Phenolic Contents of *Gleditsia triacanthos* Leaves and Evaluation of its Analgesic, Anti-inflammatory, Hepatoprotective and Antimicrobial Activities

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Abstract: This study explored the constitutive polyphenols and certain bioactivities of *Gleditsia triacanthos* leaves including analgesic, anti-inflammatory, hepatoprotective and antimicrobial activities. The chromatographic separation of 70% EtOH leaf extract resulted in isolation of eight flavonoids including six flavonoid glycosides viz Vicenin-II (**1**), Lucenin-I (**2**), Isoorientin (**3**), Orientin (**4**), Vitexin (**5**), Isovitexin (**6**) along with two aglycones viz; Luteolin (**7**) and apigenin (**8**). Their structures were established by different chromatographic and spectral techniques UV, MS, ^{1}H and ^{13}C NMR. Bioactivity studies were carried out on 70% EtOH extract of *G. triacanthos* leaves (sample A) and three fractions; II (sample B), III (sample C) and V (sample D) obtained from fractionation of 70% EtOH leaf extract on di-ion column. All the samples (A, B, C, D) showed significant analgesic, anti-inflammatory and antimicrobial activities. Additionally, all the investigated samples showed hepatoprotective activities except for sample D.

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1. Introduction

Fabaceae, (or Leguminosae), pea family, bean family is the third largest family of flowering plants after Orchidaceae and Asteraceae, with 36 tribes, 727 genera and 19,327 species [1]. The family is distributed throughout temperate and tropical regions of the world [2]. Among the Fabaceae is the genus *Gleditsia*, the Locust tree which comprises about 14 species of deciduous trees [3], and is native to North America and Asia. *Gleditsia* species have been widely used in folk medicine, they are used for treatment of carbuncle, scabies, skin diseases and for treating apoplexy, headache, |productive cough and asthma, also they are used as diuretic and expectorant [4]. *Gleditsia triacanthos* L. which is also known as Honey locust [5], is a deciduous tree, originated from the Eastern USA and is widely naturalized in the Mediterranean area, in the Middle-East, in Asia and North Australia [6]. The name Honey locust derives from the sweet taste of the legume pulp, which was used for food by Native American people and can also be fermented to produce alcoholic and non-alcoholic beverage [7].

Previous reports on *Gleditsia* species revealed that they contain bioactive compounds as phenolic compounds; phenolic acids [8,9], flavonoids [9,10-13], triterpenes and sterols[14-20], which have numerous activities including antioxidant [10,21], cytotoxic [4,14,22], anti-inflammatory, analgesic [23,24], antiallergic [25,26], antibacterial [8], hypocholesterolemic, and hypoglycemic effects [12,27,28]. Few studies report on the constitutive polyphenols and non-biological activities of *Gleditsia triacanthos* L. [10,118]. Thus, it was deemed necessary to carry out this phytochemical and biological study to throw light on this species growing in Egypt. The present study aimed to explore the isolation and identification of some phenolic constituents of *G. triacanthos* leaves and evaluation of its analgesic, anti-inflammatory, hepatoprotective, and antimicrobial activities.

2. Material and Methods

2.1. Plant materials

Gleditsia triacanthos leaves were collected from trees growing in the Zoo Garden, Cairo, Egypt. The identity of the plant was confirmed by Dr. Mohamed El Gebaly, researcher of Taxonomy, National Research Centre and Dr. Terase Labib, Taxonomist engineering, Department of Floral and Taxonomy, El-Orman Garden, Cairo, Egypt. The fresh plant leaves were washed with clean water then completely air-dried in

the shade at room temperature, reduced to powder by electric mill, and kept in dark place in closed containers until subjected to extraction process.

2.2. Instruments and materials

^{1}H-NMR (500 MHZ, DMSO-d_6) and ^{13}C-NMR (125 MHZ, DMSO-d_6) spectra were recorded on Jeol Delta2 spectrometer (NMR Department, National Research Center). The chemical shifts were expressed in δ (ppm) with reference TMS and coupling constant (*J*) in Hertz. The UV analyses for pure samples were recorded on a Shimadzu UV 240 spectrophotometer, separately as solutions in methanol and with different diagnostic UV shift reagents [29] (Faculty of Pharmacy Helwan University). ESI-MS spectra were measured on mass spectrometer connected to an ESI-II ion source (Finnigan, LC-MSLCQ^{deca}. Advantage MAX, Finnigan Surveyor LC pump) (Department of Biological Genetics, National Research Center, Cairo, Egypt) and on HPLC/MS spectrometer, the HPLC apparatus consisted of Accela 1200 LC-10AD pump, auto sampler Accela and a Hypersill gold (2.1 µm) (Phenomenex) 50×2.0mm preceded by a C_{18} security guard cartridge Gemini 5 µm C18 ((Phenomenex) 4 x 3 mm). Mobile phase A: Acetonitrile 90%, B : 0.1% formic acid 10%, Isocratic flow, Run time : 3.00 min, Flow rate : 250 µl /ml. Mass spectrometric analysis is carried out using a TSQ Quantum Access MAXtriple quadrupole system (Samples are individually tuned for each target analyte by direct injection of the individual solution (1 mg/mL),data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software, ionization mode: Heated Electrospray (HESI), polarity: negative ion mode, spray voltage: 300 V, vaporizer temperature: 400°C, sheath gas pressure: 25, aux gas pressure: 5, capillary temperature: 370°C) (Faculty of Pharmacy, Helwan University, Ain Helwan, Cairo, Egypt) and Rotary evaporator (Bűchi, G, Switzerland). Fractionation of the extracts was done by columns chromatography using Di-ion HP-20 Ion exchange and adsorbent resins (SUPELCO, Bellefonte, PA). Purification and isolation of compounds were done on Sephadex LH-20 (25-100 µm, Sigma, Sweden) columns of different dimensions and eluted with different solvent systems. Separation processes were followed up by 2D-PC and CoPC using Whatmann No. 1 and No. 3 paper sheets (Whatmann, England) with (S_1) *n*-BuOH-AcOH-H2O (BAW) (4:1:5, top layer) and (S_2) 15% aqueous AcOH as solvent systems. Spots were visualized by absorption of UV radiation using ultraviolet lamp (VL-215 LC, Marne La Vallee, France) and spraying the chromatograms with ethanolic $AlCl_3$, $FeCl_3$ (1 %) and Naturstoff reagents.

2.3. Chemicals

Sugars, reagents and solvents of analytical grade were purchased from Sigma Aldrich Co. (St Louise, Mo, USA) Authentic reference phenolic compounds were obtained from Phytochemistry Laboratory, Department of Molecular and cell Biology,University of Texas at Austin (Austin, TX) and from Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan.

Drugs and chemicals used in the pharmacological studies

Acetyl salicylic acid (aspirin) and indomethacin were obtained from Cairo Pharmaceutical Company, Egypt. Tramadol was a gift from October Pharma Pharmaceutical Company, Egypt. Carbon tetrachloride was purchased from Merck (Germany). Silymarin and all other chemicals used in the pharmacological studies were purchased from Sigma Aldrich (St. Louis, MO, USA). The kits used in the pharmacological evaluation of the hepatoprotective activities of the plant samples were obtained from Spinreact, S.A., Spain and Calbiochem, Germany.

Drugs used in the microbiological studies

Imipenem and Ciprofloxacin (Oxoid Ltd, Cairo, Egypt).

2.4. Animals used in the pharmacological studies

Male albino Swiss mice, weighing 25–30 g, were obtained from the animal house of the Research Institute of Ophthalmology, Giza, Egypt. Animals were group-housed (7-10 mice per cage) under standard environmental conditions (temperature 22±1 °C, humidity 60±5 %, 12 h light:12 h dark cycle) and allowed free access to water and standard chow pellets. The adopted protocol and implemented experiments were carried out in accordance to the international Guide for the Care and Use of Laboratory Animals.

2.5. Microorganisms

Gram positive bacteria (*Coagulase –ve Staphylococci* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumonia*). All strains were clinical isolates available in the Department of Microbiology, Research Institute of Ophthalmology, Giza, Egypt.

2.6. Culture media

Nutrient agar and a sterile cork borer (7 mm) were available in the Department of Microbiology, Research Institute of Ophthalmology, Giza, Egypt.

2.7. Extraction and isolation

The powdered air dried leaves of *Gleditsia triacanthos* (1.25 Kg) was exhaustively extracted with 70% EtOH (4 × 3 L), under reflux (50°C) and the solvent was removed under reduced pressure to give (300 g) of ethanol extract. This extract was defatted with petroleum ether. The defatted residue was extracted with chloroform (3 × 1 L) producing chloroform soluble portion (3.5 g) and aqueous ethanol extract residue (62.4 g). Both the chloroform and ethanol extracts were subjected to preliminary phytochemical screening and two dimensional paper

chromatography (2D-PC) using two solvent systems S_1 and S_2 that revealed that chloroform extract contained limited polyphenolic compounds while they were concentrated in aqueous ethanol extract. Therefore, the aqueous ethanol extract was fractionated on di-ion HP-20 column (500 g, Ø 5.5 × 120 cm) and was eluted with water followed by H_2O/MeOH mixtures with decreasing polarity affording five collective fractions (I-V). Purification of compounds was done by successive column chromatography on Sephadex LH-20 using H_2O/MeOH mixtures and also by preparative paper chromatography (PPC) followed by fractionation on Sephadex LH-20 and MeOH for elution as shown in the flow chart (Figure 1).

2.8. Preparation of plant samples and reference standard drugs for pharmacological studies

The four plant samples under investigation were given the following codes; *Gleditsia triacanthos* crude extract as 70% EtOH extract (sample A), fraction II (sample B), fraction III (sample C) and fraction V (sample D). The plant extracts were dissolved in normal saline and 2 drops of 3% Tween 80 prior to administration to mice. Based on the preliminary studies, doses of 200 mg/kg and 400 mg/kg b.wt. of each extract were selected for evaluation of analgesic and anti-inflammatory activities, whereas the higher dose (400 mg/kg) was used for investigation of the hepatoprotective activity.

The reference standards aspirin, tramadol, indomethacin, and silymarin were dissolved in normal saline and 2 drops of 3% tween 80 then administered orally as 100 mg/kg, 15 mg/kg, 100 mg/kg, and 200 mg/kg respectively. Carbon tetrachloride (CCl_4) was diluted as 0.1% CCl4 in olive oil and given in a dose of 10 ml/kg b.wt. [30].

2.9. Acute toxicity study

For acute toxicity study, groups of mice (n=7) were administered orally with the four *Gleditsia triacanthos* samples (A, B, C, D) in the range of doses 50-2000 mg/kg and the mortality rates were observed after 72 h. According to the results of acute toxicity test, doses were selected for pharmacological evaluations [31].

2.10. Determination of analgesic activity of *Gleditsia triacanthos* samples

2.10.1. Acetic acid-induced writhing in mice

The test was performed as described earlier [32]. In this experiment, mice were divided into 6 groups (n=7 mice), which were treated orally either with a solution of normal saline and 2 drops of 3% Tween 80 (control), aspirin (100 mg/kg) as a reference analgesic or *Gleditsia triacanthos* samples (A, B, C, D), each given in 200 and 400 mg/kg doses. After 1 hour of the oral treatment, 0.7% aqueous solution of acetic acid (10 ml/kg) was administered through intraperitoneal route to all groups. Immediately after injection of acetic acid, each mouse was placed in a transparent plastic observation chamber and the number of writhing movements (contraction of the abdominal muscles along with stretching of the hind limbs) was counted for 20 min commencing after injection of acetic acid. The percentage inhibition of the number of writhing movements was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{MWc - MWt}{MWc} \times 100$$

Where MWc, and MWt are the mean number of writhes of the control and test groups respectively.

2.10.2. Formalin-induced nociception in mice

The antinociceptive activity of *Gleditsia triacanthos* samples A, B, C and D were assessed using the formalin assay [33]. Groups of mice (n=7) were treated orally with either a solution of normal saline and 2 drops of 3% Tween 80 (control), plant samples A, B, C and D (200 and 400 mg/kg) or aspirin (100 mg/kg). One hour following the oral treatments, formalin 1% (20 µl) was injected into the sub-plantar space of the right hind paw of all rats and then the animals were placed in a transparent plastic observation chamber. The duration of paw licking was recorded between 0-5 min (early phase) and 15-45 min (late phase). The percentage of nociception inhibition during early and late phases was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{Mdc - Mdt}{Mdc} \times 100$$

Where Mdc and Mdt are the mean paw licking duration of the control and test groups respectively.

2.10.3. Tail flick test in mice

The apparatus used in the tail flick test consisted of a circulating immersion water heater. The thermostat was adjusted so that a constant temperature of 54±1 ^oC was maintained in the water bath. Before treatment, the terminal 3 cm of each mouse's tail was immersed in the water bath and the time in seconds taken by the animal to flick its tail was recorded. Only mice showing a pre-treatment reaction time less or equal to 4 seconds were selected for the study. Immediately after basal latency assessment, a solution of normal saline and 2 drops of 3% Tween 80 (control), *Gleditsia triacanthos* samples A, B, C, D (200 and 400 mg/kg), or tramadol (15 mg/kg) as a reference analgesic were administered by oral route to groups of mice (n=7). The reaction time was measured again 1 hour after treatment administration. The cut-off time was set as 6 seconds for tail-flick measurements in order to minimize tissue injury [34].

2.11. Determination of anti-inflammatory activity of *Gleditsia triacanthos* samples

2.11.1. Xylene-induced ear edema in mice

The xylene-induced ear edema test was performed as previously described by Zhang *et al.* [35]. Mice (n=7) were orally administered a single dose of vehicle (a solution of normal saline and 2 drops of 3% Tween 80) (control), indomethacin (100 mg/kg) (reference anti-inflammatory drug), or *Gleditsia triacanthos* samples A, B, C and D (200 mg/kg and 400 mg/kg), one hour before induction of ear edema. The latter was induced by topical application of xylene (10 µl) on the inner and outer surfaces of the right ear, while the left ear was used as a control. Mice were sacrificed by cervical dislocation 1 hour after xylene application. Ear biopsies of 9.0 mm diameter were punched out and weighed. The extent of ear edema was calculated as the weight difference between the right and the left ear biopsies of the same animal and percentage inhibition was measured using the following formula.

$$\% \text{ Inhibition} = \frac{MEWc - MEWt}{MEWc} \times 100$$

Where MEWc, and MEWt are the mean resultant edema weight of the control and test groups respectively.

2.11.2. Carrageenan-induced paw edema in mice

The anti-inflammatory activity of *Gleditsia triacanthos* samples A, B, C and D were determined using Carrageenan-induced paw edema model [36]. Groups of mice (n= 7) were used. All mice were orally treated with vehicle (negative control), indomethacin (100 mg/kg) (reference anti-inflammatory drug), or the samples A, B, C and D (200 mg/kg and 400 mg/kg). One hour after oral treatment, 1% carrageenan (30 µl) was administered into the plantar side of the right hind paw of all animals. Four hours after carrageenan administration, all animals were sacrificed by cervical dislocation and their hind paws were immediately amputated at the ankle joint and weighed. The weight of the left hind paw was subtracted from the weight of the right hind paw to obtain the weight of the resultant edema. Percentage inhibition of paw edema was calculated using the following formula:

$$EW = WR - WL$$

Where;

EW = Resultant edema weight

WR = Weight of the right hind paw

WL = Weight of the left hind paw

$$\% \text{ Inhibition} = \frac{MEWc - MEWt}{MEWc} \times 100$$

Where MEWc, and MEWt are the mean resultant edema weight of the control and test groups respectively.

2.12. Determination of hepatoprotective activity of *Gleditsia triacanthos* samples

Animals were randomly divided into seven groups (n=10). The control, *Gleditsia triacanthos* samples (A, B, C, D) and silymarin groups were orally administered either the vehicle for samples, the respective extract (400 mg/kg, orally) or standard silymarin (200 mg/kg, orally) respectively for 7 successive days [37]. At the 7th day, control group received a single i.p. dose of olive oil (vehicle for CCl_4), whereas either the samples (A, B, C, D) or silymarin groups received a single i.p. CCl_4 injection. Carbon tetrachloride (CCl_4) group received the vehicle for plant samples for 7 successive days, in addition to a single dose of CCl_4 (10 ml/kg, 0.1% CCl_4 in olive oil, i.p.) at the 7th day [30]. Twenty-four hours after the CCl_4 dose, mice were sacrificed by cervical dislocation. Blood samples were collected. Livers were excised, washed in ice-cold saline, and homogenized. Liver homogenates were used for the biochemical assays.

2.12.1. Determination of serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TBil) in mice

The activities of serum enzymes ALT, AST, ALP, and TBil were determined as markers of hepatic injury using assay kits (Spinreact, S.A., Spain) [38,39].

2.12.2. Determination of hepatic malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) levels and hepatic superoxide dismutase (SOD) activity in mice

Total lipid peroxides were measured as malondialdehyde (MDA) level in liver homogenates according to the method of Buege and Aust [40]. Hepatic nitric oxide level was measured in liver homogenate as total nitrates/nitrites according to a colorimetric assay following the method described earlier [41]. The method of Ellman [42], was adopted for the colorimetric estimation of GSH level in liver homogenate, while hepatic SOD activity was assayed by a kit obtained from Calbiochem, Germany [43].

2.13. Antimicrobial activity

The antimicrobial activity of the tested plant samples (A, B, C and D) was evaluated against six different bacterial strains using the well agar diffusion method [44]. A loopful of the tested organisms was inoculated into 5.0 ml of nutrient broth and incubated at 37 ^{0}C for 24 hours. From the 24-hours culture of the organism, 0.2 ml was dispensed into 19.8 ml sterile nutrient broth and incubated for 3-5 hours to standardize the culture to 10^6 cfu/ml. Six plates were prepared and 1 ml from the 3-hr culture bacteria was poured into each plate and 19 ml of nutrient agar was

then added. A sterile cork borer (7 mm) was used to make 6 wells in each plate (4 for the tested samples and 2 for the solvents). The base of each well was filled with molten nutrient agar to seal the bottom and allowed to gel. Few drops of the reconstituted tested sample were dispensed into each well. The plates were left to allow for diffusion of the tested sample then incubated at 37 ^{0}C for 24 hours. Two standard discs of Imipenem and Ciprofloxacin were placed on the agar plate to serve as positive control (antimicrobial reference drugs) for antimicrobial activity and few drops of solvents (methanol and ethanol) were used as a negative control. The zones of clearance produced around the wells were measured in millimeters.

2.14. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by post-hoc Tukey's test. GraphPad software Prism, version 3 (San Diego, CA, USA) was used to perform the statistical tests.

3. Results and Discussion

The dried residue of 70% EtOH extract of *Gleditsia triacanthos* leaves was fractionated on a di-ion column followed by successive separation on Sephadex LH-20 columns with different eluting solvents **(Fig. 1)**, and yielded eight flavonoids including six flavonoid glycosides viz Vicenin-II (**1**), Lucenin-I (**2**), Isoorientin (**3**), Orientin (**4**), Vitexin (**5**), Isovitexin (**6**) along with two aglycones viz; Luteolin (**7**) and apigenin (**8**) **(Fig. 2)**. The isolated pure compounds were identified by different chromatographic and spectral techniques UV, ^{1}H, ^{13}C NMR, Negative ESI-MS , by comparison with previously published data [30,45-57], as well as comparing with authentic samples (CoPC).

3.1. Characterization and identification of the isolated compounds

Compound 1

Yellowish powder (35 mg). Chromatographic properties: R_f values: 0.38 (S_1), 0.72 (S_2); it is deep purple color under UV-light turned to yellow-green on exposure to NH_3 vapour and gave grayish yellow fluorescence and green colour with Naturstoff and $FeCl_3$ spray reagents, respectively. UV-spectral data : λ_{max} (nm) (MeOH): 274, 311sh, 335; (+NaOMe): 281, 333, 398; (+NaOAc): 281, 304sh, 388; (+NaOAc /H_3BO_3): 274, 330sh, 348, 412sh; (+$AlCl_3$): 265sh, 281, 307, 353, 387; (+$AlCl_3$/HCl): 263sh, 282, 306, 347, 383. ^{1}H NMR (500 MHz,DMSO-d_6): δ ppm 13.63 (1H, s, OH-5), 8.01 (2H, d, *J* = 8.7 Hz, H-2′/6′), 6.88 (2H, d, *J* = 8.7 Hz, H-3′/5′), 6.79 (1H, s, H-3), 4.7 (1H, d, *J* = 9.5 Hz, H-1″), 4.75 (1H, d, *J* = 9.8 Hz, H-1‴), 3.3-3.9 (m, 12 sugar protons hidden by OH-group and H_2O-resonances). ^{13}C NMR (125 MHz, DMSO-d_6: δ ppm 182.36 (C-4), 164.15 (C-2), 161.25 (C-7), 160.83 (C-4′), 158.59 (C-9), 155.10 (C-5), 129.08 (C-2′/6′), 121.53 (C-1′), 116.06 (C-3′/5′), 107.49 (C-6), 105.29 (C-8), 103.93 (C-10), 102.64 (C-3), 81.93 (C-5″), 80.90 (C-5‴), 78.84 (C-3″), 77.80 (C-3‴), 74.12 (C-1″), 73.39 (C-1‴), 71.98 (C-2″), 70.98 (C-2‴), 70.59 (C-4″), 69.07 (C-4‴), 61.32 (C-6″), 59.83 (C-6‴). Negative ESI-MS: *m/z* 593 [M – H] $^-$. According to the chromatographic properties, compound **1** was expected to be a glycosyl apigenin [30]. UV-spectrum in MeOH showed the two characteristic absorption bands I and II at λ_{max} (nm) 335 and 274 of apigenin nucleus. The bathochromic shift in band II on addition of NaOAc was diagnostic for free 7-OH group. The presence of free hydroxyl groups OH-4′ and OH-5 were deduced from the bathochromic shift in NaOMe and $AlCl_3$ spectra, where the bathochromic shift remained on addition of HCl. On account of the given above data and chromatographic properties, compound **1** was expected to be 5,7, 4′-trihydroxyglycosyl flavones [30,45]. Negative ESI/MS spectrum exhibited the molecular ion peak at *m/z* 593 [M – H] $^-$ corresponding to the Mwt of 594 and molecular formula C_{27} H_{30} O_{15} to support evidence of apigenin-di- hexoside structure in comparison with ESI-MS data of compounds 5 and 6. From the ^{1}H NMR spectrum a flavone compound was confirmed by the appearance of a singlet at δ 6.79 for H-3. Additionally, the spectrum showed A_2X_2 spin coupling system of two ortho-doublets each integrated for two protons, at δ ppm 8.01 (H-2′/6′) and 6.88 (H-3′/5′) for 4′-hydroxy B-ring indicating an apigenin nucleus. The absence of H-6 and H-8 signals from the spectrum and the presence of two doublets of large *J value* 9.8 Hz and 9.5 Hz at δ ppm 4.75 and 4.7, respectively were attributed to two anomeric protons of two *C*-β-D-glucopyranoside moieties. These were indicative to 6,8-di-*C*-β-glycosyl apigenin structure.^{13}C NMR spectrum showed well resolved typical 15 signals of an apigenin aglycone moiety, including the four key signals for C-4′, C-2′/6′, C-3′/5′ and C-3 at δ ppm 160.83, 129.08, 116.06 and 102.64, respectively. The downfield shifts of ^{13}C-resonance of C-6 to 107.49 and C-8 to 105.29 (~ + 10 ppm) was confirmative evidence for the *C*-glycosidation at C-6 and C-8. Moreover the di-*C*-glycoside moiety was confirmed as 6,8-di-*C*-β-glucopyranoside depending on the intrinsic upfield location of C-1″ and C-1‴ (anomeric carbons) at δ 74.12 and 73.39, respectively and downfield location of C-5″,C-5‴, C-3″ and C-3‴ at δ ppm 81.93, 80.90, 78.84 and 77.80, respectively, with respect to those of *O*-glucoside[46]. The assignment of all other ^{13}C resonances of compound **1** was achieved by comparison with the corresponding data of structural related compounds [47,48].According to the above discussed data as well as comparison with authentic samples, compound **1** was confirmed as **apigenin-6,8-**

di-*C*-β-D-glucopyranoside (Vicenin II) which was isolated for the first time from genus *Gleditsia*.

Compound 2

Yellowish powder (30 mg). Chromatographic properties: R_f values: 0.13 (S_1), 0.38 (S_2); it is deep purple color under UV-light turned to yellow-green on exposure to NH_3 vapour. UV-spectral data : λ_{max} (nm) (MeOH): 257, 272, 349; (+NaOMe): 240sh, 266, 280, 344sh, 408; (+NaOAc): 271sh, 282, 326, 398; (+NaOAc /H_3BO_3): 266, 287sh, 382, 430; (+$AlCl_3$): 280, 303sh, 332, 430; (+$AlCl_3$/HCl): 265sh, 278, 297sh, 359, 384sh. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 13.56 (1H, s, OH-5), 7.34 (2H, m, H-2′/6′), 6.78 (1H, d, *J* = 8.1 Hz, H-5′), 6.54 (1H, s, H-3), 4.53 (1H, d, *J* = 9.9 Hz, H-1″), 4.05 (1H, d, J = 9.8 Hz, H-1‴), 4.05-3.10 (m, hidden by H_2O-signal, remaining sugar protons). ^{13}C NMR (125 MHz, DMSO-d_6): δ ppm 183.82 (C-4), 166.90 (C-2), 163.17 (C-7), 159.92 (C-5), 157.20 (C-9), 150.53 (C-4′), 146.23 (C-3′), 123.63 (C-1′), 119.99 (C-6′), 117.65 (C-5′), 114.59 (C-2′), 107.24 (C-6), 103.01 (C-10), 102.94 (C-8), 102.81 (C-3), 82.10 (C-5″/5‴), 79.27 (C-3‴), 76.49 (C-3″), 76.41 (C-1‴), 76.26 (C-1″), 72.79 (C-2‴), 71.82 (C-2″), 71.55 (C-4‴), 70.62 (C-4″), 62.82 (C-6‴), 62.11 (C-6″). Negative ESI-MS**:***m/z* 609 [M – H] $^-$. According to the chromatographic properties, compound **2** was expected to be a glycosyl luteolin [30]. UV-spectrum in MeOH showed the two characteristic absorption bands I and II of luteolin nucleus at λ_{max} 349 and 257 nm, respectively. On addition of NaOAc, a bathochromic shift of band II was observed which is diagnostic for a free 7-OH group. The bathochromic shift of band I in $AlCl_3$ together with hypsochromic shift observed after addition of HCl confirmed the presence of ortho-dihydroxyl groups at C-3′ and C-4′ in ring B, however the bathochromic shift in band II relative to MeOH remained after addition of HCl indicated the presence of a free 5-OH group. On account of the given above data and the chromatographic properties, compound **2** was expected to be 5,7,3′,4′-tetrahydroxy glycosyl flavones [30,45]. Negative ESI/MS spectrum exhibited the molecular ion peak at *m/z* 609 [M – H]$^-$, corresponding to molecular weight of 610 and molecular formula C_{27} H_{30} O_{16}, to support evidence of luteolin-di- -hexoside structure in comparison with the ESI/MS data of compounds **3** and **4**. In ^{1}H NMR spectrum a flavone compound was confirmed by the appearance of a singlet at δ 6.54 for H-3. Additionally, the spectrum showed an ABX-spin coupling system of three proton resonances at δ 7.34 (H-2′/6′) and ortho-doublet at 6.78 (H-5′) to indicate a 3′,4′-dihydroxy B ring indicating a luteolin nucleus. The absence of H-6 and H-8 signals from the spectrum and the presence of two doublets of large *J-values* 9.9 Hz and 9.8 Hz at δ 4.53 and 4.05 ppm respectively, were attributed to two anomeric protons of two *C*-β-D-glucopyranoside moieties. These were indicative to 6,8-di-*C*-β-glycosyl luteolin structure. As further confirmation, ^{13}C NMR spectrum showed well-resolved typical 15 signals of a luteolin aglycone moiety, including the three key signals of C-3′, C-4′ and C-3 at δ ppm 146.23, 150.53 and 102.81, respectively. The downfield shifts of ^{13}C-resonance of C-6 to δ 107.24 and C-8 to δ 102.94 (~ + 10 ppm) was confirmative evidence for the *C*-glycosidation at C-6 and C-8. Moreover the di-*C*-glycoside moiety was confirmed as 6,8-di-*C*-β -glucopyranoside depending on the intrinsic upfield location of C-1″and C-1‴ (anomeric carbons) at δ 76.26 and76.41 ppm, respectively and downfield location of C-5″/5‴ at δ 82.10 ppm and C-3″,C-3‴ at 76.49 and 79.27, respectively, with respect to those of *O*-glucoside [46]. The assignment of all other ^{13}C resonances of compound **2** was achieved by comparison with the corresponding data of structural related compounds [47]. According to the above discussed data as well as comparison with authentic samples, compound **2** was confirmed as **luteolin-6,8-di-*C*-β-D-glucopyranoside (Lucenin I)** which was isolated for the first time from genus *Gleditsia*.

Compounds 3 and 4

Yellow amorphous powder each, compound **3** (25 mg) and compound **4** (28 mg). Chromatographic properties: R_f –values, color under UV-light and effects on exposure to ammonia vapours, Naturstoff and $FeCl_3$ spray reagents as well as UV-spectral data (Table 1). ^{1}H and ^{13}C NMR: δ ppm and *J*-values (Hz) and Negative ESI-MS: *m/z* were recorded in (Table 2). According to their chromatographic properties compounds **3** and **4** were expected to be glycosyl luteolin [30]. UV-spectra in MeOH showed the two characteristic absorption bands for each compound at λ_{max} = 348 (I) and 269 (II) for compound **3** and 350 (I), 262 (II) for compound **4** that recommended a luteolin aglycone moiety in both structures [30]. Negative ESI-MS spectra of compounds **3** and **4** showed a molecular ion peak at *m/z* 447.2 [M-H] $^-$ as the base peak, together with a dimeric ion peak at 894.8 [2M-H] $^-$ which were consistent with Mwt of 448 for molecular formula C_{21} H_{20} O_{11}. ^{1}H NMR spectra showed an ABX-spin coupling system of three proton resonances in each compound at δ 7.34 (H-2′/6′) and ortho-doublet at 6.78 (H-5′) for compound**3** and δ 7.49 (H-6′), 7.45 (H-2′) and ortho-doublet at 6.82 (H-5′) for compound **4** to indicate a 3′,4′-dihydroxy ring-B. In the aliphatic region, the doublets at δ 4.53 and 4.63 with large *J* values (> 9Hz) for the anomeric protons of compounds **3** and **4** respectively, were intrinsic for a β-*C*-glycoside moiety in both compounds. Absence of H-6 and H-8 signals from ^{1}H NMR of compounds **3** and **4** respectively, led us to conclude that the *C*-glycosidation must be on C-6 in case of compound **3**

and C-8 in case of comound **4**. As further confirmation, ^{13}C NMR spectrum for each compound showed well-resolved typical 15 signals of a luteolin aglycone moiety, including the three key signals of C-3′, C-4′ and C-3 at δ ppm 146.44, 150.68 and 103.04 for compound 3and at δ ppm 145.7, 149.6 and 102.36 for compound **4**. Additionally, the *C*-glycoside moiety in both structures was confirmed as β-glucopyranoside depending on the characteristic upfield location of C-1″ at 73.64 and 73.30 ppm for compounds **3** and **4**, respectively, and downfield location of both C-5″ and C-3″ to δ 82.05 and 79.54 ppm for compound **3** and to δ 81.90 and 78.70 ppm for compound **4**, with respect to those of *O*-glycosides [46]. The *C*-glycosidation at C-6 in compound **3** and at C-8 in compound **4** was concluded from the downfield shift of ^{13}C-signals of C-6 to δ 109.46 and of C-8 to δ 103.90 (~ + 10 ppm) for compounds **3** and **4**, respectively. The assignment of all other ^{13}C resonances of compounds **3** and **4** was achieved by comparison with the corresponding data of structural related compounds [35,46,47,49,50,51]. According to the above discussed data as well as comparison with authentic samples, **compound 3** was confirmed as **luteolin-6-*C*-β-D-glucopyranoside (Isoorientin)** and **compound 4** as **luteolin-8-*C*-β-D-glucopyranoside (Orientin)** which were isolated for the first time from genus *Gleditsia*.

Compounds 5 and 6

Yellow amorphous powder each, **compound 5** (32 mg) and **compound 6** (20 mg). Chromatographic properties: R_f –values, color under UV-light and effects on exposure to ammonia vapours, Naturstoff and $FeCl_3$ spray reagents as well as UV-spectral data (Table 3). ^{1}H and ^{13}C NMR : δ ppm and *J*-values (Hz) and Negative ESI-MS: *m/z* were recorded in (Table 4).Based on their chromatographic properties compounds 5 and 6 were expected to be glycosyl apigenin [30]. This expectation was supported by UV-spectral data in MeOH and with different diagnostic shift reagent [30]. UV-spectrum in methanol exhibited the two characteristic absorption bands I and II at λ_{max} (nm) 336 and 270 of apigenin nucleus for both compounds. Negative ESI-MS spectra of **compounds 5** and **6** exhibited the molecular ion peak at *m/z* 431 [M – H] $^-$ together with adduct dimeric ion peak at *m/z* 863 [2M – H] $^-$ corresponding to Mwt of 432 and molecular formula C_{21} H_{20} O_{10} to support evidence of apigenin mono-hexoside structure of both compounds. ^{1}H NMR showed an A_2X_2 spin coupling system of two orthodoublets, each integrated for two protons at δ ppm 8.02 (H-2′/6′) and 6.88 (H-3′/5′) for compound **5** and at δ ppm 7.89 (H-2′/6′) and 6.88 (H-3′/5′) for compound **6** indicated 4′-hydroxyl B ring. The glycoside moiety in both compounds was identified as β-*C*-glycoside from doublets at δ 4.68 and 4.54 with large *J* values (≥ 9Hz) for compounds **5** and **6**, respectively. Absence of H-8 and H-6 signals from ^{1}H NMR spectrum of both compounds led us to conclude that the *C*-glycosidation must be on C-8 in case of compound **5** and C-6 in case of compound **6**. This evidence was confirmed from downfield shift of ^{13}C-resonance of C-8 to 104.67 and C-6 to 109.09 (~ + 10ppm) in ^{13}C NMR spectra of compounds **5** and **6,** respectively. Moreover, the *C*-glycoside moiety in both structures was confirmed as β-*C*-glucopyranoside depending on the characteristic upfield location of C-1″ at δ 73.48 ppm for compound **5** and at δ 73.32 ppm for compound **6,** and downfield location of both C-5″ and C-3″ at δ 81.88 and 78.73 ppm, respectively, for compound **5** and at δ 81.49 and 79.11 ppm, respectively, for compound **6** with respect to those of *O*-glucoside. The assignment of all other ^{13}C resonances of compounds **5** and **6** was achieved by comparison with the corresponding data of structural related compounds [47,48,52]. By comparison with authentic samples, **compound 5** was confirmed as **apigenin-8-*C*-β-D-glucopyranoside (Vitexin)** which was isolated for the first time from genus *Gleditsia* and **compound 6** as **apigenin-6-*C*-β-D-glucopyranoside (Isovitexin)**

Compounds 7 and 8

Compound **7** showed UV-spectral data: λ_{max} (nm) (MeOH): 242sh, 252, 266, 348; (+NaOMe): 266, 329sh, 400; (+NaOAc): 266, 301sh, 383; (+NaOAc/H_3BO_3): 260, 295, 371; (+$AlCl_3$): 273, 300sh, 327, 425; (+$AlCl_3$/HCl): 265sh, 275, 293sh, 354, 386. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 12.95 (1H, brs, OH-5), 7.37 (1H, dd, *J* = 2.1, 8.4 Hz, H-6′), 7.35 (1H, d, J = 2.1 Hz, H-2′), 6.84 (1H, d, *J* = 8.4 Hz, H-5′), 6.63 (1H, s, H-3), 6.4 (1H, brs, H-8), 6.15 (1H, brs, H-6). While compound **8** showed UV-spectral data: λ_{max} (nm) (MeOH): 267, 296sh, 336; (+NaOMe): 275, 324, 392; (+NaOAc): 274, 301, 376; (+NaOAc /H_3BO_3): 268, 302sh, 338; (+$AlCl_3$): 276, 301, 348, 384; (+$AlCl_3$/HCl): 276, 299, 340, 381. ^{1}H NMR (500 MHz, DMSO-d_6),: δ ppm 12.94 (1H, s, OH-5), 7.92 (2H, d, *J* = 8.79 Hz, H-2′/6′), 6.92 (2H, d, *J* = 8.8 Hz, H-3′/5′), 6.76 (1H, s, H-3), 6.46 (1H, d, *J* = 2.05 Hz, H-8), 6.17 (1H, d, *J* = 2.05 Hz, H-6).

According to the above data as well as comparison with authentic samples, compound **7** was identified as **Luteolin** [53,54,55] and compound **8** as **apigenin** [48,56,57].

3.2. Effect of *Gleditsia triacanthos* samples on acute toxicity study in mice

In the present study, oral administration of crude extract (sample A), fraction II (sample B), fraction III (sample C), and fraction V (sample D), did not induce mortality up to a dose of 2000 mg/kg in mice, as observed for 72 h after administration. The tested animals did not present any toxic manifestations. Since no fatalities or toxicities were observed at the doses

level used, the LD_{50} was assumed greater than 2000 mg/kg.

3.3. Analgesic activity

3.3.1. Effect of *Gleditsia triacanthos* samples on acetic acid-induced writhing in mice

As shown in **Table 5**, the reference drug aspirin produced a significant antinociceptive effect, with a 70.25% (P<0.001) percentage inhibition of writhing as compared to control. *Gleditsia triacanthos* sample A, at doses 200 mg/kg and 400 mg/kg, caused 58.86% and 68.35% inhibition (P<0.001) respectively. Sample B, at doses 200 mg/kg and 400 mg/kg, induced 76.58% and 87.98% inhibition (P<0.001) respectively. On the other hand, the plant sample C, at doses 200 mg/kg and 400 mg/kg, caused 67.72% and 61.39% inhibition (P<0.001) respectively. Sample D, at doses 200 mg/kg and 400 mg/kg, caused 69.62% and 78.48% inhibition (P<0.001) respectively. The mentioned inhibition percentages were calculated in comparison to the control group, indicating a pronounced antinociceptive effect against acetic acid-induced abdominal constrictions. Notably, samples B and D showed the most potent inhibition in this test.

Acetic acid-induced abdominal writhing is a reliable test for evaluation of mild analgesic non-steroidal, anti-inflammatory compounds and is a model commonly used for screening of peripheral analgesics [58]. This test represents visceral pain model, in which acetic acid produces a painful reaction and acute inflammation in the peritoneal area with consequent release of arachidonic acid via cyclooxygenase enzyme activity. Acetic acid causes an increase in prostaglandins, serotonin, and histamine, which play important roles in the nociceptive mechanism [59]. This leads to stimulation of pain nerve endings and induction of capillary permeability [60].

The analgesic effect of aspirin in this test is mediated through inhibition of cyclo-oxygenase enzyme in peripheral tissues and interference with the transduction of primary afferent nociceptors [61]. The dose-dependent inhibition of acetic acid induced-writhing offered by the investigated plant samples suggests a peripherally mediated analgesic activity based on the association of the adopted model with stimulation of peripheral receptors especially local peritoneal receptors at the surface of cells lining the peritoneal cavity [62]. The peripheral analgesic activity offered by *Gleditsia triacanthos* samples is comparable to aspirin and similarly might be attributed to the inhibition of arachidonic acid metabolites synthesis, interference with cyclo-oxygenase synthetic pathway and suppression of endogenous pro-inflammatory substances release.

3.3.2. Effect of *Gleditsia triacanthos* samples on formalin-induced nociception in mice

As shown in **Table 6**, no significant effects were observed in the first (early) phase for any of the investigated *Gleditsia triacanthos* samples at dose 200 mg/kg. Only samples B and D at 400 mg/kg dose level, showed a percentage inhibition of formalin-induced paw licking of 35.55% and 34.51% respectively as compared to control (P<0.05). Aspirin produced a significant antinociceptive effect (52%) in the second (late) phase only, compared to control group (P<0.001). Similarly, in the second (late) phase of the experiment, a marked dose-dependent inhibition in paw licking was observed for all fractions and the crude extract. *Gleditsia triacanthos* samples (A, B, C, D) at dose 200 mg/kg caused a significant inhibition in formalin-induced paw licking by 39.75%, 64.78%, 38.90%, 47.94% respectively in comparison to control group (P<0.001). At the high dose (400 mg/kg), the plant samples (A, B, C, D) showed a significant inhibition in formalin-induced paw licking by 46.99%, 71.64%, 58.12%, and 66.98% respectively in comparison to control group (P<0.001). Remarkably, samples B and D showed the most potent inhibition in both phases.

Formalin-induced nociception is a common method used to study inflammatory and non-inflammatory (neurogenic) pain [63]. This model is used to measure both centrally and peripherally mediated analgesic activities and has been reported to produce two distinct phases of nociceptive response [64]. An early phase (0–5 min), which involves non-inflammatory pain and is mediated by central nervous system via direct stimulation of sensory nerve fibres and peripheral nociceptors with marked increase in substance P levels and bradykinin. On the other hand, the late phase (15–45 min) involves peripheral release of inflammatory mediators including histamine, 5-hydroxy-tryptamine, bradykinin and prostaglandins leading to pain perception [65]. Centrally acting drugs inhibit both phases of formalin test while peripherally acting drugs inhibit the late phase only [66].

In the current study, results obtained from the formalin test revealed that *Gleditsia triacanthos* samples B and D inhibited both phases, therefore a peripheral as well as a central mechanism of their analgesic activities is probable. On the other hand, samples A and C inhibited only the late phase suggesting a peripheral analgesic effect. Moreover, samples B and D showed effectiveness against nociceptive and inflammatory pains with a more pronounced effect on the latter whereas samples A and C protected against inflammatory pain only. The inhibitory activity on inflammation-induced pain might reflect modulation of the synthesis and/or release of prostaglandins and other inflammatory mediators [65].

Similarly to aspirin, the mechanism of *Gleditsia triacanthos* samples-induced analgesic actions might probably be attributed to blockade of endogenous substances and inhibition of pain nerve endings excitation. Samples B and D might additionally exert direct effects on nociceptors.

Owing to the more pronounced effects of samples B and D in the late phase than the early one, it is probable that peripheral mechanisms are involved in their anti-nociceptive actions. Formalin develops a hyperalgesic state in the late phase of the test [67]. The pain suppression at the second phase therefore, indicates the anti-hyperalgesic activity of the studied plant samples.

3.3.3. Effect of *Gleditsia triacanthos* samples on tail flick test in mice

The inhibitory effects of all investigated *Gleditsia triacanthos* samples and the reference drug on the tail-flick reflex in mice are illustrated **(Fig. 3)**. Tramadol, the reference drug, significantly showed a 2.44 folds increase in the tail flick latency compared to the control (P<0.001). Only samples B and D at dose 200 mg/kg exerted a significant analgesic effect observed as 2.37 and 2.25 folds increase in the tail flick latency respectively as compared to control (P<0.001). At the dose of 400 mg/kg samples A, B, C and D showed a significant increase in tail flick latency observed as 2.3, 2.6, 2.4, and 2.4 folds, respectively as compared to control group (P<0.001).

It is well known that centrally acting analgesic drugs may elevate the pain threshold of rodents towards heat [68]. Therefore, the tail flick test was used to confirm the involvement of central mechanism(s) in the analgesic activity of *Gleditsia triacanthos* samples A, B, C and D. Tail-flick test is widely used to investigate the centrally acting analgesic activity [69]. The tail-flick response appears to be a spinal reflex, which is modulated by a supraspinal inhibitory mechanism and is considered to be selective for centrally acting analgesic compounds [70]. The reference narcotic analgesic drug, tramadol exhibited significant antinociceptive effects in this test. The analgesic activity of all investigated *Gleditsia triacanthos* samples seems to be dose-dependent. The high dose (400 mg/kg) of all studied plant samples produced a significant antinociceptive activity, while only samples B and D showed additional significant effectiveness at the low dose (200 mg/kg).

The present observation that samples A and C significantly inhibited thermal pain response in the tail flick test but not in the early phase of formaline-induced paw edema test may be attributed to the previously reported findings that thermal nociceptive tests are more sensitive to opioid μ-agonists while non thermal tests are more sensitive to opioid κ-agonists [71]. The present data suggests the involvement of different μ and/or κ agonistic activity for the different tested plant extracts. Interestingly, previous research studies showed that morphine analgesic effects in tail-flick test is partly mediated by brain stem serotoninergic systems [72]. This can point out the possibility of interaction with serotoninergic system by the investigated *Gleditsia triacanthos* samples.

The results obtained from the writhing response, formalin test and tail flick tests showed that samples A, B, C and D of *Gleditsia triacanthos* exhibited a dose-dependent peripheral and central analgesic activity.

3.4. Anti-inflammatory activity

3.4.1. Effect of *Gleditsia triacanthos* samples on xylene-induced ear edema in mice

The effect of *Gleditsia triacanthos* samples A, B, C and D at both doses (200 mg/kg and 400 mg/kg, p.o.) on xylene-induced ear edema in mice is shown in **Table 7**. Indomethacin, the reference drug significantly produced a 47.09% reduction in the ear edema weight as compared to control (P<0.001). Sample A at doses 200 mg/kg, and 400 mg/kg, showed a significant inhibition observed as 32.17% at P<0.01 and 46.62% at P<0.001 respectively, in comparison to control group. On the other hand, sample B (200mg/kg, 400 mg/kg) induced significant reduction in ear edema weight observed as 50.75% and 66.07%, at oral doses of 200 mg/kg and 400 mg/kg respectively, as compared to control group (P<0.001). Sample C (200 mg/kg, and 400 mg/kg) induced a reduction in ear edema weight by 37.03% and 48.88% respectively at P<0.001, while sample D (200 mg/kg, and 400 mg/kg) reduced ear edema weight by 45.20% and 59.78% respectively as compared to control at P<0.001.

Xylene is known to cause instant irritation and acute inflammation of the mouse ear, leading to severe vasodilation and increased vascular permeability with fluid exudation and subsequent edema formation [73]. Phospholipase A2 and substance P activities are also involved [74]. Pain and inflammation arise as a result of several mediators release, such as histamine, serotonin, leukotrienes, bradykinin and prostaglandins [75]. Xylene-induced ear edema is used commonly as an inflammatory model for evaluation of anti-inflammatory steroids specifically and for non-steroidal anti-inflammatory agents [76].

In this study, *Gleditsia triacanthos* samples showed anti-inflammatory efficacy evidenced by a dose-dependent inhibition of xylene-induced ear edema in rats. This antiphlogistic activity is possibly mediated via the control of vascular permeability, inhibition of phospholipase A2, and reduction in inflammatory mediators and substance P release.

3.4.2. Effect of *Gleditsia triacanthos* samples on carrageenan-induced paw edema in mice

The effects on the carrageenan-induced inflammation are represented as the percentage of

protection against edema induction **(Table 8)**. In control animals, the subplantar injection of carrageenan produced a local edema that increased progressively to reach a maximum intensity 4 h after injection of the phlogistic agent. Treatment with indomethacin, the reference drug, significantly reduced carrageenan-induced paw edema by 56.08% compared to control (P<0.001). Treatment with *Gleditsia triacanthos* sample A at dose 400 mg/kg only, produced a significant 39.69% inhibition of edema 4 h after administration of the phlogistic agent compared with control (P<0.01). Treatment with sample B at a dose of (200 mg/kg and 400 mg/kg, p.o) produced a significant inhibition of edema by 34.63% (P<0.05) and 50.77% (P<0.001) respectively, 4 h after administration of the phlogistic agent compared with control. Sample C only at a dose of 400 mg/kg, exerted a significant inhibition of edema by 41.18% (P<0.001), 4 h after administration of the phlogistic agent compared with control. Sample D at a dose of (200 mg/kg and 400 mg/kg, p.o) showed a significant inhibition of edema by 32.29% (P<0.05) and 44.94% (P<0.001) respectively, 4 h after administration of the phlogistic agent compared with control.

Carrageenan is a widely used noxious agent able to induce experimental acute inflammation, for screening of anti-inflammatory compounds. This phlogistic agent, when injected locally into the rat paw, produces a severe inflammatory reaction [77]. The development of edema induced by carrageenan is a biphasic event; the early phase of the inflammation (during the first 2 hours after carrageenan injection) is mainly mediated by chemical mediators such as histamine and serotonin, while the late phase (3-4 hours after carrageenan injection) is associated with activation of bradykinin, leukotrienes, prostaglandins and several cytokines and release of proteases and lysosomes [78]. The vascular response reaches its maximum level in this late phase, at which the present study was performed [79].

In the present study, *Gleditsia triacanthos* samples A,B,C, and D inhibited carrageenan-induced hind paw edema at the higher dose (400 mg/kg) where the greatest inhibition were observed with samples B and D that showed a dose-dependent anti-inflammatory activity after 4 hours of carrageenan injection (late phase). The anti-inflammatory activity of *Gleditsia triacanthos* samples may be mediated via inhibition of the late phase by restraining several cytokines, bradykinins, leukotrienes and prostaglandins production [80]. Previous studies revealed that the anti-inflammatory activities in carrageenan rat paw edema test might be mediated via reduction in nitric oxide and pro-inflammatory cytokines production with simultaneous elevation of anti-inflammatory cytokines levels [81,82]. The involvement of free radicals in the inflammatory processes [83], and the effectiveness of opioid receptor-mediated activities in suppressing the inflammation in carrageenan-induced paw edema were previously reported [84].

3.5. Hepatoprotective activity

3.5.1. Effect of *Gleditsia triacanthos* samples on serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TBil) in mice

As shown in **Table 9**, administration of CCl_4 to mice induced a significant increase in serum levels of serum AST, ALT, ALP, and TBil by 184.2%, 144.9%, 171.1%, and 75.3% respectively as compared to control group (P<0.001).

Pre-administration of sample A (400 mg/kg, orally) to CCl_4-treated mice showed a significant increase in the mentioned parameters by 113.0% (P<0.001), 77.2% (P<0.05), 108.5% (P<0.001), and 52.3% (P<0.05) respectively as compared to control. The same treatment group showed a significant decrease in each of AST level (25.0%), ALT level (27.7%), and ALP level (23.1%) as compared to CCl_4 group (P<0.05).

On the other hand, Pre-treatment of CCl_4-administered mice with sample B (400 mg/kg, orally) showed a significant decrease in the hepatic injury markers serum AST, ALT, ALP and TBil by 45.3% (P<0.001), 32.6% (P<0.01), 45.6% (P<0.001), and 32.4% (P<0.05) respectively as compared to CCl_4 group.

Pre-treatment of CCl_4-intoxicated mice with sample C (400 mg/kg, orally) significantly reduced serum AST, ALT, ALP and TBil by 49.3% (P<0.001), 38.7% (P<0.001), 46.1% (P<0.001), and 36.5% (P<0.01).

On the contrary, pre-administration of sample D (400 mg/kg, orally) to CCl_4-treated mice showed a marked increase in serum AST, ALT, ALP and TBil by 172.7%, 122.1%, 155.1%, and 69.5% respectively as compared to control group (P<0.001).

Pre-administration of the reference compound silymarin (200 mg/kg, orally) to CCl_4-intoxicated mice, caused a significant decrease in the serum levels of AST, ALT, ALP, and TBil by 52.3% (P<0.001), 44.7% (P<0.001), 47.4% (P<0.001), and 38.9% (P<0.01) respectively as compared to CCl_4-intoxicated group.

3.5.2. Effect of *Gleditsia triacanthos* samples on hepatic total lipid peroxides level measured as malondialdehyde (MDA) in mice

As illustrated in **Fig. 4**, administration of CCl_4 to mice caused a significant increase in liver MDA level by 146.8% as compared to control group (P<0.001). Pre-administration of *Gleditsia triacanthos* sample A (400 mg/kg, orally) to CCl_4-treated mice showed a significant increase in hepatic MDA level by 74.4% (P<0.05) as compared to control and a significant

decrease in hepatic MDA content by 29.4% (P<0.05) as compared to CCl_4. On the other hand, pre-treatment of CCl_4-administered mice with sample B or C (400 mg/kg, orally) showed a significant decrease in hepatic MDA level by 33.8% (P<0.01), 39.9% (P<0.001) respectively as compared to CCl_4 group. On the contrary, pre-administration of sample D (400 mg/kg, orally) to CCl_4-treated mice showed a marked increase in hepatic MDA level by 127.2% as compared to control group (P<0.001). Pre-administration of silymarin (200 mg/kg, orally) to CCl_4-intoxicated mice caused a significant decrease in the hepatic MDA level by 47.0% as compared to CCl_4 group (P<0.001).

3.5.3. Effect of *Gleditsia triacanthos* samples on hepatic nitric oxide (NO) level measured as total nitrates/nitrites in mice

Administration of CCl_4 to mice induced a significant increase in liver NO level by 100.1% as compared to control group (P<0.001). Pre-administration of sample A (400 mg/kg, orally) to CCl_4-treated mice showed a significant increase in hepatic NO level by 60.3% as compared to control (P<0.05). On the other hand, pre-treatment of CCl_4-administered mice with sample B or C (400 mg/kg, orally) showed a significant decrease in hepatic NO level by 28.5% (P<0.05), 35.4% (P<0.01) respectively as compared to CCl_4 group. On the contrary, pre-administration of sample D (400 mg/kg, orally) to CCl_4-treated mice showed a marked increase in hepatic NO level by 91.4% (P<0.001) as compared to control group. Pre-administration of silymarin (200 mg/kg, orally) to CCl_4-intoxicated mice caused a significant decrease in the hepatic NO level by 39.7% (P<0.01) as compared to CCl_4 group **(Fig. 5)**.

3.5.4. Effect of *Gleditsia triacanthos* samples on hepatic reduced glutathione (GSH) level in mice

As shown in **Fig. 6**, CCl_4 intoxication in mice caused a significant decrease in hepatic reduced glutathione (GSH) level by 62.8% as compared to control group (P<0.001). Pre-administration of either sample A or D (400 mg/kg, orally) to CCl_4-treated mice caused a significant decrease in hepatic GSH level by 35.9% (P<0.01), 56.6% (P<0.001) respectively as compared to control. Pre-administration of sample A to CCl_4-treated mice caused a significant elevation in hepatic glutathione level by 72.2% (P<0.05) as compared to CCl_4 group. On the other hand, pre-treatment of CCl_4-administered mice with either sample B or C (400 mg/kg, orally) caused a significant increase in hepatic GSH level by 109.9% and 118.6% respectively as compared to CCl_4-intoxicated group (P<0.001). Pre-treatment of CCl_4-administered mice with silymarin (200 mg/kg, orally) caused a significant increase in hepatic GSH level by 128.6% as compared to CCl_4-intoxicated group (P<0.001).

3.5.5. Effect of *Gleditsia triacanthos* samples on hepatic superoxide dismutase enzyme (SOD) activity in mice

CCl_4-intoxicated mice showed a significant decrease in hepatic superoxide dismutase enzyme (SOD) activity by 55.7% (P<0.001) as compared to control group. Pre-administration of either sample A or D (400 mg/kg, orally) to CCl_4-treated mice caused a significant decrease in hepatic SOD activity by 33.7% (P<0.05), 51.3% (0.001) respectively as compared to control. On the other hand, pre-treatment of CCl_4-administered mice with either sample B or C (400 mg/kg, orally) caused a significant increase in hepatic SOD activity by 76.4% (P<0.05) and 88.4% (P<0.01) respectively as compared to CCl_4-intoxicated group. Pre-treatment of CCl_4-administered mice with silymarin (200 mg/kg, orally) caused a significant increase in hepatic SOD activity by 97.8% (P<0.001) as compared to CCl_4-intoxicated group **(Fig. 7)**.

The present investigation of the hepatoprotective activities of *Gleditsia triacanthos* samples revealed that CCl_4 administration to mice induced significant hepatotoxicity evidenced by increased serum levels of AST, ALT, and ALP. These findings, in accordance with other previous studies, were attributed to hepatic tissue necrosis and loss of membrane structural integrity produced by the well-known hepatotoxin CCl_4 [85-87]. The mentioned enzymes are mainly present in the cytoplasm. However, following cellular injury, they are released into the blood stream and hence can be detected in serum [88]. The current study, similar to previous ones, showed that CCl_4-intoxication is associated with elevated serum total bilirubin level [89,90]. Removal of bilirubin from the blood into the bile is one of the normal functions of the liver. Therefore, observed elevation in serum total bilirubin concentration is considered a marker of the decreased ability of liver to process bilirubin [91].

In the present study, sample C showed the most significant hepatoprotective activity followed by sample B. On the other hand, sample A could only attenuate CCl_4-induced hepatotoxicity with a significant difference from the control group. Unfortunately, sample D did not prevent CCl_4-induced increase in serum levels of hepatic injury markers. Many plant-derived compounds were investigated for their effectiveness in reducing hepatic damage [90,92,93]. However, the present study is the first one to evaluate the hepatoprotective activities of *Gleditsia triacanthos* leaves in mice.

In order to investigate the underlying mechanisms of these hepatoprotective activities, liver contents of lipoperoxidative and nitrosative stress markers were estimated. Our findings are consistent with other previous reports about CCl_4-induced increase in MDA and NO levels in liver [94,95]. Elevated free radicals

production and oxidative stress have been implicated in acute hepatic deterioration [96]. Free radicals may interact with cellular components to induce structural damage, cellular necrosis and death [97]. Liver contains a relatively high content of polyunsaturated fatty acids, which are susceptible to peroxidation [98]. Free radicals resulting from the metabolism of CCl_4, mainly CCl3• and CCl3OO•, can covalently bind to polyunsaturated fatty acids (PUFA) leading to the formation of CHCl3 and PUFA•. The latter can cause progression in lipid peroxidation, cell membrane damage, cellular necrosis and finally death [99]. MDA interaction with amino moieties may lead to suppression of DNA and RNA synthesis, as well as, interaction with and inactivation of cellular proteins and enzymes [100].

Moreover, nitric oxide and nitric oxide free radicals may cause nitrosative stress. Reaction of nitric oxide metabolites with superoxide radicals in the mitochondria may produce peroxynitrite radicals with consequent cellular dysfunction [101]. CCl_4-induced nitrosative stress was previously reported in several experimental studies [102,103]. It was noted that nitric oxide radicals might contribute to hepatic inflammation by regulating the expression of pro-inflammatory genes [104].

The effectiveness of samples C and B to reverse or sample A to attenuate, CCl_4-induced hepatic lipoperoxidative and nitrosative stress was evidenced in this work. These effects could be directly attributed to the antioxidant activity of the samples. Flavonoids and polyphenols, which are phytochemicals with high antioxidant properties, were appreciated for their antilipoperoxidative and antinitrosative activities [105,106,107]. Silymarin, the currently used hepatoprotective agent, is a mixture of flavonolignans with marked antioxidant activity [108].

In order to examine the modulatory effects of *Gleditsia triacanthos* samples on endogenous antioxidants, hepatic GSH and SOD activity were estimated. The present data showed that CCl_4-induced decrease in hepatic GSH level was restored by pre-administration of samples C (to a greater extent) and B (to a lesser extent). Pre-administration of samples A showed significant amelioration in hepatic GSH content though without re-normalization.

Mammalian cells defend themselves against free radicals and reactive oxygen species-mediated cellular damage by means of several natural antioxidant compounds and enzymes such as GSH and SOD enzyme [109]. GSH is produced in liver and is maintained at higher concentrations in tissues than in plasma [110,111]. Marked depletion of GSH and consequent hepatic necrosis were observed after administration of hepatotoxic agents such as CCl_4, paracetamol and diethylnitrosamine [112].

On the other hand, natural antioxidants and thiol group-containing compounds have been extensively used to attenuate CCl_4-induced depletion of GSH [113-115]. Our results suggest that the hepatoprotective effects mediated by samples C and B are probably attributable to their antioxidant properties. Interestingly, it was recently found that silymarin might enhance hepatic glutathione production by shifting cysteine metabolism, which is the essential substrate for GSH, into more synthesis and less catabolism with subsequent enhancement of the antioxidant defense status of the liver [116]. Mistry *et al.* [117] revealed that plant extracts increase hepatic glutathione content by enhancement of de novo synthesis of glutathione or by increasing regeneration of the reduced form. Lately, extracts of *Gleditsia triacanthos* leaves and seeds gained attention for their non-biological antioxidant and free radical scavenging activities owing to their phenolic and flavonoid compounds content [10,118].

Similar to previous studies, CCl_4 treatment in the present investigation caused a significant inhibition of hepatic SOD activity, due to excessive generation of superoxide free radicals [95,119]. The down regulation of SOD gene expression was observed in hearts and kidneys of CCl_4-intoxicated rats [120]. SOD enzyme plays an essential role in cell salvage from detrimental effects of oxidants and free radicals via catalyzing the conversion of superoxide radicals to hydrogen peroxide plus oxygen followed by catalase enzyme, which converts hydrogen peroxide to water [121].

Fortunately, in the present study, *Gleditsia triacanthos* samples B and C showed a significant increase in hepatic SOD activity in CCl_4-intoxicated mice, comparable to that offered by silymarin. This finding emphasizes the antioxidant activity of plant samples B and C. Many compounds and plant extracts with established hepatoprotective activities against CCl_4 toxicity show strong antioxidant effects [90,92,122]. Flavonoids can protect membrane unsaturated fatty acids against oxidation by reacting with superoxide radicals and suppressing the production of peroxynitrites [123]. Moreover, the low water solubility, short intestinal retention time and low absorption rate of flavonoids grant them a distinguishable high safety margin for therapeutic use [124].

3.6. Antimicrobial activity

Results shown in (**Table 10)** revealed that all the tested plant samples (A,B,C,D) of *G. triacanthos* exerted marked antibacterial activity against the tested Gram +ve and Gram -ve bacteria. They showed higher activity against Gram +ve bacteriathan Gram -ve bacteria. Fortunately, *Pseudomonas aeuroginosa* which is one of the most common nosocomial infections (hospital acquired infection) showed sensitivity to all tested samples.

Table 1: Chromatographic properties and UV spectral data of compounds 3 and 4

No.	R_f-values		Fluorescence		Spray reagents		UV spectral data, λ_{max} (nm)					
	S_1	S_2	UV	UV/ NH_3	$FeCl_3$	NA/ PE 365 nm	MeOH	NaOMe	NaOAc	NaOAc/ H_3BO_3	$AlCl_3$	$AlCl_3$/ HCl
3	0.43	0.39	d. pr	y.	gr.	or.	269, 295sh, 348	278, 327, 403	278, 320, 385	269, 375, 430	278, 325, 415	278, 298sh, 350, 385
4	0.29	0.21	d. pr	y.	d. gr.	or.	262, 350	278, 377	278, 330sh, 385	267, 377, 425sh	278, 301sh, 353sh, 409	278, 301sh, 350, 387

d. pr. = dark purple, y. = yellow, gr. = green, d. gr. = dark green, or. = orange.

Table 2: ^1H, ^{13}C NMR and Negative ESI/MS data of compounds 3 and 4

C. No.	3		4	
	δ C	δ H	δ C	δ H
2	164.01		164.05	
3	103.04	6.54 s	102.36	6.6 s
4	182.17		181.90	
5	161.18	13.56 s (OH-5)	160.30	13.14 s (OH-5)
6	109.46		98.00	6.23 s
7	161.19		162.50	
8	94.22	6.33 s	103.90	
9	156.81		155.90	
10	103.42		104.50	
1′	121.62		121.90	
2′	113.62	7.34 m	114.03	7.45 br.s
3′	146.44		145.70	
4′	150.68		149.60	
5′	116.62	6.78 d (8.1)	115.60	6.82 d (8.4)
6′	119.42	7.34 m	119.30	7.49 m
1″	73.64	4.53 d (9.9)	73.30	4.63 d (9.8)
2″	71.12	4.03 t-like (8)	70.70	
3″	79.54	4.05-3.10 m, hidden by H_2O-signal, remaining sugar protons	78.70	3.8-3.25 m, hidden by H_2O-signal, remaining sugar protons
4″	70.64		70.60	
5″	82.05		81.90	
6″	61.97		61.60	
$[M-H]^-$	447.2			
$[2M-H]^-$	894.8			

^{13}C NMR (125 MHz, DMSO-d_6), ^1H NMR (500 MHz, DMSO-d_6)
Values between parentheses represent the *J*-values in Hz

Table 3: Chromatographic properties and UV spectral data of compounds 5 and 6

No.	R_f-values		Fluorescence		Spray reagents		UV spectral data, λ_{max} (nm)					
	S_1	S_2	UV	UV/NH_3	$FeCl_3$	NA/ PE 365 nm	MeOH	NaOMe	NaOAc	NaOAc/ H_3BO_3	$AlCl_3$	$AlCl_3$/ HCl
5	0.43	0.49	d. pr.	y. gr.	g. y.	gr.	270, 302sh, 336	279, 329, 395	280, 300, 379	271, 329sh, 344	277, 305, 350, 386;	278, 303, 343, 383
6	0.57	0.55	pr.	y. gr.	g. y.	gr.	271, 336	278, 329, 398	279, 303, 385	274, 346, 408sh	262sh, 278, 304, 352, 382;	260sh, 280, 302, 344, 380

d. pr. = dark purple, pr. = purple, y. gr. = yellowish green, g. y. = grayish yellow gr. = green

Table 4: ^{1}H, ^{13}C NMR and Negative ESI/MS data of compounds 5 and 6

C. No.	5		6	
	δ C	δ H	δ C	δ H
2	163.99		163.23	
3	102.49	6.77 s	102.60	6.75 s
4	182.03		181.63	
5	160.44		161.42	13.53 s (OH-5)
6	98.23	6.27 s	109.09	
7	162.69		161.43	
8	104.67		94.02	6.48 s
9	156.05		156.47	
10	104.07		102.60	
1′	121.66		121.06	
2′/6′	128.99	8.02 d (8.4)	128.37	7.89 d (8.1)
3′/5′	115.88	6.9 d (8.4)	116.10	6.88 d (8.1)
4′	161.18		160.71	
1″	73.48	4.68 d (9.6)	73.32	4.54 d (9)
2″	70.93		70.64	
3″	78.73	3.2-3.9 m, remaining sugar protons	79.11	3.7-3.2 m, hidden by H_2O-signal, remaining sugar protons
4″	70.63		70.27	
5″	81.88		81.49	
6″	61.38		61.47	
$[M-H]^-$	431			
$[2M-H]^-$	863			

^{13}C NMR (125 MHz, DMSO-d_6), ^{1}H NMR (500 MHz, DMSO-d_6)
Values between parentheses represent the *J*-values in Hz

Table 5: Antinociceptive effect of *Gleditsia triacanthos* samples A, B, C and D on acetic acid-induced writhing in mice

Treatment groups	Oral dose (mg/kg)	Number of writhes	Inhibition (%)
Control (vehicle)	**10 ml/kg**	22.57 ± 1.70	-
Aspirin	**100 mg/kg**	6.71 ± 0.68***	70.25%
Sample A	**200 mg/kg**	9.29 ± 1.02***	58.86%
	400 mg/kg	7.14 ± 0.51***	68.35%
Sample B	**200 mg/kg**	5.29 ± 0.57***	76.58%
	400 mg/kg	2.71 ± 0.29***	87.98%
Sample C	**200 mg/kg**	7.29 ± 0.84***	67.72%
	400 mg/kg	8.71 ± 0.75***	61.39%
Sample D	**200 mg/kg**	6.86 ± 0.74***	69.62%
	400 mg/kg	4.86 ± 0.59***	78.48%

Data is represented as mean ± SEM (n = 7 mice). *** Significantly different from control at $P<0.001$. Percentage inhibition is relative to control. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

Table 6: Antinociceptive effect of *Gleditsia triacanthos* samples A, B, C and D on formalin-induced paw licking in mice

Treatment groups	Oral dose (mg/kg)	Early phase (0-5 min.)		Late phase (15-45 min.)	
		Licking time (seconds)	Inhibition (%)	Licking time (seconds)	Inhibition (%)
Control (vehicle)	**10 ml/kg**	138.30 ± 11.24	-	150.10 ± 10.95	-
Aspirin	**100 mg/kg**	91.14 ± 7.91	34.10 %	72.57 ± 7.04***	51.65 %
Sample A	**200 mg/kg**	122.60 ± 10.58	11.35 %	90.43 ± 6.34***	39.75 %
	400 mg/kg	112.90 ± 10.46	18.37 %	79.57 ± 7.47***	46.99 %
Sample B	**200 mg/kg**	106.90 ± 11.19	22.70 %	52.86 ± 4.67***	64.78 %
	400 mg/kg	89.14 ± 7.95*	35.55 %	42.57 ± 3.90***	71.64 %
Sample C	**200 mg/kg**	126.90 ± 11.53	8.24 %	91.71 ± 6.61***	38.90 %
	400 mg/kg	98.71 ± 9.47	28.63 %	62.86 ± 6.91***	58.12 %
Sample D	**200 mg/kg**	124.40 ± 11.84	10.05 %	78.14 ± 7.39***	47.94 %
	400 mg/kg	90.57 ± 8.60*	34.51 %	49.57 ± 8.26***	66.98 %

Data is represented as mean ± SEM (n= 7 mice). *,*** Significantly different from control at $P<0.05$, $P<0.001$, respectively. Percentage inhibition is relative to control. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

Table 7: Effect of *Gleditsia triacanthos* samples A, B, C and D on xylene-induced ear edema in mice

Treatment groups	Oral dose (mg/kg)	Average ear edema weight (mg) ± SEM	Inhibition (%)
Control (vehicle)	**10 ml/kg**	15.20 ± 1.34	-
Indomethacin	**100 mg/kg**	8.04 ± 0.82***	47.09 %
Sample A	**200 mg/kg**	10.31 ± 0.78**	32.17 %
	400 mg/kg	8.11 ± 0.80***	46.62 %
Sample B	**200 mg/kg**	7.49 ± 0.76***	50.75 %
	400 mg/kg	5.16 ± 0.41***	66.07 %
Sample C	**200 mg/kg**	9.57 ± 1.06***	37.03 %
	400 mg/kg	7.77 ± 0.73***	48.88 %
Sample D	**200 mg/kg**	8.33 ± 0.84***	45.20 %
	400 mg/kg	6.11 ± 0.66***	59.78 %

Data is represented as mean ± SEM (n = 7 mice). **,*** Significantly different from control at $P<0.01$, $P<0.001$ respectively. Percentage inhibition is relative to control. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

Table 8: Effect of *Gleditsia triacanthos* samples A, B, C and D on carrageenan-induced paw edema in mice

Treatment groups	Oral dose (mg/kg)	Average paw edema weight (mg) ±SEM	Inhibition (%)
Control (vehicle)	**10 ml/kg**	54.78 ± 4.04	-
Indomethacin	**100 mg/kg**	24.06 ± 2.27***	56.08 %
Sample A	**200 mg/kg**	40.09 ± 3.63	26.82 %
	400 mg/kg	33.04 ± 2.97**	39.69 %
Sample B	**200 mg/kg**	35.81 ± 3.05*	34.63 %
	400 mg/kg	26.97 ± 3.28***	50.77 %
Sample C	**200 mg/kg**	41.14 ± 3.76	24.90 %
	400 mg/kg	32.22 ± 3.30***	41.18 %
Sample D	**200 mg/kg**	37.09 ± 4.02*	32.29 %
	400 mg/kg	30.16 ± 3.84***	44.94 %

Data is represented as mean ± SEM (n = 7 mice). *,**,*** Significantly different from control at $P<0.05$, $P<0.01$, $P<0.001$ respectively. Percentage inhibition is relative to control. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

Table 9: Effect of *Gleditsia triacanthos* samples A, B, C and D on serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (TBil) in mice

Treatment groups	AST (U/l)	ALT (U/l)	ALP (U/l)	TBil (mg/dl)
Control (vehicle)	77.59 ± 4.65	61.73 ± 4.66	109.30 ± 6.73	0.52 ± 0.05
CCl_4	220.52 ± 12.13***	151.21 ± 11.36***	296.34 ± 16.96***	0.91 ± 0.07***
Sample A + CCl_4	165.30 ± 15.12***#	109.41 ± 10.55*#	227.92 ± 21.36***#	0.79 ± 0.06*
Sample B + CCl_4	120.71 ± 10.40###	101.91 ± 8.79##	161.12 ± 12.13###	0.61 ± 0.05#
Sample C + CCl_4	111.72 ± 8.17###	92.69 ± 8.84###	159.61 ± 8.62###	0.58 ± 0.05##
Sample D + CCl_4	211.63 ± 14.61***	137.13 ± 11.94***	278.80 ± 16.36***	0.88 ± 0.07***
Silymarin + CCl_4	105.10 ± 9.14###	83.67 ± 7.65###	155.81 ± 8.65###	0.56 ± 0.05##

Data is presented as mean ± SEM (n = 10). *,*** Significantly different from control at $P<0.05$, $P<0.001$ respectively, #,##,### Significantly different from CCl_4 at $P<0.05$, $P<0.01$, $P<0.001$ respectively. CCl_4 was administered i.p. at a dose of 10 ml/kg, 0.1 % in olive oil. Samples A, B, C, and D were administered orally at a dose of 400 mg/kg. Silymarin was administered orally at a dose of 200 mg/kg. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

Table 10:Antimicrobial screening of the alcoholic samples of the leaves of *G. triacanthos*

Micro-organisms	Sample A	Sample B	Sample C	Sample D	Imipenem	Cipro-floxacin	MeOH	EtOH
***Coagulase negative Staphylococci* (G +ve)**	---	15 mm ++	18 mm +++	25 mm ++++	≥ 25 mm ++++	13 mm ++	---	---
***Staphylococcus aureus* (G +ve)**	20 mm +++	15 mm ++	15 mm ++	20 mm +++	20 mm +++	20 mm +++	---	---
***Escherichia coli* (G -ve)**	---	---	---	---	25 mm ++++	13 mm ++	---	---
***Proteus mirabilis* (G -ve)**	---	12 mm ++	---	---	24 mm ++++	20 mm +++	---	---
***Klebsiella pneumonia* (G -ve)**	---	12 mm ++	---	---	25 mm ++++	---	---	---
***Pseudomonas aeuroginosa* (G -ve)**	15 mm ++	17 mm +++	18 mm +++	20 mm +++	25 mm ++++	25 mm ++++	---	---

A: Crude extract (70% ethanol extract of leaves) **B**: Fraction II **C**: Fraction III **D**: Fraction V
G: Gram reaction--- No inhibition zone + + diameter of inhibition zone 11-15 mm.
+++ diameter of inhibition zone 16-22 mm. ++++ diameter of inhibition zone more than 23 mm.

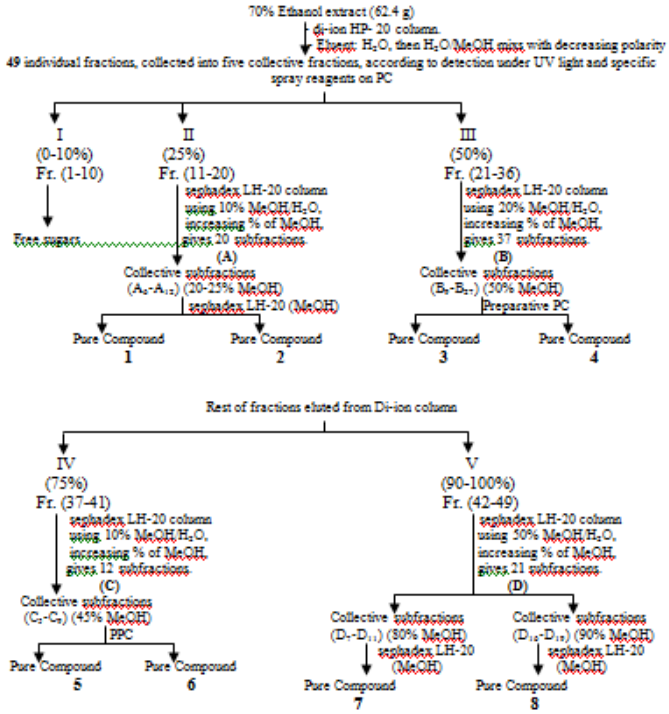

Fig. 1: Flow chart of fractionation and purification of polyphenolic compounds isolated from the leaves of *G. triacanthos*.

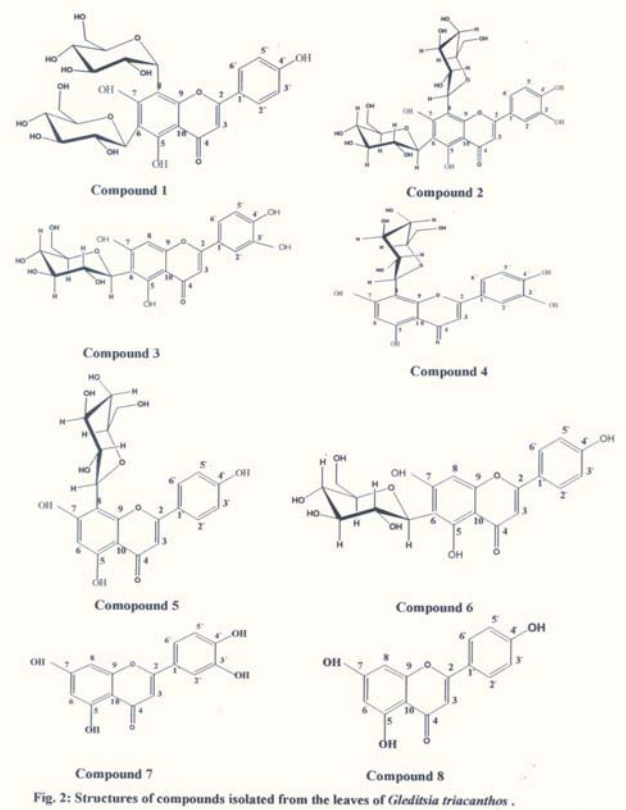

Fig. 2: Structures of compounds isolated from the leaves of *Gleditsia triacanthos* .

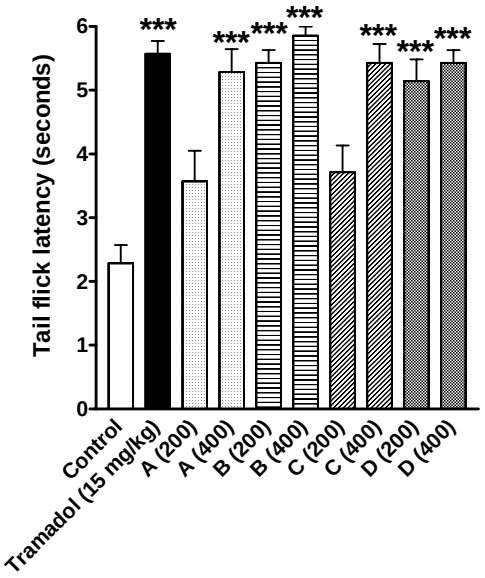

Fig. 3: Antinociceptive effect of *Gleditsia triacanthos* samples A, B, C and D on tail flick latency in mice

Data is represented as mean ± SEM (n= 7 mice). *** Significantly different from control at *P<0.001*. ANOVA and Tukey's post hoc tests were used for statistical analysis of data

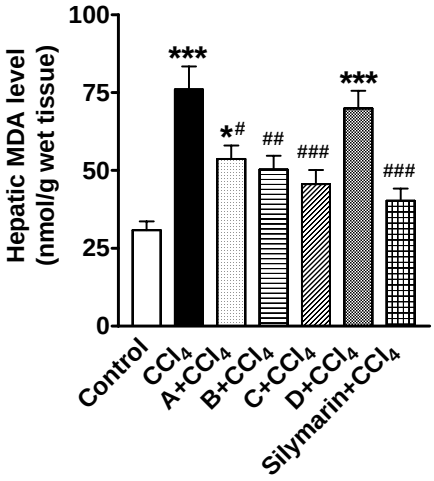

Fig. 4: Effect of *Gleditsia triacanthos* samples A, B, C and D on hepatic total lipid peroxides level measured as malondialdehyde (MDA) in mice

Data is represented as mean ± SEM (n = 10). *,*** Significantly different from control at *P<0.05, P<0.001* respectively, #,##,### Significantly different from CCl_4 at *P< 0.05, P<0.01, P<0.001* respectively. CCl_4 was administered i.p. at a dose of 10 ml/kg, 0.1 % in olive oil. Samples A, B, C, and D were administered orally at a dose of 400 mg/kg. Silymarin was administered orally at a dose of 200 mg/kg. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

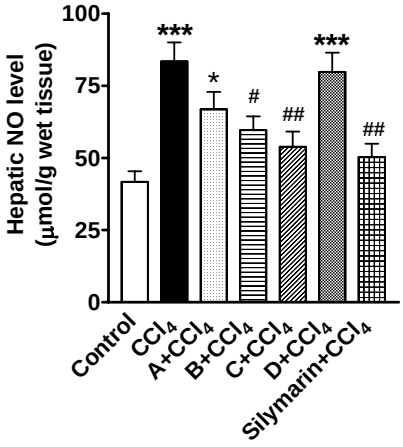

Fig. 5: Effect of *Gleditsia triacanthos* samples A, B, C and D on hepatic nitric oxide (NO) level measured as total nitrates/nitrites in mice

Data is represented as mean ± SEM (n = 10). *,*** Significantly different from control at *P<0.05, P<0.001* respectively, #,## Significantly different from CCl_4 at *P<0.05, P<0.01* respectively. CCl_4 was administered i.p. at a dose of 10 ml/kg, 0.1 % in olive oil. Samples A, B, C, and D were administered orally at a dose of 400 mg/kg. Silymarin was administered orally at a dose of 200 mg/kg. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

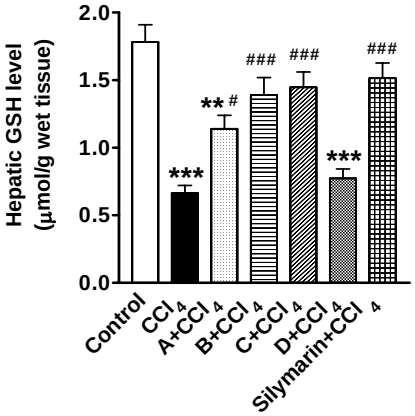

Fig. 6: Effect of *Gleditsia triacanthos* samples A, B, C and D on hepatic reduced glutathione (GSH) level in mice

Data is represented as mean ± SEM (n = 10). **,*** Significantly different from control at *P<0.01, P<0.001* respectively, #,### Significantly different from CCl_4 at *P<0.05, P<0.001* respectively. CCl_4 was administered i.p. at a dose of 10 ml/kg, 0.1 % in olive oil. Samples A, B, C, and D were administered orally at a dose of 400 mg/kg. Silymarin was administered orally at a dose of

200 mg/kg. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

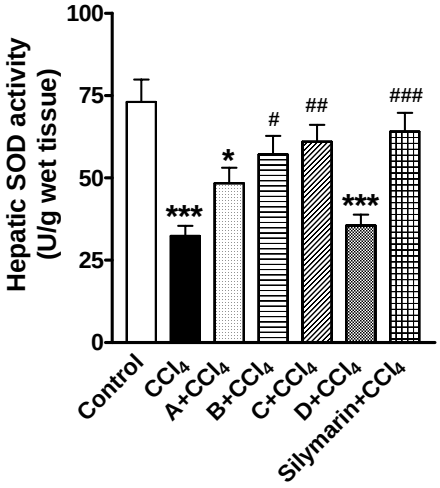

Fig. 7: Effect of *Gleditsia triacanthos* samples A, B, C and D on hepatic superoxide dismutase (SOD) activity in mice

Data is represented as mean ± SEM (n = 10). *,*** Significantly different from control at *P<0.05, P<0.001* respectively, #,##,### Significantly different from CCl_4 at *P<0.05, P<0.01, P<0.001* respectively. CCl_4 was administered i.p. at a dose of 10 ml/kg, 0.1 % in olive oil. Samples A, B, C, and D were administered orally at a dose of 400 mg/kg. Silymarin was administered orally at a dose of 200 mg/kg. ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

4. Conclusion

In conclusion, this study showed that the ethanol extract of the leaves of *Gleditsia triacanthos* contains flavonoid compounds mainly of *C*-glycosidic type, six of them are isolated for the first time from *Gleditsia* genus*:* Vicenin-II, Lucenin-I, Orientin, Isoorientin, Vitexin and Apigenin.

This study demonstrated promising analgesic, anti-inflammatory, hepatoprotective and antimicrobial activities of *Gleditsia triacanthos* leaves for the first time. These beneficial activities might be attributed to various phenolic compounds present in the examined plant samples. However, further research studies are recommended to isolate and define the bioactive analgesic, anti-inflammatory and hepatoprotective principles of *Gleditsia triacanthos* leaves. Clinical studies are encouraged to evaluate these pharmacological activities in human.

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