BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2016
VOL. 73, NO. 3, 97–103
http://dx.doi.org/10.1080/09674845.2016.1188476

The immunomodulatory activity of secondary metabolites isolated from *Streptomyces calvus* on human peripheral blood mononuclear cells

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ABSTRACT

Background: The natural products derived from micro-organisms are potential candidates for the discovery of novel drugs. *Streptomyces* bacteria are prolific sources of secondary metabolites with a wide variety of biological activities. *Streptomyces calvus* (*S. calvus*) is one strain of this genus and may be an appropriate candidate for isolating new compounds. In this study, the immunomodulatory effects of *S. calvus* secondary metabolites on the expression of various cytokine genes by human peripheral blood mononuclear cells (PBMCs) were evaluated.

Methods: A bacterial sample was inoculated in Mueller Hinton Broth and secondary metabolites were extracted. PBMCs were isolated from venous blood and were treated with *S. calvus* secondary metabolites for 48 h. The cell proliferation was assessed by Methyl tetrazolium bromide (MTT) assay and quantitative real-time polymerase chain reaction (qRT-PCR) assays to survey mRNA expressions of selected pro-inflammatory and inhibitory cytokine genes.

Results: Secondary metabolites augmented interleukin-2 and interferon-γ gene expression in PBMCs at low doses and also reduced the levels of immunosuppressive cytokine interleukin-10. In addition, the proliferation of PBMCs substantially increased in response to metabolite treatment in a concentration-dependent manner ($p < 0.001$).

Conclusion: This *in vitro* study revealed that the secondary metabolites from *S. calvus* can successfully stimulate human PBMCs. Therefore, these metabolites have the potential to serve as robust immunomodulators.

ARTICLE HISTORY
Received 27 January 2016
Accepted 4 May 2016

KEYWORDS
Human peripheral blood mononuclear cells; immunomodulation; interleukin-10; interleukin-2; interferon-γ; Streptomyces calvus

Introduction

The crucial role of immune response modulation in the alleviation of many diseases such as tumours, infections and autoimmune disease has been of interest for many years.[1,2] Recognising and characterising natural substances with immunomodulatory potency and their possible application in modern medicine are gaining attention.[2,3] Immunomodulators could decrease or augment the host's capacity to withstand infection and tumour development in a non-specific manner or to respond specifically.[4,5] Based on previous studies, immunomodulators are derived from natural sources such as biological response modifiers or synthesised chemicals.[5–8] Several disorders, such as immunodeficiency, cancer, viral infection, autoimmune diseases and allograft rejection can be alleviated by immunomodulator drugs. Different studies have described immunomodulatory properties of microbial products such as bacterial toxins, polysaccharides and secondary metabolites.[6–8]

Actinobacteria have a noteworthy role in agriculture, food production and medicine.[9] The natural products derived from micro-organisms are an important source of existing and novel therapeutic agents. *Streptomyces* and related soil Actinomycetales can be considered potential sources of new secondary metabolites. These metabolites have a wide range of biological effects and may be ultimately used as antibiotics, anticancer agents or other pharmaceutically effective compounds.[10] The bioactive compounds that originate from these non-pathogenic bacteria, such as secondary metabolites, can be used for development of new drugs and have the potential to revolutionise the pharmaceutical industry by overcoming the issue of drug resistance.[11,12] Antibiotics such as erythromycin, streptomycin, tetracycline and a variety of essential extensively used drugs can be isolated from the *Streptomyces* species.[13] Secondary metabolites are organic compounds that are not directly associated with the normal growth, development or propagation of a micro-organism, unlike primary metabolites that are essential for the growth of micro-organisms. *Streptomyces* bacteria are ubiquitous in soil and they have an important ecological role in the turnover of

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organic materials.[14] One member of this genus, which is known for its fluorinated compounds, is *Streptomyces calvus*. The anti-trypanosome and antibacterial effects of *S. calvus* against Gram-negative and Gram-positive bacteria and some pathogenic micro-organisms such as *Streptococcus pyogenes* have been described.[15] Moreover, the secondary metabolites of *S. calvus* have showed anticancer activity.[16] Therefore, we selected a native *S. calvus* in an attempt to find a new effective compound.

Since there is no previous evidence related to the immune regulatory potency of *S. calvus*, we set out to determine the effect of *S. calvus* secondary metabolites on Peripheral blood mononuclear cells (PBMCs) proliferation and the expression of interleukin 10 (IL-10), interleukin 2 (IL-2) and interferon (IFN-γ) genes.

Materials and methods

Preparation of bacterial sample

Streptomyces calvus ABRIINW673 strain (isolated from a soil sample collected in Hamedan, Iran) was obtained from the bacterial culture collections of the Department of Microbial Biotechnology, AREEO, Tabriz, Iran.[14] It was grown on a Mueller Hinton agar medium. Micro-organisms were seeded on the medium and the Petri dish was incubated at 35 °C for 10 days. The bacterial sample was harvested and suspended in a liquid medium (15 ml of Mueller Hinton broth in a 50-ml Falcon tube) and incubated at 29 °C on a shaker incubator (125 rpm) for 36 h. The turbidity of the suspension was spectrophotometrically adjusted to match the transmittance of empty medium turbidity. As previously described, the turbidity 620 nm, 0.08 O.D. was considered suitable for inoculation.[13]

Fermentation and metabolite extraction

Five hundred ml of medium (in 1000-ml Erlenmeyer flask) was inoculated with 5 ml of homogenous bacterial suspension (620 nm, 0.08 O.D.) and incubated at 29 °C on a shaker incubator (125 rpm) for 10 days. The *S. calvus* culture broth was centrifuged at 4000 rpm for 20 min. The supernatant containing secondary metabolites was filtered by Whatman paper No. 1 and secondary metabolites were extracted using an equal volume of ethyl acetate (1:1) at 175 rpm shaker for 1 h. The aquatic phase was discarded and the solvent was concentrated by evaporator at 40 °C.

Separation and preparation of immune cells

PBMCs were separated from the whole blood of healthy male donors (age range 23–28 years) by Ficoll (Sigma, St Loius, MO, U.S.A) gradient centrifugation. The PBMCs were prepared under sterile conditions in RPMI-1640 medium containing 10% foetal calf serum (Gibco, BRL Grand Island, NY, U.S.A.). By applying the adhesive characteristic of monocytes to the polyester surfaces, three cell groups of PBMCs, lymphocytes and monocytes were separated. To perform the separation, PBMCs were first suspended in 10-ml RPMI-1640 medium supplemented with 10% FBS and incubated for one hour at 37 °C and 5% CO_2 in a 75-ml flask. Following three washes with PBS, the medium containing non-adherent lymphocytes was transferred to a fresh tube and attached monocytes detached from the bottom of the flask and collected by a cell scraper.

The assessment of isolated cells purity using flow cytometry

The purity of monocytes and lymphocytes was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD14 and CD3 monoclonal antibodies (eBiosciences, San Diego, CA), respectively. In brief, 10^6 cells (monocyte and lymphocytes) were transferred to two test tubes and then stained with specific FITC-mabs, based on the manufacturer's instruction. Following one-hour incubation, the percentage of $CD14^+$ and $CD3^+$ cells was quantified using FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). The population percentage of each cell group was recognised as more than 80% (data not presented).

Dye exclusion assay

The cell viability was determined by the trypan blue exclusion test. The cell suspension was pipetted into each well of a 96-well tissue culture plate (6×10^3 cells/well). The cells were treated with four different concentrations of bacterial metabolite (between 10 and 160 µg/ml) in the presence of the negative control (untreated cells) and incubated for 36 h at 37 °C and 5% CO_2 humidified atmosphere. Cell count was performed in advance and 36 h after treatment using the trypan blue assay. Briefly, 50 µl of 0.5% trypan blue dye weight/volume in phosphate buffered saline (Merck, Darmstadt, Germany) was added to each well containing trypsinised cells resuspended in 50-µl FBS-supplemented RPMI 1640 medium. After 5 min incubation, 20 µl of mixture was pipetted into a cell-counting chamber (Neubauer hemocytometer) and subsequently the number of live cells counted under a light microscope.

Proliferation assay

In order to determine the optimal dose of the secondary metabolites, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay was performed. Two hundred µl of cell suspension was pipetted into each well of a 96-well tissue culture plate (12×10^4 cells/well)

Table 1. Primers for real-time PCR analysis.

Gene name	Primers sequence	Annealing T^a
IFN-γ Forward	CTCTGCATCGTTTTGGGTTCT	58
IFN-γ Reverse	ATCCGCTACATCTGAATGACCT	
IL-2 Forward	AACTCCTGTCTTGCATTGCACT	57
IL-2 Forward	GATGTTTCAGTTCTGTGGCCTT	
IL-10 Forward	AGCTGAGAACCAAGACCCAGAC	58
IL-10 Reverse	AAGAAATCGATGACAGCGCC	
18S Forward	GATCAGATACCGTCGTAGTTCC	57
18S Reverse	CTGTCAATCCTGTCCGTGTC	

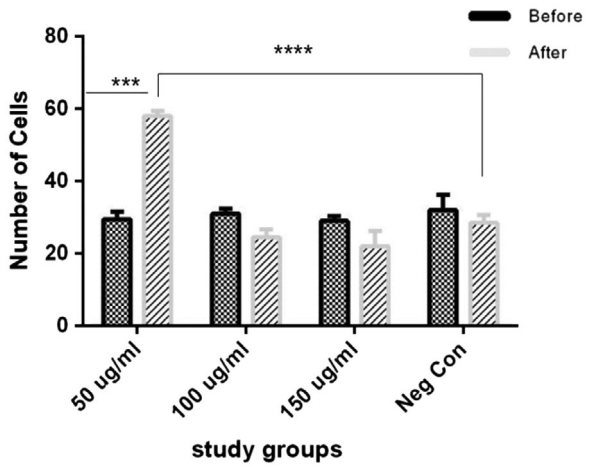

Figure 1. Number of live cells determined by dye exclusion assay pre- and post-exposure to the randomised concentrations (50, 100 and 150 µg/ml) of the secondary metabolites isolated from *S. calvus*. ****p<0001 and ***p<001 accepted as a significant difference between the test (50 µg/ml) and the negative control and two different times (before and after) of 50 µg/ml subjected group, respectively.

and incubated for 24 h at 37 °C in a 5% CO_2 incubator. After incubation, all cells were treated with six different concentrations of bacterial metabolite (0.5, 20, 50, 80, 110 and 140 µg/ml). For each cell group, one triplicate of wells was used as a positive control and treated with phytohaemagglutinin (PHA) 1.5% v/v (Gibco-BRL Grand Island, NY, U.S.A.). For the monocyte group, lipopolysaccharides (LPS)(Sigma, St. Louis, MO, U.S.A. (0.1 µg/ml)) was used as positive control and one triplicate of wells was used as a negative control (without metabolite and PHA/LPS). The plate was incubated for 48 h at 37 °C in a 5% CO_2 incubator. The proliferation of PBMCs, monocytes and lymphocytes was evaluated by the MTT assay. Following the centrifugation (400 g, 5 min) and removal of the supernatant by gently pipetting, 100 µl of fresh medium with 50 µl of 2 mg/ml MTT (Sigma, St. Louis, MO, U.S.A.) solution were added and incubated for 4 h. Then, the plate was centrifuged again and the supernatant was discarded. Finally, 200 µl of dimethyl sulfoxide and 25 µl of Sorenson's buffer (glycine 0.1 M, NaCl 0.1 M, pH:10.5 with 0.1 NaOH) was added and further incubated for 30 min. The absorbencies of specimens were spectrophotometrically quantified by ELISA reader at 570 nm.

Gene expression analysis by Real-Time PCR

The cell suspension was pipetted into each well of 6-well plates (1×10^6 cells/well). The cells were treated with non-toxic concentrations of secondary metabolites (10, 20, 40 and 50 µg/ml), in the presence of the negative and positive control (PHA). The plates were then incubated for 48 h. After incubation, RNA was extracted from the cells using an RNX-PLUS reagent (CinnaGene, Tehran, Iran) and the procedures of quantitative real-time polymerase chain reaction (qRT-PCR) analysis were carried out according to the manufacturer's instructions. Briefly, complementary DNA (cDNA) was synthesised using a reagent kit (Fermentas, Ontario, Canada) and the levels of IL-2, IFNγ and IL-10 mRNA as well as the reference gene 18srRNA were assayed by the gene-specific SYBR Green gene expression assays (Takara Bio, Shiga, Japan). Primer details are presented in Table 1 (17,18). All samples and controls were run in triplicate on a Corbett Rotor-Gene 6000 system (Corbett life science, Sydney, Australia). The qRT-PCR data were analysed by the comparative cycle number threshold method and the fold inductions of samples were compared with the untreated samples.

The amount of gene expression was calculated as the difference cycle threshold (ΔCT) between the CT value of the target gene and 18srRNA. ΔΔCT is the difference between the ΔCT values of the test sample and the control. Relative expression of target genes was calculated as $2-\Delta\Delta CT$.

Statistical Analysis

The data were analysed by t-tests and one-way ANOVA and expressed as the arithmetic mean and the standard deviation (SD). The GraphPad Prism 6 statistical software was employed. P values < 0.05 were considered to be statistically significant.

Results

Effect of S. calvus secondary metabolites on cell viability

As given in Figure 1, at 50 µg/ml concentration of the metabolites, PBMCs were efficiently stimulated compared to the negative control. At higher concentrations (100 and 150 µg/ml) the number of live cells was lower than the negative control.

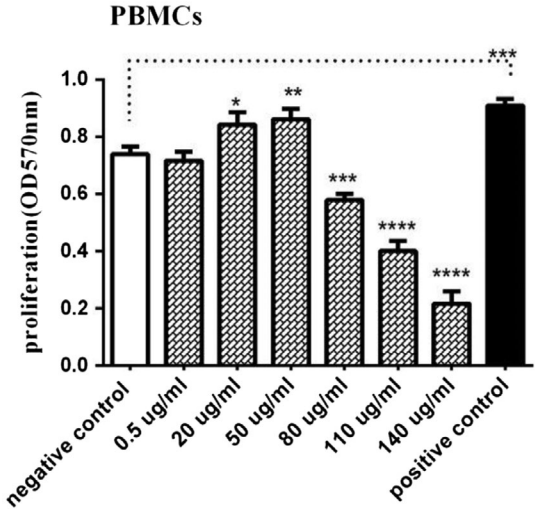

Figure 2. The stimulatory and cytotoxicity effects of *S. calvus* secondary metabolites on PBMCs. The highest stimulatory effect was observed at 50 µg/ml as indicated by an increase in proliferation of PBMCs while the toxic effect was observed at 80, 110 and 140 µg/ml concentrations where the growth of PBMCs significantly dropped (*p<0.05, **p<0.001, ****p<0.0001, compared to the negative control). Data were expressed as mean ± SD of three replicates of each treatment.

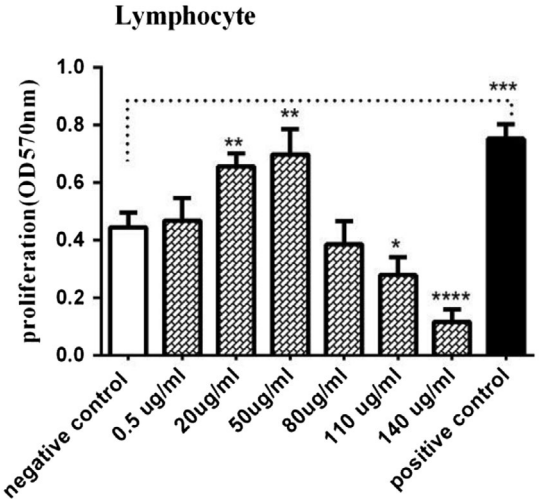

Figure 3. The stimulatory and cytotoxicity effects of *S. calvus* secondary metabolites on lymphocytes. 110 and 140 µg/ml doses of *S. calvus* exerted an inhibitory effect on the growth of lymphocytes (*$p < 0.05$, **$p < 0.001$, ****$p < 0.0001$, compared to the negative control group), whereas two doses of 20 and 50 µg/ml considerably stimulated lymphocytes (**$p < 0.001$ shows the significant level as opposed to the negative control).

Effect of S. calvus *secondary metabolites on cell proliferation*

The proliferation of PBMCs was further evaluated by MTT assay. The PBMCs were incubated with different concentrations of secondary metabolites from *S. calvus*. The results of the MTT test showed that the majority of cells survived post-treatment. In control cultures and cultures treated with 0.5, 20 and 50 µg/ml of the metabolites, cell viability was 95%. The concentrations of 80, 110 and 140 µg/ml (higher than 50 µg/ml) induced cell toxicity dose-dependently. Data for each group of cells have been represented in comparison to the negative control group (Figure 2). The lowest concentration (0.5 µg/ml) of bacterial metabolites did not significantly change growth or viability of PBMCs. However, once concentration increased up to 50 µg/ml, the stimulatory effect on proliferation reached to top levels and the highest stimulatory activity of the metabolites was observed at 50 µg/ml concentration, compared to the negative control. In this concentration, the metabolites exerted a similar efficiency to the positive control; in other words, the metabolites increased proliferation of PBMCs in a similar way to PHA. The proliferation that occurred in cells treated with 20 µg/ml was lower than 50 µg/ml of metabolite and the positive control group (treated with PHA). In contrast, the higher doses (more than 50 µg/ml) inhibited proliferation and had a toxic effect on PBMCs. The cytotoxic effect of *S. calvus* secondary metabolites is displayed in Figure 2 ($p < 0.05$).

Metabolites from *S. calvus* significantly increased the proliferation of immune cells in a dose-dependent manner. Also, the results showed no significant difference between lymphocytes and monocytes groups and the whole cells (PBMCs) group, and effects were similar. Only the toxic effect on monocytes was significantly higher than the effect on lymphocytes (Figures 3 and 4).

Effect of S. calvus *secondary metabolites on cytokine gene expression*

Based on MTT results, we exploited the non-cytotoxic concentrations of metabolites (≤50 µg/ml) to assess their effects on cytokine gene expression. According to the similarity of MTT results between cell groups (monocytes, lymphocytes and PBMCs), we continued the study with only the PBMCs group. Data are presented in Figures 5–7. The results showed that pro-inflammatory cytokines gene expression in PBMCs treated with *S. calvus* secondary metabolites was significantly increased. The level of IL-2 gene expression at 20, 40 and 50 µg/ml concentrations was increased as compared to the negative control and there was no significant difference with the positive control at 50 µg/ml (Figure 5). Three doses of *S. calvus* secondary metabolites (20, 40 and 50 µg/ml) rendered a significant increase in IFN-γ gene expression in PBMCs as compared to the negative control (Figure 6). These metabolites also significantly down-regulated IL-10 gene expression at 40 and 50 µg/ml. There were no significant differences in the level of IL-10 gene expression at 10 and 20 µg/ml as compared to the negative control (Figure 7).

Discussion

A number of studies have identified immunomodulatory compartments of microbial products such as bacterial

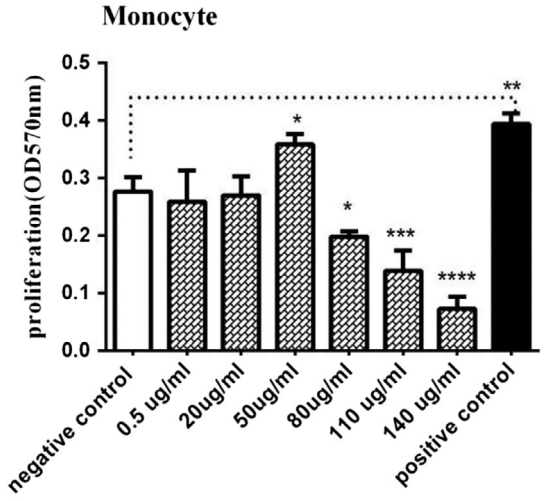

Figure 4. The stimulatory and cytotoxicity effects of *S. calvus* secondary metabolites on monocytes. A toxic effect was observed at 80, 110 and 140 µg/ml, where proliferation of the monocyte markedly reduced in response to the challenge (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0002$, ****$p < 0.0001$, compared to the negative control). In contrast, the number of monocytes decreased when exposed to the 50 µg/ml concentration (*$p < 0.05$).

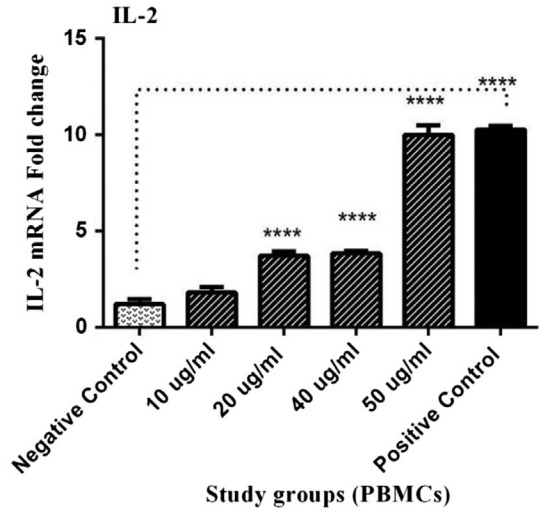

Figure 5. Effect of *S. calvus* secondary metabolites on IL-2 gene expression (**** indicates $p < 0.0001$ as compared to the negative control).

toxins, bacterial polysaccharides and secondary metabolites that have potential in treating certain pathological situations.[2,4,6,17–28] Several substances, including components of bacterial cell walls, have been reported to modulate innate immune responses.[1,7,19,20] Different studies have showed that administration of probiotic bacteria may provide beneficial effects at the intestinal epithelial level and cause release of bioactive compounds that may affect innate and adaptive immunity by activating production of cytokines from monocytes/macrophages.[29] Moreover, it has been reported that DNA of probiotic bacteria can modulate the immune responses by increasing the release of IL-10 and decreasing interleukin-1β levels.[21] Other examples of bacterial derivatives investigated as immunomodulators are bacterial polysaccharides and lipopolysaccharides, which may have modulatory effects on the immune system.[22,25] Recently, Perry et al. reported that streptozolin isolated from a specific actinomycete could substantially increase the phagocytic activity of macrophages and recruit pro-inflammatory cytokines, IL-8 and TNF-α through activating the NF-κB signalling pathway.[23] Elsewhere, the immunomodulatory potency of bacterial periodontal DNA on human and mouse immune cells was investigated. The levels of IL-1β, TNF-α and IL-6 increased following treatment of human monocytes to the DNA of *Porphyromonas gingivalis* and *Tannerella forsythia*.[24] Nonnenmacher et al. showed that DNA from *Actinobacillus actinomycetemcomitans* elicits a strong cytokine response in macrophage and fibroblast cell lines.[26] Furthermore, several studies have evaluated the potential of secondary metabolites isolated from soil samples for regulation of innate immunity in fish aquaculture.[4,19,20] In addition, secondary metabolites from marine *Pseudomonas sp.* exhibited anti-inflammatory effects through blocking P38 MAPK and JNK pathways in human activated neutrophils.[27]

In the current study, we tested the efficiency of *S. calvus* secondary metabolites on human PBMCs. We focused on finding the optimal stimulatory concentration of secondary metabolites involved in proliferation and pro-inflammatory cytokines gene expression of PBMCs. Proliferation of peripheral immune cells is an essential criterion that reflects humoral and cellular immune activity.[25] For determination of effective doses of the metabolites, a dye exclusion assay was employed, the results of which showed that approximately 50 µg/ml of the metabolites increase the number of live cells, compared to the negative control. An MTT test performed at six different concentrations suggested that at very low concentrations (<20 µg/ml) the metabolites had no effect on the survival, viability and proliferation of PBMCs, in comparison with negative control. Conversely, higher concentrations of metabolites (more than 50 µg/ml) were cytotoxic on PBMCs and this cytotoxic effect was more obvious in monocytes. Also, we found that concentrations up to 50 µg/ml were non-cytotoxic to immune cells and had stimulatory effects and promoted proliferation of both lymphocytes and monocytes. These results suggest that the metabolites contained specific compounds which can potentially trigger the production of pro-inflammatory cytokines.

Due to the prominent role of IL-2 in initiation of immune responses, the pivotal role of IFN-γ in tumour healing and the close association between IL-10 as an immunosuppressive cytokine and tumourogenesis,[2] we assessed gene expression of these cytokines. To measure cytokine gene levels in treated cells, non-toxic concentrations of metabolites were utilised. Results showed

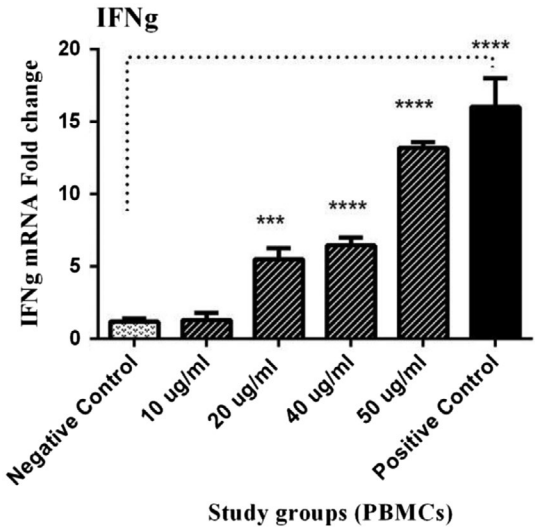

Figure 6. Effect of *S. calvus* secondary metabolites on IFN-γ gene expression (***$p < 0.004$ and ****$p < 0.001$ show significant difference between test groups and the negative control).

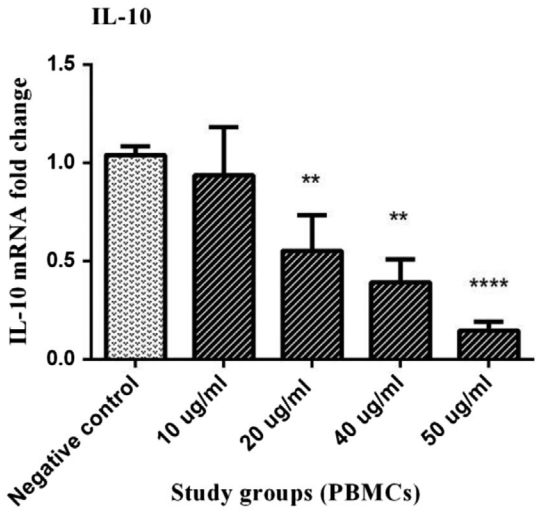

Figure 7. Effect of *S. calvus* secondary metabolites on IL-10 gene expression (**$p < 0.001$ and ****$p < 0.0001$considered as significant values in comparison with the negative control).

Table 2. Summary.

What is known about this subject:	• The bioactive compounds isolated from different micro-oganisms are used as an antibiotic and immunomodulator • The secondary metabolites extracted from *Streptomyces calvus* exert antiparasite, antibacterial and anticancer effects
What this paper adds:	• The secondary metabolites of *S. calvus* upregulates gene expression of IL-2 and IFNγ in PBMCs • The proliferation of circulating immune cells stimulated in response to treatment with secondary metabolites of *S. calvus* • Our findings confirm the potency of *S. calvus* secondary metabolites as an efficient immunostimulator

that the metabolites could successfully upregulate IL-2 and IFN-γ gene expression and also downregulate IL-10 gene expression. This compound induced a dose-related inhibition of IL-10 gene expression that can act the same as the immunostimulation process. IL-2 is essential for the proliferation, activation and action of T lymphocytes. [25] IFN- γ, which is secreted by stimulated Th1 cells, has a crucial role in modulation of various immune processes. The activated Th1 cells are not only involved in production of cytokines but also stimulate the production of different types of IgG, which are released by B lymphocytes in the immune system.[28] These results support the possible value of the metabolites for reversing pathophysiological conditions related to immunological background of diseases, particularly in immunodeficient and cancer patients or general immunosupression subsequent to chemotherapy (Table 2). The exact mechanism underlying this stimulatory effect is not clear; it may mediate through interactions between the active components of the metabolites and cell surface molecules or other compartments of the immune system.

We conclude that secondary metabolites extracted from *S. calvus* could enhance cellular immunity, thereby not only increasing the proliferation of immune cells but also heightening the expression of IL-2, IFN-γ. Our data represent an advancement in biomedical science that *S. calvus* secondary metabolites have the capacity to activate the circulating immune cells, and so indicate a potential perspective for treatment of immunocompromised patients by drug intervention, thus providing a promising basis for future biomedical studies.

Acknowledgements

The authors would like to appreciate Immunology Research Center (IRC), Tabriz University of Medical Sciences, for its kind financial and technical supports. We also acknowledge the Biotechnology Department of Agricultural Jihad Training Center (East Azerbaijan province-Iran) for helping in isolation and preparation of bacterial sample and finally thank Azadeh Montaseri for editorial assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

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