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## Exploring the immunomodulatory potential of *Clitoria ternatea* using chicken lymphocytes

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### ABSTRACT

**Background:** The growing interest in phyto-genic immunomodulators is driven by the need for natural alternatives to synthetic drugs, particularly in animal health. However, many herbal products face skepticism due to limited scientific validation. *Clitoria ternatea* L., commonly known as butterfly pea, is a traditional medicinal plant rich in diverse phytochemicals such as flavonoids, anthocyanins, alkaloids, and tannins. These compounds are known for their antioxidant, anti-inflammatory, and neuroprotective activities. Traditionally, various parts of the plant- roots, leaves, flowers, and seeds, have been used to treat ailments like fever, wounds, memory disorders, and microbial infections. **Objective:** The present study was conducted to evaluate the immunostimulatory effects of a 50% hydro-methanolic leaf extract of *Clitoria ternatea* (CTE) on chicken lymphocytes *in vitro*, and to validate its potential as a natural immunomodulator for poultry health management. **Materials and Methods:** Lymphocytes were isolated from healthy broiler chickens under aseptic conditions and cultured *in vitro*. The cytotoxicity of various concentrations of the CTE was assessed using the MTT assay to determine the maximum non-cytotoxic dose (MNCD). Subsequently, the lymphocyte proliferation assay (LPA) was performed to evaluate immunostimulatory activity. **Results:** The MNCD of CTE was found to be 0.3 mg/mL, and this concentration was used for further immunological testing in the presence of mitogenic stimulation. CTE at 0.3 mg/mL significantly enhanced lymphocyte proliferation compared to untreated control groups, indicating strong immunostimulatory activity. The results confirm the bioactivity of the extract in modulating immune cell function *in vitro*. **Conclusion:** The study demonstrates that *Clitoria ternatea* leaf extract possesses promising immunopotentiating effects on chicken lymphocytes. These findings support its potential use as a natural immune booster in poultry, and contribute to the scientific validation of *Clitoria ternatea* as a traditional medicinal herb with valuable veterinary immunopharmacological applications.

**Keywords:** *Clitoria ternatea*, Immunomodulation, Chicken lymphocytes, Lymphocytes proliferation assay.

### INTRODUCTION

Medicinal plants have historically played a vital role in human and animal health and well-being, with their applications spanning various industries such as pharmaceuticals, agriculture, cosmetics, and food processing. Ancient civilizations, including those of Egypt, India, China, and Mesopotamia, extensively documented the use of herbs for both the treatment and prevention of numerous ailments [1-3]. Long before the development of synthetic drugs, medicinal plants served as the primary source of healthcare, providing natural remedies rich in bioactive compounds responsible for diverse therapeutic effects [4]. Scientific advances in phytochemistry and pharmacognosy have since validated many of these traditional uses by identifying key secondary metabolites such as alkaloids, flavonoids, tannins, and terpenoids that exhibit pharmacological activities [5,6]. In recent years, there has been a notable global resurgence in the use of herbal medicines, a phenomenon often termed the "herbal renaissance", driven by growing public interest in natural and holistic approaches to health, concerns over the side effects of synthetic drugs, and increasing recognition of ethnopharmacological knowledge [7].

*Clitoria ternatea* Willd. (*C. ternatea*), commonly known as butterfly pea, blue pea, or cordofan pea, is a perennial herbaceous plant belonging to the family Fabaceae. It is widely distributed across tropical and subtropical regions, including India, China, the Philippines, and Madagascar [8,9]. The species thrives particularly well in humid lowland tropical climates, both in natural habitats and under cultivation. Two major floral variants of *C. ternatea*, blue and white, are found in these regions. In India, the plant is popularly referred to as "Shankpushpi" due to the conch shell-like appearance of its flowers [10,11]. *C. ternatea* has a long history of use in traditional medicine, especially within the Ayurvedic system. It is regarded as a potent nervine tonic and has been traditionally employed to improve memory and cognitive functions [11]. The plant is credited with a wide array of pharmacological activities, including nootropic, antistress, antidepressant, anticonvulsant, tranquilizing, and sedative properties [12,13].

Numerous pharmacological investigations have confirmed its therapeutic potential, reporting properties such as anthelmintic, anti-hyperglycemic, anti-inflammatory, anti-diarrheal, antioxidant, hepatoprotective, immunomodulatory, anti-histaminic, antibacterial, and cholinergic activities [8,14,15].

The leaves of *C. ternatea* are a rich source of bioactive compounds. They contain various phytochemicals including phenolics and flavonoids [9]. In addition, the leaves have been reported to contain lactones such as aparajitin and clitorin, along with essential oils, mucilage, and natural coloring matter, which contribute to the plant's therapeutic efficacy. Considering the extensive ethnomedicinal use and rich phytochemical profile of *C. ternatea*, the present investigation was designed to analyze *in vitro* immunomodulatory potential of 50% hydro-methanolic extract of the *C. ternatea* leaves (CTE) in chicken lymphocytes cell culture system.

## MATERIALS AND METHODS

### Collection of Plant Material and Preparation of Plant Extract

Fresh, authentic leaves of *C. ternatea* were collected from the Medicinal Plants Research and Development Centre at G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. A 50% hydro-methanolic extract of the leaves (CTE) was subsequently prepared following standard procedures [9]. All experimental procedures were carried out using cell culture-grade reagents to ensure sterility and consistency in downstream applications.

### Isolation of chicken lymphocytes

Blood samples were obtained from healthy broiler chickens aged 4–6 weeks, collected from a local slaughterhouse. The samples were transported under sterile conditions in chilled Dulbecco's Phosphate-Buffered Saline (DPBS). Lymphocyte isolation was carried out promptly following standard protocols under aseptic conditions to preserve cell viability and prevent contamination. More than 95% of the cells showed viability through trypan-blue dye exclusion assay. The study is *in vitro* in nature involving blood samples collected from the slaughtered healthy birds therefore do not require Institutional Animal Ethics Permission.

### Determination of Maximum Non-Cytotoxic Dose (MNCD) of CTE

The Maximum Non-Cytotoxic Dose (MNCD) of *C. ternatea* leaf extract (CTE) was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Chicken lymphocytes were seeded in a sterile 96-well flat-bottom microtiter plate at a density of  $1 \times 10^6$  cells/mL. The cells were then treated with varying concentrations of CTE, ranging from .001 to 1.6 mg/mL, in triplicate. Plates were incubated for 68 hours at 40°C in a humidified CO<sub>2</sub> incubator maintained at 5% CO<sub>2</sub>. Following incubation, the cells were examined under an inverted microscope to assess morphological signs of cytotoxicity. The culture media from each well was gently removed, and 20 µL of MTT solution (prepared as a 5 mg/mL stock and diluted in 200 µL of culture media) was added to each well. Plates were further incubated for 4 hours in the dark at 40°C under the same CO<sub>2</sub> conditions. At the end of the incubation, the media was discarded carefully, and the resulting formazan crystals were solubilized by adding 200 µL of dimethyl sulfoxide (DMSO) to each well. Absorbance was measured at 570 nm using a microplate ELISA reader. The percentage of cell viability was calculated by comparing the absorbance of treated wells with that of the untreated control group and expressed as a percentage [16,17].

### Lymphocyte Proliferation Assay (LPA)

The lymphocyte proliferation assay (LPA) was carried out to evaluate the immunomodulatory effect of *C. ternatea* extract (CTE), following standard procedures [18]. Mitogenic stimulation of chicken

lymphocytes was performed using cell culture-grade mitogens: Concanavalin A (ConA), Phytohaemagglutinin-M (PHA-M), and *Escherichia coli* lipopolysaccharide (LPS), each at a final concentration of 5 µg/mL in RPMI-1640 medium. A total of 200 µL of lymphocyte suspension ( $1 \times 10^6$  cells/mL) was dispensed into each well of a flat-bottom 96-well tissue culture plate. Cells were treated with the maximum non-cytotoxic dose (MNCD) of CTE in the presence of the respective mitogens, and each treatment was performed in triplicate to ensure reproducibility.

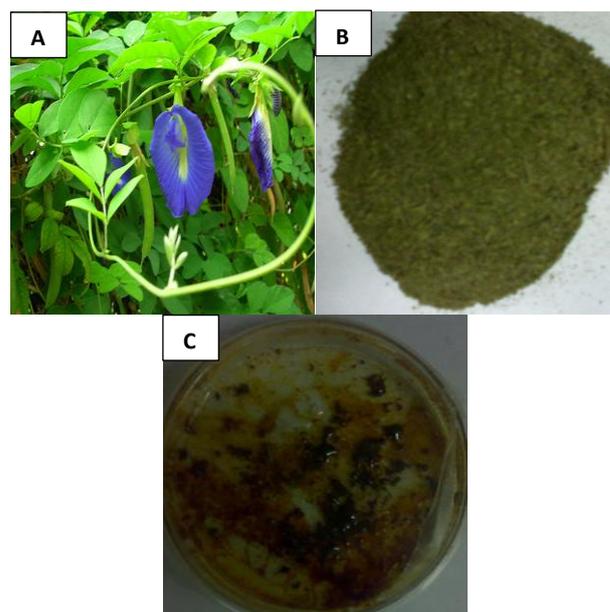
### Statistical Analysis

Statistical analysis was based on comparing the values of control group as compared to the exposed groups. The results were expressed as means ±SE. The statistical significance of the data has been determined using one way analysis of variance (ANOVA-LSD) using SPSS statistical software package. Pearson correlation test was used to determine the significant correlations between variables. The level of significance was set at  $p < 0.05$ .

## RESULT

### The Plant Extract

The dried leaf powder of *C. ternatea* was subjected to fifty percent hydromethanolic extract preparation with the percent yield of CTE obtained as 12.46% (Figure 1). CTE was evaluated for its immunomodulatory potential using chicken lymphocytes culture system.



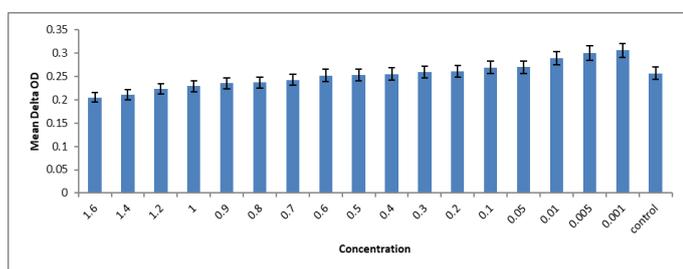
**Figure 1:** *Clitoria ternatea* plant material and extract. (A) Plant, (B) Dried leaves powder, (C) Leaf extract.

### Determination of non-cytotoxic dose of CTE

The avian lymphocytes were exposed to various concentrations of CTE to determine its maximum non-cytotoxic dose for further *in vitro* studies via MTT assay. The data indicated dose-dependent cytotoxicity induced by CTE in lymphocytes culture. Higher the concentrations of CTE i.e. ranging from 0.4 mg/ml to 1.6 mg/ml, more cytotoxicity was obtained. CTE displayed maximum cytotoxicity at the highest concentration of 1.6 mg/mL used in the study with 20.17% cytotoxicity (Table 1; Figure 2). CTE concentrations ranging from 0.3 mg/ml to 0.001mg/ml showed 100% cell viability. Since maximum concentration of CTE that showed 100% cell viability was 0.3 mg/ml, this was selected for further *in vitro* analysis.

**Table 1:** Non-cytotoxic dose determination of CTE by MTT assay in chicken lymphocytes

Concentration (mg/ml)	% viability rate*	% cytotoxicity		
1.6	79.82	20.17		
1.4	82.06	17.933		
1.2	86.74	13.255		
1	89.18	10.818		
0.9	91.61	8.3820		
0.8	92.39	7.6023		
0.7	94.54	5.4580		
0.6	98.24561	1.7543		
0.5	98.34	1.6569		
0.4	99.4152	0.584		
0.3	100.9747	0		
0.2	101.6569	0		
0.1	104.8733	0		
0.05	104.9708	0		
0.01	112.4756	0		
0.005	116.8616	0		
0.001	119.1033	0		
Control	100	0		
Cd at 1%	0.04635690	Cd at 5%	0.0348076	5.143344** (highly significant)



**Figure 2:** Non-cytotoxic dose determination of CTE by MTT assay in chicken lymphocytes

**In vitro Immunomodulatory activity of CTE**

As compared to control, CTE displayed significant enhancement in the proliferation of chicken lymphocytes. There was marked increase in LPS and ConA stimulated cells indicating enhanced B and T cell blastogenesis in case of CTE exposure as compared to respective controls (Table 2).

**Table 2:** Immunomodulatory potential due to *in vitro* exposure of CTE in chicken lymphocytes

Treatment	Un-stimulated		LPS stimulated		ConA stimulated	
	Control	CTE	Control	CTE	Control	CTE
% Viability*	100	104.7191	100	112.7522	100	102.6877
Cd value at 1 %	0.0668	9.9359**	0.1125	4.6963*	0.0597	8.7810**
Cd value at 5 %	0.0476		0.0802		0.0426	

**DISCUSSION**

Although traditional systems like Ayurveda and other complementary therapies reference a wide range of herbal remedies, their global recognition and integration into mainstream health practices remain limited. These herbal preparations contain valuable bioactive compounds, including vitamins, minerals, and essential oils, which offer notable health benefits for both humans and animals. In classical medical literature, many of these herbal formulations are described for use—either individually or in combination—in the treatment of various ailments. *C. ternatea*, commonly known as butterfly pea, is a perennial twining herb of the Fabaceae family. It is distributed throughout the tropics and subtropics, with uncertain native origin due to its widespread cultivation across Asia, Africa, the Pacific Islands, and the Americas [8,9]. *C. ternatea* exhibits a broad spectrum of pharmacological activities, including nootropic, anxiolytic, antidepressant, anticonvulsant, sedative, antipyretic, anti-inflammatory, analgesic, and hepatoprotective effects [8,10,11,19,20]. It is also found to be effective in Alzheimer’s disease condition [21]. Seeds of the plant are rich in fatty acids such as palmitic, stearic, oleic, linoleic, and linolenic acids and contain other constituents like water-soluble mucilage and delphinidin 3,3’,5’-triglucoside, a potential natural food dye [22-24]. Phytochemical investigations of CTE have revealed a diverse profile of secondary metabolites [9]. Leaves have been found to contain kaempferol derivatives, β-sitosterol, and essential oils, while petals are rich in delphinidin and malvidin glucosides. The antioxidant potential of *C. ternatea* has been extensively reported. Studies have confirmed that aqueous extracts of *C. ternatea* petals possess significant antioxidant activity, outperforming ethanolic extracts in radical scavenging and reducing power assays [21,25-27]. Oxidative stress is implicated in the pathogenesis of many chronic diseases, and plant-derived antioxidants are gaining therapeutic interest [28]. In the earlier study, GC-MS analysis of CTE revealed a complex mixture of 24 compounds, with major constituents including butyl-2-methylpropyl phthalate (20.11%), butyloctyl phthalate (11.29%), and butyl-2-ethylhexyl phthalate (30.19%) [9]. There was presence of various phytoconstituents and these compounds may contribute to the observed antioxidant and immunostimulatory properties.

**CONCLUSION**

Based on the outcomes of the present investigation, it can be concluded that the 50% hydromethanolic leaf extract of *Clitoria ternatea* exhibits noteworthy immunostimulatory potential. This was evidenced by a significant increase in lymphocyte proliferation (blastogenesis) *in vitro* when lymphocytes cultures from chickens were exposed to the extract in the presence of mitogenic stimulants. The enhanced proliferative response suggests activation of immune cells, indicating the presence of bioactive constituents in the extract capable of modulating immune function. These findings not only validate the traditional use of *Clitoria ternatea* in herbal medicine for boosting immunity but also highlight its potential as a natural immunomodulatory agent in veterinary applications, particularly for improving poultry health and reducing reliance on synthetic immunostimulants or antibiotics. Further studies involving mechanistic insights, dose optimization, and *in vivo* efficacy are warranted to support its use in commercial poultry health management.

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### Conflict of interest

The authors declared no conflict of interest.

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