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Integrated experimental and immunoinformatic study of Th1/Th2 balance by *Ferula hezarlalehzarica*: *Gata3*/*T-bet* expression, IFN- γ/IL-10 cytokine modulation, and multiorgan safety assessment

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Abstract

Objective: Anti-inflammatory and anti-cancer activities of *Ferula hezarlalehzarica* (*F. hezarlalehzarica*) are confirmed in ancient and modern studies. We explored the immunomodulatory properties of this plant and its role in the Th1/Th2 balance response.

Materials and Methods: The impact of *F. hezarlalehzarica* aerial parts total extract at doses of 50, 250, and 500 mg/kg on interleukin (IL)-10 and interferon (IFN)-γ in rats was evaluated using ELISA, alongside C-IMMSIM simulations predicting immune responses to *F. hezarlalehzarica* proteins.

Results: Our findings indicated the plant extract significantly, and dose-dependently increased IL-10 and IFN-γ cytokines ($p<0.05$), with IFN-γ showing greater elevation. IFN-γ levels in groups treated with 50, 250, and 500 mg/kg of the extract were 60.47, 117.79, and 172.14 pg/ml, respectively, compared to 32.82 pg/ml in controls ($p<0.001$). IL-10 was increased significantly. Flow cytometry demonstrated Th1 polarization in *F. hezarlalehzarica*-treated samples, boosting *T-bet* over *Gata3*, and immune simulation analyses suggested Th1-biased response.

Conclusions: This study suggests increased Th1/Th2 cytokines with a stronger boost in IFN-γ indicating a Th1-biased immune response which is potentially favorable in treatment of cancers. *F. hezarlalehzarica* can be used as an immunomodulator various disorders. However, further studies are needed to optimize *F. hezarlalehzarica* dosage, helping reduce potential adverse effects.

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Introduction

Cancer is one of the most important causes of death which approximately accounts for the mortality of 1 in 9 men and 1 in 12 women (Organization 2024). Utilizing therapeutics that modify the immune response have gained attention in recent years (Aram et al. 2024). In this regard, T helper (Th) cells have been widely studied due to their indispensable function in anti-cancer immune feedback. Cytokines are very important in directing the course of infectious and non-infectious diseases (Saleki et al. 2022a; Saleki et al. 2022b). Th1 and Th2 cells are two major subpopulations of Th cells that are at equilibrium via the excretion of cytokines and inhibit their own specific immunological feedback. In conditions where the type of cytokine in the tumor microenvironment (TME) is changed, this equilibrium may be interrupted, leading to a shift from Th1 to Th2 immune response. Th1/Th2 imbalance is a major determinant in the formation of malignant lesions. Hence, attention to the balance of Th1/Th2 anti-tumor immune responses could lead to advancement in immune-mediated cancer treatments (Shang et al. 2024).

In addition to modern drugs, herbals have shown potential to shift the imbalanced Th1/Th2 and their associated cytokine profiles in favor of Th1, as a result of boosting cancer cells elimination within the TME (Shang et al. 2024). Indeed, Interleukin-10 (IL-10) (Th2 cytokine) is an inhibitor of Interferon-gamma (IFN-γ) (Th1 cytokine). This molecule suppresses the expression of Major Histocompatibility Complex Class II (MHC-II) and co-stimulatory factors on macrophages and dendritic cells (DCs). Therefore, IL-10 could be regarded as a suppressor of cytotoxic T-cell activity (Basu et al. 2021).

Ferula is a genus of medical plants from the Apiaceae family and comprises flowering as well as fragrant plants that grow in temperate zones of the Mediterranean and Central Asia (Pimenov 1993). From 32 species of *Ferula* that have been found, certain species are endemic to Iran. Among indigenous people, the *Ferula* genus is regarded as "*Koma"* (Amiri and Joharchi 2016). Multiple species of this genus have been implemented in traditional medicine practices to treat a wide array of diseases. For instance, *Ferula assafoetida* (*F. assafoetida*), *F. gummosa*, and *F. persica* are recognized for their antispasmodic, anticonvulsant, carminative, and anti-inflammatory potential (Abd El-Razek et al. 2003; Asemani et al. 2018). *Ferula hezarlalehzarica* has been found to exert anti-cancer effects in modern medicine as it was first speculated in ancient texts. In 2020, the anti-austerity effect of secondary metabolites derived from the roots of *F. hezarlalehzarica* against the human pancreatic cancer cell line 1 (PANC-1) was shown. In this study, the dichloromethane extract derived from the roots of *F. hezarlalehzarica* demonstrated strong cell killing activity with a preferential cytotoxicity (PC50) of 0.78 μg/ml. Plant chemical analysis of this herbal extract uncovered 18 compounds with two novel molecules, a sesquiterpenoid and a monoterpenoid. All isolated compounds were evaluated for their preferential cytotoxicity against PANC-1 human pancreatic cancer cells by employing an anti-austerity strategy. Among them, ferutinin was reported as the most active compound, with a PC50 of 0.72 μM. Moreover, the real-time influence of ferutinin and compound 6 against PANC-1 cells indicated cell shrinkage, resulting in malignant cells' expiry within a short period of contact. Compounds 2 and 6 also suppressed the gathering of PANC-1 cells in the format of colonies (Alilou et al. 2020). Another study explored cytotoxic effects of *F. hezarlalehzarica* against various tumor cell lines including HepG2, A549, HT29, MCF7 and MDBK with an Inhibitory Concentration 50 (IC50) of 76.7-105.3 μg/ml for the plant extract (Hajimehdipoor et al. 2012). The toxicity of pharmaceutical and herbal plants needs to

be investigated (Saleki et al. 2023b; Vaziri et al. 2023). In addition to *F. hezarlalehzarica*, the effect of other members of *Ferula* genus on apoptosis, cytotoxicity, and caspase activation has been reported in recent literature (Afshari et al. 2022; Alharbi 2021; Bahavar and Tafrihi 2024; Bahetjan et al. 2023; Sabzehzari et al. 2020).

While cytotoxic and apoptosis-inducing characteristics of *Ferula* plants have been widely investigated, the immunomodulatory effect of *F. hezarlalehzarica* has not been properly studied. This study reports the immunomodulatory effect of *F. hezarlalehzarica* in rats. Immunoinformatics has accelerated drug and immunotherapy research in recent years by rapid screening, analysis, and design of compounds (Aram et al. 2024; Saleki et al. 2023a; Saleki et al. 2024; Saleki et al. 2022b). In line with this, this immune simulation and epitope prediction data on the role of proteins from this plant in inducing Th1/Th2 cytokines was provided in this research.

Materials and Methods

Animals housing and maintenance conditions

Rats were kept in standard cages with dimensions of 40 x 25 cm at 23±2ºC. Animals were kept and cared for in the animal house of Islamic Azad University-Babol Branch. 12 hr dark / 12 hr light cycle was maintained during the study. Animals were controlled daily for aggressive behavior, and skin or appearance changes were closely monitored. This work was approved by the Azad Islamic University, Babol Branch ethics committee (No. 1400.048).

Experimental groups and treatments

In this study, 20 male Wistar rats were randomly divided into four groups (n = 5 per group); Herbal extract of *F. hezarlalehzarica* was administered with a dose of a, 50 mg/kg; b, 250 mg/kg and c, a dose of 500 mg/kg daily, intraperitonealy (i.p.) for a duration of 4 weeks. The control group was administered with i.p. sterile normal saline.

Plant material and preparation of the methanol extract

In this experiment, the aerial portions of *F. hezarlalezarica* were gathered from Mount Hezar (Kerman province). Next, a sample was confirmed by Mr. Ajani, Institute of Medicinal Plants (IMP), Karaj, Iran. A representative sample was stored in the herbarium section of the institute (number. 2922). After drying the aerial sections of the plant, the parts were prepared in powder form (100 g), and macerated by methanol (90% conc.).

IL-10 and IFN-γ quantification by ELISA

Twenty-four hours following the last injection. Samples were taken from four groups. The rats were subjected to general anesthesia and about 3 to 4 ml of blood was collected from their axilla. Briefly, serum samples were centrifuged at x 2000 Revolutions Per Minute (RPM) for 15 min. Then, IL-10 and IFN-γ cytokines were quantified by the SEA056Ra (96 tests) and SEA049Ra (96 tests) kits, respectively. After that, samples were added to the Enzyme-Linked Immunosorbent Assay (ELISA) kit wells and processed with detection reagents A/B and the Tetramethylbenzidine (TMB) substrate according to manufacturer's instructions. Serum IFN-γ and IL-10 levels were quantified using commercial rat ELISA kits (Cloud-Clone Corp., Wuhan, China, and TX, USA). Briefly, standards and samples (100 µl) were added to antibody-precoated wells and kept at 37°C for 60 min. Following incubations with biotin-conjugated detection antibody as well as HRP-avidin, plates were washed and incubated with TMB substrate solution for a duration of 10-20 min at 37°C. The reaction was stopped with sulfuric acid, and

absorbance was measured at 450 nm by a microplate reader. Concentrations were quantified from standard curves. Results were read at the wavelength of 450 nm.

Histological assessment

After 28 days, rats were euthanized by anesthetization followed by dislocation to minimize animal suffering, and kidney, spleen, thymus, and liver specimens were collected. Also, Hematoxylin and Eosin (H&E) staining and evaluation of histological damage were performed.

Epitope prediction and Th immune simulation for other *Ferula* plants key immunomodulatory proteins

C-language IMMune system SIMulator (C-IMMSIM) server (150.146.2.1/C-IMMSIM/index.php) was used to simulate major proteins from *Ferula* genus with potential anti-tumor or anti-oxidant activity. The C-IMMSIM tool integrates genomic data and simulation of the dynamics of the immunological components in a unified utility which can reveal new avenues for a clearer comprehension of immunity. Results of this server predict immune cell subsets, cytokine production, memory response, and immunoglobulin production at any given timeframe after exposure to the aminoacidic sequence. To further explore epitopes, C-IMMSIM as well as Immune Epitopes Database (IEDB) MHC-I/II prediction tool was used to detect B-cell and T-cell epitopes.

Flow cytometry analysis of Th1/Th2 cells in treated and control groups

Splenocytes were separated and stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin with brefeldin A for about 4-6 hr; unstimulated control specimens were processed in parallel (Caraher et al. 2000). After stimulation, cells were stained with a fixable viability dye, surface-stained for CD3 and CD4, then fixed/permeabilized and intracellularly stained for IFN-γ and IL-4 (BioLegend, SanDiego, CA, USA). Appropriate controls (unstimulated, Fluorescence Minus One (FMO) for each marker, and single-stain compensation controls) were included and utilized to set gates conservatively. Specimens were acquired on a calibrated flow cytometer and analyzed in FlowJo software. Cytokine positivity was defined as appropriate, and results for CD4+ IFN-γ+ and CD4+ IL-4+ double-positive cells were analyzed separately.

Gene expression analysis of *Gata3* and *T-bet* by qRT-PCR

peripheral blood mononuclear cells (PBMCs) were separated using the gradient method from whole blood via lymphocyte separation media (Ficoll) and then, washed with phosphate-buffered saline (PBS). After the extraction of total RNA from PBMCs using the column method via RNA isolation kit (Yektatajhiz Azma, Iran), reverse transcription was accomplished by the synthesis of cDNA (Yektatajhiz Azma, Iran). The prepared cDNA was then used as a template for a qRT-PCR assay. Forward and reverse primers were designed for *T-bet* and *Gata3*, as well as the *β-actin* housekeeping gene (Table 1). The mix was prepared using cDNA template, master mix (SYBR Ampliqon, Denmark), and primers for each gene. A 3-step thermocycling program was utilized for 40 cycles, and cycle threshold (CT) values were evaluated to quantify normalized gene expression for *Gata3* and *T-bet* relative to the housekeeping gene.

Statistical analysis

In this study, data is reported as Mean ± SEM and analyzed using one-way ANOVA (Analysis of Variance) test and LSD (Least Significant Difference) Post Hoc Test. Statistical analysis and data interpretation were conducted using SPSS-18 and Prism v10.3.1 (GraphPad, USA) software. Additionally, a significance level of less than 0.05 was considered for the tests.

Table 1. Primer sequence for qRT-PCR of *Gata3* and *T-bet* genes (Chen et al. 2016)

Gene	Primer sequence	Size
T-bet^F	TCCTGTCTCCAGCCGTTTCT	20
T-bet^R	CGCTCACTGCTCGGAACTCT	20
Gata3^F	CCTACCGGGTTCGGATGTAA	20
Gata3^R	CACACACTCCCTGCCTTCTGT	21
β-actin^F	ATGCCATCCTGCGTCTGGACCTGGC	25
β-actin^R	AGCATTTGCGGTGCACGATGGACGG	25

Results

The effects of *F. hezarlalehzarica* extract on the total body and lymphatic organs weight

The effects of the plant's extract were evaluated after 28 days of treatment. Before-after analysis showed a directly dose-dependent increase in thymus, spleen, and total body weight ($p<0.05$). In the control group, rats gained 16.6 g and the weight of spleen and thymus were 0.913 g and 0.216 g, respectively. Groups treated with 500, 250, and 50 mg/Kg of the plant's extract showed a splenic weight of 1.126, 0.986, and 0.943 g, a thymic weight of 0.306, 0.286, 0.263 g, and a body weight increase of 26.4, 25.8, and 25.6 g, respectively. Lymphatic organs and body weights are provided in Table 2. Complete Blood Count (CBC) analysis of experimental groups by one-way ANOVA and LSD post-hoc showed no significant difference between the studied groups ($p>0.05$) (Table 3).

Immunomodulatory effect of *F. hezarlalehzarica* on Th1/Th2 cytokines in rats suggests a shift towards Th1

In this study, IL-10 and IFN-γ were evaluated following 28 days of intraperitoneal (i.p) injections. To explore *F. hezarlalehzarica* effects on Th1/Th2, IFN-γ and IL-10 levels were quantified by ELISA. In groups treated with 50, 250, and 500 mg/Kg of the herbal extract, IFN-γ levels were 60.47, 117.79, and 172.14 pg/ml which showed a meaningful increase compared to the control group (33.95 pg/ml) ($p<0.05$) (Figure 1). Furthermore, IL-10 levels were quantified and our results in groups treated with 50, 250, and 500 mg/Kg of the extract showed levels of 44.354, 51.056, and 110.232 pg/ml, respectively which were higher compared to the control group (33.956) ($p<0.05$) (Figure 2).

Table 2. Lymphatic organs and total body weight in experimental groups

	Control group	50 mg/Kg treated group	250 mg/Kg treated group	500 mg/Kg treated group
Initial body weight	275.6 g	267.2 g	270 g	270.4 g
Body weight at study endpoint	292.2 g	292.8 g	295.8 g	296.8 g
Spleen weight	0.913 g	0.943 g	0.986 g	1.126 g
Thymus weight	0.216 g	0.263 g	0.283 g	0.306 g

Table 3. CBC analysis of experimental groups

CBC parameter	Control group	50 mg/Kg treated group	250 mg/Kg treated group	500 mg/Kg treated group	One-way ANOVA p-value
WBC cells/µL	14533 (5130)	16767 (2436)	17800 (4349)	14100 (2875)	0.88
RBC $\times 10^6$/µL	6.547 (0.63)	7.147 (0.15)	7.647 (0.05)	7.227 (0.48)	0.31
HGB g/dL	12.50 (0.87)	13.73 (0.66)	15.13 (0.23)	13.97 (0.95)	0.17
HCT %	33.63 (2.71)	36.73 (1.58)	41.00 (0.55)	37.53 (2.41)	0.15
MCV fL	51.57 (1.51)	51.37 (1.41)	53.60 (0.58)	51.93 (0.24)	0.49
MCH pg	19.17 (0.53)	19.17 (0.62)	19.77 (0.17)	19.33 (0.24)	0.74
MCHC g/dL	37.23 (0.65)	37.37 (0.17)	36.93 (0.64)	37.20 (0.32)	0.93
Platelet cells/µL	554000 (113000)	827333 (35177)	942667 (234490)	623000 (263107)	0.54

Note: Data are presented as mean (SEM), (n = 2-3 per group)

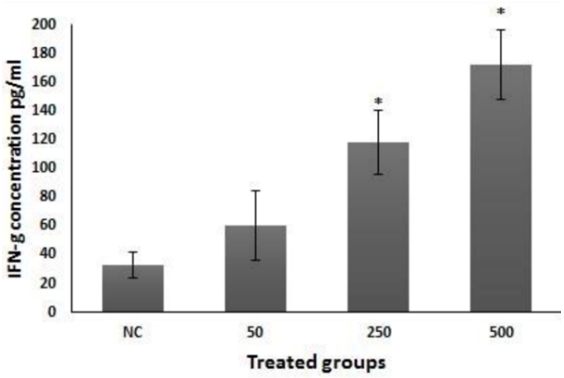


Figure 1. IFN-γ cytokine levels in the studied groups after 4 weeks; NC: Control group, 50: 50 mg/Kg treated group, 250: 250 mg/Kg treated group, 500: 500 mg/Kg treated group. * p<0.05

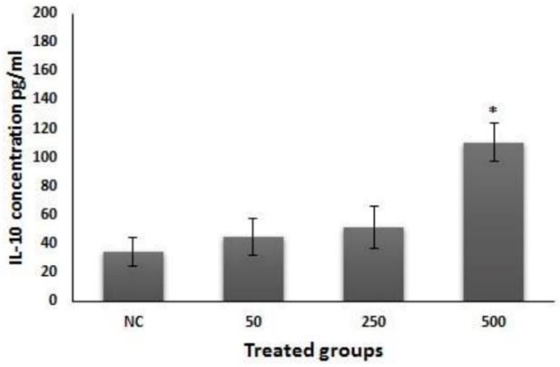


Figure 2. IL-10 cytokine levels in the studied groups after 4 weeks; NC: Control group, 50: 50 mg/Kg treated group, 250: 250 mg/Kg treated group, 500: 500 mg/Kg treated group. * p<0.05

Hepatotoxicity and nephrotoxicity of *F. hezarlalehzarica* based on histopathological analysis

1. Histopathological analysis of the liver, subsection of liver tissue from the control animals revealed that the normal histological structure of hepatic lobule was preserved; 2. Histological analysis of the liver for the 500 mg/Kg treatment group rats revealed hyperemia located at the central vein; 3. Histological examination of liver of the treated rats demonstrated hyperemia at the centrilobular and portal zones; 4. Histological examination of liver of the treated rats revealed mild sinusoidal leukocytosis, portal triad leukocytosis, hyperemia, congestion of blood sinusoids, and the mild hydropic degeneration of the hepatocytes at the centrilobular region. 1. Histological examination of the kidney from the control group revealed the normal histological structure of renal parenchyma. Nervertheless, kidney specimen of the treated rats revealed congestion of renal blood vessels. Furthermore, analysis of the kidney taken from the treatment group rats showed congestion of kidney vessels as well as tubular cellular swelling. Finally, the treatment group rats sample had marked tubular cell swelling, tubular crystals deposits, along with dilatation or congestion of renal blood vessels. Interestingly, no pathological feature was found in the spleen and thymus of the studied groups. Sections of mentioned organs with various zoom levels to better show the histological structure are included in Figure 3.

Immune simulation of *Ferula* proteins and epitope prediction

Germin-like protein from *Ferula assa-foetida* (Accession: QNQ07968.1), Peroxidase-like protein from *Ferula elaeochytris* (Accession: QIV38291.1), Beta-glucosidase-like protein from *F. assa-foetida* (Accession: XP_038842364.1), and Chitinase-like protein from *Ferula jaeschkeana* (Accession: QIB81336.1) were analyzed to detect epitopes by Immune Epitope Database (IEDB) server and simulate the immune response by C-IMMSIM server. Results indicated a high fraction of Th1-biased cells as a result of Beta-glucosidase-like protein from *F. assa-foetida* exposure without LPS (Figure 4 A-D).

Flow cytometry analysis of Th1/Th2 balance indicates a Th1-biased shift in the high-dosage *Ferula*-treated group

Flow cytometry analysis of IFN-γ+ CD4+ (Th1) and IL-4+ CD4+ (Th2) cells showed an increase in the high-dose *Ferula*-treated group compared to control rats . Additionally, the Th1/Th2 ratio was increased in the 500 mg/Kg treatment group compared to controls (Figure 5).

Gene expression analysis of *Gata3* and *T-bet* indicates increased expression in treated groups

T-bet and *Gata3* were both increased, with *T-bet* showing a more prominent increase ($p<0.05$). *T-bet* expression in PBMCs was higher in the 250 mg/Kg-treated ($p = 0.0160$) and 500 mg/Kg-treated rats ($p<0.0001$). Moreover, *Gata3* expression was higher in the 500 mg/Kg-treated group compared to control rats ($p=0.0166$) (Figures 6 and 7).

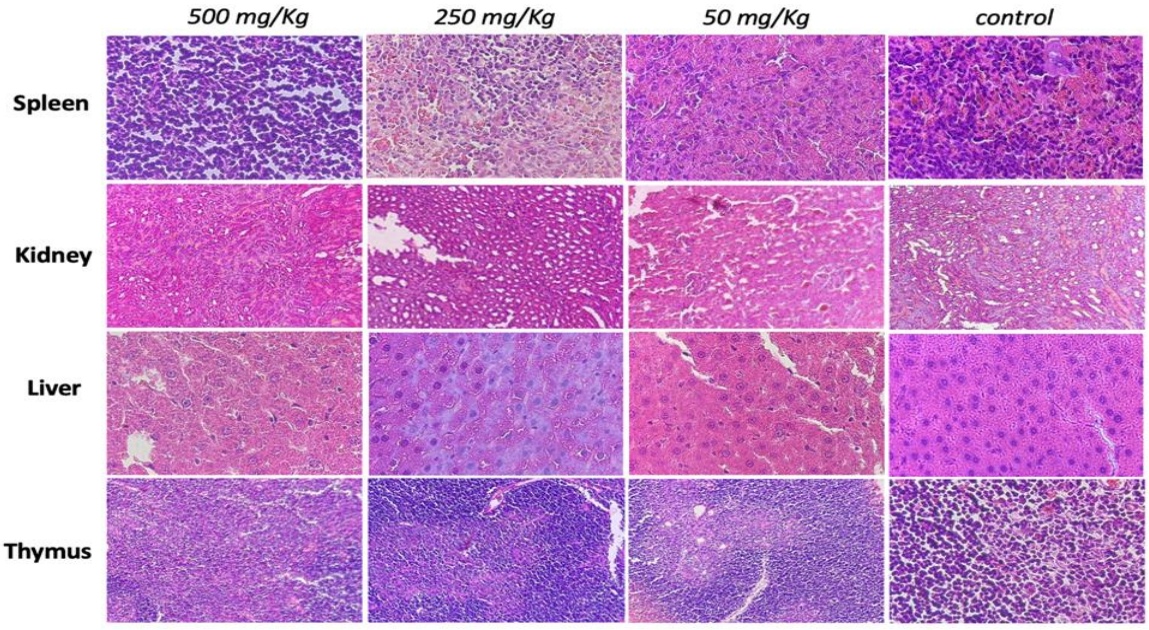
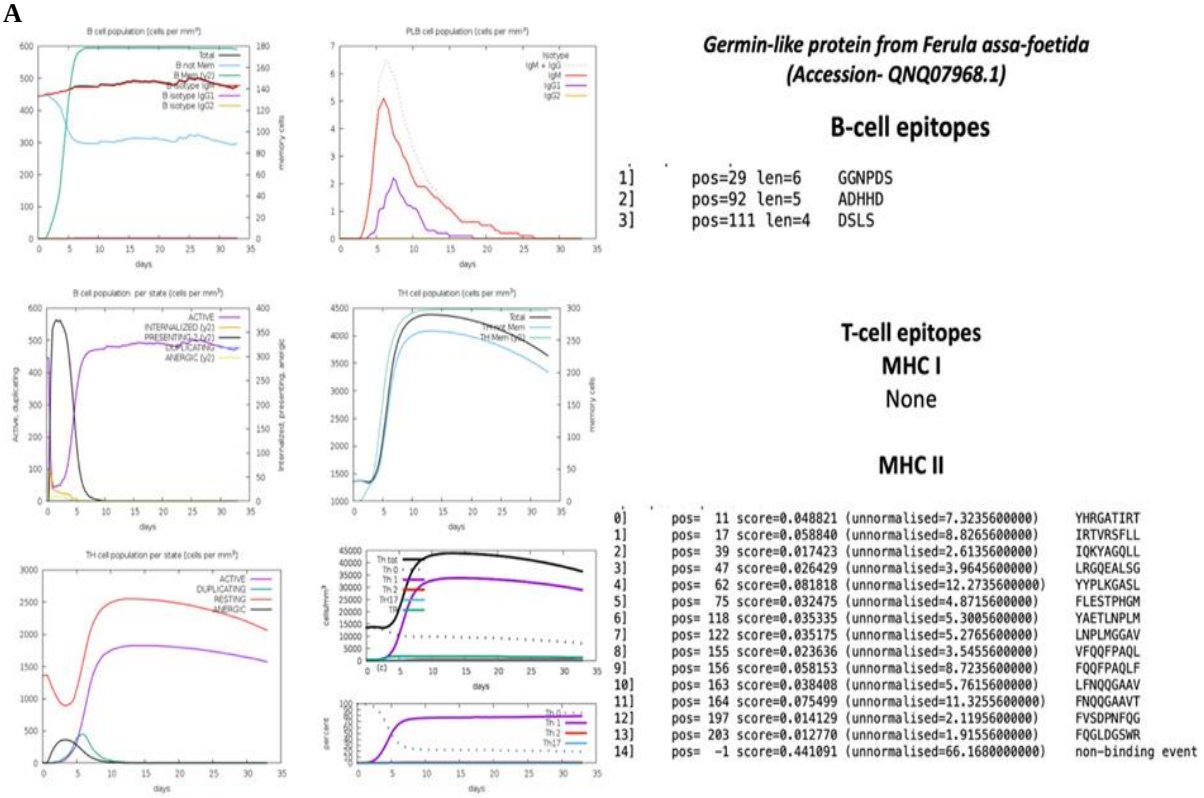


Figure 3. Histological examination of the liver, kidneys, spleen, pancreas, and thymus. Note: Panels indicate selected areas of the slides and may not reflect the complete histopathological assessment evaluated by a pathologist and suggested in the results. Magnification varies between panels.

A



Germin-like protein from Ferula assa-foetida (Accession- QNQ07968.1)

B-cell epitopes

1]	pos=29 len=6	GGNPDS
2]	pos=92 len=5	ADHHD
3]	pos=111 len=4	DSLS

T-cell epitopes

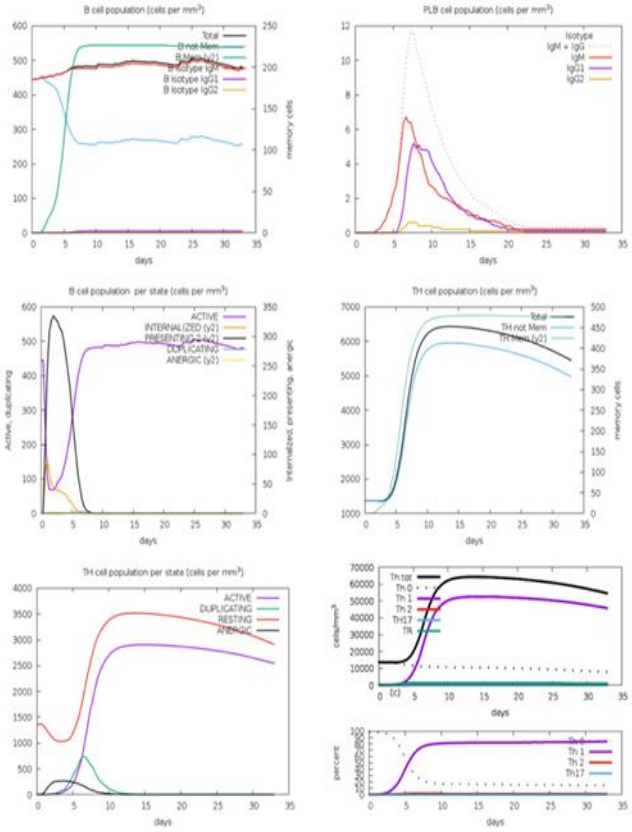
MHC I

None

MHC II

0]	pos= 11	score=0.048821 (unnormalised=7.3235600000)	YHRGATIRT
1]	pos= 17	score=0.058840 (unnormalised=8.8265600000)	IRTVRSFLL
2]	pos= 39	score=0.017423 (unnormalised=2.6135600000)	IQKYAGQLL
3]	pos= 47	score=0.026429 (unnormalised=3.9645600000)	LRGQEALSG
4]	pos= 62	score=0.081818 (unnormalised=12.2735600000)	YYPLKGASL
5]	pos= 75	score=0.032475 (unnormalised=4.8715600000)	FLESTPHGM
6]	pos= 118	score=0.035335 (unnormalised=5.3005600000)	YAETLNPLM
7]	pos= 122	score=0.035175 (unnormalised=5.2765600000)	LNPLMGGAV
8]	pos= 155	score=0.023636 (unnormalised=3.5455600000)	VFQQFPAQL
9]	pos= 156	score=0.058153 (unnormalised=8.7235600000)	FQQFPAQLF
10]	pos= 163	score=0.038408 (unnormalised=5.7615600000)	LFNQQGAAV
11]	pos= 164	score=0.075499 (unnormalised=11.3255600000)	FNQQGAAVT
12]	pos= 197	score=0.014129 (unnormalised=2.1195600000)	FVSDPNFQG
13]	pos= 203	score=0.012770 (unnormalised=1.9155600000)	FQGLDGSWR
14]	pos= -1	score=0.441091 (unnormalised=66.1680000000)	non-binding event

B



Beta-glucosidase-like protein from Ferula assa-foetida (Accession- XP_038842364.1)

B-cell epitopes

1] pos=95 len=5 PNTDT

T-cell epitopes

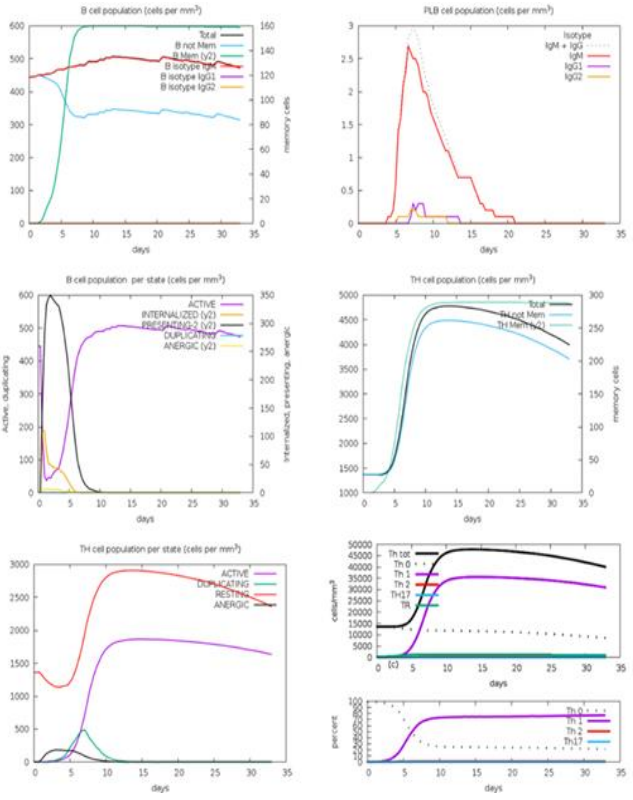
MHC I

0] pos= 72 score=0.005579 (unnormalised=0.6176000000) VPDSVLSAY
1] pos= 111 score=0.047401 (unnormalised=5.2476000000) EVDRTTIEY
2] pos= -1 score=0.947020 (unnormalised=104.8410000000) non-binding event

MHC II

0] pos= 4 score=0.093277 (unnormalised=10.0495600000) FRLAGSTFS
1] pos= 11 score=0.075011 (unnormalised=8.0815600000) FSVMPSAPP
2] pos= 13 score=0.054823 (unnormalised=5.9065600000) VMPSAPPLL
3] pos= 14 score=0.022318 (unnormalised=2.4045600000) MPSAPPLLS
4] pos= 21 score=0.054025 (unnormalised=5.8205600000) LSLLVSWGV
5] pos= 30 score=0.001583 (unnormalised=0.1705600000) LTILFGKVA
6] pos= 58 score=0.006354 (unnormalised=0.6845600000) VWGVGSNTL
7] pos= 59 score=0.000692 (unnormalised=0.0745600000) WGVGSNTLA
8] pos= 126 score=0.077767 (unnormalised=8.3785600000) MKQQYANYL
9] pos= -1 score=0.614151 (unnormalised=66.1680000000) non-binding event

C



Peroxidase-like protein from Ferula elaeochytris (Accession- QIV38291.1)

B-cell epitopes

1] pos=83 len=5 GTTPS
2] pos=101 len=4 TEGE

T-cell epitopes

MHC I

0] pos= 80 score=0.016624 (unnormalised=1.7776000000) LNDGTTPSY
1] pos= 88 score=0.002914 (unnormalised=0.3116000000) YSDIQTTFR
2] pos= -1 score=0.980462 (unnormalised=104.8410000000) non-binding event

MHC II

0] pos= 0 score=0.013276 (unnormalised=1.3795600000) MVLIRRQRL
1] pos= 3 score=0.027056 (unnormalised=2.8115600000) IRRQRLSFL
2] pos= 10 score=0.029346 (unnormalised=3.0495600000) FLITFTFLV
3] pos= 14 score=0.000390 (unnormalised=0.0405600000) FTFLVLGLI
4] pos= 16 score=0.018203 (unnormalised=1.8915600000) FLVLGLIAS
5] pos= 18 score=0.012256 (unnormalised=1.2735600000) VLGLIASHG
6] pos= 19 score=0.042790 (unnormalised=4.4465600000) LGLIASHGS
7] pos= 21 score=0.054318 (unnormalised=5.6445600000) LIASHGSRL
8] pos= 51 score=0.009484 (unnormalised=0.9855600000) FSILVTRVN
9] pos= 58 score=0.020243 (unnormalised=2.1035600000) VNQSQAFGI
10] pos= 64 score=0.029760 (unnormalised=3.0925600000) FGIYAGNLV
11] pos= 71 score=0.053693 (unnormalised=5.5795600000) LVGQTPYVV
12] pos= 95 score=0.049776 (unnormalised=5.1725600000) FRILNGTEG
13] pos= 119 score=0.002671 (unnormalised=0.2775600000) YAWEFGHKF
14] pos= -1 score=0.636740 (unnormalised=66.1680000000) non-binding event

D

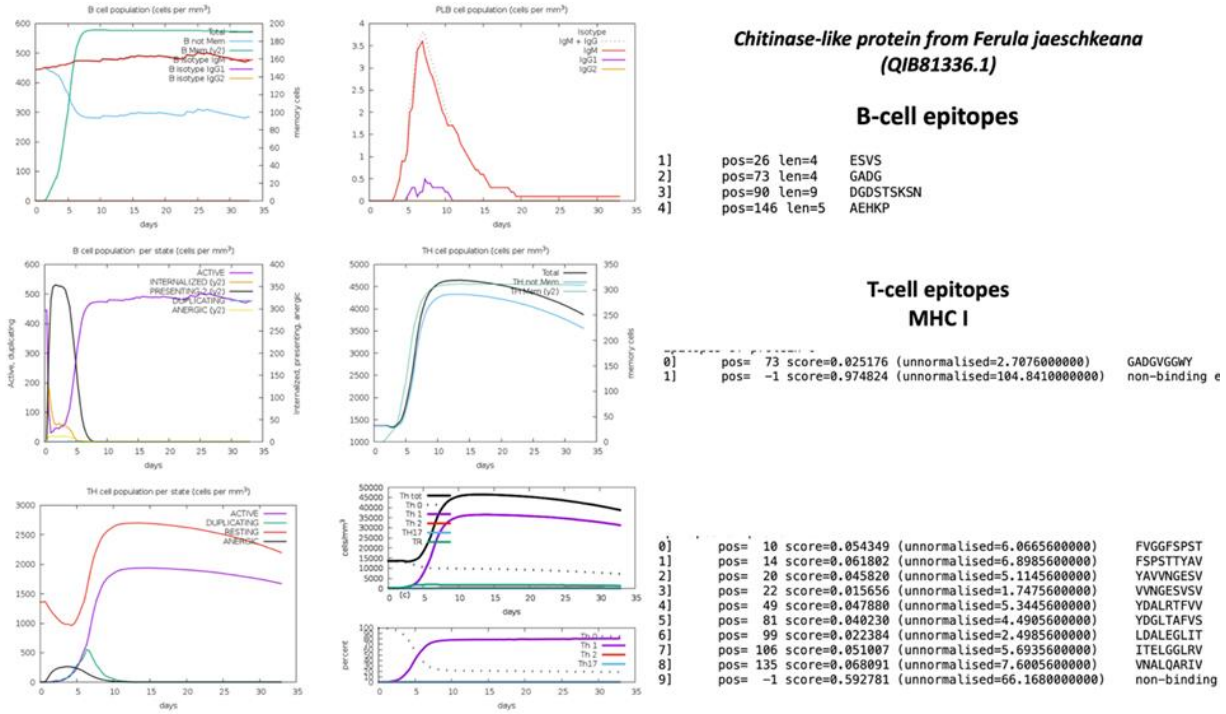


Figure 4. A-D Epitope prediction and immune simulation of potentially therapeutic ingredients from Ferula genus

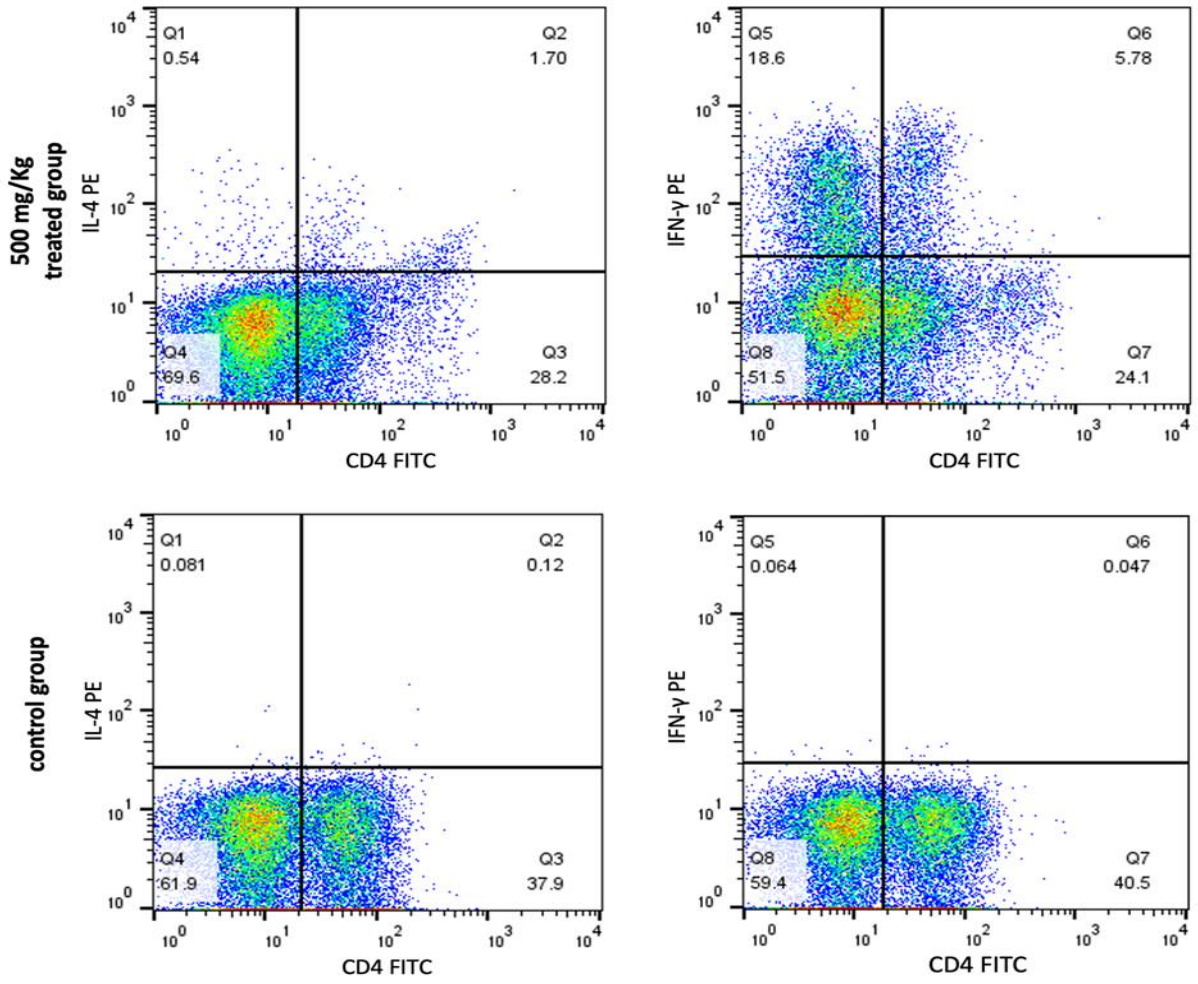


Figure 5. Flow cytometry of Th1/Th2 in Ferula-treated and control groups

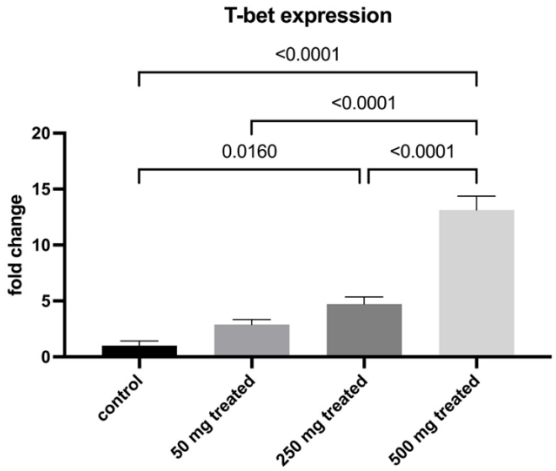


Figure 6. Expression analysis of *T-bet* in rats

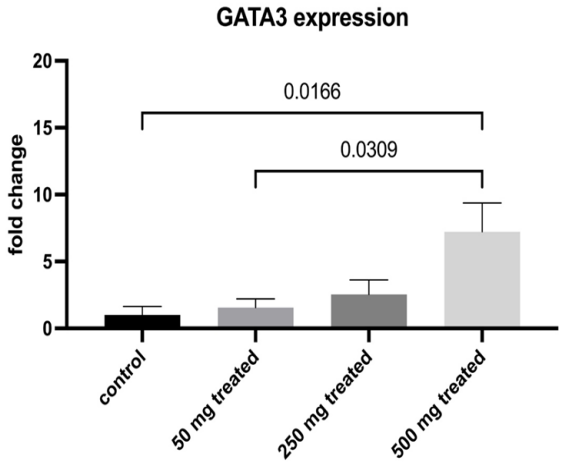


Figure 7. Expression analysis of *Gata3* in rats

Discussion

Cancer is a major cause of mortality which is on the rise globally. Using herbal medicine to modify the immune response is a novel approach which could provide better safety profiles or even diminish the resistance of cancers (Abdulridha et al. 2020; Wheat and Currie 2008).

Imbalance of Th1/Th2 response is involved in a wide range of malignant, infectious, autoimmune, cardiovascular, neurological, respiratory, and endocrine disorders (Infante-Duarte and Kamradt 1999; Jiang et al. 2021; Kidd 2003; Matia-Garcia et al. 2021; Spagnolo et al. 2022). The present study showed an increase in both Th1 and Th2 cytokines, with the predominance of IFN-γ cytokine of Th1. These results can be useful for development of a favorable immunomodulatory medicinal drug for various cancers by activating other immune response effectors, such as macrophages and antibody production against the tumor. In addition, the present study confirms a potential Th1-dominant response based on flow cytometric and qRT-PCR expression analysis of *Gata3*/*T-bet* genes which have been investigated as key Th-related genes previously (Wei et al. 2002). We also suggested a favorable safety profile as no negative effects on thymus, spleen, or body weight were observed. In line with our study, Janabi and Al-Shukri explored the influence of adding *F. asafoetida* to the drinking water or diet of broilers on their generational traits. Findings of their study indicated a significant increase of total body weight in all treatments (Al-Janabi and Al-Shukri 2019). However, mild pathological events in the liver and kidney may be associated with *F. hezarlalehzarica* and optimal dosing is highly advised to prevent these effects.

Previously, our group showed the anti-cancer effects of *F. hezarlalehzarica* Y. Ajani on different malignant cell lines, such as the Raji Burkitt's lymphoma cells (Asemani et al. 2018). However, the present study addresses a previously unknown immunomodulatory and safety profile of *F. hezarlalehzarica*. To provide first-clue for future studies we analyzed key protein sequences to estimate their epitopes and immune responses to *Ferula* proteins. Recent research reveals that *Ferula* plants have also shown anti-tumor, anti-oxidant, and cytotoxic biological activities (Dehghan et al. 2007; Dehpour et al. 2009; Naji Reyhani Garmroudi et al. 2021). Our analyses were based on *Ferula elaeochytris*, *Ferula assafoetida*, and *Ferula jaeschkeana*, because there is not enough protein sequence data for *F. hezarlalehzarica* which could be an interesting area of study for the future. Such data could be very useful for future immunotherapeutics targeting and

immunopathological studies, as most studies have focused on phytoestrogens and non-protein compounds (Naji Reyhani Garmroudi et al. 2021). In the future, immunoinformatics techniques could be utilized to design novel therapeutics based on components of *F. hezarlalehzarica* extract. In addition, our immunoinformatics findings indicate a high fraction of Th1-biased cells as a result of Beta-glucosidase-like protein from *F. assa-foetida* exposure without Lipopolysaccharide (LPS), potentially suggesting this protein could be useful for cancer treatment and immunomodulation; however, further research is necessary.

These methods offered fast structural and functional prediction as well as analysis of genetic and protein data to develop the discovery of new candidates in recent years (Alilou et al. 2020).

For the future, it is advised to explore immunomodulation by components and extracts from the *Ferula* plants. Nonetheless, as our results demonstrated potential kidney histopathological adverse effects at high *Ferula* treatment dosages, it could be suggested that regular renal function monitoring be conducted before and during clinical use of this medicinal plant in traditional medicine.

The present study offers new insights into immunomodulation by *F. hezarlalehzarica* extract, leading to a Th1-biased shift as well as increase in both Th1 and Th2 cytokines. Immunoinformatics findings of the current study be useful for cancer treatment approaches by other immune response effectors such as macrophages and antibody production against tumors. We also demonstrate favorable effect of the extract on lymphatic organ and body weight. However, caution is suggested to minimize pathological effects of *F. hezarlalehzarica* on the liver and kidneys. Further longitudinal studies and analysis of therapeutic components which may be responsible for immunomodulatory effects of *F. hezarlalehzarica* are advised. For the future, it is suggested that computational studies design or screen immunomodulatory therapeutics targeting key epitopes from *F. hezarlalehzarica* proteins. Also, extended safety analysis and dosage adjustment is advised to facilitate the progress of *F. hezarlalehzarica* into clinical trials.

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Conflicts of interest

The authors had no competing interests.

Ethical Considerations

This study was approved by Azad Islamic University, Babol Branch (no. 1400.048).

Code of Ethics

Azad Islamic University, Babol Branch (no. 1400.048).

Authors' Contributions

Mo. KR, KS, AA, ASN, RH, GM, Ma. KR, and AS conceptualized and drafted the manuscript. AA, ASN, GM, and KS critically appraised the manuscript. Mo. KR, KS, AA, and ASN performed the experiments. KS, AA, ASN, and Mo. KR performed data analysis. All authors reviewed, edited, and approved the final version of the manuscript.

Abbreviation

ANOVA: Analysis of variance. CBC: Complete blood count. CT: cycle threshold. DC: Dendritic cell. ELISA: Enzyme-linked immunosorbent assay. IEDB: Immune epitope database. IFN: Interferon. IL: Interleukin. IMMSIM: Immune simulation (server/software). LPS: Lipopolysaccharide. LSD: Least significant

difference (post-hoc test). PBS: Phosphate-buffered saline. PCR: Polymerase chain reaction. SEM: Standard error of mean. SPSS: Statistical package for the social sciences

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