Research Article

In-vitro antioxidant and hepatoprotective effect of ethanolic fraction of the leaves of *Grewia asiatica* Linn. in rats

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Abstract

Objective: The aim of this works was to estimate the total phenolic and flavonoid content and to evaluate *in vitro* antioxidant activity and *in vivo* heptaoprotective effect against Isoniazid induced liver damage in rats, of the flavonoid rich ethanolic fraction of *Grewia asiatica* Linn. leaves. **Material and methods:** Extraction of the leaves was done by ethanolic maceration for 48 h and the extract volume was reduced. Total phenolic and flavonoid content was determined in terms of gallic acid and quercetin equivalent respectively. The hepatoprotective effect was evaluated by examining the cross section of the liver and analysis of biochemical parameters involved in liver damage. **Results:** Total phenolic content in the ethanolic extract of the leaves was found to be 2.15 mg/100 g of the dried extract calculated as gallic acid equivalent (r^2=0.998)and the total flavonoids content was found to be 1.65 mg/100 g of the dried extract calculated as quercetin equivalent (r^2 = 0.999). The extract was screened by DPPH free radical scavenging activity for its antioxidant potential and was found to be possessing antioxidant potential. The extract was able to significantly reduce the levels of biochemical markers of liver damage viz, ALP, SGOT and SGPT. The histological examination examined clear prevention from injury to the tissue caused by Isoniazid. **Conclusion:** The antioxidant potential of leaves of the plant may pave way for the formulation of newer plant based antioxidant and anti-ageing neutraceutical product treating many complications of human diseases. The hepatoprotective effect found in the extract may be helpful to prepare newer liver strengthening formulations.

Keywords: Antioxidant, DPPH, free radical, *Grewia asiatica*, flavonoids, Isoniazid

Introduction

Ever since the existence, mankind has made the use of plants in the treatment of various ailments. The Indian traditional medicine like Ayurveda, Siddha and Unani are predominantly based on the use of plant materials. Herbal drugs have gained importance and popularity in the passing by decade due to their safety, efficacy and comparative low cost. Herbal-based therapeutics for liver disorders has been use in India for a long time and has been popularized world over by leading pharmaceuticals (Agrawal, 2001). Even after gaining significant popularity by several herbal medicines they are still unacceptable treatment modalities for liver diseases.

Grewia asiatica, popularly known as phaalsa has been known for its antioxidant potential (Sharma and Patni, 2013) and various medicinal properties including immunomodulatory (Singh and Yadav, 2014), antimicrobial (Shukla et al., 2016), anticancer (Kakoti et al., 2011), radioprotective (Zia-Ul-Haq et al., 2013), antihyperglycemic (Joshi et al., 2013), analgesic and anti-inflammatory (Pavaiya et al., 2013).

Oxidative stress is known to be associated with liver diseases and is considered to play a role in the pathogenesis of chronic hepatitis C, alcoholic liver disease and Wilson's disease. Antioxidant therapy has thus been considered to have the possibility of beneficial effects in the management of these liver diseases. Owing to its antioxidant potential, it was envisioned that *Grewia asiatica* could be beneficial in management of hepatic damage induced due to different pathological conditions.

Materials and methods

Procurement and authentication of plant

The leaves of *G. asiatica* were collected from interiors of Bhopal (M.P.) in the month of Feb. 2017. The plant has been authenticated by Safia College of Science, Peer Gate,

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Bhopal (Madhya Pradesh). The authenticated leaves were dried under shade and then coarsely powdered with the help of mechanical grinder. The powdered leaves were passed through sieve number 40 and stored in airtight container for extraction.

Flavanoids rich extraction of *G. asiatica*

Extraction of the powdered leaves of the plant was performed using ethanol by maceration method for 48 h (AleMeshal et al., 1985). The ethanolic extract was then filtered and the filtrate obtained was evaporated to $1/4^{th}$ of the total volume of the solution and then further evaporated to get crystalline powder of the flavanoids rich extract.

Total phenolics content

The total phenols present in the macerated extract were measured at 765 nm by Folin Ciocalteu reagent (McDonald et al., 2001). 1 ml of ethanolic extract or standard was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (1M) sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30 min at 40°C for colour development. The total phenols were determined by spectrophotometer at 765 nm using a standard curve prepared using 10, 20, 30, 40 and 50 µg/mL solutions of gallic acid in methanol.

Total flavonoid content

An aliquot of 1 mL of 2% $AlCl_3$ solution was added to 1 mL of the extract in methanol or to a standard solution of quercetin (Zengin et al., 2011). The mixture was allowed to stand for 60 min at room temperature and the absorbance was determined at 420 nm against solvent blank. The results are expressed as mg quercetin/g dry weight, calculated using the calibration curve of quercetin (5, 10, 15, 20 & 25 25 µg/mL).

DPPH free radical scavenging assay

Various concentration of the plant extract was prepared in methanol and each of this was separately mixed with 0.5 mL DPPH solution (15 mg in 10 mL methanol). The test solution was incubated for 30 min at room temperature in dark condition. The absorbance of the test solutions was recorded at 517 nm using methanol as blank (Zhang et al., 2009). The percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition of DPPH} = \frac{\text{Control Absorbance} - \text{Test absorbance}}{\text{Control Absorbance}} X100$$

Animals

Adult male wistar rats weighing from 200 to 225 g were used in the present study. The animals were housed in controlled conditions of temperature (22 ± 2 °C) and humidity (57 ± 2 %) under a 12: 12 h dark and light photocycle. The animals were fed with standard pelletized diet and drinking water ad libitum. All the experimental protocols were carried out according the guidelines of the Institutional Animal Ethical Committee. The animals were monitored for the signs of acute toxicity according to the OECD guidelines.

Experimental Setup

After acclimatization for a period of one week, the animals were randomly segregated into five groups each consisting of six rats. Group I served as the vehicle control and was administered only with normal saline (10 mL/Kg, po). For inducing hepatotoxicity, Groups II, III, IV and V were administered with Isoniazid (100 mg/Kg, i.p). After intoxification with Isoniazid, Group II served as the Isoniazid control. Groups III was administered with Liv-52 (200 mg/Kg po) and the Groups IV and V were orally administered the extract of *G. asiatica* dissolved in distilled water at doses of 100 mg/Kg and 200 mg/Kg respectively, daily throughout the period of study (15 days).

Biochemical evaluation

After the end of the treatment period, blood was collected from all animals by retro orbital puncture using sterile capillaries. The serum was separated out by centrifuging at 3000 rpm for 15 min and was stored at -20 °C. The animals, after collecting blood samples were sacrificed and the liver of each animal was removed and a section of around 2 mm thickness of liver was cut and fixed in 10% neutral formalin solution for histopathology study. The remaining portion of liver was homogenized and assessed for liver damage by estimating the serum activities of SGPT, SGOT, ALP and total bilirubin using commercially available test kits.

Histopathological examination

The portions of liver fixed in formalin solution were then embedded in paraffin wax, sectioned in thin slices of 5 µm thickness with the help of semi automatic rotary microtome and stained with hematoxylin and eosin (H&E). The liver sections were examined under microscope for architectural alterations (such as congestion, hemorrhage, necrosis, inflammation, infiltration, kuffer cells and sinusoids) induced in the tissue due to Isoniazid toxicity and also to evaluate the improved architecture of tissue due to pretreatment with the extract and the standard drug (Valeer, 2003).

Statistical Analysis

Statistical analysis was performed using the SPSS for Windows statistical package version 10.0. All the data were expressed as mean ± SD. The effect of drug treatments were evaluated statistically using one way ANOVA followed by Borforonie test for comparison of results. Statistical significance was set at the $p < 0.05$ level.

Results

Molecules with unpaired electron, involved in bacterial and parasitic infections, inflammation, ageing and neoplastic conditions are known as free radicals. It was very evident from the results that the ethanolic extract of the leaves of *G. asiatica* possessed the ability to inhibit free radicals generated by DPPH.

Total phenolics content

The phenolic compounds are known to be powerful chain breaking antioxidants and have the ability to inhibit the free radicals involved in chain formation. The ethanolic extract of *G. asiatica* leaves was found to contain 2.15 mg/100 g of the dried extract in terms of gallic acid equivalent. The standard curve of gallic acid is shown in figure 1.

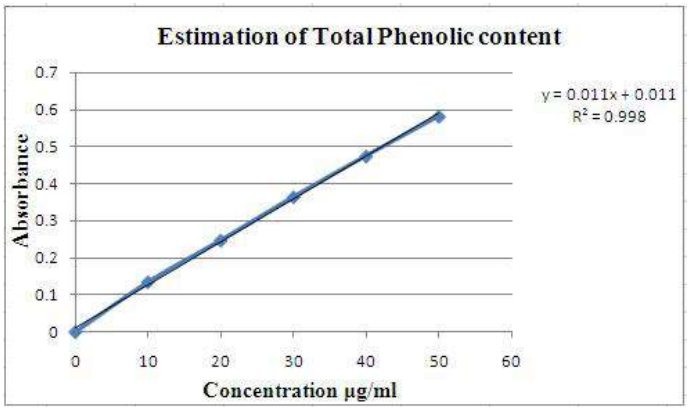

Figure 1. Graph of estimation of total Phenolic content

Total flavonoids content

Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: **$Y=0.040X+0.009$, $R^2=0.999$**, where X is the absorbance and Y is the quercetin equivalent (QE). Flavonoids have the ability of scavenge the reactive oxygen species due to the presence of phenolic hydroxyl group in their structures. The total flavonoid content in G. asiactica leaves was found to be 1.65 mg /100 g dried extract in terms of quercetin equivalent. The standard curve of quercetin is shown in figure 2.

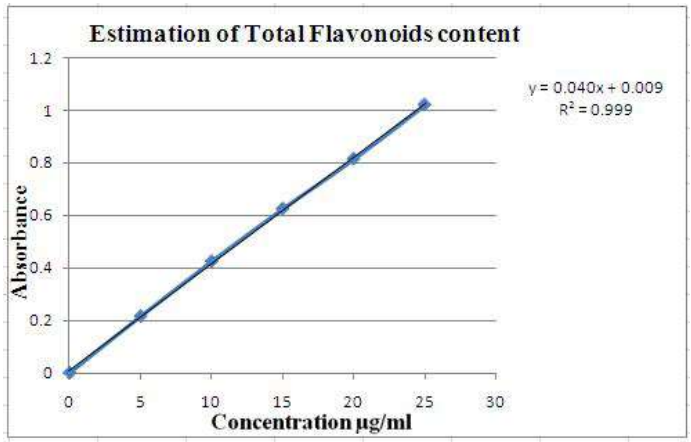

Figure 2. Graph of estimation of total flavonoids content

DPPH free radical scavenging activity

DPPH scavenging activity has been used by widely as a rapid, easy and reliable parameter for screening the *in vitro* antioxidant activity of plant extracts. DPPH is a stable free radical and accepts an electron to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was at 517 nm. It is observed that with the increase of concentration, there is decrease of absorbance value (figure 3). The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidants molecules and radical, progresses, which results in the scavenging of the radical by electron donation. IC_{50} for standard ascorbic acid was found to be 63.62 µg/ml and that for ethanolic extract of *G. asiatica* Linn. was 157.69 µg/ml (table 1). From the results it is known that the species, *G. asiatica* Linn. possess hydrogen donating capabilities and does scavenging of the free radicals.

Table 1. DPPH free radical scavenging activity

S. No	Conc. (µg/mL)	% Inhibiton
1	20	50.1775
2	40	52.8994
3	60	58.1065
4	80	63.0769
5	100	69.9408
IC_{50}(µg/ml)		157.6929

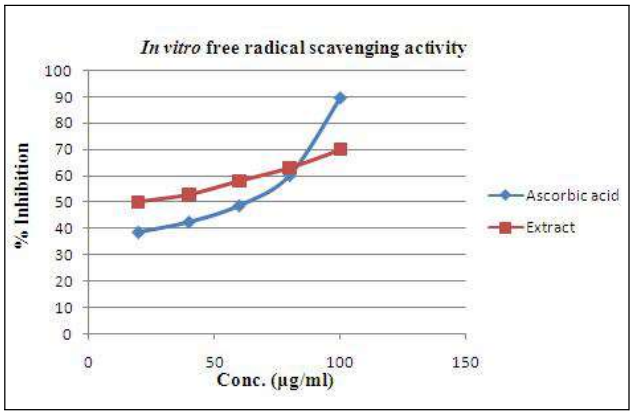

Figure 3. Graph of *in vitro* free radical scavenging activity

Biochemical Analysis

Isoniazid induces significant elevation in the levels of SGOT, SGPT, ALP and bilirubin content as compard to the normal control group. However, pretreatment with Liv-52 and *G. asiatica* extracts (100 mg and 200 mg) produces dose dependent significant decrease in these serum biochemical

Table 2. Effects of *G. asiatica* leaf ethanolic extract on serum and liver biochemical indices in Isoniazid induced hepatotoxicity in rats

Parameters	Control	Isoniazid	Isoniazid + Liv-52	Isoniazid + Grewia extract (mg/Kg body weight)	
				100	200
SGOT	44 ± 19.700**	94.40 ± 8.930	58.180 ± 5.300**	77.080 ± 10.550(ns)	68.500 ± 8.900*
SGPT	56.700 ± 6.140**	113.70 ± 13.190	70.550 ± 5.630**	92.330 ± 7.210*	81.410 ± 11.210**
ALP	110.60 ± 8.510**	199.15 ± 21.700	141.410 ± 6.680**	162.367 ± 11.410**	151.700 ± 6.790**
Total Bilirubin	44.00 ± 19.700**	94.400 ± 8.930	58.180 ± 5.300**	77.080 ± 10.550ns	68.500 ± 8.900*

*P<0.50; **P<0.001; ns – non significant

indices of liver damage as compared to the Isoniazid treatment group. The results of biochemical analysis are represented in table 2.

Histopathological studies

The central vein, hepatocyte and the portal space were found to be normal in control group. As Isoniazid induces hepatic damage, the derangement of the hepatocyte cord and peripheral necrosis were visible in the Isoniazid treated animals. The histological characteristics of the treated groups of animals were found to be quite similar to that of the control group. In these groups, minimal tissue degeneration was observed around the periphery of the central vein (figure 4 A-E).

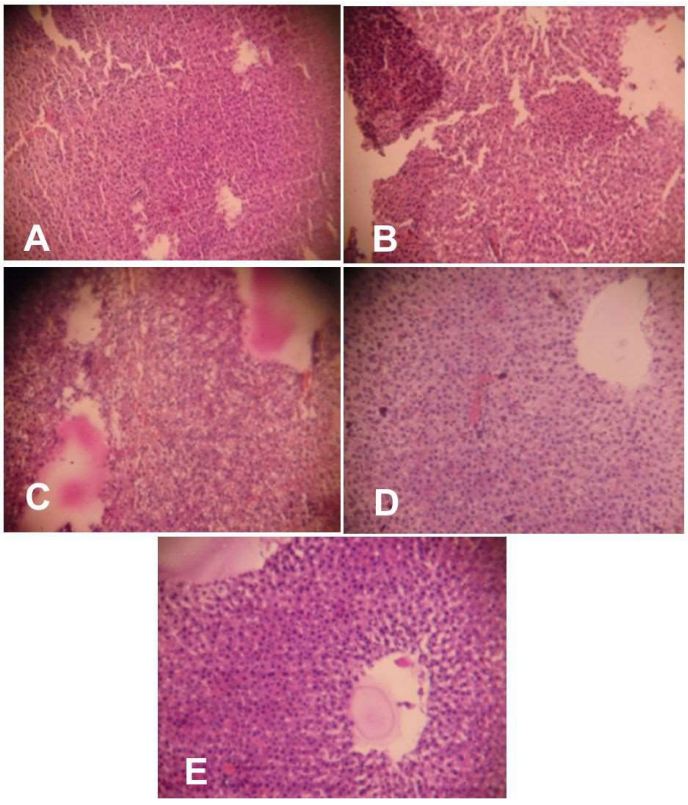

Figure 4. Light microscopic examination of rat liver sections: (A) Vehicle control; (B) Isoniazid treated; (C) Standard treated; (D) Extract treated (100 mg/kg); (E) Extract treated (200 mg/kg)

Discussion

The present study exhibits the hepatoprotective, curative and antioxidant capabilities of ethanolic extract of the leaves of *Grewia asiatica* against Isoniazid induced liver damage in rats. As the liver is the organ responsible for detoxification of drugs and chemicals, it is the first target for all toxic chemicals. A number of studies have revealed the role of Isoniazid in inducing damage to the liver tissue leading to lipid peroxidation and thereby liver fibrosis. The histological fearutes of severe Isoniazid induced liver damage includes hepatocellular injury along with multilobular necrosis and a mononuclear cell infiltrate (Maddrey and Boitnott, 1973).

The results obtained from the study reflected that the extract of leaves of *G. asiatica* were effectively able to protect the loss of antioxidants caused due to Isoniazid administration and preventive the formation of reactive acetyl hydrazine and hydrazine metabolites responsible for liver damage (Stepan et al., 2011). Increase in levels of ALP, SGOT and SGPT in serum of the Isoniazid-treated animals indicate liver damage as these enzymes leak out from liver into the blood due to tissue damage (Naik and Panda, 2008; Ree and Spector, 1961). Following the treatment of ethanolic extract, the levels of these marker enzymes were near normal or only slightly elevated, indicating protection against liver damage.

The phytochemical analysis of *G. asiatica* extract has revealed the presence of flavonoids in the extract. A number of studies have been reported which suggest that flavnoids function as antioxidants and may protect against oxidative stress caused due to environmental conditions (Tattini et al., 2004; Gould and Lister, 2006). The antioxidant potential of flavones has been attributed to the higher reactivity of the hydroxyl substituents leading to its radical scavenging capacity (Heim et al., 2002).

Conclusion

The ever persistent search of humanity for treatment of ailments since the ancient times has brought many new

natural products in the pharmaceutical, cosmetic and nutraceutical market. The in-vitro antioxidant study provides scientific evidence to the traditional claims of *Grewia asiatica* Linn leaves as capable of decreasing ageing and stress. The presence of phenolics and flavonoids in adequate amounts was consistent with the fact. The findings in the current study suggest this plant to be good source of natural antioxidants. The hepatoprotective potential would be helpful in formulating newer plant based products for fighting with liver related ailments. The isolation and characterization of the antioxidant compounds and *in vivo* studies to ascertain the efficacy in humans are warranted in the future.

Conflict of Interests

None declared

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