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Evaluation of the Anti-inflammatory Properties of Traditional Herbal Medicines in Macrophage Cells Stimulated with High Glucose Levels

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A B S T R A C T

Type 2 diabetes (T2DM) causes inflammation and organ dysfunction as well as oxidative stress. Chronic inflammation is strongly linked to diabetes complications. Medications to treat T2DM are available but have limitations and harmful side effects. Traditional herbal therapies (THT) offer a promising solution due to their safety, affordability, and wide acceptance. Objective: The study aimed to examine the anti-inflammatory properties of Artemisia afra, Cinnamon verum, and Trigonella foenum-graecum, commonly used in African countries for treating diabetes and its progression. Method: In stock extracts of plant extracts (Artemisia afra leaves, Cinnamon verum bark, and Trigonella foenum-graecum seeds) were prepared in ethanol solutions 70%. A macrophage cell line (RAW 264.7) was exposed to high-glucose levels, followed by exposure to plant extracts. Results: The results were compared with those of pharmaceutical-grade metformin. The present study revealed that Artemisia afra, Cinnamon verum, and Trigonella foenum-graecum extracts significantly inhibited the expression of several mediators of inflammation by the cell, including TNF-α, IL-6, G-CSF, RANTES and NO. Metformin had no effect on RANTES or NO levels. Results indicated that different herbs inhibited inflammatory mediators at different concentrations. Artemisia afra and metformin had the lowest cytotoxicity compared to Cinnamon verum and Trigonella foenum-graecum. Conclusion: Artemisia afra was found to exhibit a higher anti-inflammatory effect than the other herbs tested. These findings suggest that Artemisia afra plant extracts may be used to regulate low-grade/chronic inflammation related to diabetes and prevent its complications. Therefore, it is necessary to prevent the early onset of diabetes and the progression of the disease individually by using pharmacological and anti-inflammatory traditional therapies.

Keywords: Type 2 diabetes mellitus - Artemisia afra- Cinnamon verum - Trigonella foenum-graecum – Metformin - Macrophage cell - Inflammatory markers.

تقيم خصائص الأعشاب التقليدية الطبية المضادة للالتهابات لدى الخلايا البلعمة المحفزة بمستويات عالية لسكر الجلكوز

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الملخص

مرض السكري من النوع الثاني (T_2DM) يسبب الالتهابات وأختلال وظائف الأعضاء بالإضافة إلى الإجهاد التأكسدي يرتبط الالتهاب المزمن بقوة بمضاعفات مرض السكري. تتوفر أدوية لعلاج T_2DM ولكن لها قيود وآثار جانبية ضارة. تقدم العلاجات العشبية التقليدية (THT) حلاً واعدًا نظرًا لسلامتها والقدرة على تحمل تكاليفها وقبولها على نطاق واسع. **الهدف:** هدفت الدراسة إلى فحص الخصائص المضادة للالتهابات لنباتات Artemisia afra و Cinnamon verum و Trigonella foenum–graecum، المستخدمة عادة في البلدان الأفريقية لعلاج مرض السكري وتطوره. **الطريقة:** تم تحضير المستخلصات النباتية المتوفرة (أوراق الأرتيميسيا أفرا " الشيح"، ولحاء القرفة، وبذور تريجونيلا فوينوم–جريكوم " الحلبة") في محلول الإيثانول بنسبة 70%. تم تعريض خطوط الخلايا البلعمية نوع (RAW 264.7 لمستويات عالية من الجلوكوز، يليها التعرض للمستخلصات النباتية. **النتائج:** تمت مقارنة النتائج مع نتائج الميتفورمين الصيدلاني. كشفت الدراسة الحالية أن مستخلصات Artemisia afra و Cinnamon verum و Trigonella foenum–graecum تمنع بشكل كبير التعبير عن العديد من وسطاء الالتهاب بواسطة الخلية، بما في ذلك TNF–α و IL–6 و G–CSF و RANTES و NO. لم يكن للميتفورمين أي تأثير على مستويات RANTES أو NO. أشارت النتائج إلى أن الأعشاب المختلفة تمنع وسطاء الالتهابات بتركيزات مختلفة. كان لـ Artemisia afra و metformin أ سمية للخلايا مقارنة بالقرفة الحقيقية و Trigonella foenum–graecum. **الخلاصة:** وجد أن نبات الأرتيميسيا أفراء يظهر تأثيرًا مضادًا للالتهابات أعلى من الأعشاب الأخرى التي تم اختبارها. تشير هذه النتائج إلى أنه يمكن استخدام مستخلصات نبات الأرتيميسيا أفرا لتنظيم الالتهابات المنخفضة الدرجة/المزمنة المرتبطة بمرض السكري ومنع مضاعفاته. ولذلك، فمن الضروري منع ظهور مرض السكري في وقت مبكر وتطور المرض باستخدام العلاجات التقليدية الدوائية والمضادة للالتهابات.

الكلمات المفتاحية:

الخلايا البلعمية, الأريتميسيا افرا، القرفة الحقيقية، الميتفورمين، تريجونيلا فوينوم جريكوم ، داء السكري من النوع 2، علاما الالتهابات .

Abbreviations

Type 2 diabetes mellitus (T2DM), Traditional herbal therapies THT, Tumour necrosis factor α (TNFα), Interleukin-6 (IL-6), Granulocyte colony-stimulating factor (G-CSF), Regulated on activation, normal T cell expressed and secreted (RANTES), Oxidative stress (OS), Diabetes mellitus (DM), Reagent kit for cytotoxicity (XTT), Enzyme-linked immunosorbent assay (ELISA), Mouse macrophages cells (RAW 264.7), Dulbecco's modified Eagle's medium (DMEM), low glucose (LG) , high glucose (HG), lipopolysaccharides (LPS), Analysis of Variance (ANOVA), Half-maximal inhibitory

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concentration (IC_{50}), Inducible nitric oxide synthase (iNOS), interferon-gamma (IFN-γ), Interleukin-1 beta (IL-1β), insulin resistance (IR).

Introduction

Type 2 diabetes mellitus (T2DM) is a common pathological disorder associated with metabolic syndrome – carbohydrate, lipid, and protein metabolism changes are often related to obesity, chronic inflammation, and oxidative stress (OS). [1], [2] Chronic inflammation and lipid accumulation in skeletal muscle cells also contribute to OS activation, impairing insulin signalling pathways. [3], [4] The effects of hyperglycaemia on the body's OS have been studied, enhancing the pro-inflammatory modulator responses leading to IR and resulting in diabetic complications. [5] Several studies have shown that inflammation plays an essential role in the development of diabetes and the progression of complications associated with this disease. [6], [7]

Therapies that reduce and control glucose levels are ineffective in preventing the development of diabetes complications. [8] In a prospective diabetes study, [9] evaluated three glucose-lowering drugs (sulfonylurea, metformin, and insulin) but could not establish which drug was superior regarding diabetes complications. The use of herbal medicines in the treatment of diabetes is safe, inexpensive, widely available, and acceptable compared with standard Western medicines. [10], [11] the efficacy of traditional medicines to treat diabetes is currently being evaluated scientifically and medically. [12], [13] Earlier studies suggest that *Artemisia afra* (A. afra), *Cinnamon verum* (C. verum*)* and Trigonella foenum-graecum *(T. foenum-graecum)* can be used for treating diabetes. [14], [15]

Macrophage cells contribute to diabetes complications through immune cells interacting with inflammatory mediators to cause cardiovascular and neuropathy, nephropathy, and retinopathy diseases, as suggested in some studies. [7] Therefore, activated macrophages are considered a potential therapeutic target for treating chronic complications associated with diabetes mellitus (DM). [16]

The aim was to determine the *in vitro* effectiveness and safety of plant remedies commonly used in African countries to regulate the pro-inflammatory markers associated with chronically high glucose levels in mimic-diabetic cells. May offer a new strategy for using

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traditional inhibitor agents to synthesis pro-inflammatory mediators as potential novel treatments for diabetes and its complications.

Materials and Methods

Preparation of plant extracts

In stock extracts of *A. afra* leaves (20 g/100 ml), *C. verum* bark (16.6 g/100 ml) and *T. foenum-graecum* seeds (16.6 g/100 ml) were prepared in ethanol solutions 70%. XTT reagent Kit (Roche Diagnostic GmbH, Germany), nitrite standard and Griess reagent (Sigma-Aldrich), Mouse ELISA kits (R&D Systems, USA) and eBioscience, USA) and a microplate reader Multiskan Ex, (Thermo Electron Corporation).

Cell cultures

Mouse macrophages cells (RAW 264.7) were cultured in Dulbecco's modified Eagle's medium (DMEM). DMEM was supplemented with 10% heat-inactivated of fetal bovine serum (FBS) (HyClone) (v/v), 1% L-glutamine (v/v) (Gibco), 1% antibiotic-antimycotic mix (v/v) (Danvers, MA, USA) and 0.5% gentamycin (v/v). Cells were cultured in 75 cm^2 flasks at 37°C in a humidified atmosphere containing 5% carbon dioxide. Upon reaching 70–80% confluence, the cells were suspended in the medium and centrifuged (10 minutes at 2000 rpm). RAW 264.7 cells were seeded in four treated 96-well microtiter plates (Nunc) at 6×10^5 cells per well, and the cells were grown in a humidified atmosphere of 5% CO_2 at 37°C for approximately 48 hours until they reached 80–90% confluence.

The cells were then pre-exposed to low (5 mM) and high D-glucose (25 mM) concentrations {low glucose (LG) and high glucose (HG)} (v/v), which were incubated for 24 hours. The cell plate medium was replaced with a fresh exposure medium containing LG and HG plant extracts. Varying concentrations of *A. afra*, *C. verum* and metformin solution (0–1000 µg/ml) and exposure medium were supplemented with *T. foenum-graecum* (0–5000 g/ml). The cells were left unstimulated or stimulated with lipopolysaccharides (LPS) (0.2 µg/ml) (v/v) to induce inflammation. The plates were incubated for 24 hours under standard tissue culture conditions. Then, 100 µl of culture supernatant was collected from each culture well and used for nitric oxide (NO) and cytokine biomarker determination.

Statistical analysis

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Data were analysed using averages and ± SDs. The graph data and concentration parameters for each treatment were compared with the controls (no treatment) for each experiment. Sigma-Plot 12.0 was used to assess significant differences using a one-way Analysis of Variance (ANOVA). Significant differences compared to the controls were considered for samples with a $P < 0.01$.

Results

Cytotoxicity assay

Effects of high-glucose and LPS on cell viability

Results showed that glucose, with and without LPS, significantly decreases cell viability at 45 mM glucose concentration ($P < 0.001$) compared to 5 mM. There were no effects of glucose with and without LPS at the 25-mM glucose concentration as shown in figure (1).

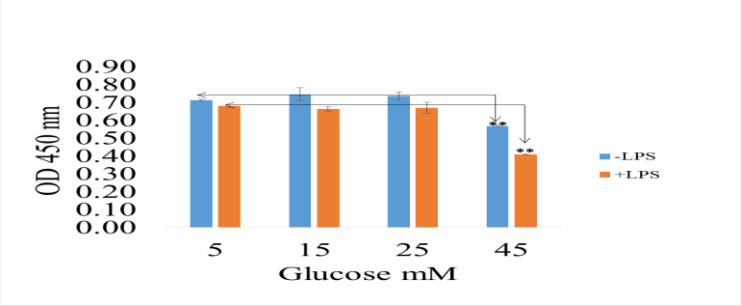

Figure 1. The effects of glucose with LPS on the viability of RAW 264.7 cells at 45-mM glucose concentration

Effects of herb extracts on pro-inflammatory biomarkers secretion and cell viability

In this study, we compared the effects of three THT with standard Western medicine on RAW 264.7 cell cytokine secretion profiles exposed to HG and evaluated the inflammatory response to THT and possible cytotoxicity. The results showed that HG treatment of cells stimulated with LPS markedly induced the expression of the protein markers in cell culture supernatants.

Culture media containing 5 mM glucose concentration is used as normal glucose and 25 mM glucose concentration as high glucose.

Effects of *A. afra* and *C. verum* extracts on cell viability and nitric oxide secretion

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The XTT assay was used to assess the toxicity of the *A. afra* extract on RAW 264.7 cells. Cells were exposed to HG for 24 hours, then treated with increasing concentrations of *A. afra* and *C. verum* extracts (up to 1000 µg/ml) for 24 hours.

A reduction in cell viability was also observed at *A. afra* concentrations > 125 µg/ml compared to the control at 0 µg/ml (Figure 2 (i)). The viability of RAW 264.7 cells declined significantly at an *A. afra* concentration of ≥ 250 µg/ml ($P < 0.001$). Thus, the cell viability in LG vs HG, with and without LPS-stimulation, is clearly dependent on the concentration of *A. afra* (Figure 2 (i)). The Griess assay was used to determine nitric acid (NO) in the supernatant of RAW 264.7 cell culture supernatants after treatment with various *A. afra* concentrations under LG and HG conditions in the presence of LPS. Results showed that NO secretion was significantly inhibited ($P < 0.001$) by increasing the concentration of *A. afra* (Figure 2 (ii)). *C. verum* decreased cell viability at concentrations > 250 µg/ml, compared to the control at 0 µg/ml. The effects of *C. verum* were only observed at concentrations ≥ 500 µg/ml, and cell viability was significantly reduced ($P < 0.001$) (Figure 3 (i)). *C. verum* concentrations showed a low inhibition of NO in HG and LG media-containing LPS. The *C. verum* effects reduced the NO secretion-dependent concentration and at higher concentrations because of a decrease in cell proliferation (Figure 3 (ii)).

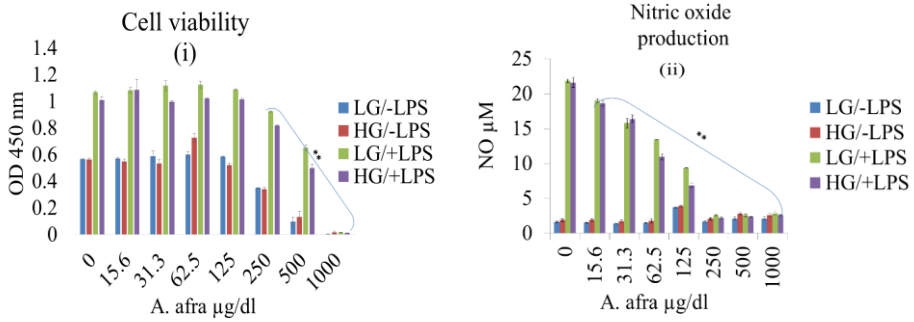

Figure 2. Cell viability of RAW 264.7 cells treated with various concentrations of *A. afra* under LG and HG with and without LPS-stimulation. Bars marked with ** indicate a statistically significant difference ($P < 0.001$) compared to 0 µg/ml *A. afra* extract (i). Effects of *A. afra* extract on NO production in LG and HG with and without LPS-stimulated RAW 264.7 cells.

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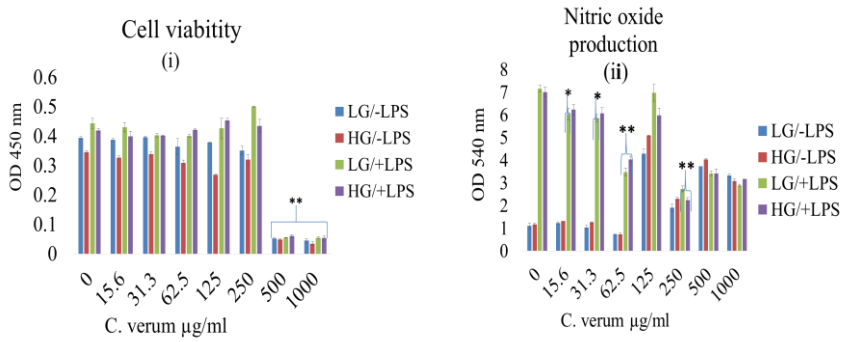

Figure 3. Cell viability of RAW 264.7 cells treated with *C. verum*. Statistically significant difference, ** ($P < 0.001$) is shown at the higher concentrations compared to 0 µg/ml *C. verum* (i). Effects of *C. verum* extract on NO production in LG vs HG with and without LPS-stimulated RAW 264.7 cells compared to 0 µg/ml of *C. verum* concentration (ii).

Effects of A. afra on TNF-α, IL-6, G-CSF, and RANTES expression levels

A. afra extracts significantly decreased tumour necrosis factor α (TNFα) levels at 62.5 µg/ml (statistical significance, * $P < 0.01$) and at ≥ 125 µg/ml (** $P < 0.001$) in LG and HG media under LPS-stimulated conditions (Figure 4 (i)). *A. afra* at 13.3 µg/ml significantly decreased interleukin-6 (IL-6) secretion by RAW 264.7 cells when incubated in LG with LPS ($P < 0.01$) and HG with LPS ($P < 0.001$). *A. afra* at ≥ 62.5 µg/ml significantly decreased IL-6 secretion ($P < 0.001$) in LG and HG media supplemented with LPS-stimulation (Figure 4 (ii)). *A. afra* significantly decreased ($P < 0.001$) the synthesis of granulocyte colony stimulating factor (G-CSF) at a concentration of 13.3 µg/ml in LG medium containing LPS and by $P < 0.01$ in HG medium containing LPS-stimulation. At ≥ 62.5 µg/ml, *A. afra* significantly decreased G-CSF ($P < 0.001$) in LG and HG media containing LPS-stimulation (Figure 5 (i)). Exposure of LPS-stimulated RAW 264.7 cells to *A. afra* significantly decreased ($P < 0.001$) regulated on activation, normal T cell expressed and secreted (RANTES) secretion levels at ≥ 31.25 µg/ml. RANTES secretion was inversely proportional to the *A. afra* concentration (Figure 5 (ii)).

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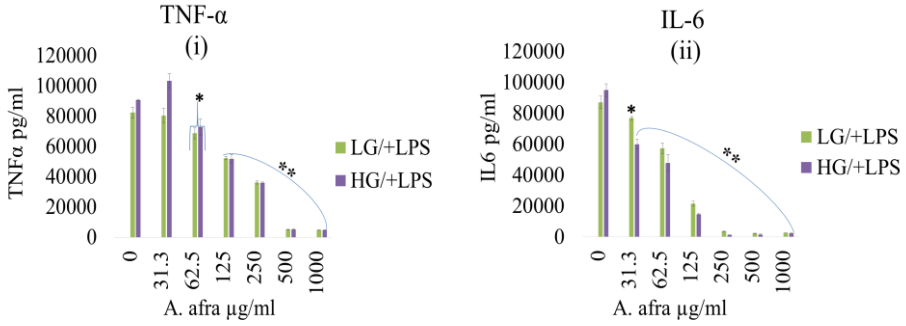

Figure 4. The effects of the *A. afra* extract on TNF-α and IL-6 secretion by RAW 264.7 cells incubated with LG and HG media containing LPS-stimulation (i and ii). Statistically significant difference levels are indicated as * ($P < 0.01$) and ** ($P < 0.001$).

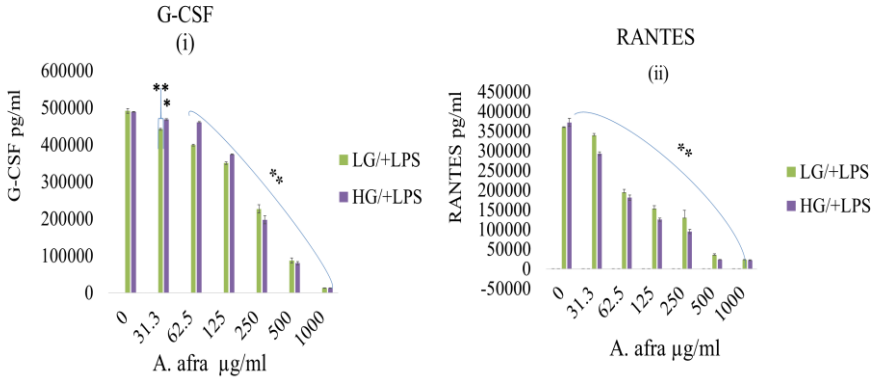

Figure 5. The effect of the *A. afra* extract on G-CSF secretion in LG and HG media containing LPS-stimulation (i). The effect of the *A, afra* extract on RANTES secretion in LG and HG media containing LPS compared to the control 0 µg/ml (ii).

Half-maximal inhibitor concentration (IC_{50})

In Table 1, we present half-maximal IC_{50} of *A. afra* extract for the various biomarker markers.

Table 1. IC_{50} of the *A. afra* extract for the secretion of various inflammatory and pro-inflammatory biomarkers by RAW 264.7 cells.

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Markers		TNF α	IL6	G-CSF	RANTES	NO	Cell viability
A. afra IC_{50} µg/ml	LG/-LPS						305
	HG/-LPS						751
	LG/+LPS	242	76	187	83	88	591
	HG/+LPS	207	50	207	59	75	539

Effects of C. verum on TNF-α, IL-6, G-CSF and RANTES expression levels

C. verum extract exposure of RAW 264.7 cells at 62.5 µg/ml in HG medium containing LPS significantly decreased TNF-α secretion levels ($P < 0.01$) and by $P < 0.001$ at ≥ 125 µg/ml extract in LG and HG media containing LPS-stimulation (Figure 6 (i)). *C. verum* extract exposure of RAW 264.7 cells in LG and HG media containing LPS significantly decreased IL-6 secretion ($P < 0.001$) (Figure 6 (ii)). *C. verum* exposure had no effects on G-CSF secretion by RAW 264.7 cells cultured in LG and HG media containing LPS, while it inhibited G-CSF secretion because the cell proliferation decreases at concentrations of 500 and 1000 µg/ml (Figure 7 (i)). Exposure to *C. verum* significantly decreased RANTES secretion levels by RAW 264.7 cells at concentrations ≥ 62.5 µg/ml by $P < 0.001$ in LG and HG media containing LPS-stimulation (Figure 7 (ii)).

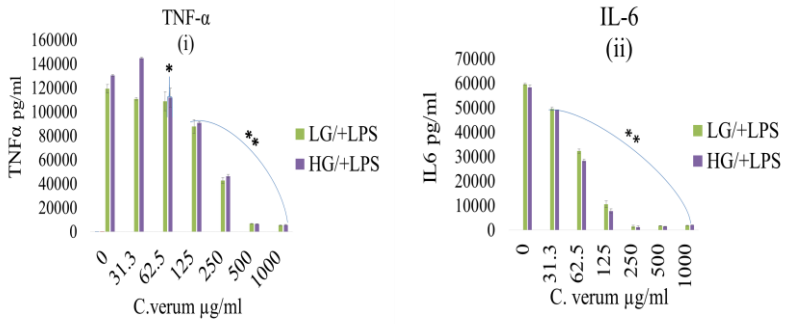

Figure 6. The effect of the *C. verum* extract on TNF-α and IL-6 secretion levels in LG vs HG media containing LPS-stimulation (i and ii).

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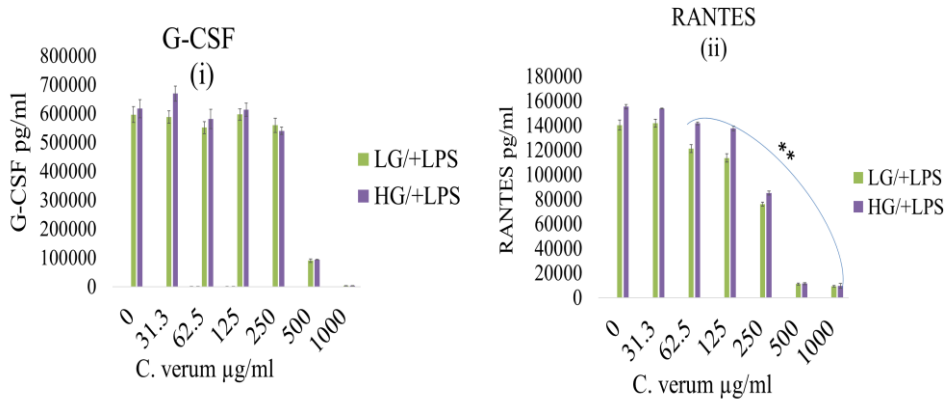

Figure 7. The effect of the *C. verum* extract on G-CSF secretion in LG and HG media containing LPS-stimulation (i). The effects of the *C. verum* extract on RANTES secretion by RAW 264.7 cells cultured in LG and HG media containing LPS-stimulation (ii).

Half-maximal inhibitory concentration (IC_{50})

The IC_{50} values of the *C. verum* extract for the various biomarkers analysed, were calculated, as presented on Table 2.

Table 2. IC_{50} of the *C. verum* extract for the secretion of various inflammatory biomarkers by RAW 264.7 cells.

Markers		TNF α	IL6	RANTES
C. verum IC_{50} µg/ml	LG/-LPS			
	HG/-LPS			
	LG/+LPS	183	60	274
	HG/+LPS	172	54	287

Effects of *T. foenum-graecum* and metformin extracts on RAW 264.7 cells

Mouse macrophage cells were exposed to HG for 24 hours, then treated with increasing concentrations of T. foenum-graecum extracts (up to 5000 µg/ml) and metformin extracts (up to 1000 µg/ml) for 24 hours.

T. foenum-graecum had no effect at levels ≤ 2500 µg/ml but reduced cell viability at concentrations higher than 2500 µg/ml compared to the control 0 µg/ml *T. foenum-graecum* control. Cell viability decreased significantly ($P < 0.001$) at the higher concentration of 5000

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µg/ml *T. foenum-graecum* (Figure 8 (i)). The NO production assay was performed on the cell culture medium supernatant of RAW 264.7 cells exposed to *T. foenum-graecum* in LG and HG media containing LPS. The *T. foenum-graecum* significantly inhibited ($P < 0.001$) the production of NO at the higher concentration (2500 µg/ml) and inhibited NO secretion because the cell proliferation decreases at a concentration of 5000 µg/ml, compared to 0 µg/ml in LG and HG media containing LPS-stimulation (Figure 8 (ii)). Metformin significantly decreased cell proliferation at concentrations ≥ 250 µg/ml ($P < 0.001$) (Figure 9 (i)). Nitric oxide was measured in RAW 264.7 cell culture medium after exposure to metformin. Metformin at ≤ 125 µg/ml had no effect on NO secretion by RAW 264.7 cells incubated with LG and HG media containing LPS. Higher metformin concentrations inhibited NO secretion because of cell proliferation that decreased at concentrations ≥ 250 µg/ml (Figure 9 (ii)). Data also show that metformin had no affect NO secretion by cells incubated in LG and HG media without LPS.

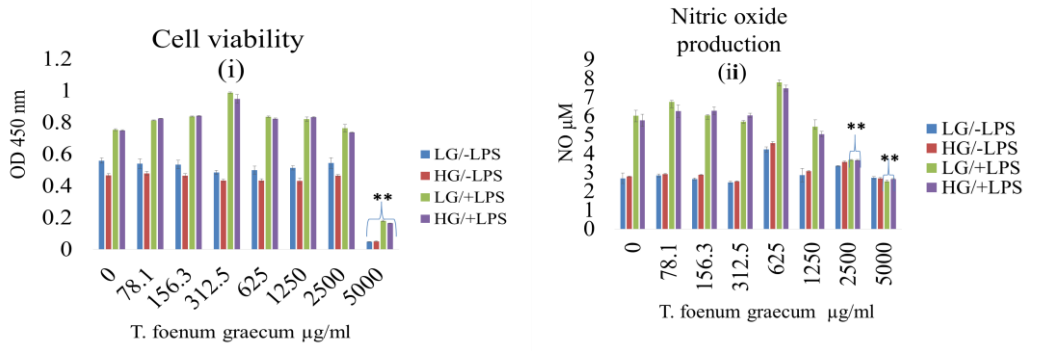

Figure 8. The effect of the *T. foenum-graecum extract* on RAW 264.7 cells viability. Statistical significance is indicated by ** ($P < 0.001$) at the higher concentration compared with 0 µg/ml *T. foenum-graecum* extract (i). The effects of *T. foenum-graecum* extract on NO production by RAW 264.7 cells cultured in LG and HG media with and without LPS-stimulation (ii). Data represent the mean ± SEM. Differential significance was denoted by $P < 0.01$ and ($P < 0.001$) (indicated by **) at the higher concentration in LG and HG media containing LPS compared to 0 µg/ml *T. foenum-graecum*.

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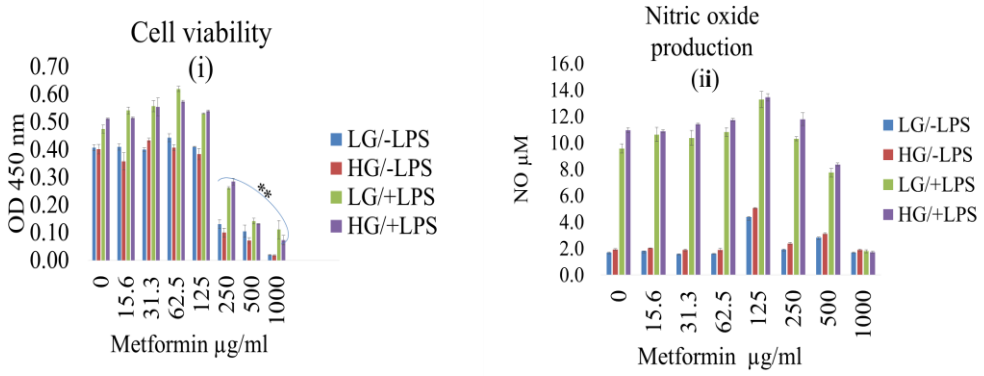

Figure 9. Cell viability of RAW 264.7 cells treated with metformin solution. The effect of metformin on cell proliferation was significant ($P < 0.001$) compared to 0 µg/ml metformin solution (i). No effects of metformin solution on RAW 264.7 cell in LG and HG with and without LPS-induced NO production (ii).

Effects of T. foenum-graecum on TNF-α, IL-6, G-CSF and RANTES expression levels

The *T. foenum-graecum* extract significantly decreased secretion of TNF-α ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media containing LPS at concentrations ≥ 625 µg/ml (Figure 10 (i)). *T. foenum-graecum* significantly decreased IL-6 secretion ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media containing LPS at concentrations ≥ 156.3 µg/ml (Figure 10 (ii)). Exposure to *T. foenum-graecum* extracts significantly decreased the synthesis of G-CSF ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media-containing LPS at concentrations ≥ 625 µg/ml compared to the 0 µg/ml *T. foenum-graecum* control (Figure 11 (i)). The *T. foenum-graecum* extract down-regulated RANTES significantly at a concentration of 1250 µg/ml ($P < 0.01$) and less so (at a concentration ≥ 2500 µg/ml $P < 0.001$) in LG medium containing LPS-stimulation (Figure 11 (ii)).

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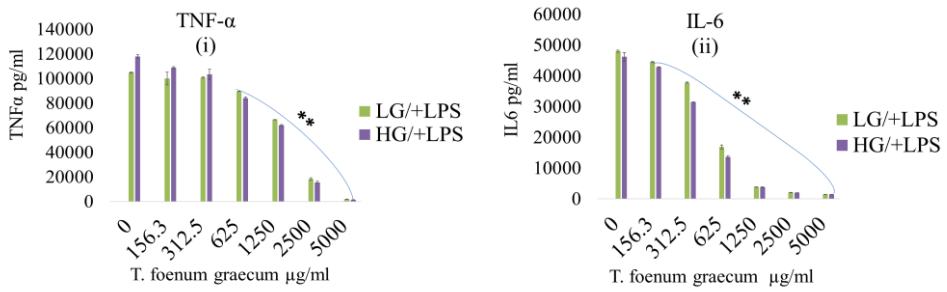

Figure 10. The effects of *T. foenum-graecum* extract on TNF-α and IL-6 secretion levels by RAW 264.7 cells cultured in LG and HG media containing LPS (ii and ii). ** P < 0.001.

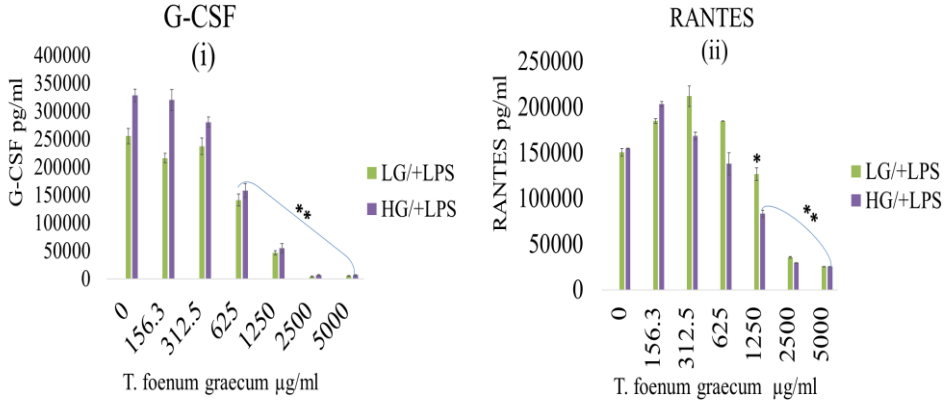

Figure 21. The effects of *T. foenum-graecum* extract on G-CSF secretion levels by RAW 264.7 cells cultured in LG and HG media containing LPS (i). ** P < 0.001. The effects of *T. foenum-graecum* extract on RANTES synthesis by RAW 264.7 cells cultured in LG and HG media containing LPS-stimulation (ii).

Half-maximal inhibitory concentration (IC_{50})

In Table 3, the IC_{50} values of the *T. foenum-graecum* extract for the various biomarkers analysed are presented.

Table 3. IC_{50} of *T. foenum-graecum* extract for the secretion of various inflammatory biomarkers by RAW 264.7 cells.

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Markers		TNF α	IL6	G-CSF	RANTES	NO	Cell viability
T. foenum-graecum IC_{50} µg/ml	LG/-LPS						2680
	HG/-LPS						2372
	LG/+LPS	1107	747	697	1337	3184	3878
	HG/+LPS	1023	514	658	1121	3178	2800

Effects of metformin on TNF-α, IL-6, G-CSF and RANTES expression levels

Exposure of RAW 264.7 cells to metformin in an LG medium containing LPS inhibited TNF-α secretion significantly at ≥ 31.3 µg/ml ($P < 0.001$). RAW 264.7 cells cultured in an HG medium containing LPS showed a significant decrease in TNF-α secretion ($P < 0.001$) at a metformin concentration of ≥ 125 µg/ml (Figure 12 (i)). There was no significant decrease in IL-6 secretion levels at ≥ 125 µg/ml metformin (Figure 12 (ii)). Increasing the concentration of metformin decreased the expression level of G-CSF ($P < 0.001$) in LG medium containing LPS-stimulated cells, whereas at the concentration (125 µg/ml) and over significantly decreased G-CSF expression ($P < 0.001$) in HG medium containing LPS-stimulated cells (Figure 13 (i)). Metformin caused no significant decrease in RANTES secretion levels in LG and HG media treated with LPS-stimulated cells, as shown in (Figure 13 (ii)). Reductions appeared at ≥ 500 µg/ml metformin because of the cell proliferation that decreased in LPS-containing media.

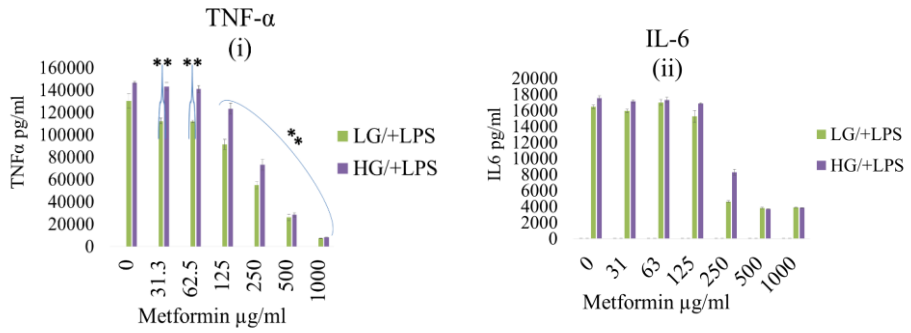

Figure 12. Metformin inhibited the TNF-α expression level, which significantly decreased ($P < 0.001$) in LG with LPS-stimulated cells (i). There was no significant decrease in IL-6 secretion levels in LG- and HG-treated LPS-stimulated cells (ii).

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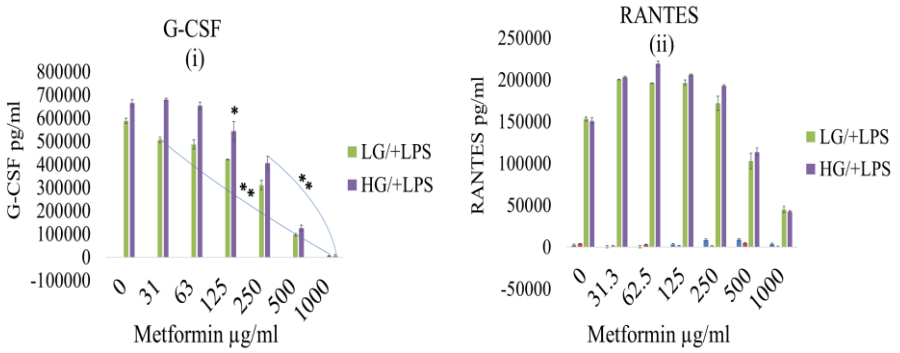

Figure 13. Metformin significantly decreased G-CSF secretion levels by $P < 0.001$ (**) in LG and HG with LPS-stimulated cells (i). Metformin had no effect on RANTES secretion levels at increasing concentrations in LG- and HG-treated cells (ii).

Half-maximal inhibitory concentration (IC_{50})

The IC_{50} values of metformin extract for the various biomarkers analysed are calculated and presented in Table 4.

Table 4. IC_{50} of the metformin extract for the secretion of various inflammatory factors by RAW 264.7 cells.

Markers		TNF α	IL6	G-CSF	RANTES	NO	Cell viability
Metformin IC_{50} µg/ml	LG/-LPS						277
	HG/-LPS						206
	LG/+LPS	262	192	198	576*	431*	335
	HG/+LPS	243	175	208	544*	473*	374

Discussion

Pharmaceutical agents such as metformin, which lower glucose levels and enhance glucose uptake, are insufficient for maintaining or preventing the development of DM. [8], [17] Therefore, it is necessary to handle early diabetes individually and to prevent its progression by using both pharmacological and traditional therapies.

It has been suggested that the use of proteome profiles is a convenient approach for evaluating mechanisms involved in the anti-inflammatory role in diabetes complications. [18], [19] In the present study, biomarker proteins were used – findings indicated that plants

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used as traditional medicines inhibit pro-inflammatory cytokines such as TNF-α, IL-6, G-CSF and RANTES associated with HG.

In this study, the IC_{50} effects of THT and standard Western medicine on RAW 264.7 cell viability were evaluated. *A, afra, C. verum, T. foenum-graecum* extracts and metformin reduced cell proliferation at different concentrations. The results indicated increased cell proliferation in LG and HG media containing LPS compared to cell proliferation in cells cultured in LG and HG media without LPS. The effects of *C. verum* and *T. foenum-graecum* on cell viability were observed at higher concentrations. The findings in the current study showed that *A. afra* and metformin were significantly more cytotoxic than *C. verum* and *T. foenum-graecum.*

The different herbs were found to have different IC_{50} values on the inflammatory marker expression. These results showed that the effects of *A. afra* extracts significantly inhibited TNF-α, IL-6, G-CSF and RANTES and NO. *C. verum* extract significantly inhibited TNF-α, IL-6 and RANTES, and *T. foenum-graecum* extract significantly inhibited TNF-α, IL-6, G-CSF, RANTES and NO at higher concentrations and metformin solution significantly inhibited TNF-α and G-CSF.

Results also indicated that *A. afra* had the highest anti-inflammatory activity compared to the other herbs in the study. The effects of *A. afra* may be a novel therapeutic target for the treatment of progression diabetes complications with minimal side effects.

The pro-inflammatory mediators related to the pathogenesis of T2DM have been identified in several studies. TNF-α and IL-6 play an essential role in activating other inflammatory cytokines, such as interferon-gamma (IFN-γ) and interleukin-1 beta (IL-1β), at the onset of diabetes-related obesity. [20], [21] TNF-α is the first pro-inflammatory cytokine associated with the pathogenesis of obesity-related impaired insulin signalling. [22] Increased TNF-α levels are also associated with renal complications in patients with T2DM. [23], [24]

Chronic wounds are one of the severe side effects of inflammatory and metabolic disorders like T2DM. [16] Several autoimmune diseases are associated with the dysregulation of the IL-6 signalling pathway, including T2DM. [25] Therefore, the regulation of IL-6 levels may be used in the treatment and maintenance of chronic wounds derived from diabetes. [26]

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The present study showed that *A. afra, C. verum* and *T. foenum-graecum* decreased TNF-α and IL-6 levels in a concentration-dependent manner in an HG medium containing LPS. Conversely, metformin showed a minimal effect on the TNF-α level and no effect on the IL-6 concentration. In a recent study, the benefits of *metformin* combined with atorvastatin were reported to inhibit inflammatory markers, such as TNF-α and IL-6, in diabetic rats. [27] Furthermore, polysaccharides extracted from *T. foenum-graecum* seeds have an effective wound healing potential through their antioxidant activities, a finding that may improve insulin secretion/sensitivity and wound healing. [28]

Chronic inflammation in T2DM upregulates macrophage production of inducible nitric oxide synthase (iNOS), causing tissue damage and impaired wound healing. [29] The iNOS has an essential role in the inflammatory response, as activated iNOS produces NO that mediates the cytotoxic effect involved in the pathogenesis of cell damage and insulin resistance (IR). [30] In earlier studies, it was demonstrated that the inhibition of NO production is essential in preventing the inflammatory response and improving endothelial dysfunction and chronic inflammation. [31], [32]

The results also showed that *A. afra* decreased the NO concentration, while *C. verum* and *T. foenum-graecum* had effects at higher concentrations on NO in LG and HG with and without LPS stimulation of cells. Metformin had no effect on NO production in LG and HG with or without LPS. This finding shows that the above plants may be used as therapies for preventing or managing impaired wound healing in patients with diabetes.

The hematopoietic cytokine G-CSF enhances the proliferation and differentiation of neutrophil progenitor cells. [33] Therefore, G-CSF is crucial for neutrophil survival and the induction of inflammation to attack pathogen invasion. [34] The G-CSF levels are increased in T2DM patients with free fatty acid induced inflammation, IR and atherosclerosis. [35] Therefore, regulating G-CSF levels may be an effective strategy for preventing diabetic cardiomyopathy and the progression of diastolic dysfunction. [36] Our findings in the present study showed that *A. afra* and *T. foenum-graecum* effectively inhibit G-CSF expression through LPS-stimulated macrophages cultured in an HG medium. Metformin inhibits

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macrophage expression of G-CSF at higher concentrations (LG with LPS), while *C. verum* did not affect G-CSF synthesis.

RANTES is a chemokine expressed by many cell types, such as endothelial cells, macrophages, smooth muscle cells and platelets, in chronic inflammation. It activates T-cell adhesion and transmigration through the endothelial wall. [37] RANTES has been associated with pro- and anti-inflammatory effects and potentially regulates angiogenesis mediators. It may contribute to chronic inflammation and the process of inflammatory angiogenesis. [38], [37] During angiogenesis, a new blood vessel is formed from an existing one which plays a critical role in inflammation and wound healing. [39] Therefore, inhibiting this inflammatory mediator may be used to target and stimulate endothelial cells for wound healing in DM. [40] In this study, *A. afra, C. verum* and *T. foenum-graecum* extracts effectively inhibited RANTES expression, while *metformin* did not affect RANTES levels in HG with LPS-stimulated cells.

In an earlier study that evaluated the effects of metformin on trophoblast cells (Sw.71), metformin was shown not to affect the HG modulation of G-CSF and RANTES secretion. [41] However, the efficacy of these plants in inhibiting G-CSF and RANTES may be potential angiogenic drugs. These plants may be potential therapeutic drugs to treat microvascular complications associated with DM by inhibiting angiogenic modulating effects.

Conclusion

There is a strong evidence that chronic inflammation is involved in the pathogenesis of the onset and progression of diabetes complications. This study focused on the benefits of plant-based therapies for inflammation and DM. The findings of this study indicated the effects of *A. afra, C. verum* and *T. foenum-graecum* on pro-inflammatory cytokines related to the progression of T2DM compared to metformin. The results suggest that the plants could be used as anti-inflammatory agents to prevent or delay the onset of DM and its complications. *A. afra* may be a novel therapeutic target for treating progressive diabetes complications with minimal side effects. The prevention and management of chronic inflammatory conditions linked to diabetes may indeed benefit from plant-based therapies. This study may

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offer a new strategy for using traditional inhibitor agents to synthesize pro-inflammatory mediators as potential novel treatments for diabetes and its complications. Further studies are required to look for the bioactive molecules of *A. afra*, *C. verum*, and *T. foenum-graecum* as diabetes-related pro-inflammatory response inhibitors.

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