

Adjuvanting Property of Aqueous Extract of the Indian Mistletoe, *Dendrophthoe falcata*

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Research Article

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Abstract

The Indian mistletoe, *Dendrophthoe falcata*, has been traditionally used in Ayurvedic medicine for its various therapeutic properties. Adjuvants are substances that enhance the immune response to antigens, thereby improving the effectiveness of vaccines or immunotherapies. In this study, the aqueous extract of *D. falcata* was evaluated for its adjuvanting potential in Nile tilapia, *Oreochromis niloticus*. An aqueous extract (AE) was prepared from the dried leaves of *D. falcata*. The adjuvant activity was assessed by co-administering the extract with a heat inactivated *Streptococcus agalactiae* vaccine in *O. niloticus*. The immune response was evaluated by measuring the serum lysozyme activity and the serum antibody levels. Results showed that the AE significantly enhanced the specific and nonspecific immune responses to the model antigen. It increased the serum lysozyme activity and elicited a robust production of specific antibodies, suggesting a potentiated nonspecific and specific immune response. The adjuvanting property of the aqueous extract of *D. falcata* suggests its potential utility in enhancing the effectiveness of vaccines or immunotherapies.

Introduction

Dendrophthoe falcata, commonly known as the Indian mistletoe, is a parasitic plant belonging to the Loranthaceae family. It is widely distributed throughout various regions of India and has been used in traditional Ayurvedic medicine [1]. Some of the diverse pharmacological activities of *D. falcata* include anti-inflammatory, antioxidant, immunomodulatory [2], and anticancer effects [3]. Its therapeutic potential is attributed to the presence of bioactive compounds such as flavonoids, alkaloids, tannins, and phenolic compounds [4] in their leaves and stems. Given its traditional use and reported pharmacological properties, *D. falcata* has gained attention as a potential source of natural compounds for various applications in medicine, particularly in the field of immunotherapy. Understanding the adjuvanting properties of its aqueous extract can provide valuable insights into its immunomodulatory potential and further support its utilization in vaccine development and immunotherapeutic approaches.

Oreochromis niloticus, commonly known as the Nile tilapia, is often regarded as a good model organism for various research purposes. It shows rapid growth, high reproductive ability, and is easy to breed in captivity. Nile tilapia is known for its broad environmental adaptability, tolerating a wide range of water temperatures and salinities [5]. Nile tilapia is susceptible to various infectious diseases, making it a valuable model for studying fish immunology, host-pathogen interactions, and developing strategies for disease prevention and control. Additionally, its immune system shares similarities with other fish species, allowing for broader insights into fish immunology [6].

Adjuvants are utilized to enhance the efficacy of vaccines by augmenting the immune response. In the context of aquaculture, whole cell inactivated vaccines commonly exhibit a suboptimal immunologic response, necessitating multiple booster doses or the incorporation of adjuvants [7]. Mineral oil and squalene-based adjuvants are commonly employed in aquaculture practices [8]. Adjuvants can sometimes lead to adverse reactions in individuals receiving vaccines. Mineral oils, such as paraffin oil,

have been associated with localized reactions at the injection site, including granuloma formation. Squalene, although well-tolerated, has been linked to disorders like Autoimmune/Inflammatory Syndrome Induced by Adjuvants [9]. Researchers are continuously exploring and developing new adjuvants with improved safety profiles and enhanced efficacy. The search for alternative adjuvants aims to address the limitations and challenges associated with currently used adjuvants like mineral oils and squalene. The present study aims to investigate the adjuvanting activity of the aqueous extract (AE) derived from *D. falcata*, focusing on its impact on serum lysozyme activity and serum antibody response against the heat-killed *Streptococcus agalactiae* vaccine.

Materials and methods

Fish maintenance

A total of 100 tilapia fish were procured from a commercial fish farm located in Chengalpattu, India. The fish were carefully transported to the fish keeping facility at Vels University's Centre for Fish Immunology. Prior to initiating the experiments, the fish underwent an acclimation period to ensure their adaptation to the laboratory conditions. Fish with a weight of 40 ± 5 g and displaying no external signs of illness or abnormalities were selected for the study. Throughout the experimental period, regular assessments of water quality parameters were conducted to maintain optimal conditions for the tilapia. Parameters such as dissolved oxygen levels (4 mg/l), and total dissolved solids (400 mg/l) were measured on a weekly basis. These measurements were found to be in accordance with the guidelines provided by the Food and Agriculture Organization (FAO, 2005) for tilapia aquaculture. Fish were housed in 150 l fibre reinforced plastic tanks fitted with external canister filters (Eheim, Germany) and maintained under a natural light-dark cycle and ambient water temperature ($27 \pm 2^\circ\text{C}$) conditions. The fish were fed commercial tilapia feed (Grobest™, Chennai, India).

Plant material and AE preparation

Whole *D. falcata* plants growing on neem trees (*Azadirachta indica*) were gathered from the premises of Madurai Kamaraj University, Madurai (coordinates: 9.94°N and 78.01°E). The collected plants were authenticated by Dr. Stephen of the Department of Botany at The American College in Madurai, Tamil Nadu, India. To establish a reference, a voucher specimen (Specimen No: CFI MP5) was deposited in the herbarium of the Department of Botany at Lady Doak College in Madurai, Tamil Nadu, India.

AE was prepared by adapting the protocols of [10]. *D. falcata* leaves were handpicked and subjected to a shade-drying process for two weeks. Subsequently, the dried leaves were finely ground into a powder using a blender (Havells, India). To obtain the AE, 20 g of the powdered leaves were soaked in 100 ml of sterile double-distilled water at 4°C with continuous stirring for 24 hours. After 24 hours, the mixture was centrifuged at 5000 rpm for 15 minutes at 4°C to remove debris. The resulting supernatant (approximately 50 ml) was transferred to a 250 ml conical flask, and powdered ammonium sulphate was added until a 70% saturation level was reached, following the protocol outlined by Evans et al. (2009). Gentle stirring with a magnetic stirrer for 2 hours at 4°C facilitated the precipitation of the desired

components. The resulting precipitate was collected by centrifugation at 2500 rpm for 10 minutes at 4°C, yielding a pellet that was subsequently dissolved in 15 ml of sterile Tris-buffered saline (TBS, pH 7.6) containing 50 mM trisaminomethane, 100 mM sodium chloride, and 10 mM calcium chloride.

A 10 kDa centrifugal ultrafiltration unit (Amicon, Cork, Ireland) was used to concentrate the AE, and TBS buffer exchange was carried out to remove residual ammonium sulphate. Subsequently, the concentrated AE was passed through 0.2 µm syringe filters, resulting in 2 ml of the concentrated AE and stored at -20°C till further use (HiMedia, Mumbai, India).

Heat inactivated vaccine preparation

A colony of *S. agalactiae* was aseptically inoculated into tryptone soya broth and incubated at 37°C overnight with continuous agitation. Following the overnight incubation, the bacterial culture was subjected to heat killing by immersing it in a water bath set at 60°C for a duration of 1 hour. Subsequently, the heat-killed bacteria were pelleted by centrifugation at 3000 rpm for 15 minutes, followed by three washes with TBS. The resulting pellet of bacterial cells was resuspended in TBS to achieve the desired concentration of 10^9 cells/ml and called as the vaccine.

Administration of adjuvanted vaccine to fish

To explore the degree of adjuvant efficacy, the vaccine was mixed in a 1:1 dilution with either AE (in different dilutions of 1:1, 1:10, and 1:100 v/v in TBS) or Freund's complete adjuvant (Sigma, Bengaluru). The prepared adjuvanted vaccine formulations were administered intraperitoneally to four groups of fish (n = 6). Furthermore, an unadjuvanted vaccine group was maintained and used as a negative control for comparative analysis.

Blood collection and serum separation

Blood is collected from the common cardinal vein of the vaccinated fish according to our earlier procedures [2]. The blood was allowed to clot at 4°C for 24 hours and then centrifuged at 2000 rpm for 15 minutes at 4°C to obtain the serum. Serum samples were stored at -20°C till further use.

Serum lysozyme activity

The measurement of serum lysozyme activity was conducted in microplates following the methods outlined by Hutchinson and Manning, 1996 [11], with slight modifications outlined in our previous protocol [12]. Test sera samples (10 µl) were combined with *Micrococcus lysodeikticus* (Sigma, Bengaluru, India) in a 96-well U-bottomed microplate. The absorbance at 490 nm was then assessed at 0.5 and 4.5 minutes using a microplate reader (Bio-Rad, Hercules, USA). A reduction in absorbance of 0.001/min corresponded to one unit of lysozyme activity.

ELISA

The ELISA was performed following the procedures described by Binuramesh et al., 2005 [13] which in turn is an adaptation of procedures initially outlined by Delamare et al., 2002 [14]. First, 96 well plates

(Nunc, Roskilde, Denmark) were coated with *S. Agalactiae* at a concentration of 10^8 cells/ml in carbonate-bicarbonate buffer and incubated overnight at 4°C. Following the coating, diluted tilapia antisera (1:10 in PBS) obtained from the experimental fish were added to the plates. The plates were then incubated at 37°C for 1 hour. Subsequently, the plates were washed three times with PBS-Triton X100 (PBS-T, HiMedia, Mumbai, India). Next, anti-tilapia Ig antisera (1:100) raised in rabbits, produced in our laboratory, was added to the plates, and incubated at room temperature for 1 hour. Following this incubation, the plates were washed three times with PBS-T. An HRP-conjugated goat anti-rabbit IgG antibody (1:10,000) from Sigma (Bengaluru, India) was then added to the plates. The plates were incubated at room temperature for 1 hour and subsequently washed three times with PBS-T. To initiate the colour development, 100 µl of 3,3',5,5'-Tetramethylbenzidine (Sigma, Bengaluru, India), was added to each well and incubated for 2 minutes. The reaction was stopped by adding 25 µl of 2 N sulfuric acid, and the optical density of the wells was measured at 450 nm using a microplate reader (Bio-Rad, USA).

Result

Lysozyme activity

Figure 1 shows the changes in serum lysozyme activity of fish sera following the administration of the AE adjuvanted vaccine. In the first week after injection, an increase in lysozyme activity was observed. Notably, both the high and mid doses of the vaccine exhibited a significant elevation in lysozyme activity. The positive control group (FCA) also displayed a significant increase in lysozyme activity. However, no noticeable difference in lysozyme activity was observed between the treated and control groups at two- and three-weeks post-vaccination.

ELISA

Figure 2 presents the antibody response of the injected fish during the three weeks following the administration of the vaccine. Notably, a significant increase in antibody response was observed after 3 weeks post-vaccination. The antibody response in all doses of AE was significantly higher than that observed in the unadjuvanted control group. The positive control group treated with FCA exhibited a significant elevation in antibody response at this time point. However, no significant difference in antibody response was observed during the initial two weeks post-vaccination.

Discussion

Adjuvants play a crucial role in enhancing the efficacy of vaccines. However, in the context of farmed animals, more potent adjuvants are needed as the currently available vaccines, such as whole cell inactivated vaccines, often fail to elicit sufficient protective immunity [15]. Existing options like mineral oil and alum have certain limitations, necessitating the exploration of novel adjuvants [16]. One major concern is the potential for adverse effects associated with adjuvants, including local reactions at the injection site or systemic reactions. Moreover, specific formulation techniques are often required to

maintain the stability and effectiveness of adjuvants, thereby adding complexity and cost to the vaccine manufacturing process. Additionally, adjuvants do not have universal effectiveness, as certain antigens may require specific adjuvants or formulations to elicit the desired immune response [17]. To address these challenges, ongoing research and development endeavours are focused on improving the safety, efficacy, and versatility of adjuvants. Advances in adjuvant technology aim to overcome these limitations and optimize their role in vaccine development and implementation [18, 19]. By addressing these concerns, scientists aim to develop more effective and safer adjuvants that can significantly enhance vaccine effectiveness in farmed animals [20].

Lysozyme is an enzyme that plays a role in the innate immune response and acts as a natural antimicrobial agent. It is found in various bodily secretions and tissues, including blood, tears, and saliva [21]. Adjuvants can activate the nonspecific immune cells, such as macrophages and dendritic cells, leading to the production of various immune mediators and cytokines [22, 23]. In this study, lysozyme activity increased several folds in the AE adjuvanted fish. Studies have shown that adjuvants can enhance lysozyme production or increase lysozyme activity in fishes [24–26]. The activation of immune cells by adjuvants can result in the release of lysozyme and other antimicrobial peptides as part of the immune response. The interaction between adjuvants and lysozyme is an active area of research, and further studies are needed to fully understand the specific mechanisms and implications of this relationship. However, adjuvants can modulate the immune system, which can affect the production and activity of lysozyme and other components of the immune response.

The antibody response serves as an important endpoint measure when evaluating the efficacy of adjuvants. Our study reveals a significant increase in antibody response among the adjuvanted groups after 3-week post-vaccination. Several studies show that adjuvants primarily exert their effects through the depot effect, which is the enhancement of the persistence of the antigen within the system. Additionally, there is evidence showing that adjuvants can augment the antibody response by stimulating various immune cells [27]. Earlier research has shown that adjuvants can enhance the antibody response in fish when used in conjunction with heat-killed bacterial vaccines [28]. However, it is important to note that the results obtained in our study are preliminary, and further investigation is needed to elucidate the specific mechanisms by which AE functions as an adjuvant. The current findings provide initial insights, but a more comprehensive understanding of AE's mode of action requires additional research.

Several plant extracts have been proven to enhance the lysozyme activity and antibody response in fish [29]. It has been observed that polysaccharides [30], and terpenoids (saponins) [31] are the primary components responsible for this effect [32–34]. Many of these compounds were also found to be present in *D. falcata* [35]. However, in this study, the compounds present in AE were not purified or characterized, making it challenging to predict the specific compounds responsible for the observed adjuvanting activity. Further investigation is necessary to identify and characterize the active components within AE and understand their role in enhancing the immune response.

Conclusion

The injection of AE adjuvanted *S. agalactiae* vaccine resulted in a significant increase in lysozyme activity and antibody response in fish. These findings highlight the potent adjuvant properties of AE and suggest its potential as an effective adjuvant. AE offers an environmentally friendly alternative to conventional vaccine adjuvants, chemotherapeutics, or antibiotics, thereby aiding in the mitigation of disease issues in aquaculture. To establish AE as a reliable adjuvant for aquaculture vaccines, further studies are needed, including field trials involving various fish species and pathogens. Similarly, for human vaccination, AE must undergo additional characterization and testing through clinical trials. The utilization of advanced proteomics and genomics techniques could expand our understanding of AE and its potential applications in the field of vaccination. In conclusion, the results obtained thus far demonstrate the promising adjuvanting capabilities of AE and highlight its potential for use in aquaculture and beyond. Continued research and evaluation are necessary to fully harness its benefits and explore its broader applications in vaccine development and disease prevention.

Declarations

Conflict of interest

The authors unequivocally state that there are no conflicts of interest pertaining to the research, data collection, analysis, and publication of this paper. They confirm that no financial support was received from any organization that might have a vested interest in the study's outcomes. Furthermore, all authors have thoroughly reviewed and granted their approval for the final manuscript's publication.

Use of language model

ChatGPT's role in this manuscript is strictly limited to providing language assistance and should not be regarded as a traditional author. The authors diligently review and curate the responses generated by ChatGPT to guarantee their accuracy and relevance. They assume full responsibility for verifying the content, upholding scholarly integrity, and complementing ChatGPT's answers with authenticated data and authoritative sources whenever required.

The study protocol was approved by the Centre for Advanced Research and Development (CARD) at Vels Institute of Science, Technology, and Advanced Studies (VISTAS). Collection of blood samples from fish was carried out by the researchers, following established ethical regulations and guidelines. The procedures for sample collection ensured adherence to animal welfare considerations and were in line with the ethical considerations in fish research as outlined by Sloman et al. (2019).

Reference

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Figures

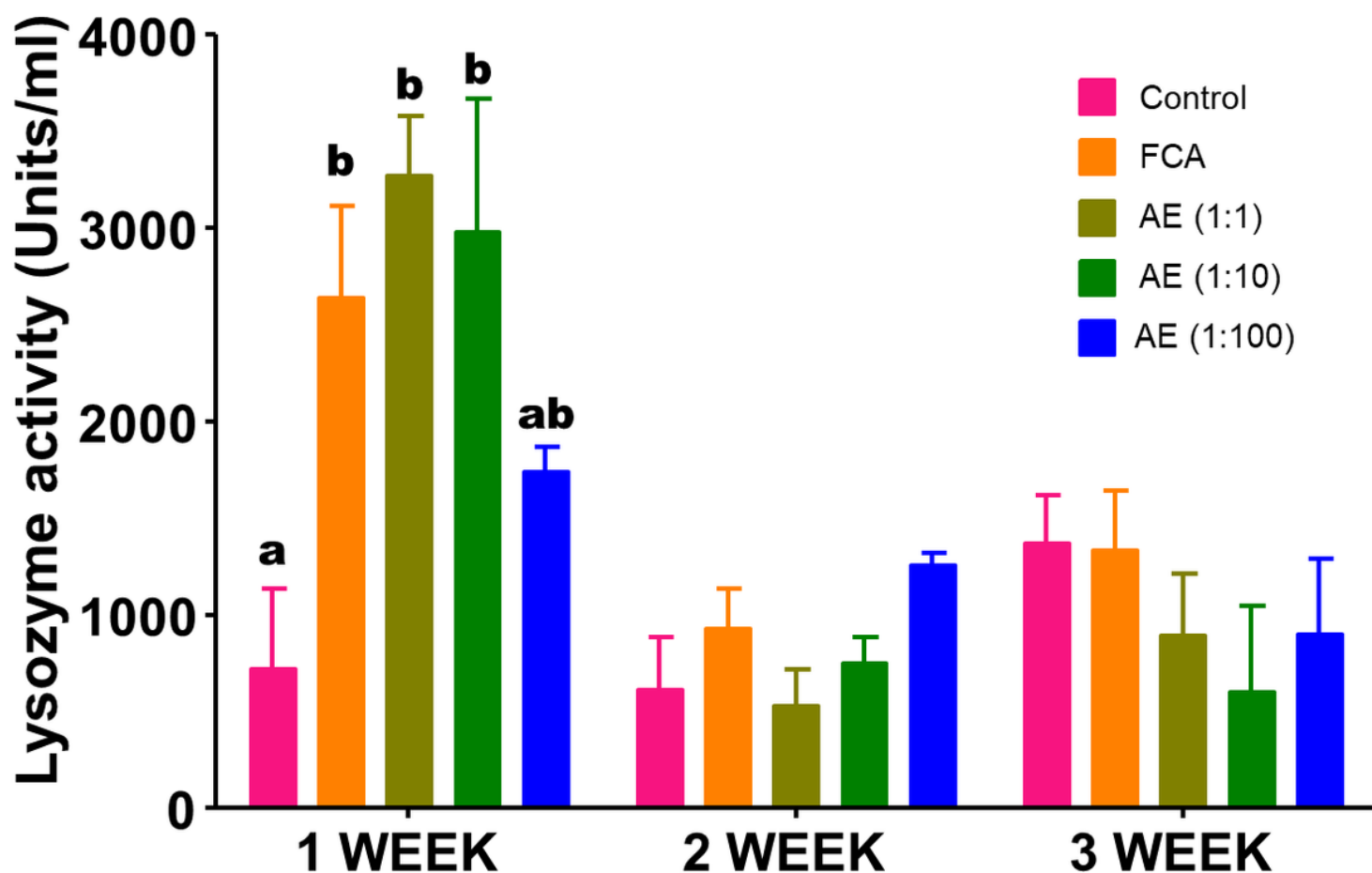


Figure 1

Lysozyme activity of fish injected with AE adjuvanted *Streptococcus agalactiae* vaccine. Each column represents mean $\pm$ SE of data obtained from 6 fish in triplicates. Different alphabets represent significant difference between the means ($p < 0.05$).

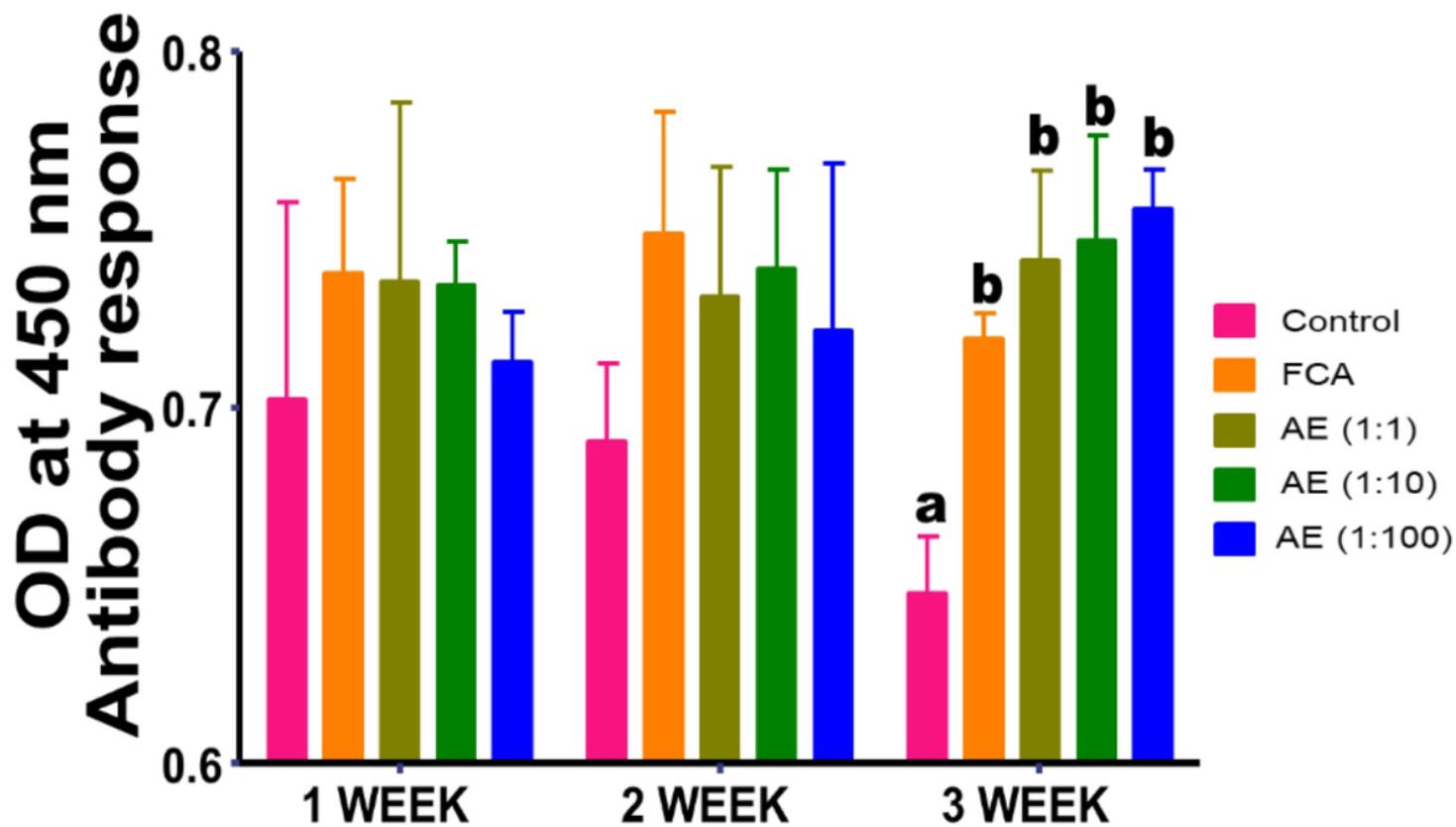


Figure 2

Antibody response of fish injected with AE adjuvanted *Streptococcus agalactiae* vaccine. Each column represents mean $\pm$ SE of data obtained from 6 fish in triplicates. Different alphabets represent significant difference between the means ($p<0.05$).