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Original Article

Hepatoprotective Activity of Morin and its Semi-Synthetic Derivatives Against Alcohol Induced Hepatotoxicity in Rats

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Abstract

Background: Morin is natural flavonoids obtained from *Moraceae* family, found to be associated with many therapeutic properties like anti-inflammatory, anti-cancer and cardio-protective activities.

Aim: The aim of present study is to synthesise flavone (JPG-I), a derivative of Morin (JPG-II) by simple acetylation and to investigate their comparative hepatoprotective activity with Morin.

Methods: Standard (Silymarin 100 mg/kg), morin (40 mg/kg), JPG-I (40 mg/kg), JPG-II (40 mg/kg), were administered per orally for 21 days in 40% alcohol (2 ml/100 g/day, p.o.) treated groups. Body weight and urine analysis were assessed on 7th, 14th, and 21st day. On 21st day animals were sacrificed and blood was collected for assessment of liver profile (SGOT, SGPT, ALP, TB) and liver was isolated to carry out the evaluation of biochemical (LPO, GSH, nitrate).

Results: The present study demonstrated that there was decrease in body weight, increase in liver function enzymes (SGOT, SGPT, ALP, TB) and oxidative stress parameter level in alcohol treated group on 7th, 14th, and 21st day.Whereas reverse was observed with test compounds (morin, JPG-I and JPG-II).

Conclusion: Compound JPG-II treated group was found to be more effective as compared to morin and JPG-I treated group.

Introduction

Liver is one of the largest organs in human body that plays a key role in, maintaining and regulating homeostasis by metabolizing all foreign compounds (1). Hepatotoxicity is defined as liver dysfunction or liver damage that is related with an overload of drugs or xenobiotic (2). Despite of aggressive research in this field there are very less hepatoprotective preparation currently licensed for human use Hence, the development of effective hepatoprotective therapies is an urgent medical need because of the high prevalence of liver disorder.

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Long-term excessive alcohol consumption can lead to ALD (3) and causes oxidative stress in the liver due to the imbalance between the prooxidant and the antioxidant systems (4). Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring in all plant parts but mostly they are major coloring component of flowering plants. Morin Hydrate (3, 5, 7, 2', 4' pentahydroxyavone), (Fig. 1), was a yellow crystalline polyphenolic compound isolated from *Maclurapomifera* (Osage orange *Macluratinctoria* (old fustic) (5) leaves of *Psidiumguajava* (common guava), almond (*Prunusdulcis*, family *Rosaceae*), sweet chest nut (*Castaneasativa*, family *Fagaceae*) and other fruits also (6). Due to its potent antioxidant and metal ion chelating capacities, Morin is reported to perform various therapeutic effects such as anti-inflammatory, anti-cancer and cardio-protective activities (7). Our main objective of present study is to investigate the hepatoprotective activity of Morin, flavone (JPG-I) and derivative of Morin (JPG-II). The newly synthesized compounds JPG-I and JPG-II were characterized by means of Fourier transform infrared (FTIR) spectroscopies and ^{1}H NMR.

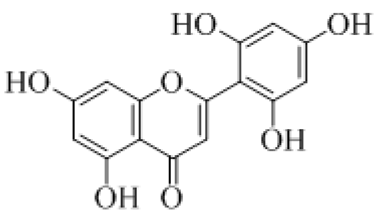


Fig. 1 : Chemical structure of Morin hydrate.

Materials and Methods

Chemical

Morin was purchased from Himedia, Mumbai, India and JPG-I & JPG-II has been synthesized in our department (Department of Pharmaceutical chemistry, ISF College of Pharmacy, Ferozepur road, GhalKalan, Moga-142001, Punjab, India). SGOT, SGPT, ALP, Total Bilirubin kits (Coral Clinical System Pvt. Ltd, Goa, India) were used for the estimation of liver enzymes.

Synthesis of the JPG-I and II

Synthesis of (JPG-I)

Step (i) o-Benzoyloxyacetophenone

The *o*-hydroxyacetophenone (6 ml, 6.79 g, 0.05 mole), benzoyl chloride (10.6 g, 8.75 ml, 0.075 mole) and dry pyridine (20 ml) were placed in a 50 ml conical flask fitted with calcium chloride tube. The exothermic reaction was takes place and temperature of reaction mixture rises, further when no heat was evolved (10-15 min) than mixture was transferred on the boiling water bath for 15 min, poured this mixture stirringly into diluted hydrochloride acid (3%, 250 ml) containing crushed ice (100 g). After filtration the separated product was washed with methanol (20 ml) and water. The white crystals was obtained when crystallized with methanol (Yield 10 g (83%); m.p. 87-8°C).

Step (ii) o-Hydroxydibezoylmethone

The pulverized potassium hydroxide (3.5 g) was added to a warm solution (50°C) of *o*-benzoyloxyacetophenone (10 g, 0.042 mole) which was prepared in pyridine (35 ml) and the mixture was stirred for 15 min. The viscous mass so obtained is cooled to room temperature and acidified with acetic acid (50 ml, 10%). The separated diketone was filtered and crystallised with ethanol to produce light yellow crystals (Yield 9 g (90%). M.p. 119-20°C).

Step (iii) Flavone (JPG-I):

The glacial acetic acid (40ml) was added to diketone end product (8.8 g, 0.03 mole) which was obtained from the above step with constant shaking and than add 1.8 ml of concentrated sulphuric acid in the same. The mixture is heated on a steam bath for 15 min and then poured over crushed ice (200 g) with continous stirring. The separated product was filtered and washed with water (until free from acid). It was crystallised from petroleum ether as white needles shaped crystals (Yield 7.9 g (97%). M.p. 96-7°C). Purity of the product was checked up by TLC using ethyl acetate: hexane (0.5:0.5) as mobile phase.

Mol. Formula: $C_{15}H_{10}O_2$; MW: 222.24;Colour: white; IR; ^{1}HNMR

Synthesis of JPG-II

Morin (500 mg) was dissolved in acetic anhydride (6 ml) and catalytic amount (One drop) of H_2SO_4 was added and than warm the mixture for 5 min at 40°C and left it for 24 h. Purity of product was checked up by TLC using ethyl acetate:hexane (0.5:0.5) as mobile phase (Yield 500 mg (99%) M.p. 110-111°C.)

Mol. formula: $C_{25}H_{20}O_{12}$; mw: 512.42; Colour: light brown

IR (KBr) cm^{-1}: 1645 (C=O); 1495 (C=C); 3058 (C-H); 1078 (C=O).

Animals

Wistar rats weighing 200-250 g of either sex were used. Animals were obtained from Central Animal House facility of I.S.F. College of Pharmacy, Moga, Punjab, India. They were housed at ambient temperature (21±10°C) and relative humidity (55±5%) with fixed 12 h light/dark cycle. All the behavioral assessments were carried between 9:00 and 17:00 h. The experimental protocol was approved as ISFCP/IAEC/CPCSEA/2015/245 by Institutional Animal Ethical Committee (IAEC) as per the guidance of committee for the purpose of control and Supervision of Experiments on Animals (CPCSEA).

Experimental design

Acute Toxicity Study

Healthy mice maintained under standard laboratory conditions were used for acute oral toxicity test. Animals were observed individually at least once during first 30 min after dosing, periodically during first 24 h and daily thereafter for period of 3 days. Doses of JPG-I, and JPG-II were selected from acute toxicity studies performed according to OECD (Organization for Economic Co-operation and Development) guidelines; Section 423. On the basis of the toxicity study, 40 mg/kg doses were taken for *in-vivo* studies.

Animals were observed individually at least once during first 30 min after dosing, periodically during first 24 h (with special attention during the first 4 h) and daily thereafter for period of 3 days (OECD, 1996). The acute toxicity studies were done routinely with reference to change in skin texture and fur loss, eyes, mucus membrane (nasal), respiratory rate, circulatory signs (heart rate), autonomic effect (salivation, lacrimation, perspiration, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsion) changes.

Experimental grouping

The experimental rats were randomly divided into six groups, each group consisting of six rats (n=6) given in Table I.

Treatment Schedule

Hepatotoxicity was induced by administering 40% alcohol at dose of 2 ml/100 g/day, p.o. for 21 days (8). Standard (Silymarin 100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg), JPG-II (40 mg/kg), were suspended in 0.5% CMC (Carboxy methylcellulose) solution and administered per orally for 21 days in alcoholtreated groups, one and half hour prior to alcohol dosing. On 7th, 14th, and 21st day body weight, and urine analysis were assessed. On 21st day, blood was collected for assessment of liver function enzymes (SGOT, SGPT, ALP, TB**)**, animals were sacrificed and liver was isolated to carry out the evaluation of biochemical (LPO, GSH, nitrate). ELISA kit was used to estimate the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6).

Measurement of liver and body weight

Animal body weight was measured on 1st, 7th, 14th & 21st day of experimentation. Percent change in body weight was calculated. Besides, liver weight and morphological changes were examined on 21st day of the experimentation.

Measurement of bilirubin (BIL), urobilinogen (URO), protein albumin (PRO), ketone (KET) by urine analysis

Urine analysis was done by Orinasys^{TM} kit (Span

Diagnostics. Ltd, Surat) on 1st, 7th, 14th & 21st day of experimentation. This kit contains reagent strips which provide a reliable method for diagnosis of pathological changes in the composition of urine.

Biochemical assays

Assessment of liver function enzymes

All animals were sacrificed on 21st day under light isoflurane anesthesia. The blood samples were collected by puncturing retro-orbital plexes and allowed to coagulate for 30 min at 37°C, centrifuge it for 10 min at 2500 rpm. The clear serum was taken for assessment of liver function enzymes such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TB). All estimation was carried out using commercial biochemical enzymatic diagnostic kits (Coral Clinical System Pvt. Ltd, Goa, India).

Assessment of oxidative stress parameters

Liver tissue homogenate preparation

Animals were sacrificed immediately after behavioral observations on 21st day by cervical dislocation under light anesthesias and the liver were removed and rinsed with ice-cold isotonic saline. Liver were separated out and weighed. Liver tissue were then homogenized with ice-cold 0.1 mol/l phosphate buffer (pH 7.4) 10% w/v. The homogenate was centrifuged at 10000×g for 15 min at –4ºC and aliquots of supernatant were separated and used for biochemical estimation.

Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation in liver was performed according to the method of Will's (9). The amount of malondialdehyde (MDA), a measure of lipid peroxidation was assayed in the form of thiobarbituric acid reacting substances (TBARS). TBARS were quantified using an extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} and expressed as nmol of MDA per mg protein.

Glutathione Estimation

Reduced glutathione was estimated according to the method described by Ellman (10). Reduced Glutathione levels were measured at 412 nm using a Perkin Elmer Lambda 20 spectrophotometer were calculated using molar extinction co-efficient of the chromophore (1.36 × 104 $(mol/L)^{-1}$ cm^{-1})

Nitrite estimation

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent [0.1% N- (1-naphthyl) ethylenediamedihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid] as described by Green (11).

Protein estimation

Protein estimation was done by Lowry method (12).

Assessment of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) levels

The quantifications of TNF-α, IL-1β, and IL-6 were done by rat TNF-α, IL-1β, and IL-6 immunoassay kit (KRISHEGEN BioSystems, Ashelyct Whittier, CA, USA). The TNF-α, IL-1β, and IL-6 immunoassay is a 4.5 h solid phase ELISA designed to measure TNF-α, IL-1β, and IL-6 levels. It is solid phase sandwich enzyme linked immunosorbent assay (ELISA) using a microtitre plate reader. This was followed by successive seven steps that is (1) Sample (100 µl) was added to the pre-coated plate. Plate was sealed and incubated at room temperature for 2 hours. (2) Plate was washed 4 times with wash buffer. (3) Diluted Detection antibody solution (100 µl) was added to each well; plate was sealed and incubated at room temperature for 2 hours. (4) Plate was washed 4 times with wash buffer. (5) Diluted Straptavidin-HRP solution (100 µl) was added to each well and plate was sealed and incubated at room temperature for 30 minutes. (6) Plate was washed 4 times with wash buffer. (7) Diluted TMB substrate solution (100 µl) was added to each well and plate was incubated in the dark for 15 minutes. Bluish color appeared in positive wells. (9) Reaction was stopped by adding 100 µl of stop solution to each well. Positive well turned yellow from blue. (10) Absorbance was noted at 450 nm within 15 minutes of stopping reaction.

Statistical analysis

All the results obtained were expressed as mean±SD. The data obtained for behavioral parameters were analyzed by using two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. All biochemical parameters were analyzed by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. P<0.05 was considered statistically significant.

Results

Synthesis of JPG-I

Synthesis of the JPG-1 (Flavone) was achieved by reacting *o*-hydroxyacetophenone (**1**) with benzoyl chloride (**2**) using pyridine as medium. Pyridine also played a role of base to scavenge the librated HCl and push the reaction in the forward direction. The benzylatedacetophenone (**3**) obtained was high in yield and purity and it was used for the rest step after recrystalization. The next step of the reaction involves the treatment of *o*-benzoylacetophenone (**3**) with KOH in pyridine to give the o-hydroxydibenzoylmethone (**4**) which was subsequently cyclized in acidic condition to give the desire flavone [**5** (JPG-1)] Scheme-1 (Fig. 2).

Synthesis of JPG-II

Synthesis of JPG-II (**8**) was achieved by reacting Morin (**6**) with acetic anhydride (**7**) in acidic medium (H_2SO_4) followed by Scheme 2 (Fig. 3). We have synthesized JPG-I and JPG-II and their physical characterization is given in Table I. The FTIR spectra of JPG-I, JPG-II and 1H NMR of JPG-I, JPG-II is shown in Figs. 4, 5, 6, 7, respectively.

In-vivo Results

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on body weight in 40% alcohol induced hepatotoxicity in rats

There was a significant decrease in body weight in 40% alcohol (2 ml/100 g) treated group as compared

TABLE I: Experimental grouping.

S.No.	Groups
1)	Control (0.5% CMC, p.o.)
2)	Disease control (Alcohol 40% 2 ml/100 g, p.o.)
3)	Standard (Silymarin 100 mg/kg, p.o.) + alcohol (40% 2 ml/100 g, p.o.)
4)	Morin (40 mg/kg, p.o.) + alcohol (40% 2 ml/100 g, p.o.)
5)	JPG-I (40 mg/kg, p.o.) + alcohol (40% 2 ml/100 g, p.o.)
6)	JPG-II (40 mg/kg, p.o.) + alcohol (40% 2 ml/100 g, p.o.)

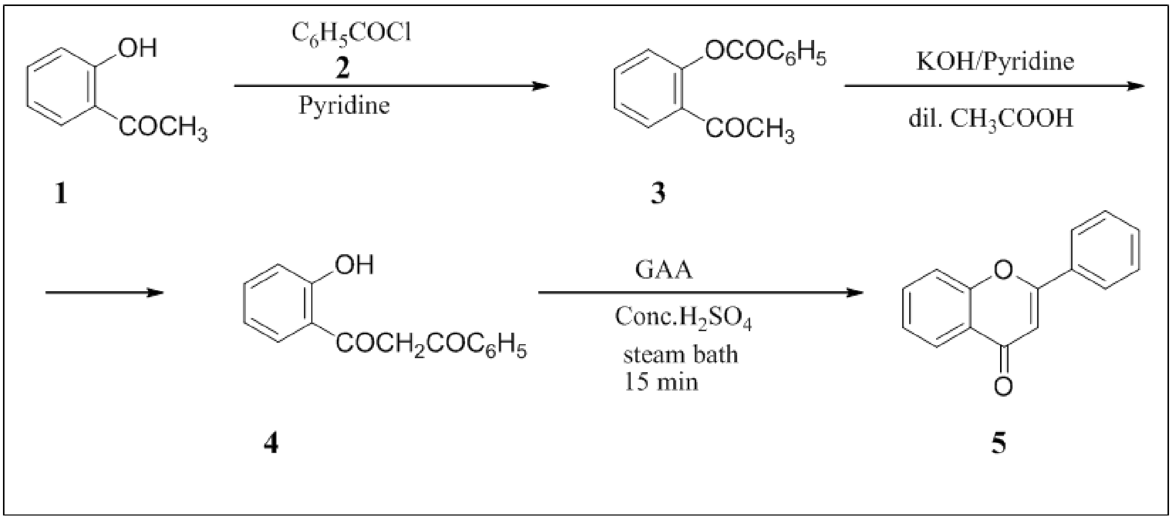


Fig. 2: Schematic prestentaion of JPG-1(Synthesis of the Flavone).

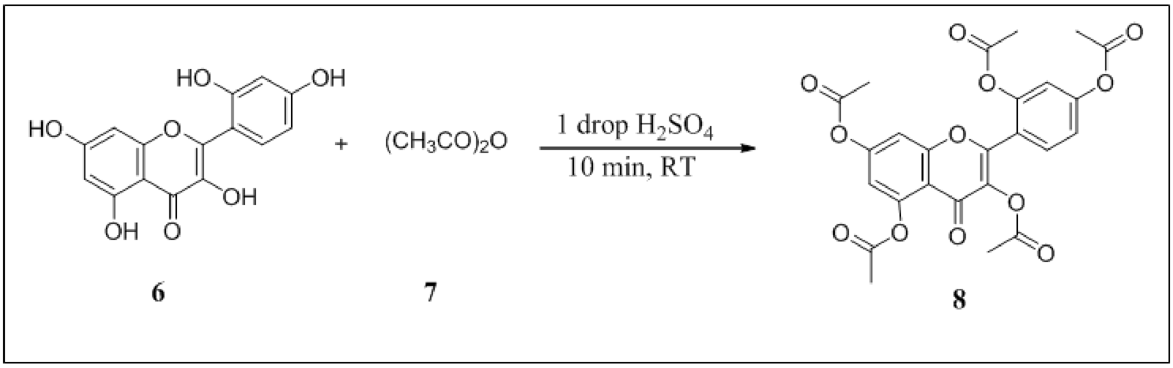


Fig. 3 : Schematic prestentaion of JPG-1 (Synthesis of JPG-II).

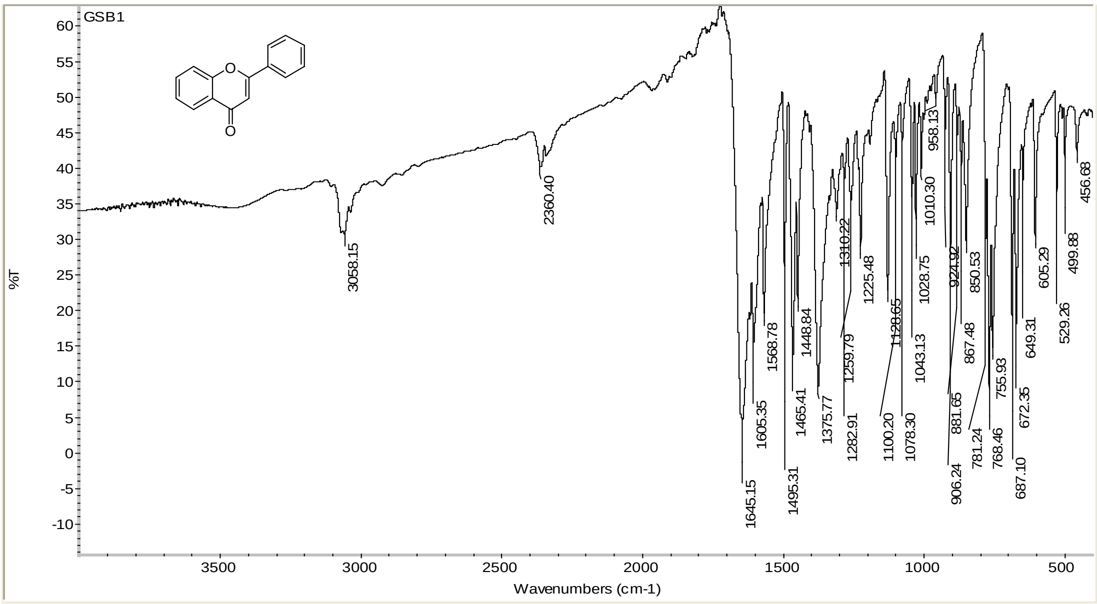


Fig. 4 : FTIR spectra of JPG-I (IR (KBr) cm^{-1}: 1645 (C=O), 1495 (C=C), 3058 (C-H), 1078 (C=O).

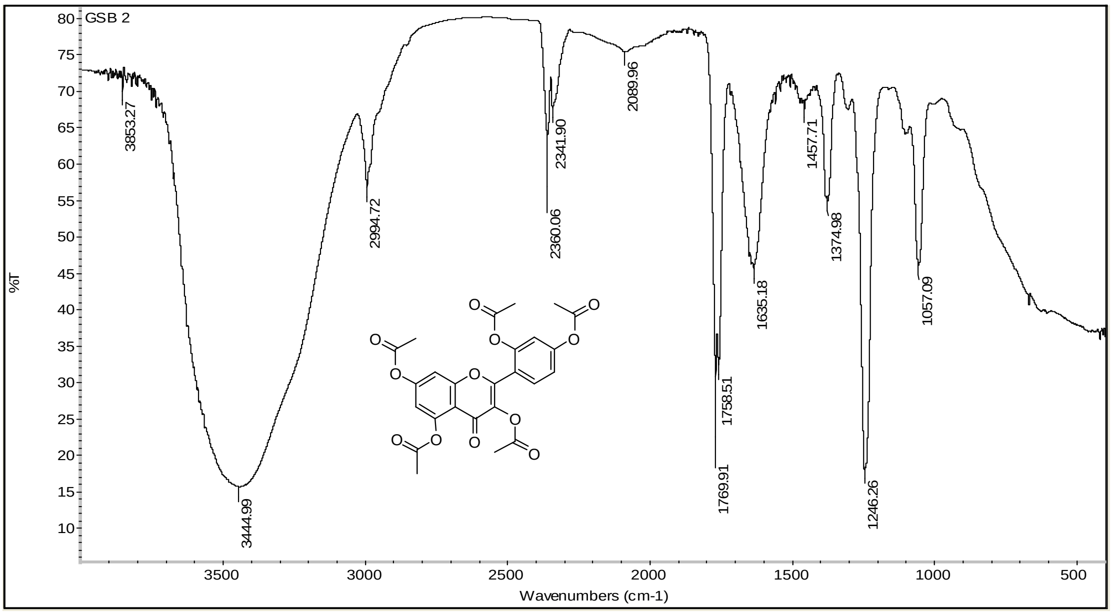


Fig. 5 : FTIR spectra of JPG-II (IR (KBr) cm^{-1}: 1774 (C=O), 1072 (C-O), 1654 (C=O), 3095 (Ar C-H), 2939 (=C-H).

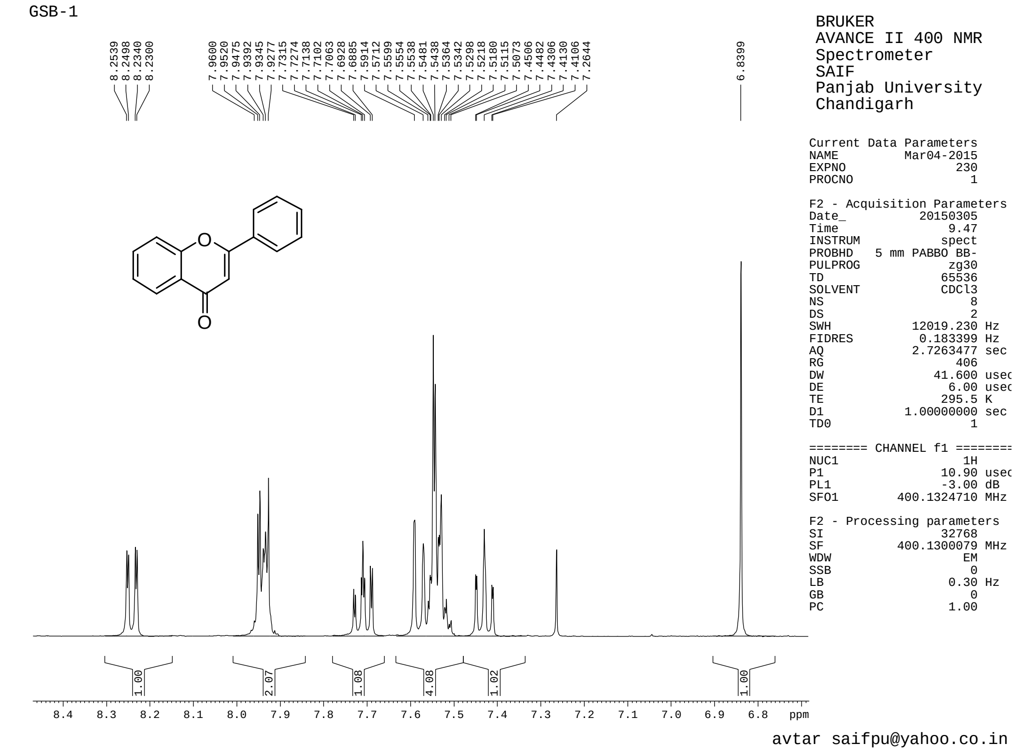


Fig. 6 : ¹H NMR of JPG-I (¹H NMR: 8.24 (1H d, J = 6.32 Hz), 7.94 (2H d, J =7.8 Hz), 7.71(1H t, J = 7.8 Hz), 7.59(4H, m), 7.43(1H, t, J = 7.52 Hz), 6.83 (1H, s).

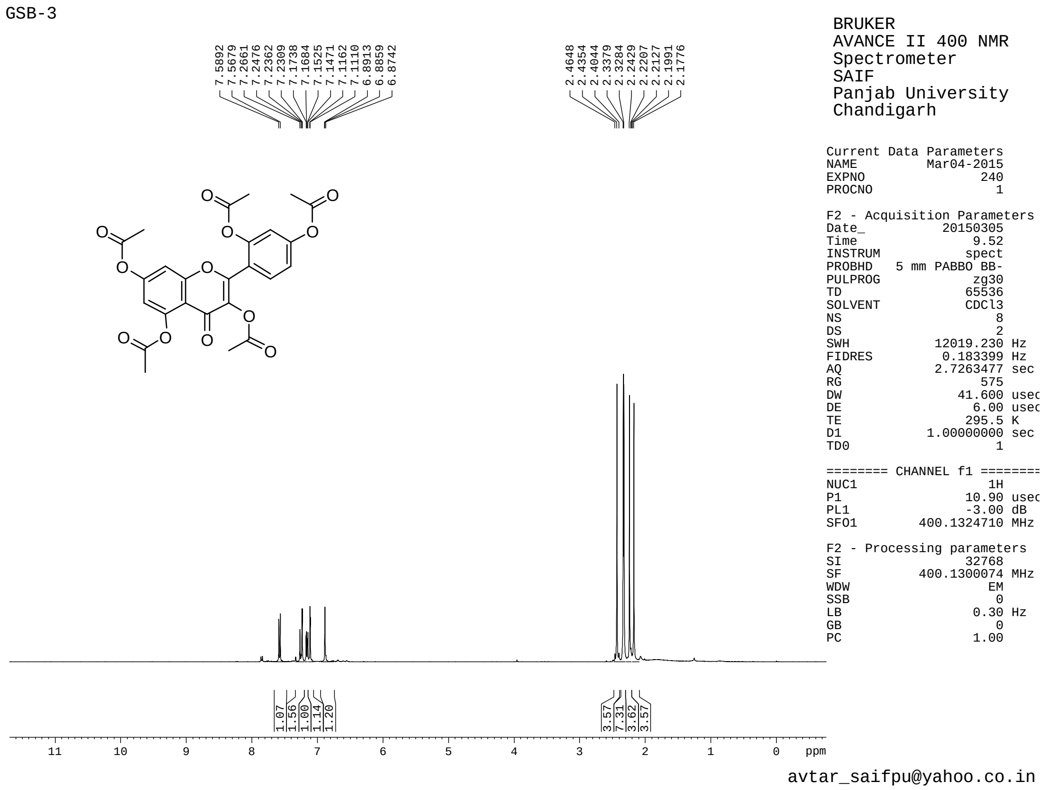


Fig. 7 : ¹H NMR of JPG-II (¹H NMR: 7.58(1H, s), 7.24(1H, s), 7.12(1H, d, J = 8.5 Hz), 7.11 (1H, s), 6.88 (1H, d, J = 6.84Hz), 2.43(3H, s), 2.33(6H, s), 2.24(3H, s), 2.17(3H, s).

to normal control groups which indicates the development of hepatotoxicity in rats. Further, silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) were administered orally for 21 days along with 40% alcohol in respective group which shown a significant increase in body weight as compared to diseased control group on 14th and 21th day. The effect of JPG-II (40 mg/kg) proved to be more significant ($p<0.05$) as compared to Morin and JPG-I (Fig. 8).

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver weight in 40% alcohol induced hepatotoxicity in rats

There was significant increase in the size of liver in 40% alcohol (2 ml/100 g) treated group on 21st day as compared to control group. Treatment with silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) showed significant improvement in liver weight and their morphology when compared with diseased control group on 21th day as shown in Fig. 9.

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on bilirubin (BIL), urobilinogen (URO), protein albumin (PRO), ketone (KET) level in 40% alcohol induced hepatotoxicity in rats

Oral administration of 40% alcohol (2 ml/100g) treatment caused a significant ($p<0.001$) increase in urobilinogen (URO), protein albumin (PRO), ketone (KET), bilirubin (BIL) level on 21st day as compared to control group. Treatment with silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) showed significantly ($p<0.05$) attenuation in urobilinogen (URO), protein albumin (PRO), ketone (KET), bilirubin (BIL) level on 21st day when compared with 40% alcohol treated group (Table III).

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver function enzymes Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) level, Total bilirubin (TB) and alkaline phosphatase (ALP)in 40% alcohol induced hepatotoxicity in rats

Oral administration of 40% alcohol (2 ml/100 g) treatment caused a significant ($p<0.001$) increase in SGOT, SGPT, TB and ALP level on the 21st day as compared to control group. Treatment with silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) showed significant ($p< 0.05$) decrease in SGOT, SGPT, TB and ALP level as compared to 40% alcohol treated group. The effect of JPG-II (40 mg/kg) showed significant ($p< 0.05$)

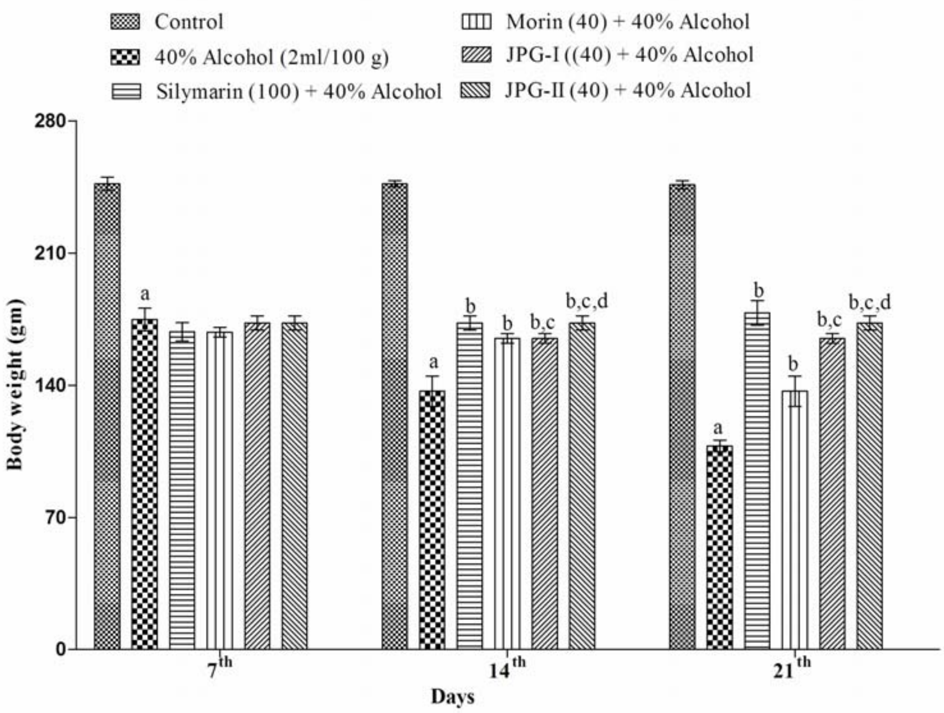


Fig. 8 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on body weight in alcohol treated rats. Values are expressed as Mean±S.D. $^{a}p<0.001$ vs control; $^{b}p<0.05$ vs 40% alcohol; $^{c}p< 0.05$ vsMorin (40 mg/kg); $^{d}p< 0.05$ vsMorin (40 mg/kg) and JPG-I (40 mg/kg) treated group on 7th, 14th, 21st day. Data were analyzed using two-way ANOVA followed by Bonferroni's test.

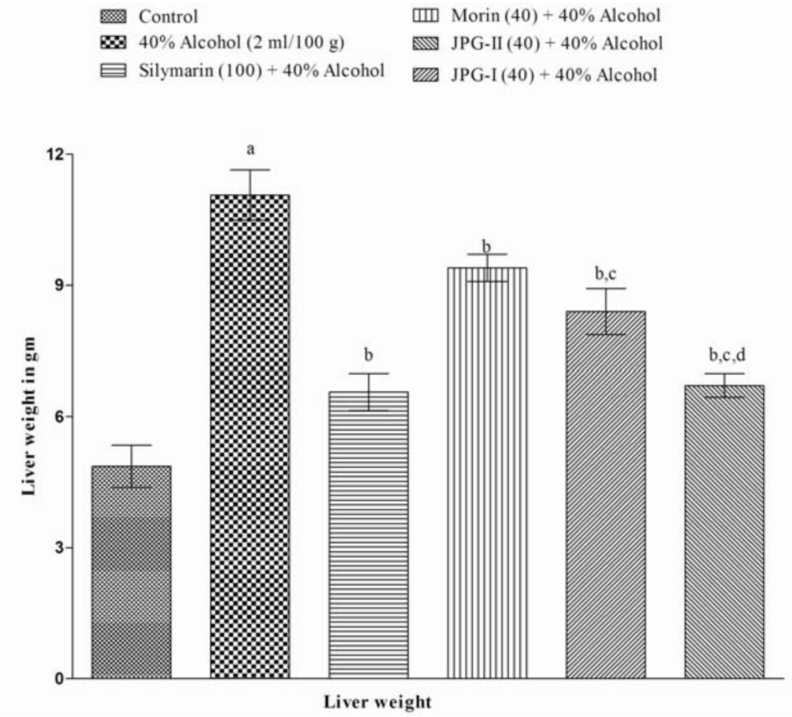


Fig. 9 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver weight in alcohol treated rats. Values are expressed as Mean±S.D. [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p< 0.05 vsMorin (40 mg/kg); [d]p< 0.05 vsMorin (40 mg/kg) and JPG-I (40 mg/kg). Data were analyzed using one-way ANOVA followed by Bonferroni's test.

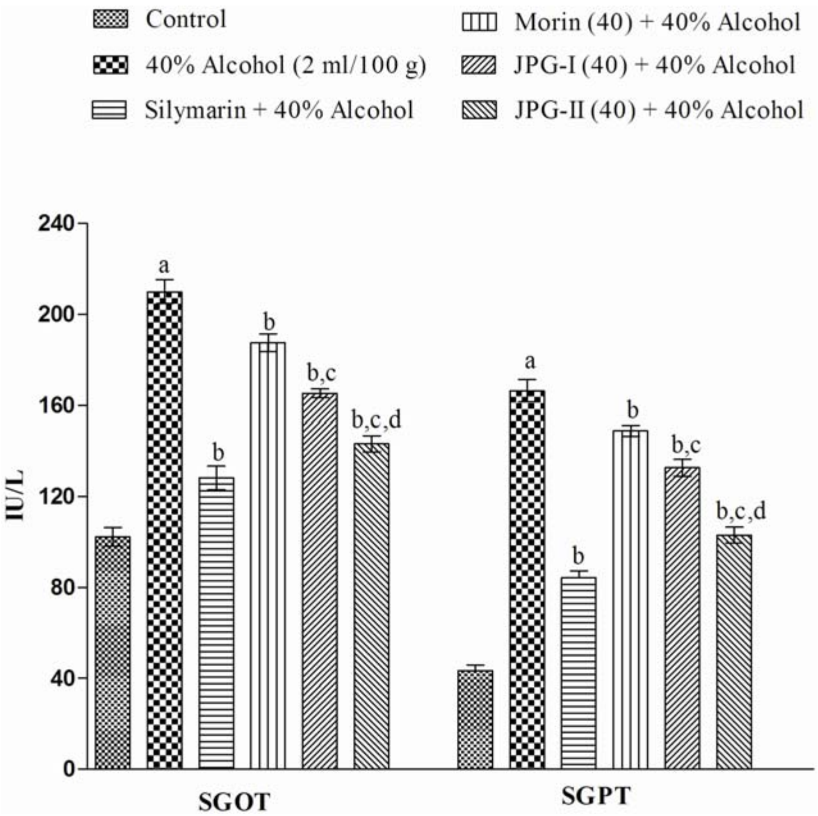


Fig. 10 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on alcohol induced change in SGOT and SGPT in rats. Values are expressed as Mean±S.D. [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p<0.05 vsMorin (40 mg/kg); [d]p< 0.05 vsMorin (40 mg/kg) and JPG-I (40 mg/kg). Data were analyzed using one-way ANOVA followed by Bonferroni's test.

TABLE II : Physical characterization of JPG-I and JPG-II.

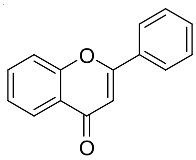
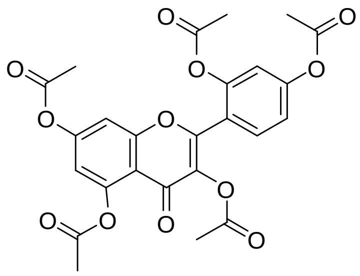
S. No.	*Compound*	*Mol. formula*	*Mol. Wt. (Calculated)*	*m.p. (°C)*	*% yield*
1.		$C_{15}H_{10}O_2$	222.24	95-97	97%
2.		$C_{25}H_{20}O_{12}$	512.42	110-112	98%

TABLE III : Effect of morin and its derivatives on level in bilirubin (BIL), urobilinogen (URO), protein albumin (PRO) and ketone (KET) 40% alcohol induced hepatotoxicity in rats.

Treatment	*7th Day*	*14th Day*	*21st Day*
Bilirubin (BIL) 0.4 mg/dL			
Control (0.5% CMC)	0.38±0.04	0.38±0.04	0.38±0.04
Disease control (alcohol 40% 2 ml/100 g)	2.15±0.50[a]	2.08±0.70[a]	2.65±0.46[a]
Standard silymarin (100 mg/kg)	1.50±0.43[b]	1.20±0.44[b]	0.71±0.11[b]
Morin (40 mg/kg)	2.01±0.52[c]	2.18±0.67[b]	2.36±0.48[b]
JPG-I (40 mg/kg)	2.13±0.64	1.76±0.59[b,c]	1.31±0.49[b,c]
JPG-II (40 mg/kg)	1.96±0.64	0.91±0.27[b,c,d]	0.95±0.28[b,c,d]
Urobilinogen (URO) 0.2 mg/dL			
Control (0.5% CMC)	0.2±0.09	0.2±0.09	0.2±0.09
Disease control (alcohol 40% 2 ml/100 g)	0.67±0.05[a]	0.77±0.07[a]	2.13±0.52[a]
Standard silymarin (100 mg/kg)	0.53±0.06[b]	0.56±0.20[b]	0.37±0.08[b]
Morin (40 mg/kg)	0.64±0.10	0.70±0.05	0.70±0.03[b]
JPG-I (40 mg/kg)	0.71±0.05	0.64±0.17	0.71±0.02[b]
JPG-II (40 mg/kg)	0.67±0.30	0.54±0.05[b,c]	0.51±0.05[b,c]
Protein(PRO) (albumin) 7.5-15mg/l			
Control (0.5% CMC)	13.8±0.68	13.8±0.56	13.80±0.61
Disease control (alcohol 40% 2 ml/100 g)	75.5±3.81[a]	82.68±5.28[a]	90.87±0.32[a]
Standard silymarin (100 mg/kg)	48.75±1.57[b]	39.12±5.38[b]	28.57±2.22[b]
Morin (40 mg/kg)	49.12±5.35	48.16±7.35	41.26±7.45[b]
JPG-I (40 mg/kg)	48.65±1.56	42.16±7.48	38.16±4.41[b]
JPG-II (40 mg/kg)	39.22±5.37[b,c]	35.25±5.25[b,c]	31.25±1.25[b,c]
Ketone (KET) 2.5-5 mg/dL			
Control (0.5% CMC)	3.7±0.7	3.7±0.4	3.7±0.4
Disease control (alcohol 40% 2 ml/100 g)	14.5±0.67	14.2±0.78[a]	12.8±0.66[a]
Standard silymarin (100 mg/kg)	10.43±0.66[b]	9.61±0.60[b]	5.08±0.98[b]
Morin (40 mg/kg)	9.88±0.71[b]	8.43±0.85[b]	7.41±0.64[b]
JPG-I (40 mg/kg)	9.28±0.91[b]	9.78±0.42[b]	8.31±1.02[b,c]
JPG-II (40 mg/kg)	10.27±0.59[b]	9.05±0.94[b,]	6.50±1.05[b,c,d]

Effect of morin and its derivative in alcohol induced change in bilirubin (BIL), urobilinogen (URO), protein albumin (PRO) and ketone (KET) in rats. Values are expressed as Mean±S.D. [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p<0.05 vs Morin (40 mg/kg); [d]p<0.05 vs Morin (40 mg/kg) and JPG-I (40 mg/kg) treated group on 7th, 14th, 21st day. Data were analyzed using two-way ANOVA followed by Bonferroni's test.

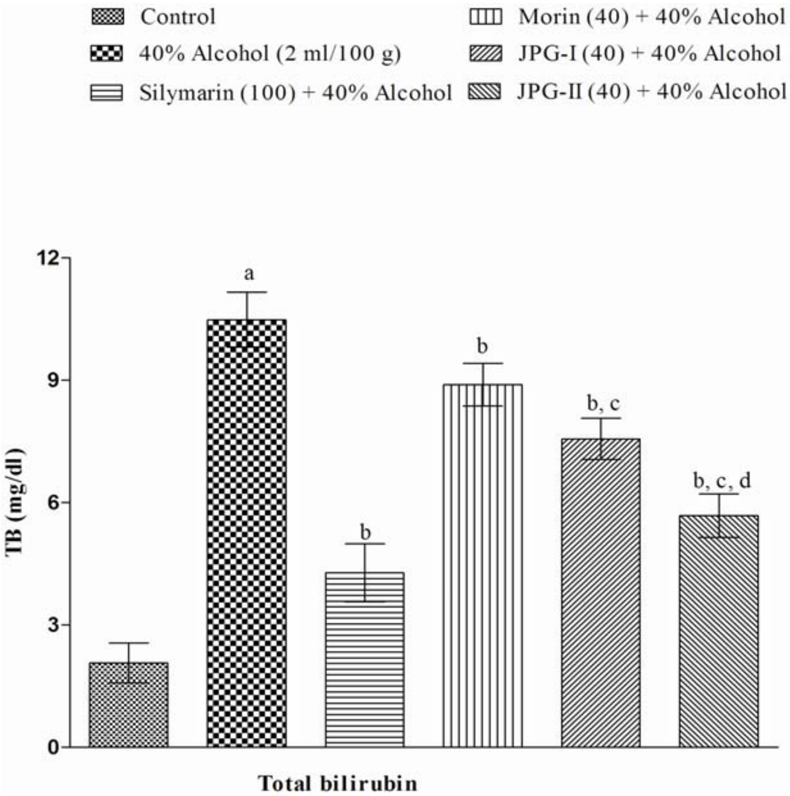


Fig. 11 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on alcohol induced change in Total Bilirubin in rats. Values are expressed as Mean±S.D. [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p<0.05 vs Morin (40 mg/kg); [d]p<0.05 vsMorin (40 mg/kg) and JPG-I (40 mg/kg). Data were analyzed using one-way ANOVA followed by Bonferroni's test.

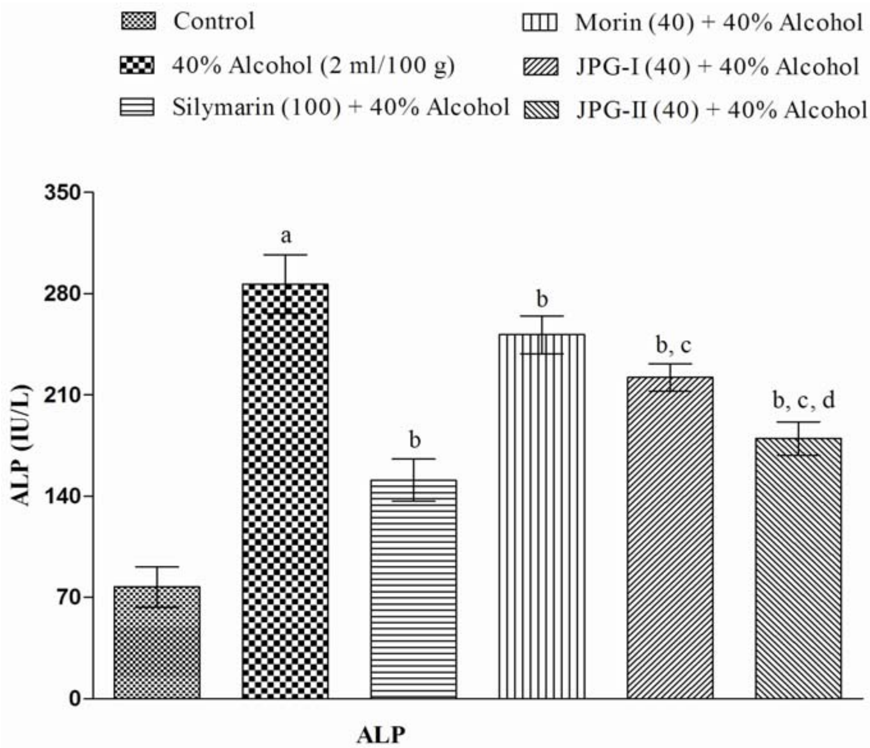


Fig. 12 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on alcohol induced change in ALP in rats. Values are expressed as Mean±S.D. [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p<0.05 vs Morin (40 mg/kg); [d]p<0.05 vsMorin (40 mg/kg) and JPG-I (40 mg/kg). Data were analyzed using one-way ANOVA followed by Bonferroni's test.

decrease in SGOT and SGPT level as compared to Morin and JPG-I as shown in Figs. 10, 11 and 12.

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver peroxide (MDA), nitrite and serum antioxidant enzyme (GSH) levels in 40% alcohol induced hepatotoxicity in rats

Oral administration of 40% alcohol (2 ml/100 g) treatment caused a significant ($p<0.001$) increase in lipid peroxidation, nitrite concentration and depleted in glutathione enzyme activity in liver as compared to control treated group. Treatment with silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) showed significantly ($p<0.05$) attenuated lipid peroxidation, nitrite concentration and restored levels of antioxidant enzyme glutathione as compared to 40% alcohol treated group. The effect of JPG-II (40 mg/kg) showed significantly ($p<0.05$) attenuation of lipid peroxidation, nitrite concentration and restored levels of antioxidant enzyme glutathione when compared to Morin and JPG-I as shown in Table IV.

Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver cytokine (TNF-α, IL-1β, IL-6) level in 40% alcohol induced hepatotoxicity in rats

Oral administration of 40% alcohol (2 ml/100 g) treatment caused a significant ($p<0.001$) elevation in liver TNF-α, IL-1β, IL-6 levels as compared to control group. Treatment with silymarin (100 mg/kg), Morin (40 mg/kg), JPG-I (40 mg/kg) & JPG-II (40 mg/kg) showed significantly ($p< 0.05$) attenuated liver TNF-α, IL-1β, IL-6 levels as compared to 40% alcohol

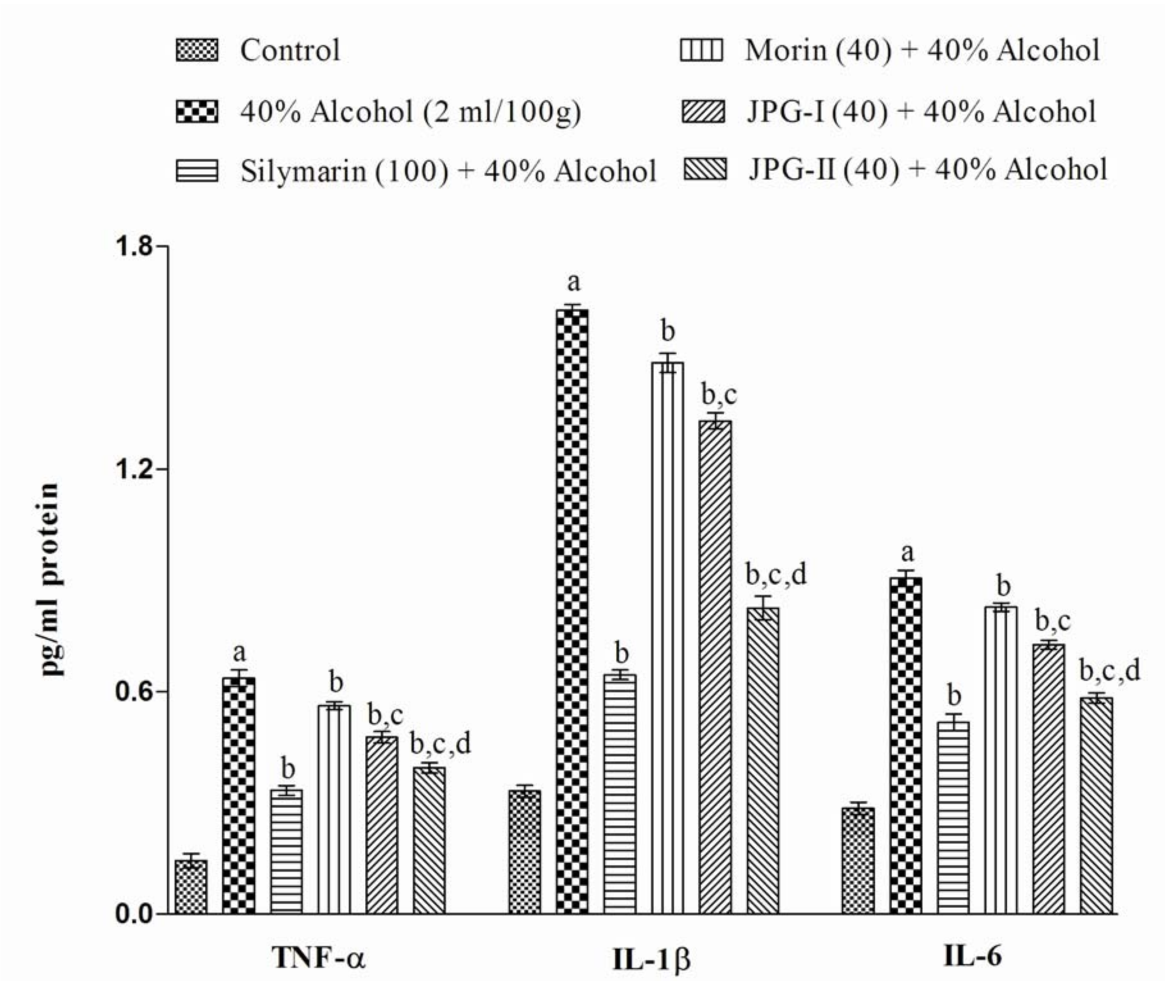


Fig. 13 : Effect of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) on liver cytokine (TNF-α, IL-1β, IL-6) level in 40% alcohol induced hepatotoxicity in rats. Values are expressed as Mean±S.D. [a]$p<0.001$ vs control; [b]$p<0.05$ vs 40% alcohol; [c]$p< 0.05$ vs Morin (40 mg/ kg); [d]$p< 0.05$ vsMorin (40 mg/kg) and JPG-I (40 mg/kg). Data were analyzed using one-way ANOVA followed by Bonferroni's test.

TABLE IV : Effect of morin and its derivatives on liver peroxide (MDA), nitrite and serum antioxidant enzyme (GSH) level in 40% alcohol induced hepatotoxicity in rats.

Parameter	*LPO*	*GSH*	*Nitrite*
Control group	1.192±0.4650	0.2118±0.01178	89.83±13.54
Disease control(Alcohol 40% 2 ml/100 g)	10.16±0.6461[a]	0.08447±0.01514[a]	862.3±30.10[a]
Silymarin (100)+40% alcohol	5.166±0.4168[b]	0.2018±0.01321[b]	425.8±23.22[b]
Morin (40)+40% alcohol	9.008±0.5729[b]	0.1082±0.007348[b]	767.3±25.14[b]
JPG-I (40)+40% alcohol	7.806±0.5514[b,c]	0.1344±0.00562[b,c]	666.0±28.15[b,c]
JPG-II (40)+40% alcohol	6.833±0.4128[b,c,d]	0.1771±0.0100[b,c,d]	499.3±19.05[b,c,d]

Value are expressed as Mean±S.D. (n=6). Statistical signicance at [a]p<0.001 vs control; [b]p<0.05 vs 40% alcohol; [c]p<0.05 vs Morin (40 mg/kg); [d]p<0.05 vs Morin (40 mg/kg) and JPG-I (40 mg/kg).
LPO-MDA levels are expressed as nmol MDA/mg of protein, nitrite levels are expressed as m mol/mg of protein, GSH levels are expressed as nmol of GSH/mg of protein.

treated group. The effect of JPG-II (40 mg/kg) showed significantly ($p<0.05$) attenuation of liver TNF-α, IL-1β, IL-6 levels when compared with Morin and JPG-I as shown in Fig. 13.

Discussion

The present study demonstrates the hepatoprotective activities of Morin and their newly synthesized derivatives (JPG-I and JPG-II) in alcohol induced hepatotoxicity model in rats. Alcohol induced hepatotoxicity in the experimental rats within 21 days produced impairment in morphological parameters (body weights, urine analysis), biochemical parameters (serum liver functional enzymes profile). The targeted JPG-I and JPG-II were successfully synthesized and obtained in moderate to high yield which were found to be analytically pure and used for the *in-vivo* studies without further purification.

The etiology of liver disorder varies due to different reasons like autoimmune disorder, viral infection, toxic chemical and unhealthy diet style. It is a key organ to regulate homeostasis within the body & also involved in all the biochemical pathway related to metabolism such as metabolism of fats, carbohydrates, proteins, hormones, synthesis of vitamins, formation of bile, excretion of bilirubin, detoxification of drugs and other toxins (13, 14). Several mechanisms like oxidative stress, antioxidants depletion, nitric oxide synthase activity etc. are responsible for the damage of liver cells (15). The pathogenesis of acute and chronic alcohol consumption is complex and involves multiple mechanisms of cell injury. Alcohol causes liver abnormalities ranging from steatosis (fat deposition), steatohepatitis (fat plus inflammation to cirrhosis) and hepatocellular carcinoma. The alcohol induced liver toxicity produce harmful effect on both parenchymal and non-parenchymal cells of the liver which leads to the progression of liver fibrosis (16). In the present study, alcohol 40% was administered at dose of 2 ml/100 g/day, p.o. for 21 days and the effect of this systemic administration was investigated on body weight, urine analysis, liver function enzyme level, biochemical parameter, and proinflammatory cytokine level in rats. Body weight was significantly decreased as compared with normal control groups. Treatment with silymarin, Morin, JPG-I, JPG-II significantly reversed the decline in body weight.

Assay of aspartate aminotransferase (AST/SGOT) and alanine aminotransferase (ALT/SGPT) activities have long been considered as sensitive indicator of hepatic injury. Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells. This leakage causes a decrease in levels of AST and ALT in hepatic cells, but increase in levels of AST/SGOT with AST and ALT/SGPT with ALT in the blood (17). The raised levels of cytoplasmic hepatic enzymes due to hepato-bile duct damage are considered as an index of the extent and severity of hepatocellular damage.

Alkaline phosphatase (ALP) is a membrane bound enzyme but not a liver specific enzyme (18) and elevated levels of ALP may reflect impaired biliary tract function (19). The level of serum ALP might be increased due to the presence of increased biliary pressure and cholestasis (20). The elevation of enzymatic activities in the blood is associated with high level of bilirubin content. Indirect bilirubin is elevated by pre-hepatic causes such as hemolytic disorders or liver diseases resulting in impaired entry, transport, or conjugation within the liver. A typical pattern of bilirubin content therefore, reflects the pathophysiology of liver (18). Total bilirubin is elevated in obstructive conditions of bile duct, hepatitis, cirrhosishemolytic disorders and several inherited enzyme deficiencies (21). The significant elevated levels of serum SGOT, SGPT, ALP and bilirubin in alcohol treated groups is consistent with these findings and confirm the hepatocellular damage in the present study. Oral administration of 40% alcohol (2 ml/100 g) treatment caused a significant ($p<0.001$) increase in SGOT, SGPT, ALP and TB level on 21st day as compared to control group. The pre-treatment with JPG-I & JPG-II produce significant ($p<0.05$) suppression of the increased serum SGOT, SGPT, and ALP activities with the significant ($p<0.05$) depletion of raised serum bilirubin, suggest the hepatoprotective activity of newly synthesized compounds as compared to Morin.

Oxidative stress plays a major role in the etiology of liver disoders mainly by the action of substance endotoxin, which activate the kuffer cells to generate reactive oxygen species (ROS) (22). In present study oral administration of 40% alcohol (2 ml/100 g) treatment caused a significant ($p<0.001$) increase in the levels of MDA, nitrite, and decrease in the levels of glutathione antioxidant enzyme in the liver. Treatment with silymarin, Morin, JPG-I & JPG-II showed significantly ($p<0.05$) attenuated lipid peroxidation, nitrite concentration and restored levels of antioxidant enzyme glutathione as compared to 40% alcohol treated group. The effect of Morin derivative (JPG-II) showed significantly ($p< 0.05$) attenuated lipid peroxidation, nitrite concentration and restored levels of antioxidant enzyme glutathione as compared to Morin and JPG-I.

Inflammation is commonly associated with fibrogenesis during chronic liver diseases. Inflammation plays an important role in the pathophysiology of cellular injury in liver. Hepatotoxins rapidly induced release of proinammatory cytokines by Kupffer cells and have been linked to liver injury (24). A previous report indicated the inhibitory effect of Morin on TNF-α induced activation of NF-κB in various cancer cells (25). The scientific data suggested that pro inflammatory cytokines like IL-6, IL-1β and TNF-α produce devastating effect in the progression of liver inflammation and liver fibrosis in rodents (26). Wang et al., in 2013 demonstrated that the novel mechanism of morin through inhibition of hepatic SphK1/S1P signaling pathway which exerts hepatoprotection in high fructose-fed rats, possibly involving liver inflammation inhibition and lipid accumulation recovery (27). We found that oral administration of 40% alcohol (2 ml/100 g) caused a significant ($p<0.001$) elevation in liver TNF-α, IL-1β, IL-6 levels as compared to control group and reverse was takes place in silymarin, Morin, JPG-I & JPG-II treated groups. The effect of JPG-II showed significantly ($p<0.05$) attenuated in liver TNF-α, IL-1β, IL-6 levels as compared to Morin and JPG-I.

Morin has been the subject of a number of experimental studies dealing with its pharmacological activities, such as anti-inammatory activity (28), antioxidant properties (29), anticancer activity (25), neuroprotective activity (30), ameliorative potential in neuropathic pain (31). It is highly soluble in aqueous media. However, it is barely absorbed by rats, because it merely passes through the gastrointestinal tract to be degraded by the intestinal microflora. Therefore, we synthesized a novel derivative named JPG-II. The exact mechanisms explaining their biological activities are poorly understood and largely unknown, but it is possible that different types of biochemical events are involved. The mechanisms underlying these promising effects of Morin, JPG-I and JPG-II (40 mg/kg) could be through attenuating oxidative stress as well as decreasing the expression of proinammatory cytokine production. This study suggested that these compounds act as a hepatotoprotective through the inhibition of free radical, and anti-inflammatory mechanism.

Conclusion

In conclusion, we hypothesized that Morin, JPG-I and JPG-II (40 mg/kg) showed their protective effect against ethanol-induced hepatotoxicity due to their antioxidants, anti-inflammatory and antifibrotic effect. The ROS scavenging and *proinflammatory cytokines (*TNF-α, IL-1β, IL-6*) inhibitory activity* of Morin and its synthetic derivative might be benificial in the treatment of alcoholic liver injury. Further it is documented that the Morin derivative JPG-II (40 mg/kg) proved to be more beneficial as compared to Morin and JPG-I (40 mg/kg) against ethanol induced hepatotoxicity in rodents. The findings of the present study clearly demonstrate the potential role of Morin, JPG-I (40 mg/kg) and JPG-II (40 mg/kg) against ethanol induced hepatotoxicity in rodents through inhibition of oxidative stress and Inflammatory mediators as shown in the concluded diagram (Fig. 14) and there is need to explore more about the novel pharmacological intervention for the therapeutic treatment of alcoholic liver disorders.

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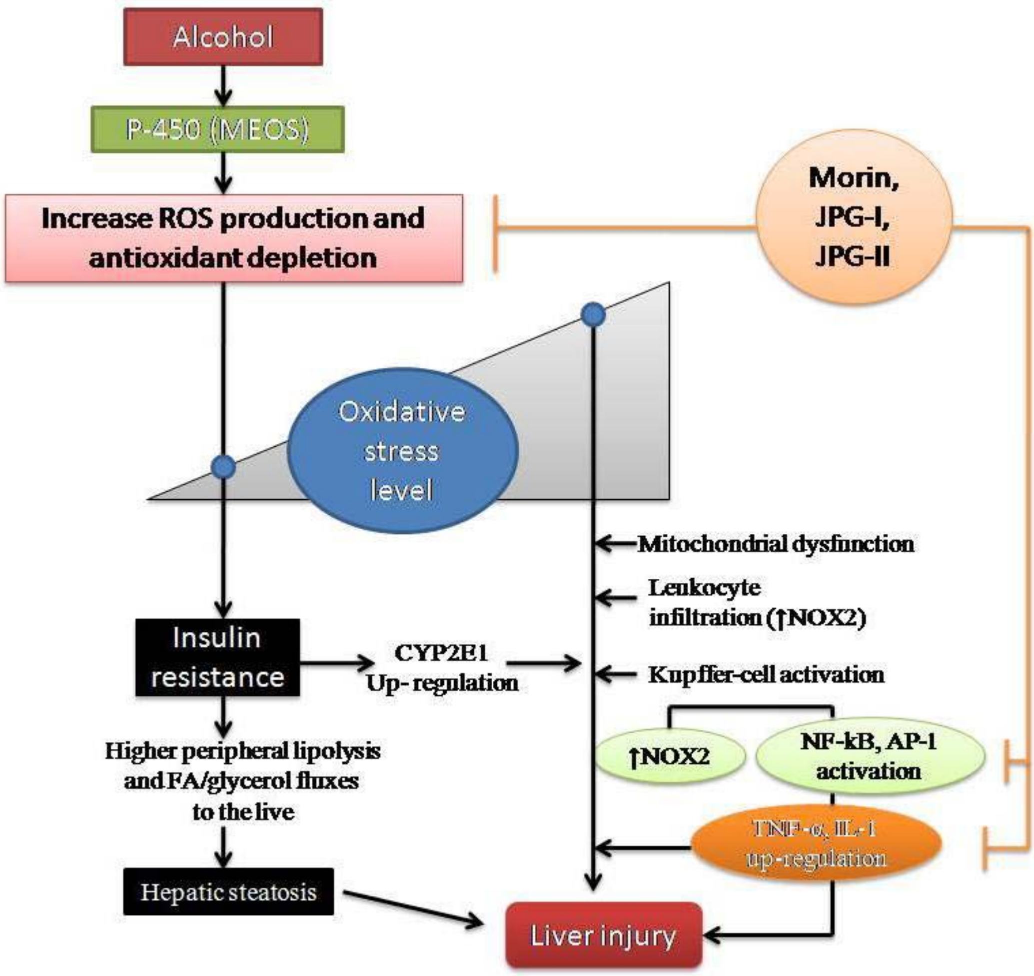

Fig. 14 : Proposed Mechanism of Morin, flavone (JPG-I) and derivative of Morin (JPG-II) as an antioxidant and anti-inflammatory in alcohol induced hepatotoxicity.

References

1. Sharma A, Chakraborti KK, Handa SS. Antihepatotoxic activity of some Indian herbal formulations as compared to silymarin. *Fitoterapia* 1991; 62: 229–235.
2. Navarro VJ, Senior JR. Drug-related hepatotoxicity. *N Engl J Med* 2006; 354: 731–739.
3. Pari L, Karthikesan K. Protective role of caffeic acid against alcohol induced biochemical changes in rats. *Fundam Clin Pharmacol* 2007; 21: 355–361.
4. Nordmann R, RibiÃ¨re C, RouachHln. Implication of free radical mechanisms in ethanol-induced cellular injury. *Free Radic Biol Med* 1992; 12: 219–240.
5. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* 2006; 71: 1397–1421.
6. Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Montesano D, et al. Antibacterial and allelopathic activity of extract from Castanea sativa leaves. *Fitoterapia* 2000; 71: S110-S116.
7. Sivaramakrishnan V, Shilpa PNM, Kumar VRP, Devaraj SN. Attenuation of N-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol Morin. *Chem Biol Interact* 2008; 171: 79–88.
8. Sharma A, Sangameswaran B, Jain V, and Saluja MS. Hepatoprotective activity of Adina cordifolia against ethanol induce hepatotoxicity in rats. 2012.
9. Wills ED. Mechanisms of lipid peroxide formation in animal tissues. *Biochem J* 1966; 99(3): 667.
10. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70–47.
11. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. *Anal Biochem* 1982; 126: 131–138.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J biol Chem* 1951; 193: 265–275.
13. Bhakuni GS, Bedi O, Bariwal J, Deshmukh R, Kumar P. Animal models of hepatotoxicity. *Inflamm Res* 2016; 65: 13–24.
14. Modi H, Patel V, Patel K. Hepatoprotective activity of Aeglemarmelos against ethanol induced hepatotoxicity in rats. *Asian J Pharm Clin Res* 2012; 5: 164–167.
15. Lewis JH. Drug-induced liver disease. *Medical Clinics of North America* 2000; 84: 1275–1311.
16. Mandrekar P, Ambade A. Cellular Signaling Pathways in Alcoholic Liver Disease. 2012.
17. Nkosi CZ, Opoku AR, Terblanche SE. Effect of pumpkin seed (Cucurbitapepo) protein isolate on the activity levels of certain plasma enzymes in CCl4•induced liver injury in lowβ protein fed rats. *Phytotherapy Research* 2005; 19: 341–345.
18. Sherlock S, Dooley J. Diseases of the liver and biliary system: John Wiley & Sons; 2008.
19. Wolf PL, Williams D, Von der Muchll E. Practical Clinical Enzymology, Techniques and Interpretations and Biochemical Profiling. A Wiley-Interscience Publication. John Wiley and Sons, New York, London, Sydney, Toronto; 1973.
20. Sanmugapriya E, Venkataraman S. Studies on hepatoprotective and antioxidant actions of Strychnospotatorum Linn. seeds on CCl 4-induced acute hepatic injury in experimental rats. *J Ethnopharmacol* 2006; 105: 154–160.
21. Plaa GL, Hewitt WR. Detection and evaluation of chemically induced liver injury. Principles and methods of toxicology. 1989; 3: 841–846.
22. Gujrati V, Patel N, Rao VN, Nandakumar K, Gouda TS, Shalam M, et al. Hepatoprotective activity of alcoholic and aqueous extracts of leaves of Tylophoraindica (Linn.) in rats. *Indian J Pharmacol* 2007; 39: 43.
23. Marra F, Tacke F. Roles for chemokines in liver disease. *Gastroenterology* 2014; 147(3): 577–594. e1.
24. Shito M, Balis UJ, Tompkins RG, Yarmush ML, Toner M. A fulminant hepatic failure model in the rat. *Digestive Diseases and Sciences* 2001; 46: 1700–1708.
25. Manna SK, Aggarwal RS, Sethi G, Aggarwal BB, Ramesh GT. Morin (3, 5, 7, 2β, 4β-Pentahydroxyflavone) Abolishes Nuclear Factor-ÎºB Activation Induced by Various Carcinogens and Inflammatory Stimuli, Leading to Suppression of Nuclear Factor-ÎºBβ Regulated Gene Expression and Up-regulation of Apoptosis. *Clin Cancer Research* 2007; 13: 2290–2297.
26. Yin M, Wheeler MD, Kono H, Bradford BU, Gallucci RM, Luster MI, et al. Essential role of tumor necrosis factor Î± in alcohol-induced liver injury in mice. *Gastroenterology* 1999; 117942–117952.
27. Wang X, Zhang DM, Gu TT, Ding XQ, Fan CY, Zhu Q, et al.Morin reduces hepatic inflammation-associated lipid accumulation in high fructose-fed rats via inhibiting sphingosine kinase 1/sphingosine 1-phosphate signaling pathway. *Biochem Pharmacol* 2013; 86: 1791–1804.
28. Galvez J, Coelho G, Crespo ME, Cruz T, RodrÃ-guezβ Cabezas ME, Concha A, et al. Intestinal anti β inflammatory activity of Morin on chronic experimental colitis in the rat. *Alimentary Pharmacology & Therapeutics* 2001; 15: 2027–2039.
29. McPhail DB, Hartley RC, Gardner PT, Duthie GG. Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy. *J Agric Food Chem* 2003; 51: 1684–1690.
30. Zhang Z-t, Cao X-b, Xiong N, Wang H-c, Huang J-s, Sun S-g, et al. Morin exerts neuroprotective actions in Parkinson disease models in vitro and in vivo. *Acta Pharmacologica Sinica* 2010; 31: 900–906.
31. AlSharari SD, Al-Rejaie SS, Abuohashish HM, Aleisa AM, Parmar MY, Ahmed MM. Ameliorative potential of Morin in streptozotocin-induced neuropathic pain in rats. *Trop J Pharm Res* 2014; 13: 1429–1436.