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IMMUNOSTIMULATORY EFFECT OF *Laurus nobilis* LEAF POWDER ON *Labeo catla* AND ITS RESISTANCE AGAINST *Aeromonas hydrophila*

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ABSTRACT

The present study was conducted to evaluate the effect of bay laurel (*Laurus nobilis*) leaf powder on haematology, immune response and disease resistance of *Labeo catla* fry against *Aeromonas hydrophila*. Feeding trial was conducted for 60 days where the fish were fed with five different diets supplemented with *L. nobilis* leaf powder at the rate of T0 (control diet 0%), T1 (0.5%), T2 (1%), T3 (1.5%), and T4 (2%) followed by challenge study. The protein level in all the diets was maintained at approximately 30%. *L. catla* fry (initial weight: 0.4±0.03g) were stocked at twenty rectangular plastic tanks having dimension of 2×1×1 feet with a stocking density of 15 nos./tank. The result showed that at the end of the feeding trial, haematological and immunological parameter such as total erythrocyte count (1.64±0.007), total leukocyte count (28.05±0.132) and respiratory burst activity (0.23±0.009) were found to be significantly higher ($p<0.05$) at T3. In the challenge study against *A. hydrophila*, the treatment T3 showed significantly ($p<0.05$) higher relative percentage survival (64.28%). The results suggests that supplementing *L. nobilis* leaf powder at 1.5% in the diet of *L. catla* significant ($p<0.05$) enhance haematology, immune response and resistance against *A. hydrophila*.

Keywords **:** Aquaculture, *Labeo catla*, *Laurus nobilis*, haematology, immune response, *Aeromonas hydrophila*.

Introduction

Aquaculture is one of the fastest growing foods producing sector in the world. It plays a crucial role in addressing the global demand for food and high-quality animal protein. This sector not only contribute to food security and livelihood but also support the economic development in many countries. To meet the global demand for food, aquaculture has shifted from the extensive or traditional farming system to semi-intensive and intensive systems to increase the productivity from the available resources (Magouz *et al*., 2021).

Unfortunately, this intensive culture system where the fish are culture under high stocking density and dependence on formulated feed under crowded condition has adversely affect the fish immune system and has make them susceptible to infectious agents (Dawood *et al*., 2020). The disease outbreak in the farm poses a major threat to the fish health affecting the farm productivity and the economic sustainability. With the outbreak of disease in the farm, there arise a need to improve the immunity of fish and its ability to resist infection (Cristea *et al*., 2012). In order to boost the health status of the fish, feed additives are required but the use of this feed additive has concern for the public health (Caipang *et al*., 2019).

Under the stressful farming conditions of fish, a wide range of infectious disease exists which affect the aquaculture productivity and profitability (Ahmadifar *et al*., 2019). *Aeromonas hydrophila* is one of the dominant infectious pathogens which is responsible for mass mortality of fish in farms every year (Jiang *et al*., 2012). *A. hydrophila* is a free-living opportunistic pathogen that can cause higher level of infection in fish when fish are in stress condition or with poor immunity which can lead to heavy mortality of fish in the aquaculture farm (Harikrishnan & Balasundaram, 2005). *A. hydrophila* is a gram-negative bacterium with anaerobic rod shape in structure and they do not form spore (Roberts *et al*., 1996). The infection by these bacteria in fish cause symptoms such as dropsy, haemorrhagic septicemia, exophthalmia, rotting in the region of fins and tail (Austin & Austin, 1993).

In order to control the infection, antibiotics are overused by the farmers and this continuous unregulated use of antibiotics has led to resistance against the pathogenic bacteria leading to concern about the development of antimicrobial resistance among the fish farm and also their effect on environment and safety to the consumers (Sutili *et al*., 2018). Considering this, the aquaculture industry has a growing attention in using medicinal plants as alternatives to antibiotics and chemotherapeutics in diseases prevention and treatment (Awad and Awaad, 2017). The herbal medicines are reported to have essential nutrients such as high content of protein, lipids, amino acids, vitamins and other components that could enhance the growth and metabolic activity of fish and other aquatic animals (Lin *et al*., 2006; Yousefi *et al*., 2020).

Labeo catla is the fastest growing species among the three Indian major carp growing upto 1.0 to 1.5 kg in one year. It is considered as an integral component in polyculture of carp in the country. It attains maturity in the second year of its life (Raizada, 2019). As Catla is a surface feeder, it plays an important role in carp polyculture system as they primarily consume the plankton present in the upper layer which reduces the competition with the other species of fish feeding upon the column and bottom layer of the water body.

The bay laurel (*Laurus nobilis*) commonly known as bay leaf is a plant belonging to the family Lauraceae. The bay laurel plant is a multibranched shrub or tree that can grow upto 10 m in height and is a plant of industrial importance that are used in foods, drugs and cosmetics. Chemically they contain sesquiterpene lactones such as 10-epigazaniolide, gazaniolide, spirafolide, costunolide, reynosin, santamarine, flavonoid glycosides and essential oil. The bay laurel plant has been reported to possess wound healing, neuroprotective, antioxidant, antiulcerogenic, anticonvulsant, antimutagenic, antiviral, anticholinergic, antibacterial and antifungal properties (Patrakar *et al*., 2012; Ramling *et al*., 2012).

Materials and Methods

Experimental Set-Up

A 60-days feeding experiment was conducted at the Department of Aquaculture, College of Fisheries Science, Kamdhenu University, Veraval, Gujarat, India. The experimental design consists of 5 treatments with each treatment having 4 replicates and twenty rectangular plastic tanks having dimension of about 2×1×1 feet with 50L capacity of water was used.

Bay Leaf Powder

The procured bay leaf was sun dried and grounded into fine powder in grinder and sieving was done so as to get uniform sized particles. The prepared ingredients were then stored in air tight container.

Experimental Fish

Healthy seed of *Labeo catla* having average initial body of (0.4 ± 0.03) were procured from the Aqua hatchery, Surendranagar, Gujarat, India. The seed was transported in oxygen filled polyethylene bags and was acclimatized upon arrival at the experimental site.

Experimental Feed Formulation

The concentration of different composition of the feed ingredients was formulated using Pearson's square method to ensure the dietary protein content of 30%. The treatment diet T0 was prepared without the bay leaf powder. The other treatment diets, T1, T2, T3 and T4 was prepared with the ingredient containing 0.5%, 1%, 1.5%, 2% bay leaf powder. The required quantities of all the ingredient were accurately weight according to the composition requirements for the five experimental diets and mixed together (except bay laurel leaf powder & vitamins-minerals mix) thoroughly with the addition of small quantity of water to form a dough.

The prepared dough was steam-cooked after which the dough was allowed to cool down for a short period of time. Then the required amount of bay leaf powder and vitamin-mineral mix for each treatment was added and mixed well to make the final dough. The final dough was then processed using hand pelletizer to form pellet feeds and was allowed to dry. The prepared dried experimental feed was then packed separately in air tight container and was labelled and stored at the room temperature in cool and dry place.

Table 1: Composition of experimental diet

Ingredients	Composition (%)				
	T0	**T1**	**T2**	**T3**	**T4**
Bay laurel	0	0.5	1	1.5	2
Sterilized fish meal	30	30	30	30	30
Groundnut oil cake	39	39	39	39	39

Wheat flour	15	14.5	14	13.5	13
Tapioca powder	12	12	12	12	12
Fish oil	1	1	1	1	1
Sunflower oil	1	1	1	1	1
Vitamins & minerals	2	2	2	2	2
Total	100	100	100	100	100

Experimental Procedure

The experimental fish was fed twice a day with the prepared experimental feed at the rate of 5% per body weight per day. Cleaning of the tank and regular exchange of water was carried out to maintain the water quality parameter at the ideal range.

Haematological Parameters

Total Erythrocyte Count (TEC)

Total erythrocyte count was estimated following the standard procedure using a Neubauer haemocytometer (Shah, 2010). Blood was drawn into RBC pipette upto 0.5 mark. It was then dilute with Hayem's solution (1:200 dilution) and was shaken gently for proper mixing. The prepared sample was loaded in Neubauer haemocytometer and the erythrocyte count was carried out under a compound microscope.

Total Leukocyte Count (TLC)

Total leukocyte count was determined using a Neubauer haemocytometer (Shah and Altindag, 2005). The experimental blood sample was drawn into WBC pipette upto 0.5 mark. It was then dilute with Turk's solution (1:20 dilution). The content was allowed to mix by gentle shaking and was loaded in Neubauer haemocytometer chamber to carry out the leukocytes count.

Respiratory Burst Activity

Respiratory Burst Activity was carried out using Nitroblue tetrazolium (NBT) assay (Secombes and Fletcher, 1992). 0.1 ml of the collected blood was mixed with 0.2% NBT solution and was incubate at room temperature for 30 minutes. After incubation, 1 ml methanol was added and was mixed gently and centrifugation was done at 3000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed 2-3 times with methanol. After washing, 1 ml each of 2 M KOH and DMSO was added ad mixed thoroughly in order to dissolve formazan. The prepared sample was then measured in spectrophotometer at 540 nm.

Challenge Study Against *Aeromonas hydrophila*

After the 60 days culture period, challenge study of *L. catla* fry against *A. hydrophila* was evaluated for 10 days. Ten fish from each treatment group was randomly selected and was injected with 0.1 ml *A. hydrophila*. The dead fish was removed and the daily survival rate for each treatment group was documented.

Preparation of *Aeromonas hydrophila* Active Stock Culture

A. hydrophila (MTCC 1739) subspecies hydrophila was obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. The bacteria were incubated in 1.5% tryptone soya broth in a shaker incubator for 12 hours at 37 °C. The culture was harvested at 0.9 optical density (OD) at 680 nm and was centrifuged at 3000 × g for 10 min. The bacterial pellet was prepared by washing three times with sterile phosphate buffer saline (PBS, pH 7.2). The standard plate count using ten-fold serial dilution was performed following Surendran *et al.* (2003).

Determination of LD_{50} of *Aeromonas hydrophila*

Lethal dose (LD_{50}) for the experimental fish was determined following Reed and Muench (1938). The 10 Catla fry per tank was maintained in aquarium glass tank which were provided with aeration. 0.1ml *A. hydrophila* was injected to each fish using 1 ml sterile syringe with varying dose ranging from 10^3 to 10^8 CFU/ml. Regular feeding was done throughout the 10 days observation period. The dead fish was removed daily and mortality of the fish was recorded for 10 days.

Challenge Study

After the 60 days culture period, the challenge study was carried out in triplicate by injecting 0.1 ml of *A. hydrophila* suspension (LD_{50}= 1.76 × 10^6 CFU/ml) to 30 fishes each, 10 fish in each replicate, taken from treatment tank T1, T2, T3, T4. In the control treatment (T0) where the feed was made without bay leaf powder, the fish were injected with 0.1 ml phosphate buffered saline (PBS). Fed was given and for 10 days, mortality was recorded. The relative percentage survival (RPS) was estimated following the procedure of Amend et at. (1981).

$$RPS = 1 - \left(\frac{\%\ mortality\ of\ treatment}{\%mortality\ of\ control}\right) \times 100$$

Statistical Analysis

The data was analysed using the standard statistical methods and was applied the Completely Randomized Design (CRD) experimental design (Snedecor & Cochran, 2014). The data obtained was analysed by using IBM SPSS version 22 software and all the results were evaluated by one-way Analysis of Variance (ANOVA). The significant difference between the treatments was analysed by Duncan's multiple range tests (DMRTs) at $p < 0.05$.

Results and Discussions

Dietary Effect of Bay Laurel (*Laurus Nobilis)* Leaf Powder on Haematological and Immunological Response of *Labeo Catla* Fry

After the feeding period of 60 days, the analysis of total erythrocyte count, total leukocyte count and respiratory burst activity was conducted to evaluate the properties of immune responses exhibited by supplementation of bay laurel leaf powder on the fry of *L. catla*.

Total Erythrocyte Count (TEC)

The analysis reported the highest count in the fish fed with the treatment diet T3 (1.64 ± 0.007^{a}) which were followed by T2 (1.62 ± 0.007^{a}), T1 (1.59 ± 0.006^{b}), T4 (1.57 ± 0.005^{b}) and T0 (1.53 ± 0.009^{c}). The results of the statistical analysis indicate that the treatment T3 (1.64 ± 0.007^{a}) and T2 (1.62 ± 0.007^{a}) exhibited higher significant difference ($p<0.05$) when compared with the other treatments but are not significant ($p>0.05$) with each other. T1 (1.59 ± 0.006^{b}) and T4 (1.57 ± 0.005^{b}) also showed higher significant ($p<0.05$) then the control treatment T0 (1.53 ± 0.009^{c}).

Overall, the study shows that the total erythrocyte count was enhanced when bay laurel leaf powder was supplemented in the diets of *L. catla* as shown in Table 2 and Figure 1 respectively. This increased in TEC may be due to enhancement in oxygen carrying capacity and also improvement in physiological condition of fish.

Similar results were found by Swamy *et al*. (2023) in Nile Tilapia (*O. niloticus*) where the fish fed with diet supplemented with *L. nobilis* leaf powder at varying concentration of 0, 10, 15, 20 and 25 g/kg for 90 days and the highest erythrocyte count was found in fish fed with 15 g/kg *L. nobilis* leaf powder supplemented diet group while the lowest count was found in fish fed with the control diet.

Total Leukocyte Count (TLC)

The total leukocyte count was significantly ($p<0.05$) higher in the treatment T3 (28.05 ± 0.132^{a}) then the other treatments. The highest total leukocyte count was observed in T3 (28.05 ± 0.132^{a}) followed by T2 (26.55 ± 0.065^{b}), T1 (25.78 ± 0.048^{c}), T4 (25.60 ± 0.041^{c}) and T0 (24.28 ± 0.085^{d}) as shown in Table 2 and Figure 2 respectively. No significant difference was found between the treatment T1 (25.78 ± 0.048^{c}) and T4 (25.60 ± 0.041^{c}). The observed higher TLC in treatment T3 and other bay laurel diet supplemented group indicate stimulation of the immune system which may be due to immunomodulatory effect of the diet.

Similar to the finding in the present study, Ananda *et al*. (2019) reported that the incorporation of bay laurel (*L. nobilis*) leaf powder to the diet of rohu (*L. rohita*) resulted in increase in the erythrocyte and leukocyte count and the highest count was observed in fish fed with treatment diet containing 1.5% bay laurel leaf powder.

Further report by Swamy *et al*. (2023) in Nile Tilapia (*O. niloticus*) where diet supplemented with *L. nobilis* leaf powder at different concentration of 0, 10, 15, 20 and 25 g/kg was fed for 90 days suggest that the highest leukocyte count was reported in fish fed with 15 g/kg *L. nobilis* leaf powder supplemented diet group while the lowest was found in fish fed with the control diet.

Respiratory Burst Activity (NBT assay)

In this study, the maximum value of NBT activity was reported in fish fed with treatment diet T3 (0.23 ± 0.009^{a}) and the minimum value of NBT activity was observed in treatment T0 (0.14 ± 0.004^{e}) as shown in Table 2 and Figure 3. Significant difference ($p<0.05$) was reported in all the treatment. There was higher significant difference ($p<0.05$) observed in the treatment T3 (0.23 ± 0.009^{a}) which indicate enhanced immune response. This observed enhancement in NBT activity indicate the improvement in respiratory burst activity of the phagocyte which reflects improvement in non-specific immunity.

The results from the present study for the haematological and immunological analysis shows dose-dependent pattern where improvement was observed up to T3 after which there was slight decline at higher level of inclusion of the supplemented leaf powder. In this present study, the observed results indicate that T3 represents the optimum dietary inclusion level of *L. nobilis* leaf powder in *L. catla.*

The observed results align with the work conducted by Shehata *et al*. (2025) where the dietary inclusion of *L. nobilis* leaf extract (LNLE) results in the enhancement of NBT activity in all the LNLE supplemented groups when compared with the control treatment. Swamy *et al*. (2023) also reported similar results where the inclusion of *L. nobilis* leaf powder at 15g/kg in Nile Tilapia (*O. niloticus*) shows the highest observed NBT and the lowest was observed in control group. However, Bilen and Bulut (2010) found no significant effect on respiratory burst activity in rainbow trout when they were fed with diet supplemented with 0.5% and 1% bay laurel powder.

Dietary Effect of Bay Laurel (*Laurus Nobilis)* Leaf Powder on Challenge Study Against *Aeromonas Hydrophila* in *Labeo Catla* Fry.

After the 60 days culture period, challenge study against *A. hydrophila* was carried out to study the mortality rate (%) and Relative percentage survival (RPS) when supplemented with bay laurel leaf powder in the diets of *L. catla* fry. The highest mortality rate was observed in control treatment T0 (46.67±3.333[a]) while the lowest mortality was observed in T3 (16.67±3.333[c]). The highest RPS was reported in treatment T3 followed by T2, T1, T4 and T0 as shown in Table 3 and Figure 4 respectively.

The results indicate that T3 (16.67±3.333[c]) shows higher significant difference than the other treatment group. No significant difference (p>0.05) was observed between the treatment T0 (46.67±3.333[a]) and T4 (40.00±0.000[a]) and also between the treatment T1 (26.67±3.333[b]) and T2 (26.67±3.333[b]).

The present study coincides with the results reported by Ananda *et al*. (2019) where the diet of rohu (*L. rohita*) were incorporated with bay laurel (*L. nobilis*) leaf powder which results in increase resistance against *A.* hydrophila in the challenge study.

Similarly, Swamy *et al*. (2023) reported that in Nile Tilapia (*O. niloticus*) when supplementation of *Laurus nobilis* leaf powder in the fish diet at different concentration (T0, T1, T2, T3, T4) of 0, 1, 1.5, 2 and 2.5 % for 90 days, the highest survival after the challenge study against *A. hydrophila* was observed in treatment T2 (1.5 g/kg) followed by T1, T3, T4 and T0. Moreover, Yousefi *et al*. (2025) reported that the dietary inclusion of *L. nobilis* essential oil at 50 mg/kg and 100 mg/kg enhance the survival of Nile Tilapia (*O. niloticus*) when challenged with *A. hydrophila.*

Table 2 : TEC, TLC and NBT assay of *Labeo catla* recorded in different treatment after 60 days (n=4, M±SE)

	T0	T1	T2	T3	T4
TEC	1.53±0.009[c]	1.59±0.006[b]	1.62±0.007[a]	1.64±0.007[a]	1.57±0.005[b]
TLC	24.28±0.085[d]	25.78±0.048[c]	26.55±0.065[b]	28.05±0.132[a]	25.60±0.041[c]
NBT assay	0.14±0.004[e]	0.18±0.005[c]	0.21±0.003[b]	0.23±0.009[a]	0.16±0.005[d]

Table 3 : Mortality rate (%) and Relative percentage survival rate (RPS) of *Labeo catla* after the challenged study against *Aeromonas hydrophila* (n=3 {10}, M±SE)

	T0	T1	T2	T3	T4
Mortality %	46.67 ±3.333[a]	26.67 ±3.333[b]	26.67 ±3.333[b]	16.67 ±3.333[c]	40.00 ±0.000[a]
RPS	0.00	42.85	42.85	64.28	14.29

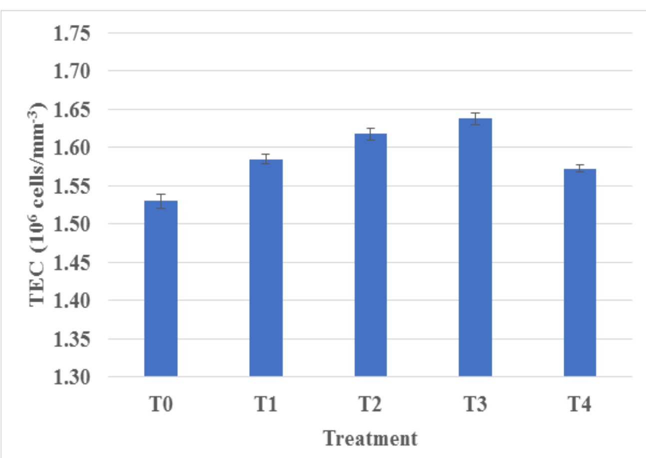

Fig. 1 : Total erythrocyte count (10^6 cells/mm^{-3}) of *Labeo catla* recorded in different treatment after 60 days (n=4, M±SE)

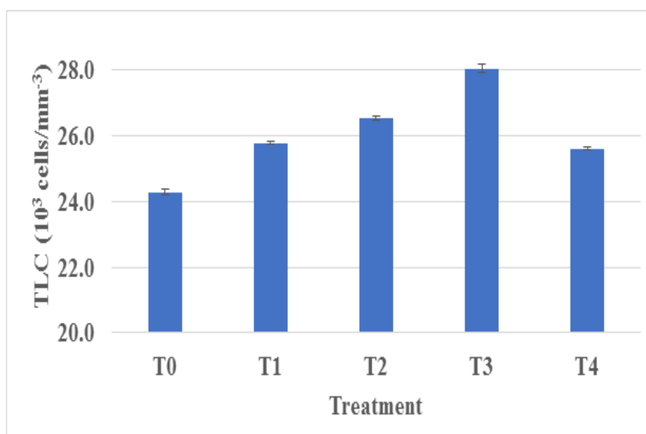

Fig. 2 : Total leukocyte count (10^3 cells/mm^{-3}) of *Labeo catla* recorded in different treatment after 60 days (n=4, M±SE)

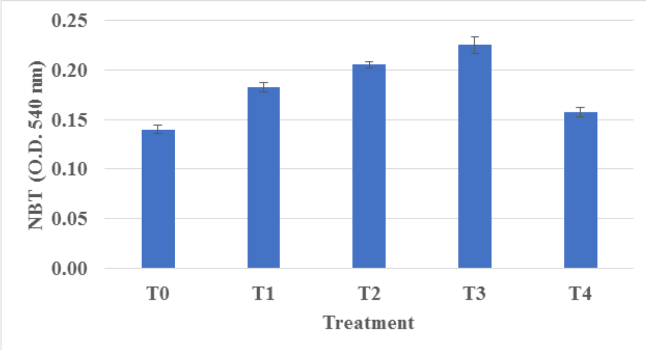

Fig. 3 : Respiratory burst activity (NBT assay) of *Labeo catla* recorded in different treatment after 60 days (n=4, M±SE)

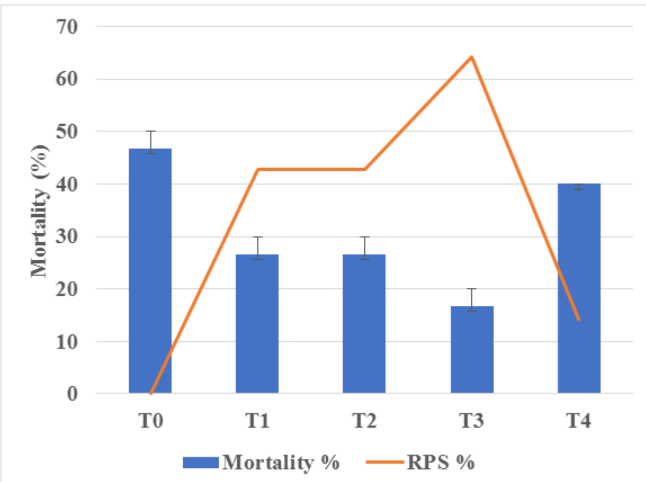

Fig. 4 : Mortality rate (%) and Relative Percentage Survival rate (RPS) of *Labeo catla* after the challenged study against *Aeromonas hydrophila* (n=3 {10}, M±SE)

Conclusion

The supplementation of *L. nobilis* leaf powder in the diet of *L. catla* resulted in significantly higher values of TEC, TLC and NBT which suggest that the supplementation of *L. nobilis* leaf powder at 1.5% (T3) had positive effect on immunity and health status of the fish. The observed higher relative percentage survival in the challenge study further indicates that the fish in treatment T3 had better resistance against disease which could be due to improvement in immune status and overall health condition of the fish.

It can be concluded that the inclusion of *L. nobilis* leaf powder at 1.5% enhance the immunity and disease resistance of *L. catla*. The study highlights the use of *L. nobilis* leaf powder as potential immunostimulant for enhancing the health status and overall productivity in sustainable culture of Catla.

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Ethical Approval

All experimental procedures involving live fish were conducted following standard guidelines for the care and use of laboratory animals in aquaculture research. Appropriate measures were taken to minimize stress and discomfort throughout the experimental period under the supervision of the Department of Aquaculture, College of Fisheries Science, Kamdhenu University, Veraval.

Conflict of Interest

The author declares no conflict of interest.

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