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Pharmaco-Chemical Studies on the Aqueous Methanolic Extract of *Diospyros lotus* Leaves

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Abstract: This study evaluated phytochemical and some biological activities of the aqueous methanolic extract of the leaves of *Diospyros lotus* L. Eight phenolic compounds were isolated and identified as gallic acid, methylgallate, ellagic acid, kaempferol, quercetin, myricetin, myricetin 3-O-β-glucuronide and myricetin 3-O-α-rhamnoside. The structures were established on the basis of chemical and spectral analysis. The extract was tested for antioxidant, analgesic, anti-inflammatory and hepatoprotective activities. The 70% methanolic extract of *D. lotus* up to 5 g kg^{-1} b. wt. revealed no obvious toxic effects in mice and its antioxidant activity showed a marked significant scavenging activity. On the other hand, the extract indicates a central and peripheral analgesic effect. The acute paw oedema response was significantly reduced. The protective effect of the extract against acetaminophen induced liver damage was indicated by the reduction of the elevation of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT) that caused by acetaminophen. The phenolic compounds isolated from *Diospyros lotus* in this study were found to be isolated for the first time from the genus *Diospyros*.

Key words: Analgesic, anti-inflammatory, antioxidant, *Diospyros lotus* leaves, hepatoprotective, phenolic compounds

INTRODUCTION

The Ebenaceae are a family of flowering plants, which include ebony and the persimmons. The family has approximately 500 species of trees and shrubs in two genera, being Diospyros and Euclea (Brummitt, 1992). The genus *Diospyros* is the largest with ca 80% of all species of the family distributed in the humid tropics of Asia, Africa and Central South America (Cronquist, 1981; Ng, 1986; Whitmore, 1978). This genus is home to the persimmons. The uniqueness of the genus is the elaboration of a large number of pentacyclic triterpenes, phenolic, naphthalene glycoside and juglone based 1,4-naphthoquinone metabolites (Mallavadhani *et al*., 1998; Pathak *et al*., 2004).

Diospyros lotus L. is a tree growing to about 15 m and it is native to Middle East and South Asia, especially from China and Japan (Chittendon, 1956; Hedrick, 1972). In traditional medicine, *D. lotus* fruits are used as a sedative, antitussive, antiseptic, antidiabetic, Antitumor, astringent, laxative, nutritive and as a febrifuge (Simmons, 1972; Chopra *et al*., 1986; Ebrahimzadeh *et al*., 2008). In addition, *D. lotus* fruits are used to treat diarrhea, dry coughs and hypertension (Bown, 1995). The previous phytochemical investigation of *D. lotus* plant revealed the presence of some fatty acids and

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non volatile acids in the fruits (Ahmet and Kadioglu, 1998), total phenol and flavonoid content (Ebrahimzadeh *et al*., 2008), terpens (Khasan *et al*., 1976), naphthoquinones (Yoshihira *et al*., 1971).

In the course of phytochemical investigation on Egyptian plants (Hawas, 2007), the isolation and structure elucidation of the phenolic compounds from aqueous methanolic extract of the *Diospyros lotus* leaves were described. Antioxidant, anti-inflammatory, analgesic and hepatoprotective activities were evaluated for the investigated extract, as well.

MATERIALS AND METHODS

Experimental

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR spectra: Varian Unity Inova 400 (400 MHZ); ^{13}C-NMR spectra: Varian Unity 400 (100 MHZ) (Graz University, Austria). MS (Finnigan MAT SSQ 7000, 70 eV). Polyamide (MN-polyamide SC6, Machery Nagel, for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Solvent mixtures, BAW (n-butanol:acetic acid:water 4:1:5 upper phase). Paper Chromatography (PC), Whatman No. 3 MM (46×57 cm).

Plant Material

The leaves of *Diospyros lotus* L. were collected from the Agricultural Research Centre, Giza, Egypt in April 2006 during flowering and identified by Dr. Kamal El-Batanony, Professor of Taxonomy and Botany, Faculty of Science, Cairo University. Voucher specimens are deposited in the herbarium of the National Research Centre (CAIRC), Cairo, Egypt.

Animals

Adult rats of both sexes weighing 150-200 g and adult rat weighing 20-25 g (Laboratory Animal Colonies, NRC, Cairo, Egypt) were used in the experiments. The animals were allowed access to standard rat chow and tap water *ad libitum*. They were maintained in a controlled environment at 22-25 °C and 60±5 % relative hnmidity with a 12 h dark/light cycle and acclimatized for at least one week prior to use. Animal were randomly assigned to different experimental groups, each kept in separate cage.

All animal procedures were performed after approval from the Ethics Committee of the National Research Centre and in accordance with the recommendations for the proper care and use of laboratory animals.

Drugs and Chemicals

Acetaminophen (BDH Chemicals, England); Silymarin (SEDICO, Cairo, A.R.E), Indomethacin capsules (Khahira Pharmaceutical and Chemical Company, Cairo, Egypt); Tramadol (Octobar Pharma, Cairo, Egypt); DPPH (Diphenyl picrylhydrazyl, Sigma Chemical Company, USA); Carrageenan (Sigma-Aldrich Chemical Company, USA); Diagnostic kits (Quimica Clinica Aplicada S.A., Spain). The used kit's included sernm gamma glutamyltransferase (GGT) aspartate and alanine aminotransferase (AST and ALT) were used in the experiments. The doses employed were based upon the hnman dose after conversion to that of rat (Paget and Barnes, 1964).

Extraction and Isolation

The air-dried powdered leaves of *D. lotus* (500 g) were repeatedly extracted in the room temperature with 70% methanol until exhaustion. The extracts were combined and concentrated under reduced pressure to give 115 g. The soluble crude extract (74 g) was subjected to a polyamide column chromatography (200 g) and the column was eluted in the gradient mode from H_2O to MeOH. One

hundred and fifty fractions were collected. Fractions showing similar PC profiles in BAW and 15% acetic acid were pooled to provide combined fractions (I, II, III, IV). The fractions were further purified on preparative paper and Sephadex LH-20 column chromatography. Fraction (I) was subjected to paper chromatography (3 mm) using 15% acetic acid as eluent. Two dark purple bands appeared under UV light, changed to yellow when fumed with ammonia vapour. Each band was cutted off and eluted with 70% methanol to yield compounds **(7)** (5.2 mg) and **(8)** (10.8 mg) as flavonoid glycoside constituents. Soluble fraction II was purified on paper chromatography (3 mm) using BAW as eluent to get mauve band along with two yellow bands under UV light. Each band was separated, dissolved in methanol and loaded directly to Sephadex LH-20 column eluted with ethyl alcohol to get compounds **(1)** (6.5 mg), **(5)** (4.8 mg) and **(6)** (7.5 mg). Compounds **(2)** (6 mg) and **(4)** (5.5 mg) were isolated from fraction III by subjecting the soluble fraction on preparative paper chromatography (3 mm) using BAW as eluent, while compound **(3)** (4.2 mg) was purified from fraction IV through column Sephadex LH-20 eluted by methanol.

Determination of Median Lethal Dose (LD_{50})

The LD_{50} of the aqueous methanolic extract of *D. lotus* leaves was determined using mice. Six groups of six mice were treated orally with different doses of the soluble extract. One group was used as a control and given orally the respective volume of the vehicle (1% Tween 80 in distilled water). The tested extract of the plant was suspended in the vehicle immediately before use and were orally administered to the groups, through increasing doses from 100 mg till 5000 mg/100 g b. wt. All groups were kept under observation for 24 h after administration of the extracts to record any gross behavioral changes, symptoms of toxicity and mortality (Litchfield and Wilcoxon, 1949).

Evaluation of Antioxidant Effect (*in vitro*)

The antioxidant activity of the *D. lotus* extract was studied *in vitro* using the DPPH method. The activity of DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging was investigated according to method of Peiwu *et al*. (1999). In this method, a methanolic solution of DPPH (2.95 mL) was added to 50 µL sample of different concentrations of the extracts (10-100 mg mL^{-1}) in disposable cuvette. The absorbance was measured at 517 nm at regular intervals of 15 sec for 5 min. Ascorbic acid was used as a standard (0.1 M concentration) (Govindaragan *et al*., 2003).

$$\text{Inhibition (\%) (Reactive reaction rate)} = \frac{\text{Abs. (DPPH solution)-Abs. (sample)}}{\text{Abs. (DPPH solution)}} \times 100$$

Test on Analgesia

Hot-Plate Test

The hot-plate test was performed on mice by using an electronically controlled hot plate (µgo Basile, Italy) (50±0.1°C) for possible centrally mediated analgesics effect of the extract according to the method of Woolfe and MacDonald (1944). Four groups of 6 mice each were given vehicle and/or the extract (50 and 25 mg/100 g b. wt.) and the last group received Tremadol (2 mg/100 g b. wt.) 60 min prior for testing. Latency to lick a hind paw or jnmping was recorded sequentially before and at 1 and 2 h post treatment (Eaton, 2003).

Acetic Acid-Induced Writhing

Four separate groups of mice (6 mice each) were administered the vehicle, indomethacin (2 mg/100 g b. wt.) and/or the tested extract (50 and 25 mg/100 g b. wt.). After 60 min interval, an i.p. injection of 0.6% acetic acid was administered (Koster *et al*., 1959; Chakraborty *et al*., 2004). Each mouse was then placed in an individnal clear plastic observational chamber and the total nnmber of writhes made by each rat was counted for 20 min.

Anti-Inflammatory Effect

The carrageenan was used to evaluate the anti-inflammatory properties of the tested extract (Winter *et al*., 1962). Rats were randomly assigned to treatment groups and sterile carrageenan lambda (100 µL of a 1% solution in saline) was injected sub-planter into the right hind paw of the rat. The contralateral hind paw received the same volume of saline and served as a normal control. Carrageenan caused visible redness and pronounced swelling that was well developed by 4 h and persisted for more than 48 h (Vinegar *et al*., 1976). Hind footpad thickness was measured with a micrometer caliber (Obukowics *et al*., 1998; Meng *et al*., 1999) before and at 1, 2, 3 and 4 h after carrageenan injection. Four groups of rats each of six animals were administered either saline (1 mL) and served as control or the tested extract (50 or 25 mg/100 g b. wt. orally) and Indomethacin (2 mg/100 g b. wt.). The tested extract or indomethacin was given 1 h before carrageenan injection.

Hepatoprotective Effect

The Acetaminophen-Induced Hepatic Damage

The hepatoprotective effect of the aqueous methanolic extract of *D. lotus* leaves was evaluated in normal and liver damaged rats whose livers have been partly damaged by the administration of acetaminophen in a dose of 1 g kg, p.o., to induce hepatic injury and fibrosis (Beales and McLean, 1996). Rats were divided into four groups of rats (6/group). Group I, the normal control group was given a single daily dose of the vehicle saline while group II and III were received the extract orally at doses of 25 or 50 mg/100 g and group III, the animals received Silymarin (22 mg kg^{-1}, p.o.) daily for 10 days, at the end of this period all groups received acetaminophin (1 g kg^{-1}, p.o.). Rats had free access to food and driuking water during the study period.

Biochemical Assessment

At the end of the experiments, blood samples were obtained from the retro-orbital vein plexus, under ether anaesthesia. The activities of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were measured according to the colourimetric method described by Bergmeyer *et al*. (1986), whereas gamma glutamyltransferase (GGT) activity was assayed according to standard method of Szasz (1969).

Statistical Analysis

Data were expressed as Mean±SE. The differences between control and treated groups were determined by one-way ANOVA followed by the least significant difference (LSD) (Armitage, 1971).

RESULTS

Chemical Characterization of Isolated Compounds

The isolated compounds led to the identification of eight phenolic compounds, as two hydrolyzable tannins, gallic acid **(1)**, its methyl ester **(2)** and ellagic acid **(3)** (Foo, 1993; Nawwar *et al*., 1994); three flavonol aglycones, kaempferol **(4)**, quercetin **(5)** and myricetin **(6)** (Mabry *et al*., 1970) and two flavonol glycosides, myricetin 3-O-β-glucuronide **(7)** and myricetin 3-O-α-rhamnoside **(8)** (Markham, 1982; Harborne and Mabry, 1982) from aqueous methanolic extract of *D. lotus* leaves.

The structures of the pure isolated compounds were established by means of UV and NMR spectroscopy and MS spectrometry. Complete acid hydrolysis of flavonol glycosides 7 and 8 was carried out for 1 h in methanol at 100°C using 2N HCl, to yield myricetin as a flavonol aglycone and glucronic acid and rhamnose, respectively, as sugar moieties.

Gallic Acid (1)

White amorphous powder, PC R_f 0.67 (BAW). UV λmax (MeOH): 273. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 7.15 (2H, s, H-2,6). ^{13}C-NMR (DMSO-d_6, 100 MHZ): δ 167.2 (-COOH), 145 (C-3,5), 137.7 (C-4), 121 (C-1), 109.1 (C-2,6).

Methyl Gallate (2)

White amorphous powder, PC R_f 0.76 (BAW). UV λmax (MeOH): 275. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 6.94 (2H, s, H-2,6), 3.73 (3H, s, -OCH_3). ^{13}C-NMR (DMSO-d_6, 100 MHZ): δ 166.8 (-COO), 146 (C-3,5), 138.9 (C-4), 119.8 (C-1), 109 (C-2,6), 52 (-OCH_3).

Ellagic Acid (3)

White amorphous powder, PC R_f 0.34 (BAW). UV (λ_{max}, nm): 255, 362. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 7.44 (2H, s, H-4,9). ^{13}C-NMR (DMSO-d_6, 100 MHZ): δ 158.8 (5,10-CO), 147.8 (C-3,8), 139.3 (C-2,7), 136.1 (C-1a,6a), 112 (C-4b,9b), 110.2 (C-4,9), 107.3 (4a,9a).

Kaempferol (4)

Yellow powder, PC R_f 0.82 (BAW) and 0.05 (15% HOAc). UV λmax (MeOH): 265, 320, 366; (NaOMe): 276, 317, 406; ($AlCl_3$): 262sh, 269, 310sh, 367; ($AlCl_3$/HCl): 263sh, 268, 320sh, 344, 425; (NaOAc): 274, 306, 382; (NaOAc/H_3BO_3): 267, 368. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 8.11 (2H, d, J = 8 Hz, H-2',6'), 6.96 (2H, d, J = 8 Hz, H-3',5'), 6.47 (1H, d, J = 2 Hz, H-8), 6.19 (1H, d, J = 2 Hz, H-6). EI-MS: m/z 286.

Quercetin (5)

Yellow powder, PC R_f 0.91 (BAW) and 0.12 (15% HOAc). UV λmax (MeOH): 255, 267, 371; (NaOMe): 270, 320, 420; ($AlCl_3$): 270, 455; ($AlCl_3$/HCl): 264, 303sh, 315sh, 428; (NaOAc): 257, 274, 318, 383; (NaOAc/H_3BO_3): 259, 387. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 7.74 (1H, d, J = 8, 2 Hz, H-2'), 7.55 (1H, d, J = 2 Hz, H-6'), 6.92 (1H, d, J = 8 Hz, H-5'), 6.42 (1H, d, J = 1.2 Hz, H-8), 6.15 (1H, d, J = 1.2 Hz, H-6). EI-MS: m/z 302.

Myricetin (6)

Yellow powder, PC R_f 0.45 (BAW) and 0.27 (15% HOAc). UV λmax (MeOH): 254, 272sh, 374; (NaOMe): 262sh, 285sh, 322sh, 423(Dec.); ($AlCl_3$): 271, 316sh, 450; ($AlCl_3$/HCl): 266, 275sh, 308sh, 360sh, 428; (NaOAc): 269, 335(Dec.); (NaOAc/H_3BO_3): 258, 304sh, 392. ^{1}H-NMR (MeOD, 400 MHZ): δ 7.35 (2H, s, H-2',6'), 6.43 (1H, d, J = 1.5 Hz, H-8), 6.18 (1H, d, J = 1.5 Hz, H-6). ^{13}C-NMR (MeOD, 100 MHZ): δ 177.5 (C-4), 165.8 (C-7), 162.6 (C-5), 158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5 (C-3), 137.1 (C-4'), 123.3 (C-1'), 108.8 (C-2',6'), 104.7 (C-10), 99.5 (C-8), 94.6 (C-6). EI-MS: m/z 316.

Myricetin 3-O-β-Glucuronide (7):

Yellow amorphous powder; PC R_f 0.34 (BAW) and 0.48 (15% HOAc). UV λmax (MeOH): 262, 298sh, 349; (NaOMe): 272, 324, 392; ($AlCl_3$): 272, 312, 428; ($AlCl_3$/HCl): 270, 310, 404; (NaOAc): 270, 318, 366; (NaOAc/H_3BO_3): 260, 300, 374. ^{1}H-NMR (MeOD, 400 MHZ): δ 7.42 (2H, s, H-2',6'), 6.45 (1H, d, J = 1.2 Hz, H-8), 6.22 (1H, d, J = 1.2 Hz, H-6), 5.47 (1H, d, J = 7.5 Hz, H-1''). ^{13}C-NMR (MeOD, 100 MHZ)): δ 177.5 (C-4), 174 (C-6''), 165.8 (C-7), 162.6 (C-5), 158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5 (C-3), 137.1 (C-4'), 123.3 (C-1'), 108.8 (C-2',6'), 104.7 (C-10), 104 (C-1''), 99.5 (C-8), 94.6 (C-6), 78.2 (C-3''), 78 (C-5''), 75.6 (C-2''), 73.4 (C-4'').

Myricetin 3-O-α-Rhamnoside (8)

Yellow amorphous powder; PC R_f 0.51 (BAW) and 0.43 (15% HOAc). UV λmax (MeOH): 262, 298sh, 353; (NaOMe): 273, 321, 392; ($AlCl_3$): 272, 312, 420; ($AlCl_3$/HCl): 270, 310, 404; (NaOAc): 270, 317, 364; (NaOAc/H_3BO_3): 260, 303, 376. ^{1}H-NMR (DMSO-d_6, 400 MHZ): δ 6.85 (2H, s, H-2',6'), 6.35 (1H, d, J = 1.2 Hz, H-8), 6.15 (1H, d, J = 1.2 Hz, H-6), 5.15 (1H, b, H-1''), 0.9 (1H, d, J = 6 Hz, CH_3-rhamnosyl). ^{13}C-NMR (DMSO-d_6, 100 MHZ)): δ 178 (C-4), 165.8 (C-7), 162.6 (C-5), 158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5 (C-3), 137.1 (C-4'), 123.3 (C-1'), 108.8 (C-2',6'), 104.7 (C-10), 102.8 (C-1''), 99.5 (C-8), 94.6 (C-6), 72 (C-5''), 71.8 (C-3''), 71 (C-2''), 70.5 (C-4''), 18 (CH_3-rhamnosyl).

Pharmacological Results

LD_{50} (Toxicity)

Oral administration of different doses of the methanolic extract of *D. lotus* up to 5 g kg^{-1} b. wt. revealed no obvious toxic effects and all the treated animals were still alive after 24 h.

Antioxidant Effect (*in vitro*)

The antioxidant activity of methanolic extract of *D. lotus* was studied *in vitro* using DPPH method. The results of the kinetics of DPPH scavenging reaction of the tested extracts and L-ascorbic acid were demonstrated in *D. lotus* methanolic extract in different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg mL^{-1}) and showed a strong scavenging activity (antioxidant activity). The maximum reactive reaction rate after 5 min were 82.5, 79.3, 77.9, 73.6, 72.4, 65.2, 61.7, 62.5, 66.1 and 66.9, respectively when compared to L-ascorbic acid (89.8). The lower doses seem to be more active than the higher one as shown in Fig. 1. Scavenging activity % for 10 mg mL^{-1} was 82.5% and 66.9% for 100 mg mL^{-1} when compared to L-ascorbic acid (89.8).

Effect on Analgesia

Hot Plate Test

The mean reaction time on the hot plate showed significant delay after the administration of the *D. lotus* extracts (25 and 50 mg/100 g b. wt.). After 1 h, the percentages of change were 26.5, 31.9% and 51.5, 58%, after 2 h. Tremadol showed a significant delay by percent of change 48 and 68% after 1 and 2 h indicating a central analgesic effect (Table 1).

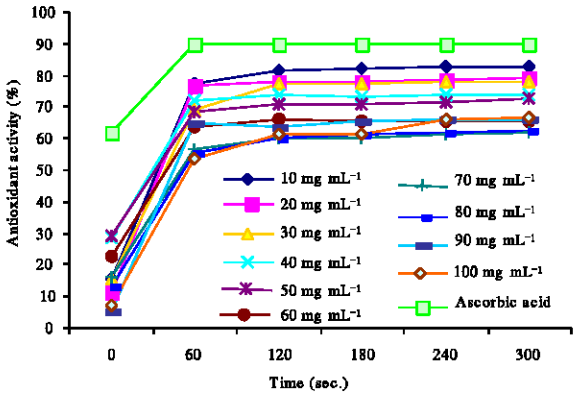

Fig. 1: The antioxidant activity of the methanolic extract of *D. lotus* leabes *in vitro*

Table l: Analgesic effect of oral administration of *D. lotus* aqueous methanolic extract on thermal pain by using hot plate

Groups	Dose (mg/100 g b. wt.)	Pre-drug value	1 h		2 h	
			Mean±SE	Change (%)	Mean±SE	Change (%)
Control	1 mL saline	6.1±1.5	5.7±1.5	-6.6	5.7±1.5	-6.6
D. lotus extract	25	6.8±0.1	8.6±0.3**	26.5	10.3±0.7**	51.5
D. lotus extract	50	6.9±0.6	9.1±0.6**	31.9	10.9±0.8**	58.0
Tremadol	2	5.0±0.4	7.4±0.5**	48.0	8.4±0.3***	68.0

Data are presented as Mean±SE. Percentage of change from basal (pre-drug) value for each group. Value is denoted by *p<0.05, **p<0.01 ***p<0.001

Table 2: Analgesic effect of *D. lotus* aqueous methanolic extract on abdominal constrictions caused by injection of acetic acid in mice

Groups	Dose (mg/100 g b. wt.)	Number of contraction	Change (%)	Potency as compared
Control	1 mL saline	56.7±1.4	-	0.40
D. lotus extract	25	33.3±0.8***	41.3	0.65
D. lotus extract	50	23.5±0.6***	58.6	0.93
Indomethacin	2	20.8±0.9***	63.3	1.00

Data are presented as Mean±SE; Percentage of change from control value; Significant change from control group is denoted by ***p<0.001

Table 3: Anti-inflammatory effect of *D. lotus* aqueous methanolic extract at doses of 50, 25 mg/100 g b. wt. on carrageenan-induced rat pawoedema

Groups	After 1 h		2 h		3 h		4 h	
	Oedema (%)	Oedema inhibition (%)	Oedema (%)	Oedema inhibition (%)	Oedema (%)	Oedema inhibition (%)	Oedema (%)	Oedema inhibition (%)
Control	67.1±5.6	-	82.1±2.8	-	85.6±3.6	-	87.03±3.3	-
D. lotus extract (25 mg/100 g b. wt.)	41.1±4.3*	38.7	53.7±3.2*	34.6	59.9±2.7*	30.0	61.50±3.2*	29.3
D. lotus extract (50 mg/100 g b. wt.)	42.4±1.7*	36.8	48.4±5.6*	41.0	59.5±4.0*	30.5	58.40±4.1	32.9
Indomethacin (2 mg/100 g b.wt)	44.2±3.1*	34.1	45.5±1.7*	44.6	52.5±2.5*	38.7	44.30±1.0	49.1

Data are presented as Mean±SEM; Significant change from control values at respective time; Points are denoted by *p<0.05 (one way ANOVA and Duncan test)

Acetic Acid Induced Writhing

Writhing induced by acetic acid revealed significant reduction in mice received the two dose of the plant extract. The antinociceptive activity of the *D. lotus* extract (25, 50 mg/100 g b. wt.) was 41.3 and 58.6%, respectively indicating peripheral analgesic effect. The positive control indomethacin inhibited the writhing response by 63.3% (Table 2).

Anti-Inflammatory Effect

Sub-planter injection of carrageenan into the rat hind paw elicited an inflammation (swelling and erythma) and a time dependant increase in paw thickness compared with the precarrageenan control value. The acute paw oedema response induced by sub-planter carrageenan was significantly reduced at 1, 2, 3 and 4 h by 38.7, 34.6, 30.0 and 29.3% for 25 mg of *D. lotus* extract and by 36.8, 41.0, 30.5, 32.9% for 50 mg of the same extract and 34.1, 44.5, 38.7 and 49.1% for indomethacin respectively vs. the control group at the corresponding time (Table 3).

Biochemical Results

Administration of acetaminophen induced a marked increase in the serum hepatic enzyme levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma aminotransferase (GGT) as compared to normal controls indicating liver damage. Alanine aminotransferase increased by 154.8%, AST by 130.1% and GGT by 24.0%. The *D. lotus* extract at doses 25 and 50 mg g^{-1} b. wt.

Table 4: Effect of the oral administration of the aqueous methanolic extract of *D. lotus* (25 and 50 mg/100g. b.wt) on serum ALT, AST and GGT of normal and acetaminophen rats

Groups	ALT (U L^{-1})	Change (%)	AST (U L^{-1})	Change (%)	GGT (U L^{-1})	Change (%)
Control	47.1±1.5	-	38.9±0.3	-	12.9±0.1	-
25 mg kg^{-1} b.wt *D. lotus* Ext.	43.3±0.5	-8.1	37.5±0.3**	-3.6	13.2±0.4	2.3
50 mg kg^{-1} b.wt *D. lotus* Ext.	44.5±0.5	-5.5	37.2±0.2**	-4.4	13.0±0.2	0.8
Silymarin	41.4±0.7*	-12.1	36.8±0.1***	-5.4	11.6±0.6	10.1
Acetaminophen	120.0±2.7	154.8	89.5±1.3	130.1	24.0±0.5	-
25 mg kg^{-1} b.wt *D. lotus* Ext.	96.5±2.9***	-19.6	76.6±2.8**	-14.4	15.1±0.4	37.1
50 mg kg^{-1} b.wt *D. lotus* Ext.	90.6±2.0***	-24.5	64.9±3.6***	-27.5	14.1±0.6	41.3
Silymarin	84.0±1.6***	-30.0	72.9±1.0***	-18.5	13.0±0.6	45.8

Results are Means±SE; $p<0.05$ was considered significant

reduce the elevated ALT levels in rats administered with acetaminophen by 19.6 and 24.5%, respectively. Silymarin administration caused significant reduction in plasma ALT (30.0%) and reduce the elevated AST levels by 14.4 and 27.5%. Rats treated with silymarin (22 mg kg^{-1}) exhibited significant reduction in their plasma AST (by 18.5%). As concern GGT serum levels, oral administration of acetaminophen caused a significant increase by 86% as compared with the control one. A significant decrease by 37.1 and 41.3%, for 25 and 50 mg of the extract and by 45.8% for silymarin vs. acetaminophen control group was recorded (Table 4).

DISCUSSION

The aqueous methanolic (70%) extract of *D. lotus* leaves was shown by two-dimensional paper chromatography to contain a complicated phenolic mixture. Eight known phenolic compounds were isolated and purified by standard methods. Compounds 1-3, an off-white amorphous powder, showed chromatographic properties and color reactions (positive $FeCl_3$ and KIO_3 tests) indicative of galloyl esters. Compounds **4-6** detected as yellow spots on PC under UV light did not change by ammonia vapour while compounds **7** and **8** appeared as dark purple spots under UV light, change to yellow when fumed to ammonia. The chemical investigation of **7** and **8** was followed by paper chromatography to identify the hydrolytic flavonoid-O-glycoside products whether aglycone (myricetin) and sugar moieties, glucuronic acid and rhamnose, respectively (Fig. 2). The identification of the isolated compounds was confirmed by co-chromatography with authentic samples, UV and NMR spectroscopy and MS spectrometry. The spectral data of the isolated compounds were compared with the literature data (Fig. 1).

Antioxidant activity of the aqueous methanolic (70%) extract of *D. lotus* leaves was studied for its free radical scavenging property on (DPPH). The extract showed a high effective free radical scavenging property and exhibited a remarkable antioxidant effect at low doses, but this effect decreased with the increase of concentration. Anti-oxidant property of the extract may be due to the isolated phenolic compounds which serve as derivatives of conjugated ring structures and hydroxyl groups that have the potential to function as antioxidant in *in-vitro* cell culture or cell free systems (Robak and Gryglewski, 1988) and this was in agreement with Maige *et al*. (2005) who proved that the extract of *D. Abyssinica* showed high activity as radical scavengers and lipoxygenase inhibitors. Flavonoids and other polyphenolic compounds have powerful antioxidant effect *in vitro* in many test systems, but can act as pro-oxidants in some others (Halliwell, 2007).

Pharmacological studies were conducted with *D. lotus* extract on experimental animals for evaluating the analgesic and anti-inflammatory activities. Significant anti-inflammatory and analgesic activities were observed in the tested experimental models indicating inhibition of all phases of inflammation.

The development of oedema in the paw of the rat after injection of carrageenan is a biphasic event. The initial of the oedema is due the release of histamine and serotonin and the oedema is maintained

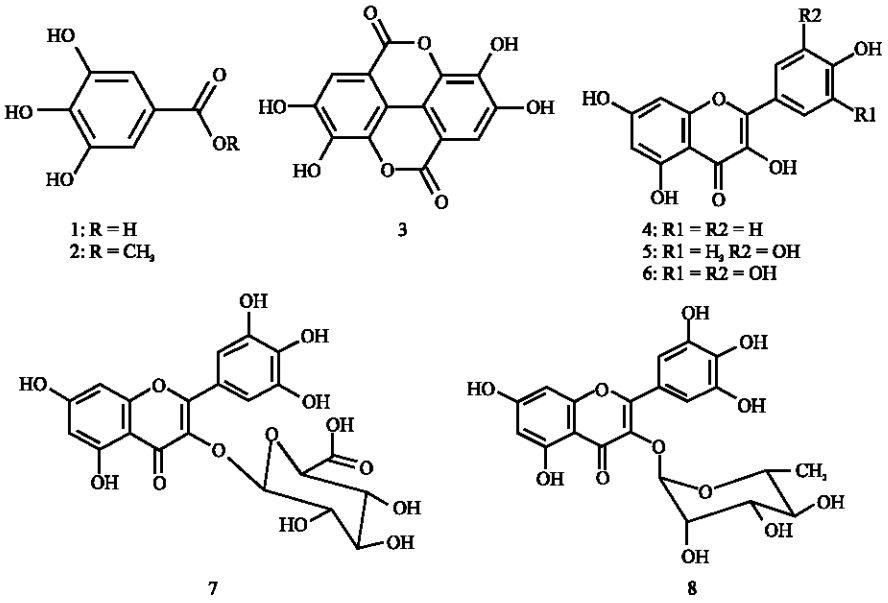

Fig. 2: Phenolic compounds isolated from *Diospyros lotus*

during the plateau phase by kinin like substance (Chauhan *et al*., 1998) and the second accelerating phase of swelling due to the release of prostaglandin like substances. Inhibition of oedema observed in carrageenan models may be due to the ability of *D. lotus* leaves extract constituents (flavonoids) to inhibit these chemical mediators of inflammation, or astabilizating effect on lysosomal membranes (Samuelsen, 2000).

The central analgesic activity of *D. lotus* leaves extract was studies using hot plate method and peripheral activity in acetic acid induced writhing test. *D. lotus* leaves extract significantly increased the reaction time in hot-plate test and also reduced the writhing response in mice injected with acetic acid indicating its ability to inhibit the permeability of the small blood vessels. Hence, it is speculated that apart from inhibition of chemical mediators of inflammation, may also modulate the pain response in the central nervous system.

Increased oxidative stress is important factor in the development of liver injury. It is worthy to note that in acetaminophen-induced liver damage, there is excessive lipid peroxidation leading to functional and structural disruption. The hepatic toxicity of acetaminophen is ascribed to its toxic metabolite N-acetyl-p-benzoquinone imine which formed when apart of paracetamol is activated by hepatic cytochrom P-450 (Vermeulen *et al*., 1992). This is normally inactivated by coupling to glutathione. Toxicity which occurs after ingestion of large doses of the drug is the result of depletion of the glutathione stores (Amimoto *et al*., 1995).

In the model of acetaminophen-induced hepatic toxicity, results indicate that the *D. lotus* leaves extract reduces the degree of hepatic injury and significantly reduced the elevated serum ALT, AST and GGT levels caused by acetaminophen intoxication in the rat. The *D. lotus* leaves extract protective effects have largely been ascribed to its known ability to scavenge free radicals and function as an antioxidant. The hepatoprotective effect of the extract may be due to flavonoid constituents. Such flavonoids, as quercetin prevented the glutathione depletion and lipid peroxidation induced by an acute intoxication with carbon tetrachloride, ethanol, acetamenophen and bromobenzene in the liver and in the rates with biliary obstruction (Peres *et al*., 2000; Yao *et al*., 2007).

CONCLUSION

Eight phenolic compounds from *Diospyros lotus* leaves in this study were found to be isolated for the first time from the genus *Diospyros*. It is assumed that these phenolic constituents can participate on observed antioxidant, anti-inflammatory, analgesic and hepatoprotective activities in the plant. Further studies are in progress to determine the mechanism of action.

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