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Original Article

IMMUNOMODULATORY EFFECT OF DIFFERENT PROPORTIONS OF THE HERBAL MIXTURE IN TRIPHALA ON HUMAN T LYMPHOCYTES (MOLT-4)

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ABSTRACT

Objectives: To investigate effect of different proportions of *Terminalia bellerica* Roxb (TB):*Terminalia chebula* Retz (TC):*Phyllanthus emblica* L. (PE) in triphala extract on immunological activity and to determine the possibility of applying the appropriate extract for health and immune disorders.

Methods: The extracts with different proportions of TB: TC: PE (w/w/w), i.e.12:8:4 (F1), 4:12:8 (F2), 8:4:12 (F3) and 8:8:8 (F4), were prepared by decoction in water and dried under vacuum. Gallic acid, a major compound in triphala, was detected by high performance liquid chromatography (HPLC). The effect of the extracts on IFN-γ and IL-10 cytokine production produced by MOLT-4 cells was determined by ELISA.

Results: The results show that F1, F4 and TB extracts significantly stimulated IFN-γproduction but no alteration in IL-10 expression was observed. With LPS induction, F1, F2, F3 and PE extracts significantly inhibited IFN-γproduction, while F2, F3, TB and TC extracts inhibited IL-10 production. By determining the IFN-γ/IL-10 cytokine ratio, we found that the Th1/Th2 balance after treatment with triphala extract was mainly skewed toward a Th1-like response. With LPS induction, only the F1 extract could restore the balance of immunity by shifting the Th2 response to a normal level.

Conclusion: Our investigation indicates that different proportions of triphala extracts and induction conditions affect cytokine production, with a predominant Th1 response. F4, the equal proportion triphala extract, could be applied as a healthy herbal drink. F1, containing a high proportion of *T. bellerica*, was a promising extract as an effective therapeutic intervention against Th2 imbalance diseases such as allergy and autoimmune disease or for use with cancer vaccines.

Keywords: Triphala, Immunomodulatory activity, MOLT-4 cells, Interferon-γ, Interleukin-10, Th1/Th2 balance.

INTRODUCTION

Herbs have been used for food and medicinal purposes for centuries. Traditional, complementary, alternative or non-conventional medicines are used by 70–95% of the global population, particularly in developing countries [1]. Nowadays, alternative medicine for the treatment of various diseases, including immunological disorders, is becoming more popular. Research interest has been focused on various herbs that possess immunomodulatory properties that may be useful in reducing the risk of various diseases and cancers [2]. Immune activation is an effective as well as a protective approach against emerging infectious diseases [3]. There have been many reports on the effects of herbal medicines as immunomodulators which alter immune function. Some effective plants have been developed into clinical therapeutics. Many of these traditional herbs are prepared as combined formulas, which may influence various molecular pathways. Therefore, the pharmacological activity may be different from combinations of individual herbs. Therapeutic success using these formulas may be partially due to their effects on cytokines. Although some mechanisms of action of herbs are unclear and remain to be elucidated, they are worth further study as potential therapeutic agents for immunological applications [2].

Triphala is an herbal formulation consisting of the dried and powdered fruits of three plants, *Terminalia bellerica* (family Combretaceae), *Terminalia chebula* (family Combretaceae) and *Emblica officinalis* (family Phyllanthaceae). Chemically, the fruit of *T. bellerica* had been found to contain gallic acid as an active component, as well as other phytochemical compounds such as ellagic acid, ethyl gallate, chebulagic acid and β-sitosterol [4, 5]. *T. bellerica* has been scientifically shown to possess antibacterial [6], antifungal [7], antioxidant [8] and hypotensive [9] effects. Moreover, our previous study showed that the methanolic extract of *T. bellerica* affected the mouse immune system, both in terms of the cellular and humoral immune responses *in vitro*, corresponding to its folklore applications [10]. *T. chebula* fruit is reported to possess the phytocompounds responsible for various activities such as antimicrobial, antioxidant, antiviral, anticarcinogenic, hypocholesterolemic and antispasmodic [11]. The fruit extract of *P. emblica* contains phenolic compounds such as anthocyanins, flavonols, ellagic acid and its derivatives [12] and is also a rich source of ascorbic acid [13]. It exhibits various biological activities, including antioxidant, anti-tumour, anti-inflammatory, anti-bacterial and hepatoprotective activity [14, 15].

Triphala is an Ayurvedic formulation rich in antioxidants which is believed to promote health, immunity and longevity [16, 17]. It is used to treat many diseases such as inflammation, infection, obesity, anaemia, fatigue, tuberculosis, AIDS [18], jaundice, constipation, asthma, fever and chronic ulcers [19]. Gallic acid has been identified as a major ingredient of triphala [20]. Typically, triphala is an herbal formulation containing the fruits of *T. bellerica*, *T. chebula* and *P. emblica* in equal proportions [21]. In Thailand, several proportions of the three fruits in triphala are used, such as 1:1:1, 12:8:4, 4:12:8 and 8:4:12, recommended for various disorders. Triphala juice sold in Thailand and abroad, in particularly in India, is usually an equal proportion of *T. bellerica*, *T. chebula* and *P. emblica* in the formulation. The Department of Thai Traditional and Complementary Medicines in Thailand introduced triphala at equal proportion for a healthy balance and at ratio of 100:200:400 g (1:2:4) for immunity improvement. In Thai traditional medicine, different proportions of the three components in triphala are defined according to aetiology and season. In this study, we investigated the immunomodulatory activity of triphala extracts prepared in different proportions of *T. bellerica*, *T. chebula* and *P. emblica*. The possibility of developing an effective triphala extract or juice for health promotion or immune disorder was assessed.

MATERIALS AND METHODS

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO),

antibiotic-antimycotic solution (100 U penicillin, 100 µg streptomycin and 0.25 µg/ml amphotericin B), phosphate buffered saline (PBS) and gallic acid were purchased from Sigma-Aldrich (Germany). RPMI-1640 medium, L-glutamine and foetal bovine serum (FBS), were purchased from GIBCO (UK). Human IFN-γ and IL-10 ELISA kits were purchased from eBioscience Inc. (USA). 2-propanol, acetic acid, acetonitrile and methanol (HPLC grade) were purchased from Merck (Germany).

MOLT-4 cell culture

The human leukemic T cell line (MOLT-4) was used in this study. The cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS at 37 °C in a 5% CO_2 atmosphere.

Plant material

Dried fruits of *Terminalia bellerica* Roxb (TB), *Terminalia chebula* Retz (TC) and *Phyllanthus emblica* L. (PE) were purchased from an herbal medicine store in Phitsanulok, Thailand, and identified by a botanist at the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand. A specimen was prepared and deposited at the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

Preparation of extracts

The dried powder (500 g) of TB, TC and PE or triphala extract (TB: TC: PE of 12:8:4 (F1), 4:12:8 (F2), 8:4:12 (F3) and 8:8:8 (F4) (w/w/w)) were extracted by decoction in 3 l of distilled water and concentrated to 1 l, then filtered. The filtrate was evaporated under reduced pressure until dryness and the percentage yield was calculated. The extract was dissolved in 0.1% DMSO in PBS solution. Insoluble material was centrifuged and the extract was sterilised using a 0.2 µm filter. 0.1% DMSO in PBS solution was used as the control in all experiments.

Determination of the gallic acid content by HPLC analysis

Gallic acid in the extract was analysed by HPLC according to previous study [22] with appropriate modifications. The retention time of authentic gallic acid (30 µg/ml) was 4.8 min. The HPLC system consisted of a LC-20AT pump (Shimadzu, Kyoto, Japan), a SPD-20A UV detector (Shimadzu, Kyoto, Japan), equipped with a SPD-20 A system controller (Shimadzu, Kyoto, Japan) and a SIL-10ADVP sample injector (Shimadzu, Kyoto, Japan) fitted with a 20 µl sample loop. The chromatographic separations were carried out on a Luna C18 column (250 mm×4.6 mm i.d., 5µm, 250 °A from Phenomenex, USA). The mobile phase was acetonitrile-water-acetic acid (10:88:2, v/v/v). All separations were performed isocratically at a flow rate of 1 ml/min. Column temperature was maintained at room temperature (27±2 °C). The peaks were determined using a UV detector set at a wavelength of 270 nm. Identification was based on comparing retention times and UV–Vis spectral data of the peaks detected with the gallic acid peaks. Quantification was accomplished using external calibration with pure standards. The calibration curves were linear with $r^2 = 0.999$. The amount of gallic acid in the extracts was calculated using the area under the curve in the chromatograms. Standard aqueous solutions of gallic acid were prepared, at concentrations of 0.05–0.5 µg/ml. The dried extract was dissolved in the mobile phase. After filtering through filter paper and a 0.45 µm membrane filter, the extract was injected directly.

Cytokine production assay

The production of IFN-γ and IL-10 by a human leukemic T cell line (MOLT-4) was measured by ELISA according to the instructions of the manufacturer (eBioscience, Inc. San Diego, USA). T cells (3×10^5 cell) induced with 5 µg/ml LPS were treated with TB, TC and PE or triphala extracts (0.01, 0.1, 1 mg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. Cytokine secretion into the culture supernatant was analysed. Briefly, a 96-well microtiter plate was pre-coated overnight with the capture antibody. This was followed by blocking and several washings, then the working standards and samples were added and incubated for 2 h. After further washing, the working detector solution containing biotinylated anti-cytokine monoclonal antibody and avidin-horseradish peroxidase conjugate was added to each well and incubated for 30 min. The substrate solution was then added, followed by the addition of the stop solution, and the absorbance was read using a microtiter plate reader at 450 nm. To determine Th1/Th2 balance, the IFN-γ/IL-10 ratio was calculated.

Cell viability assay

Cell viability assays were assessed using a modified MTT assay [23]. Briefly, cell suspensions (3×10^5 cell) were treated with TB, TC and PE or triphala extracts (0.01, 0.1, 1 mg/ml) for 24 h at 37 °C in a humidified 5% CO_2 incubator. Subsequently, 5 mg/ml MTT was added and incubation was continued for a further 4 h. The culture medium was removed by aspiration; DMSO was then added to dissolve the formazan crystals. The absorbance was measured at 595 nm using a microplate reader (Bio-Tek Instrument Inc., USA). Viability was measured as the percentage of viable treated cells relative to untreated cells.

Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean±SE Statistical differences (significance level of $P<0.05$) between groups were assessed using one-way analysis of variance, followed by multiple comparisons using Tukey's method.

RESULTS

Plant extraction

T. bellerica (TB), *T. chebula* (TC), *P. emblica* (PE) extracts and their mixtures in different proportions as triphala extract (F1-F4) were obtained. The physical appearance of the seven extracts was similar, i.e. a dark brown viscous gum. The percentage yield of each extract is shown in table 1.

Determination of the gallic acid content by HPLC analysis

The gallic content in *T. bellerica*, *T. chebula*, *P. emblica* and triphala extracts was analysed using HPLC. The acetonitrile-water-acetic acid (10:88:2, v/v/v) system as the mobile phase demonstrated good separation of gallic acid from the extracts. Comparison of the retention times of the peaks detected on the HPLC chromatograms of the extracts with the reference standard gallic acid revealed that gallic acid was the major compound in all extracts. The maximum amount of gallic acid was detected in *P. emblica* extract. For triphala extracts, the gallic acid level in F4 was higher than in F3, F1 and F2, respectively. The percentage yield of gallic acid content of each extracts is shown in table 1. The HPLC chromatograms of the *T. bellerica*, *T. chebula*, *P. emblica* extracts are shown in fig. 1, and those of the triphala extracts are shown in fig. 2.

Table 1: Percentage yield of extracts and their containing gallic acid content analysed by HPLC technique

Extract	Plant/ingredient of formulation (ratio by weight)	%yield of extract (by weight of dried plant)	%yield of gallic content (by weight of dried material) (n = 4)
F1	TB: TC: PE (12:8:4)	30.26	0.64±0.003
F2	TB: TC: PE (4:12:8)	21.25	0.57±0.005
F3	TB: TC: PE (8:4:12)	21.10	0.71±0.006
F4	TB: TC: PE (8:8:8)	26.42	0.87±0.014
TB	*T. bellerica*	33.00	0.41±0.003
TC	*T. chebula*	36.03	0.31±0.003
PE	*P. emblica*	33.34	2.20±0.015

Cytokine production assay

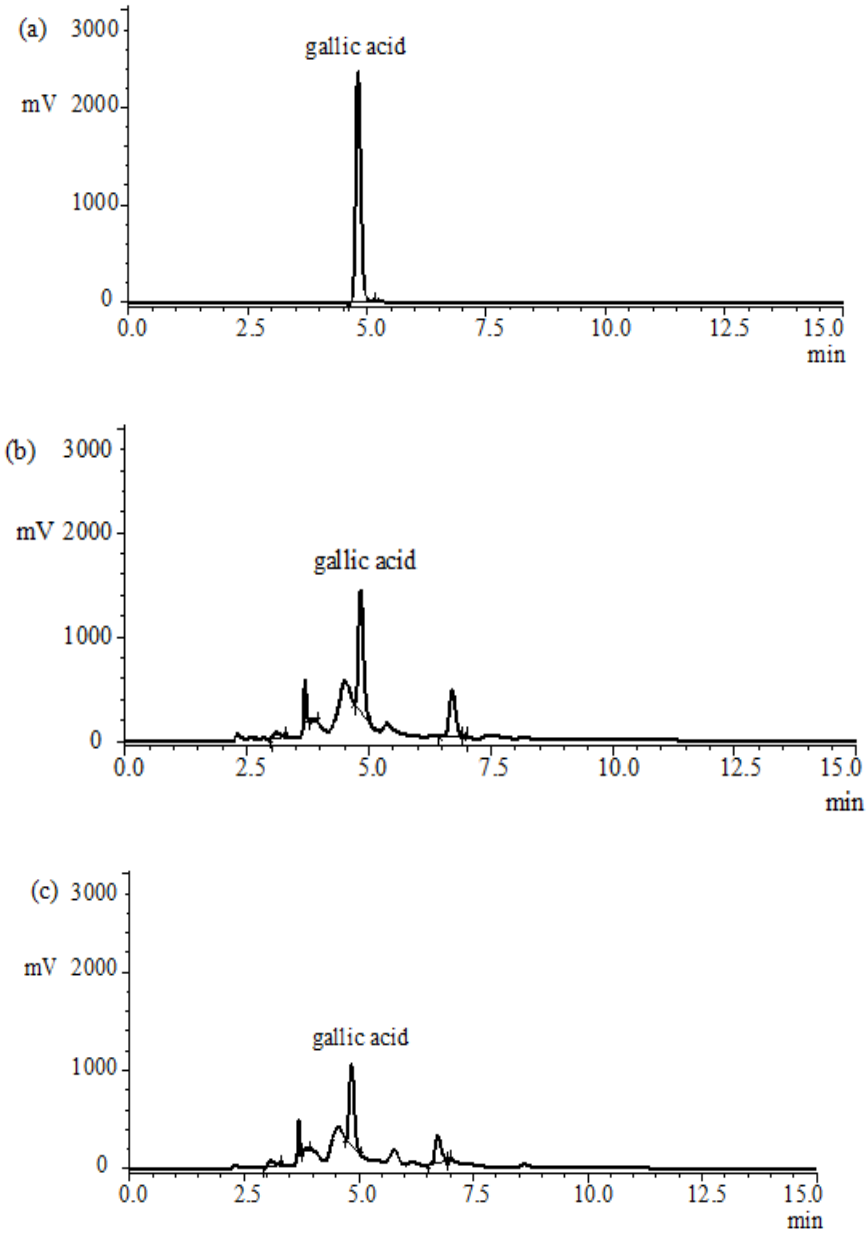
MOLT-4 cells without or with LPS induction were treated with four triphala extracts, as well as *T. bellerica*, *T. chebula* and *P. emblica* extracts. The production of IFN-γ and IL-10 was measured by ELISA. The results show that F1 (0.01 and 0.1 mg/ml), F4 (1 mg/ml) and *T. bellerica* extract (0.01 mg/ml) significantly enhanced IFN-γ production. The maximal IFN-γ production of approximately 166.58±36.95 pg/ml was observed with cells treated with F1 at 0.01 mg/ml. However, none of the extracts affected IL-10 production compared with basal secretion.

LPS alone markedly increased IFN-γ and mildly increased IL-10 production, significantly. With LPS induction, F1, F2 and F3 extracts suppressed IFN-γ production, of which the F1 extract (0.01 mg/ml) provided the maximum suppression to 20.00±4.85 pg/ml. Of the pure extracts, the *P. emblica* extract (1 mg/ml) showed the maximum inhibition of IFN-γ production following LPS treatment, to 56.00±5.40 pg/ml. Mild suppression of IL-10 was observed when cells were treated with F2, F3, *T. bellerica* and *T. chebula* extracts. The results are shown in fig. 3.

The Th1/Th2 balance was determined by the IFN-γ/IL-10 ratio. It was found that all extracts mainly affected the Th1 response. Compared to normal secretion, the IFN-γ/IL-10 ratio in cells treated with the triphala extracts F1, F3 and F2, F4 was increased by approximately 14-fold and 10-fold, respectively, which was higher than that of the three single extracts. With LPS induction, only the F1 extract decreased the IFN-γ/IL-10 ratio to close to the normal level. The results are shown in fig. 4.

Cell viability assay

The effect of *T. bellerica*, *T. chebula*, *P. emblica* and triphala extracts on MOLT-4 cell viability was assessed. All extracts allowed cell viability greater than 90%, i.e. the extracts were not toxic to the cells (data not shown). Interestingly, *P. emblica*, *T. bellerica* and F1 extracts at high concentrations tended to slightly increase cell proliferation by approximately 122%, 114% and 134%, respectively.



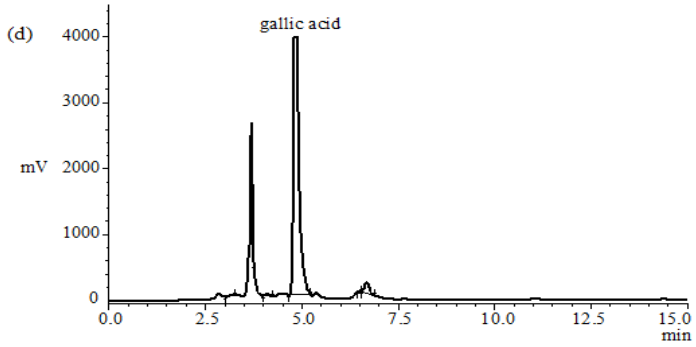


Fig. 1: **HPLC chromatogram of (a) 0.3 mg/ml gallic acid and 10 mg/ml extracts; (b) *T. bellerica*, (c) *T. chebula* and (d) *P. Emblica***

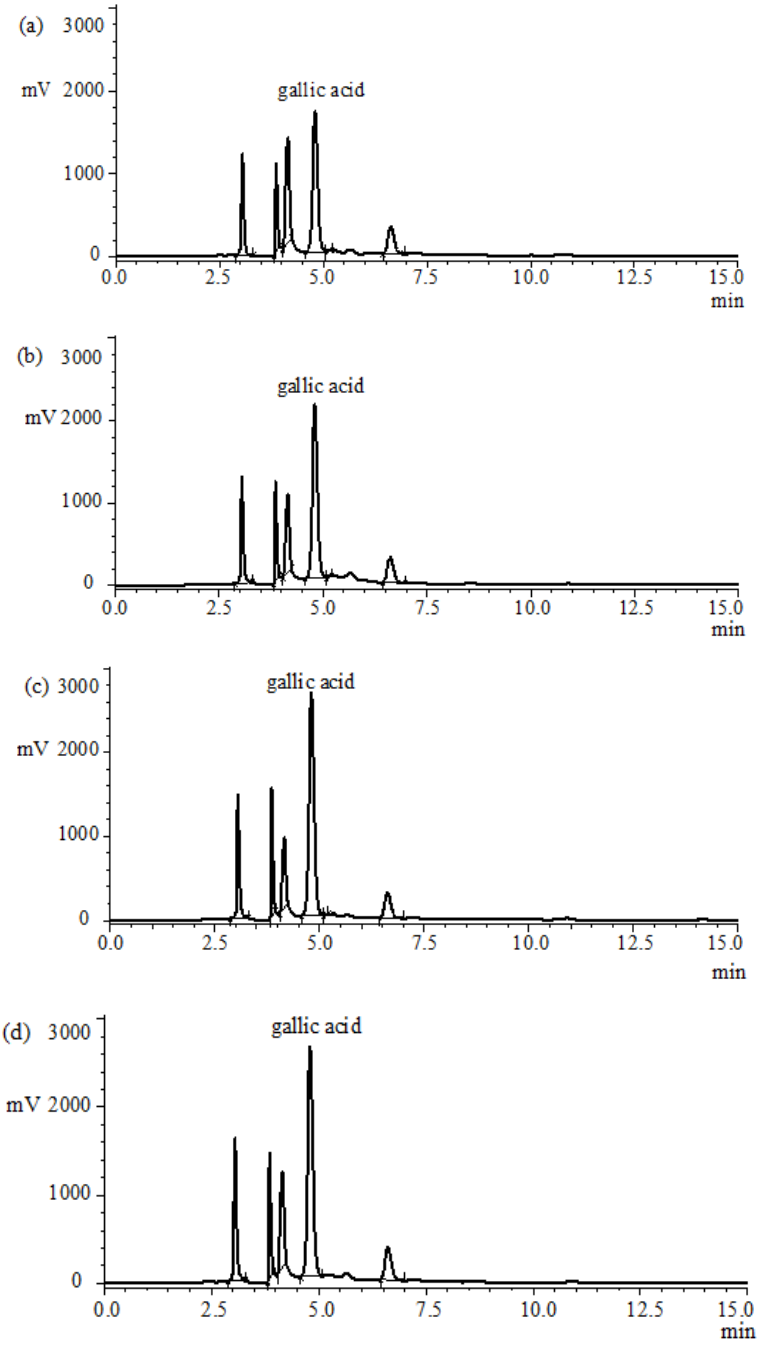


Fig. 2: **HPLC chromatogram of triphala extracts with different ratios (w/w/w) of 10 mg/ml *T. bellerica*, *T. chebula*, *P. emblica* extracts; (a) F1 (12:8:4), (b) F2 (4:12:8), (c) F3 (8:4:12) and (d) F4 (8:8:8)**

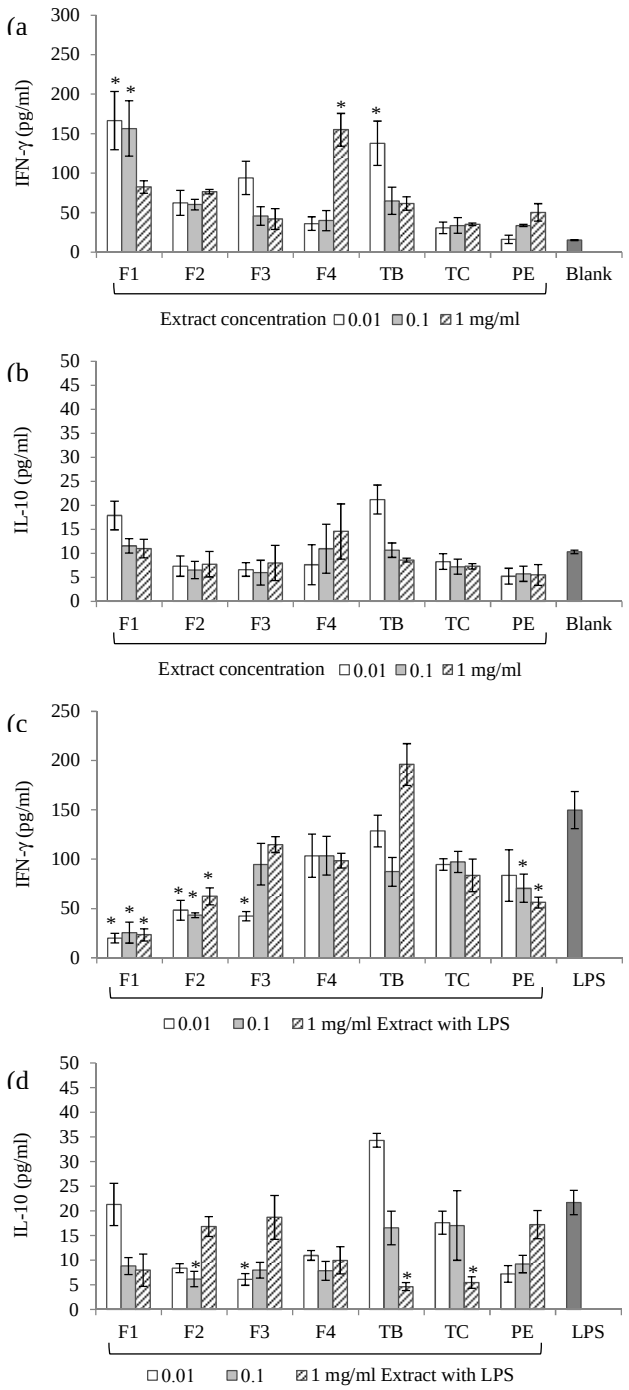


Fig. 3: Effects of triphala extracts (F1, F2, F3 and F4), *T. bellerica* (TB), *T. chebula* (TC) and *P. emblica* (PE) on the production of (a) IFN-γ and (b) IL-10 by MOLT-4 cells compared with (c) IFN-γ and (d) IL-10 following induction with 5 µg/ml LPS. Each value represents the mean±SE of triplicates compared to LPS; *$P<0.05$

DISCUSSION

To demonstrate the pharmacological activity of triphala in correlation with folklore remedies, four triphala extracts containing *T. bellerica* (TB), *T. chebula* (TC) and *P. emblica* (PE) in different proportions were mixed and extracted by decoction in boiling water, as in the folklore preparation. Gallic acid, a major compound in triphala, has been reported to possess various biological activities and was used as a marker for analysis. Gallic acid was detected at higher levels in *P. emblica* than that in the *T. bellerica* and *T. chebula* extracts. Certainly, the F3 and F4 extract composed of a high proportion of *P. emblica* contained a high amount of gallic acid.

Cytokines are peptides and pleiotropic glycoproteins which are produced from various sources and have effects on various targets. Cytokines are significant mediators in innate and adaptive immunity and have effects on cell growth, cell differentiation, cell death, and angiogenesis and repair processed [24]. The cytokine IFN-γ is an important driver in resisting viral and bacterial infections and also induces various immune responses. IL-10 is an inflammatory cytokine with effects on immunoregulation; it also downregulates the expression of Th1 cytokines, MHC class II antigens and co-stimulatory molecules on macrophages. Our study shows that the triphala F1 and F4 extracts, containing high proportions of *T. bellerica*, maximally increased IFN-γ production of about 9 to 11-fold. At high concentrations, F1 and the *T. bellerica* extract tended to decrease IFN-γ expression.

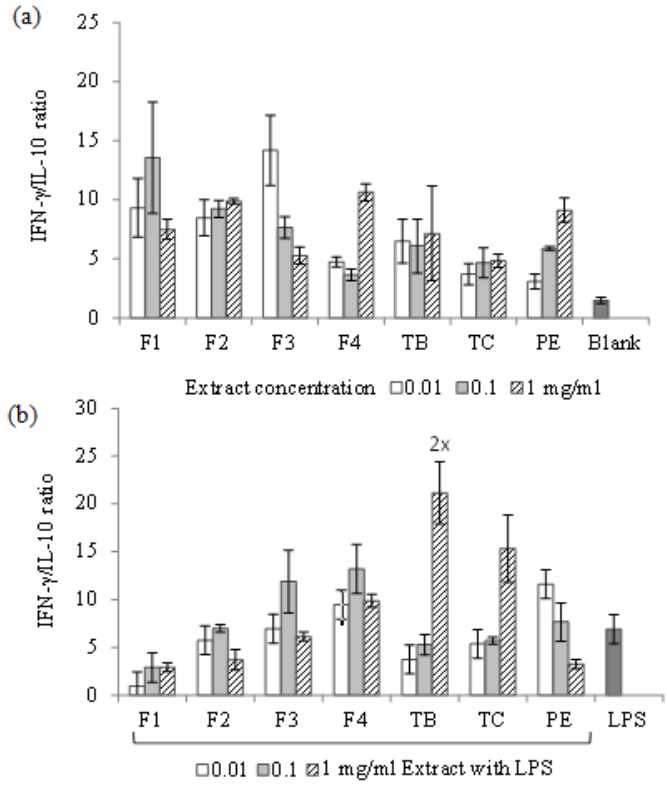


Fig. 4: Effects of triphala extracts (F1, F2, F3 and F4), *T. bellerica* (TB), *T. chebula* (TC) and *P. emblica* (PE) (a) without LPS and (b) with 5 µg/ml LPS on the IFN-γ/IL-10 ratio in MOLT-4 cells. Each value represents the mean±SE of triplicates

This variation in the cytokine production pattern was similar to a previous report [25]. However, the extracts did not clearly alter IL-10 levels, since its basal secretion is normally low. The effects of triphala extracts on LPS-induced cytokine production were also investigated. F1, F2, F3 and *P. emblica* extracts following LPS treatment significantly decreased IFN-γ production, compared to levels with LPS alone. The F1 extract provided the maximum inhibition of IFN-γ production (7.5-fold) while the F2, F3, *T. bellerica* and *T. chebula* extracts significantly decreased IL-10 production (3.5-fold). Our investigations indicate that different proportions in the triphala extract and the experimental conditions affect the cytokine production pattern. The Th1/Th2 cytokine balance is one of the most important regulatory mechanisms of the immune system and can be evaluated by measuring certain cytokine patterns, i.e. Th1 and Th2 profiles [26]. The Th1/Th2 ratio is a significant value for determining the effects of herbs or herbal formulas on immune

function. Cytokines affect both cell-mediated immunity (CMI) and humoral-mediated immunity (HMI). Naïve T cells are activated to the Th0 cell state, which expresses both Th1 and Th2 cell characteristics by expressing various cytokines such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and IFN–γ. After activation, cytokines which induce an immune response through CMI such as IL-2, IL-3 IL-12, IFN-γ and TNF-α (Th1 cytokines) lead to a shift from HMI to CMI. In contrast, cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2 cytokines) induce to a shift from CMI to HMI [27]. Moreover, IFN-γ produced from Th1 cells can suppress Th2 cell progression and the HMI response, while Th2 cells suppress the progression and stimulation of Th1 cells [28,29]. Thus, pharmacologically active substances that can affect the Th1 or Th2 balance may be beneficial. However, this may depend on the cell environment or the nature of the pathogen (for example, the Th1 response for intracellular parasites and viruses and the Th2 response for extracellular bacteria). Moreover, Th2 cytokines are important in the immune response to cancer, but also lead to immune pathology such as allergy and autoimmune diseases [27].

The Th1/Th2 balance, determined by the IFN-γ/IL-10 ratio in this study, presented the maximum ratio for the F1 and F3 extracts (14-fold), which was greater than that with the F2 and F4 extracts (10-fold). These results indicate that triphala extracts mainly stimulate the Th1 response and might lead to a shift from HMI to CMI. Stimulation of the CMI response by triphala extract might have advantages in terms of resistance to intracellular infections by parasites and viruses and may be beneficial in the treatment of allergy and autoimmune disease.

It has been noted that therapeutic activity of herbs is due to their chemical complexity. Two bioactive compounds acting at the same site might have an additive effect, while two or more bioactive compounds acting at different sites might have a synergistic effect and possibly improved higher activity by 50 to 200-fold comparing to the equivalent concentrations of the isolated compounds [30]. Our results show that the change in the Th1/Th2 ratio by triphala was greater than that caused by the three single extracts. Thus, it might be stated that the complexity of mixed herbs could promote effectiveness regarding the immunological response over the use of single herbs. After simulating infection by LPS induction, only the F1 extract decreased the IFN-γ/IL-10 ratio to close to the normal level. These findings suggest that the F1 extract could restore the balance of immunity by shifting to a Th2 response. Anti-allergy activity of triphala extract has been shown to decrease sensitivity to bronchial allergen exposure in ovalbumin-sensitised mice [31]. Triphala also has anti-inflammatory activity on the inhibition of the complement system, HMI response (assessed by the delayed type hypersensitivity (DTH) assay in rat) and phytohaemagglutinin-induced T-lymphocyte proliferation [32]. Therefore, triphala could be a good candidate for anti-allergy and anti-inflammatory treatment of immune deficiency disorders.

The immunomodulatory effect of triphala (in equal proportions) was also reported in albino rats with noise-induced stress. An increase in serum antibody titres and IL-4, a reduction in IL-2 and IFN-γ expression, and a decline in the pan-T, CD4+/CD8+lymphocyte phenotype in the spleen were observed [33]. Triphala also inhibited the CMI response, reduced foot pad swelling and decreased leukocyte migration inhibition (LMI) [34], as well as the activation of neutrophils [35]. Different experimental models, the conditions of cell induction and the tested concentrations might cause variations in the results.

Increases in the production of the cytokines IL-2, IFN-γ, TNF-α and IL-12 could lead to a Th1-type cellular response, while increased expression of IL-4 and IL-6 could lead to a Th2-type humoral response [36]. It had been found that several cancer vaccines, especially those with immune adjuvants, significantly activate the CMI response, resulting in IL-2, IFN-γ, TNF-α and IL-10 expression [37]. Therefore, triphala extracts, especially F1, might be a promising extract for further investigation as cancer vaccine adjuvants. The cell viability assay by using the MTT technique showed that none of the tested extracts were toxic to MOLT-4 cells (viability>90%). Moreover, F1, *T. bellerica* and *P. emblica* extracts led to T-cell proliferation, which was possibly related to the increase in IFN-γ production. A previous study reported that a triphala aqueous extract had a cytotoxic effect on the human breast cancer cell line MCF-7 and barcl-95 transplantable mouse thymic lymphocytes, but it was not toxic to normal cells such as breast epithelial cells, MCF-10F cells, human peripheral blood mononuclear cells, and mouse liver and spleen cells. The possible mechanism might correlate with the response to ROS production in cancer cells [38]. These studies support our conclusion that triphala is safe for normal cells and might have an anti-cancer effect. Gallic acid and other phenolic compounds might be responsible for this activity [39].

A mixture containing several components could have broad specificity and low affinity at the site of action. In some cases, this would have be more efficacious and safer than a substance with high affinity and specificity [40, 41]. Herbal mixtures may reduce adverse effects by buffering the toxicity that may occur when using a single extract or specific compounds [42]. Therefore, the complexity of the triphala mixture may provide greater pharmacological efficacy, lower toxicity and fewer side effects compared to single extracts.

Herbal or mixed herbal formulations which have anti-inflammatory effects and are used for the treatment of immune-related diseases might be defined as immunomodulators [43]. Immunomodulators also include herbs that affect various immunological molecules such as cytokines, adhesion molecules, nitric oxide, hormones, neurotransmitters and peptides [44]. Since triphala extracts affect IFN-γ and IL-10 production by T lymphocytes (MOLT-4 cells), we can state that triphala extract is an immunomodulator.

We can conclude that triphala extract has immunomodulatory activity, supporting the Th1 response more than the Th2 response. The present study confirms the traditional uses of triphala related to its immunological applications. Preparation of triphala with various proportions of *T. bellerica*, *T. chebula* and *P. emblica* affected the immunological pattern. F4, an equal proportion of triphala extract which is recommended for daily use, could be applied as a healthy herbal drink. Interestingly, the triphala extract F1, which contained a high proportion of *T. bellerica*, could be recommended for the treatment of disorders with a Th2 imbalance such as allergy and autoimmune disease, or applied with a cancer vaccine to stimulate a shift to a Th2 response. However, the concentration of each component in the product should be carefully determined. Detailed insight into the molecular mode of action of triphala extract leading to the observed immunomodulatory activity is lacking and further study is required.

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CONFLICT OF INTERESTS

Declared None

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