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A methanolic extract of *Trigonella foenum-graecum* (fenugreek) seeds regulates markers of macrophage polarization

***Nurudeen Hassan, Cathryn Withycombe, Maninder Ahluwalia, Andrew Thomas, and Keith Morris**

Cardiff School of Health Sciences, Cardiff Metropolitan University, Western Avenue, Cardiff, CF5 2YB, United Kingdom

***Corresponding Author:** Nurudeen Hassan, Cardiff School of Health Sciences, Cardiff Metropolitan University, Cardiff, CF5 2YB, United Kingdom

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ABSTRACT

Background: Macrophages are key cellular mediators in diabetes-related inflammation. Molecular cues such as cytokines found in the tissue microenvironment regulates the polarization of macrophages into an M1 (pro-inflammatory) or M2 (immunoregulatory) phenotype. Recent evidence suggests that M1 macrophages in diabetic patients may contribute to the complications associated with the disease such as atherosclerosis. *Trigonella foenum- graecum* (*Tfg:* fenugreek) seeds have been used in traditional medicine in Asia, Africa and the Middle-East for their alleged anti-diabetic properties.

Objective: To identify the molecular mechanism(s) through which *Tfg* seeds exert their effects, we investigated the role of a crude methanolic extract of *Tfg* seeds (FME) on macrophage polarization *in vitro*.

Method: THP-1 macrophages (Mϕ) were treated with gBSA in the presence/absence of FME and the release and expression of M1 and M2 markers/cytokines were analysed. The role of FME on NF-κB activity was also explored using transfected HEK-293T cells.

Results: This study found that the *FME* significantly ($P<0.05$) decreased gBSA-induced secretion of M1 cytokines (TNF-α, IL-1β, IL-6 and IL-8) in THP-1 Mϕ cells. In the presence of gBSA, FME also significantly increased the gene expression of the M2 marker Dectin-1, but had no effect on IL-10, IL-1Ra. FME also significantly decreased TNF-α induced NF-kB reporter activity.

Conclusion: These results suggest that FME can regulate the expression of M1 and M2 markers in THP-1 Mϕ cells. This may be potentially through the modulation of NF-kB activity. Further work should be carried out to identify precise mechanism(s) involved in the effects of FME and *Tfg* seeds in diabetes and other conditions.

Keywords: chronic inflammation, macrophage polarization, diabetes, glycated BSA, THP-1 cells, *Trigonella foenum graecum*, fenugreek seeds, NF-κB,

INTRODUCTION

Chronic low-grade inflammation is involved in the pathogenesis of obesity-related type-2-diabetes and its complications[1]. Macrophages (Mϕ) are key cellular mediators in diabetes-related inflammation [2] and in response to different stimuli e.g. cytokines are polarized into classically activated (M1) or alternatively activated (M2) cells [3].

The inflammatory M1 sub-type is activated by IFN-γ and bacterial lipopolysaccharide (LPS) and is characterised by the production of proinflammatory cytokines e.g. IL-1, IL-6 and TNF-α. In contrast, the M2 macrophages are activated by IL-4 and IL-13 and have a major role in immune regulation and tissue repair [4]. M2 macrophages produce cytokines and cell-surface receptors such as IL-10, IL-1 receptor antagonist (IL-1Ra) and Dectin-1, and down-regulate inflammatory processes associated with the M1 phenotype [5]. It is now understood that an imbalance in population of M1 and M2 and a predominance of M1 macrophages is a contributing factor to dysregulation of glucose metabolism in adipose tissue and leads to insulin resistance [6]. In line with this, studies have found that adipose tissue macrophages adopt an M1-like phenotype and produce large amounts of pro-inflammatory cytokines [4].A recent study also found that M2 macrophage polarization was associated with improved insulin resistance in diabetic mice [7].

Trigonella foenum graecum (Tfg) seeds are used in traditional Indian, Chinese and Middle-Eastern medicine for managing conditions including diabetes [8], metabolic disorder [9] and cancer [10]. More recently, *Tfg* seeds have been shown to decrease the release of pro-inflammatory cytokines such as TNF-α and Interleukin-1β in tissues of diabetic mice [11].

Neelakantan *et al.*, conducted a meta-analysis on the effects of *Tfg* seeds on glycaemic control and found a strong association between *Tfg* intake and hypoglycaemic effect in diabetic patients [12]. In line with that, a saponin-rich water soluble extract of *Tfg;* Fenfuro™ was recently patented in the US as an antidiabetic agent [13]. Taken together, the literature suggests a role for *Tfg* seeds in modulating diabetes and related inflammation.

In this study, we aimed to investigate the role of a methanolic extract of *Tfg* (FME) in regulating markers of M1 and M2 macrophages using an *in vitro* THP-1 Mϕ cell model.

MATERIALS AND METHODS:

Cell culture: Human promyelocytic cells (THP-1) were cultured in RPMI 1640 (Gibco, UK) supplemented with 10% heat inactivated Foetal Calf Serum; FCS (Labtech International), 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin (100 IU/ml), streptomycin (100µg/ml) (Gibco, UK). For *in vitro* differentiation into macrophages, THP-1 monocytes (6 x 10^5cells/ml) were treated with 8nM phorbol-myristate-acetate; PMA (Sigma) for 48hours then maintained in fresh media without PMA for an additional 48hours before all experiments. In order to achieve a diabetes-related pro-inflammatory phenotype in THP-1 macrophages, cells were treated with 500µg/ml of glycated-BSA (Sigma, UK). HEK293T (ATCC, Middlesex, UK) epithelial cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM), 100U/ml penicillin/100µg/ml streptomycin mix and 4mM L-glutamine (Gibco, UK).

Cell viability assay: Cells were cultured in a 96-well micro-titre plate for 48 hours. Varying concentrations of the FME was incubated with the cells for 24 hours and cell viability was determined using Cell Titer-Blue® (Promega) according to manufacturer's guidelines.

Extract preparation: 40g of *Tfg* seeds were ground to fine powder and transferred into 100ml of 100% Methanol for incubation in a rotary mixer overnight at 25°C. The solvent fraction was recovered by centrifugation (6000g for 15mins) and the methanol evaporated *in vacuo* using a rotary evaporator (Bibby RE100, Staffordshire, UK). The residue was further subjected to N_2 to obtain a dry residue. The residue (extract) was dissolved in dimethyl sulfoxide (DMSO) and reconstituted in culture media, filtered through a 0.22µm PVDF filter and stored at -20°C. For experiments, the extract was thawed and reconstituted in fresh media at the respective concentrations before adding to cells.

Quantification of Cytokines using ELISA: TNF-α, IL-6 and IL-1β ELISA kits were obtained from R & D systems (Abingdon, Oxford) and the assay undertaken using protocols outlined by manufacturer.

Quantitative reverse transcription-PCR (qRT-PCR): Total RNA was extracted from cell pellets using TRIzol® reagent (Life technologies). RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK). Gene expression was analysed by RT-PCR method using Fast SYBR® Green in combination with an Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, Warrington UK). The comparative CT method ($2^{-\Delta\Delta CT}$) was used to calculate relative gene expression. The primer sequences used were; GAPDH: 5'-CATTGACCTCAACTACATG-3' and 5'-TCTCCATGGTGGTGAAGAC-3'; IL-10: 5'- ACGGCGCTGTCATCGATT- 3' and 5'-TGGAGCTTATTAAAGGCATTCTTC- 3'; IL-1Ra: 5'-GGCCTCCGCAGTCACC-TAATCAC- 3' and 5'-GGACAGGCACATCTTCCCTCCAT-3', Dectin-1; 5'-GGAAGCAACACATTGGAGAATGG -3' and 5'- CTTTGGTAGGAGTCACACTGTC -3'.

NF-kB reporter assay: HEK293T cells were grown in a 24-well plate to a confluence of 60-80%. Cells were transfected with 50ng of NF-κB plasmid DNA and 5ng of the *Renilla* luciferase reporter DNA (Pr6tk; Promega, Southampton, UK) diluted in Opti-MEM® I Reduced-Serum Medium (Invitrogen Ltd, Paisley, UK) using Lipofectamine® transfection reagent (Invitrogen Ltd, Paisley, UK) for 4hours. The media was replaced with fresh media for another 20hours before experiments.

Statistical analysis: all data are presented as the mean $\pm$ standard deviation. One-way analysis of Variance (ANOVA) was conducted for within group comparisons. The statistical package, *Graphpad Prism 5* was used for all statistical analyses. Statistical significance is represented as: * (P-value <0.05); ** (P<0.01); *** (P<0.001). Error bars represent standard deviation of at least three replicates.

RESULTS

The effect of FME on THP-1 Mϕ cell viability. To determine the cytotoxic effect of FME on THP-1 Mϕ, the cells were exposed to a range of concentrations (30-460µg/ml) for 24 hours and then the viability of the cells was assessed. At the concentration of 120µg/ml and above FME significantly reduced cell viability compared to untreated cells (Figure 1).

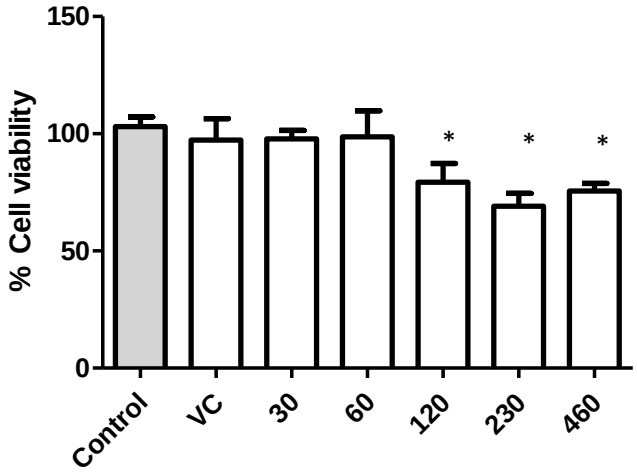

Figure 1. Effect of FME on cell viability. THP-1 Mϕ cells were treated with 30-460µg/ml of FME and Vehicle Control (VC: DMSO 0.1%) for 24 hours. The difference between control and treatment groups was determined by One-way ANOVA with Dunnett's *post hoc* test.

The effect of Fenugreek seed extract on gBSA induced TNF-α secretion in THP-1 Mϕ cells.

Using non-cytotoxic concentrations of FME, the effect of a range of concentrations 1-50µg/ml FME on gBSA-induced TNF-α release was studied in order to determine the optimal and range of bioactive concentrations. In the absence of gBSA, there was no significant increase in TNF-α secretion compared to the control and VC. All concentrations tested (1-50µg/ml) significantly reduced TNF-α secretion in the cells (Figure 2). The most potent concentration was 25µg/ml FME, reducing g-BSA induced TNF-α secretion (1008.429± 99.5pg/ml vs. 532.1± 94.2pg/ml). There was no significant difference in TNF-α levels in the treatment groups.

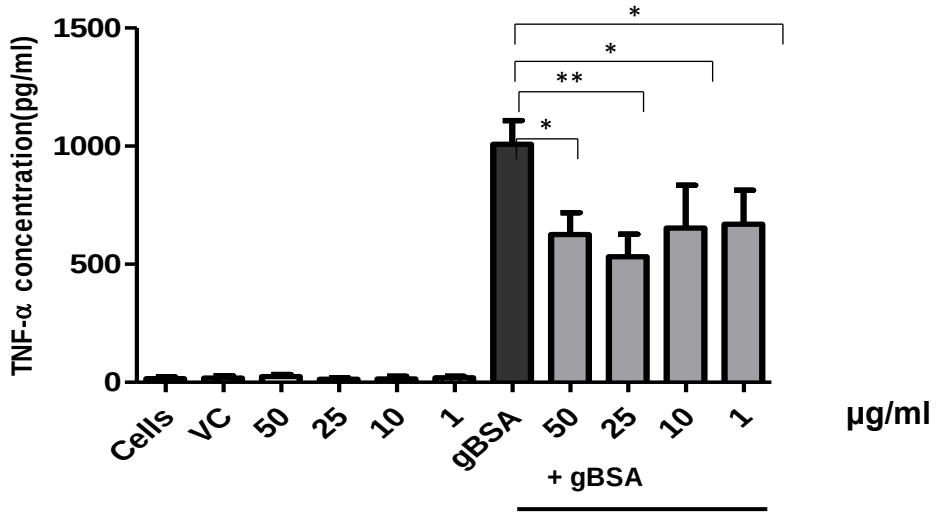

Figure 2. Effect of FME on gBSA-induced TNF-α release in THP-1 Mϕ cells. Cells were pre-treated with 1-50µg/ml FME for 1 hour and then the cells were treated with 500µg/ml gBSA for 6 hours. The level of TNF-α secretion was analysed in the cell-free media using ELISA. The difference between all treatment groups was determined by One-way ANOVA with Tukey's *post hoc* test.

FME reduces the secretion of M1 cytokines in THP-1 Mϕ cells.

It has been previously demonstrated that THP-1 macrophages express a number of M1(proinflammatory) cytokines such as TNF-α, IL-1β and IL-6 upon stimulation of by proinflammatory stimuli such as bacterial LPS and Interferon-γ (IFN-γ) [14]. Upon activation, M1 macrophages are characterised by the ability to secrete high levels of TNF-α, IL1-β and IL-6 cytokines [15]. Having demonstrated the effect of FME on TNF-α secretion in the presence of gBSA (figure 2), we further investigated the role of FME in regulating the secretory pattern of a panel of M1 cytokines within an inflammatory milieu at an early and later time point. At a concentration of 29µg/ml, we found that FME was able to significantly reduce gBSA induced levels of TNF-α (figure 3), IL-1β (figure 4) and IL-6 (figure 5) at 6 and 24hours.

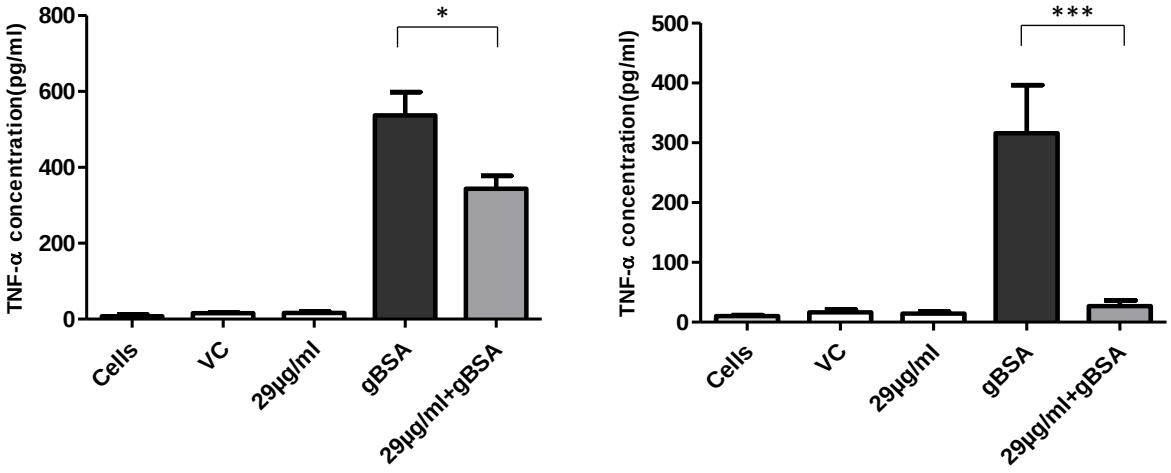

Figure 3. Effect of FME on gBSA-induced TNF-α release in THP-1 Mϕ cells. Cells were pre-treated with 29µg/ml FME for 1 hour and then the cells were treated with 500µg/ml gBSA for a) 6 hours and b) 24 hours. The level of TNF-α secretion was analysed in the cell-free media using ELISA. The difference between treatment groups was determined by One-way ANOVA with Tukey's *post hoc* test.

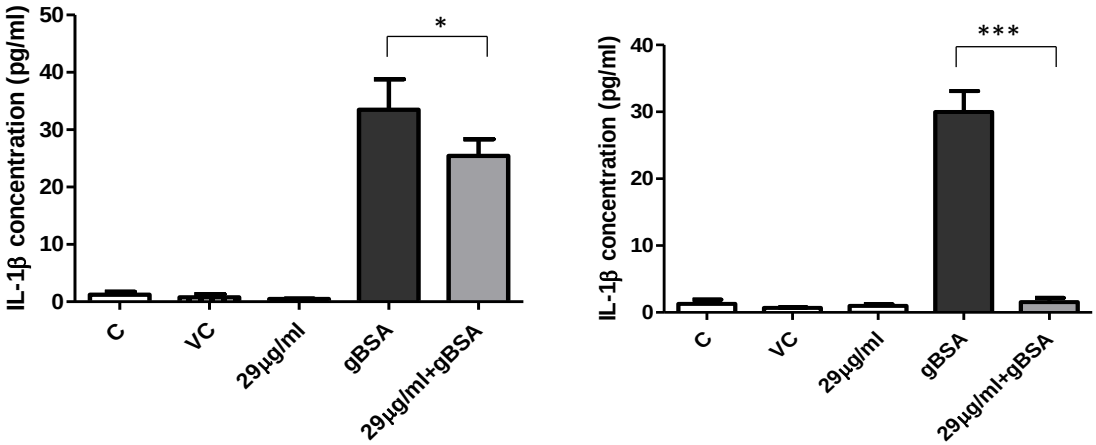

Figure 4. Effect of FME on gBSA-induced IL-1β release in THP-1 Mϕ cells. Cells were pre-treated with 29µg/ml FME for 1 hour and then the cells were treated with 500µg/ml gBSA for a) 6 hours and b) 24 hours. The level of IL-1β secretion was analysed in the cell-free media using ELISA. The difference between treatment groups was determined by One-way ANOVA with Tukey's multiple comparison *post hoc* test.

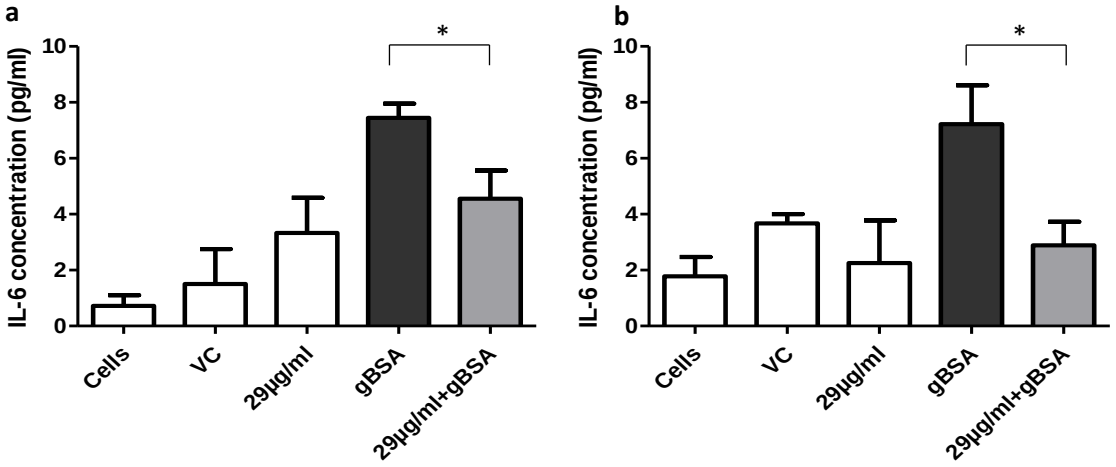

Figure 5. Effect of FME on gBSA-induced IL-6 release in THP-1 Mϕ cells. Cells were pre-treated with 29µg/ml FME for 1 hour and then the cells were treated with 500µg/ml gBSA for a) 6 hours and b) 24 hours. The level of IL-6 secretion was analysed in the cell-free media using ELISA. The difference between treatment groups was determined by One-way ANOVA with Tukey's multiple comparison *post hoc* test.

The effect of FME on M2 markers in THP-1 macrophages

After demonstrating that FME reduces the secretion of M1 cytokines (figure 3-5), we further investigated whether FME up-regulates the expression of M2 markers and can favour an M2 phenotype in the presence of pro-inflammatory gBSA. THP-1 Mϕ cells were pre-treated with FME and then stimulated with gBSA and the expression of M2 genes was analysed using qRT-PCR. There was a significant increase in the gene-expression of IL-10 and IL-1Ra (figure 6a & 6b) in cells treated with gBSA alone and a combination of FME and gBSA, however, there was no significant difference between gBSA and FME-gBSA. Dectin-1 demonstrated a different expression pattern; in the presence of FME-gBSA, there was a significant increase in Dectin-1 expression at mRNA level (figure 6c).

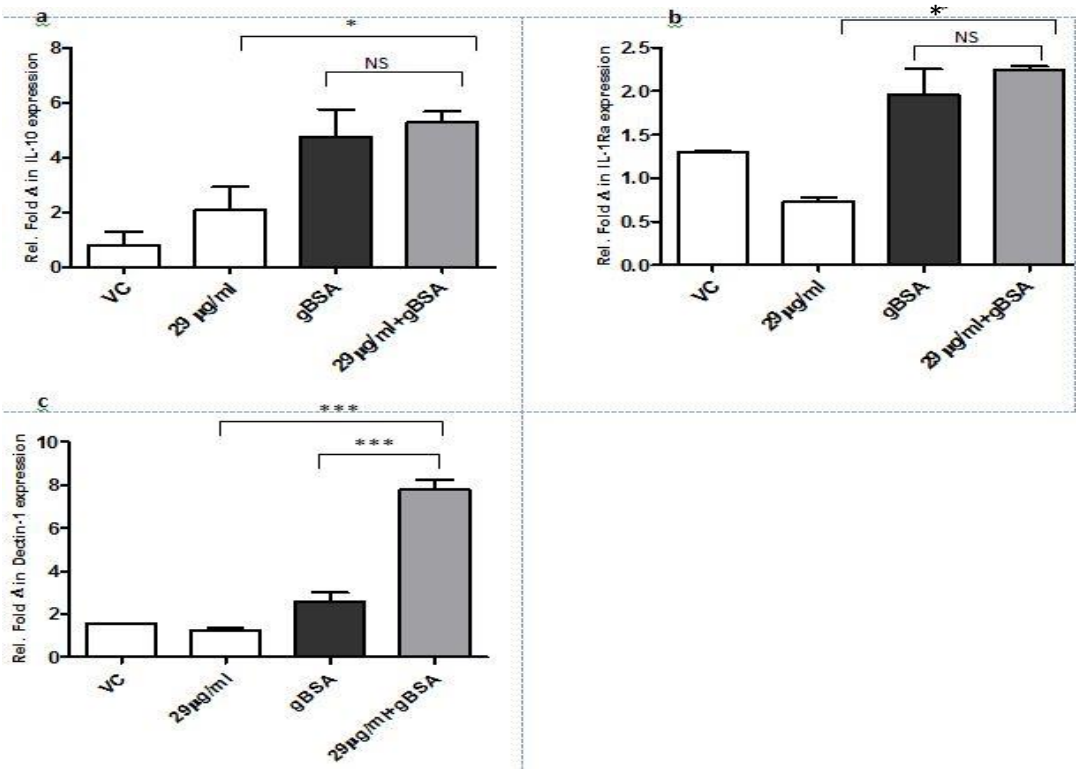

Figure 6. Effect of FME on M2 markers: IL-10, IL-1Ra and Dectin-1 expression in THP-1 Mϕ cells. Cells were pre-treated with 29μg/ml FME for 1 hour and then the cells were treated with500μg/ml gBSA for 24 hours. The RNA from the cell pellets was collected and the expression of M2 makers a) IL-10, b) IL-1Ra, and c) Dectin-1 was analysed using qRT-PCR. The difference between treatment groups was determined by One-way ANOVA with Tukey's multiple comparison *post hoc* test. NS: not significant.

FME significantly reduces TNF-alpha induced NF-kB reporter activity.

To determine the mechanism through which the observed effects FME may be mediated, NF-κB gene reporter assay was carried out. Due to the fact that FME reduced TNF-α secretion (fig 2-3) and TNF-α is a known target gene of NF-kB [16], the effect of FME on NF-kB activity was explored as a potential target for the anti-inflammatory activity of the extract. Many nutraceutical agents are known to exert anti-inflammatory effects through inhibition of NF-kB signalling [17]. In the presence of the TNF-α, there was a significant increase in NF-kB reporter activity which was significantly reduced by pre-treatment with FME (figure 7).

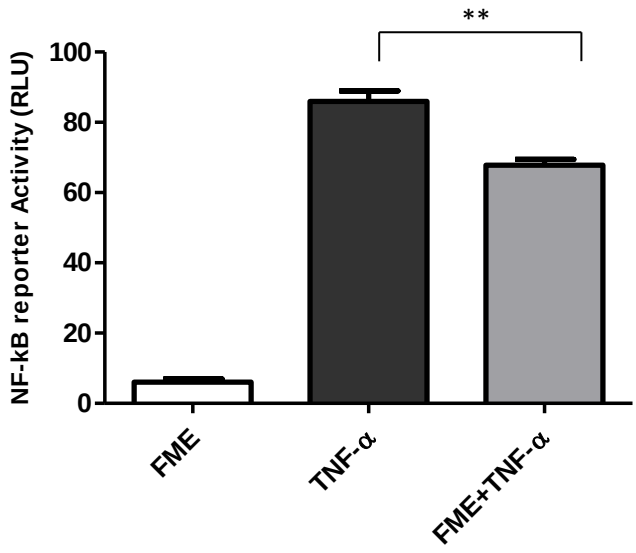

Figure 7. FME significantly attenuates TNF-alpha stimulated NF-kB activity**.** HEK-293T cells were co-transfected with NF-kB and Renilla control plasmids for 24hr. The cells were then pre-treated with FME for 1hour. The cells were then stimulated with 10ng/ml TNF-alpha. After 24hours post-stimulation, the cells were lysed and gene-reporter activity was analysed using the dual-glo luciferase assay.

DISCUSSION

The role of macrophage polarisation in obesity-linked metabolic disorders such Type-2-diabetes is currently an area that is being extensively explored. The state of chronic low-grade inflammation as found in obesity can lead to polarization of macrophages from an immune-regulatory M2 to a proinflammatory M1 phenotype [18].

In this study, the role of a methanolic extract of *Tfg* seeds on macrophage polarization was explored using the human THP-1 macrophage cells. Previous studies have demonstrated that THP-1 macrophages can be polarized into M1 and subtypes of M2 macrophages (M2a and M2c) *in vitro* [19] and hence was an appropriate model for a study whose primary aim was the regulation of polarization of these immune cells. For instance, in a recent paper,

Chanput and colleagues explored the polarizing ability of LPS and food compounds such as vitamin D3 and Lentinan in THP-1 macrophages [14]. Although their study found that the food components (Vitamin D3 and Lentinan) had no effect on macrophage polarization, in contrast another paper by Gao *et al.* reported an M2 phenotype switch in macrophages mediated through release of IL-4 and/or IL-13 following treatment with Curcumin, a widely used food product [20].

Tfg seeds have been associated with many beneficial effects including antidiabetic, antibacterial, hypocholesterolaemic, anti-inflammatory, anticarcinogenic and hepatoprotective (see review [21]). We hypothesised that the reported clinical effects of *Tfg* may be in part attributable to the role of its bioactive flavonoid compounds in attenuating diabetes-related inflammatory processes such as M1 macrophage polarization. In our study we demonstrated that FME significantly reduced gBSA induced secretion of TNF-α, IL-1β and IL-6 by THP-1 macrophages.

Chemical analysis of a hydromethanolic extract of *Tfg* seeds by Benayad *et al.*, found that the main compounds present were flavonoid glycosides; mainly, apigenin and luteolin glycosides [22]. In concordance with their study, we found that chemical analysis of FME exhibited a similar profile of chemical compounds (data unpublished).

Along with a reduction in M1 cytokines, FME also induced a significant upregulation in the gene expression of an M2 marker; Dectin-1, in the presence of gBSA (fig. 6). There was no difference in IL-10 and IL-1Ra expression compared to the gBSA treatment.

Although inconclusive, our results suggest that FME has the potential to induce a shift towards an M2 phenotype that is characterised by a high Dectin-1 and low IL-10 and IL-1Ra expression. Dectin-1 is a marker that is associated with the M2b subtype of macrophages [23]. Consequently, we propose that other M2b signature markers such as MR and SIGNR3 should be studied to confirm if FME can specifically induce M2b-like phenotype.

Our work also revealed that FME attenuates TNF-α induced NF-κB gene reporter activity. NF-κB plays a central role in the signalling of gBSA (an advanced glycation end product; AGE) [24] and M1 macrophage polarization [25]. Upon binding of AGEs to the receptor for AGE (RAGE), the transcription factor NF-kB is activated, initiating a cellular signalling cascade that results in the production of proinflammatory cytokines such as TNF-α, and IL-1 and IL-6 [26]. The ability of FME to reduce NF-κB activity in the presence of a proinflammatory cytokine (TNF-α) suggests that FME may exert Mϕ polarization through modulating the activity of cellular and transcription factors.

There are a number of other molecular and cellular mechanisms that play a major role in Mϕ polarization. This study only explored a select group of cytokines, cellular receptors and transcription factor involved in this process. Other major molecular mechanisms involved in M1-M2 switch include Nod-like receptors [27], cytokine signal inhibitors (suppressor of cytokine signalling; SOCS [28]) and transcription factors such as Peroxisome Proliferator-Activated Receptors (PPARs), Activated Protein-1 (AP-1), Glucocorticoid Receptors, IFN regulatory factors (IRFs), Signal Transducers and Activators of Transcription (STATs) and Krüppel-Like Factors (KLFs) (see review [25]). Due to the regulatory role of transcription factors in M1-M2 polarization in macrophages and disease, the effect of FME on these transcription factors should be explored.

Conclusion: The current work has demonstrated that the methanolic extract of *Tfg* seeds exert potent anti-inflammatory effects and can modulate the release and expression of M1

and M2 markers in a human *in vitro* macrophage model. These effects appear to be mediated through the regulation of the transcription factor NF-kB, however, further work needs to be carried out to confirm the exact mechanisms through which these effects are mediated and whether these effects would also be observed *in vivo*.

Abbreviations: *Tfg (Trigonella foenum-graecum);* FME (methanolic extract of *Tfg)*; TNF-α (tumour necrosis factor- α); IL-1β (Interleukin-1β); NF-κB (Nuclear factor-κB); IL-10 (Interleukin-10); IL-1Ra (Interleukin-1 Receptor Antagonist); qRT-PCR (quantitative-reverse transcription-polymerase chain reaction).

Competing Interests: The authors declare that they have no conflicts of interest.

Author's contributions: All authors contributed to this study.

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