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Original Article

# Phytochemical analysis of *Juglans regia* oil and kernel exploring their antinociceptive and anti-inflammatory potentials utilizing combined bio-guided GC–FID, GC–MS and HPLC analyses

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ABSTRACT

*Juglans regia* L., Juglandaceae, is broadly used due to its immunomodulatory effects, potentials in protecting against many sever-disorders, and high safety-profile. The aim of this work is to make a phytochemical analysis of *J. regia* oil and kernel exploring their antinociceptive and anti-inflammatory potentials utilizing combined bio-guided gas chromatography with mass spectrometer (GC–MS), gas chromatography with flame ionization detection (GC–FID) and reversed-phase high-performance liquid chromatography (RP–HPLC) analyses. Combined bio-guided GC–MS, GC–FID and RP–HPLC analyses is an innovative-combined-technique aiming at efficiently analyzing various-extracts phytochemical and biological characters. The *J. regia* oil and kernel ethyl-acetate extract were monitored during exploring their possible acute-antinflammatory, antidiabetic and antidiabetic-neuropathy. Glycated-hemoglobin, serum-insulin, serum-catalase and lipid-peroxidation levels have been also monitored. Combined bio-guided GC–FID, GC–MS and HPLC analyses have shown to be an efficient analyzing-method through identifying the most active compound, linoleic acid. Linoleic acid has shown the highest improvement of the acute inflammatory-pain, chronic blood-glucose level reduction, serum-insulin elevation, and normalization of glycated-hemoglobin levels. *J. regia* oil has shown more lipid-peroxidation reduction, while kernel ethyl-acetate extract has shown more acute-blood-glucose level reduction and serum-catalase levels elevation. Compared to tramadol, the highest-doses of *J. regia* oil, kernel ethyl-acetate extract, and linoleic acid have shown higher antinociceptive-potentials in amelioration of thermal-hyperalgesic and anti-allodynic neuropathic-pain. Thus, the antinflammatory, the reduction of oxidative-stress, and the insulin-secretagogue potentials might be among the possible mechanisms of improvement of neuropathic-pain. In correlation to conventional-techniques, the combined bio-guided analyses have shown to be an efficient innovative-combined technique. After further clinical studies, *J. regia* might be utilized as a possible-remedy for various painful-syndromes.



## Introduction

Many studies have established medicinal plants efficacy in the amelioration of many serious and chronic disorders including inflammation, diabetes, and painful diabetic neuropathy (Wu and Liang, 2007; Xu et al., 2012). *Juglans regia* L., Juglandaceae, was used since ancient times because of its immunomodulatory effects, potentials in protecting against many sever disorders, and high safety profile (Paudel et al., 2013). *J. regia is* rich in unsaturated fatty acids, plant sterols, and polyphenols. Recent investigations reported that it can reduce serum-cholesterol, improve blood-vessels function and has hypoglycemic potentials (Hayes et al., 2016).

Currently, the majority of phytochemical investigations focus on single chemical or analytical technique to standardize and identify the content of oils or kernels (Mathias and Halkar, 2004). Whereas, the combination of analytical and biological methods would give a more clear and accurate view of the oils and kernels active constituents. This will help in identifying the most active compounds with the aid of the bio-guided fractionation studies.

The prevalence of painful diabetic neuropathy (DN) is affecting *ca.* 366 million patients worldwide (Tesfaye, 2013). DN occurs in *ca.* 50% of patients with diabetes, with *ca.* 15% being painful (Boulton et al., 2004; Wallace et al., 2015). Patients often complain of pain, hyperalgesia and tactile allodynia (Galer et al., 2000). Despite the presence of several useful drugs for the management of neuropathic pain, still there is a number of medical-cases that are under-treated or un-treated (Raafat et al., 2017). This matter has raised the concern to find an alternative therapies for the better

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management of neuropathic pain. One of the promising phytotherpies that can ameliorate neuropathic pain is *J. regia.* Both *J. regia* leaves and kernels are reported to have hypoglycemic activities (Pan et al., 2013; Hosseini et al., 2014). The leaves are the only one reported to have neuroprotective effects (Nasiry et al., 2017). No reports were found about the neuroprotective effects of oils or kernels.

Therefore, the aim of the current work is to make phytochemical analysis of *J. regia* oil and kernel exploring their antinociceptive and anti-inflammatory potentials utilizing combined bio-guided GC–FID, GC–MS and HPLC analyses.

## Materials and methods

### *Plant samples*

*Juglans regia* L., Juglandaceae, kernels were obtained from Ibn Al-Nafees herbalist (Beirut, Lebanon), and the kernels were authenticated by comparing to a reference-sample, and a sample was kept in the faculty-herbarium with a voucher number (PS-17-55).

### *Extraction of* Juglans regia *kernel ethyl acetate extract and oil*

The kernels were size-reduced using Sichuan mill (China). The powdered kernels were divided into two portions. The first portion was extracted utilizing ethyl acetate (Analytical grade, Sigma–Aldrich, Germany) and was sonicated (Jeken Sonicator, China) for 3 h. The ethyl acetate kernel extract (JREA) was dried under reduced pressure using Buchi rotary-evaporator (Germany) at 40 °C, and JREA was kept at −4 °C until further experimentation. The *J. regia* oil (JRO) was extracted from the second portion utilizing Elemental Scientific Soxhlet apparatus (USA) and hexane (Analytical grade, Sigma-Aldrich, Germany) as a solvent for 6 h. The hexane was removed from JRO under reduced pressure using rotary-evaporator at 40 °C, and JRO was preserved at −4 °C until testing time.

### *Combined bio-guided GC–FID, GC–MS and RP–HPLC analyses*

Analytical gas chromatography with flame ionization detection (GC–FID), and mass spectrometry (GC–MS), and RP–HPLC combined by biological diabetic and diabetic neuropathy models were used for the characterization of the JREA and JRO active constituent(s).

### *Preparation of fatty acid methyl esters*

Fatty acids were determined by a gas chromatography with trans esterification procedure (AOAC, 1998). In brief, JREA or JRO were separately methylated with sulphuric acid:toluene:methanol 1:1:2 (v/v/v), for 12 h in 50 °C bath; then de-ionized water was added. The methylated products were recovered by vortexing with diethyl-ether. To remove water, the diethyl-ether phase was dried utilizing anhydrous sodium sulfate and filtered.

### *Sterols isolation*

The JREA or JRO sterols were separately isolated utilizing a saponification method; using potassium hydroxide solution in ethanol. The non-saponifiable part was isolated by diethyl-ether, and the sterols were isolated after purification using preparative-TLC (acetic acid:diethyl ether:hexane; 1:30:70; v/v/v). The JREA or JRO sterols were then converted to volatile TMS-derivatives suitable for GC-system detection.

### *GC–FID and GC–MS analyses*

JREA or JRO pure, methylated and isolated sterols separately analyzed utilizing GC system. GC–FID analysis was carried out on a GC-apparatus fitted to flame-ionization detector (FID) and quadrupolar mass-spectrometer selective-detector (MSD) utilizing an Agilent 6890N Network (Agilent Technologies, USA) in *m/z* range 40–450 in EI mode (70 eV). The GC system was supplied with automatic sampler and split-splitless injector, connected to HP-5MS column (30 m * 0.25 mm, 0.25 μm). At a split-ratio of 1:30, the flow rate of the carrier gas was adjusted to 1 ml/min and the injector temperature was adjusted to 250 °C, and the temperature of the detector was adjusted to 300 °C, while the column temperature was programmed at a ramp of 4 °C/min beginning from 40 °C to 260 °C, and then kept isothermally for 10 min at 260 °C. In various types of samples (pure, methylated and isolated sterols), constituents were identified by correlation of their mass-spectra with those present in Wiley (Wiley, Chichester, West Sussex, England) and NIST (NIST 11.0, National Institute of Standards and Technology, Gaithersburg, MD) library-databases.

### *HPLC analysis*

The pure JREA extract was RP–HPLC analyzed to determine the phenolic compounds. At 40 °C, RP-C18 end-capped Lichrospher-column (250 9 4.6 mm I.D.; 5 μm particle size) (Merck), was utilized. Spectra of the compounds were recorded between 200 and 600 nm. The mobile phase was combined of a mixture of formic-acid (0.1%) in double-distilled water (A) and formic-acid (0.1%) in acetonitrile (B). Samples were analyzed utilizing a gradient elution from 95% to 80% A in the first 15 min, followed by a gradient-elution beginning from 80% to 70% A for 5 min, then maintained at 70% A mixture for 5 min, followed by a gradient from 70% to 10% A for 5 min, and then maintained at 10% A for 5 min, then gradient from 95% to 80% A in the last 5 min. The injection amount was 5 μl and 0.5 ml/min flow rate. The RP–HPLC system was equipped by fraction-collector. After fractionation and concentration, each fraction was injected separately to Nano-ESI MS instrument (110–1500 *m/z*). The identification of compounds was confirmed by comparison of retention times, fragmentation, and steepping methods utilizing standard solutions and standard calibration curves.

### *Bio-guided fractionation, separation, and identification of the most active constituent*

Both JREA and JRO was fractionated separately using preparative-chromatography column (50 mm * 1000 mm). Gradient elution was done utilizing one bed-volume (BV) diethyl ether/*n*-pentane (25/75, v/v), then one BV *n*-pentane/diethyl ether (50/50, v/v), then one BV *n*-pentane/diethyl ether (25/75, v/v), and finally with one BV of diethyl-ether, utilizing silica gel as the stationary-phase. Throughout the chromatographic process, the column-eluent was collected by time to over 200 fractions. Each fraction was investigated for its antidiabetic and antinociceptive activities, the same way as JREA and JRO utilizing *in vivo* alloxan-diabetic animals. The most active fraction was investigated utilizing GC-system.

### *Animals*

Male Albino mice weighing 22–34 g were obtained from the faculty animal house (BAU, Lebanon). Animals were preserved under temperature (20 ± 1 °C) and alternating 12 h light–dark cycle, standard environmental conditions, and were fed mice standard pellets and had open access to water. This study was done according

to the international ethical standards and certified by the BAU-Institutional Review Board (2017-A-0045-P-R-0218).

### *Acute carrageenan-induced inflammatory-pain*

The carrageenan-solution (1% in saline; Sigma–Aldrich, Germany) 100 μl was intraplantarly injected into the left hind paw. Ibuprofen 100 mg/kg was orally administered 30.0 ± 1.5 min prior to carrageenan (100 μl, 1%) as a positive control, as done before (Gardmark et al., 1998; Salama et al., 2016). The control mice were intraplantarly injected with 100 μl saline only. Behavioral measurements were performed 2 h post carrageen-injection.

### *Diabetes induction*

After one week of animal acclimatization, diabetes was induced utilizing intraperitoneal injection of alloxan (Sigma–Aldrich, Germany; 180 mg/kg in saline). Animals showing blood glucose level ≥200 mg/dl, seven days after alloxination, were considered diabetic and were included in the further experimentation.

### *Blood glucose level, HbA1c and body weight*

The experimental design was summarized in Table 1. In order to solubilize various non-polar solutions, 5 mg tween 80 was added to 1 ml test-solution, as done before in literature (Koleilat et al., 2017). The blood was obtained by pricking the animal tail, as done before (Bakirel et al., 2008). The blood glucose level (BGL) was monitored, for various doses of all test compounds, acutely (6 h), subchronically (eight days) and for a longer time (eight weeks) utilizing Sigma glucometers (Germany) and Analyticon HbA1c analytical columns (Germany). During the subchronic antidiabetic experiments, extracts and linoleic acid were administered every other day and the body weights (BW) of the animals were also monitored.

### *Serum insulin level*

For a longer time, the serum insulin levels were recorded prior-to and eight weeks post-test administration using an RP–HPLC method utilizing reversed phase-C18 endcapped Lichrospher-column (250 × 9 × 4.6 mm i.d.; 5 μm particle size) (Merck) with a 1 ml/min flow-rate, and 40 °C column temperature. The eluent was composed of 0.1% trifluoroacetic acid in Milli-Q water (A) and acetonitrile (B). The gradient-elution-conditions started from 0 to 5 min 70% (A) and then from 5 to 15 min 60% (A), at 214 nm (Raafat et al., 2018).

### *Serum catalase and lipid peroxide levels*

During the subchronic antidiabetic assessment, serum-catalase (CAT) and lipid peroxidation (LPO) levels were also monitored. The CAT levels were measured prior to and each other day post-treatment for eight days in kU/I (Yasmineh et al., 1995). The LPO levels were determined by thiobarbituric-acid test modified from a previously described method (Ohkawa et al., 1979). Briefly, prior to and on the eighth-day post-treatment, 0.8% thiobarbituric acid was added to serum (0.2 ml), 8.1% sodium lauryl sulphate and 20% diluted acetic acid in Milli-Q water. After 1 h of heating at 95 °C and cooling, the mixture was extracted with (1:15, v/v) methanol/isopropyl-alcohol and the product was measured by JASCO spectrophotometer (Japan) at 532 nm (Ohkawa et al., 1979).

### *Assessment of nociceptive responses*

After eight weeks of induction of diabetes, mice were tested for diabetic neuropathy success-rate (significant sensory loss of thermal-sensitivity under 10S) (Sullivan et al., 2007) was *ca.* 90% and their thermal and mechanical neurological functions were evaluated weekly for eight weeks.

**Table 1**
Protocol of experimental design.

| Groups | *n* | Tested substance(s) | Description |
|---|---|---|---|
| **A. Acute (0, 0.5, 2 and 6 h) and subchronic (1, 3, 5, 8 days) effect of *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA), and linoleic acid (LA) on blood glucose, body weights and CAT serum levels:** | | | |
| *I* | 7 | Control | Normal mice: vehicle [sterile cold saline (0.9%)], oral gavages (PO) |
| *II* | 7 | Diabetic Control | Diabetic mice: vehicle, PO |
| *III* | 7 | GB | Diabetic mice: GB 5 mg/kg, PO |
| *IV* | 7 | JRO | Diabetic mice: JRO 12.5 mg/kg, PO |
| *V* | 7 | JRO | Diabetic mice: JRO 25 mg/kg, PO |
| *VI* | 7 | JRO | Diabetic mice: JRO 50 mg/kg, PO |
| *VII* | 7 | JREA | Diabetic mice: JREA 12.5 mg/kg, PO |
| *VIII* | 7 | JREA | Diabetic mice: JREA 25 mg/kg, PO |
| *IX* | 7 | JREA | Diabetic mice: JREA 50 mg/kg, PO |
| *X* | 7 | LA | Diabetic mice: LA 1.6 mg/kg, PO |
| *XI* | 7 | LA | Diabetic mice: LA 3.3 mg/kg, PO |
| *XII* | 7 | LA | Diabetic mice: LA 7.5 mg/kg, PO |
| **B. Effect of JRO, JREA, and LA for longer times (0, 2, 4, 6 and 8 weeks) on hot plate and tail withdrawal latencies, and von Frey paw withdrawal thresholds:** | | | |
| *XIII* | 7 | NORM | Normal mice: vehicle [sterile cold saline (0.9%)], PO |
| *XIV* | 7 | VEH | Diabetic mice: vehicle, PO |
| *XV* | 7 | TRA | Diabetic mice: TRA 10 mg/kg, PO |
| *XVI* | 7 | JRO | Diabetic mice: JRO12.5 mg/kg, PO |
| *XVII* | 7 | JRO | Diabetic mice: JRO 25 mg/kg, PO |
| *XVIII* | 7 | JRO | Diabetic mice: JRO 50 mg/kg, PO |
| *XIX* | 7 | JREA | Diabetic mice: JREA 12.5 mg/kg, PO |
| *XX* | 7 | JREA | Diabetic mice: JREA 25 mg/kg, PO |
| *XXI* | 7 | JREA | Diabetic mice: JREA 50 mg/kg, PO |
| *XXII* | 7 | LA | Diabetic mice: LA 1.6 mg/kg, PO |
| *XXIII* | 7 | LA | Diabetic mice: LA 3.3 mg/kg, PO |
| *XXIV* | 7 | LA | Diabetic mice: LA 7.5 mg/kg, PO |

*Evaluation of thermal-nociceptive latencies*

The thermal-hyperalgesia in Diabetes mellitus-animals were evaluated by the thermal tail-flick and hot-plate latency experiments (Micov et al., 2015). Briefly, the mice tails exposed to Hugo Sachs Elektronik tail-flick apparatus (Germany) or Ugo-Basile hot-plate analgesia-meter (Italy). The thermal intensity was adjusted to provide a baseline latency-time of 1.5–2.5 s for the tail-flick test and 4–5 s for the hot-plate test, for normal non-diabetic mice (Norm). To prevent tissue-damage, 10 s cut-off time was utilized.

*Evaluation of mechanical-nociceptive latencies*

The allodynia in Diabetes mellitus-animals was monitored by evaluating the paw-withdrawal thresholds utilizing OptiHair Von-Frey filaments (Germany) (Ohsawa et al., 2011). Briefly, animals were separately placed in inverted plastic cages on a mesh floor. An increasing force was applied to the left hind paw plantar surface until the mouse withdrew the paw. For animal safety, a 32 g cut-off force was utilized.

*Statistical analysis*

All results (mean ± S.E.M) were statistically evaluated by ANOVA followed by Fisher post hoc test using OriginPro® statistics program. The $p$-value <0.05 was considered statistically-significant.

## Results and discussions

The present results have shown the phytochemical and biological profile of *J. regia* kernel ethyl acetate extract (JREA) and oil (JRO) in different types of pain.

*Combined bio-guided GC–FID, GC–MS and RP–HPLC analyses*

The yield after extraction for JRO and JREA was 853.6 g/kg and 449.9 g/kg, respectively. Fatty acid composition of JRO and JREA (Table 2) identified seven fatty acids by combined chromatographic analysis. JRO has shown six fatty acids; palmitic acid (8.3%), palmitoleic acid (0.8%), stearic acid (1.9%), oleic acid (10.7%), linoleic acid (55.0%), and gamma-linolenic acid (16.7%). JREA has shown seven fatty acids; palmitic acid (5.6%), palmitoleic acid (0.1%), stearic acid (2.8%), oleic acid (14.2%), linoleic acid (45.0%), gamma-linolenic acid (8.1%), and arachidic acid (0.1%). The phytochemical screening as well as GC–FID and GC–MS analysis of JRO and JREA has identified sixteen volatile oils summarized in Table 3. The major volatile oil ingredients identified in JRO was hepta-decane (14.9%), while that of JREA was myristicin (11.8%). Sterols were also identified in minor amounts (Table 4). Phenolic compounds were also identified utilizing RP–HPLC. JRO has shown eight major HPLC peaks; from which Glansreginin B (11.5%) and Glansreginin A (10.4%) were identified after isolation via HPLC fraction collector and identification via Nano-ESI-MS. On the other hand, JREA has shown twenty-one major HPLC peaks; from which gallic acid (1.2%), isochlorogenic acid (5.9%), catechin (3.1%) and ellagic acid (8.4%) were identified by comparison of retention times, and steepping methods utilizing standard solutions and standard calibration curves (Fig. 1).

After JEO and JREA fractionation separately utilizing preparative-chromatography column and examination of each fraction for antidiabetic and antinociceptive activities, the most active compound identified was linoleic acid (LA).

**Table 2**
Fatty acid identified in *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) identified by gas chromatography (%, w/w).

| Constituent | JRO | JREA |
|---|---|---|
| Palmitic acid | 8.3 | 5.6 |
| Palmitoleic acid | 0.8 | 0.1 |
| Stearic acid | 1.9 | 2.8 |
| Oleic acid | 10.7 | 14.2 |
| Linoleic acid | 55.0 | 45.0 |
| Gamma-linolenic acid | 16.7 | 8.1 |
| Arachidic acid | n.i.[a] | 0.1 |

[a] n.i., not identified.

**Table 3**
Volatile oils identified in *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) identified by gas chromatography (%, w/w).

| KIL[a] | Constituent | JRO | JREA |
|---|---|---|---|
| 1107 | Iso-amyl iso-butyrate | tr.[b] | n.i.[c] |
| 1248 | *E*-Pinocarveol | tr. | 3.3 |
| 1314 | Myretenal | tr. | 6.3 |
| 1459 | Iso-butyl benzoate | tr. | n.i. |
| 1540 | Tetra-decanes | 13.3 | 0.6 |
| 1558 | (*E*-) beta-caryophyllene | tr. | 8.3 |
| 1650 | Penta-decanes | 4.1 | n.i. |
| 1668 | Myristicin | 10.6 | 11.8 |
| 1740 | Caryophyllene oxide | tr. | 6.1 |
| 1760 | Hexa-decane | 13.2 | 5.2 |
| 1870 | Hepta-decane | 14.9 | 10.2 |
| 1980 | Octa-decane | 12.8 | n.i. |
| 2010 | Iso-propyl tetra-decanoate | tr. | tr. |
| 2090 | Nona-decane | 11.0 | 7.3 |
| 2200 | Eicosane | 7.7 | n.i. |
| 2310 | Heneicosane | 6.8 | 2.0 |

[a] KIL, Kovats retention index.
[b] tr., traces (≤0.1%).
[c] n.i., not identified.

**Table 4**
Sterols identified in *J. regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) identified by gas chromatography (%, w/w).

| Constituent | JRO | JREA |
|---|---|---|
| Cholesterol | 0.009 | 0.001 |
| Campesterol | 0.063 | 0.010 |
| Stigmasterol | n.i.[a] | 0.001 |
| Clerosterol | 0.021 | 0.012 |
| Beta-sitosterol | 0.110 | 0.220 |
| Delta-5-avenasterol | 0.027 | 0.100 |
| Delta-9-stigmasterol | n.i. | 0.001 |
| Delta-7-avenasterol | n.i. | 0.001 |

[a] n.i., not identified.

*JEO, JREA and LA potentials toward inflammatory pain*

To assess JEO, JREA and LA possible anti-inflammatory potentials, inflammation acute phase was evaluated utilizing paw-edema induced by carrageenan in the mouse model. The carrageenan-experiments are standard-models for evaluation of the antinflammatory lead-drugs and are broadly utilized for assessment of the natural or synthetic acute antinflammatory activities. The first phase (0–2 h post-carrageenan administration) has shown to be due to the liberation of pro-inflammatory mediators from damaged-tissues, including serotonin, histamine, and bradykinins (Willoughby and DiRosa, 1972).

Two h post-carrageenan injection, mice have adopted a significant mechanical hypersensitivity ($p < 0.05$, $n = 7$/group) (Fig. 2). The paw withdrawal threshold (PWT) on the paw reduced from 7.9 ± 0.2 g normal untreated mice (Normal) to 3.5 ± 0.1 g vehicle treated carrageenan-injected mice (VEH). JRO has significantly reversed the carrageenan-induced edema in a dose dependant manner ($p < 0.05$, $n = 7$/group). When compared to VEH, JRO (12.5, 25 and 50 mg/kg) has increased PWT (*i.e.* decreased the mechanical hypersensitivity) by 34.3, 45.7 and 60.0%, respectively. JREA

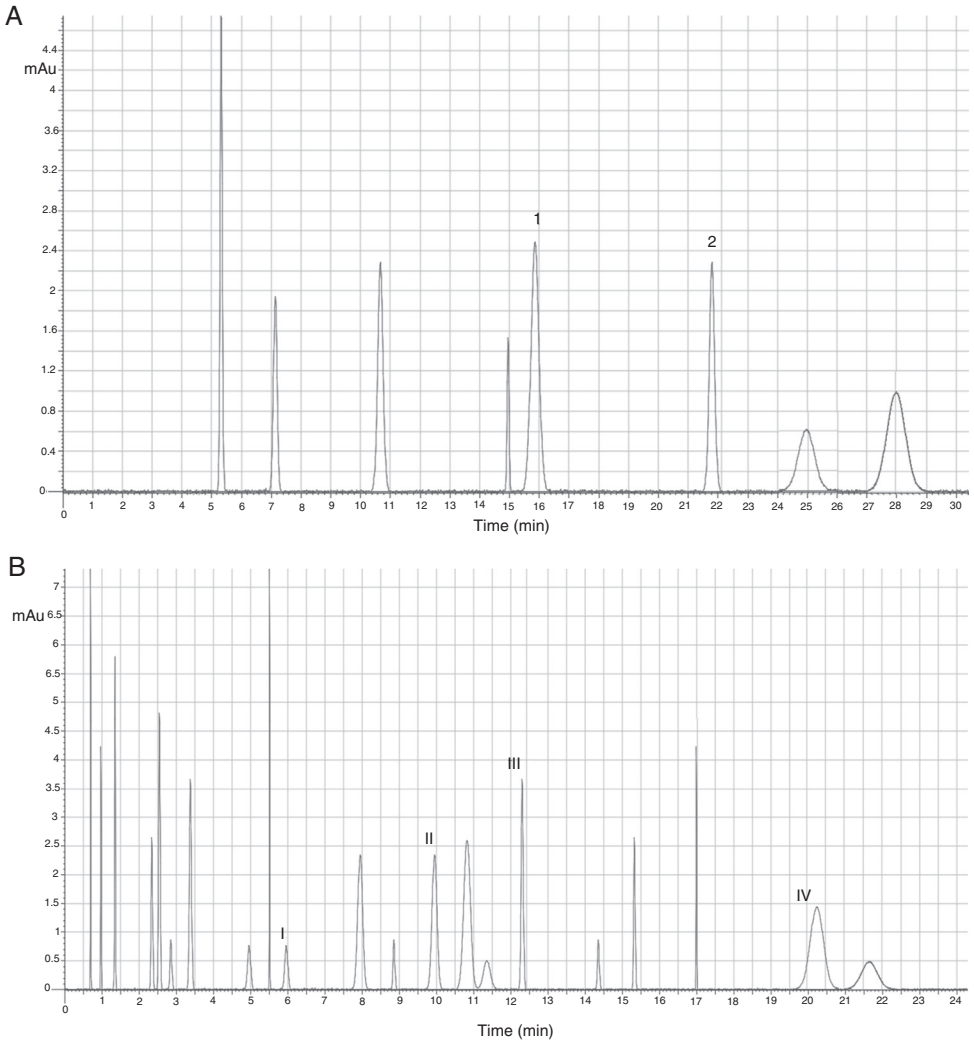


**Fig. 1.** HPLC chromatogram of phenolic compounds in (A) *Juglans regia* oil (JRO) major peaks: 1, Glansreginin B; 2, Glansreginin A. (B) *J. regia* ethyl acetate extract (JREA) major peaks: I, gallic acid; II, isochlorogenic acid; III, catechin; IV, ellagic acid at 200–600 nm at 40 °C and flow rate 0.5 ml/min.

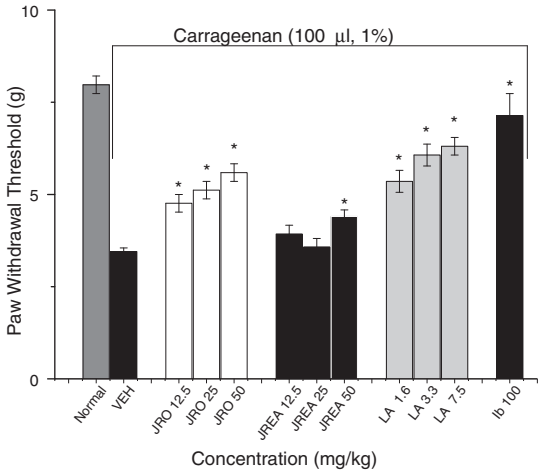


**Fig. 2.** Inflammatory pain. Carrageenan (100 µl, 1%) was injected intraplantarly 2 h prior the pain threshold measurement by the paw pressure test. Oral administration (gavages) of *Juglans regia* oil (JRO), *J. regia* ethyl acetate extracts (JREA) and linoleic acid (LA) 30 min before the test. Oral administration of ibuprofen 100 mg/kg (Ib 100) was used as positive control. "Normal" designates normal mice. "*" means $p < 0.05$, $n = 7$ compared with vehicle control (VEH).

highest dose (50 mg/kg) was the only dose that showed a significant increase in PWT by 22.8%. The highest dose of LA (7.5 mg/kg) has shown comparable antinflammatory results to those of the positive control, ibuprofen 100 mg/kg (Ib 100). As compared to VEH, JREA (12.5, 25 and 50 mg/kg) has increased PWT by 54.3, 74.3 and 80.0%, respectively, while that of Ib 100 has shown 105.7% increase in PWT (Fig. 2).

The efficacy of JRO, JREA, and LA on decreasing mechanical hypersensitivity, suggests their ability to potentially control the acute inflammatory pain.

### *The effects of JEO, JREA and LA on blood glucose levels, body weights, and HbA1c*

Acutely, JRO (12.5, 25 and 50 mg/kg) has decreased the blood glucose levels (BGL) by 37.2, 40.1 and 40.2%, 6 h post-administration respectively, when compared to vehicle-treated diabetic control mice (DIA Control) (Fig. 3A). While in correlation to DIA Control, JRE (12.5, 25 and 50 mg/kg) has decreased the BGL by 37.4, 48.5 and 61.9%, 6 h post-administration respectively (Fig. 3B), at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has reduced BGL by 26.1, 32.7 and 40.1%, 6 h post-administration respectively, while the positive control, glibenclamide 5 mg/kg (GB), has had 34.4% decrease in BGL (Fig. 3C).

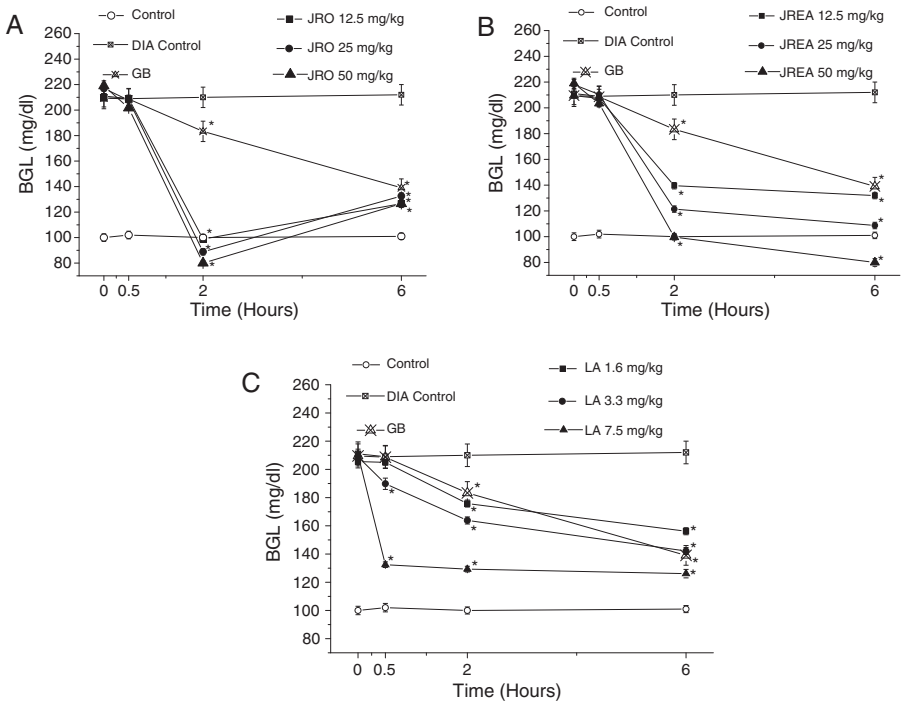


**Fig. 3.** The acute effect of (A) *Juglans regia* oil (JRO), (B) *J. regia* ethyl acetate extract (JREA) and (C) linoleic acid (LA) on blood glucose levels, utilizing glibenclamide 5 mg/kg (GB) as positive control. "Control" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (DIA Control).

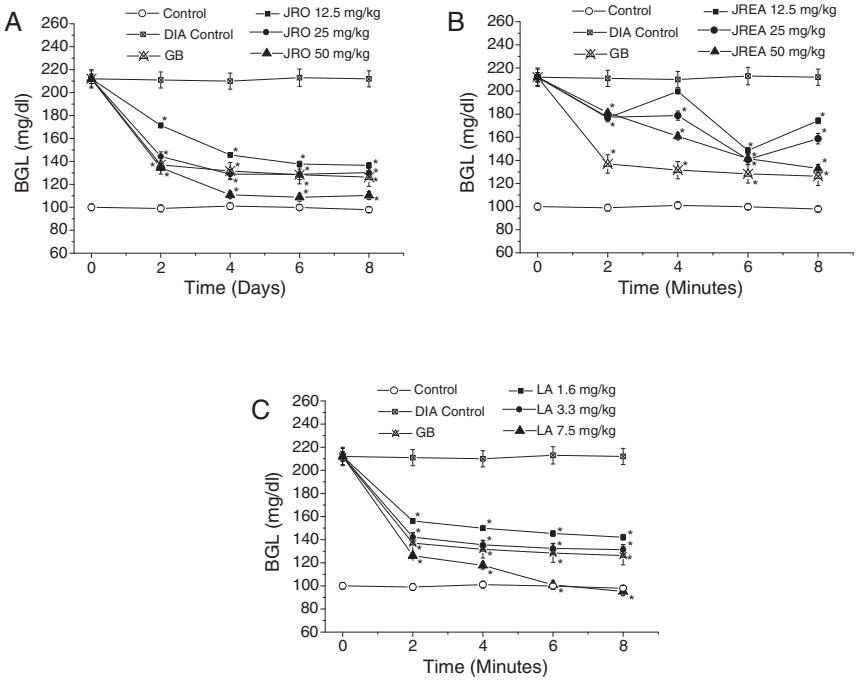


**Fig. 4.** The subchronic effect of (A) *Juglans regia* oil (JRO), (B) *J. regia* ethyl acetate extract (JREA) and (C) linoleic acid (LA) on blood glucose levels, utilizing glibenclamide 5 mg/kg (GB) as positive control. "Control" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (DIA Control).

Subchronically, JRO (12.5, 25 and 50 mg/kg) has reduced BGL by 35.6, 38.4 and 48.0%, 8 days post-administration respectively, when compared to DIA Control (Fig. 4A). While in correlation to DIA Control, JRE (12.5, 25 and 50 mg/kg) has decreased the BGL by 17.8, 25.0 and 37.0%, 8 days post-administration respectively (Fig. 4B), at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has decreased BGL by 32.9, 38.3 and 55.1%, 8 days post-administration respectively, while GB has had 40.2% decline in BGL (Fig. 4C).

Moreover, subchronically JRO (12.5, 25 and 50 mg/kg) has increased body weight (BW) by 29.5, 75.7 and 97.6%, 8 days post-administration respectively, when compared to vehicle-treated control group (VEH) (Fig. 5). While in correlation to VEH, JRE (12.5,

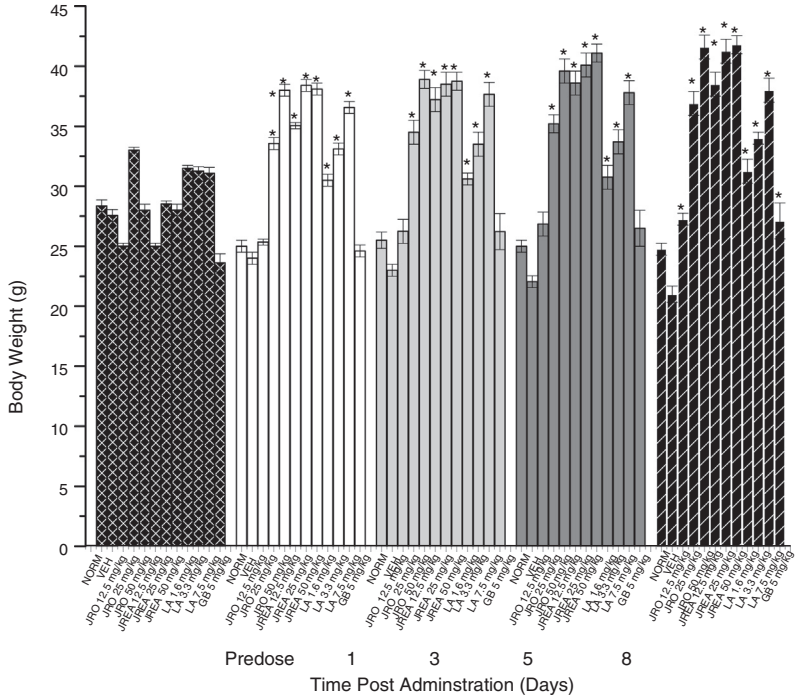


**Fig. 5.** The subchronic effect of (A) *Juglans regia* oil (JRO), (B) *J. regia* ethyl acetate extract (JREA) and (C) linoleic acid (LA) on body weights, utilizing glibenclamide 5 mg/kg (GB) as positive control. "NORM" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (VEH).

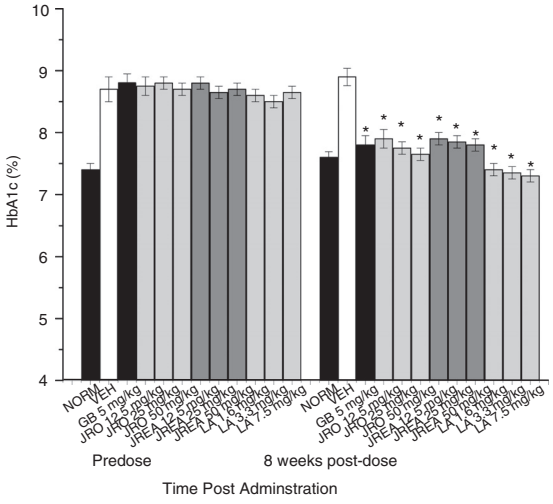


**Fig. 6.** The effect of *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) and linoleic acid (LA) on HbA1c levels before (predose) and 8 weeks after administration (8 weeks post-dose), utilizing glibenclamide 5 mg/kg (GB) as positive control. "NORM" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (VEH).

25 and 50 mg/kg) has increased the BW by 82.9, 96.7 and 99.1%, 8 days post-administration respectively, at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has increased the BW by 48.1, 61.9 and 81.4%, 8 days post-administration respectively, while GB has had 29.5% increase in BW (Fig. 5).

For a longer time, various doses of JRO, JREA, and LA have significantly decreased the stored glycated hemoglobin levels (HbA1c) 8 weeks post-administration (Fig. 6). As, JRO (12.5, 25 and 50 mg/kg) has significantly decreased HbA1c by 11.2, 13.5 and 14.6%, respectively, when compared to VEH (Fig. 6). While in correlation to VEH, JRE (12.5, 25 and 50 mg/kg) has declined the HbA1c levels by 11.2, 12.6 and 13.5%, respectively, at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has decreased the HbA1c by 16.9, 18.0 and 19.1%, 8 weeks post-administration respectively, while GB has had 12.4% decrease in HbA1c level (Fig. 6).

The potencies of JRO, JREA, and LA on decreasing BGL and normalizing HbA1c levels, suggests their sustain ability to significantly control diabetes and their possible potentials in controlling diabetes-induced neuropathy.

### *Serum insulin level*

To facilitate the identification of JRO, JREA and LA antidiabetic mechanism, serum insulin levels were monitored. For longer time, JRO, JREA and LA have significantly increased serum insulin level (SIL) in a dose-dependent manner, eight weeks post-administration (Fig. 6). Where, JRO (12.5, 25 and 50 mg/kg) has significantly increased SIL by 3.2, 4.5 and 5.8 folds, respectively, when compared to VEH (Fig. 7). While in correlation to VEH, JRE (12.5, 25 and 50 mg/kg) has elevated the SIL by 3.5, 4.1 and 5.1 folds, respectively, at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has increased the SIL by 5.1, 7.1 and 8.7 folds, eight weeks post-administration respectively. On the other hand, GB has not shown a significant increase in SIL (Fig. 7).

The efficacy of test compounds to significantly increase serum insulin levels suggests that the insulin secretagogue potentials is one of the possible mechanisms by which JRO, JREA, and LA control diabetes.

### *Serum-catalase and lipid-peroxide levels*

Serum-catalase (CAT) and lipid-peroxide (LPO) levels were monitored subchronically for 8 days. On the 8th day, JRO (12.5, 25 and 50 mg/kg) has significantly increased CAT level by 27.6, 36.9 and 70.1%, respectively, and decreased LPO level by 19.2, 50 and 69.2% respectively, when compared to VEH (Figs. 8 and 9). While in correlation to VEH, JRE (12.5, 25 and 50 mg/kg) has elevated the CAT level by 29.9, 54.7 and 85.5%, respectively, and decreased LPO

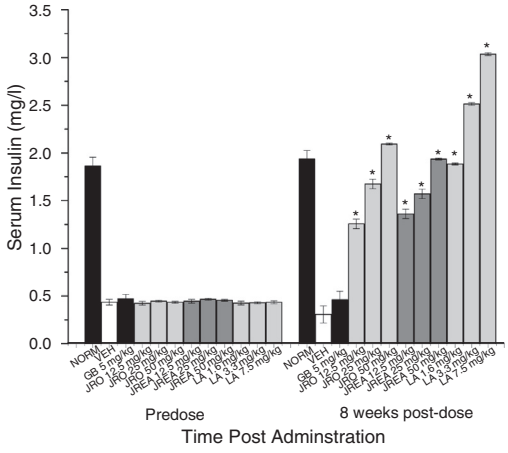


**Fig. 7.** The effect of *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) and linoleic acid (LA) on serum insulin levels before (predose) and 8 weeks after administration (8 weeks post-dose), utilizing glibenclamide 5 mg/kg (GB) as positive control. "NORM" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (VEH).

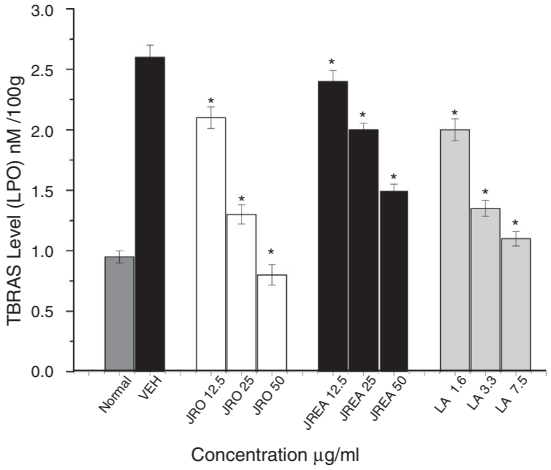


**Fig. 9.** The level of LPO (TBARS) in *Juglans regia* oil (JRO) and *J. regia* ethyl acetate extract (JREA) and LINOLEIC acid (LA). "NORM" designates normal non-diabetic mice. "*" means $p < 0.05$, $n = 7$ compared with vehicle control (VEH).

level by 7.7, 23.0 and 42.3% respectively, at the same time, LA (1.6, 3.3 and 7.5 mg/kg) has increased the CAT level by 32.2, 42.5 and 72.4%, respectively, and decreased LPO level by 23.1, 50.0 and 57.7%, respectively (Figs. 8 and 9).

The antioxidant potentials of JRO, JREA, and LA on increasing CAT and decreasing LPO levels, suggests their possible potentials in controlling hyperglycemia-induced neuropathy.

*Assessment of nociceptive responses*

Thermal and mechanical neurological functions were evaluated weekly for eight weeks utilizing tail-flick, hot plate and von Frey filament methods (Figs. 10–12).

*Assessment of thermal-nociceptive responses*

The JRO administrated at doses of 12.5, 25 and 50 mg/kg induced a significant elevation in the reaction-time to thermal-stimuli when correlated to the vehicle-treated diabetic control group (DIA + VEH) by 0.6, 1.1 and 2.1 folds for tail-flick latency (TFL) and by 1.2, 1.3 and 1.4 folds for hot-plate latency (HPL), on the eighth week respectively (Figs. 10A and 11A). On the eighth week compared to DIA + VEH, JREA administration at doses of 12.5, 25 and 50 mg/kg increased TFL by 1.6, 1.9 and 2 folds, and increased HPL by 1.0, 1.3 and 1.4 folds, respectively (Figs. 10B and 11B). At the same time, LA administration in doses of 1.6, 3.3 and 7.5 mg/kg elevated TFL by 1.8, 2.1 and 2.3 folds, and elevated HPL by 1.2, 1.3 and 1.4 folds, respectively (Figs. 10C and 11C). These results were measured uti-

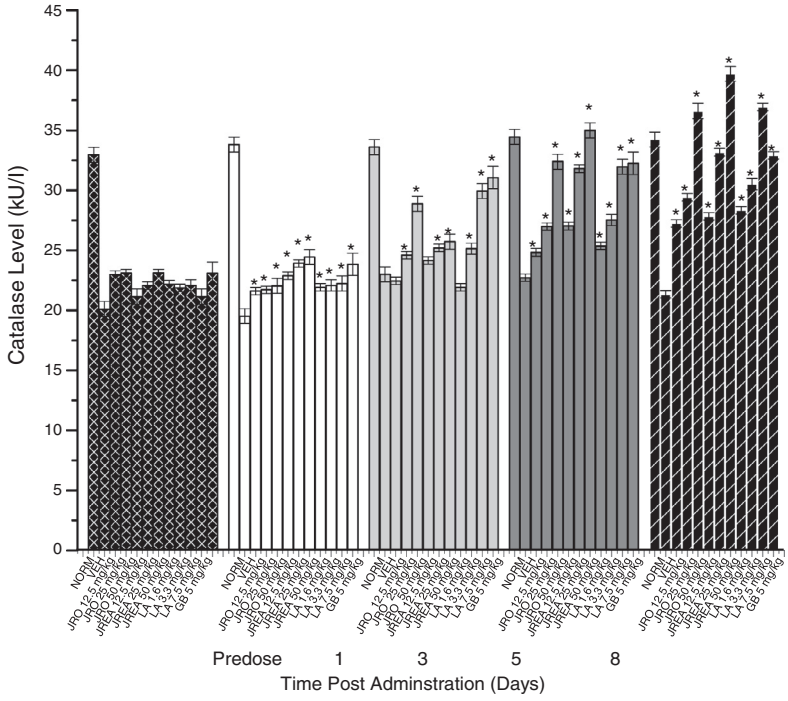


**Fig. 8.** The subchronic effect of *J. regia* oil (JRO) *and Juglans regia* ethyl acetate extract (JREA) and linoleic acid (LA) on serum catalase level (CAT), utilizing glibenclamide 5 mg/kg (GB) as positive control. "NORM" designates normal non-diabetic mice. "*" designates significant results ($p < 0.05$, $n = 7$/group) when compared to vehicle treated diabetic control (VEH).

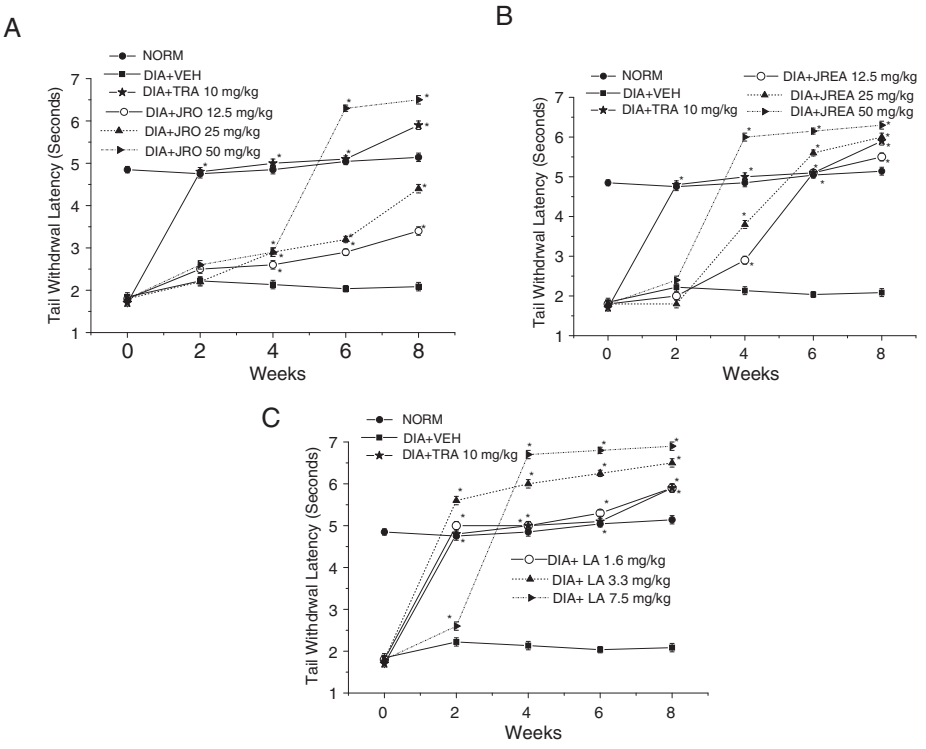


**Fig. 10.** Effect of (A) *Juglans regia* oil (JRO), (B) *J. regia* ethyl acetate extracts (JREA) and (C) linoleic acid (LA) on the tail flick in alloxan treated mice with tramadol (TRA 10 mg/kg) as a positive control. "NORM" designates normal non-diabetic mice. "*" means $p < 0.05$, $n = 7$ compared with vehicle diabetic control (DIA + VEH).

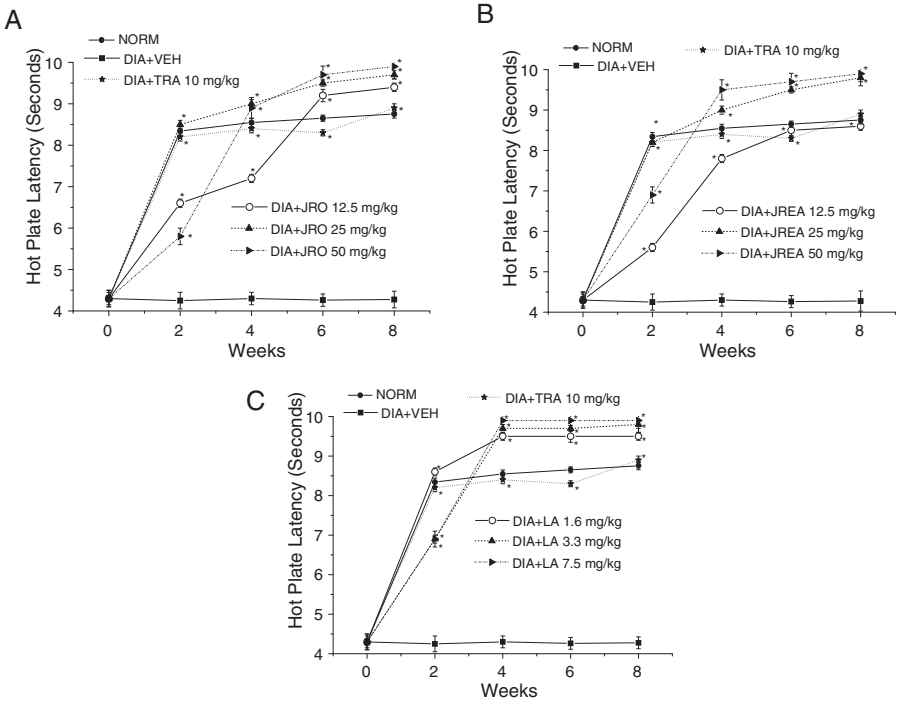


**Fig. 11.** Effect of (A) *Juglans regia* oil (JRO), (B) *J. regia* ethyl acetate extracts (JREA) and (C) linoleic acid (LA) on the hot plate in alloxan treated mice with tramadol (TRA 10 mg/kg) as a positive control. "NORM" designates normal non-diabetic mice. "*" means $p < 0.05$, $n = 7$ compared with vehicle diabetic control (DIA + VEH).

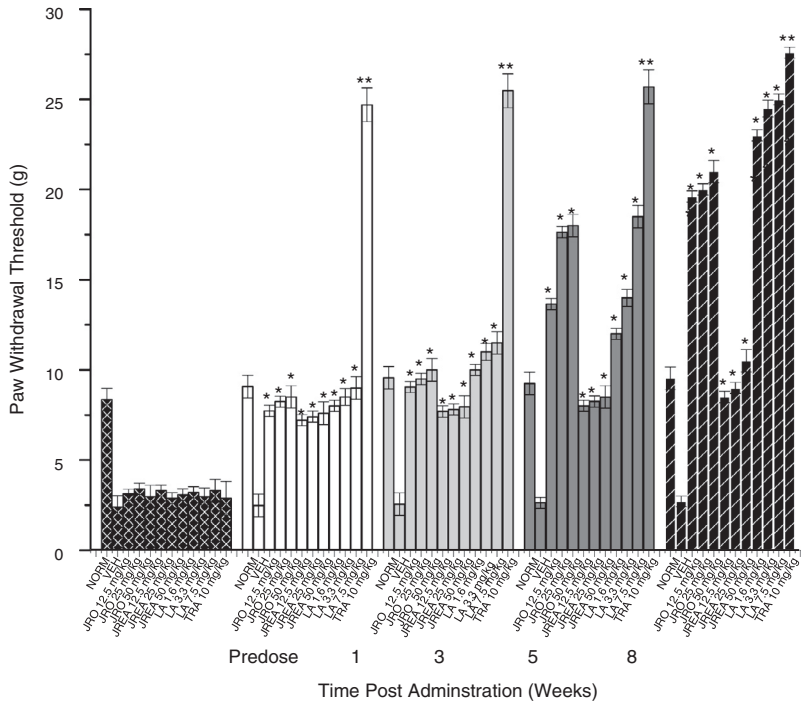


**Fig. 12.** The effect of *Juglans regia* oil (JRO) *and J. regia* ethyl acetate extract (JREA), linoleic acid (LA) and tramadol (TRA) 10 mg/kg on tactile allodynia in neuropathic model in alloxan-induced diabetic mice. Paw withdrawal thresholds to von Frey filaments were determined on hind paw prior to (Predose) and up to 8 weeks. (NORM) normal non-diabetic untreated mice. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ compared to vehicle treated animals (VEH) ($n = 7$ animals/group).

lizing tramadol 10 mg/kg (TRA) as a positive control. TRA has shown an increase in TFL by 1.8 folds and HPL by 1.1 folds (Figs. 10 and 11).

The efficacy of JRO, JREA, and LA on decreasing thermal hyperalgesia, suggests their antinociceptive potentials to diabetes-induced hyperalgesic pain.

*Evaluation of mechanical-nociceptive latencies*

The allodynia in diabetes mellitus-animals was monitored by evaluating the paw-withdrawal thresholds (PWT) utilizing Von-Frey filaments (Fig. 12). The JRO administrated at doses of 12.5, 25 and 50 mg/kg provoked a significant elevation in PWT when compared to DIA + VEH by 6.5, 6.6 and 6.8 folds, on the eighth week respectively (Fig. 12). On the eighth week compared to DIA + VEH, JREA administration at doses of 12.5, 25 and 50 mg/kg increased PWT by 2.4, 2.5 and 3.0 folds, respectively (Fig. 12). Simultaneously, LA administration in doses of 1.6, 3.3 and 7.5 mg/kg elevated PWT by 7.8, 8.4 and 8.6 folds, respectively (Fig. 12). TRA has shown an increase in PWT by 9.6 folds (Fig. 12).

JRO, JREA and LA potentials on decreasing mechanical-nociceptive potentials, suggests their antinociceptive potentials to diabetes-induced allodynic pain.

The combined bio-guided GC–FID, GC–MS and HPLC analyses has shown to be a potential tool in the assessment of *J. regia* phytochemical and biological characters. After fractionation, the most active compound identified was linoleic acid. *J. regia* oil *and J. regia* ethyl acetate extract, and linoleic acid have shown significant antinflammatory, antidiabetic and antinociceptive potentials to diabetes-induced neuropathic pain. LA has shown more significant and dose-dependent amelioration of acute inflammatory pain, chronic blood glucose level reduction, serum-insulin elevation, and normalization of HbA1c levels. On the other hand, JRO has shown more LPO reduction, while JREA has shown more acute BGL reduction and CAT levels reduction. JRO and JREA have shown an equipotent effect in increasing body weights. Compared to tramadol, the highest doses of JRO, JREA, and LA have shown higher antinociceptive potentials in amelioration of thermal hyperalgesic and anti-allodynic neuropathic pain. The antinflammatory, the reduction of oxidative stress, and the insulin secretagogue potentials might be among the possible mechanisms by which JRO, JREA, and LA ameliorate neuropathic pain. After further clinical studies, *J. regia oil*, ethyl acetate extract and linoleic acid might be utilized in the future as a possible remedy for various painful syndromes.

## Conflicts of interest

The author declares no conflicts of interest.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Confidentiality of data.** The authors declare that they have followed the protocols of their work center on the publication of patient data.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

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