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Efficacy of Doob grass, *Cynodon dactylon* against white spot syndrome virus in *Penaeus monodon*

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Abstract
White spot syndrome virus (WSSV) has been considered one of the deadliest pathogens of penaeid farmed shrimp. A doob grass, *Cynodon dactylon* was tested against deadly white spot syndrome virus *Penaeus monodon*. The trial was to optimize the crude extraction and to mix the powder to the shrimp feed. The obtained formulation was tested against WSSV at a small scale in bioassay challenge laboratory, medium scale trial in cages and large-scale trial in the commercial ponds. The trial showed heat deactivates the active ingredient/s present in *C. dactylon*. The formulations using 150 g/kg of feed and 200 g/kg of *Cynodon dactylon* in feed gave 100% survival. Similar results were obtained in the cage trial. Large scale trials were conducted in 60 commercial ponds of the same size (area 5,000 m2 and depth 1.2 m), shape and locations. The treatment ponds' productivity was higher (12.7 ton/ha) than that of the control ponds (11.79 ton/ha). WSSV cases were not recorded in the control and treatment ponds. This study showed that *C. dactylon* extract has anti-WSSV properties and has no negative impact on shrimp growth.

Keywords: WSSV, *Cynodon dactylon* plant extract, bioassay trial, cage trial, pond culture

1. Introduction
White spot syndrome virus (WSSV) is considered as the most pathogenic for the cultured shrimp which cause substantial economic losses in the shrimp culture industry worldwide [1, 2, 3]. It causes high mortality in the shrimp. The mortality rate often reaches 100% in 4-7 days in the laboratory and 10-14 days in the ponds [3]. The presence of circular white spots on the shrimp cephalothorax's inner side is generally considered the principal clinical sign of this disease.

Several groups of researchers have been continuously working on exploring the solution for the deadly virus. Various methods, like, development of a recombinant vaccine [4] an immunostimulant [1, 5], and natural herbs, such as *Sargassum polycystum* extracts [6], the fruit of *Duriozibethinus* [7] and the root bark of *Psidium guajava* [8] proven successful at laboratory scale.

The Doob grass, *Cynodon dactylon* is widely available all over the world. This herb is well known for its characteristics to purify the blood and cure anuria, biliousness, conjunctivitis, diarrhoea and gonorrhoea [9]. (Balasubramanian *et al*., 2008 [1] identified the anti-WSSV properties in the ethanolic extract of *Cynodon dactylon*. The extraction method of *C. dactylon* found unaffordable for the shrimp industry. A series of trials were conducted to simplify the extraction process, mixing it with the raw material of feed in the feed mill and optimize the maximum tolerant temperature to keep the active ingredient of *Cynodon dactylon* viable. The produced feed showed its efficacy against WSSV at lab scale, and in the cages. Trial in commercial culture ponds showed that *Cynodon dactylon* enriched feed was palatable, enhanced productivity, and reduced the incidence of WSSV without damaging the water quality parameters.

2. Materials and Methods
2.1 Study Area
The study was conducted in the Disease Research Centre, Bandar Lampung of P.T. Central Pertiwi Bahari. The bioassay and cage trials were conducted in a biosecure facility, whereas the pond trial was conducted in a commercial level research farm.

2.2 Preparation of anti-WSSV formula from *Cynodon dactylon*

The *Cynodon dactylon* formula was prepared using two methods: the ethanolic extraction method and crude powdered extract. The ethanolic extract was top-dressed on the feed and used initially in the laboratory trials. The crude powdered extract was prepared by grinding *C. dactylon* dried in the shade and mixing in the feed as raw material during production.

2.3 Preparation of *Cynodon dactylon* powder*: Cynodon dactylon* (leaf and shoot) was collected from the fields of P.T. Central Pertiwi Bahari, Lampung. The clean and fresh specimens were dried in the shade for 7-10 days. The dried grass was carefully screened to discard mould and other abnormalities. The dried material was powdered using an electrical blender. The powder was sieved, weighed, packed in sealed bags and stored in a cool place for further use. *C. dactylon* powder was used to conduct the trials on the cage and commercial pond levels.

2.4 Feed preparation using crude powdered extract of *C. dactylon*

The experimental feed was produced in a feed mill, P.T. Central Proteina Prima, Surabaya. Two doses of the powdered extract, including 150 g/kg of feed or 15% and 200 g/kg of feed or 20%, were mixed. The treatment feed was named RAV-S 15% and RAV-S 20%. The required concentration of the extract powder was mixed with other raw materials of the feed, and all components were mixed together in a feed mill. The feed was produced at lower temperature (80 °C) to avoid degeneration of the active ingredients of *C. dactylon*.

2.5 Bioassay challenge of feed prepared using *C. dactylon* before and after heating

The *Cynodon dactylon* powdered extracts were divided into two groups, one was stored at room temperature and the second one was heated at 85 ^{0}C for 30 minutes in the oven. The powders were used to produce feed and use during bioassay trial.

2.6 GCMS detection of bioactive compounds of *C. dactylon*

Detection and quantification of bioactive compounds of *C. dactylon* were performed by gas chromatography-mass spectrometry analysis by the improved method of [10]. GCMS was performed at P.T. Charoen Phokphand Indonesia, Jakarta. The powdered samples of *Cynodon dactylon* (prepared as described above) were mixed with the solvents (MeOH, ACN and hexane) by adding 0.1 g dried sample per 2 mL of a solvent. The mixture was sonicated at room temperature for 30 mins and injected into GCMS. The mass spectra obtained by GC-MS were assigned by comparing the result with the databased on the reports of the literature.

Two sets of grass powders were taken, one prepared at room temperature and the second one, heated at 85^{0}C. Both were tested for the ingredients present to compare before and after heating. During feed production, it was made sure that processing temperature should not exceed 80^{0}C in all feed types.

2.7 WSSV infected tissue preparation for bioassay *per os* challenge

WSSV-infected shrimp were collected, and the presence of the virus was confirmed. The head, shell and gut were removed, and the muscle was cut into small pieces by scissors and finely minced using a sterilized manual blender. The obtained semisolid gel-like tissue was filtered through an 80µm filter and recovered. The minced muscle containing WSSV was homogenized, and RT-PCR determined the presence and levels of the virus. WSSV-containing minced meat was packed in small blocks and stored at -20 °C before use. The tissue was thawed gradually by moving to warmer temperature (from -20 °C to 4 °C and finally to 18-20-25 °C). The complete procedure took 4-5 hours.

2.8 Laboratory scale experiment

The laboratory-scale experiment was set-up using plastic tanks for the experimental and control groups. Monodon shrimp, the average weight of 2.5 g was collected from the Marine Research Centre, Lampung, and each tank contained 20 animals. The plastic tank was cleaned, sun-dried, and disinfected with 70% alcohol. Each tank was filled with chlorinated marine water obtained from C.P.B. hatchery and provided with the required dissolved oxygen (D.O.) supply. The water temperature was maintained at 25±1 °C. The water salinity was maintained at 20 ppt; pH was 7.6-8, dissolved oxygen was 5.1-6 ppm, and water exchange was performed at a rate of 20% daily. Siphoning was performed once daily.

2.8.1. Bioassay challenge for temperature and active ingredient identification

In the negative control group, shrimp were fed standard pellet feed on regular monodon CP-P feed throughout the experimental period. In the positive control group, the shrimp were fed regular monodon pellet feed. Four sets of treatment feed were produced, two doses of *C. dactylon* extracts were tried, 15% (RAV-S 15%) and 20% (RAV-S 20%). The extracts were mixed in the feed during feed production as raw material. The control feeds were not added any *C. dactylon* extracts.

2.8.2. Challenge method: Experimental shrimps were challenged *per os* with 1% WSSV-infected muscle.

2.8.3. Post challenge observation: The behaviour, feeding rate and cumulative mortality during the trial were monitored in the challenged shrimp. The feeding rate and tank bottom siphoning schedule were the same for the control and treatment groups throughout the experiment. Uneaten food and waste matter were removed before feeding. The experimental animals were examined twice daily for gross signs of the disease, and the number of deaths was recorded. Dead and moribund shrimp were removed.

2.9. Cage trial

2.9.1. Trial set-up: The shrimp were divided into five groups of 40 shrimp per cage or 40 pieces per m3, and each group was tested in three replicates. The trial duration was 45 days, including ten days of acclimatization and 14 days of feeding; at day 15, shrimp were challenged with WSSV; feeding and observation were continued for ten days starting from day 16. The details of the method are as follows.

2.9.2. Cage and water preparation: Water for the experiment was prepared by the standard procedure of culture pond preparation. Three cages in each group (1 m^3 size and 4 mm mesh size) were set in 150 m^2 ponds. The water depth was maintained at 1 m.

2.9.3. Experimental groups: Specific pathogen-free (SPF) shrimp groups were stocked with juvenile shrimp (average weight of 4 g) at 40 animals/m^3. The treatment and control groups were located in different ponds.

2.9.4. Treatment and control feed: Cage trials were conducted using feed was produced by mixing with the *C. dactylon* powder (15% and 20%). The regular feed was used as the control feed.

2.9.5. Challenge method: Shrimp were challenged *per os* with 1% WSSV-infected muscle. No artificial food was given on the day of the challenge. Artificial food was used starting from day 16.

2.9.6. Post challenge observation: Monitoring of the challenged shrimp was performed similar to that in the lab experiment. The behaviour, feeding rate and mortality rate were monitored.

2.10. Pond level trial

2.10.1. Trial Set-up: The 60 units, 30 units in two rows, each row for control feed and RAV-S 20% feed of commercial ponds in the WSSV-prone area in Lampung were selected for the trial and observation. The ponds had a size of 5,000 m2 and full HDPE lining. P.T. Central Pertiwi Bahari's standard operating procedure was followed in pond preparation, water preparation, and culture. Sludge and barnacles were removed from the bottom, washed, sun-dried and disinfected with 100 ppm chlorine. Water for the experiment was treated with one ppm CuSO4, one ppm Pondfos (dichlorvos) and 20 ppm (active) chlorine. The SPF shrimp were stocked at a density of 40 animals/m2 in 60 ponds**.** Autotrophic culture system with low water exchange and high aeration was practised. Water was maintained at 120 cm depth during the culture with an average 20% daily water exchange. The treatment and control feed were produced on the same day at a feed mill (PT. Central Proteina Prima, Surabaya) to avoid dissimilarities.

3. Results

3.1. Analysis of *C. dactylon* extract by GCMS

Identification and quantification of individual phenolic compounds before and after heating at 85 ^{0}C of the ethanol extract of *C. dactylon* was performed by GCMS analysis (Figure1a and 1b). The results indicated that few of the ethanolic fraction, acetonitrile fraction, methanol fraction, and hexane fraction of *C. dactylon* were missing after heating.

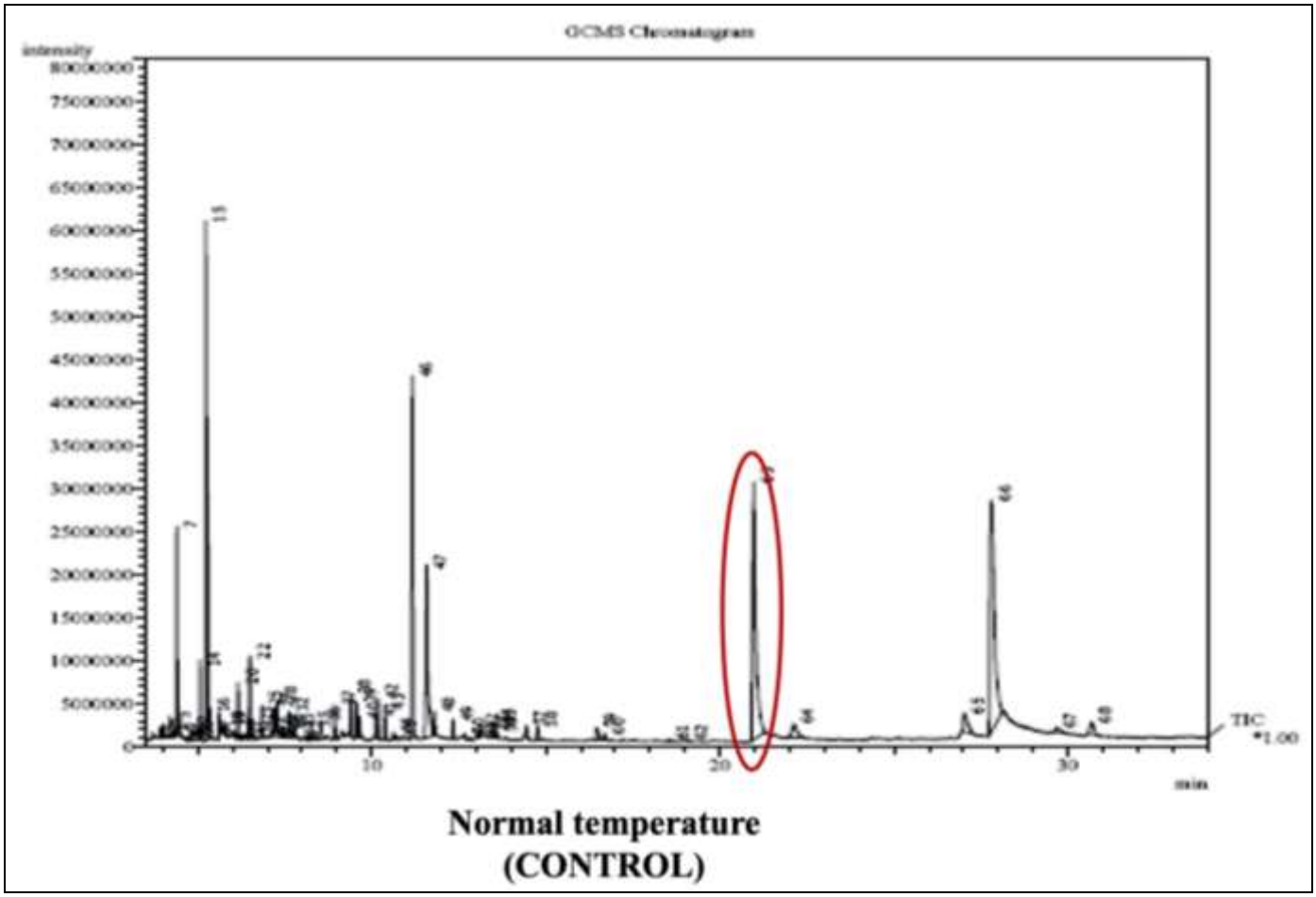

Fig 1a: *GCMS chromatogram of Cynodon dactylon* extract showing fraction peaks at normal (30^{0}C) temperature. The peak in circle shows the original presence of ingredients in the C. dactylon before heating at higher temperature.

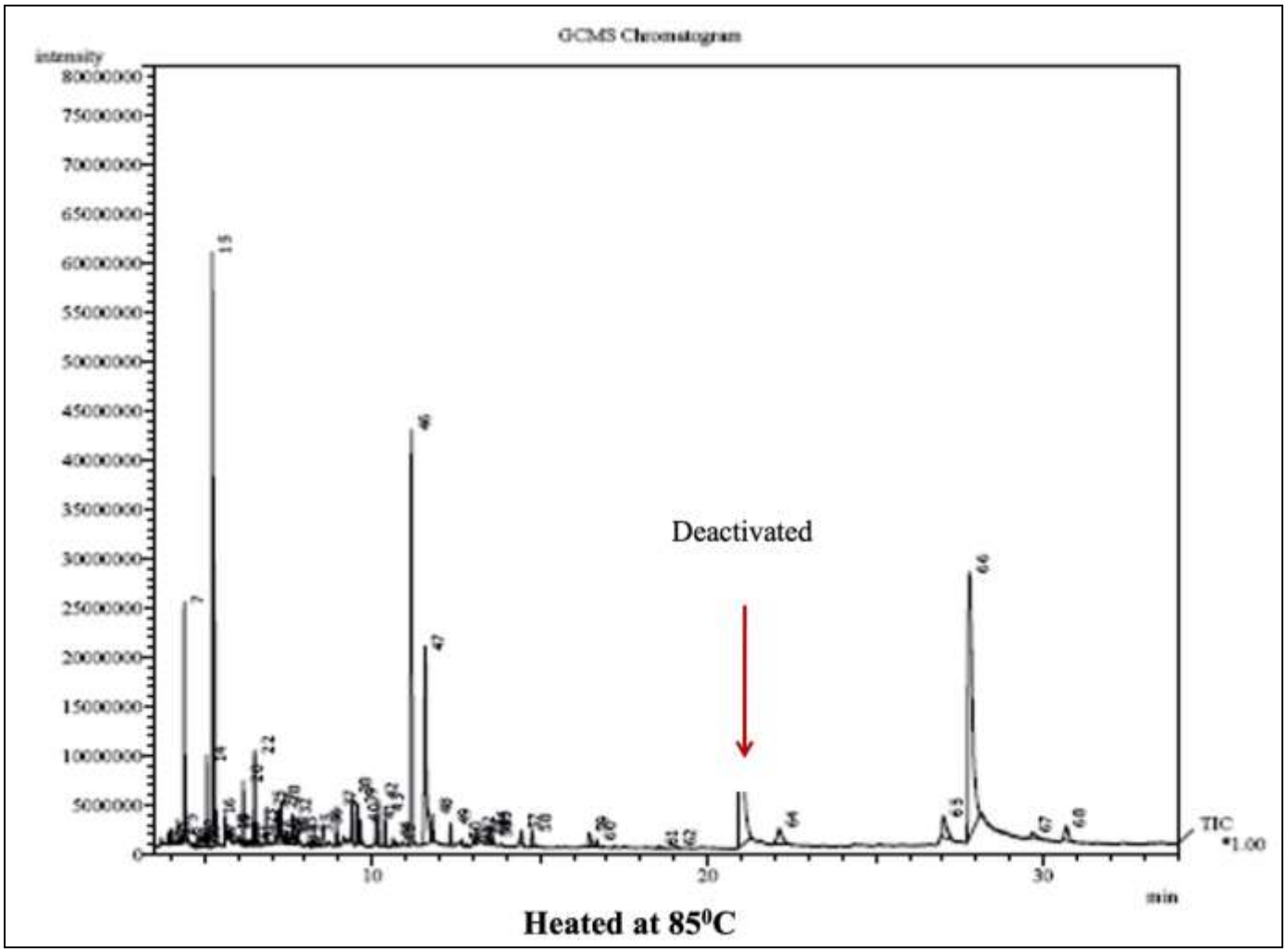

Fig 1b: GCMS chromatogram of *Cynodon dactylon* extract showing fraction peaks at higher (85 ^{0}C) temperature. The red arrows indicate the disappearance of active ingredient peak after heating at 85 ^{0}C which indicates the non-effectiveness of feed produced above 85 ^{0}C.

A clear pick could be seen missing after heating of the *Cynodon* extract in GCMS chromatogram. The number of potential compounds marked were about 246 in number, acetonitrile fraction 36, methanol fraction 117 and hexane fraction 103. Any of them or combination of certain compounds have anti-WSSV effect.

3.2. Bioassay trial using feed produced by normal *C. dactylon* extract and heated *C. dactylon* extracts.

This was proven by conducting bioassay trials using not heated (30 ^{0}C) and heated (85 ^{0}C) cynodon. The non-heated cynodon feed showed efficacy and protected the shrimp from WSSV infection whereas the heated didn't show any protection (Tables 1a and 1b).

Table 1a: Cumulative mortality percentage (%) of control feed and treatments feeds i.e., RAV-S 15% and RAV-S 20% fed group of shrimps in lab trial. The feed was produced at 80 ^{0}C. The data shows that treatment feed was effective against WSSV.

Experimental Tan	Group	Population	Day Post Infection (%) / Cumulative Mortality percentage										
			0	1	2	3	4	5	6	7	8	9	10
B3		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B11		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B19	RAV-S 15%	20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Average			**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
B4		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B12		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B20	RAV-S 20%	20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Average			**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
B6		20	0,0	0,0	0,0	22,2	44,4	77,8	100,0	100,0	100,0	100,0	100,0
B14	Positive Control	20	0,0	0,0	0,0	5,6	72,2	100,0	100,0	100,0	100,0	100,0	100,0
B22		20	0,0	0,0	0,0	0,0	0,0	5,6	66,7	66,7	66,7	66,7	66,7
Average			**0,0**	**0,0**	**0,0**	**9,3**	**38,9**	**61,1**	**88,9**	**88,9**	**88,9**	**88,9**	**88,9**
B1		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B9	Negative Control	20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B17		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Average			**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**

Table 1b: Cumulative mortality percentage (%) of control feed and treatments feeds i.e., RAV-S 15% and RAV-S 20% fed group of shrimps in lab trial. The feed was produced at 85^{0}C. The data shows that treatment feed was not effective against WSSV.

Experimental Tank	Group	Population	Day Post Infection (%) / Cumulative Mortality percentage										
			0	1	2	3	4	5	6	7	8	9	10
B3	RAV-S 15%	20	0,0	0,0	0,0	45,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
B11		20	0,0	0,0	0,0	5,3	15,8	36,8	84,2	84,2	84,2	84,2	84,2
B19		20	0,0	0,0	0,0	21,1	68,4	100,0	100,0	100,0	100,0	100,0	100,0
AVERAGE			**0,0**	**0,0**	**0,0**	**23,8**	**61,4**	**78,9**	**94,7**	**94,7**	**94,7**	**94,7**	**94,7**
B4	RAV-S 20%	20	0,0	0,0	0,0	10,5	84,2	100,0	100,0	100,0	100,0	100,0	100,0
B12		20	0,0	0,0	0,0	5,3	63,2	89,5	100,0	100,0	100,0	100,0	100,0
B20		20	0,0	0,0	0,0	21,1	84,2	100,0	100,0	100,0	100,0	100,0	100,0
AVERAGE			**0,0**	**0,0**	**0,0**	**12,3**	**77,2**	**96,5**	**100,0**	**100,0**	**100,0**	**100,0**	**100,0**
B6	Positive Control	20	0,0	0,0	0,0	22,2	44,4	77,8	100,0	100,0	100,0	100,0	100,0
B14		20	0,0	0,0	0,0	5,6	72,2	100,0	100,0	100,0	100,0	100,0	100,0
B22		20	0,0	0,0	0,0	0,0	0,0	5,6	66,7	66,7	66,7	66,7	66,7
AVERAGE			**0,0**	**0,0**	**0,0**	**9,3**	**38,9**	**61,1**	**88,9**	**88,9**	**88,9**	**88,9**	**88,9**
B1	Negative Control	20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B9		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
B17		20	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
AVERAGE			**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**

3.3 Anti-WSSV activity of *C. dactylon* in the cage

Then, the efficacy of *C. dactylon* was determined on a larger scale, i.e., in the small ponds with cages. In the laboratory scale experiment, total mortality was detected in the positive control group within six days after the infection. The 100% cumulative mortality was observed in the positive control on day 6 after the challenge. The RAV-S 15% and RAV-S 20% treatment groups showed 100% survival. No mortality was detected in the negative control groups (Table 2). The feed RAVs C containing 20% *C. dactylon* was effective against WSSV in the cage level trial.

Table 2: Cumulative mortality percentage (%) of control feed and treatments feeds i.e., RAV-S 15% and RAV-S 20% fed group of shrimps in cage trial. The feed was produced at 80^{0}C. The data shows that treatment feed was effective against WSSV.

Treatment	Pond	Cage replicates	shrimps (n)	DPI 1	DPI 2	DPI 3	DPI 4	DPI 5	DPI 6	DPI 7	DPI 8	DPI 9	DPI 10	DPI 11	No. of Dead shrimp	No. of live shrimp	Survival rate percentage	Average SR percentage
Negative control	1	1	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	100,00
		2	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
		3	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
RAV-S 15%	2	1	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	100,00
		2	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
		3	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
RAV-S 20%	3	1	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	100,00
		2	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
		3	40	0	0	0	0	0	0	0	0	0	0	0	0	40	100	
Positive control	7	1	40	0	0	1	63	0	16	0	0	0	0	0	40	0	0	0,00
		2	40	0	0	0	0	6	9	17	12	28	8	0	40	0	0	
		3	40	0	0	1	21	0	31	25	2	0	0	0	40	0	0	

3.4 Commercial pond trial using *C. dactylon* feed

The final step was to determine the efficacy of *C. dactylo*n in commercial ponds. The treatment feed formula RAV-S 20% (20 mg/kg of *C. dactylon* powdered extract) was selected for the pond level trial. The trial was conducted in *Penaeus vannamei* species. The ponds in the WSSV-prone red zone area of the farm, were used for the trial. The results showed that *C. dactylon* has a positive effect against WSSV (Figures 5). The use of *C. dactylon* enhanced the productivity of the ponds (Table 3).

Table 3: Efficacy of *C. dactylon*-enriched RAV-S 20% on *P. monodon* harvest performance in commercial ponds.

Ponds	MBW	ADG	SR	FCR	BIO	Prod/Ha	Prod/HP
A. Treatment RAV-S (20%) feed	19.31	0.17	84.66	1.46	3,307	12,719	551
B. Control Regular Feed	17.86	0.16	85.07	1.57	3,066	11,791	511

No WSSV cases were recorded in the control and treatment ponds. The yield of the treatment ponds was approximately 1,000 kg/ha higher than that of the control ponds. There was no negative impact on water quality parameters due to *C. dactylon* feed. The obtained parameters were similar in the control and treatment ponds.

4. Discussion

The pathogenicity level of White spot syndrome virus is still highest among all the known shrimp pathogens. The unique immune system and aquatic nature of crustaceans limit vaccines and immune boosters [11-14]. The previously developed and successfully reported recombinant DNA and protein vaccines against WSSV infection [4] did not achieve success in the field due to the high cost of production and adjustment. Some of the herbal extracts of natural origin contain anti-viral properties. These herbs could play a significant role in reducing the risk of white spot syndrome virus. A successful effort was made to use the extracts of *Cynodon dactylon* against white spot syndrome virus of monodon shrimp. The active ingredients were obtained using the crude extraction method and provided 100% protection in the bioassay trial. The reason for using crude extracts was cost-effectiveness. The ethanol extraction method, followed by [10], was not suitable for commercial-scale production. This result validates the concept and agrees with the data obtained previously [1].

The use of ethanol increased the cost of the extract to not be utilized in shrimp culture to protect against the virus. The crude powdered extract was optimized to mix in the feed and

produce shrimp feed with anti-WSSV properties. The doses of both 15% and 20% *C. dactylon* powder mixed in shrimp feed as raw material provided the best protection compared to other tested doses. The pond level trial showed no negative impact of *C. dactylon* powder on feed palatability or water quality parameters. The components detected in the GCMS analysis of *C. dactylon* is in align with [15]. The *C. dactylon* extract's active ingredients were tested and compared before and after heating at 85^0C found that some of the critical components deactivated after the heating. It proves the concept that some of these deactivated components alone or in combination, have the anti-WSSV properties. It also limited quality feed production with better palatability as heating provides better water stability to the shrimp feed.

5. Conclusions

A successful attempt was made using the well-known herb *Cynodon dactylon* to deactivate the deadly white spot syndrome virus. This experiment developed shrimp feed supplemented with various extracts of *C. dactylon*, commonly called Rajeev Anti-Viral -Sanjivani (RAV-S). The results at the laboratory, cage and commercial pond levels are encouraging. Feeding of shrimp with RAV-S provided 100% protection of *P. monodon*. Feeding did not reduce, indicating that the virus did not multiply and did not induce shrimp stress. The results indicate that the extracts of *C. dactylon* contain chemicals with anti-WSSV properties and act independently or in combination. These chemicals can provide complete protection of the animals against WSSV. High temperature reduced the herb's efficacy, which indicates that the anti-viral component/s present in the doob grass is heat sensitive.

Since *C. dactylon* has potent activity against WSSV, further studies on the isolation, characterization and purification of active compounds of this plant should be carried out to develop their applications in the shrimp culture industry.

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