The Journal of Phytopharmacology 2024; 13(6):464-469
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2024; 13(6): 464-469
November- December
Received: 16-10-2024
Accepted: 06-12-2024

doi: 10.31254/phyto.2024.13607

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Comparative analysis of *Albizia lebbeck* and *Lawsonia inermis*: Phytochemical estimation and their immunomodulatory effects

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ABSTRACT

Indigenous knowledge holds valuable insights into medicinal plants and their traditional uses. Incorporating products derived from medicinal plants as natural antioxidants could play a crucial role in enhancing immunity and overall health. This study investigates the immunomodulatory effects of two plant hydromethanolic extracts viz., *Albizia lebbeck* (ALE) and *Lawsonia inermis* (LIE), utilizing a chicken lymphocytes culture model. Both plants were extracted using a hydromethanolic method, revealing significant phytochemical constituents, including flavonoids and phenolics. The percent yield of LIE (6.08%) was higher than that of ALE (5.44%). Phytochemical analysis indicated that ALE contained 124 µg rutin equivalents (RE)/mg of extract for flavonoids and 3.93 µg gallic acid equivalents (GAE)/mg for phenolics, while LIE showed 104.5 µg RE/mg and 1.84 µg GAE/mg, respectively. The maximum non-cytotoxic dose (MNCD) was established at 0.4 mg/ml for ALE and 0.2 mg/ml for LIE, beyond which both the extracts exhibited dose-dependent cytotoxicity. Both extracts enhanced T and B cell proliferation, significantly. These findings suggest that ALE and LIE possess significant immunomodulatory properties, potentially offering therapeutic applications in managing immunity-related conditions. Further research is warranted to elucidate their mechanisms of action and establish optimal dosages for clinical use.

Keywords: Immunomodulation, *Albizia lebbeck*, *Lawsonia inermis*, Chicken, Lymphocytes Proliferation assay.

INTRODUCTION

Vertebrates have an extremely complex defence mechanism called the immune system that keeps them safe from invaders. Any alteration in the immune response, including the induction, expression, amplification, or diminution of any component or stage of the immunological response, is called immune system modulation [1]. Thus, an immunomodulator is a substance used to affect the immune system. They can mount an immune response or defend against pathogens or tumours [2]. Plants as immunomodulatory agents have been widely investigated in different parts of the world for their possible immunomodulatory properties [3]. Plant derived compounds were investigated for immunomodulatory properties such as sterols and sterolins, Cannabinoids, polysaccharides, flavonoids, and polyphenols [2]. The Food and Agriculture Organization of the United Nations estimated that more than 50 billion chickens are raised annually as a source of food, for their meat and eggs. Ministry of Agriculture, Department of Animal Husbandry, Dairy and Fisheries, Government of India declared that India with 59.84 billion eggs production ranks third in the world. But poultry industry is surrounded by obstacles such as economic losses due to poultry diseases, capacity to diagnose the causes, of disease in poultry and to recognize an emerging disease rapidly is essential. Application of biogenic (natural) immunomodulators to the feed or water of chickens is a natural potential method of achieving pathogen control without using drugs. Such immunomodulators (nucleotides, beta-glucans, probiotics, and prebiotics) are expected to enhance the development of the intestinal immune system (which is typically immature at the time the chick hatches), thereby improving resistance to intestinal pathogens. Intestinal pathogens of significant economic concern to the poultry industry include *Salmonella* spp (United State Department of Agriculture USDA). Approximately 80% of people in developing countries utilize traditional and folk medicines, according to the World Health Organization (WHO). Plants are still a major source for the development of new drugs, even with the tremendous advances in modern medicine [4]. The two medicinal plants chosen for this study are *Albizia lebbeck* (*A. lebbeck*) commonly known as siras and *Lawsonia inermis* commonly known as mehndi. These plants are widely employed in African and Arab folklore medicine systems as well as the Ayurvedic traditional medical system in India. According to a study, *Albizia lebbeck* demonstrates remarkable properties in the areas of wound healing, nootropics, neuroprotection, anti-inflammatory, anti-cancer, anti-malarial, anti-allergic, antihyperglycemic, anti-diabetic, and anti-Alzheimer [5].

Two novel phenolic glycosides, called Albibrissinosides A and B, have also been identified from the bark, leaves, seeds, and pods of *A. lebbeck* and the stem bark of the plant Albizia. These compounds exhibit cytotoxic activity against cell lines that represent liver, colon, larynx, cervical, and breast cancer [6]. Recent research has demonstrated that the various parts of the plant exhibit a multitude of properties, including anti-inflammatory, antibacterial, antifertility, antifungal, anthelminthic, antiulcer, immunomodulatory, antiarthritic, antispasmodic, antidiarrheal, nootropic, mast cell stabilizer, antitumor, etc. These advancements in the field of study will aid in future research by determining appropriate dosages and forms that will aid in the treatment of numerous diseases that are fatal to humans [7]. *A. lebbeck's* leaf and flower oils have a high concentration of hydrocarbons, with linalool (32.3%) and heneicosaine (23.2%) serving as the primary ingredients, respectively. In West Africa, it's customary to use vaporized steam derived from plant components to treat illnesses and disorders [8]. *Lawsonia inermis* (*L. Inermis*) was found to include carbohydrates, polyphenols, flavanoids, saponins, proteins, alkaloids, terpenoids, quinones, coumarins, xanthones, fat, resin, and tannins according to a phytochemical examination. Moreover, 2-hydroxy-1,4- naphthoquinone (lawsone) was present. Al-Snafi (2019) [9] lists several pharmacological effects, including those that are antibacterial, antifungal, antiparasitic, molluscicidal, antioxidant, hepatoprotective, central nervous system, analgesic, anti-inflammatory, antipyretic, wound and burn healing, immunomodulatory, antiurolithiatic, antidiabetic, hypolipidemic, antiulcer, antidiarrheal, diuretic, and anticancer. The stimulation of T-lymphocyte proliferative responses indicated that the methanolic extract of henna leaves, at a concentration of 1 mg/ml, possessed immunomodulatory properties. Significant immunomodulatory effects were also demonstrated by naphthoquinone derived from leaves [10,11]. Therefore, this study was conducted using a chicken splenocyte model to assess the potential immunomodulatory effects of hydromethanolic leaves extracts of *A. lebbeck* (AIE) and *L. inermis* (LIE).

MATERIALS AND METHODS

Plant collection

Leaf sample of *A. Lebbeck and L. Inermis* used in this study were collected from the Medicinal Plant Research and Development Center (MRDC), GBPUA&T, Pantnagar. Plant material was identified and authenticated by Dr. D. S. Rawat, Department of Biological Sciences, C.B.S.H., G.B.P.U.A.&T., Pantnagar. Fresh leaves were thoroughly washed, shade dried, grounded to powder, and utilized for 50 percent hydromethanolic extract preparation.

Extraction of plant material and sample preparation

Plant material was extracted by the maceration method [12] with slight modification. Briefly, this process is conducted by soaking the 50g of plant materials (coarse or powered) in a closed stopper container in a solvent (water+methanol in 1:1) for 48 hours under continuous agitation at 40ºC in a shaking incubator to obtain plant extracts. At atmospheric pressure, evaporation of solvent is prevented by using a sealed extractor. The goal of the procedure is to liberate the soluble phytoconstituents by breaking down and softening the plant's cell walls. After a certain amount of time, the mixture is filtered or decanted to press or strain it. Filtered material is then evaporated under hot circulating air at 40ºC and residue is taken finally for lyophilization.

Percent Yield of AIE and LIE was calculated after lyophilization as per the following formula:

$$\text{Per cent Yield of the extract} = \frac{\text{weight of the extract after lyophliziation}}{\text{Dry weight of the plant material}} \text{ x } 100$$

Phytochemical analysis

Phytochemical analysis of AIE and LIE was carried out by estimation of total flavonoid and total phenolic content.

Total flavonoid content

Total flavonoid content in AIE and LIE was determined by a colorimetric method [13]. Briefly, 0.5 ml of the extract was diluted with 2.5 ml of distilled water. Then 150 µl of a 5% $NaNO_2$ solution was added, and the mixture was kept at room temperature. After 6 min, 300 µl of a 10% $AlCl_3\_6H_2O$ solution was added, and the mixture was allowed to stand for a further 5 min. After that, 1.0 ml of 1 M NaOH was added. Finally, 0.55 ml distilled water was added to the mixed solution. The final solution was mixed thoroughly and absorbance was immediately measured against the prepared blank (all the reagents except extract which was replaced by distilled water) at 510 nm wave length. Rutin was used as a standard in the concentration range of 200-1000 µg/ml to construct the standard curve. Results are expressed as micrograms of rutin equivalents (RE) per milligram of extract.

Total phenolic content

Total phenolic contents in the extracts were determined by the Folin-Ciocalteu reagent [14]. Gallic acid was used as standard in the experiment in the concentration range of 50 µg/ml – 350 µg/ml. 1 ml of the plant extracts, *viz.* AIE and LIE (200 µg/ml) and standard of different concentrations were mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 (v/v) and 4 ml (7.5% sodium carbonate) of sodium carbonate. For colour construction, the tubes underwent vortexes for a few seconds and then let to stand at 20°C for 30 minutes. The absorbance of samples and standard were measured at 765 nm using a spectrophotometer against blank.

In vitro cell culture

Chicken spleens were collected from healthy chickens from a local slaughter house in sterile Dulbecco's phosphate buffer saline (DPBS), brought to the laboratory, and processed immediately to isolate lymphocytes. Lymphocytes isolated from healthy chicken spleens were cultured in Roswell Park Memorial Institute 1640 media (RMPI-1640) in particular cell concentration ($1x10^7$ cells/ml) that was maintained after viable cell counting by dye exclusion method (trypan blue staining) [15]. Seeding of the cells was carried out as per method described by Creed, *et al.* (2009) [16]. The cells were incubated for 24 hours at 37°c in a CO_2 incubator, providing an optimal environment for cell growth and preparation for further experimental procedures.

Determination of maximum non-cytotoxic dose

The cytotoxic potential of AIE and LIE was evaluated using the MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) assay, which measures cell viability. To determine the maximum non-cytotoxic dose (MNCD), cells were exposed to various concentrations of AIE and LIE in triplicate and incubated for 24 hours at 37°C in a CO_2 incubator. After this period, the media was replaced with MTT dye and incubated for an additional 4 hours in darkness under the same conditions. The resulting formazan crystals were then dissolved using dimethyl sulfoxide (DMSO). Following a 15-minute incubation, the absorbance was measured at 570nm using a computerized micro-scan ELISA reader, allowing for quantification of cell viability [17]. Optical density was measured by microplate reader at the wave length of 570 nm. Using the following formula, the percentage of cell viability was determined:

$$\text{Per cent cell viability} = (\text{treated cells/control}) \text{ X } 100$$

Evaluating immunomodulatory activity by lymphocyte proliferation assay

Immunomodulatory properties of the plant extracts were evaluated through Lymphocytes proliferation assay by exposing cells to maximum non-cytotoxic dose of the AIE and LIE in presence of various mitogens, *viz*. Concanavalin A (Con A), phytohaemagglutinin (PHA) and lipopolysaccharide (LPS). Lymphocyte Proliferation

Assay (LPA) was carried out as per the method described [15]. T cell proliferation was studied by treating the cells with mitogens (Con A and PHA) while lipopolysacchrides (LPS) was used to study B cell proliferation. The isolated lymphocytes were mixed with RPMI 1640 media and the cell density was adjusted to $1x10^7$ cells/ ml. Flat bottom 96 well tissue culture plates were used. Each well was seeded with 200µl of lymphocyte cell suspension and was exposed to MNCD of AIE and LIE separately in presence of various mitogens. AIE and LIE untreated cells with mitogens were kept as respective controls for assessing influence of individual plant extract on cells proliferation.

Statistical analysis

Results were expressed as mean ± standard deviation. Data were analyzed by one-way ANOVA followed by Tukey's test and P values<0.05 were considered statistically significant.

RESULT

Percent yield of extract

Per cent yield of hydromethanolic extract of *Albizia lebbeck* (ALE) and *Lawsonia inermis* (LIE) was calculated. The results exhibited higher yield in case of LIE thanALE. LIE per cent yield was found to be 6.08% while it was found to be 5.44% for ALE (Table 1; Fig. 1).

Total Flavonoid content

Rutin was used as standard for total flavonoid estimation. ALE showed higher flavonoid content that is 124 µg RE/mg of the extract, while in case of LIE the total flavonoid content is 104.5 µg RE/mg of the extract. Total flavonoid content of ALE and LIE is presented in table 2.

Total Phenolic content

Gallic acid was used as standard for total phenolic estimation. In ALE total phenolic content was estimated to be 3.93 µg GAE/mg of extract which was found to be higher than the LIE total phenolic content which was estimated to be 1.84 µg GAE/mg of extract. Total phenolic content of ALE and LIE is presented in table 3.

Non-cytotoxic dose of hydromethanolic extract of *Albizia lebbeck* (ALE) and *Lawsonia inermis* (LIE) in chicken lymphocytes culture system

In order to study the immunomodulatory property of ALE and LIE, experiment was conducted to determine maximum non-cytotoxic doseon chicken lymphocyte culture system by giving exposure of different concentrations of the extract. MNCD of ALE was found to be 0.4mg/ml whereas LIE exhibited dose dependent cytotoxicity beyond 0.2 mg/ml. Results are depicted in figure 2 and 3.

Lymphocyte proliferation assay

Lymphocyte proliferation was studied to determine immunomodulatory potential of ALE and LIE with respect to control. Cells treated with mitogens were used as respective controls for co-treatment of mitogens and individual plant extracts. Compared to respective controls, the plant extracts treated cells displayed higher T and B cell proliferation. LPS treated cells were evaluated for B cells and highest proliferation was found to be 24% in case of ALE exposed cells. ConA and PHA treated cells were assessed for T cells and LIE treated cells displayed higher T cell proliferation of 22.8% when stimulated with ConA whereas ALE displayed increased T cell proliferation of 11.8% when stimulated with PHA (figure 4).

Table 1: Percent yield of hydromethanolic extracts of *Albizia lebbeck* (ALE) and *Lawsonia inermis* (LIE)

S. No.	Plant Extracts	Dry powder (gm)	Weight of hydromethanolic extract (gm)	% Yield
1.	*Albizia lebbeck* (ALE)	50	2.72	5.44
2.	*Lawsonia inermis* (LIE)	50	3.04	6.08

Table 2: Total flavonoid content of ALE and LIE

S. No.	Plant Extracts	Conc. (µg/ml)	Total Flavonoid (µg RE/ mg extract)
1.	*Albizia lebbeck* (ALE)	200	12.4
2.	*Lawsonia inermis* (LIE)	200	10.45

Table 3: Total phenolic content of ALE and LIE

S. No.	Plant Extracts	Conc. (µg/ml)	Total Phenolic (µg GAE/ mg extract)
1.	*Albizia lebbeck* (ALE)	200	39.39
2.	*Lawsonia inermis* (LIE)	50	18.40

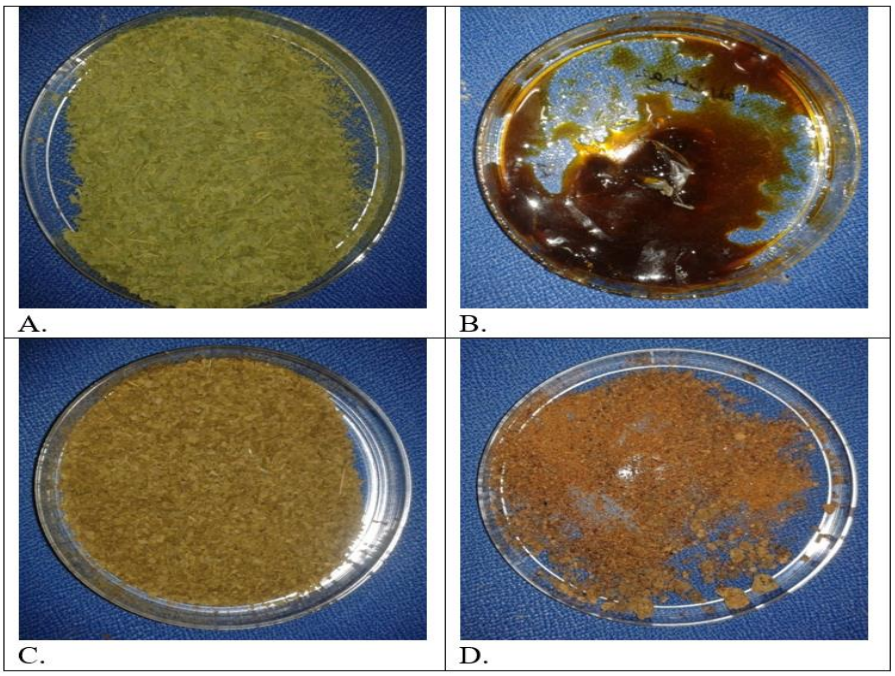

Figure 1: Dried plant leaves powders and hydromethanolic extracts. **A**. *Albizia lebbeck* dried leaves powder, **B**. Hydromethanolic extract of *Albizia lebbeck* (ALE), **C**. *Lawsonia inermis* dried leaves powder, **D**. Hydromethanolic extract of *Lawsonia inermis* (LIE)

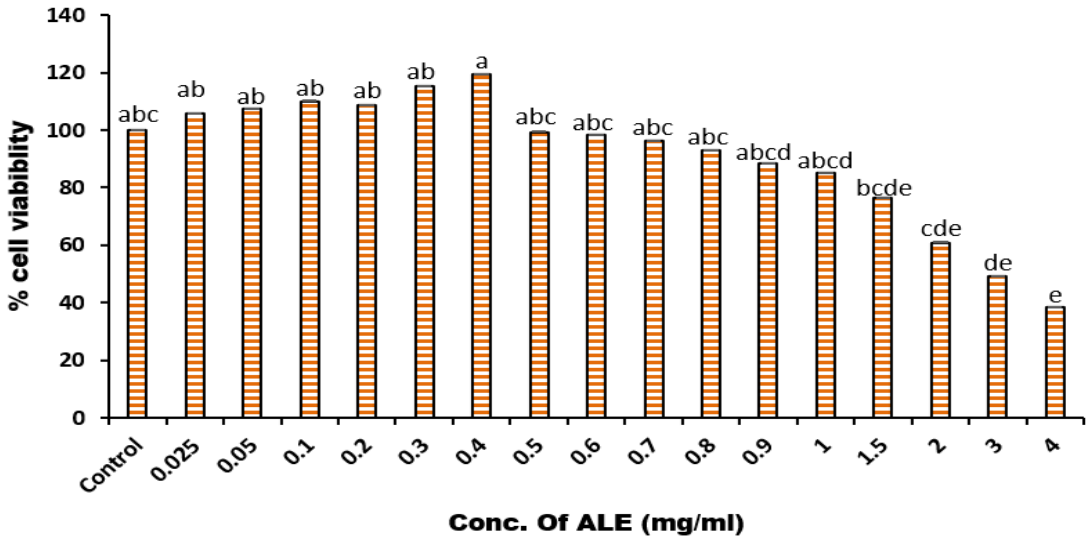

Figure 2: Non-cytotoxic dose of hydromethanolic extract of *Albizia lebbeck* (ALE) on chicken lymphocyte culture system

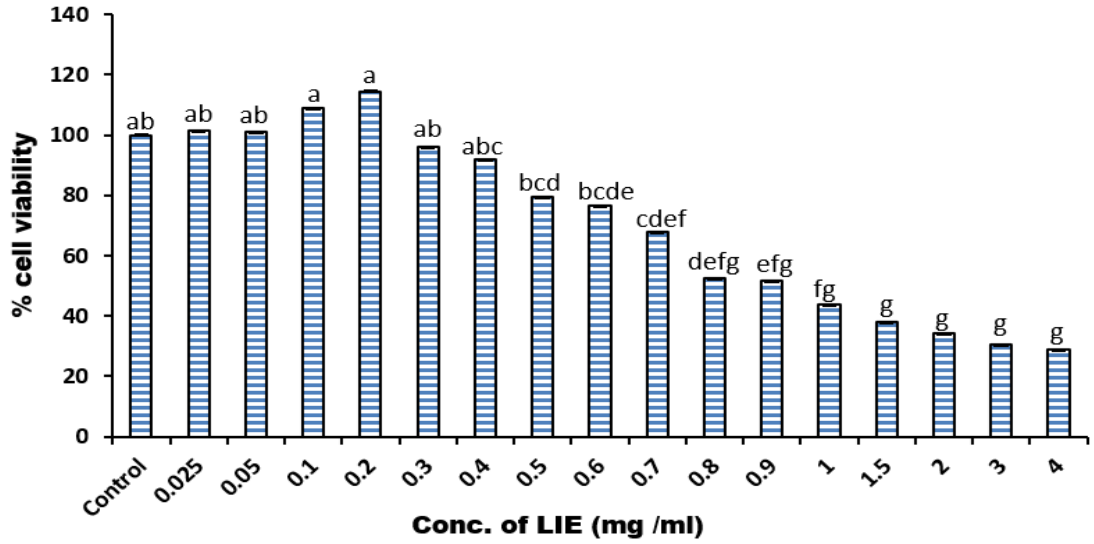

Figure 3: Non-cytotoxic dose of hydromethanolic extract of *Lawsonia inermis* (LIE) on chicken lymphocyte culture system

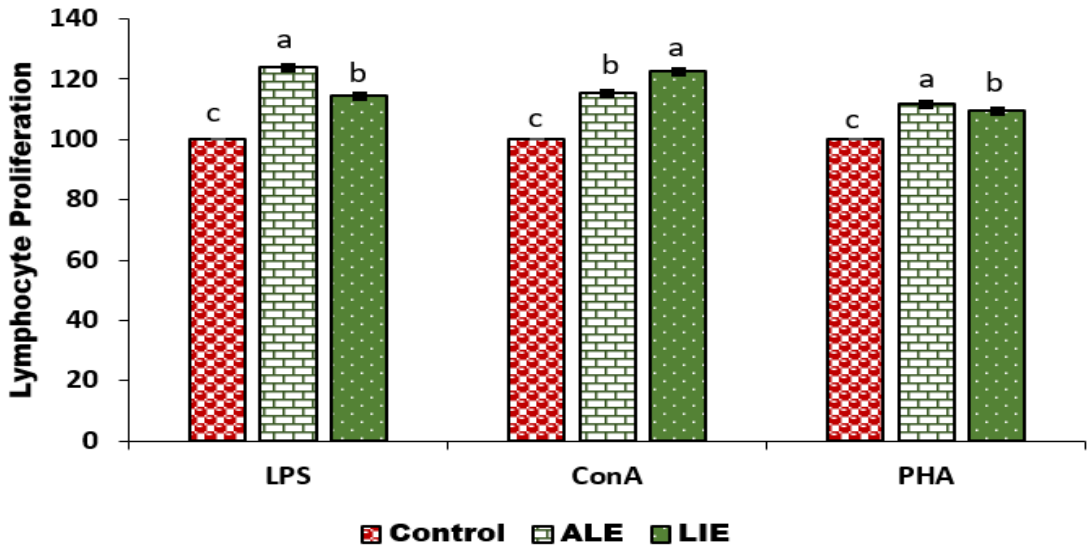

Figure 4: Effect of *A. lebbeck* and *L. inermis* hydromethanolic extracts on lymphocyte proliferation upon mitogenic stimulation

DISCUSSION

Numerous herbal remedies are mentioned in ayurveda and other complementary medicine, but their acceptance and application in improving the general health of people worldwide are still in their infancy. The active ingredients, minerals, vitamins, and oils found in herbal remedies are extremely beneficial to both people and animals. Numerous herbal remedies are employed in medical texts to treat a variety of illnesses, either singly or in combination. One of them, frequently utilized in the Ayurvedic medical systems is *A. Lebbeck* [18]. Alkaloids, anthraquinones, flavonoids, glycosides, phenolics, phytosterol, saponins, steroids, and triterpenoids are among the many of the phytochemicals found in *A. Lebbeck* [19,20]. Apart from that, a study revealed that leaves have a significant amount of essential oil that contains 2-pentylfuran (16.4%), (E)-geranyl acetone (15.46%), (E)-α-ionone (15.45%), and 3-Octanone (11.61%)[8].Worldwide, a wide range of conditions, including anorectal, ocular, gastrointestinal, genital, inflammatory, neurological, oral, respiratory, skin, urinary, and venereal diseases, have been treated using *A lebbeck* [21]. As a result of an injury or infection brought on by a physical, chemical, infectious, or immunological factor, a mammal's live tissue will express an inflammatory response. That's one of the body's natural defence mechanisms [22]. Any modification that affects a protein molecule's three-dimensional shape and causes peptide bond breaking is known as denaturation of protein. Inflammatory diseases like rheumatoid arthritis, diabetes, and cancer have been linked to protein denaturation. Defending against inflammatory illnesses involves preventing protein denaturation [23]. A study conducted in 2016 reported that aqueous and ethanolic leaf extracts demonstrated anti-inflammatory effects at 200 mg/kg, reducing the production of granulomas by 38.55% and 42.33%, respectively, and exhibiting percentage inhibitions of 39.36% and 42.55% in paw edema generated by carrageenan [24]. In an experimental model of bronchial asthma, treatment with *A lebbeck* bark extract at several doses reduced the levels of neutrophils and eosinophils. IgE has a significant role as a mediator in allergic asthma, especially when it comes to the initial reaction to antigen and the spread of airway inflammation in bronchial asthma [25]. Practitioners of traditional medicine have reported that certain bacteria and fungi are particularly dangerous, capable of seriously infecting humans. In the 20th century, a resolution was achieved with the development of antibiotics to fight these infections [26]. Nonetheless, a significant portion of the issue stems from our growing overuse and abuse of already available antibiotics in veterinary and human medicine [27]. Contemporary pharmacological investigations on henna and its components have validated its anti-inflammatory, antipyretic, and analgesic properties while also uncovering its potential to prevent cancer [28]. Two distinct fractions of leaf ethanolic extract were found to have strong anti-inflammatory and antidiabetic properties in a study [29], as a result, the extract, and its phytochemical constituents, which include flavonoids, polyphenols, and alkaloids, may offer an alternative. There have been reports of these chemicals' analgesic and anti-inflammatory properties. Steroids are vital medications that reduce inflammation [16,31]. Tannins, or tannic acid, are water-soluble polyphenols that have strong antioxidant properties and are present in a variety of plants [31,32]. Secondary metabolites called flavonoids have a variety of uses, including being analgesics and antioxidants [33,34]. Therefore, the above findings are consistent with the current analysis that displays the immunomodulatory moreover, anti-inflammatory effects of both the plant extract.

CONCLUSION

The immunomodulatory properties of *Lawsonia inermis*, commonly known as henna, have garnered interest due to its bioactive compounds. Current finding indicates that extracts from *L. inermis* can modulate immune responses, potentially enhancing the activity of immune cells and exhibiting anti-inflammatory effects. These properties may be attributed to various phytochemicals, including flavonoids and tannins, which have demonstrated the ability to influence immune cell proliferation. In conclusion, *Albizia lebbeck* showed significant promise as a natural immunomodulator, with potential applications in the treatment of immunity-related conditions. However, further investigation is essential to fully understand its mechanisms of action, optimal dosages, and safety profiles for further utility of these bioresources.

Conflict of interest

The authors declared no conflict of interest.

Financial Support

None declared.

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HOW TO CITE THIS ARTICLE
Osman SOM, Paliwal L, Ambwani S, Ambwani TK. Comparative analysis of *Albizia lebbeck* and *Lawsonia inermis*: Phytochemical estimation and their immunomodulatory effects. J Phytopharmacol 2024; 13(6):464-469. doi: 10.31254/phyto.2024.13607