

Protective and therapeutic activities of *Mayodendron igneum* Kurz against paracetamol induced liver toxicity in rats and its bioactive constituents

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ABSTRACT

The total ethanol extract of *Mayodendron igneum* leaves and its successive extracts (pet. Ether, chloroform and ethyl acetate combined with ethanol) were examined for their protective and therapeutic effects against paracetamol induced liver damage in rats. The evaluation was done through measuring the liver function enzymes; aspartate and alanine aminotransferases (AST and ALT) as well as cholestatic marker; alkaline phosphatase (ALP). Liver histopathological analysis were also evaluated. Treatment with the selected extracts significantly decreased AST, ALT and ALP. The biochemical results were in accordance with histopathological results. From the successive ethyl acetate extract, three flavonoids were isolated, purified and identified as 6-methoxyapigenin; fisetin-3-O- β -glucopyranoside and 3-methoxyquercetin-7-O- β -glucopyranoside(6 \rightarrow 8'')^{3''}-methoxykaempferol-7''-O- β -glucopyranoside. Five phytoconstituents were isolated from successive ethanol extract and were identified as 4-methoxybenzoic acid; 6,7-dihydroxycoumarin; apigenin-7-O- β -neohesperidoside; 6-methoxyluteolin-7-O- β -neohesperidoside and luteolin-7-O- β -neohesperidoside. *Mayodendron* leaves extracts resulted in an attractive candidate for the protection and the treatment of liver damage induced by paracetamol. The flavonoids and coumarin present in the plant may be responsible for the pharmacological activity.

INTRODUCTION

Mayodendron igneum Kurz, also known as *Radermachera ignea*, is a member of family Bignoniaceae which includes about 750 species of plants in 120 genera within 8 tribes. Species within the Bignoniaceae have yielded a number of phytochemicals especially terpenoids and flavonoids. Aliphatic hydroxy-ketones compounds have been isolated from the petroleum ether extract of *Adenocalymma alliaceum* leaves, (Misra *et al.*, 1989). A Study of *Newbouldia laevis*, *Markhamia acuminata*, *Spathodea campanulata* and *Kigelia africana* revealed that the principal constituents were a homologous series of n-alkanes (C₂₃-C₃₃), n-alcohols (C₁₈-C₃₀) and related carboxylic acids (C₁₆-C₃₆), (Gormann *et al.*, 2004). The aqueous ethanol extract of *Jacaranda caucana* yielded β -sitosterol and triterpene acids:

Jacarandic acid, ursolic acid, 2 α -hydroxyursolic acid and 2 $\alpha,3$ α -dihydroxyurs-12-en-28-oic acid, (Ogura *et al.*, 1977). *Tanaecium jaroba*, a Bolivian plant used in traditional medicine, was reported to contain 2 α -hydroxy-12 β -hydroxy-isopimar-8(14),15-diene (diterpene), triterpenes, sterols and fatty acids, (Mitaine-Offer *et al.*, 2002).

Work by Jin *et al.*, (2004) led to the isolation of pentacyclic triterpenoids from leaves of *Campsis grandiflora* that have been characterized by spectroscopic methods as oleanolic acid, hederagenin, ursolic acid, tormentic acid and myrianthic acid. Harborne, (1967) reported that most species of bignonioids contained flavones rather than flavonols. 5,6,7,3',4'-pentahydroxyflavone (6-hydroxy-luteolin) was identified in leaves of *Catalpa bignonioides*, *C. speciosa*, *C. bungei* and *Tecoma australis*. Subramanian and Nair, (1972) isolated from the leaves of *Oroxylum indicum*: baicalein, scutellarein and baicalein-6-glucuronide.

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The same authors isolated quercetin, kaempferol, kaempferol-3-sophorosides and dihydrokaempferol-7-glucoside from leaves of *Pajanelia longifolia*. Tillequin *et al.*, (1977) isolated 5-hydroxy-6,7,4'-trimethoxy flavone from *Phylarthrone madagascariense* leaves. Takemura *et al.*, (1995) isolated a flavone from leaves of the Brazilian plant *Arrabidaea chica* f. *cuprea*, its structure was established by spectroscopic analysis. Hase *et al.*, (1995) isolated from *Millingtonia hortensis* 5,7-dihydroxy-6,4'-dimethoxy flavone (Pectolinarigenin), hispidulin, apigenin-7-*O*-glucuronide, hispidulin 7-*O*-glucoside and hispidulin 7-*O*-glucuronide methyl ester. Chen *et al.*, (2003) isolated four flavonoids from the seeds of *Oroxylum indicum*: chrysin, baicalein, baicalein-7-*O*-glucoside, baicalein-7-*O*-diglucoside (Oroxylin B) by high-speed counter-current chromatography.

From *Mayodendron igneum* Kurz bark a new diterpenoid, igeumone, was isolated together with the following flavonoids: quercetin, naringenin and naringin, (Guo *et al.*, 2007). While Hashem *et al.*, (2007a) isolated four apigenin glycosides from the ethyl acetate extract of *M. igneum* leaves, identified as apigenin 7-*O*-glucoside, 6-methoxy apigenin 7-*O*-glucoside, 6-methoxy apigenin-7-*O*-rhamnoglucoside and 6-hydroxy apigenin-7-*O*-rhamnoglucoside.

Many plants in the Bignoniaceae family have been reported to possess anti-inflammatory (Alguacil *et al.*, 2000), (Munoz-Mingarro *et al.*, 2003) and (Silva *et al.*, 2006), antioxidant (Kumar *et al.*, 2005) and (Hashem *et al.*, 2007b), antimicrobial (Pereira *et al.*, 2006) and (Eyong *et al.*, 2006), cytotoxic (Alguacil *et al.*, 2000) and (Marzouk *et al.*, 2006), antimalarial (Onegi *et al.*, 2002) and antimutagenic activities (Nakahara *et al.*, 2002). Earlier studies have shown the anti-inflammatory, analgesic and antipyretic activities of 80% ethanol extract of *M. igneum* Kurz leaves, (Hashem *et al.*, 2007a).

In a study of the total alcohol and successive extracts of *M. igneum* leaves against human cancer cell lines, (Hashem *et al.*, 2011), the total ethanol extract demonstrated strong cytotoxic activity against the cervix cancer cell line (IC₅₀=0.60 µg/mL), breast cancer cell line (IC₅₀=0.54 µg/mL) and liver cancer cell line (IC₅₀=0.87 µg/mL) when compared to doxorubicin (anticancer drug).

Antioxidant activity of total ethanol extract and successive fractions of *M. igneum* was measured by electron spin resonance (ESR) and using DPPH as the stable free radical, (Hashem *et al.*, 2012). The strongest inhibition of DPPH stable free radicals was exhibited by the successive ethanol fraction (equal to 100% of free radical scavenging activity relative to ascorbic acid), followed by ethyl acetate fraction which had 64.96% of the antioxidant activity of ascorbic acid. The petroleum ether and chloroform fractions had 12.46% and 0%, respectively, of the antioxidant activity of ascorbic acid. Therefore, in this study, the successive ethanol and ethyl acetate extracts were subjected to a phytochemical investigation to determine the active constituents that contribute to the strong antioxidant activity. In addition, the total ethanol extract and successive extracts were tested for their

prophylactic and therapeutic activities against paracetamol-induced liver damage in rats.

EXPERIMENTAL

Materials and methods

Plant material

Fresh leaves of *Mayodendron igneum* Kurz plant (Bignoniaceae) growing in Giza Zoo, Giza, Egypt, were collected. The plant was identified by Dr. M. El-Gibaly, a plant taxonomist, and Mrs. Trease Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of Orman Botanical Garden, Giza, Egypt. A voucher specimen has been placed in the herbarium at the Orman Botanical Garden (Voucher number M.20).

Plant extracts

total ethanol extract was prepared from 400 g of air-dried, powdered leaves of *M. igneum* by repeatedly extraction in a Soxhlet apparatus with 80% ethanol until exhaustion. The extract was filtered, concentrated under reduced pressure at 55°C to dryness, yielding 95 g of crude extract, divided to 90g for successive extraction with different solvents and the remaining 5g total ethanol extract used for biological study (hepatoprotection). Successive fractions were made using 90 g of the crude extract suspended in water and partitioned successively with petroleum ether (60-80°C), chloroform, and ethyl acetate. The solvents were evaporated to dryness to yield fractions of 25 g of petroleum ether extract, 19 g of chloroform extract, 10 g of ethyl acetate extract, and 30 g of remaining residue considered as successive ethanol extract.

Isolation and purification of phytoconstituents

The successive ethyl acetate fraction was examined by paper chromatography (Whatmann No 1) using the solvent systems: *n*-butanol; acetic acid; water (3:1:1) (S₁), 15% acetic acid (S₂) and *n*-butanol; acetic acid; water (4:1:5 upper phase) (S₃). Chromatograms were visualized under UV, UV & NH₃ and UV & AlCl₃. The presence of the detected spots, their R_f values and their colours were recorded. Isolation of detected compounds were carried out by preparative paper chromatography using Whatmann No.3MM sheets using S₃ solvent system. The eluted substances were further purified by repetitive chromatography on Sephadex LH-20 column and eluted with methanol to yield compounds **1-3**. The successive ethyl alcohol fraction of *M. igneum* was fractionated over a polyamide 6S column. The elution process occurred in a gradient mode using water/methanol mixtures of decreasing polarities as eluent. Fractions of similar PC profiles in S₁ and S₂ were pooled together, concentrated and further purified on preparative paper chromatography (Whatmann No.3MM) using S₁, S₂ and S₃ solvent systems. The separated bands were cut off and eluted with 70% methanol to get compounds **4-8**, which were further purified by rechromatography on Sephadex LH-20 column using methanol as eluent.

Median lethal dose determination (LD₅₀)

Male albino mice (25-30 g) were divided into groups, each of six animals. One group was used as a control and given an oral dose of the vehicle (oil or water). Preliminary experiments were done to determine the minimum dose of the total ethanol extract that kills all animals (LD₁₀₀) and the maximum dose of the same extract that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between and were injected into a group of six animals by subcutaneous injection. The mice were then observed for 24 hours after administration of the extract to record the symptoms of toxicity and mortality rates in each group and LD₅₀ was calculated, (Karber, 1931).

Hepatoprotective activity of *M. igneum* Kurz

The study was undertaken to detect the prophylactic and the therapeutic effects of petroleum ether, chloroform, ethyl acetate combined with ethanol extract and total ethanol extracts of *M. igneum* against the hepatotoxicity induced by paracetamol.

Experimental design

A total number of 72 female rats (120-150 g) were used in this study. Rats were obtained from the animal house colony of National Research Centre, Cairo, Egypt. They were acclimatized to the new laboratory conditions before using for experimentation for a period of one week in a quiet room at temperature of 20-25°C with a lighting schedule of 12 hours light and 12 hours dark. Rats were provided with food and water *ad libitum* throughout the period of the experiment. Animal procedures in this study were approved by the Ethics Committee for the use of animals in biological research of the National Research Centre (Approval Certificate no. 09/117).

Rats were divided into 12 groups (6 rats / group).

Group I: The control group- treated with 1 ml vehicle (water or oil) for eight days

Group II: Rats received oral dose of vehicle (water or oil) for 7 days prior to a single oral dose of Paracetamol (2 g/kg body weight) on the eighth day (Mitra *et al.*, 1998).

Group III: Rats received oral dose of Silymarin (50 mg/kg body weight) for 7 days prior to a single dose of Paracetamol (2 g/kg body weight), (Jain *et al.*, 2008).

Group IV, V, VI, VII: Animals received total ethanol, petroleum ether, chloroform and ethyl acetate combined with ethanol extracts of *Mayodendron igneum* (0.67 g/kg body weight) for 7 days prior to a single dose of Paracetamol (2 g/kg body weight) on the eighth day.

Group VIII: Rats received oral dose of Silymarin (50 mg/kg body weight) for 7 days after a single dose of Paracetamol (2 g/kg body weight), (Jain *et al.*, 2008).

Group IX, X, XI, XII: Rats received total ethanol, petroleum ether, chloroform and ethyl acetate combined with ethanol extracts of *Mayodendron igneum* (0.67 g/kg body weight) for 7 days after a single dose of Paracetamol (2 g/kg body weight). Paracetamol and Silymarin solutions were prepared by dissolving

in distilled water. All extracts were dissolved in distilled water as a vehicle except petroleum ether extract was dissolved in oil. All the doses were given by a gastric tube.

Biochemical analysis

At the end of the experiment, blood samples were obtained using the orbital sinus technique, (Sanford, 1954). Blood was collected in dry tube and left to clot at room temperature, then centrifuged at 3000 r.p.m for ten minutes. Serum was separated and kept for subsequent evaluation of biochemical parameters. Determination of serum alanine aminotransferase (glutamate pyruvate transaminase) (ALT, SGPT), aspartate aminotransferase (glutamate oxaloacetate transaminase) (AST, SGOT) and alkaline phosphatase (ALP) were carried out using colorimetric methods, (Begmeyer *et al.*, 1986) and (Tietz *et al.*, 1983).

Histopathological examination

After blood samples were obtained, rats were decapitated and slices of liver were removed out and fixed instantaneously in normal saline for 24 hours, the specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax. Sections of 6µm thickness were prepared and stained with Haematoxylin and eosin (Drury and Wallington, 1980). The prepared sections of liver were examined by light microscope.

Statistical Analysis

All obtained values were evaluated as mean ± Standard Error. The data was statistically analyzed using the unpaired Student's "t" test (Snedecor and Cochran, 1971). For all statistical evaluations, p < 0.05 were recognized as statistically significant.

RESULTS

Chemical characterization of isolated compounds:

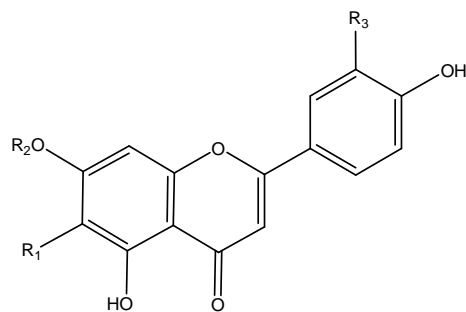
Eight compounds (**Figure. 1**) were isolated and identified as 6-methoxyapigenin (**1**), fisetin 3-*O*-β-glucopyranoside (**2**) and 3-Methoxy quercetin-7-*O*-β glucopyranoside(6→8'') 3''-methoxy kaempferol-7''-*O*-β-glucopyranoside (**3**). These three flavonoids were isolated from ethyl acetate fraction. Five compounds were isolated from ethyl alcohol fraction and identified as 4-methoxy benzoic acid (**4**), 6,7 dihydroxycoumarin (**5**) and the following flavonoids: Apigenin-7-*O*-β- neohesperidoside (**6**), 6-methoxyluteolin-7-*O*-β- neohesperidoside (**7**) and luteolin-7-*O*-β- neohesperidoside (**8**). (Mabry *et al.*, 1970; Markham, 1982; Harborne and Mabry, 1982; Harborne, 1994). These compounds were isolated for the first time from *Mayodendron igneum* plant and even from the genus. The structures of these compounds were established by their chromatographic properties (Table.1), by means of UV-Visible spectrophotometer (UV-VIS double beam UVD-3500, Labomed, Inc.), Mass spectrometers (Finnigan Model 3200 at 70 eV & Thermo-Finnigan LCQ Advantage MAX) and NMR spectrometers (JEOL ECA- 500 MHz & Varian Mercury VX-300- 300 MHz) as follows:

Table 1: Chromatographic properties of isolated compounds:

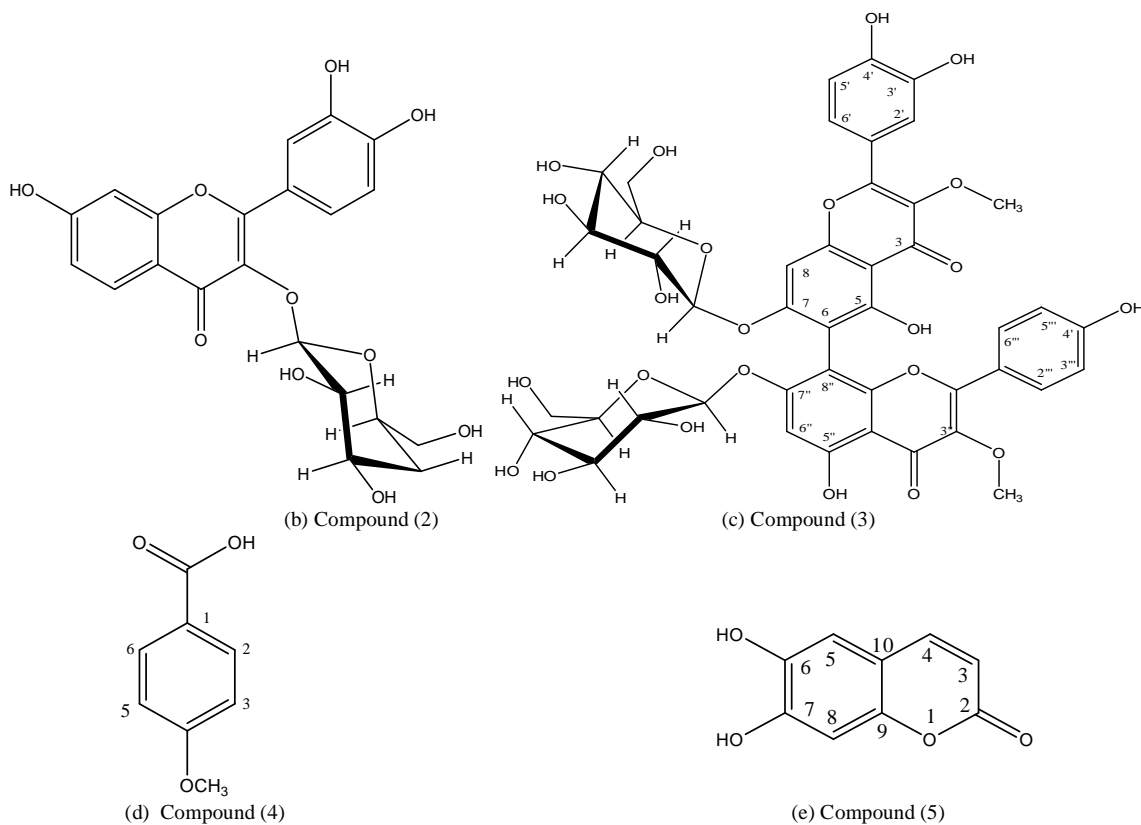
Compound No.	Solvent system S ₂	Solvent system S ₁	UV	UV/NH ₃	AlCl ₃ / UV
1	0.15	0.75	Purple	Yellow	Yellow
2	0.50	0.48	Blue	Yellow green	Yellow
3	0.16	0.29	Purple	Yellow	Yellow
4	0.49	0.85	Blue	Blue	—
5	0.52	0.45	Blue	Blue	—
6	0.48	0.42	Purple	Yellow	Yellow
7	0.17	0.37	Purple	Yellow	Yellow
8	0.32	0.23	Purple	Yellow	Yellow

Table 2: ¹H-NMR of isolated compounds in DMSO, 500MHz:

Proton No.	1	2	4	6	7	8
3	6.77(s)	-----	-----	6.78 (s)	6.69 (s)	6.70 (s)
5	-----	7.68 (d,J=9Hz)	-----	-----	-----	-----
6	-----	6.69 (m)	-----	6.36 (d,J=2Hz)	-----	6.36 (d,J=2Hz)
8	6.85(s)	6.69 (m)	-----	6.84 (d,J=2Hz)	6.94(s)	6.74 (d,J=2Hz)
2'	7.93 (d,J=8.5Hz)	7.28 (m)	7.50 (d,J=8.5Hz)	7.92 (d,J=8.7Hz)	7.38 (d,J=2Hz)	7.41 (d,J=2Hz)
6'	7.93 (d,J=8.5Hz)	7.28 (m)	7.50 (d,J=8.5Hz)	7.92 (d,J=8.7Hz)	7.38 (dd,J=2&8.4Hz)	7.41 (dd,J=2&8.4Hz)
3'	6.92 (d,J=8.5Hz)	-----	7.07 (d,J=8.5Hz)	6.95 (d,J=8.7Hz)	-----	-----
5'	6.92 (d,J=8.5Hz)	7.00 (d,J=8.5Hz)	7.07 (d,J=8.5Hz)	6.95 (d,J=8.7Hz)	6.83 (d,J=8.4Hz)	6.94 (d,J=8.4Hz)
Glucose H-1''	-----	5.87 (d,J=6.9Hz)	-----	5.20 (d,J=7Hz)	5.38 (d,J=7Hz)	5.20 (d,J=6.9Hz)
Rhamnose H-1'''	-----	-----	-----	5.13 (d,J=2Hz)	5.09 (d,J=2Hz)	5.12 (d,J=2Hz)
H-6'''	-----	-----	-----	1.13 (d,J=6Hz)	1.12 (d,J=6Hz)	1.13 (d,J=6.3Hz)
O-CH ₃	3.66 (s) at 6-position	-----	3.75 (s) at 4-position	-----	3.72 (s) at 6-position	-----



(a) Compound (1): R₁=OMe, R₂=H, R₃=H; Compound (6): R₁=H, R₂=neohesperidose, R₃=H; Compound (7): R₁=OMe, R₂=neohesperidose, R₃=OH
Compound (8): R₁=H, R₂=neohesperidose, R₃=OH

**Fig. 1:** Structures of compounds isolated from *M. igneum*.

The successive ethyl acetate fraction yielded:

6-Methoxyapigenin (1) (Fig. 1-a)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 260, 330; (NaOMe): 272, 383; (AlCl₃): 275, 300(sh), 355; (AlCl₃ / HCl): 220(sh), 276, 300(sh),350; (NaOAc): 271, 392; (NaOAc / H₃BO₃): 267,332. ¹H-NMR: (Table.2).

Fisetin 3-O-β-D-glucopyranoside (2) (Fig. 1-b)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 220(sh), 252(sh), 300, 330; (NaOMe): 270, 324, 378; (AlCl₃): 240(sh), 273, 320, 372; (AlCl₃ / HCl): 220(sh), 252, 300, 328; (NaOAc): 250(sh), 310, 327, 380; (NaOAc / H₃BO₃): 260, 300,351. ¹H-NMR: (Table.2).

Complete acid hydrolysis of compound (2): Compound (2) was hydrolysed with 1N HCl for 5 hours. The mixture was diluted with water and shaken with ethyl acetate. The ethyl acetate (containing fisetin) and water (containing glucose) were co-chromatographed on PC with authentic samples.

3-Methoxy quercetin-7-O-β glucopyranoside(6→8'') 3''-methoxy kaempferol-7''-O-β-glucopyranoside (3) (Fig. 1-c):

Dark yellow powder, chromatographic properties and R_f (Table.1). UV λ_{max} (MeOH): 255, 271,335; (NaOMe): 274,395; (AlCl₃): 275,299(sh), 340,415; (AlCl₃ / HCl): 275, 297 (sh),370 ; (NaOAc): 271, 401; (NaOAc / H₃BO₃): 269,359. ESI-MS (3) exhibited molecular ion peaks at m/z 937, corresponding to molecular weight [M⁺] 938 and molecular formula (C₄₄H₄₂O₂₃) of biflavonoid structure. ¹H-NMR (DMSO-d₆-300MHz): δ: 7.95 (*d*, *J*= 9 Hz) (H-2'',H-6''); 7.40 (*dd*, *J*= 9&2 Hz, H-2', H-6'); 6.95 (*d*, *J*= 9 Hz, H-3'', H-5'' and H-5'); 6.68 (*s*, H-8); 6.42(*s*, H-6''); 5.00(*d*, *J*= 7 Hz, 2x(H-1 of glucose at 7 and 7''- O-glucosyl); 3.76 (*s*, OCH₃-3 and OCH₃-3''); 3.17-3.50 (remaining two glucosyl protons).

The successive ethyl alcohol fraction of *M. igneum* yielded:

4-methoxy benzoic acid (4) (Fig. 1-d)

White amorphous powder, PCR_f (Table.1). MS: m/z 153 [M⁺ +H] corresponding to molecular weight [M⁺] 152 and molecular formula (C₈H₈O₃). ¹H-NMR (DMSO-d₆-300MHz): δ: 7.50 (*d*, *J*= 8.5 Hz, H-2,6); 7.07 (*d*, *J*= 8.5 Hz, H-3,5); 3.75 (*s*, OCH₃-4).

6,7 dihydroxycoumarin (5) (Fig. 1-e)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 226, 283, 297, 334; (NaOMe): 230, 280, 298, 386; (AlCl₃): 229 (sh), 275, 369; (AlCl₃ / HCl): 229 (sh), 282, 333 ; (NaOAc): 268,365(sh), 400; (NaOAc / H₃BO₃): 261,359. ESI-MS: m/z 179 [M⁺ +H] corresponding to molecular weight [M⁺] 178 and molecular formula (C₉H₆O₄). ¹H-NMR (DMSO-d₆-500MHz): δ:7.78 (*d*, *J*= 9.5 Hz, H-4); 6.93 (*s*, H-5); 6.70 (*s*, H-8); 6.10 (*d*, *J*= 9.5 Hz, H-3).

Apigenin-7-O-β- neohesperidoside (6) (Fig. 1-a)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 268, 334; (NaOMe): 240(sh), 269, 295(sh), 389; (AlCl₃): 276,300, 350, 385; (AlCl₃ / HCl): 277, 300,345,382; (NaOAc): 267, 345, 393; (NaOAc / H₃BO₃): 267,339. ¹H-NMR: (Table.2). Complete acid hydrolysis of compound (6): Compound (6) was hydrolysed with 1N HCl for 5 hours. The mixture was diluted with water and shaken with ethyl acetate. The ethyl acetate (containing apigenin) and water (containing glucose and rhamnose) were co-chromatographed on PC with authentic samples.

6-methoxyluteolin-7-O-β- neohesperidoside (7) (Fig. 1-a)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 255 (sh), 272,346; (NaOMe): 273, 400; (AlCl₃): 261,273, 300,423; (AlCl₃ / HCl): 261, 276, 296,363; (NaOAc): 272, 409; (NaOAc / H₃BO₃): 266,370. ¹HNMR (DMSO-d₆-500MHz): δ: (Table.2). Complete acid hydrolysis of compound (7): Compound (7) was hydrolysed with 1N HCl for 5 hours. The mixture was diluted with water and shaken with ethyl acetate. The ethyl acetate (containing 6-methoxyluteolin) and water (containing glucose and rhamnose) were co-chromatographed on PC with authentic samples.

Luteolin-7-O-β- neohesperidoside (8) (Fig. 1-a)

Yellow powder, PCR_f (Table.1). UV λ_{max} (MeOH): 263,341; (NaOMe): 266,300(sh), 397; (AlCl₃): 273,299(sh), 342,424; (AlCl₃ / HCl): 270, 300 (sh),357,390; (NaOAc): 263,357, 406; (NaOAc / H₃BO₃): 260,366. ¹HNMR (DMSO-d₆-300MHz): δ: (Table.2).

Complete acid hydrolysis of compound (8): Compound (8) was hydrolysed with 1N HCl for 5 hours. The mixture was diluted with water and shaken with ethyl acetate. The ethyl acetate (containing luteolin) and water (containing glucose and rhamnose) were co-chromatographed on PC with authentic samples.

Median lethal dose (LD₅₀)

The total ethanol extract of leaves of *M. igneum* was found to be practically non toxic when administered orally to mice and its LD₅₀ values was found to be 6.7 g/kg body weight and explained its use as vegetable. One tenth of LD₅₀ was selected for evaluation of hepatoprotective activity "i.e. 0.67 g/kg weight of total ethanol and successive extracts were used".

BIOCHEMICAL RESULTS

Prophylactic test

[a] Serum alanine aminotransferase (ALT, SGPT):

A significant increase (p< 0.05) of ALT level was recorded in paracetamol treated group as compared with the control group. Significant decrease (p< 0.05) was recorded in ALT level of groups III, IV, V, VI, VII as compared with paracetamol group (Table.3).

Table. 3: Liver function of rats given different extracts of *M. igneum* Kurz and paracetamol. (Prophylactic test).

Groups	ALT U/l	AST U/l	ALP U/l
Group I	30.2 ± 3.8	93 ± 1.6	65.5 ± 1.2
Group II	152.8 ± 1.8*	223.1 ± 2.8*	255.6 ± 4.5*
Group III	50.3 ± 0.7**	104.3 ± 4.3**	78.1 ± 2.8**
Group IV	34.2 ± 2.7**	123.1 ± 9.1**	123.6 ± 3.3**
Group V	55.7 ± 1.2**	116.1 ± 6.5**	91.4 ± 8.1**
Group VI	39.5 ± 2.1**	134.3 ± 3.7**	107.1 ± 7.4**
Group VII	30.5 ± 7.3**	132.9 ± 5.5**	84.1 ± 4.7**

Data presented as means ± standard error

* Significant at $p < 0.05$ as compared with control group

** Significant at $p < 0.05$ as compared with paracetamol group

[b] Serum aspartate aminotransferase (AST, SGOT)

The results showed significant increase ($P < 0.05$) of AST level for the paracetamol group as compared with the control group, while groups III, IV, V, VI and VII revealed significant decrease in the AST level when compared with the paracetamol group (Table.3).

[c] Alkaline phosphatase (ALP)

A significant increase ($P < 0.05$) in the serum ALP was recorded for paracetamol group as compared with the control group. Groups III, IV, V, VI and VII revealed significant decrease in the level of serum ALP as compared with the paracetamol group (Table.3).

Therapeutic test

a] Serum alanine aminotransferase (ALT, SGPT)

Paracetamol group showed significant increase in the level of ALT as compared with the control group. The following groups, VIII, IX, X XI and XII exhibited significant decrease in the level of ALT as compared with the paracetamol groups (Table.4).

b] Serum aspartate aminotransferase (AST, SGOT)

Serum AST obtained from the paracetamol group showed significant increased level, as compared with the control one. The comparison of the above results showed significant decrease ($P < 0.05$) in AST level for groups VIII, IX, X XI and XII when compared with the paracetamol group (Table.4).

[c] Alkaline phosphatase (ALP)

Concerning the paracetamol group, a significant increase ($p < 0.05$) in the serum ALP was recorded as compared with the control group. On the other hand, groups VIII, IX, X XI and XII revealed significant decrease in the level of serum ALP as compared with the paracetamol group (Table.4).

HISTOPATHOLOGICAL RESULTS

Histopathological examination of rat's liver of the control group, showed the hepatic lobules (the structural units of the liver); each is formed of cords of hepatocytes (HC) and blood sinusoids (HS) in between (**Figure.2a**). Histopathological examination of liver of rats after 24 hrs of administration of one

dose of paracetamol equivalent to 2 g/kg showed mild lymphocytic infiltration in the portal and periportal area (**Figure.2b**, **small arrow**), that associated with dilatation and congestion of the veins (**Figure.2b**, **big arrow**). Notice the presence of focal necrosis and vacuoles (**Figure.2b**).

Prophylactic effect

Oral administration of silymarin at a dose of 50 mg/kg p.o. for seven days prior to one oral dose of paracetamol equivalent to 2 g/kg on the eighth day revealed that the hepatocytes of the treated rats appeared more or less as normal. In some rats, slight inflammatory infiltration was occurred in the portal and periportal tract (**Figure.2c**, **big arrow**).

In rats receiving daily doses of total ethanol extract of *M. igneum* at a dose of 0.67 g/kg body weight/day, p.o. for seven days and on the eighth day given one dose of paracetamol equivalent to 2 g/kg, the liver sections showed hepatocytic degeneration around the central vein (**Figure.2d**, **big arrow**) and some vacuoles (**Figure.2d**, **small arrow**). Examination of liver of rats given oral dose of petroleum ether extract of the plant (0.67 g/kg body weight/day, p.o.) for seven days and on the eighth day given one dose of paracetamol (2 g/kg), the liver showed normal structure. Dilatation of the hepatic sinusoids (**Figure.2e**, **big arrow**) and activated Kupffer cells were detected (**Figure.2e**, **small arrow**).

Microscopic examination of liver sections of rats treated with chloroform extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) for seven days prior to a single oral dose of paracetamol (2 g/kg body weight) on the eighth day showed normal structure of the liver except for the presence of some vacuoles in the hepatocytes (**Figure.2f**, **big arrow**) and hepatic sinusoids were dilated (**Figure.2f**, **small arrow**). Rats given oral dose of ethyl acetate combined with ethanol extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) for seven days followed by one dose of paracetamol equivalent to 2 g/kg on the eighth day, the liver showed normal structure except for the presence of a few necrotic hepatocytes (**Figure.2g**, **small arrow**).

Therapeutic effect

Histopathological examinations of rat's liver given one dose of paracetamol equivalent to 2 g/kg followed by oral dose of silymarin (50 mg/kg body weight/day, p.o.) for seven days, showed normal hepatocytes except for some dilatation of the hepatic sinusoids (**Figure.2h**, **big arrow**). Examination of liver sections of rats given one dose of paracetamol (2 g/kg) followed by a daily dose (7 days) of total ethanol extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) showed normal structure except for dilatation of hepatic sinusoids (**Figure.2i**, **big arrow**). Histopathological examinations of liver of rats given one dose of paracetamol (2 g/kg) followed by oral dose of petroleum ether extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) for seven days, showed normal structure of hepatocytes. Some of the hepatocytes showed swelling or ballooning (**Figure.2j**, **big arrow**).

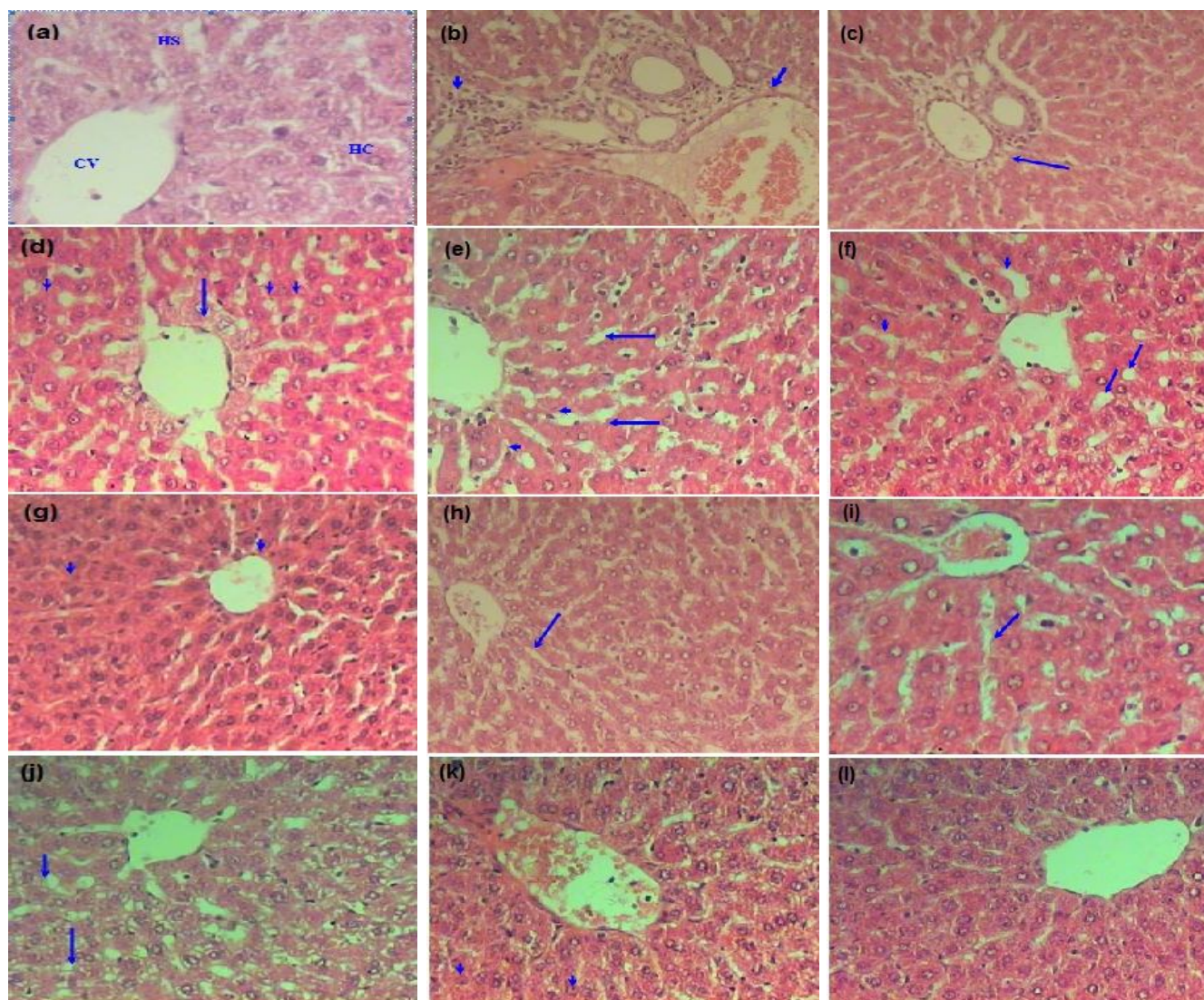


Fig. 2: A photomicrograph (H&E stained) of liver sections of control group(a), control intoxicated with paracetamol (b), Prophylactic effect: control treated with silymarin for seven days prior to a single dose of paracetamol on the eighth day (c), control treated with total ethanol extract for seven days prior to a single dose of paracetamol on the eighth day (d), control treated with petroleum ether extract for seven days prior to a single dose of paracetamol on the eighth day (e), control treated with chloroform extract for seven days prior to a single dose of paracetamol on the eighth day (f), control treated with ethyl acetate combined with ethanol extract for seven days prior to a single dose of paracetamol on the eighth day (g), Therapeutic effect: paracetamol group treated with silymarin for seven days (h), paracetamol group treated with total ethanol extract for seven days (i), paracetamol group treated with petroleum ether extract for seven days (j), paracetamol group treated with chloroform extract for seven days (k), paracetamol group treated with ethyl acetate combined with ethanol extract for seven days (l). CV: Central vein; HC: Hepatocytes; HS: Hepatic sinusoids.

Examination of liver sections of rats given one dose of paracetamol (2 g/kg) followed by oral dose of chloroform extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) for seven days, showed foci of necrotic cells (**Figure.2k, small arrow**).

Liver sections of rats given one dose of paracetamol (2 g/kg) followed by oral dose of ethyl acetate combined with ethanol extract of *M. igneum* (0.67 g/kg body weight/day, p.o.) for seven days, showed normal structure (**Figure.2l**)

DISCUSSION

Biological screening of total ethanol extract of *M. igneum* leaf revealed significant antioxidant activity. The strongest inhibition of DPPH stable free radicals was exhibited by the

successive ethanol fraction followed by the successive ethyl acetate fraction (Hashem *et al.*, 2012). Phytochemical investigations and hepatoprotective testing on these phytoactive, antioxidant extracts were continued in this study.

The combined successive ethyl acetate and ethanol extracts of *M. igneum* leaf contained a complicated phenolic mixture. Eight phenolic constituents were isolated and purified by standard methods. These constituents were six flavonoids, one coumarin and one phenolic acid. Complete acid hydrolysis for O-glycosides was carried out, followed by paper co-chromatography with authentic samples to identify the hydrolytic products (aglycones and sugar moieties). The spectral analysis of the isolated compounds included UV, MS and NMR. The biflavonoid (**3**) is a new compound isolated for the first time from nature,

unfortunately ^{13}C NMR could not be done in the current study as the small amounts left no material for ^{13}C NMR. The total ethanol extract of *M. igneum* was found to be of low toxicity and explained its use as vegetable ($\text{LD}_{50} = 6.7 \text{ g/Kg}$ body weight). This is the first study to show the hepatoprotective activity of *M. igneum* on rats using paracetamol-induced hepatotoxicity.

Paracetamol administration causes severe liver damage in rats, demonstrated by remarkable elevation of serum ALT, AST and ALP levels. However, hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites, (Vermeulen *et al.*, 1992) which lead to liver injury. Due to liver injury, the transport function of the hepatocytes gets disrupted, resulting in the leakage of plasma membrane, thereby causing an increased enzyme level in the serum (Zimmerman and Seeff, 1970). Administration of the different extracts of *M. igneum*, before (Prophylactic action) or after (therapeutic action) paracetamol provided significant hepatoprotective effect. The biochemical results of the present work were in accordance with histopathological results confirming that the different extracts of *M. igneum* possess both prophylactic and therapeutic effects against experimentally induced hepatic damage by paracetamol.

CONCLUSION

The results showed that paracetamol administration causes severe acute liver damage in rats, demonstrated by remarkable elevation of serum ALT, AST and ALP levels. The increased serum levels of ALT, AST and ALP have been attributed to the damaged structural integrity of the liver. The biochemical parameters revealed that all the extracts showed significant decrease in the liver enzymes when paracetamol was given before the extracts (therapeutic effect) or given after the extracts (prophylactic effect).

By scrutinizing the histopathological results, it could be concluded that: the different extracts of *M. igneum* possesses both prophylactic and therapeutic activity effects against paracetamol induced liver injury. Administration of different extracts of *M. igneum* Kurz, especially ethyl acetate combined with ethanol extracts, resulted in a significant decrease in serum ALT, AST and ALP levels and also regeneration of hepatic architecture as compared with the paracetamol-treated group. This is the first study to show the hepatoprotective activity of *M. igneum* on rats using paracetamol-induced hepatotoxicity method. The biochemical results were in accordance with histopathological results. It can be concluded that *M. igneum* has a promising activity as hepatoprotective against liver damage.

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