



Original Article

Phytochemical and anti-neuropathic investigations of *Crocus sativus* via alleviating inflammation, oxidative stress and pancreatic beta-cells regeneration

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ABSTRACT

Objective: The aim of this study is to investigate the phytochemical and the long-term anti-neuropathic potentials of *Crocus sativus* cultivated in the University botanical garden, and explore its most bioactive compounds and their underlying mechanisms of action.

Methods: Phytochemical analysis and bio-guided isolation-procedures including RP-HPLC and ^1H and ^{13}C NMR utilizing biological models of diabetes, inflammation, and diabetic-neuropathy were used. Cultivated saffron (S-RCED) and Spanish-saffron stigma (S-SP) alone or in combination with *Camellia sinus* (CS) were investigated.

Results: The RP-HPLC analyses showed the presence of picrocrocin, crocin I, crocin II, crocin I', crocin II', and safranal (SAF) in both S-SP and S-RCED extracts with higher-concentrations. It had been shown that SAF was the most bioactive-compound in *Crocus sativus*. Both S-SP and S-RCED possessed significant ($P < 0.05$) anti-diabetic activities in acute (6 h), subchronic (8 d) and chronic (8 weeks) models. S-RCED had been proven with more hypoglycemic potentials when compared to S-SP and SAF. S-SP, S-RCED, and SAF produced significant anti-inflammatory and anti-nociceptive activities against carrageenan-induced inflammatory, hyperalgesic and tactile diabetic-neuropathy models, respectively. S-SP, S-RCED, and SAF elevated serum catalase, reduced glutathione, and insulin serum levels, ameliorated lipid peroxidation and HbA1c levels, and histopathologically regenerated the pancreatic beta-cells. Combinations with CS showed more significant efficacy than the single component.

Conclusion: The oxidative stress reduction, insulin secretagogue, and pancreatic beta-cells regeneration potentials might be responsible for the mechanism underlying the anti-diabetic, anti-inflammatory and anti-diabetic neuropathy activities. Thus, the cultivated *Crocus sativus* might be clinically useful for protecting against many serious-disorders.

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1. Introduction

The stigma of *Crocus sativus* L. (Iridaceae) is one of the most expensive natural alternative medications. It is broadly utilized as a food additive and as anxiolytic, sedative, and as immunomodulatory drug in the Asian and European traditional medications (Samarghandian & Borji, 2014). One of the main components of essential oil of saffron responsible for its aroma is safranal, which is an aldehydic monoterpene, formed in saffron by hydrolysis from picrocrocin (Sobolev et al., 2014).

The high cost of *C. sativus* limited its exploration of its biological activities (Mardani, Sekine, Azizi, Mishyna & Fujii, 2015). The cultivation of *C. sativus* in research centers might overcome this economic burden and facilitate the identification of its most active phytochemicals as well as their phytotherapeutic effects.

The inflammatory pain responses are very essential protective reactions to trauma, noxious-stimuli, or infection. The current synthetic treatments of these responses cause many gastrointestinal and neurological side-effects (Katanić et al., 2018). Therefore, it is of high need to focus on finding an effective alternative therapy with lesser side effects.

Diabetes is a major chronic disorder characterized by hyperglycemia, hypoinsulinemia and increased oxidative stress, which

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causes many complications (Raafat & Omar, 2016). Painful diabetic-neuropathy is one of the major complications of diabetes which is very difficult to treat, and the available treatments are not so efficacious and possess many undesirable effects (Daugherty, Marquez, Calcutt & Schubert, 2018). Oxidative-stress plays a big-role in the pathogenesis and complications of diabetes and diabetic neuropathy (Shakeel, 2015). Thus, the exploration of novel effective anti-nociceptive alternative medications with high antioxidant potentials and long-term neuroprotective effects is of extreme importance.

Previous studies concerning *C. sativus* have focused mainly on exploring the short-term biological effects on plants that differ in cultivation, bioactive compound composition and concentration from that cultivated in the University botanical gardens (Srivastava, Ahmed, Dixit & Dharamveer, 2010). Currently, long-term biological effects of *C. sativus* are of increasing importance to study in order to observe the full picture and to ensure its long-term efficacy.

Therefore, the aim of this study is to investigate the phytochemical and the anti-neuropathic potentials of *C. sativus* cultivated in the University botanical garden long-term effects, and explore its most bioactive compounds possessing anti-neuropathic potentials and their underlying mechanisms of action.

2. Materials and methods

2.1. Chemicals

Chemicals, solvents, and standards have been purchased from Sigma-Aldrich (Germany).

2.2. Plant materials

Stigmas of *Crocus sativus* L. were obtained from the Botanical Garden, Research Center for Environment and Development (RCED), Beirut Arab University, Bekaa, Lebanon. Another sample of *C. sativus* stigmas was a gift from Mrs. D. Kanj, obtained from Spain (SP). Both specimens (RCED and SP) were identified by comparison with an authentic sample, and reference samples were deposited in the Faculty-herbarium with voucher numbers FP17-011 and FP17-012, respectively.

2.3. Extraction procedures

The powder of Spanish saffron stigmas (S-SP) and saffron stigmas from Research Center for Environment and Development (S-RCED) were suspended separately in 50% ethanol at 0 °C, vortexed and filtered. The procedure was repeated four times for each extract (S-SP or S-RCED). Extracts were dried under vacuum utilizing Buchi rotary-evaporator (Germany). S-SP and S-RCED were kept separately at –40 °C until further experimentation in thick-walled brown glass containers in darkness.

2.4. Quantitative RP-HPLC phytochemical analysis

Chromatographic determination of components was operated on an Agilent HPLC system with C₁₈ RP-column (4.6 mm × 250 mm, 5 μm), consisting of mobile phase delivery module, mobile phase mixing vacuum-degasser, column oven adjusted at 40 °C, a multiple wavelength DAD-detector (adjusted at different maxima including 254 nm, 308 nm, 440 nm for the identification of SAF-piccrocin-crocins and crocetin, respectively). The peaks have been quantified and identified by using reference-standards and steeping method utilizing standard calibration-curves. The flow rate and the injection volume were 1 mL/min and 20 μL, respectively. A gradient mobile-phase was

utilized consisting of a mixture of acetonitrile (A) and double distilled water (B). First, 10% B for 5 min; increased to 80% in 20 min and held for more than 10 min.

2.5. Bio-guided fractionation procedures

The S-SP extract has been fractionated utilizing RP-preparative column-chromatography (0.5 m × 3.0 m) with a gradient mobile-phase mixture of acetonitrile (A) and double distilled water (B). First two bed volume (BV) of 90% A, then two BV of 80% B, and finally two BV of 100% B. During the whole fractionation period, the fractions were collected by time, then similar fractions were combined and concentrated. Each portion was examined the same-way as the whole extracts and the most bioactive fraction had been identified using ¹H and ¹³C NMR analysis.

2.6. ¹H and ¹³C NMR analysis

NMR-spectra of the saffron extract most bioactive fraction was measured in CD₃OD at 26 °C on a Bruker 300 NMR-spectrometer working at a proton-frequency of 300 MHz and operated with a Bruker-multinuclear z-gradient inverse probe-head capable of making gradients in the z-direction. ¹H-spectra have been referenced to the TSP methyl-signal at 0.00 × 10⁻⁶, whereas ¹³C-spectra have been referenced to the deuterated methanol CD₃ resonance (δ = 50.0 × 10⁻⁶).

2.7. Animals

Male albino mice (28–32 g) were accommodated in the faculty animal house for one week prior to experimentation and had free access to water and food (unless the contrary is stated). All animal tests were done according to animal-care regulations and under BAU Institutional Review Board approval (2018A-0057-P-R-0280).

2.8. Acute inflammatory-pain

The acute inflammatory-pain was induced by injecting 100 μL of carrageenan-solution (1%) intraplantarly into the animal (*n* = 7/group) left hind-paw (Boukhary, Raafat, Ghoneim, Aboul-Ela & El-Lakany, 2016). Ibuprofen 100 mg/kg, utilized as positive-control, test compounds, or vehicle control were separately administered *Per-os* (P.O.) 0.5 h before carrageenan administration as described before (Gardmark, Hoglund & Hammarlund-Udenaes, 1998a, 1998b; Salama, El Gayar, Georgy & Hamza, 2016). Behavioral-measurements have been done 2 h after carrageenan-administration.

2.9. Diabetes and diabetic neuropathy induction

Diabetes induction was done by injecting 180 mg/Kg alloxan in animals for 3 d. The animal blood-glucose levels (BGL) have been monitored by animal-tail pricking method (Raafat, 2018) utilizing Accu-check glucometers (Germany). The glycated hemoglobin (HbA1c) levels were measured utilizing Analyticon HbA1c kits (Germany). Mice having HbA1c more than 8, BGL more than 200 mg/dL, and serum insulin level less than 0.5 μg/L have been considered diabetic. After 8 weeks of induction of diabetes, diabetic neuropathy was confirmed by a basal nociceptive-reaction of around 2.5 s (Raafat & Wael, 2018; Saleh et al., 2016).

2.10. Experimental protocol

Diabetic mice were randomized to various groups with seven animals per group, and subjected to pre-dose and 8 weeks post-oral treatment evaluation, as follows:

- Experimental test groups: The hypoglycemic, anti-inflammatory, antinociceptive effects of S-SP (15, 200, and 250 mg/kg), S-RCED (150, 200, and 250 mg/kg), SAF (15, 20, and 25 mg/kg), the first combination of S-SP 150 mg/kg and CS 200 mg/kg, and the second combination of S-RCED 150 mg/kg and CS 200 mg/kg were evaluated in experimental animals.
- Positive controls: The consecutive positive-standards were utilized: glibenclamide (5 mg/kg) (GB) in serum insulin and HbA1c experiments, tramadol (TRA) 10 mg/kg in diabetic-neuropathy investigations, metformin (MTF) 25 mg/kg in the biochemical analysis, and ibuprofen (Ib) 100 mg/kg in inflammatory pain evaluation.
- Vehicle control (VEH or DIA+VEH): Alloxan-induced vehicle-treated diabetic/diabetic neuropathy control mice group.
- Normal control (NORM): Untreated normoglycemic mice group.

2.11. Serum-insulin, HbA1c levels, and body-weights

In order to identify diabetic mice and to recognize the test compounds mechanism of hypoglycemia, serum-insulin levels (SIL) have been evaluated. After being discovered the diabetic animals, SIL was monitored at pre-dose and eight weeks post-treatment utilizing HPLC reversed phase method at 40 °C utilizing C₁₈ end-capped Lichrospher-column (Merck) with a 1 mL/min flow-rate. The eluent was composed of TFA (0.1%) in HPLC water (A) and acetonitrile (B). The gradient-development-conditions were as follows: zero min 70% (A), and then 60% (A) at 214 nm after 5 min, as priority described (Raafat & Wael, 2018; Raafat et al., 2017). Furthermore, the glycosylated-hemoglobin levels (HbA1c) were carefully evaluated utilizing Analyticon HbA1c analytical-columns at pre-dose and 8 weeks post-treatment. Additionally, in order to monitor the amelioration of hyperglycemia, animals body weight were recorded pre- and subchronically (1st, 3rd, 5th, and 8th day) post-treatment.

2.12. Biochemical oxidative stress analysis

Estimation of oxidative stress functional markers was determined by evaluating serum catalase (CAT) levels, lipid peroxidation via thiobarbituric acid test (TBARS), and reduced glutathione (GSH) levels. The CAT levels were determined as kU/L via a method described before (Raafat & El-Lakany, 2017). The TBARS levels have been monitored as nmol/L/100 g through a procedure priority described (Raafat & El-Lakany, 2018). Furthermore, GSH content was also evaluated as µg/mg by a protocol described in a previous account (Raafat et al., 2017).

2.13. Evaluation of diabetic-induced nociception

After the confirmation of provoking diabetic neuropathy 8 weeks post-alloxination, the sensory nerve function was evaluated using tail-flick, hot-plate, and Von-fray tests pre-treatment, and

chronically (1st, 3rd, 5th, and 8th week) post-treatment. The tail-flick method consisted of a radiant heat manual tail flick analgesimeter was tested to evaluate reaction latencies with a cut-off time of 10 s to avoid tissue-damage (Raafat & Omar, 2016). Moreover, the hot-plate approach was used by locating the neuropathic mice on a (52 ± 0.2) °C adjusted heating plate (Germany) to evaluate reaction latencies with a 10 s cut off time as reported before in literature (Raafat & El-Lakany, 2015). Additionally, the Von fray method was done to evaluate the mechanical thresholds utilizing Von fray filaments (Germany). The thresholds were recorded in grams when the animal hind paw was withdrawn from a particular filament (Raafat & Hdaib, 2017).

2.14. Histopathological assessments

At the conclusion of this study, all animal groups have been sacrificed; The pancreatic-tissues have been harvested and promptly fixed-in formalin (10%) for 2 d. Fixed pancreatic tissue samples have been then embedded in paraffin and stained with eosin and hematoxylin (Brown, 2001). The samples were evaluated utilizing a compound microscope and photographed using a Nikon digital camera (Japan).

2.15. Statistical analysis

All data have been represented as (mean ± S.E.M). Statistical-differences between tests and controls have been evaluated by one-way analysis of variance (ANOVA), followed by Fisher post-hoc test via the “OriginPro” statistic computer software. *P* < 0.05 have been considered statistically-significant.

3. Results and discussion

In order to phytochemically and biologically investigate *C. sativus* cultivated in the botanical garden at Beirut Arab University, Lebanon the subsequent outcomes were evaluated.

3.1. Quantitative RP-HPLC phytochemical analysis

The RP-HPLC analyses of both S-SP and S-RCED extracts showed the identification of six major peaks with different concentrations. S-SP major peaks identified were (1) picrocrocin (8.1%), (2) crocin I (13.5%), (3) crocin II (11.3%), (4) crocin I' (9.0%), (5) crocin II' (8.3%), and (6) safranal (7.7%) (Fig. 1A), while that of S-RCED were (1) picrocrocin (3.1%), (2) crocin I (5.2%), (3) crocin II (2.6%), (4) crocin I' (3.7%), (5) crocin II' (3.3%), and (6) safranal (2.5%) (Fig. 1B). It was observed that S-SP had superiority in the amounts of the six major peaks identified when compared to those of S-RCED. Safranal was noticed to be presented in both extracts.

3.2. Bio-guided fractionation procedures

The bio-guided fractionation and isolation procedures have resulted in the separation of the most active fraction. Based on

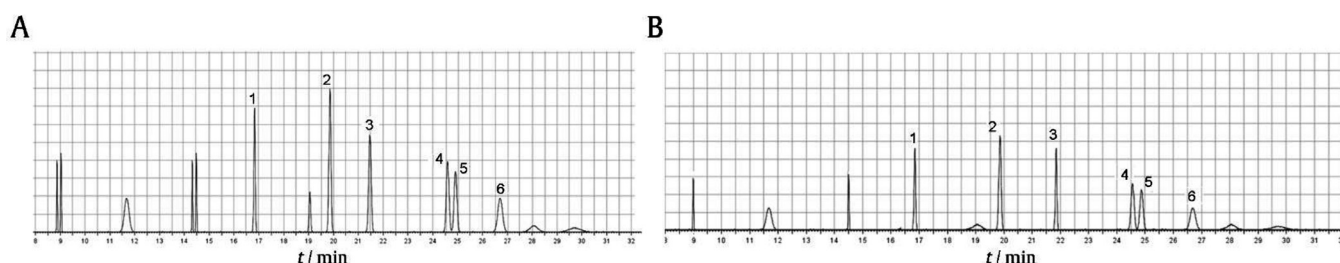


Fig. 1. HPLC-DAD analysis for S-SP and S-RCED. (A) S-SP: (1) Picrocrocin (8.1%), (2) Crocin I (13.5%), (3) Crocin II (11.3%), (4) Crocin I' (9.0%), (5) Crocin II' (8.3%), and (6) Safranal (7.7%). (B) S-RCED: (1) Picrocrocin (3.1%), (2) Crocin I (5.2%), (3) Crocin II (2.6%), (4) Crocin I' (3.7%), (5) Crocin II' (3.3%), and (6) Safranal (2.5%).

the ^1H NMR and ^{13}C NMR evaluations, the most bioactive compound isolated from the most active fraction was characterized as safranal (SAF) (Fig. 2 and Table 1). Therefore, the isolated SAF might be considered as the most anti-inflammatory, anti-diabetic, and anti-nociceptive compound isolated from *C. sativus* utilizing carrageenan-induced inflammatory and alloxan-induced diabetes and diabetic neuropathy models, respectively.

3.3. Acute inflammatory-pain analysis

The amelioration of acute inflammatory pain was investigated after the oral administration of S-SP (150, 200, and 250 mg/kg), S-RCED (150, 200, and 250 mg/kg), SAF (15, 20, and 25 mg/kg), combination of S-SP 150 mg/kg and CS 200 mg/kg, combination of S-RCED 150 mg/kg and CS 200 mg/kg, combination of SAF 15 mg/kg and CS 200 mg/kg, or positive control, ibuprofen 100 mg/kg (Fig. 3). When correlated to vehicle-control (VEH), S-SP at 150, 200, and 250 mg/kg demonstrated 1.15, 1.60, and 1.66 folds improvement in the pain paw withdrawal thresholds (PWT), respectively, while that of S-RCED at 150, 200, and 250 mg/kg showed 1.09, 1.45, and 1.68 folds, respectively, showing amelioration of PWT. While that of SAF at 15, 20, and 25 mg/kg confirmed to increase PWT by 1.06, 1.12, and 1.19, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg demonstrated 1.68, 1.71, and 1.28 folds improvement in PWT, respectively. The positive control, Ib showed significant improvement of PWT, when compared to VEH, by 1.77 folds.

Our results showed that S-SP and S-RCED produced significant anti-inflammatory effects in the carrageenan-induced inflammatory nociception model. Thereby, our results supported that S-SP and S-RCED might have anti-inflammatory potentials since they blocked heat and pain, which are essential-signs of inflammation (Simões et al., 2018). SAF had shown significant improvement of PWT but with comparatively lower magnitude than the whole extracts, implying that whole extract compounds work in synergism with SAF giving higher analgesic effects than SAF alone, as

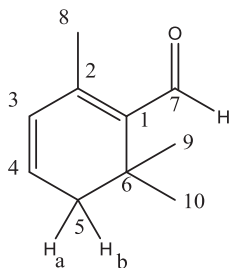


Fig. 2. Structure of safranal (SAF).

Table 1

^1H and ^{13}C - NMR data (δ , ppm) of safranal (SAF).

Position	δ_{C}	δ_{H} (J in Hz)
1	146.9	—
2	137.4	—
3	129.9	6.62 (d, $J=10.43$)
4	134.5	6.54 (ddd, $J=10.43, 11.07, 9.31$)
5	41.0	a 2.13 (dd, $J=15.82, 9.31$) b 2.14 (dd, $J=15.82, 11.07$)
6	32.7	—
7	191.8	9.70 (s)
8	17.1	2.34 (s)
9	26.2	1.23 (s)
10	26.2	1.26 (s)

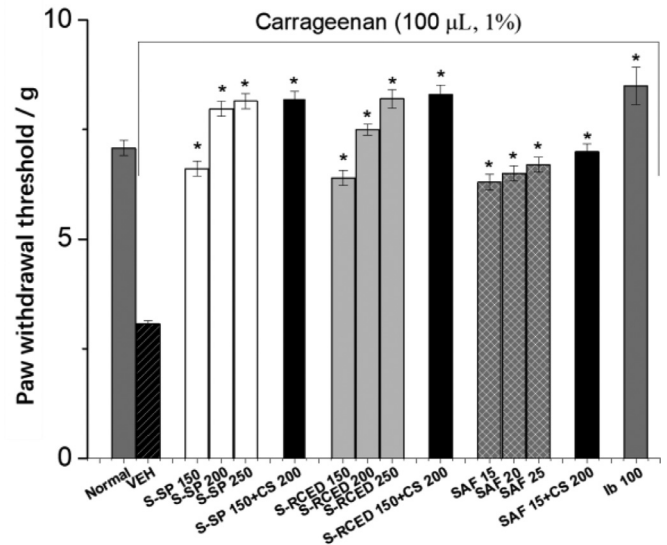


Fig. 3. Effect on acute inflammatory pain (Mean \pm SEM, $n = 7$). * $P < 0.05$ vs vehicle control (VEH) group.

seen before with similar compounds (Raafat, Wurglics & Schubert-Zsilavec, 2016). Results also showed the superiority in amelioration of inflammatory pain of the tested compounds when tested in their lower doses (IC_{50}) in combination with *C. sinu* 200 mg/kg. Although no serious side-effects were observed from the tested compounds throughout the course of the study, decreasing the dose of *C. sativus* in the combination has shown many benefits like improvement of the efficacy, decrease side-effects, if any, associated with saffron higher doses, and decreasing the economic burden by decreasing the concentration of the comparatively expensive saffron treatment.

3.4. Effect on diabetes and diabetic-induced nociception

3.4.1. Acute, subchronic and chronic effects on diabetes

In the favor of accessing the antihyperglycemic activity of the tested compounds, the effect of various doses of the single extract/SAF or combination with CS were evaluated for their acute (6 h), subchronic (8 d), and chronic (8 weeks) anti-diabetic activities using Sigma-Performa glucometers (Germany), and HbA1c Analyticon micro-columns (Germany) (Figs. 4 and 5).

In the acute evaluation and after 6 h post-treatment, S-SP (150, 200, and 250 mg/kg) exhibited 21.13%, 27.75%, and 39.10% lowering in blood glucose levels (BGL), respectively, whereas that of S-RCED (150, 200, and 250 mg/kg) displayed 31.38%, 36.50%, and 40.1%, respectively, reduction in BGL, when compared to diabetic control (DC) (Fig. 4). At the same time, that of SAF (15, 20, and 25 mg/kg) showed the decrease of BGL by 27.50%, 35.10%, and 37.50%, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg showed 46.88%, 45.20%, and 46.00% BGL amelioration, respectively. Glibenclamide (5 mg/kg) (GB) showed significant declining in BGL, when compared to DC, by 32.50% 6 h post-treatment (Fig. 4).

Subchronically (8 d post-oral treatment), S-SP (150, 200, and 250 mg/kg) showed 39.11%, 43.10%, and 45.78% reduction in BGL, respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 41.33%, 42.22%, and 49.34% of BGL reduction, respectively, when compared to DC (Fig. 4). Simultaneously, SAF (15, 20, and 25 mg/kg) showed 40.00%, 42.67%, and 43.56% of BGL decrease, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 49.33%, 63.11%, and 55.56% BGL

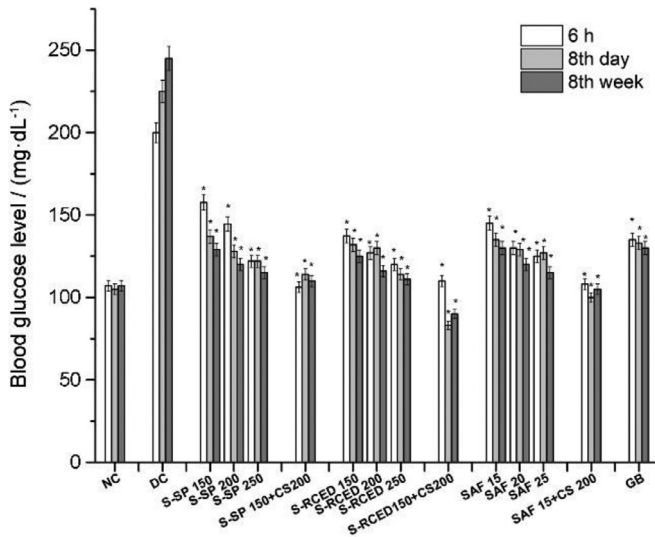


Fig. 4. Effect on acute (6 h), subchronic (8th day) and chronic (8th week) blood glucose levels (Mean \pm SEM, $n = 7$). * $P < 0.05$ vs diabetic control (DC) group.

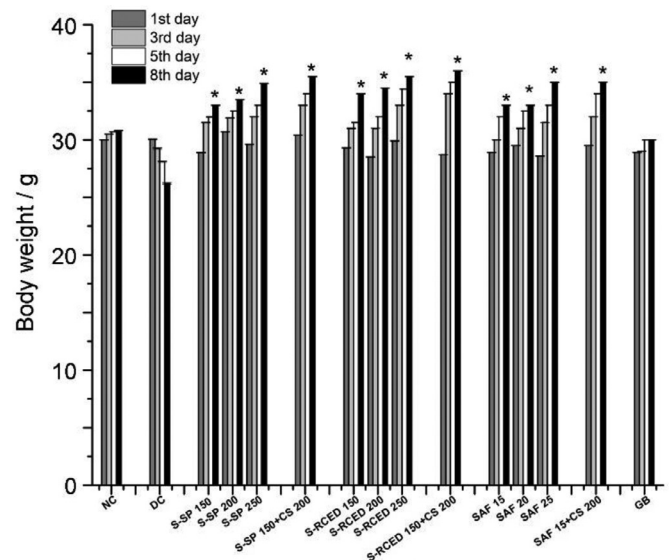


Fig. 6. Effect on body weight (Mean \pm SEM, $n = 7$). * $P < 0.05$ vs diabetic control (DC) group.

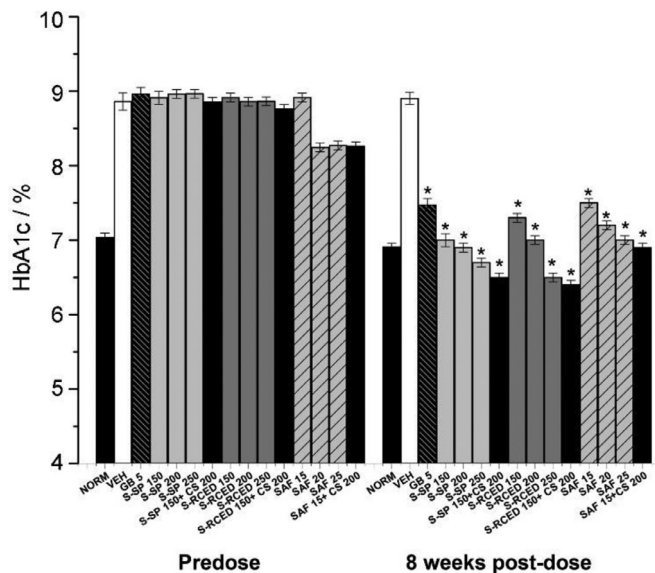


Fig. 5. Effect on HbA1c before giving test compounds (predose) and 8 weeks post-dose (Mean \pm SEM, $n = 7$). * $P < 0.05$ vs vehicle control (VEH) group.

normalization, respectively. Moreover, GB showed a significant reduction in BGL, when compared to DC, by 40.89% 8 d after oral administration (Fig. 4).

During the subchronic assessment, mice body weights (BW) were observed, where the elevation of diabetic mice body-weights are one of the indicators of alleviation of diabetes symptoms (Raafat & El-Lakany, 2018). S-SP (150, 200, and 250 mg/kg) displayed 25.86%, 27.77%, and 33.10% elevation in BW, respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured BW increase of 29.67%, 31.58%, and 35.39%, respectively, when compared to DC (Fig. 6). SAF (15, 20, and 25 mg/kg) elevated BW by 25.86%, 25.90%, and 33.49%, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 35.39%, 37.30%, and 33.50% BW elevation, respectively. Additionally, GB displayed a significant improvement in BW by 14.40% 8 d post-treatment, when compared to DC (Fig. 6).

In the chronic studies (8 weeks post-treatment), S-SP (150, 200, and 250 mg/kg) displayed 47.35%, 51.02%, and 53.06% reduction in BGL, respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 48.98, 52.65, and 54.69%, respectively, BGL reduction, when compared to DC (Fig. 4). Concurrently, SAF (15, 20, and 25 mg/kg) decreased BGL by 46.94%, 51.02%, and 53.06%, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 55.10%, 63.27%, and 57.14% BGL amelioration, respectively. Furthermore, GB displayed a significant reduction in BGL by 46.94% 8 weeks post-treatment, when compared to DC (Fig. 4).

In addition, during the chronic evaluation HbA1c levels were monitored 8 weeks post-test administration and compared to vehicle control (VEH) before treatment (predose) (Fig. 5). S-SP (150, 200, and 250 mg/kg) showed 21.36%, 22.48%, and 24.7% reduction in HbA1c level, respectively, whilst that of S-RCED (150, 200, and 250 mg/kg) displayed 17.99%, 21.36%, and 26.98% of HbA1c level reduction, respectively, when compared to VEH (Fig. 5). Concomitantly, SAF (15, 20, and 25 mg/kg) decreased HbA1c level by 15.74%, 19.11%, and 21.36%, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 26.98%, 28.10%, and 22.48% HbA1c level amelioration, respectively. GB displayed significant reduction in HbA1c level by 16.05% 8 weeks post-treatment, when compared to VEH (Fig. 5).

Our research showed that the oral-administration of S-SP (150, 200, and 250 mg/kg) or S-RCED (150, 200, and 250 mg/kg) produced a stable, significant, and dose-dependent normalization of acute, subchronic and chronic models of diabetes, as observed before with similar compounds (Raafat & Samy, 2014). S-RCED has proven more hypoglycemic potentials when compared to S-SP and SAF acutely, subchronically and chronically. Moreover, the gradual increase in experimental mice body weights and the normalization of HbA1c levels, after treatment with tested compounds, indicated the alleviation and long-term management of diabetes symptoms and their possible efficacy in amelioration and prevention of diabetes-related complications. The Moreover, the combination with the EC₅₀ of S-SP (150 mg/kg), S-RCED (150 mg/kg), or SAF (15 mg/kg) and CS 200 mg/kg have shown superiority over the highest doses of the single components. This apparent additive effect between S-SP, S-RCED, or SAF and CS might be bene-

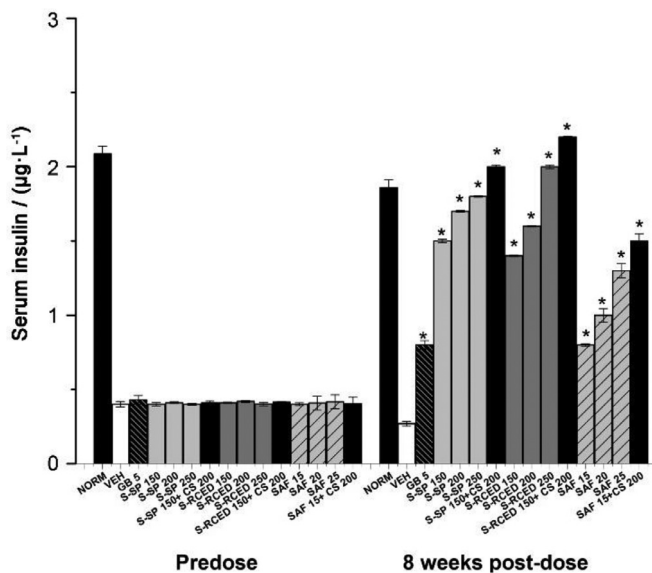


Fig. 7. Effect on serum insulin before giving test compounds (predose) and 8 weeks post-dose (Mean ± SEM, n = 7). *P < 0.05 vs vehicle control (VEH) group.

ficial therapeutically and economically in the long-term management of diabetes, and their potentials in amelioration and prevention of diabetes-related complications.

To identify the hypoglycemic mechanism of the tested-compounds, serum insulin levels were monitored.

3.5. Serum insulin levels

The serum insulin levels (SIL) were significantly elevated in mice after treatment with S-SP (150, 200, and 250 mg/kg) displaying 4.56, 5.30, and 5.67 folds increase in SIL, respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 4.19, 4.93, and 6.41 folds of SIL elevation, respectively, when compared to VEH (Fig. 7). SAF (15, 20, and 25 mg/kg) raised SIL by 1.96, 2.70, and 3.81 folds, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 6.41, 7.15, and 4.56 folds SIL augmentation, respectively. Furthermore, GB displayed significant elevation in SIL by 1.96 folds, 8 weeks post-treatment, when

compared to VEH (Fig. 7). Thus, it is evident that S-SP, S-RCED, and SAF promote insulin release to ameliorate diabetes hyperglycemic effects in the diabetic mouse model with superiority when combined with CS. Moreover, a number of studies have regarded that insulin-secretagogue therapy, mainly when focused on glucose-regulation, was a rationale therapeutic path for the diabetic patients (Leu et al., 2009; Raafat, 2016). Therefore, *C. sativus* possesses an insulin secretagogue mechanism of action throughout the long-term amelioration of hyperglycemia.

3.6. Effects on hyperalgesic nociception

After 8 weeks of induction of diabetes, the results of the hot-plate test showed that the response-latency in the diabetic group was much lower, by 45.65% than the response latency in the normal animals (P < 0.05) indicating the provoking of diabetic neuropathy (Fig. 8A). As shown in Fig. 8A, 8 weeks post-treatment with S-SP (150, 200, and 250 mg/kg) displayed 2.60, 2.90, and 3.24 folds increase in hot plate pain thresholds (HPPT), respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 2.32, 2.82, and 3.17 folds of HPPT elevation, respectively, when compared to DC (Fig. 8A). Furthermore, SAF (15, 20, and 25 mg/kg) raised HPPT by 2.40, 2.62, and 2.78 folds, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 3.17, 3.21, and 2.83 folds HPPT elevation, respectively. Furthermore, the positive control, tramadol 10 mg/kg (TRA), displayed significant raise in HPPT by 3.15 folds, 8 weeks post-treatment, when compared to DC (Fig. 8A).

In addition, the results of tail flick test showed that the response-latency in the diabetic group was 44.18% lower than the response-latency in the normal mice (P < 0.05), indicating another proof of stimulation of diabetic neuropathy (Fig. 8B). After induction of diabetic neuropathy and after 8 weeks post-treatment with S-SP (150, 200, and 250 mg/kg) neuropathic mice displayed 1.06, 1.33, and 2.28 folds increase in tail flick pain thresholds (TFPT), respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 1.11, 1.72, and 2.22 folds of TFPT elevation, respectively, when compared to DC (Fig. 8B). Furthermore, SAF (15, 20, and 25 mg/kg) raised TFPT by 1.27, 1.66, and 1.61 folds, respectively. The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg displayed 1.89, 2.05, and 1.78 folds of TFPT elevation, respectively. When compared to DC, TRA displayed significant raise in TFPT by 3.39 folds, 8 weeks post-treatment (Fig. 8B).

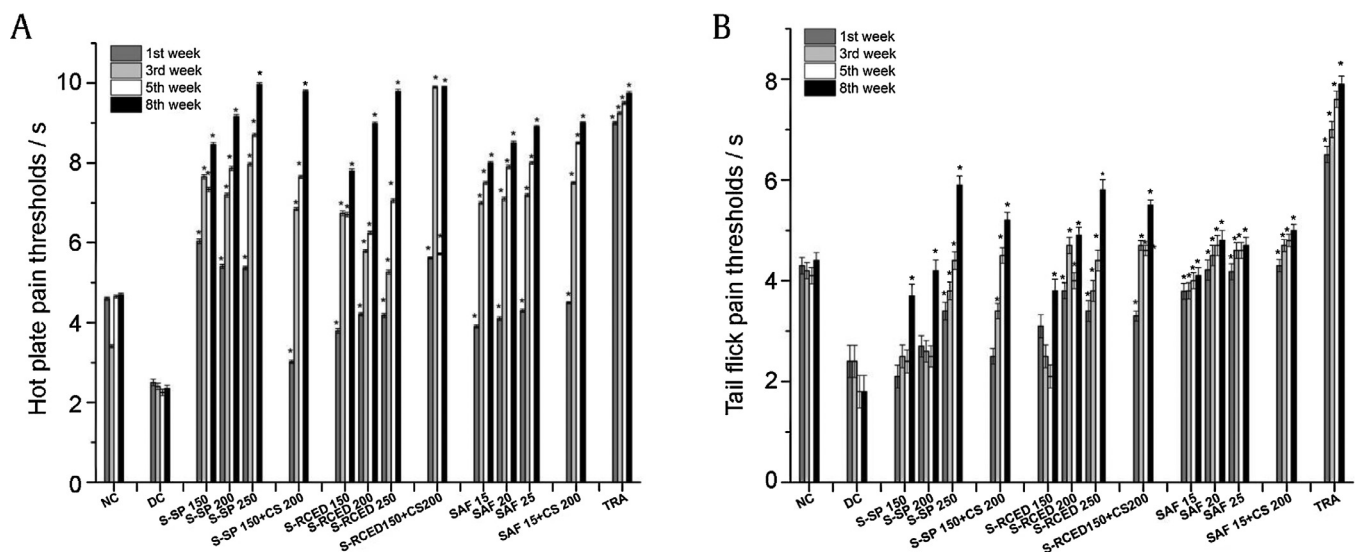


Fig. 8. Effect on hot plate pain thresholds (A) and tail flick pain thresholds (B) (Mean ± SEM, n = 7). *P < 0.05 vs diabetic control (DC) group.

After 8 weeks of tested-compounds treatment, HPPT and TFPT were significantly elevated in the diabetic animals with clear dominance to S-SP and the combinations with CS, which suggests their efficacy towards amelioration of diabetic hyperalgesia nociception, as shown with similar compounds previously (Raafat & El-Lakany, 2015, 2017).

3.7. Effects on allodynic nociception

To determine the anti-allodynic effects of the tested compounds, von Frey filament method was utilized to determine the paw withdrawal thresholds (PWT), 8 weeks after induction of diabetes and provoking diabetic neuropathy, all results were compared to DC (Fig. 9). After the chronic administration of S-SP (150, 200, and 250 mg/kg) to neuropathic mice, these animals displayed 7.00, 7.10, and 10.33 folds increase in PWT, respectively, while that of S-RCED (150, 200, and 250 mg/kg) featured 6.88, 6.90, and 10.20 folds of PWT elevation, respectively, when compared to DC (Fig. 9). Furthermore, SAF (15, 20, and 25 mg/kg) raised PWT by 4.50, 5.00, and 5.50 folds, respectively. The combinations of CS 200 with S-

SP 150, S-RCED 150, and SAF 15 mg/kg displayed 10.35, 10.30, and 6.50 folds PWT elevation, respectively. Eight weeks post-treatment, TRA displayed significant raise in PWT by 10.25 folds (Fig. 9).

Our results indicated that the tested-compounds exhibited antiallodynic effects with a superiority to S-SP and combinations with CS. The nociception relieving effects featured after a significant declining of pain thresholds, indicating some specificity of *C. sativus* against chronic-pain. Moreover, the studied extracts demonstrated more or less equieffective effects in relieving diabetic neuropathy like TRA, an atypical-opioids known for its efficacy towards diabetic neuropathy (Czerwińska et al., 2018). These results were similar to other bioactive plants preciously discovered (Raafat & Omar, 2016).

In order to study the tested compounds antinociceptive mechanism, oxidative stress markers were studied.

3.8. Effects on oxidative stress markers

Oxidative stress markers including serum catalase (CAT), glutathione reduction (GSH), and lipid peroxidation (LPO) levels were monitored at pre-dose and 8 weeks after treatment, and compared to vehicle control (Table 2). S-SP (150, 200, and 250 mg/kg) significantly elevated CAT level by 1.17, 1.65, and 1.79 folds respectively, increased GSH level by 37.84%, 54.91%, and 64.21%, respectively, and reduced LPO level by 87.03%, 88.47% and 90.20% respectively (Table 2). S-RCED (150, 200, and 250 mg/kg) raised the CAT level by 1.07, 1.75 and 1.83 folds, respectively, increased GSH level by 33.37%, 59.40%, and 70.44%, respectively, and reduced LPO level by 77.23%, 88.76% and 90.78%, respectively. Simultaneously, SAF (15, 20 and 25 mg/kg) raised the CAT level by 1.25, 1.49, and 1.56 folds, respectively, increased GSH level by 26.78%, 35.46% and 41.54%, respectively, and reduced LPO level by 73.49%, 85.59% and 88.76%, respectively (Table 2). The combinations of CS 200 with S-SP 150, S-RCED 150, and SAF 15 mg/kg raised the CAT level by 1.82, 1.48 and 1.63 folds, respectively, increased GSH level by 69.47%, 57.49%, and 45.73%, respectively, and reduced LPO level by 91.64%, 88.47% and 85.30%, respectively (Table 2).

The antioxidant potentials of S-SP, S-RCED, and SAF on elevating CAT and GSH, and reducing LPO levels, presents their potentials in ameliorating painful-neuropathy with a superiority of S-SP and combinations with CS. Previous accounts using other-compounds exhibiting similar antioxidant potentials proved to have antinociceptive properties (Muthuraman, Diwan, Jaggi, Singh & Singh, 2008; Nishiyama & Ogawa, 2005).

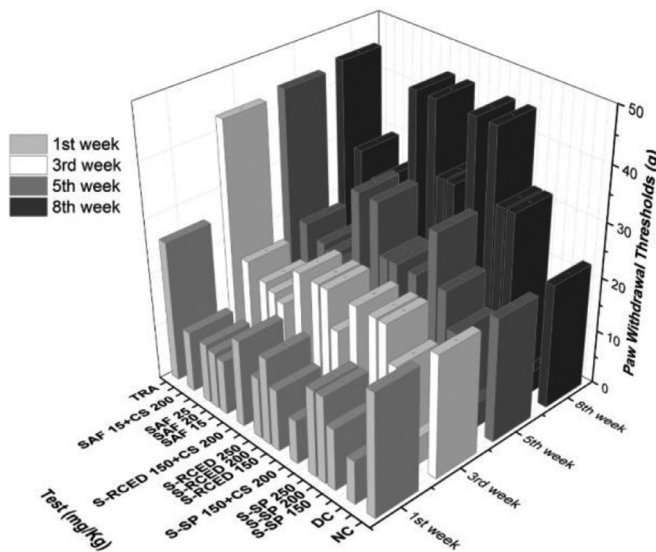


Fig. 9. Effect on paw withdrawal thresholds.

Table 2

In vivo assessment of antioxidant activities on CAT levels in serum, alterations in TBARS and reduced GSH (Mean \pm SD, $n = 7$).

Groups (mg·kg ⁻¹)	Catalase level (kU·L ⁻¹)		TBARS Level (nmol·L ⁻¹ ·100 g ⁻¹)		GSH (μ g·mg ⁻¹)	
	Predose	8th weeks	Predose	8th weeks	Predose	8th weeks
Normal control	29.77 \pm 1.58	29.98 \pm 1.12	0.70 \pm 0.02	0.72 \pm 0.02	61.39 \pm 1.89	61.80 \pm 1.25
Vehicle control	20.20 \pm 1.59	19.20 \pm 1.75	1.14 \pm 0.04	3.47 \pm 0.03	54.63 \pm 1.50	45.40 \pm 1.90
MTFa	20.07 \pm 1.42	21.28 \pm 1.45	1.08 \pm 0.02	1.65 \pm 0.04	52.52 \pm 1.56	52.20 \pm 1.45
S-SP 150a	20.11 \pm 1.59	41.74 \pm 1.45*	1.00 \pm 0.01	0.45 \pm 0.02*	57.56 \pm 1.45	62.58 \pm 1.45*
S-SP 200a	21.25 \pm 1.75	50.85 \pm 1.45*	0.99 \pm 0.02	0.40 \pm 0.03*	56.00 \pm 1.65	70.33 \pm 1.42*
S-SP 250a	21.33 \pm 1.18	53.50 \pm 1.26*	0.97 \pm 0.04	0.34 \pm 0.06*	58.13 \pm 1.45	74.55 \pm 1.55*
S-SP 150+CS 200a	21.24 \pm 1.22	54.13 \pm 1.81*	0.97 \pm 0.04	0.29 \pm 0.06*	58.12 \pm 1.66	76.94 \pm 1.44*
S-RCED 150a	21.27 \pm 1.78	39.84 \pm 1.12*	0.94 \pm 0.03	0.79 \pm 0.01*	58.10 \pm 1.70	60.55 \pm 1.90*
S-RCED 200a	20.19 \pm 1.26	52.76 \pm 1.36*	0.96 \pm 0.02	0.39 \pm 0.02*	59.16 \pm 1.60	72.36 \pm 1.32*
S-RCED 250a	21.07 \pm 1.36	54.33 \pm 1.66*	0.93 \pm 0.05	0.32 \pm 0.03*	59.12 \pm 1.50	77.38 \pm 1.27*
S-RCED 150+CS 200a	20.30 \pm 1.49	47.56 \pm 1.65*	1.15 \pm 0.01	0.40 \pm 0.03*	57.18 \pm 1.40	71.50 \pm 1.24*
SAF 15 a	20.22 \pm 1.44	43.20 \pm 1.23*	0.95 \pm 0.04	0.92 \pm 0.02*	58.15 \pm 1.60	57.56 \pm 1.45*
SAF 20 a	20.50 \pm 1.22	47.80 \pm 1.22*	0.94 \pm 0.03	0.50 \pm 0.02*	57.23 \pm 1.80	61.50 \pm 1.26*
SAF 25 a	21.20 \pm 1.56	49.10 \pm 1.38*	0.94 \pm 0.05	0.39 \pm 0.03*	56.17 \pm 1.90	64.26 \pm 1.80*
SAF 15+CS 200a	21.28 \pm 1.42	50.45 \pm 1.45*	0.93 \pm 0.03	0.51 \pm 0.05*	58.12 \pm 1.25	66.16 \pm 1.46*

* $P < 0.05$ significant from vehicle control animals.

^a Compared to vehicle control.

3.9. Histopathological examinations

The histopathological assessment demonstrated representative photo-micrographs of histological bio-analyses of pancreatic-tissues from non-diabetic normal control animals (Fig. 10A), vehicle-treated diabetic control mice (Fig. 10B), and diabetic mice treated with S-SP (150, 200, and 250 mg/kg) (Fig. 10C–E, respectively), S-RCED (150, 200, and 250 mg/kg) (Fig. 10G–I, respectively), and combinations of CS 200 with S-SP 150 mg/kg and S-RCED 150 mg/kg (Fig. 10F and J, respectively) were assessed. A decline in the density of the cells, an injury of the beta-islets with vacuolated-cells and number of blood-vessels decreased was detected for the diabetic control group compared to normal non-diabetic control group. The beta-islets from the different S-SP-treated mice demonstrated a remarkable enhancement in cells re-granulation and density. Moreover, S-RCED-treated group was less

injured compared to the diabetic control group. Both combinations with CS demonstrated more or less equipotent improvements in the number of beta-islets than single components and ensured re-generation of the beta-islets. When compared to the diabetic control, alloxan-induced diabetic mice in all treated groups showed a significant increase in beta-islet density and restoration of the blood-vessels density.

Histopathological pancreatic assessment in diabetic animals showed an evidence for the decline in langerhans cells, necrosis, and de-granulation as observed before (Saleh, El-Darra & Raafat, 2017). After treatment with S-SP and S-RCED, the langerhans cells demonstrated improvement in the histological architecture. These improvements were augmented when the tested compounds were combined with CS 200 mg/kg. S-SP-treated mice demonstrated a remarkable enhancement in cells re-granulation and density. Moreover, S-RCED-treated group was less injured, when correlated to the diabetic control group, and returned back to its normal structure. In extract treated groups, these bio-effects were associated with significant amelioration of BGL, and insulin secretagogue potentials. The histopathological pancreatic studies revealed significant beta-cell regeneration and might be responsible for the anti-diabetic and antidiabetic neuropathy mechanism of the tested extracts. These results were in line with previous studies showing the regeneration potentials of other natural phytochemicals (Kanter, Coskun, Korkmaz, & Oter, 2004; Saleh et al., 2017).

4. Conclusion

The findings of the current study indicate that *C. sativus* cultivated in the botanical garden at Beirut Arab University possessed significant long-term anti-diabetic, anti-inflammatory, and anti-nociceptive activities against diabetic neuropathy and that its oxidative stress reduction, the insulin secretagogue, and pancreatic beta-cells regeneration potentials might be responsible for the mechanism for these activities. The bio-guided fractionation and the phytochemical analysis, including RP-HPLC and ^1H and ^{13}C NMR, have shown that safranal is the most bioactive compound responsible for saffron bio-activities. The combination of *C. sativus* with *C. sinus* has shown superiority in efficiency, and lowering *C. sativus* dose, which has a great economic value in decreasing the overall price of the expensive saffron treatment. Consequently, *C. sativus* may be clinically useful for protecting against many serious disorders including inflammation and painful diabetic neuropathy.

Declaration of Competing Interest

Authors state no conflicts of interest.

Authors' contribution

Karim Raafat is the main author who did the experimental work, made the analysis of data, and wrote the manuscript. Maha Aboul-Ela and Abdalla El-Lakany revised the manuscript.

References

- Boukhary, R., Raafat, K., Ghoneim, A. I., Aboul-Ela, M., & El-Lakany, A. (2016). Anti-inflammatory and antioxidant activities of *Salvia fruticosa*: An HPLC determination of phenolic contents. *Evidence-based Complementary and Alternative Medicine*, 6 2016.
- Czerwińska, M. E., Gasińska, E., Leśniak, A., Krawczyk, P., Kiss, A. K., & Naruszewicz, M. (2018). Inhibitory effect of *Ligustrum vulgare* leaf extract on the development of neuropathic pain in a streptozotocin-induced rat model of diabetes. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, 49, 75–82.
- Daugherty, D. J., Marquez, A., Calcutt, N. A., & Schubert, D. (2018). A novel curcumin derivative for the treatment of diabetic neuropathy. *Neuropharmacology*, 129, 26–35.

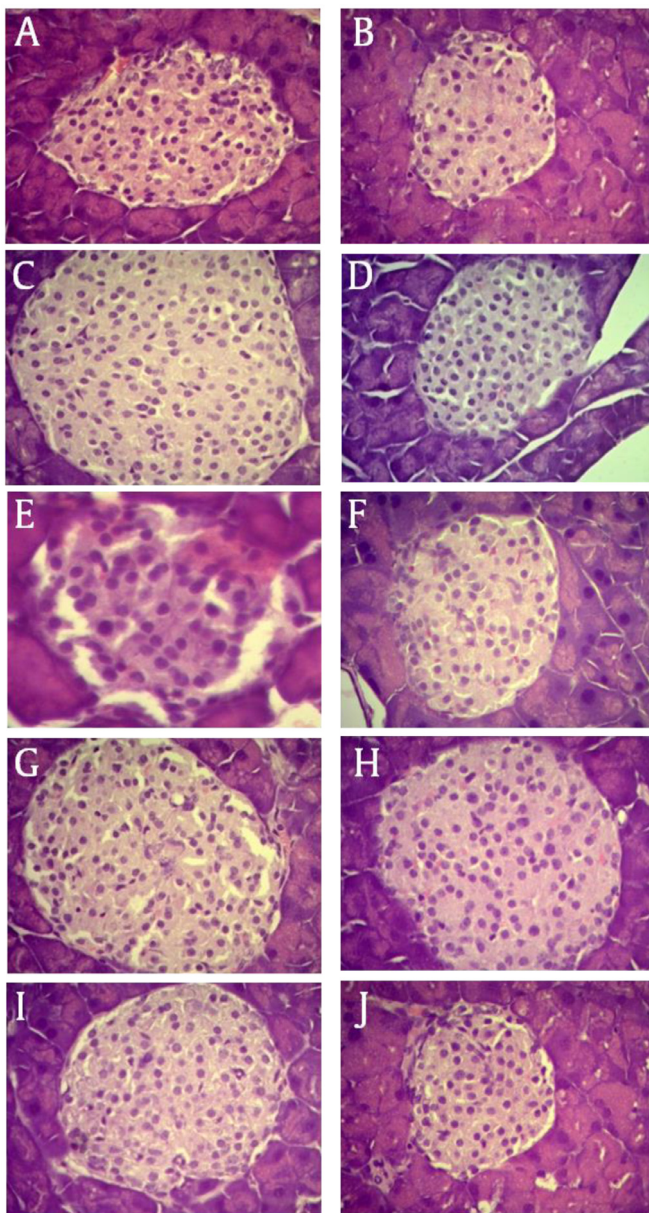


Fig. 10. Pathohistology of pancreas of each group. (A) Normal control; (B) diabetic control; (C) S-SP 150 mg/kg; (D) S-SP 200 mg/kg; (E) S-SP 250 mg/kg; (F) S-SP 150 and CS 200 mg/kg; (G) S-RCED 150 mg/kg; (H) S-RCED 200 mg/kg; (I) S-RCED 250 mg/kg and (J) S-RCED 150 mg/kg and CS 200 mg/kg.

- Gardmark, M., Hoglund, A. U., & Hammarlund-Udenaes, M. (1998a). Aspects on tail-flick, hot-plate and electrical stimulation tests for morphine antinociception. *Pharmacology and Toxicology*, 83(6), 252–258.
- Gardmark, M., Karlsson, M. O., Jonsson, F., & Hammarlund-Udenaes, M. (1998b). Morphine-3-glucuronide has a minor effect on morphine antinociception. Pharmacodynamic modeling. *Journal of Pharmaceutical Sciences*, 87(7), 813–820.
- Kanter, M., Coskun, O., Korkmaz, A., & Oter, S. (2004). Effects of *Nigella sativa* on oxidative stress and β -cell damage in streptozotocin-induced diabetic rats. The anatomical record part A: Discoveries in molecular. *Cellular, and Evolutionary Biology*, 279A(1), 685–691.
- Katanić, J., Pferschy-Wenzig, E.-M., Mihailović, V., Boroja, T., Pan, S.-P., & Nikles, S. (2018). Phytochemical analysis and anti-inflammatory effects of *Filipendula vulgaris* moench extracts. *Food and Chemical Toxicology*, 122(1), 151–162.
- Leu, Y. L., Chen, Y. W., Yang, C. Y., Huang, C. F., Lin, G. H., & Tsai, K. S., Yang, R. S. & (2009). Extract isolated from *Angelica hirsutiflora* with insulin secretagogue activity. *Journal of Ethnopharmacology*, 123(2), 208–212.
- Mardani, H., Sekine, T., Azizi, M., Mishyna, M., & Fujii, Y. (2015). Identification of safranal as the main allelochemical from saffron (*Crocus sativus*). *Natural Products Communications*, 10(5), 775–777.
- Muthuraman, A., Diwan, V., Jaggi, A. S., Singh, N., & Singh, D. (2008). Ameliorative effects of *Ocimum sanctum* in sciatic nerve transection-induced neuropathy in rats. *Journal of Ethnopharmacology*, 120(1), 56–62.
- Nishiyama, T., & Ogawa, M. (2005). Intrathecal edaravone, a free radical scavenger, is effective on inflammatory-induced pain in rats. *Acta Anaesthesiologica Scandinavica*, 49(2), 147–151.
- Raafat, K. (2016). *The top 100 phytoremedies and caregiving* (1st ed.). Beirut: Dar Al-Nahda Al-Arabia.
- Raafat, K. (2018). 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. *Revista Brasileira de Farmacognosia*, 28(3), 358–368.
- Raafat, K., El-Darra, N., Saleh, F. A., Rajha, H. N., Maroun, R. G., & Louka, N. (2017a). Infrared-assisted extraction and HPLC-analysis of *Prunus armeniaca* L. pomace and detoxified-kernel and their antidiabetic effects. *Phytochemical Analysis*, 1–12.
- Raafat, K., El-Haj, R., Shoumar, D., Alaaeddine, R., Fakhro, Y., & Tawil, N. (2017b). Neuropathic pain: Literature review and recommendations of potential phytotherapies. *Pharmacognosy Journal*, 9(4), 425–434.
- Raafat, K., & El-Lakany, A. (2015). Acute and subchronic *in-vivo* effects of *Ferula hermonis* L. and *Sambucus nigra* L. and their potential active isolates in a diabetic mouse model of neuropathic pain. *BMC Complementary and Alternative Medicine*, 15, 257.
- Raafat, K., & El-Lakany, A. (2017). Combination of *Rheum ribes* and Metformin against diabetes, thermal hyperalgesia, and tactile allodynia in a mice model. *Alternative Therapies in Health and Medicine*, 24(5), 33–43.
- Raafat, K., & El-Lakany, A. (2018). Phytochemical and antinociceptive investigations of *Anemone coronaria* active part ameliorating diabetic neuropathy pain. *Planta Medica International Open*, 5(1), e5–e13.
- Raafat, K., & Hdaib, F. (2017). Neuroprotective effects of *Moringa oleifera*: Bio-guided GC-MS identification of active compounds in diabetic neuropathic pain model. *Chinese Journal of Integrative Medicine*, e4, 1–8.
- Raafat, K., & Samy, W. (2014). Amelioration of diabetes and painful diabetic neuropathy by *Punica granatum* L. extract and its spray dried biopolymeric dispersions. *Evidence-Based Complementary and Alternative Medicine*, 2014, 180495.
- Raafat, K., & Wael, S. (2018). Phytochemical and biological evaluation of ultrasound-assisted spray dried *Lonicera etrusca* for potential management of diabetes. *Records of Natural Products*, 12(4), 367–379.
- Raafat, K., Wurglics, M., & Schubert-Zsilavec, M. (2016). *Prunella vulgaris* L. active components and their hypoglycemic and antinociceptive effects in alloxan-induced diabetic mice. *Biomedicine Pharmacotherapy*, 84, 1008–1018.
- Raafat, K. M., & Omar, A. G. (2016). Phytotherapeutic activity of curcumin: Isolation, GC-MS identification, and assessing potentials against acute and subchronic hyperglycemia, tactile allodynia, and hyperalgesia. *Pharmaceutical Biology*, 54(8), 1334–1344.
- Salama, R. A. M., El Gayar, N. H., Georgy, S. S., & Hamza, M. (2016). Equivalent intraperitoneal doses of ibuprofen supplemented in drinking water or in diet: A behavioral and biochemical assay using antinociceptive and thromboxane inhibitory dose-response curves in mice. *PeerJ*, 4, e2239.
- Saleh, F. A., El-Darra, N., & Raafat, K. (2017). Hypoglycemic effects of *Prunus cerasus* L. pulp and seed extracts on alloxan-induced diabetic mice with histopathological evaluation. *Biomedicine and Pharmacotherapies*, 88, 870–877.
- Saleh, H. M., Soliman, O. A., Elshazly, M. O., Raafat, A., Gohar, A. K., & Saleh, T. A. (2016). Acute hematologic, hepatologic, and nephrologic changes after intraperitoneal injections of 18 nm gold nanoparticles in hamsters. *International Journal of Nanomedicine*, 11, 2505–2513.
- Samarghandian, S., & Borji, A. (2014). Anticarcinogenic effect of saffron (*Crocus sativus* L.) and its ingredients. *Pharmacognosy Research*, 6(2), 99–107.
- Shakeel, M. (2015). Recent advances in understanding the role of oxidative stress in diabetic neuropathy. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 9(4), 373–378.
- Simões, R. R., Kraus, S. I., Coelho, I. S., Dal-Secco, D., Siebert, D. A., Mücke, G. A., & Alberton, M. D. (2018). *Eugenia brasiliensis* leaves extract attenuates visceral and somatic inflammatory pain in mice. *Journal of Ethnopharmacology*, 217, 178–186.
- Sobolev, A. P., Carradori, S., Capitani, D., Vista, S., Trella, A., & Marini, F. (2014). Saffron samples of different origin: An NMR study of microwave-assisted extracts. *Foods (Basel, Switzerland)*, 3(3), 403–419.
- Srivastava, R., Ahmed, H., Dixit, R. K., & Dharamveer, S., S. A. (2010). *Crocus sativus* L.: A comprehensive review. *Pharmacognosy Reviews*, 4(8), 200–208.