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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