, lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria that act as endotoxin, and a well-known inflammatory agent[3]. It has been reported that D-GalN treated mice is shown to increase the sensitivity of LPS in inducing hepatic damage[4]. The liver damage caused due to combination of D-GalN with LPS is similar to a human hepatic diseases[5]. LPS/D-GalN induced liver damage is a widely accepted model to study the mechanism of fulminant hepatitis (FH), also known as acute liver damage, and to identify the potential therapeutic drugs as it exhibit similar symptoms of hepatotoxicity in human being[6,7]. D-GalN is a liver specific toxin and LPS increase the sensitivity and lethality to liver damage[8]. The possible mechanism of LPS/D-GalN-induced liver damage is due to the inflammatory responses and oxidative stress[9,10]. LPS/D-GalN activates Kupffer cells (KCs) that release the proinflammatory cytokines causing increase in the hepatic necrosis and decreases in the antioxidant enzyme activities[9,10].

Thus, LPS/D-GalN triggers the formation of reactive oxygen species (ROS) by promoting inflammasome, which induces the oxidative stress in liver[7,11,12]. Therefore, antioxidant therapy could be an effective therapeutic pathway for LPS/D-GalN induced liver damage[7]. Several natural compounds from plants, vegetables and fruits showed hepatoprotective activity through anti-inflammatory and antioxidant properties[13-15].

Trianthema porulacastrum L., commonly called the Giant or Black Pigweed, is widely used since time immemorial to treat several diseases such as liver ailments, anaemia, heart disease, asthma, inflammation, rheumatism, corneal ulcers, beri-beri, and migraine, etc.[16]. In our previous study, free radical scavenging, antioxidant and anti-inflammatory properties of *T. portulacastrum* were evaluated[17]. The hepatoprotective activity of *T. portulacastrum* was also demonstrated against toxicity of chemicals like CCl_4, paracetamol and thioacetamide[16]. The extract of *T. portulacastrum* have also been found to exhibit hepatoprotective activity against the radiation induced liver damage *in vitro*[17] as well *in vivo* model[18]. In continuation of this, here, we have evaluated the hepatoprotective effect of *Trianthema portulacastrum* extract against LPS/D-GalN-induced hepatotoxicity in Swiss albino mice.

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Materials and Methods

Chemicals

Lipopolysaccharides (LPS) and D-galactosamine (D-GalN) were purchased from M/s Sigma Aldrich, and 5,5′-dithiobisnitro benzoic acid (DTNB), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced (NADPH), 5-methylphenaziniummethosulfate (PMS), reduced glutathione (GSH), oxidized glutathione (GSSG), 2,4-dinitrophenyl-hydrazine (DNPH), N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDD), 1-chloro-2,4-dinitrobenzene (CDNB), nitrobluetetrazolium (NBT) were procured from M/s SRL Chemical Co.; Folin–Ciocalteu's phenol reagent, sodium pyro-phosphate and gallic acid from M/s. Merck India Ltd.; and transaminase kits from M/s. Coral clinical systems.

Trianthema portulacastrum extract preparation

Trianthema portulacastrum (TP) stem extract was prepared as described elsewhere[17]. In brief, dry powder of stem (100 g) was extracted with petroleum ether (500 mL) for 24 h with constant stirring and then filtered. The same procedure was repeated. Ethyl acetate, acetone and ethanol solvents (100 mL in each case) were used sequentially followed by petroleum ether. Solvents were evaporated and dried using lyophilizer. For the treatment, the extract was dissolved in 1X PBS at different concentrations for oral administration with the dose of 50, 100 and 200 mg/kg body wt.

Animal model system

Male Swiss mice, 8-10 weeks old and weighing 20-25 g, were purchased and maintained under standard conditions of temperature (25±2°C) and humidity. Animals were provided with food and water *ad libitum*. Five animals were housed in each sterile polypropylene cage containing sterile paddy husk as bedding. The study was performed with the approval (dated 27/03/2019) of the Institutional Animal Ethics Committee of the University of Kalyani regarding the experiments, maintenance and dissections of small animals.

Treatment protocol

Mice were divided into following eight groups of five mice in each group as follows: Gr. I: PBS treated as control group; Gr. II: treated with *T. portulacastrum* extract @200 mg/kg body wt.;Gr. III: treated with lipopolysaccharide @10 µg/kg body wt.; Gr. IV: treated with D-galactosamine @400 mg/kg body wt.; Gr. V: treated with LPS @10 µg/kg body wt. and D-GalN @400 mg/kg body wt.; and Gr. VI-VIII: treated with TP extract@50, 100 and 200 mg/kg body wt., respectively for five days followed by LPS/D-GalN. Treated groups (II, VI, VII and VIII) received *T. portulacastrum* (TP) extract at the above mentioned concentrations orally for five days consecutively and the groups VI, VII and VIII were administered with LPS/D-GalN intraperitoneallyon the 6th day. Groups III and IV received LPS or D-GalN accordingly on the 6th day. After 6 h of LPS/D-GalN treatment[19], blood samples were collected from retro-orbital plexus to evaluate the activities of liver function enzymes, such as aspartate amino transferase (AST) and alanine amino transferase (ALT). Subsequently, the mice were sacrificed, and a part of liver was homogenised in 1X PBS to study the antioxidant parameters of the organ; while the remaining part of the liver was stored in formalin solution for histopathology.

Homogenized liver tissue in 1X PBS solution was used to estimate the antioxidant non-enzymes such as reduced glutathione (GSH) content[20], lipid peroxidation[21] and nitric oxide (NO) content[22]; and activities of antioxidant enzymes such as glutathione peroxidise (GPx)[23], glutathione reductase (GR)[24], glutathione s-transferase (GST)[25] catalase (CAT)[26] and superoxide dismutase (SOD)[27].

Liver tissue specimens that were fixed in 10% formalin PBS solution, processed routinely and embedded in paraffin. 4 µm thick sections were stained with hematoxylin and eosin to evaluate morphological changes under light microscope.

Statistical analysis

All experiments with *T. portulacastrum* extract were performed five times, and all results were expressed as mean ± Standard Error. Significant differences were assessed using the one-way analysis of variance (ANOVA), followed by the Tukey test for individual differences using the Statistical Package for Social Science (SPSS, Chicago, Illinois) software, version 23. A value of P <0.05 was considered statistical significant.

Results and Discussion

Our results demonstrated enhanced activities of ALT and AST after 6 h of LPS or D-GalN administration independently (Table 1), indicating their heptotoxic property. However, treatment with their combination (LPS/D-GalN) further enhanced the

Table 1 — Effect of *Ttianthema portulacasrum* on aspartate amino transferase (AST) and alanine aminotransferase (ALT) in mice serum after 6 h of treatment with lipopolysaccharide (LPS), D-galactosamine (D-GalN) and LPS/D-GalN

Groups	AST (IU/L)	ALT (IU/L)
I: Control (PBS treatted)	25.6±2.79	20.4±1.81
II: *Trianthema portulacasrum* (200 mg/ kg body wt.)	24.4±2.29	19.6±2.25
III: Lipopolysaccharide (10 µg/ kg body wt.)	74.6±2.32*	66.6±3.33*
IV: D-GalN (400 mg/ kg body wt.)	64.4±3.14*	56.2±3.61*
V: LPS/ D-GalN (LPS: 10 µg/ /kg body wt. + D-GalN 400 mg/kg body wt.)	90.8±2.35*	88±4.57*
VI: *T. portulacasrum* (50 mg/ kg body wt.) and LPS/D-GalN	79.2±3.12	77.2±2.82
VII: *T. portulacasrum* (100 mg/ kg body wt.) and LPS/D-GalN	66±4.48#	60.4±3.27#
VIII: *T. portulacasrum* (200 mg/ kg body wt.) and LPS/D-GalN	51.2±3.73#	44.2±3.34#
F Value	60.59	59.26

[Values are Mean ± standard error (SE) of 5 experiments. *$P \leq 0.05$ compared to control, and # $P \leq 0.05$ compared to LPS/D-Gal N treatment]

Table 2 — Effect of *Trianthema portulacasrum* on content of reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) and nitric oxide (NO); and activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione s-transferase (GST), catalase and superoxide dismutase (SOD) in mice liver after 6 h of treatment with lipopolysaccharide (LPS), D-galactosamine (D-GalN) and LPS/D-GalN

Groups	GSH[a]	TBARS[b]	NO[c]	GPx[d]	GR[e]	GST[f]	Catalase[g]	SOD[h]
I	5.34±0.3	1.02±0.11	9.8±1.02	31.8±1.88	20.2±1.77	16.8±1.24	32.4±1.5	30.4±1.5
II	5.46±0.23	0.97±0.12	9.2±0.86	31.4±1.94	20.6±1.86	17.4±1.54	32.8±1.36	29.8±1.86
III	4.86±0.46	1.16±0.14	28.4±1.81*	21.2±1.16*	11.4±1.03*	10.4±0.93*	27±1.27	21.8±1.28*
IV	4.4±0.38	1.38±0.15	22.6±1.54*	22.6±1.03*	12.6±1.08*	11.6±1.44	24.8±1.69*	19.4±1.33*
V	3.26±0.27*	2.22±0.14*	32.6±1.78*	15±1.41*	9.2±1.02*	7.6 ±1.03*	18.4±1.6*	13.8±1.2*
VI	3.76±0.33	1.94±0.09	27.4±1.99	18.2±1.28	11±1	9.6±1.03	22.2±1.69	18±1.38
VII	4.32±0.23	1.56±0.1#	21.2±2.42#	22.8±1.86#	13.4±1.08	11.4±1.33	25.6±1.86	22.4±1.63#
VIII	4.96±0.3#	1.28±0.14#	16.6±1.81#	27±1.23#	17±1.14#	14.4±1.03#	29.8±2.15#	26±1.41#
F Value	5.55	13.04	25.01	15.63	11.21	8.25	8.99	15.62

[Gr. I: Control (PBS treated); Gr. II: *Trianthema portulacasrum* (200 mg/ kg body wt.); Gr. III: Lipopolysaccharide (10 µg/ kg body wt.); Gr. IV: D-GalN (400 mg/ kg body wt.); Gr. V: LPS/ D-GalN (LPS: 10 µg/ /kg body wt. + D-GalN 400 mg/kg body wt.); Gr. VI: VI: *T. portulacasrum* (50 mg/ kg body wt.) and LPS/D-GalN; Gr. VII: *T. portulacasrum* (100 mg/ kg body wt.) and LPS/D-GalN; and Gr. VIII: *T. portulacasrum* (200 mg/ kg body wt.) and LPS/D-GalN. Values are Mean ± standard error (SE) of 5 experiments. Units: [a]µM/mg protein, [b]µM/mg protein, [c]µM/mg protein, [d]µM NADPH breakdown/min/mg protein, [e]nM NADPH breakdown/min/mg protein, , [f]mM CDNB conjugate formed/min/mg protein, [g]mM H_2O_2 decomposed/min/mg protein, [h]One Unit of SOD enzyme is defined as the enzyme concentration required to inhibit the OD at 560 nm of chromogen produced by 50%/min under assay condition. *$P \leq 0.05$ compared to control, and # $P \leq 0.05$ compared to LPS/D-Gal N treatment]

activities of ALT and AST in mice (Table 1), suggesting that combination of LPS and D-GalN is more heptotoxic than that of the independent dose. However, treatment with *T. portulacastrum* extract showed significant attenuation of activities of liver enzymes dose dependently (Table 1).

Our experiments further demonstrated that independent treatment of LPS or D-GalN caused significant increase in lipid peroxidation and nitric oxide level, while decreased GSH level and reduced activities of antioxidant enzymes studied (Table 2). All these changes were further aggravated when combined(LPS/D-GalN) (Table 2). Dose dependent attenuation in these parameters were observed with *T. portulacastrum* extract and the maximum restoration of GSH level was noticed at highest concentration i.e. 200 mg/kg bw (Table 2).

Histopathology evidence revealed that LPS induced liver stress as observed by the infiltration of monocytes (Fig. 1B), whereas D-GalN increased the sinusoid hyperaemia and damaged the architecture of liver (Fig. 1C). The combination of LPS/D-GalN altered the morphological structure of liver further (Fig. 1D), which is responsible for the increased number of infiltrations compared to only LPS treated group and increased number of hepatic sinusoid hyperaemia compared to only D-GalN treated group. *T. portulacastrum* extract treatment was found to reduce the number of neutrophil infiltration as well as the sinusoid formation and able to maintain the liver architecture. *T. portulacastrum* extract protected the liver dose-dependently with the number of infiltrations was less with very few sinusoids at higher concentration of *TP* extract (Fig. 2).

Natural compounds can scavenge ROS and exert a pivotal role against inflammation due to their antioxidants properties[28,29]. Liver damage is a lethal clinical condition, where rapid decline of liver

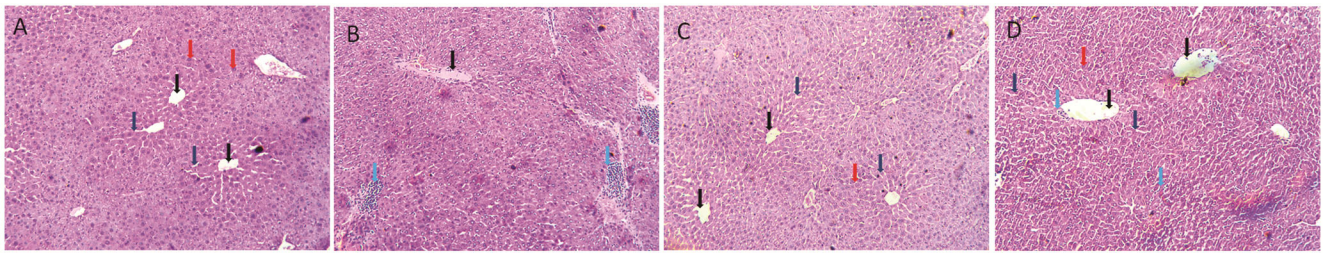

Fig. 1 — Effect of LPS, D-GalN and LPS/D-GalN on liver was observed through the histopathology of liver in mice. (A) Control; (B) LPS treated; (C) D-GalN treated; and (D) LPS/D-GalN treated. Black arrow shows the vein, red arrow shows the hepatocytes, blue arrow shows the sinusoid and green arrow shows Infiltration.

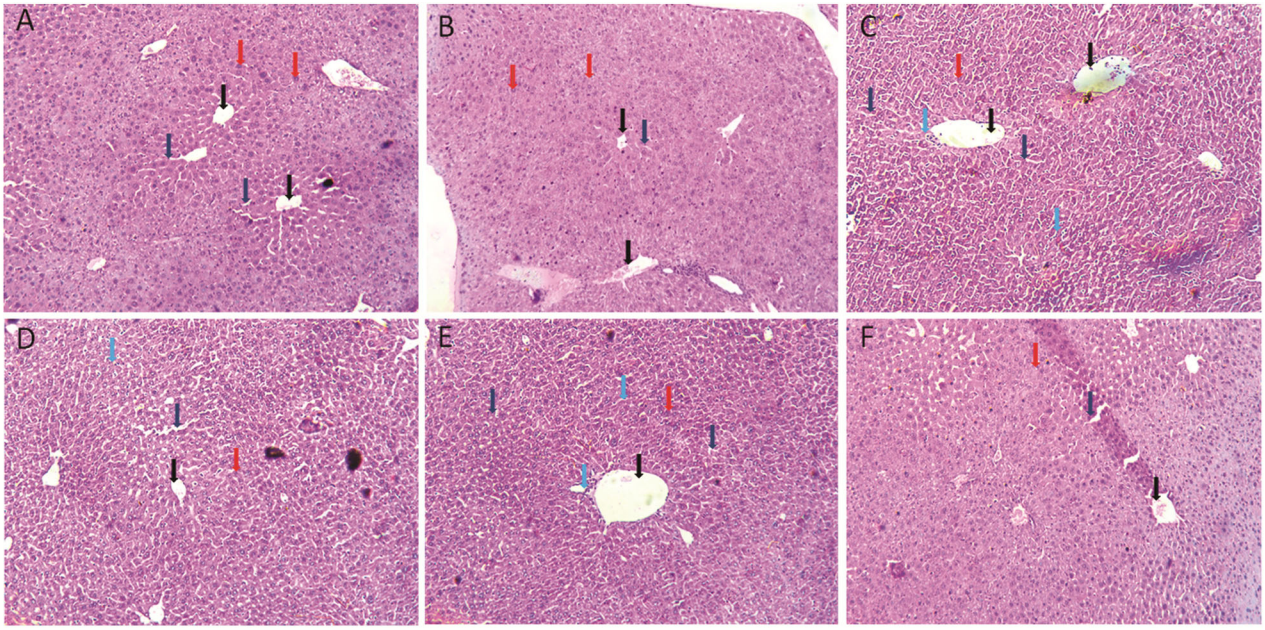

Fig. 2 — Effect of *T. portulacastrum* on liver was observed through the histopathology analysis from LPS/D-GalN injected mice, pre-treated with different concentration of the extract and compared with control mice. (A) Control; (B) mice treated with stem 200 mg/kg body wt.; (C) LPS/D-GalN; (D-F) LPS/D-GalN + stem 50, 100 and 200 mg/kg body wt., respectively.Black arrow shows the vein, red arrow shows the hepatocytes, blue arrow shows the sinusoids and green arrow shows Infiltration.

function is common. This is responsible for hepatic encephalopathy and inflammatory responses that may lead to multi organ failure in patients[30]. Liver function test (LFT) is an early method for detection of liver damage where aminotransferase (ALT and AST) activities are monitored along with other parameters. The cytosolic enzyme ALT is found in high concentration in liver tissue specifically; while, AST is found in the cytosol and mitochondria of liver, as well in muscle, lungs, kidneys, pancreas, red cells and leucocytes. Therefore, AST is non-specific marker for liver function[31]. This study demonstrated that the sensitivity of liver is increased in the presence of LPS or D-GalN, while their combination (LPS/D-GalN) can worsen the condition.

LPS/D-GalN triggers ROS that leads to oxidative stress, cytotoxicity and pathogenesis in liver damage that lead to acute liver failure (ALF)[7,32]. ROS neutralizes the antioxidant capacity to further enhance the oxidative stress[33,34]. In normal condition, hepatocytes overcome oxidative damage by antioxidant enzymes of liver[35]. The enzymatic antioxidants such as SOD, catalase, GR and GPx and non-enzymatic antioxidant GSH are responsible for counteracting the oxidative stress[6,36]. Therefore, the antioxidant therapy could be an important therapeutic approach for LPS/D-GalN induced liver damage as well as for the ALF[7].

Reduced glutathione (GSH), the most abundant antioxidant of cells, plays pivotal role in detoxification of liver[37]. GSH protects the cells from excessive quantity of exogenous and endogenous electrophiles by scavenging hydroxyl and superoxide radicals directly[37]. LPS or D-GalN independently or

in combination lowered the level of GSH in liver tissues of mice in our study, which is in agreement with other study[38].

Stress induced membrane damage is mediated through the free radicals by acquiring electrons from membranes, which is further responsible for oxidation of protein and enzymes[7,39]. It has been reported that the Significant increase in lipid peroxidation due to LPS or D-GalN administration in our study is in agreement with other reports[38,40].

Nitric oxide (NO) is an important inflammatory mediator that plays a key role in death or survival of liver cells, exhibits cytotoxicity and paradoxical functions[41,42]. Production of NO is correlated with cytotoxicity. Increased production of NO in the presence of LPS or D-GalN in mice liver after 6 h of treatment in our study, might be due to the upregulation of iNOS[42].

Glutathione peroxidise (GPx) and glutathione reductase (GR) are critical enzymes for GSH metabolism. Conversion of H_2O_2 to O_2 by GPx oxidizes GSH and form GSSG. The decline in GSH content is replenished by GR action[36]. Glutathione s-transferase (GST) in conjugation with GSH also protects cells from electrophilic compounds and xenobiotics by eliminating them from body[43]. Several studies have reported decreased GSH content and GPx, GR and GST activities in mice liver due to LPS and/or D-GalN exposure similar to our study[44,45].

A combined action of superoxide dismutase (SOD) and catalase protects cells from free radicals more efficiently. SOD protects cells from superoxide radicals by converting the superoxide anion ($O^{\bullet}$) to H_2O_2 and O_2[46,47]. H_2O_2 is further decomposed by catalase and GPx. Decreased activity of SOD and catalase in LPS and D-GalN treated mice observed in our study corroborates with other findings[48].

Liver histology reveals several clinical phenotypes and strengthens the medical conditions through laboratory outcomes[49]. In this study, histopathological findings have revealed that the infiltration of neutrophils in mice treated with LPS/D-GalN. The anti-inflammatory properties of the *T. portulacastrum* extract observed in our previous study[17] further got strengthened by the histopathological findings of the liver here, where the *T. portulacastrum* extract dose-dependently reduced the neutrophil infiltrations and maintained the structure of liver, demonstrating the hepatoprotective activity of *T. portulacastrum.* Moreover, our results have demonstrated that *T. Portulacastrum* extract not only reduced the elevated activities of liver specific marker enzymes, but also attenuated the altered activities of antioxidant enzymes or levels of antioxidant non-enzymes due to exposure of LPS/D-GalN in mice liver dose-dependently. The protective activity of *T. portulacastrum* stem extract was mainly mediated through free radical scavenging properties and anti-inflammatory activities.

Polysaccharide isolated from *Hippophae rhamnoides* L. (sea buckthorn) protected liver from LPS/D-GalN induced hepatic damage, where the mice were treated for 14 consecutive days with the extract doses of 50, 100 and 200 mg/kg body wt.[11]. Our study has further demonstrated that five days pre-treatment with the same doses of *T. portulacastrum* extract was sufficient to provide protection against LPS/D-GalN induced hepatic damage in mice. This implies that pre-treatment with *T. portulacastrum* extract may be beneficial to reduce the toxic effect of different drugs like paracetamol, responsible for hepatic damage after a certain threshold level[50]. In another report, a formulation of 13 different herbs of Korea named CGX is in use since 2001 against LPS/D-GalN induced hepatic damage[51]. In this context, our findings are significant as *T. portulacastrum* extracts also provide protection against liver damage.

Conclusion

The above study has demonstrated that the common Giant Pigweed or Black Pigweed *Trianthema portulacastrum* provides protection against the lipopolysaccharide LPS/D-GalN induced hepatotoxicity also by reducing the oxidative stress and inflammation. Thus, it suggests that *T. portulacastrum* may be considered as a potential source for therapeutic drug against bacterial/drug induced liver damage.

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Conflict of interest

Authors declare no competing interests.

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