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Assessment of immunomodulatory properties of *Acacia catechu* leaf extract employing chicken lymphocyte culture system

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ABSTRACT

Background: Medicinal plants have long been integral to traditional healthcare systems, offering a rich source of bioactive compounds beneficial for both human and animal health. Their accessibility, low toxicity, and therapeutic potential make them valuable alternatives or complements to modern pharmaceuticals. *Acacia catechu* (L.f.) Willd. (*Senegalia catechu*) (family: Fabaceae), a widely used plant in traditional Ayurvedic and folk medicine, has garnered attention for its diverse pharmacological properties. Various parts of the plant particularly the bark, heartwood, and leaves are traditionally employed for treating ailments such as diarrhea, cough, skin diseases, and inflammation. Recent studies have highlighted the immunomodulatory potential of *Acacia catechu*. **Objective:** The fifty percent hydromethanolic leaf extract of *Acacia catechu* (ACLE) was prepared and evaluated for its in vitro immunomodulatory potential using animal cell-culture model of avian lymphocytes. *Materials and Methods:* In this study, lymphocytes were isolated from peripheral blood of healthy chickens using density gradient centrifugation. The isolated cells were cultured and treated with varying concentrations of ACLE under standard *in vitro* conditions. Mitogens such as Concanavalin A (ConA) and lipopolysaccharide (LPS) were used to stimulate T and B cell proliferation, respectively. Cell viability and proliferation were assessed using the MTT assay. **Results:** MTT assay revealed a dose-dependent enhancement of lymphocyte proliferation in the presence of the extract. Considering the outcome of MTT assay, 0.3 mg/mL dose was chosen as the maximum non-cytotoxic dose (MNCD) for further immunological evaluations, as it maintained cell viability without inducing cytotoxic effects. Lymphocytes proliferation assay (LPA) demonstrated the immunomodulating potential under mitogenic stimulation. **Conclusion:** *In vitro* experiments using chicken lymphocytes have demonstrated significant immunomodulatory activity. These findings underscore the potential of *Acacia catechu* as a natural immunomodulator in poultry health management, contributing to improved disease resistance and reduced reliance on synthetic antibiotics. Further exploration and validation of such traditional botanicals may support the development of sustainable and effective herbal therapeutics for both veterinary and human applications.

Keywords: *Acacia catechu*, *Senegalia catechu*, Immunomodulation, Chicken lymphocytes, Lymphocytes proliferation assay.

INTRODUCTION

Medicinal plants have long been integral to maintaining the health of both humans and animals, with their benefits extending across multiple sectors, including medicine, agriculture, food, and cosmetics. Historical records from ancient civilizations such as those in India, China, Mesopotamia, and Egypt reveal extensive use of botanical remedies for the prevention and treatment of various diseases [1,2]. Prior to the advent of modern synthetic pharmaceuticals, these plants formed the foundation of traditional healthcare systems, offering natural treatments rich in biologically active substances. Advances in fields like phytochemistry and pharmacognosy have provided scientific validation for many traditional practices, linking specific plant-derived compounds—such as alkaloids, flavonoids, terpenoids, and tannins—to therapeutic effects [3,4]. In recent decades, there has been a marked revival of interest in herbal medicine, often referred to as a "herbal renaissance," driven by a preference for natural remedies, awareness of adverse effects associated with synthetic drugs, and renewed appreciation for traditional ethnomedical knowledge [4].

Acacia catechu (L.f.) Willd (*Senegalia catechu*) (family: Fabaceae), commonly referred to as khair, black cutch, catechu, or cachou, is a deciduous tree native to India and widely distributed across other parts of Asia, including Nepal, Bangladesh, Pakistan, Myanmar, Thailand, and Indonesia, as well as regions of East Africa [5]. Botanically, *Acacia catechu* is a medium-sized tree ranging from 5 to 15 meters in height. Its straight stem is grayish-brown in color, and the bark is dark brown with reddish

inner layers that exfoliate in narrow strips [6]. The leaves are bipinnately compound, comprising 10–30 pairs of pinnae, each bearing 20–50 pairs of small leaflets. The plant also features short, hooked spines and axillary pedunculate spike inflorescences. A decoction made from the heartwood is traditionally consumed as a health beverage in southern India, believed to purify the blood, enhance skin health, and strengthen the immune system [7]. In various traditional medicinal systems, *Acacia catechu* has been employed for its antimicrobial, anti-inflammatory, antidiarrheal, astringent, antimycotic, and coagulant properties [8,9,10,11]. It is also known for its vermifuge action and has been traditionally used to manage conditions such as diabetes, obesity, and to promote wound healing and dental hygiene [8,12,13,14]. The heartwood extract, commonly known as black catechu, is used to treat respiratory conditions such as asthma and bronchitis, as well as gastrointestinal disorders like colic and diarrhea [15]. It is also applied in the treatment of skin ailments, boils, ulcers, and stomatitis [14]. Pharmacological studies have confirmed the bark's anthelmintic, antipyretic, and anti-inflammatory effects [16,17]. Recent research has highlighted the immunomodulatory potential of the bark extract of *Acacia catechu*, suggesting its role in enhancing host defense mechanisms [7,18]. In traditional veterinary medicine, the bark decoction (500 ml) of *Senegalia modesta* (*Accacia modesta*) is mixed with 200 g butter oil and is given orally twice a day for 5 days to cows and buffaloes for easy parturition and for expelling the placenta [19]. Furthermore, in Chinese traditional medicine, a formulation known as "Ercha," derived from the heartwood extract of *Acacia catechu*, is widely used in the treatment of dysentery, Ulcerative colitis [20]. Extracts of the heartwood showed positive results for tannins, terpenoids, triterpenoids, alkaloids, ascorbic acid, and carbohydrates, while leaf extracts also exhibited tannin and saponins [21,2223,24].

The leaves of *Acacia catechu* have been reported to contain significant structural and nutritional components, including hemicellulose, lignin, cellulose, and essential minerals. Phytochemical analysis of its ethanolic leaf extract revealed the presence of various flavonoids such as quercetin, poriferasterol-β-D-glucoside, quercetin-3-O-arabinofuranoside, quercetin-3-O-rhamnoside, and quercetin-3-O-galactoside [25,26]. Considering above, this study aimed to evaluate the *in vitro* immunomodulatory activity of a 50% hydro-methanolic extract derived from *Acacia catechu* leaves (ACLE) using a chicken lymphocyte cell culture model.

MATERIALS AND METHODS

Collection and Preparation of the Plant Extract

Fresh and taxonomically authenticated leaves of *Acacia catechu* were harvested from the Agroforestry Research Centre (AFRC) at Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. A 50% hydro-methanolic extract of the leaves (ACLE) was prepared following the previously described method [23,27]. To maintain consistency and sterility across all downstream applications, only cell culture-grade reagents and solvents were used throughout the experimental procedures.

Evaluation of *In vitro* Immunomodulatory Properties of ACLE

Isolation of Chicken Lymphocytes

Peripheral blood samples were obtained from healthy broiler chickens aged between 4–6 weeks, procured from a local poultry processing facility. Samples were immediately transferred to the laboratory in sterile condition. Lymphocytes were isolated under aseptic conditions using standard density gradient separation techniques to ensure high cell viability. More than 95% cell viability was confirmed via the trypan blue exclusion assay, indicating the integrity of the isolated lymphocyte population. 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) and Lymphocyte Proliferation Assay (LPA)

The cytotoxicity of *Acacia catechu* leaf extract (ACLE) was assessed using the MTT assay, which measures mitochondrial-dependent cellular activity [28,29]. Chicken lymphocytes were seeded in 96-well flat-bottom plates at a density of 1×10^6 cells/mL and exposed to a gradient of ACLE concentrations ranging from 0.001 to 1.6 mg/mL in triplicates following 68 hours of incubation at 40°C in a humidified 5% CO_2 incubator. Subsequently, 20 µL of MTT solution (5 mg/mL) was added to each well, and incubation was continued for 4 hours in the dark. Formazan crystals formed by viable cells were dissolved by adding 200 µL of DMSO per well. Absorbance was recorded at 570 nm using an ELISA microplate reader. Cell viability was expressed as a percentage relative to the untreated control group.

To assess the immunomodulatory potential of ACLE, a lymphocyte proliferation assay (LPA) was performed following established protocols [30]. Chicken lymphocytes (1×10^6 cells/mL) were seeded in 96-well tissue culture plates with or without mitogenic stimulation. The mitogens used included Concanavalin A (ConA) and *E. coli* lipopolysaccharide (LPS), each at a final concentration of 5 µg/mL. The MNCD of ACLE was added to the respective wells, and all treatments were conducted in triplicate to ensure reproducibility and statistical reliability.

Statistical Analysis

Experimental data were statistically analyzed using one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) post-hoc test to compare control and treated groups. Results are presented as mean ± standard error (SE). Pearson correlation analysis was employed to examine associations between variables. Statistical significance was defined at $p < 0.05$. All analyses were performed using SPSS statistical software.

RESULTS

The Plant Extract (ACLE)

The percent yield of fifty percent hydromethanolic plant extract *i.e.*, ACLE obtained was 10.46% that was prepared using dried leaf powder of *Acacia catechu* (Figure 1).

Evaluation of Immunomodulatory properties of ACLE

To determine the maximum non-cytotoxic dose (MNCD) suitable for *in vitro* immunological studies, chicken lymphocytes were exposed to a range of concentrations of the 50% hydro-methanolic extract of *Acacia catechu* leaves (ACLE). The viability of the cells was assessed using the MTT assay, which revealed a dose-dependent cytotoxic effect. Higher concentrations of the extract, particularly between 0.5 mg/mL and 1.6 mg/mL, were associated with a notable decline in cell viability. At the highest tested concentration (1.6 mg/mL), cell viability decreased significantly, showing 32.58% cytotoxicity. In contrast, lower concentrations till 0.3 mg/mL maintained 100% viability of the lymphocytes. Based on these observations, 0.3 mg/mL the highest concentration that did not impair cell viability was selected as the MNCD for subsequent immunological assays.

When lymphocytes were treated with the MNCD of ACLE, a slight increase of 5.33% in overall lymphocyte proliferation was observed compared to the untreated control, indicating potential immunostimulatory activity. Notably, treatment with ACLE enhanced lipopolysaccharide (LPS)-stimulated lymphocyte proliferation by 14.75%, suggestive of augmented B-cell activation and blastogenesis. Conversely, a slight reduction in Concanavalin A (ConA)-stimulated proliferation was noted, with cell viability maintained at 94.15%, indicating a marginal suppression of T-cell responsiveness. These findings suggest a differential modulatory effect of *Acacia catechu*

leaf extract on T and B lymphocytes, with a stronger stimulatory influence on B-cell activity.

Table 1: Immounomodulatory potential due to *in vitro* exposure of CTE in chicken lymphocytes

Treatment	Un-stimulated		LPS stimulated		ConA stimulated	
	Control	ACLE	Control	ACLE	Control	ACLE
% Viability*	100	105.34	100	114.75	100	94.15
Cd value at 1 %	0.046622	4.577562*	0.122522	4.596257*	0.054151	11.65769**
Cd value at 5 %	0.033269		0.080395		0.038642	

Figure 1: *Acacia catechu* plant material and extract. (a) *Acacia catechu*, (b) Dried leaf powder of *Acacia catechu*, (c)Fifty percent hydromethanolic leaf extract of *Acacia catechu*

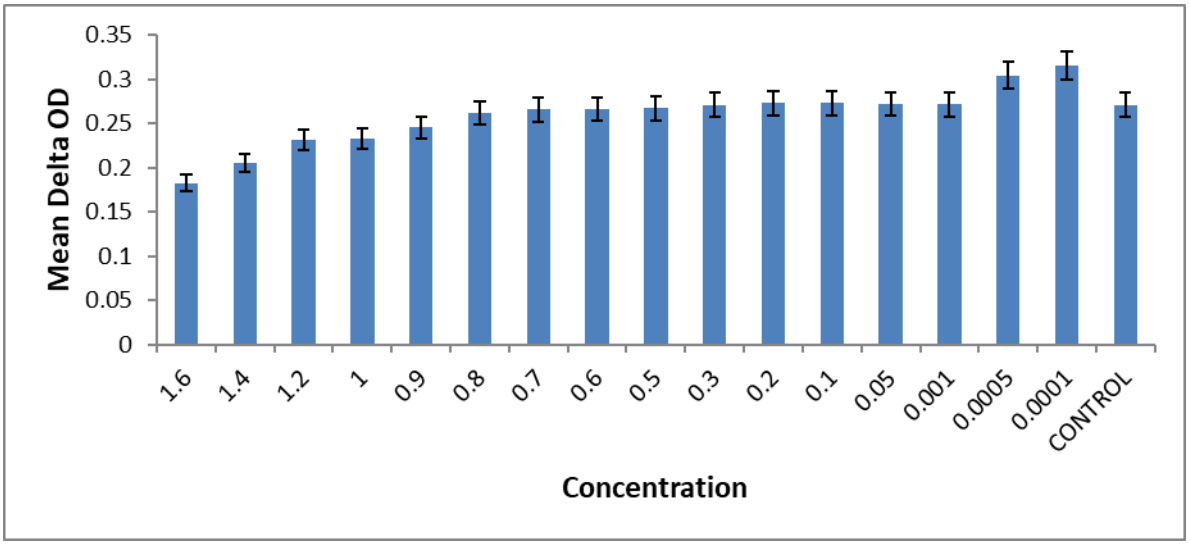

Figure 2: Non-cytotoxic dose of *Acacia catechu* extract by MTT cytotoxicity assay

DISCUSSION

The global resurgence in the use of herbal medicine over recent decades is driven by a growing interest in plant-based therapeutic agents with fewer side effects and broader biological activities. *Acacia catechu* (commonly known as Black Cutch or Black Kutch, khair or kath) is a well-recognized medicinal plant employed in both traditional and contemporary medical systems [31,32]. This species has been used extensively in Ayurvedic and Unani practices for the treatment of various ailments, including diarrhea, cough, ulcers, and skin infections [33]. Phytochemical Composition *Acacia catechu* is notably rich in secondary metabolites such as flavonoids, tannins, polyphenols, and terpenoids. Almost all parts of the plant—including the heartwood, bark, leaves, roots, and flowers—harbor biologically active compounds; however, the heartwood and leaves are particularly pharmacologically significant [14,21,22,24].

The heartwood of *Acacia catechu* is a source of key phytochemicals including catechin, epicatechin, epigallocatechin, catechin tetramers, taxifolin, kaempferol, quercetin, quercitrin, phlobatannin, gossypectin, protocatechuic acid, phloroglucin, and various glycosides and sugars such as L-arabinose and D-galactose [10,21,22,25]. In addition to flavonoids and tannins, GC-MS analysis of leaf extracts has identified terpene compounds such as camphor (76.40%) and phytol (27.56%), which contribute to the plant's antimicrobial, DNA protective and antifungal properties [10,34,35,36]. Further GC-MS profiling revealed the presence of Caprylic acid methyl ester (19.77%), Lauric acid methyl ester (27.42%), 2-ethyl-3-methyl-1-butene (42.09%), and Myristic acid methyl ester (10.72%), highlighting the chemical diversity of the species [23].

The therapeutic potential of *Acacia catechu* is strongly correlated with its antioxidant activity, mainly due to its high phenolic and flavonoid

content. Quercetin, a prominent phenolic flavonoid, plays a significant role in neutralizing free radicals and contributing to the plant's antioxidative defense mechanism [23,37,38]. Taxifolin, another key flavonoid, has demonstrated a broad range of biological activities including antifungal, antiviral, anti-inflammatory, and antioxidant effects [26,38,39]. Taxifolin isolated from the leaf extract has been found to exert potent antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus*, with inhibition zones of 23 mm and 14.5 mm, respectively at 2.5 mg/mL, underscoring its efficacy as an antimicrobial agent [25]. Saha *et al*. [17] reported substantial antioxidant activity in the leaf extract of *Acacia catechu*, suggesting that the pronounced scavenging effects against various reactive oxygen species were due to the rich flavonoid and phenolic content. *In vitro* assays using rat liver post-mitochondrial supernatant systems showed that the extracts of *Acacia catechu* significantly inhibited both the initiation and propagation of lipid peroxidation [40]. The extract also demonstrated potent hydrogen peroxide scavenging capacity [23]. In a related study, Hédi *et al*. [41] observed that *Acacia salicina*, a species of the same genus, also exhibits anti-genotoxic and antioxidant activity, attributed primarily to its sterol and phenolic contents. They noted that the antioxidant potential of leaf extracts was relatively lower compared to bark and heartwood, a finding that aligns with observations in *Acacia catechu*. Similarly, Stohs & Bagchi [16] further substantiated its antioxidant, anti-inflammatory and chemoprotective benefits—key to reducing cellular damage. Our findings displayed *in vitro* immunomodulatory properties of ACLE in cell culture model system of lymphocytes that can be attributed to the presence of various phytochemicals present in the extract. Ismail & Asad [18] and Sunil *et al*. [7] provided compelling *in vitro* and *in vivo* evidence that *Acacia catechu* extracts can modulate both innate and adaptive immunity. The plant also exhibits a variety of other pharmacological effects including antipyretic, hypoglycemic [17,42], antidiarrheal, antiulcer, and hepatoprotective properties [8,42]. Saha *et al*. [17] elucidate the plant's antioxidant capabilities, which may counteract oxidative stress implicated in cognitive disorders. These properties enhance the plant's value as a traditional medicine source.

CONCLUSION

On the basis of the outcome of this study, it can be concluded that the fifty percent hydro-methanolic leaf extract of *Acacia catechu* exhibited significant *in vitro* immunomodulating properties in chicken lymphocytes. There was increase in lymphocytes proliferation under LPS stimulation while under ConA stimulation marked decrease was observed. The wide-ranging pharmacological properties of *Acacia catechu* are largely due to its complex and diverse phytochemical profile. Its antioxidant, antimicrobial, immunomodulatory, and hepatoprotective properties make it a valuable plant for the development of herbal therapeutics. While traditional uses support its medicinal relevance, further advanced analytical and clinical studies are essential to fully explore and validate the therapeutic potential of this important medicinal species.

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Conflict of interest

The authors declared no conflict of interest.

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