natans L was identified and authenticated by Dr. Anjula Pandey, Principal Scientist, National Bureau of Plants Genetic Resource (NBPGR), New Delhi, and given the voucher no. NHCP/NBPGR/2013-7.

Extraction process

The plant materials were shade dried at normal room temperature (25°C) for 15 days till constant weight was attained. After that, the plant materials (500 g) were crushed using an electric grinder, and the powdered material was kept in an airtight container before the extraction. The powdered material was extracted using methanol in the Soxhlet apparatus at 70°C for 72 h. Finally, the solvent of the extract was evaporated by a rotary evaporator. The obtained extract (65.12 g) fractionation with petether, chloroform and ethyl acetate. The ethyl acetate fraction (25.5 g).was collected for the father isolation process[11].

A concentrated ethyl acetate fraction of *T. natans* was mixed with silica gel (200-400 mesh) and evaporated by rotary evaporator until the mixture of silica gel and extract became completely dried and in free-flowing mass condition. The ethyl acetate fraction-coated silica gel was poured into the column. *T. natans* extract coated silica settled completely on the upper surface of the silica gel of the column. The compounds slowly came out from the column along with the eluting solvent, n-hexane/EtOH (9:1, 8;2, 7:3, 6:4, 1:9 v/v). The rate of elution was 35 drops per min. At the beginning of elution, about 500 mL of the solvent (n-hexane) was allowed to pass through the column[12,13]. Then the solvent eluting from the column was collected in the 10 mL conical flask at 15 min intervals. The TLC was carried out for each collection. The yellow crystalline compound was isolated from the fraction. The compound was characterized by UV, IR, NMR and Mass spectroscopy[14,15].

Acute toxicity study

The acute toxicity study was approved by the Animal Ethical Committee (Protocol No. IAEC/ NIET/2019/01/08) and performed following OECD guideline 423.

For the acute toxicity study, 15 Wistar albino female rats were taken and the animals were divided into four groups, each containing three animals. Group I, the control group, received distilled water; the animals of Gr. II-IV received 5, 50 and 300 mg/kg isolated compounds, respectively. After administration of the drug, the animals were kept under observation at 30 min intervals for the first 4 h and then twice a day for the next 14 days, after the administration of the drug. Every day, the animals were observed twice daily to find out the mortality of the rats, if any. The changes in the behaviour of the experimental animals and their other body parameters, like body wt., urination, respiration, intake of food and water, body temperature and the skin colour were observed[16,17].

Experiment design for hepatoprotective activity

For hepatoprotective activity, the animals were divided into four groups each containing 6 animals. The animals were subjected to the following treatments for 9 days. Before administration of paracetamol (3 g/kg p.o) to the experimental rat. Group I serve as normal control received water (2 mL/kg), Gr. II, serves as negative control, received water 2 mL/kg; Gr. III treated with silymarin (30 mg/kg); and Gr. IV had 30 mg/kg isolated flavonoids obtained from *Trapa natans* L extract. On the 10th day, paracetamol 3 g/kg,p.o was administered to all the groups except Gr. I. Food was withdrawn 12 h prior to paracetamol administration. After 24 h of paracetamol administration, the rats were sacrificed by cervical dislocation[18,19].

Determination of AST, ALT, ALP, and serum bilirubin

The collected blood from the experimental rats was kept in a clean, dry tube and centrifuged for 10 min at 2000 rpm. After that, the supernatant serum sample was transferred into the Eppendorf tube. Biochemicals analysis was performed by a semi-automated biochemical analyzer (Microlab MC300) for determination of liver enzymes such as alkaline aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum bilirubin using the common biochemical analyzer kit (Roche Diagnostics GmbH, Mannheim, Germany)[20,21].

Determination of liver enzyme level

The isolated livers were immediately washed with the help of saline (ice-cold) to remove the blood as much as possible. The required quantity of liver homogenates (5% w/v) was prepared using ice-cold potassium phosphate buffer (pH7.4) with the help of a homogenizer. The cell fragments and other insoluble substances were removed by centrifugation method (1000 rpm)[22]. The supernatant was collected and used for the testing of superoxide dismutase (SOD) adopting the method described by Vincent *et al*.[23], catalase (CAT) was determined by following the method of Gupta *et al*.[24] Malondialdehyde was determined according to Khan *et al*.[25] and hydro peroxide was tested according to Mondal *et al*.[26].

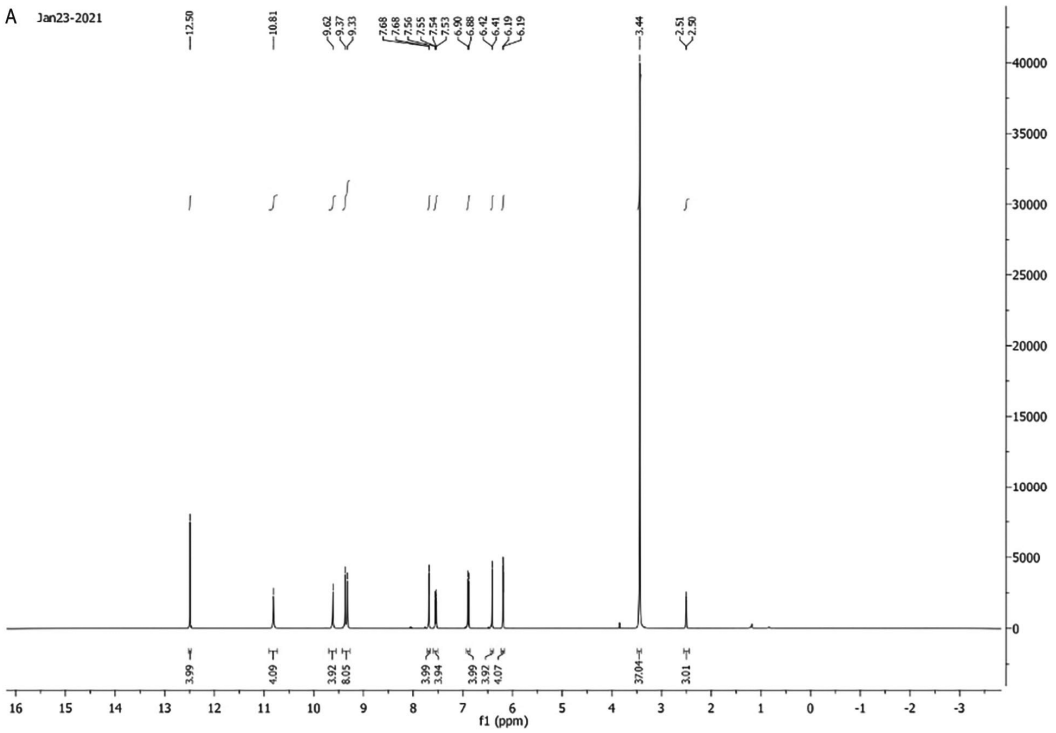

Fig. 2 — (A) H NMR; and (B) 13 C NMR spectra of the isolated compound.

(silymarin 30 mg/kg) and isolated flavonoids (30 mg/kg body wt.), and on the other hand level of total protein was increased as given in Table 1. The standard drug and isolated compound gave approximately the same result.

Effect of lipid peroxidation and oxidation enzyme and total protein

In the case of the control group, the SOD level was decreased to45.42±1.91 mg/dL, which was very low as compared to the normal control group

Table 1 — Effect of the isolated compound on liverenzyme and biochemicals

Study Group	ALT(U/L)	AST(U/L)	ALP (U/L)	Total Bilirubin (mg/dL)	Total Protein (mg/dL)
Gr. I Normal control	42.80±2.92	110.52±2.19	95.15±1.84	0.34±0.018	6.92±0.12
Gr. II Paracetamol	218.57±3.40	300.4±6.12	146.7±1.87	0.64±0.027	5.51±0.15
Gr. III Paracetamol+silymarin (30 mg/kg)	74.20±1.42***	153.2±3.15***	87 ±1.91***	0.35±0.005***	7.15±0.32***
Gr. IV Paracetamol+isolated compound (30 mg/kg)	63.80±1.39***	160±1.53***	98.20±1.59***	0.39±0.01***	7.38±0.16***

[Value expressed by mean ± standard error mean; significance value *** P <0.001 as compared to model control (only paracetamol)]

Table 2 — Antioxidant levels of various treatment groups

Treatment Group	SOD (mg/dL)	GSH (mg/dL)	CAT U/mg Protein	MDA nmol/mL	Hydroperoxides (mmol/100 g)
Gr. I Normal Control	70.83±1.13	60.33±1.49	80.50±1.23	201.5±2.1	59.2±2.5
Gr. II Paracetamol	45.42±1.91	36.83±1.20	25.45±1.76	360±4.5	90.5±5.5
Gr. III Paracetamol+Silymarin (30 mg/kg)	65.50±1.58***	52.67±2.71***	59.83±1.79***	220.5±3.2***	71.3±2.3***
Gr. IV Paracetamol+Isolated compound (30/mg/kg)	63.80±1.39***	62±2.53***	58.20±1.59***	240.3±9.52***	73.8±2.16***

[Value expressed by mean ± standard error mean; significance value *** P <0.001 as compared to model control (only paracetamol)]

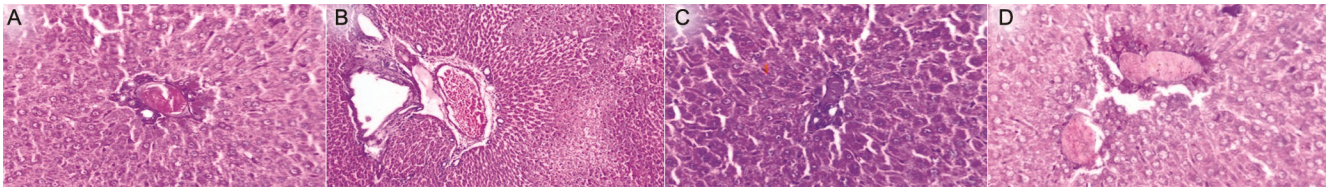

Fig. 3 — (A) Liver section of Normal control; (B) Model control (only paracetamol); (C) Standard (treated with silymarin 30 mg/kg); and (D) Test group (Treated with the isolated compound @30 mg/kg).

(70.083±1.13 91 mg/dL). The standard drug and isolated compound significantly (P <0.001) increased the SOD level by 65.50±1.58, and 63.80±1.39, respectively. The same result was also observed in the case of GSH levels. The standard and test drugs were able to enhance the level of GSH 52.67±2.7 mg/dL and and 62±2.53mg/dL, respectively which were very high as compared to the model control group (36.83±1.20). The level of CAT was very low in the model control group (25.45±1.76 U/mg) as compared to the normal control group (80.50±1.23). With the administration of the test drug (isolated compound) the level of CAT increased (58.20±1.59). The isolated compound showed a similar result as that of the standard drug (59.83±1.79). The serum MDA level in the model control group increased remarkably (360±4.5 nmol/mL) whereas in the case of normal control groups the level of MDA was (201.5±2.1 nmol/mL). After administration of the isolated flavonoids, the MDA level ware found 240.3±9.52. The results were given in Table 2.

Histopathology of liver

The case of the normal control group (only water 1 mL/kg) revealed normal hepatoccellular structure through the majority of the tissue section, with prominent nucleolus visible in the central vein. In the case of the model control group, massive fatty change, in some area cell necrosis, increased sinusoidal spaces which were either filled with blood or edematous fluid, and loss of cellular boundaries were observed in rats' liver histogram. In the case of the standard group (silymarin 30 mg/kg) the histogram of the liver revealed normal cellular and architectural structure throughout the majority of the tissue section in some areas mild fatty change was observed. A few hepatocyte binucleations were also observed. The group treated with isolated flavonoids (30 mg/kg) liver histopathology showed normal cellular and architectural structure throughout the majority of the tissue section. However, in a few multifocal areas, mild fatty changes in hepatocytes along with increased sinusoidal space and congestion were observed (Fig. 3 A-D).

Discussion

Paracetamol is a very common drug and relatively nontoxic when administered between therapeutic doses, but in a high single dose or repeated doses cause hepatotoxicity. In the liver, the small portion of paracetamol (5-10%) is converted to N-acetyl-para-benzoquinone–imine (NAPBI) a relative metabolite by the isoform of P450 (CYP2E1, CYP2A6)[30]. Generally, it is produced in small quantities and

immediately detoxified by the liver enzyme. But when NAPBI is present in a high amount it does not detoxify properly in the liver and causes severe liver damage[31].Rising the level of aminotransferase compounds (ALT and AST) is the primary indication of hepatotoxicity due to the overdose of paracetamol. The level of AST and ALT is elevated when the tissue and the cells are damaged or injured. The degree of AST increments in the blood after the six-hour of cell damage[32]. In this experiment, administration of paracetamol increases the serum ALT, AST, ALP and bilirubin levels.

For this experiment, silymarin (30 mg) was chosen as a standard hepatoprotective drug as it is known to protect the plasma membrane of liver cells. Silymarin has the ability to reduce free radicals. Additionally, it prevents toxins from entering the liver cells. It also stimulates protein synthesis The isolated flavonoids identified as morin reduces the elevated level of the enzyme. The effectiveness of the isolated compound 30 mg/kg is comparable to the silymarin. The hepatoprotective activity of isolated flavonoids is associated with antioxidant potentiality[33,34].

The elevated level of MDA indicates the enhancement of lipid peroxidation which leads to tissue damage and reduces the antioxidant defense mechanism which prevents the formation of excess numbers of free radicals[35,36]. The morin acts as a natural protector against lipid peroxidation and reduces the elevated level of MDA. SOD and CAT are important scavengers of hydrogen peroxide and superoxide ions. These enzymes protect the cellular constituents from oxidative damage by preventing the generation of hydroxyl radicals. An isolated compound is able to increase the level of SOD and CAT and protect the liver from hydroxyl radicals[37,38].

Paracetamol is metabolized in the liver where the toxic byproduct of the paracetamol will be produced and the toxic product removed from the liver by forming the conjugation with glutathione[39]. Reduced Nrf2 activity, lower GSH levels, and greater oxidative stress were linked to hepatocyte cell death in mice. Reduced Nrf2 activity, lower GSH levels, and greater oxidative stress were linked to hepatocyte cell death in mice. This finding led to the observation that liver necrosis occurs when the level of GSH levels is considerably low[40]. In the case of paracetamol induce model control groups the level of GSH markedly reduces. The test drug can able to elevate the level of GSH.

Conclusion

High doses of paracetamol cause liver damage and change the biochemical parameter in comparison to the control group. According to the experimental findings, the isolated flavonoids from *Trapa natans* plant extract treated group resists the change in the typical biochemical parameter after the administration of paracetamol. The isolated flavonoids also protect and prevent damage to liver cells. The isolated flavonoid proved effective that was comparable to the standard compound silymarin. The inhibition of reactive oxygen species by a boost in antioxidant enzymes like SOD, CAT, and GSH may be the foundation for the possible mechanism of isolated flavonoids. Based on experimental findings, it may be concluded that isolated flavonoids from plant extract have potent hepatoprotective potentiality and in the future will be used as effective drugs to treat liver problems in human beings.

Conflict of interest

Authors declare no competing interests.

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