Research Article

The Effect of Chia Seed Extracts against Complete Freund’s Adjuvant-Induced Rheumatoid Arthritis in Rats

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Background and Objectives. It is known that the oxidation of chia seeds is minimal or absent, due to the presence of bioactive compounds, having a great potential in the foods and pharmacological industry. This investigation was done to estimate the nutrition values and anti-oxidant activity of chia seed extracts.

Materials and Methods. The protective effects of chia seed extract against complete Freund’s adjuvant (CFA) rheumatoid arthritis in rats were investigated with 100, 200, 300, and 400 ppm/kg BW rat/day chia seeds aqueous extract for 4 weeks. Results. The results obtained revealed that chia seed extract contained high proportions of protein and total dietary fiber values of 21.35% and 27.24%, respectively. Moreover, the results reported that the mineral contents were the greatest in phosphorous, potassium, magnesium, calcium, and sodium (450.09, 410.46, 245.22, 218.69, and 120.34 mg/100 g, respectively). Meanwhile, iron, zinc, and copper were the lowest in chia seeds (9.26, 4.67, and 3.66 mg/100 g, respectively). Besides, vitamins C and E reported 1.65 and 0.82 mg/100 g, respectively. Chia seed extracts were effective in vitro for the bioactive components such as phenolics, flavonoids, and anti-oxidant activities. The biomarkers of complete blood picture, lipid profile, anti-oxidant enzymes, TNF-α, and IL-10 had been improved. Histopathological examination of the rat knee confirmed health amelioration, revealing that chia seed extract consumption can lower pathological changes in injured rheumatoid arthritis rats. Conclusion. It could be seen that the chia seed extracts alleviated the harmful effect of rheumatoid arthritis CFA-induced rats.

1. Introduction

Rheumatoid arthritis (RA) is a disease of the articular cartilage that results from a defect in the immune regulation that either directly or indirectly affects the physiology of the cartilage cells [1]. It is also distinguished by inflammation and injury to the bone joints, and if not treated, its complications increases [2, 3]. The etiology of rheumatoid arthritis involves the infiltration of multiple inflammatory cells and crosstalk with cytokines [4]. Genetic and environmental agents that can ultimately converge in an overactive immune system are the basic idea of the etiology of rheumatoid arthritis [5]. Arthritis collectively points out to more rheumatic illness described by inflammation, pain, and stiffness in the musculoskeletal system [6].

Rheumatoid arthritis affects women being affected more often than men; rheumatoid arthritis is also evaluated with a high mortality rate [7, 8]. Oliveira-Alves et al. [9] suggested that chia seeds could contribute to improving better the consumers’ health. Therefore, phenolic compounds found in
chia seeds, mainly caffeic acid and salvianolic acids, act as protective agents for illnesses, which may be caused by oxidative overwork [10–12]. Likewise, bioactive ingredients present in chia seeds are associated with lowering the risk of chronic heart disease, liver influence, rheumatoid arthritis, plasma oxidative stress, and obesity-associated illness [13, 14].

Despite the fact that various studies have been conducted on rheumatoid arthritis prevention, much work has been done using chia seeds as a preventive or remedial measure. Therefore, the purpose of the current research work was to evaluate chia seed extract as a chemical analysis and antioxidant compounds. In addition, estimate the biological experiment with chia seed aqueous extract at different levels to treat rheumatoid arthritis in rats’ chronic inflammatory disorders.

2. Material and Methods

2.1. Study Area. This research project was conducted from September 2021 (Starting date) to January 2022 (Ending date) according to the regulations and rules laid down by the committee of animals’ experimentation of Taif University, Kingdom of Saudi Arabia.

2.2. Materials. Chia seeds (Salvia hispanica L.) were purchased from the local market in Taif City, Saudi Arabia. Kits for all different parameters were obtained from Sigma-Aldrich Corp., MO. Also, complete Freund’s adjuvant (CFA) was purchased from Sigma, St. Louis, Mo. Male albino rats (n = 36 rats), 150–160 g per each, were obtained from Pharmacy Faculty at King Saud University and fed on the basal diet.

2.3. Preparation of Chia Seeds. Chia seeds were washed under tap water to remove foreign particles, dried at 50–60°C, and milled to a fine powder. The powder was stored at 5°C until analysis.

2.4. Determination of the Chemical Composition of Chia Seeds. Chemical compositions such as protein, fat, crude fiber, and ash content were determined in the chia seeds according to Sami et al. [15, 16]. Minerals content as magnesium, sodium, potassium, zinc, phosphorus, iron, calcium, and copper were estimated in chia seeds according to Sami et al. [17].

2.5. Vitamin Analysis (HPLC). The vitamin C and E determinations were detected according to the modified method of Rokayya et al. [18]. Chromatographic analyses were detected for vitamin C by an Agilent HPLC system (2000 ECOM, Chrastany u Prahy, CZ 252 19, Czech) at 254 nm with UV detection. Analytical column YMC-Triart C18 (150 × 4.6 mm) was used as the mobile phase of A/B 33/67; A: 0.1 M potassium acetate, distilled water 50:50, pH (4.9), and 1 mL/min for the flow rate at the ambient temperature [19].

2.6. Determination of Anti-Oxidant Assays (ABTS, DPPH, and FRAP). ABTS assay was evaluated according to the stabilization of the ABTS+ radical cation [20]. The DPPH test was to assess the anti-oxidant capacity of the DPPH radical in ethanol. FRAP reagent ensured the anti-oxidant potential by reducing iron (Fe²⁺) and iron (Fe³⁺) in chia seed extracts [21]. The results were considered at Mmol TEAC g⁻¹.

3. Bioactive Components Determination

3.1. Total Polyphenol Contents (TPC). Approximately 100 µg/mL chia extracts were mixed thoroughly with the corresponding Folin reagent, and 1.0 mL 7% of Na₂CO₃ solution was added and incubated for 90 min, detected at 765 nm, and calculated as gallic acid equivalents (GAEs) mg GAE/g DW [22].

3.2. Total Flavonoid Contents (TFC). Approximately 0.2 mL of chia extracts and 0.2 mL of 30% ethanolic AlCl₃·6H₂O were immediately added and incubated for 5 min. The absorbance was detected at 430 nm and calculated as quercetin equivalents mg QE/g DW [23].

3.3. Total Tannin Contents (TTC). Approximately 1 mL of chia extracts and 5 mL of potassium iodide (2.5%) were mixed, closed, and placed in a ~30°C water bath (10 min). The absorbance was assessed at 590 nm and calculated as tannic acid equivalents (TAEs) mg TAEs /100 g DW [24].

3.4. Biological Experimental. The experimental rats were fed on a basal diet for 7 days and randomly divided into 6 groups, 6 rats for each. The first main group was fed on a basal diet for four weeks, namely the control negative rats group. The rest of the rats were injected with 100 µL of CFA into the left hind knee joint to induce rheumatoid arthritis in rats. After 7 days, secondary arthritis was induced by injecting 50 µL of CFA under the left hind knee joint according to Narendhirakannan et al. [25] and then divided into 5 groups. A positive control group was also fed the basal diet only, while the other 4 groups were fed basal diets and taken 100, 200, 300, and 400 ppm/kg BW rat/day chia aqueous extract were taken orally by a stomach tube during the experimental period.

After the end of the experiment, the blood was pulled withdrawn from different rat groups, centrifuged to obtain serum, and kept at −20°C until analysis. Blood hemoglobin (Hb), hematocrit (Ht), platelets, red blood cells (RBCs), and white blood cells (WBCs) were estimated [26]. Lipid profiles such as triglycerides, total lipids, total cholesterol, high-density lipoprotein (HDL), and low-density lipoproteins (LDL) were estimated according to Zollner and Kirsch [27]. Superoxide dismutase (SOD), nonenzymic glutathione (GSH), malondialdehyde (MDA), serum-selected cytokines IL-10, and TNF-α were determined by Kandir and Keskin [28].
3.5. Histopathology Evaluations. The animals were sacrificed (after four weeks following CFA injection), and the knee joint was excised and kept in 10% buffered formalin then sectioned and embedded in paraffin. Slides were stained with hematoxylin and eosin according to Laste et al. [29].

3.6. Statistical Analysis. All experimental data were applied to the (ANOVA) test, and the results were shown as means of standard deviation (±SD). Significant differences were evaluated as \( P \leq 0.05 \).

4. Results

4.1. Chemical Composition. The chemical compositions and dietary fiber levels of chia seeds were determined and published in (Figure 1). Chia seeds have elevated high amounts of protein, total lipid, and crude fibers by 31.35%, 27.64%, and 10.96%, respectively. Moreover, the total dietary fiber, insoluble and soluble dietary fibers detected were 27.24%, 18.98%, and 8.26%, respectively. Furthermore, the results of the mineral and vitamin contents in chia seeds were presented in Table 1. The results reported that the mineral contents were the greatest in phosphorous, potassium, magnesium, calcium, and sodium (450.09, 410.46, 245.22, 218.69, and 120.34 mg/100 g, respectively). Meanwhile, iron, zinc, and copper were the lowest in chia seeds (9.26, 4.67, and 3.66 mg/100 g, respectively). Vitamins C and E in chia seeds contained 1.65 and 0.82 mg/100 g, respectively.

4.2. Anti-Oxidant Profile. The research findings on the anti-oxidant activity of chia seed extracts were reported in Figure 2. Chia seed extract had high ABTS, DPPH, and FRAP activities by 3.21, 2.43, and 3.39 mmol TEAC/g DW, respectively. Total phenolic acids, flavonoids, and total tannins contents recorded 0.98 mg GAE/g DW, 0.23 mg QE/g DW, and 16.11 mg TAEs g/100 g DW, respectively (Table 2).

4.3. Effect of Chia Seed Extract on Complete Blood Picture. Data that are obtained from the research work and presented in Table 3 showed that the hemoglobin was the highest in the rat group that was fed on a basal diet as a control negative by 12.7 g/dL. The positive control rats that induced rheumatoid arthritis reported 9.8 g/dL, while the different groups rats' induced rheumatoid arthritis that was fed on a basal diet and taken orally with extracts at 100, 200, 300, and 400 ppm were gradually increased to 11.2, 11.8, 12.3, and 12.5 g/dL, respectively. Therefore, Omoigui et al. [30] confirmed that the treatment with chia extract resulted in a hemoglobin increase. The results observed that the hematocrit was lower in positive control (30.3%) compared with negative control (38%), and it was increased in the group that was fed on chia extract at 40 ppm by 37.8%. In addition, the red blood cells increased at different extracts by 5.22, 5.84, 6.27, and 6.81 m/cm compared with negative control (4.45 m/cm), respectively. White blood cells reported an increase in the positive control group by 10.14 cm compared with negative control (5.67 cm), while the other groups that were taken 100, 200, 300, and 400 ppm were gradually decreased by 9.33, 8.53,
4.4. Effect of Chia Seed Extract on Lipid Profile. The results of the total lipid of the chia seeds were presented in Table 4. The results depicted that the negative control was 0.64 g/dL, and a significant increase in the positive control rats group induced rheumatoid arthritis by 1.42 g/dL. Moreover, chia extracts at levels 100, 200, 300, and 400 ppm were 1.21, 1.00, 0.83, and 0.60 g/dL, respectively. Results of triglycerides, total cholesterol, and LDL were 112.3, 86.3, and 25.0 mg/dL in the negative control, while in the positive control group increased to reach 245.7, 196.3, and 131.7 mg/dL, respectively. Meanwhile, the other rat groups were gradually decreased to 212.0, 179.7, 146.4, and 113.3 mg/dL in triglycerides, as well as for total cholesterol the results indicated decreasing to 169.0, 142.3, 115.26, and 88.18 mg/dL, respectively; in addition, the LDL was decreased to 105.67, 79.3, 53.42 and 27.16 mg/dL, respectively. The HDL determination reported a significant increase in serum rats’ negative control to reach 73.73 mg/dL compared with positive control (27.3 mg/dL). Moreover, the HDL was decreased in the other groups with chia extracts to reach 38.0, 49.0, 61.28, and 72.73 mg/dL, respectively.

4.5. Effect of Chia Seed Extract on Oxidative Stress. Findings obtained from this study on the effect of chia seed extract on oxidative stress were presented in Figure 3. The lipid peroxidation as malondialdehyde (MDA), and the activity of glutathione (GSH) and superoxide activity (SOD) were determined in different rat groups induced rheumatoid arthritis at different levels 100, 200, 300, and 400 ppm taken orally, and the results are shown in Figure 3. The results reported that the positive control rats were decreased to reach 3.16 and 4.39 m.mol/mg protein, compared with negative control rats that increased to reach 11.32 and 15.34 m.mol/mg protein, respectively. Rats with chia seed extracts gave the gradually best results for GSH and SOD; especially, the groups 5 and 6 were taken orally at 300 and 400 ppm levels that increased in GSH to 9.44 and 10.50 m. mol/mg protein, as well as in SOD more increased to 11.91 and 13.98 m. mol/mg protein, respectively.

4.6. Effect of Chia Seed Extract on Cytokines IL-10 and TNF-α. Results presented in Figure 4 revealed the effect of chia seed extract on cytokines. Figure 4 illustrated that the TNF-α in the positive control group was the highest (48.53 pg/ML$^{-1}$) compared with the negative healthy rat group (30.25 pg/ML$^{-1}$). Meanwhile, the best results were detected for the group that had 400 ppm/kg body weight/rat/day from chia extracts, which reported 32.15 pg/ML$^{-1}$. These results were confirmed by Shanahan and St[31] who found that when the TNF-α value is lowered, the variety of inflammation is decreased. From the same table, IL-10 acts as an anti-inflammatory cytokine that had the highest value of 25.86 pg/ML$^{-1}$ in positive control rats, while the negative control reported 13.28 pg/ML$^{-1}$. Meanwhile, the best results were detected for the group that had 400 ppm/kg body weight/rat/day from chia extracts, which reported 15.12 pg/ML$^{-1}$. IL-10 can control the development of rheumatoid arthritis and inhibit cytokine production, released by activated macrophages[32].

4.7. Histopathology Evaluations. Microscopic examinations of joints for the negative control group were given in Figures 5(a)–5(f). The pictures in the plates revealed normal histology of the joint and free from inflammatory or degenerative changes; it appeared to consist of two cartilages covered bone heads with synovial membrane lining the joint capsule internally and histologically normal trabecular bone of the epiphysis.

On the other hand, microscopic examinations of joints for the positive control group are given in Figures 5(a)–5(f) and Figures 6(a)–6(e). There were various alterations; the articular cartilage was thinned, and the synovium and the joint capsule were greatly...
thickened due to expansion by inflammatory edema and exudate. The trabecular bone forming the head was decreased in size and number.

Concerning the group fed on a basal diet and taken orally 100 ppm/kg body weight/day from chia seed extract, the microscopic examinations of joints are presented in Figures 7(a)–7(e). The photographs in Figures 7(a)–7(e) depicted that both articular surfaces and the synovium were normal. The trabecular of the epiphysis was normal as well.

Regarding the group fed on a basal diet and taken orally 200 ppm/kg body weight/day from chia seed extract, their microscopic joint examinations are presented in Figures 8(a)–8(e). As revealed, the joints exhibited mild thinning in the articular cartilage covering the articular surface. The synovium and the joint capsule appeared normal, while the trabecular of the epiphysis was thinner compared to the negative control group.

Lastly, the microscopic joint examinations of the rats fed on a basal diet and taken orally 400 ppm/kg body weight/day from chia seed extract are shown in Figures 9(a)–9(e). As presented, the rats exhibited a good recovery, as the joint was free from degeneration.

The group fed on a basal diet and taken orally 400 ppm/kg body weight/day from chia seed extract (Figure 10(a)–10(c)) exhibited the best recovery with no inflammatory lesions and appeared histologically normal.

### 5. Discussion

The goal of this study was to characterize chia seeds that had a high concentration of chemical components (protein, oil content, dietary fiber, and total carbohydrates), minerals content (phosphorous, potassium, magnesium, calcium, and sodium), vitamins (E and C), and total dietary soluble and insoluble dietary. These results were in agreement with Ixtaina et al. [33] who reported that chia seed protein, fats, carbohydrates, high dietary fiber, and ash range from 15% to 25%, 30 to 33%, 26 to 41%, 18 to 30%, and 4.0 to 5.0%, respectively. In addition, minerals, vitamins, and dry matter were from 90% to 93%. The chia seeds have contented the highest total dietary fiber ranging from 36% to 40%, which is greater than grains and vegetables [34]. The insoluble dietary fiber is associated with the gradual elevation in blood sugar levels after eating and lowering insulin resistance [35]. The results were confirmed by De et al. [36]. Drusch and Mannino concluded that chia flour is rich in protein, with

### Table 4: Lipid profile.

<table>
<thead>
<tr>
<th>Groups</th>
<th>T. Lipid (g/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>T. cholesterol (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.64 ± 0.03</td>
<td>112.3 ± 6.1</td>
<td>86.3 ± 1.1</td>
<td>73.7 ± 10.0</td>
<td>25.0 ± 5.56</td>
</tr>
<tr>
<td>Positive control</td>
<td>1.42 ± 0.17</td>
<td>245.7 ± 27.9</td>
<td>196.3 ± 6.5</td>
<td>27.3 ± 17.2</td>
<td>131.7 ± 20.2</td>
</tr>
<tr>
<td>Group, 100 ppm</td>
<td>1.21 ± 0.13</td>
<td>212.0 ± 30.0</td>
<td>169.0 ± 7.0</td>
<td>38.0 ± 5.3</td>
<td>105.67 ± 10.0</td>
</tr>
<tr>
<td>Group, 200 ppm</td>
<td>1.00 ± 0.06</td>
<td>179.7 ± 9.07</td>
<td>142.3 ± 3.5</td>
<td>49.0 ± 3.0</td>
<td>79.3 ± 6.03</td>
</tr>
<tr>
<td>Group, 300 ppm</td>
<td>0.83 ± 0.07</td>
<td>146.38 ± 8.35</td>
<td>115.26 ± 4.12</td>
<td>61.28 ± 4.29</td>
<td>53.42 ± 3.17</td>
</tr>
<tr>
<td>Group, 400 ppm</td>
<td>0.60 ± 0.04</td>
<td>113.28 ± 6.58</td>
<td>88.18 ± 2.31</td>
<td>72.73 ± 4.78</td>
<td>27.16 ± 1.56</td>
</tr>
</tbody>
</table>

Mean values in each raw having different superscript (a, b, c, and d) are significantly different at 0.05 levels.

![Oxidative stress](image)

**Figure 3: Oxidative stress.**

![Cytokines IL-10 and TNF-α](image)

**Figure 4: Cytokines IL-10 and TNF-α.**
Figure 5: (a) Negative control group showing normal cartilage (black arrow), normal synovium (black star), and bone (red star); (b) higher magnification showing normal cartilage (black arrow); (c) normal synovial membrane and joint capsule (black arrow); (d) higher magnification showing synovial membrane and joint capsule (black arrow); (e) magnification showing synovial membrane and joint capsule (black arrows); and (f) normal trabecular bone (arrows) of the head of the bone.

Figure 6: (a) Positive control group, showing mild thinning in the cartilage coating the head of the bone (arrow); (b) showing inflamed joint capsule and synovium (arrows); (c) higher magnification showing perivascular inflammatory cells infiltration (arrows) and edema in the synovial membrane; (d) higher magnification showing inflammatory cells infiltration (arrows) and edema in the synovial membrane; and (e) showing mild thinning in the trabecular bone (arrows) of the epiphyses.
eight essential amino acids and high amounts of calcium, iron, ascorbic acid, omega [30], and anti-oxidants; therefore, it can be called food for healthy skin, hair, and nail.

The anti-oxidant as total phenolic, total flavonoids, and tannic acid content, as well as anti-oxidant activity, had the highest in chia seeds. Sargi et al. [37] indicated that chia seeds can inactive ABTS radicals and scavenge synthetic

Figure 7: (a) Group (100 ppm) showing normal articular cartilage, (b) normal articular cartilage (arrow) and synovium (star), (c) showing normal synovium and joint capsule, (d) higher magnification showing normal synovium and joint capsule, and (e) showing normal bone of the epiphysis.

Figure 8: (a) Group (200 ppm) showing mild thinning in the articular cartilage (arrow), normal synovial membrane, and joint capsule (star); (b) mild thinning in the articular cartilage (arrow); (c) normal synovial member joint capsule; (d) synovial membrane and joint capsule; and (e) higher in higher magnification, showing normal synovial membrane and joint capsule.
DPPH radicals. Furthermore, Coelho and Salas-Mellado [38] validated the anti-oxidant efficacy of these findings. Chia’s high anti-oxidant activity can enhance the benefits for human health. Besides, the Brazilian Chia seeds had contented phenolic compounds by 0.97 and 0.99 mg GAE/g DW33.

Results from biological experiments, it could be found that the chia seeds improved the complete blood picture, lipid profile anti-oxidant enzymes, and cytokine in rat groups with complete Freund’s adjuvant-induced rheumatoid arthritis. Therefore, Omoigui et al. [39] confirmed that the treatment with chia extract resulted in a hemoglobin increase. These results were in a similar trend to the previous study of Anderson et al. [40], who reported the hematological results of rheumatoid arthritis could due to activating the immune response to assistance to the body in fighting infection by producing antibodies that circulate in the bloodstream.

These results investigated that have shown that the highest level of polyphenols in chia extract may play a great significant role in contributing to health and chronic heart disease [41]. The anti-inflammatory influence of treatment with chia extract is to adverse endothelial trouble by inhibiting LDL oxidation [42]. Also, the biologically active contents of chia extract such as natural anti-oxidants can act locally as a cytokine and defend the body against micro-organisms [43]. Treating rats with chia extract reduced elevated levels of triglycerides, LDL, and cholesterol in the positive group. In addition, the treatment of chia extract enhanced the lipid abnormalities that may be due to the
rheumatoid arthritis-like TG and TC values that returned to normal while HDL and LDL were significantly better [31].

Hendawy et al. [32] observed that the activity of enzymes such as GSH was increased in the blood in animals that fed with chia supplementation. Regarding the other enzymes, no variations in their activity were shown. The results reported that the MDA increased in the positive control group by 4.23 m.mol/mg protein, while negative control reported 0.45 m.mol/mg protein. In addition, the orally fed groups of 100 and 200 ppm chia seed extracts reported 2.81 and 2.11 m.mol/mg protein. Meanwhile, the best results were for groups 5 and 6 that reported the lowest values of 1.68 and 0.78 m.mol/mg protein, respectively. MDA is a product of lipid peroxidation, whereas SOD is a function to remove reactive oxygen species (ROS). These factors can affect the vitality of the body as when tissue damage, large ROS amounts are produced and occur to oxidative stress (OS) [44].

TNF-α is a pleiotropic cytokine as it plays a serious function for each from acute and chronic inflammation by enhancing the adhesion of neutrophils and lymphocytes to endothelial cells [45]. These results were confirmed by Shanahan and St [46] who found that when the TNF-α value is lowered, the variety of inflammation is decreased. From the same Table, IL-10 acts as an anti-inflammatory cytokine that had the highest value of 25.86 pg/ML−1 in positive control rats, while the negative control reported 13.28 pg/ML−1. Meanwhile, the best results were detected for the group which had 400 ppm/kg body weight/rat/day from chia extracts that reported 15.12 pg/ML−1. IL-10 can control the development of rheumatoid arthritis and inhibit cytokine production, released by activated macrophages [47, 48].

This study reported that chia seeds have high values of bioactive compounds and effective anti-oxidant activities besides the pharmacological effect against complete Freund’s adjuvant-induced rheumatoid arthritis in rats. The formulation approach was used in this study to focus on the pharmacological potential benefit of food extracts.

6. Conclusion
The purpose of this study was to determine how chia seed extract affected the development of bones and joints in animals. The researchers discovered that chia seed extract had a significant favorable impact on the growth and development of rats’ bones. It was concluded that chia seeds have a high amount of nutrients, phenolic acids, and flavonoids that scavenge the free radicals in the blood. Therefore, when fed the rats on a basal diet and taken orally at levels of 300 and 400 ppm chia seed extracts, it led to the best results for oxidative stress, complete blood picture, lipids profile, and also cytokines (TNF-α and IL-10) in rheumatoid arthritis in rats’ chronic inflammatory disorders.

7. Significance Statement
This study reported that chia seeds have high values of bioactive compounds and effective anti-oxidant activities besides the pharmacological effect against complete Freund’s adjuvant-induced rheumatoid arthritis in rats. The formulation approach was used in this study to focus on the pharmacological potential benefit of food extracts.

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

References


