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PHYTOCHEMICAL ANALYSIS OF AQUEOUS LEAF EXTRACT OF *PSIDIUM GUAJAVA* AND ITS INFLUENCE ON GROWTH AND HEALTH PARAMETERS OF *ETROPLUS SURATENSIS*

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ABSTRACT: The present study was done to observe the effect of aqueous leaf extract of *Psidium guajava* (ALG) on growth performance, hematological parameters, serum parameters, antioxidant enzyme activity and histological changes of liver and intestine tissues of *Etroplus suratensis*. Qualitative analysis of ALG revealed the presence of 49 pharmacologically active compounds. Antibacterial property of ALG was also checked using the microdilution method and got a MIC value of 0.31mg/ml against *A. hydrophila*. The fish fingerlings (4±0.03g) were fed four experimental diets containing 0.5%, 1%, 1.5%, 2% ALG and one control diet having 0% ALG for 60 days in triplicates. At the end of the experiment, the result showed that there is a significant increase in growth parameters like weight gain, weight gain percentage, specific growth rate and a decrease in FCR in fishes fed with ALG incorporated diet. The activity of SOD and catalase in the liver showed significantly higher values in groups having 1.5% and 2% ALG ($P<0.05$). Blood parameters like RBC, WBC, HGB, HCT, MCH, MCV, MCHC and thrombocytes showed a significant increase among treatment groups ($P<0.05$) and the highest value was obtained with diet containing 2% ALG. The serum parameters like total protein, albumin, A/G ratio and alkaline phosphatase also showed significant increase with ALG ($P<0.05$). Serum glucose showed significant decrease when compared to control. The parameters like alanine transaminase (ALT) and aspartate transaminase (AST) showed no significant difference among treatment and control ($P>0.05$). Histological analysis of liver and intestine showed changes like increase in hepatocyte diameter and increase in villi length among treatment groups when compared to control. Therefore, ALG can be used as a growth promoter, immunostimulant, an antistress and antibacterial agent for *E.suratensis*. The results of this study confirm the positive effect of aquous leaf extract of *P. guajava* on the growth and health parameters of *E. suratensis.*

Keywords: *Etroplus suratensis*, leaf extract, phytotherapy, *Psidium guajava*

1. Introduction

Aquaculture production has significantly increased through species diversification and the intensification of culture systems (Hodar et al., 2021). However, higher stocking densities, even under optimal husbandry conditions, impose stress on cultured animals. Disease outbreaks in aquaculture are primarily linked to poor husbandry and environmental conditions, leading to the emergence of new diseases and the resurgence of known

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pathogens, resulting in substantial economic losses. It is estimated that diseases account for nearly 50% of production losses in the aquaculture sector (Gabriel, 2019). To mitigate these losses, antibiotics are commonly used (Van Doan et al., 2020; Lieke et al., 2020). Nevertheless, the frequent and indiscriminate use of antibiotics negatively affects fish metabolism, compromises environmental health, and poses risks to public health due to the development of antimicrobial resistance. In response, prophylactic strategies are gaining attention (Vijayan, 2017). These include vaccination, immune stimulation, probiotics, and best management practices, all aimed at disease prevention. Ideally, these strategies should be cost-effective, environmentally friendly, growth-promoting, immunostimulatory, and possess broad-spectrum antimicrobial activity (Gabriel, 2019).

In recent decades, plant-derived supplements have emerged as sustainable alternatives due to their low cost, accessibility, minimal side effects, and eco-friendly nature (Reverter et al., 2014; Van Hai, 2015; Gabriel, 2019). Plants contain a variety of bioactive secondary metabolites such as alkaloids, flavonoids, tannins, saponins, phenolics, and terpenoids (Citarasu, 2010; Chakraborty & Hancz, 2011; Reverter et al., 2014; Hashimoto et al., 2016), which have been shown to enhance growth, stimulate immunity, and increase disease resistance in aquatic animals (Ghosh et al., 2017; Sethiya, 2016; Citarasu, 2010; Ji et al., 2007). Moreover, these natural compounds are biodegradable, reduce treatment costs (Mustafa et al., 2017), and do not contribute to antibiotic resistance, making them environmentally sustainable (Haq, 2004).

The present study evaluates the phytogenic potential of the aqueous leaf extract of *Psidium guajava* (guava) in the culture of *Etroplus suratensis*, the state fish of Kerala, India, and a widely consumed local delicacy. Guava, a member of the Myrtaceae family, is a tropical tree that can grow up to 35 feet tall. Its leaves and bark have been traditionally used for medicinal purposes (Joseph & Priya, 2011). Guava leaves are rich in beneficial compounds such as tannins, phenols, triterpenes, flavonoids, essential oils, and saponins (Pandey & Shweta, 2012), and they are also known for their high antioxidant and vitamin content (Tee et al., 1997; Hobert & Tietze, 1998).

Previous studies have demonstrated the antibacterial (Puntawong et al., 2012; Francisco et al., 2020; Gobi et al., 2016), immune-stimulatory, and growth-promoting effects of guava leaf extract in fish and shrimp (Abdel-Tawwab et al., 2020; Giri et al., 2015). Accordingly,

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this study aims to identify the pharmacologically active compounds in the aqueous leaf extract of P. guajava, assess its antibacterial activity against fish pathogens, evaluate its acute oral toxicity in fish, and examine its effects on growth, survival, and health parameters in *E. suratensis*.

2. Materials and methods:

2.1. Preparation of aqueous leaf extract of *P. guajava* (ALG)

The leaves of *P. guajava* were collected from a 15-year-old tree from Kollam district (9.060 latitude, 76.722 longitude) during the month of January, 2022. It was authenticated by National Institute of Science Communication and Information Resources, New Delhi (NISCAIR). After collection, it was cleaned thoroughly with distilled water, shade dried at room temperature to a constant mass. The dried materials were powdered very finely in a kitchen-grade grinder and sieved through a fine mesh. 100g of powdered leaves were heated for 2h in 500ml distilled water, at 90°C with a magnetic stirrer (Labquest, Borosil, India). It was then filtered using filter paper into a clean and dry conical flask and aqueous filtrate was concentrated by evaporation at 40°C in pre-weighed petri dishes. After complete evaporation of the solvent, the final weight was taken to calculate the yield. The ALG thus produced was stored at 4°C until used (Ndebia et al., 2007).

2.2. Qualitative analysis of aqueous leaf extract of *P. guajava*

Qualitative phytochemical analysis of ALG was done as per standard methods described by Shaikh and Patil (2020) with some modifications. Initially 10gm ALG extract was dissolved in 40ml of distilled water and was used as stock solution. The compounds like saponin, tannin, alkaloids, flavonoids, phenolic compounds, and terpenoids were analysed.

Apart from this qualitative phytochemical analysis using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) was also done to understand the different pharmacogenic compounds present in ALG. The analysis was done at the Indian Institute of Technology, Mumbai, India using Agilent 1260 Infinity Ultra High-Performance Liquid Chromatography (UHPLC) system (Agilent Technologies, USA) attached to a quadrupole time-of-flight (Q-TOF) mass spectrometer. Chromatographic separation was carried out on a Phenomenex Gemini C18 column (2 mm × 100 mm, 3 µm particle size). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid

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in acetonitrile), with a gradient elution program as follows: starting at 5% B, linearly increasing to 100% B over 55 minutes, held at 100% B for 10 minutes, and then returning to initial conditions over the final 10 minutes. The flow rate was maintained at 0.3 mL/min, with an injection volume of 5 µL, and the column temperature was kept constant at 35°C. High-resolution mass spectrometry (HRMS) was performed in full MS/dd-MS^2 mode. The mass range for the full scan acquisition was set from m/z 120 to 1200.

2.3. Antibacterial activity of the plant extract

In order to find out the minimum inhibitory concentration (MIC) of ALG on *A. hydrophila*, broth microdilution technique was done by the resazurin based method (Elshikh et al., 2016) with slight modifications. The isolated colonies from a 24 hour old fresh culture of *A. hydrophila* obtained from Aquatic Animal Health Microbiology lab, KUFOS, were suspended in saline solution (0.9%) and turbidity was visually compared with 0.5 McFarland standard to get a concentration of 1.5×10^8 CFU/ml. Then it was serially diluted in Muller Hinton Broth (MHB) to a concentration of 1.5×10^5 CFU/ml. Serial double dilution was done to get the required concentration of ALG in each well of the sterile 96 well plate. For this, 50µl of sterile MHB was added into each well and added 50µl of ALG solution (10mg/ml) into the first well and 50µl from this was sequentially transferred to subsequent wells, and 50µl from the last well was finally discarded. The concentration of extract thus maintained in each well was 5000, 2500,1250, 625, 312, 156, 78, 39, 19.5, 9.75 and 4.8µg/ml. There were three replications for each concentration. Then, 50µl of already prepared bacterial suspension (1.5×10^5 CFU/ml) was added to all wells containing extract. There were control wells which contained 100µl of sterile broth which served as sterility control and 100µl of diluted standardised inoculum as growth control. The plate was incubated for 24h at 37°C and 30µl resazurin (0.015g resazurin in 100ml, Himedia) was added to all wells and further incubated for 2-4h for observing colour change. On completion of incubation, if no bacterial growth is there, the colour remained blue and if bacterial growth is present, the colour changed to pink. Accordingly, wells with no colour were scored to determine the MIC value.

2.4. Experimental feed preparation

Four treatment diets were prepared with ALG incorporated at the rate of 0.5%, 1%, 1.5% and 2% (w/w) in commercial feed (Growel, India, point 8) in the Nutrition Laboratory of

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KUFOS. For acute toxicity test separate feed was made incorporating ALG at the rate of 2gm per kg body weight of the fish.

2.5. Acute oral toxicity test (Limit test-2000mg/kg)

Acute oral toxicity of extract was tested as per test no. 425 of OECD guidelines with some modifications. Seven fishes weighing approximately 50gm were maintained as control and another seven as treatment in FRP (fibreglass reinforced plastic) tanks of 80l capacity. The quarantined and acclimatised fishes were starved for 24 hours before starting the experiment. The feed prepared for the purpose was given for 7 fishes at the rate of 3% of body weight, in the treatment tanks on the first day of experiment. The 7 fishes in control tank were fed commercial feed. For the next 13 days, both the groups received normal diet (OECD, 2022). The feed was given two times daily. The water quality parameters were maintained optimum by continuous aeration and 50% water was exchanged daily. The fish were closely monitored everyday for any sign of stress.

2.6. Experimental set up for feeding experiment

The experiment was conducted in the laboratory facilities of the Department of Aquatic Animal Health Management (AAHM), Kerala University of Fisheries and Ocean Studies (KUFOS), Kerala, India. About 200 numbers of apparently healthy uniform sized *E. suratensis* (4.2±0.2g) were procured from a private hatchery in Vaikom, Kottayam, Kerala. The fish were acclimated and quarantined in 200l FRP (fiberglass reinforced plastic) tanks, for a period of one week. After 1 week, 10 numbers of *E. suratensis* fry were stocked in each glass tank of 30l capacity with 20l of water. There were four treatments with 0.5%, 1%, 1.5% and 2% ALG incorporated in feed (w/w) and a control with 0% ALG in triplicate. Good quality potable water was used for the experiment. The experimental tanks were provided with sponge filters and aeration. The faecal matter was removed daily, the water quality parameters were checked, and 50% of water was exchanged on a weekly basis. The fishes were fed twice a day so that the total feed given equals 3 percentage of body weight. The experiment was done for a period of 60 days.

2.7. Determination of growth and feed utilization of fish

Fish were weighed at the beginning and at the end of the feeding trial. Mortality was recorded daily. Growth performances and survival rate of fish were calculated using the following formula.

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Weight gain (g) = Final weight (g) – initial weight (g)

Specific growth rate (SGR) = 100× [ln Final weight (g) – ln Initial weight (g)] × days of experiment

Feed conversion ratio (FCR) = Feed intake / (Final weight – Initial weight)

Survival % = (Number of fish on final sampling / Number of fish at initial stocking) x 100

2.8. Antioxidant Enzyme Assays

2.8.1. Superoxide dismutase (SOD)

Liver tissue (75 mg) was homogenized with 1.5 ml EDTA buffer and then centrifuged at 5000rpm for 5 min and the supernatant was taken as test sample. Equal amounts of sodium bicarbonate (5mM) solution and EDTA (0.1mM) buffer solution was mixed. From that 120µl of solution was taken and mixed with 96mM NBT (50µl). Then Triton X (10µl) was added to it. The reaction was started by adding 20mM hydroxylamine hydrochloride (10µl). After 1.5 minutes, 10µl of test sample was added. Initial reading was taken after 0.5 min (0-minute reading). Second reading was taken after 3 minutes (3-minute reading). A blank was simultaneously run along with the test. (Madesh and Balasubramanian, 1998)

Blank reading (B) was calculated as B= (A550 of blank at 3min-A550 of blank at 0 min)/ 3. Test reading (T) was calculated as T= (A550 of test at 3min-A550 of test at 0 min)/ 3. % inhibition is calculated as = (B-T) x 100/B, the values were expressed as Unit/mg of protein.

2.8.2 Catalase

Liver tissue (75mg) was homogenised with 1.5ml phosphate buffer and then centrifuged for 5000rpm for 5min. The supernatant (0.5ml) was added to a mixture of 0.5ml H_2O_2 (0.2M), 1ml of phosphate buffer and 0.4 ml of distilled water. After 60s of incubation, 2ml of dichromate /acetic acid reagent (Mixed 5% potassium dichromate (Himedia) solution with glacial acetic acid (Himedia) in the ratio 1:3 by volume) was added for termination. Tubes were then heated for 10min in boiling water bath and the colour developed was read at 610nm in a spectrophotometer (Hach,U.S.A). (Sinha, 1972). H2O2 (micro mole consumed /min) = optical density /0.000265. Total protein the the liver homogenate was determined by the method of Fenk et al., (2007)

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2.9. Blood sampling, haematological and serum analysis

At the end of the experiment, blood samples were collected using sterile 2 ml syringes with 24 gauge needle by caudal severance. The blood samples were collected both with the addition of EDTA (Himedia) (1g EDTA in 10ml of distilled water, pH 8), and without anticoagulant. Samples with coagulant were used for haematological analysis and those without coagulant were used for serum analysis. The haematological parameters like WBC (white blood corpuscles), RBC (red blood corpuscles), HGB (haemoglobin), HCT (haematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), and PLT (thrombocyte count) were analysed using the haematological analyser (Benesphera, India).The blood samples for serum analysis were centrifuged for 20 min at 3000 rpm after it is coagulated at room temperature. The serum was stored at -20ºC for further analysis using serum analyser (Fuji, Japan) using respective slides supplied along with equipment. The amount of alkaline phosphatase, albumin, glucose, total protein, aspartate transaminase (AST) and alanine transaminase (ALT) in the serum were measured.

2.10. Histological studies

In order to assess the effect of ALG in tissues, liver and intestine tissues at a distance of 1cm from anal point of the fish were dissected out and fixed in 10% neutral buffered ormalin (NBF). The tissues were processed, sectioned and stained in hematoxylin and eosin as per Roberts, (2012). The slides were observed under a trinocular microscope (Leica, Germany) using 100X and 450X objectives.

2.11. Statistical analysis

The data were expressed in mean ± standard deviation (Mean± SD). The statistical analysis was done using one-way analysis of variance (ANOVA). The mean differences were compared for significance using Tukey Honestly Significant Difference Test. The significance level was set at $p< 0.05$. Statistical analyses were performed with the software package SPSS v. 26

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3. Results

3.1. Qualitative analysis of the aqueous leaf extract of *P. guajava* (guava)

The extraction of ALG gave a yield of 13%. The major bioactive compounds present in the water extract of guava leaves were alkaloids, flavonoids, phenolic compounds, tannin, saponin, presented in table 1. Qualitative phytochemical analysis using LC-HRMS revealed the presence of 49 pharmacologically active compounds of which 11 were unclassified group. The details are given in table 2

Table 1. Qualitative analysis of *P.guajava* (guava) aqueous leaf extract.

Bioactive compounds	**Aqueous extract**
Alkaloid	+
Flavanoid	+
Phenol	+
Tannin	+
Saponin	+
Terpenoid	+

(+= present)

Table 2. Qualitative analysis report of LCHRMS of ALG.

SL NO.	**Compound name**	**Formula**	**Type**
1	Quinic acid	C7H12O6	Cyclohexane
2	Gallic acid	C7H6O5	Phenol
3	Gentisic acid	C7H6O4	Phenol
4	Syringic acid	C9H10O5	Phenol
5	1-O-Galloylglycerol	C10H12O7	Unclassified
6	(-)-Epicatechin	C15H14O6	Flavanoid
7	Catechin-4beta-ol	C15H14O7	Flavanoid
8	Dihydroferulic acid 4-O-glucuronide	C16H20O10	Phenol
9	m-Coumaric acid	C9H8O3	Unclassified
10	Vanillic acid	C8H8O4	Phenol
11	Quercetin 3-(2-galloylglucoside)	C28H24O16	Flavanoid

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12	Isoacetoside	C29H36O15	Hydroxycinnamic acid
13	Maclurin 3-C-(6”-p-hydroxybenzoyl-glucoside)	C26H24O13	benzophenones
14	Myricitrin	C21H20O12	Flavanoid
15	Shoyuflavone B	C19H14O10	Flavanoid
16	4-Methylumbelliferyl sulfate	C10H8O6 S	Unclassified
17	8-Hydroxyluteolin 8-sulfate	C15H10O10 S	Unclassified
18	Herbacetin	C15H10O7	Flavanoid
19	Guajavarin	C20H18O11	Unclassified
20	Cynaroside	C21H20O11	Flavanoid
21	2”,4”,6”-Triacetylglycitin	C28H28O13	Isoflavanoid
22	Bikoeniquinone A	C27H20N2 O3	Unclassified
23	Astringin	C20H22O9	Poly phenol
24	Piceatannol 4’-galloylglucoside	C27H26O13	Glycoside
25	Caryoptin	C26H36O9	Diterpenoid
26	Karwinskione	C32H32O7	Unclassified
27	Saccharocin	C21H40N4O12	aminoglycoside
28	Protobassic acid	C30H48O6	Triterpenoid
29	4’-O-methyl-(-)-epicatectin-3’-O-beta-glucuronide	C23H26O12	Flavanoid
30	2-Descarboxy- cyclo- dopa	C8H9NO2	Unclassified
31	Kiwiionoside	C19H34O9	Terpene
32	(5alpha, 8 beta, 9 beta)- 5,9- Epoxy-3,6-megastigmadien	C31H2002	Unclassified
33	Fukinolic acid	C20H18O11	Poly phenol
34	8- Acetoxy-4-acoren-3-one	C17H26O3	cyclohexane
35	3-O-p-trans-Coumaroylalphitolic acid	C39H54O6	triterpenoid
36	Ursolic acid	C30H48O3	Triterpenoids
37	Glucosyl (2E,6E,10x)10,11-dihydroxy-2,6-farnesadionoate	C21H36O9	Unclassified

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38	Luvangetin	C15H14O4	coumarin
39	Militarinone C	C26H33 N O4	alkaloid
40	Hericerin	C27H33 N O3	Unclassified
41	(E,E,E)-Sylvatine	C24H33 N O3	Unclassified
42	Sphinganine	C16H35 N O2	2- aminooctadecane
43	Icaceine	C22H33 N O4	Diterpenoid alkaloid
44	Karakoline	C22H35 N O4	Organonitrogen
45	Citronellyl hexanoate	C16H30O2	Carboxylic ester
46	Asterosterol	C26H42O	Hydroxysteroids
47	Ganderic acid F	C32H42O9	Triterpenoid
48	Hexadecyl ferulate	C26H42O4	Hydroxycinnamic acid
49	Pheophytin a	C55H74 N4 O5	Unclassified

3.3. Antibacterial activity of *P.guajava* leaf extract

The minimum inhibitory concentration (MIC) based on the standard protocol for determination of MIC described in guidelines of Clinical and Laboratory Standard Institute revealed an MIC value of (0.31±0.0)mg/ml against the gram-negative bacteria *A. hydrophila*.

3.4. Acute oral toxicity test

In the acute oral toxicity test as per OECD guidelines, mortality or any abnormal symptoms were not observed in fish during test. Therefore, further experiment to measure LD_{50} was not conducted and the extract was assumed to be non-toxic to fish.

3.5. Growth performance and antioxidant enzyme status

The parameters such as percentage weight gain, specific growth rate (SGR), and survival rate (%) and liver antioxidant enzymes like SOD and catalase of *E. suratensis* fed diet incorporated with aqueous leaf extract of *P. guajava* over a period of 60 days are presented in the table 3. The highest value of percentage weight gain was shown in treatment 3 (T3) (122.5±0.01%) fed 1.5% of guava leaf aqueous extract, which was significantly higher ($P<0.05$) than control and other treatment groups. Specific growth rate also showed the

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same pattern as percentage weight gain. There was no significant difference between the groups in the case of survivability ($P > 0.05$). The lowest FCR value (1.46±0.01) was shown by treatment 3 (T3) having 1.5% of aqueous guava leaf extract. Liver antioxidant enzymes like SOD and catalase also showed significant ($P<0.05$) dose dependent increase in the treatment groups.

Table 3. Growth performance and liver antioxidant enzyme status of *E. suratensis* fed ALG incorporated diets

Parameters	**Control**	**T1**	**T2**	**T3**	**T4**	**Significant value**
Weight gain %	114.6 ± 1.4^{a}	115.7 ± 0.01^{a}	120.1 ± 0.0^{b}	122.5 ± 0.01^{c}	121.2 ± 0.01^{c}	<0.05
SGR (%)	1.25 ± 0.01^{a}	1.26 ± 0.005^{a}	1.31 ± 0.005^{ab}	1.33 ± 0.005^{b}	1.32 ± 0.005^{b}	<0.05
Survival (%)	100±0.0	100±1.00	99.3±0.15	100±0.0	99.6±0.3	0.62
FCR	1.53 ± 0.005^{d}	1.51 ± 0.005^{cd}	1.5 ± 0.01^{bc}	1.46 ± 0.01^{b}	1.48 ± 0.005^{a}	24.64
SOD (U/mg)	0.37 ± 0.05^{a}	0.41 ± 0.01^{ab}	0.43 ± 0.03^{bc}	0.45 ± 0.07^{bc}	0.47 ± 0.005^{c}	<0.05
Catalase (U/mg)	0.21 ± 0.05^{a}	0.28 ± 0.004^{b}	0.315 ± 0.07^{c}	0.34 ± 0.01^{d}	0.38 ± 0.007^{e}	<0.05

Control group of fish fed with normal diet, T1-Group of Fish fed with diet containing 0.5% ALG, T2- Group of Fish fed with diet containing 1% ALG, T3- Group of Fish fed with diet containing 1.5% ALG, T4- Group of Fish fed with diet containing 2% ALG. Different superscripts denote significant differences between the means ($P<0.05$)

3.7. Hematological parameters

The hematological parameters of *E. suratensis* fed diets supplemented with aqueous *P. guajava* leaf extract over a 60-day period are presented in Table 4. All measured parameters were significantly higher ($P < 0.05$) in the treatment groups compared to the control. Among the treatments, T3 and T4—corresponding to diets containing 1.5% and 2% aqueous leaf extract (ALG), respectively—demonstrated superior hematological responses.

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Table 4. Hematological parameters of *E. suratensis* fed with diet containing aqueous extract of *P. guajava* leaf over a period of 60 days.

Parameters	Control	T1	T2	T3	T4	Sig. value
WBC×10^3/µL	180.2±0.01^a	192.1±1.3^b	196.3±0.25^c	199.8±0.45^d	202.4±0.80^d	<0.05
RBC×10^6/µL	0.7±0.04^a	1.12±0.03^b	1.24±0.05^b	1.39±0.03^c	1.46±0.02^c	<0.05
HGB (g/dl)	9±0.05^a	10.3±0.2^b	11.1±0.2^c	12.6±0.15^d	12.9±0.05^d	<0.05
HCT (%)	14.2±0.2^a	15.6±0.0^a	16.8±0.1^b	17.30±0.09^c	18.2±0.10^d	<0.05
MCV (fL)	138.8±0.5^a	158.6±0.2^b	162.2±0.6^c	168.9±0.35^d	171.4±0.1^e	<0.05
MCH (pg)	55.2±0.65^a	61.8±0.5^b	64.7±0.29^c	67.5±0.19^d	70.8±0.05^e	<0.05
MCHC (g/DI)	40.5±0.46^a	44.6±0.10^b	45.5±0.25^c	47.9±0.11^d	48.91±0.01^e	<0.05
PLT×10^3 /µL	31.3±0.15^a	33.8±0.15^b	38.5±0.63^c	40.7±0.75^d	44.9±0.06^e	<0.05

(WBC – White Blood Corpuscles, RBC – Red Blood Corpuscles, HGB – Haemoglobin, HCT – Hematocrit, MCV- Mean Corpuscular Volume, MCH – Mean Corpuscular Hemoglobin, MCHC – Mean Cell Haemoglobin Concentration, PLT- thrombocytes, T1- Treatment 1, T2- Treatment 2, T3- Treatment 3, T4- Treatment 4, Different superscripts denote significant difference P<0.05).

3.8. Serum parameters

The serum parameters of *E. suratensis* fed diet incorporated with *P. guajava* aqueous leaf extract over a period of 60 days were represented in the table 7. The immune parameters tested like total serum protein, albumin, albumin globulin ratio, and alkaline phosphatase increased significantly ($P < 0.05$) in all the treatments compared to control. The liver health indicators such as ALT and AST values showed no significant difference between control and treatments. The serum glucose decreased significantly in fish fed ALG incorporated diet compared to control ($P < 0.05$).

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Table 5. Serum parameters of *E. suratensis* fed with diet containing aqueous extract of *P. guajava* leaf over a period of 60 days.

Parameter	Control	T1	T2	T3	T4	P value
Total protein (g/dl)	3.05 ± 0.05^{a}	3.57 ± 0.02^{b}	3.82 ± 0.02^{c}	4.05 ± 0.05^{d}	4.45 ± 0.05^{e}	<0.05
Albumin (g/dl)	1.2 ± 0.005^{a}	1.62 ± 0.03^{b}	1.8 ± 0.005^{c}	1.9 ± 0.015^{d}	2.25 ± 0.04^{e}	<0.05
A/G	0.65 ± 0.01^{a}	0.83 ± 0.03^{b}	0.91 ± 0.005^{c}	0.93 ± 0.01^{c}	1.07 ± 0.04^{d}	<0.05
ALP (U/l)	44.6 ± 0.5^{a}	47.45 ± 0.15^{b}	50.2 ± 0.11^{c}	51.7 ± 0.4^{d}	52.5 ± 0.1^{e}	<0.05
ALT (U/l)	29.6±0.66	32.1±1.4	32.3±1.2	33.0±2.08	34.6±2.02	0.79
AST(U/l)	29.4±1.2	30±2.3	30.3±0.12	32.3±1.4	34.1±2.0	0.85
Glucose (mg/dl)	89.8 ± 0.3^{e}	85.3 ± 0.1^{d}	79.1 ± 0.4^{c}	71.3 ± 0.15^{b}	66.3 ± 0.1^{a}	<0.05

(A/G – albumin: globulin ratio, ALT - alanine transaminase, AST – aspartate transaminase, ALP- alkaline phosphatase, T1- treatment 1, T2- treatment 2, T3- treatment 3, T4- treatment 4, Different superscripts denote significant difference P<0.05)

3.9. Histology

3.9.1. Histology of liver

Histological examination of hepatic tissue of control and experimental groups showed differences in hepatocyte diameter. The hepatocyte diameter increased in the treatment groups which received ALG when compared to control. The hepatocyte diameter of control group ranged from 10µm to 17µm, and that of the experimental group from 25µm to 40µm.

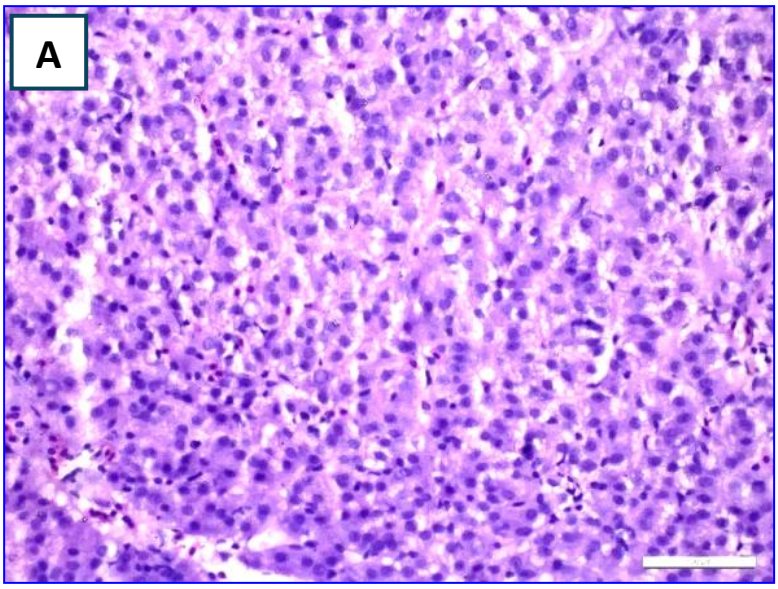

Figure 1A. Histological section of *E. suratensis* liver (A) 45X (control)

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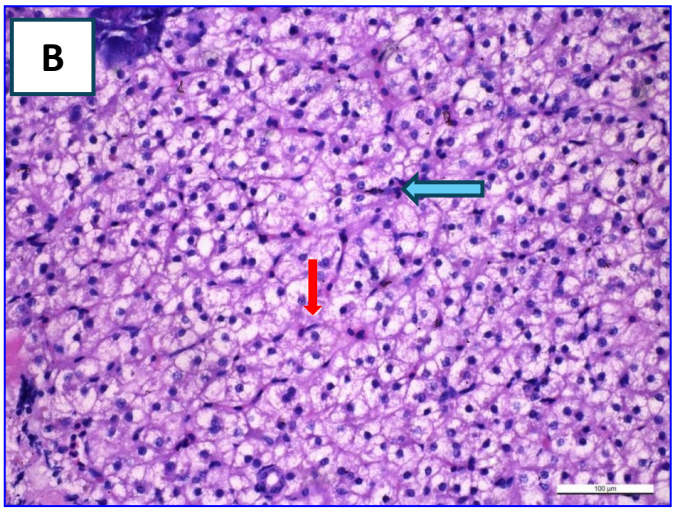

Figure 1B. Histological section of *E. suratensis* liver (B) 40X (Treatment 3) showing uniformly arranged hepatocytes with nucleus more densely stained compared to control

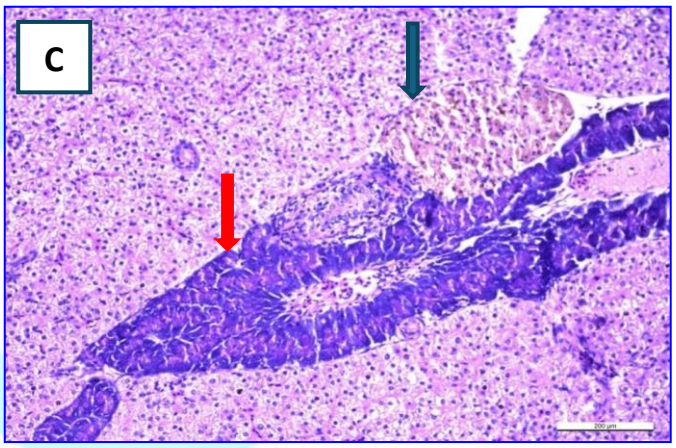

Figure 1C. Histopathological section of *E. suratensis* liver (C) 20X treatment 3. Hepatopancreas (red arrow), Melano macrophage centre (Blue arrow)

The size of the nucleus was also analysed and showed no change in size. The diameter of nucleus for both control and experimental group ranged between 9-11µm. There was an increase in the intensity of staining of nucleus showing that the cells become more metabolically active. The oil reserves in the hepatocytes were also more in treatment groups.

3.9.2. Histology of intestine

The histological examination of intestine was also done among control and experimental group. The villi in the intestine of control group were shorter and narrower and ranged from 180µm to 230µm. Whereas in experimental groups the length of villi increased

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showing a range from 350µm to 670µm. All the experimental groups (T1, T2, T3, T4) showed similar longer villi in comparison with the control group.

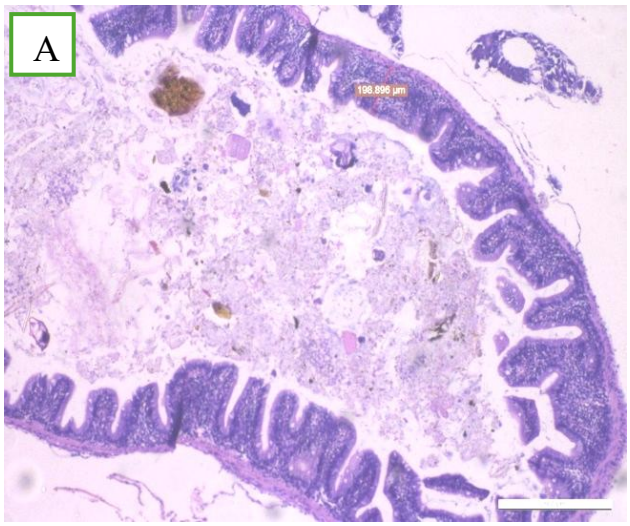

Figure 2A. Histology of *E. suratensis* intestine (D) 10X control

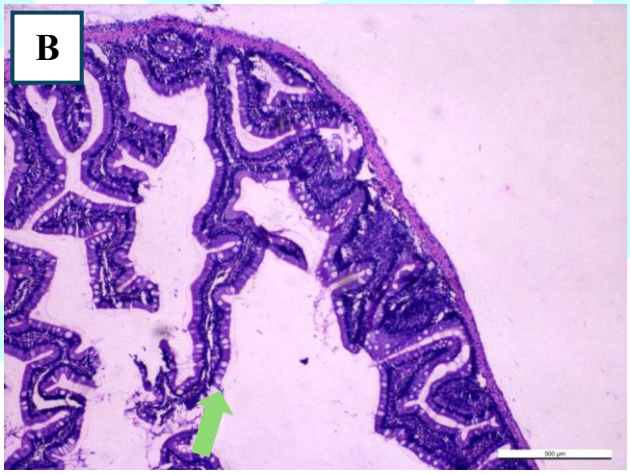

Figure 2B. Histology of *E. suratensis* intestine (E) 10X treatment 3. Green colour shows villi of increased size.

4. Discussion

Qualitative phytochemical screening of guava leaves done in our laboratories showed the presence of alkaloids, flavonoids, phenol, tannin, saponin and terpenoids. Similar results were obtained by Pandey and Shweta (2012), who showed the presence of tannins, saponins, terpenoids, alkaloids in the leaves of guava. Das and Goswami, (2019) reported that the guava leaf contains large amounts of tannins, phenols and flavonoids whereas alkaloids, saponin and terpenes are present in lesser amount. Tannin present in the guava

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leaves act as antimicrobial agent (Mailoa *et al*., 2013). In the present study, using aqueous extract of leaf, apart from these compounds, terpenoids were also seen. Phytochemical analysis of ALG using LC-HRMS revealed the presence of more than 100 compounds. Among these compounds, nearly 50 compounds were pharmacologically active. Ryu *et al*., (2012) identified 60 compounds in a hexane fraction of *P. guajava* including many flavonoids, phenols etc. In the present study most of the compounds identified came under the group phenols, flavonoids and triterpenoids. Compounds like gallic acid, syringic acid, astringin, etc., come under phenolic compounds. The presence of phenolic compounds in guava leaves is well known for anti-inflammatory, antimicrobial and antioxidant activities (Xie *et al*., 2015). The flavonoid compounds in guava include epicatechin, quercetin, myricitrin, herbacetin, cymaroside, etc., and these flavonoids are proved to have an antibacterial and antioxidant effect (Joseph and Priya, 2011). Arima *et al*., (2002) and Soman *et al*., (2010) have identified quercetin which is a flavonoid in guava leaves. The antioxidant property of quercetin has also been reported by Jimenez- Escrig *et al*. (2001). Begum *et al*. (2002) have done isolation of triterpenes from guava leaves and they got many pharmacologically active secondary metabolites like coumaric acid, ursolic acid, etc. Shaheena *et al*. (2019) have analysed the leaf extract of guava and revealed the presence of gallic acid, epicatechin, guajaverin, quercetin, etc., and reported that they have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant properties. Nayeem *et al*., (2016) reported the presence of gallic acid in guava leaf extract and also its functions like antibacterial, antioxidant, antifungal, antiviral, and anti-inflammatory. Thus, the present study indicated the presence of many pharmacologically active compounds in the aqueous extract of guava leaf, which proved its health benefits.

The antibacterial effect of ALG was evaluated in the present study using microdilution method against *A. hydrophila* and the MIC value obtained was 0.31mg/ml. A similar work was done by Hoque *et al*. (2007), using ethanolic and aqueous extract of guava leaf and got MIC value of 4mg/ml against *A. hydrophila*. They also found that the MIC varies with bacterial species and it was 0.1mg/ml for both *S. aureus,* and *V. parahaemolyticus.* The aqueous extracts of guava leaf also showed antibacterial property and the MIC value ranged from 0.9mg/ml to 7mg/ml for *A. faecalis* and *L. monocytogenes.* In this study the aqueous extract of guava leaf showed an MIC value of 2mg/ml to *A. hydrophila*. Olatunde *et al*., (2018) checked the MIC values of ethanolic extract from guava leaf powder without

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and with prior dechlorophyllisation against *P. aeruginosa, E. coli, V. parahaemolyticus, L . monocytogenes, S. aureus* and got MIC values ranging from 0.51 to 1.25 mg/ml. Many works have reported that the MIC values of guava leaf extract range from 150µg/ml to 4mg/ml for inhibition of bacterial pathogens (Prabu *et al*., 2006, Sanches *et al*., 2005, Hidetoshi and Danno, 2002). In the present study, the aqueous extract gave a much lower concentration for MIC, which may be because of the extraction method employed in the present study that might have eluted more compounds.

In the present study, the growth performance of *E. suratensis* is evaluated by giving four treatment diets having 0.5%, 1%, 1.5% and 2% of guava leaf aqueous extract and a control containing no leaf extract. The values like weight gain percentage and SGR were higher for T3, with 1.5% ALG. In a similar study conducted by Giri *et al*., (2015), in *Labeo rohita* the highest growth was observed with leaf extract was included at the rate of 0.5%. Gobi *et al*., (2016) did a study to find the effect of aqueous and ethanolic extract of guava leaves in *O. mossambicus* and obtained similar growth rate using both extracts.in a period of 30 days. Increased growth rate in *O.niloticus* is reported by Omitoyin *et al*. (2019) and Abdel-Tawwab and Hamed, (2020) using the leaf extract. In the present study, similar results were obtained for *E. suratensis* also using aqueous leaf extract, using a procedure which can be used by the farmer himself, thus reducing the cost of adding a commercial immunostimulant product. Since the amount of extract that can be incorporated into the feed without affecting the feed intake depends on the feeding habits of species cultured, further research is required to find its suitability and effect in carnivorous fish as well.

The haematological parameters are used to provide information about the health and physiological status of fish and feeding conditions (Fazio *et al*., 2013). WBC, RBC, HCT, and HGB values are particularly recommended to monitor the health of a stock. When herbs are given as immunostimulants, it has been reported that bioactive substances in the plants cause an increase in blood cell counts and thus trigger immune system and natural defense in fish (Aly *et al*., 2008, Nya and Austin, 2009, Talpur *et al*., 2013, Ajeel and Faragi., 2013). In the present study, haematological parameters like RBC, WBC, HGB, HCT, MCH, MCHC, MCV and PLT were measured to analyze the health status of fish. The ALG incorporated diet showed a positive effect on all these parameters in four treatment groups. A similar work was done by Goda (2008) to observe the effect of ginseng

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herb in *O. niloticus* and RBC, hematocrit and haemoglobin significantly increased among treatments. Das *et al*. (2013) also reported an increase in RBC, WBC and Hb counts in treatment groups compared to control when *Ocimum sanctum* was given to *L. rohita.* Yonar *et al*. (2019) showed that the effect of curcumin in *O. Oncorhynchus mykiss* increased the RBC, HGB and HCT. Sahan *et al*., (2016) showed the effect of ginger in Nile tilapia. Gabriel *et al*., (2019) in *C. gariepinus* gave similar results. Adel *et al.* (2021) showed that there is an increase in these values in Siberian sturgeon when diet incorporated with lemon verbena was given. These findings indicate that guava leaf extract exerts effects on fish blood cellular parameters comparable to those of other medicinal herbs. Furthermore, all eight hematological parameters assessed in this study showed significant increases in the treatment groups, underscoring the relevance and impact of this investigation.

Serum parameters like ALP, alanine aminotransferase, aspartate aminotransferase, glucose, total protein, albumin, A/G ratio were analysed using blood collected from the fishes of control and treatment groups. In the present study, parameters like total protein, albumin, A/G ratio increased with increase in the content of ALG in diet. The total protein estimation is helpful in differentiating normal and damaged liver function as the majority of plasma proteins like albumin and globulin are produced in liver (Thapa and Walia, 2007). Globulin are essential for maintaining a healthy immune system. Serum albumin and globulin values in the fish treated with immunostimulants were higher than control (Choudhary *et al*., 2005). An increase in serum protein, albumin and globulin are linked with stronger innate response in fishes (Wiegertjes *et al*., 1996). The alkaline phosphatase (ALP) is a hydrolase enzyme present mainly in cell membrane of liver (Ramaiah, 2007). In the present study, ALP values also showed a significant increase in treatment groups than control, indicating better immune status of treatment fishes. In shrimp, *P. monodon* also ALP was increased when guava leaf extract was fed along with diet (Yin *et al*., 2014). The level of ALT and AST in serum can strongly be correlated with hepatic dysfunction. During liver damage hepatocytes release these enzymes and it will enter into circulation increasing their levels in serum (Drotman, 1978, Amacher, 1998). In the present experiment, there was no significant difference in the ALT and AST between treatment and control groups of *Etroplu*s. Adel *et al*., (2020) have done a work to study the effect of *Polygonum minus* extract on rainbow trout. The result showed that there is an increase in total protein, albumin, and globulin levels and a decrease in ALP and ALT. Another similar

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work is done by Adel *et al.*, (2021) in Persian sturgeon with dietary *Gracilaria persica* and the results showed that there is an increase in total protein and albumin in fish fed with 5 and 10g/kg of diet. But no significant difference was observed in parameters like ALT, AST, ALP and glucose in treatment groups. In the present study, the ALP increased in treatment groups suggesting an enhancement in the immune status. The serum glucose was less in treatment groups compared to control indicating that the fish is experiencing less stress when fed with guava leaf extract. The AST and ALT values were lower in control group and the values were almost similar, pointing to the fact that the guava leaf extract has a hepatic protective function as well.

Various studies have reported that guava is a strong antioxidant having a protective, regenerative features by maintaining degradation of free radicals (Olatunde *et al.*, 2018; Giri *et al.*, 2020). Fishes affected by stress factors undergo oxidative stress and produce free radicals and the antioxidant system in fish protects it from these free radicals. The important indicators of these activities are the increase in SOD and CAT enzyme levels (Ritola *et al.*, 2002). In our study, the levels of SOD and catalase were high among treatments, compared to control. Gobi *et al.*, (2016) also supplemented guava leaves to *O. mossambicus* and found an increase in SOD and catalase. Giri *et al.*, (2020) showed a significant decrease in SOD and catalase value in *C. carpio* when fed with feed having guava leaf extract. While comparing other phytogenics, Metwally (2009) showed an increase in SOD and catalase in tilapia which was incorporated with garlic. Sahan *et al.*, (2016) done a similar study using ginger in Nile tilapia and reported that the SOD and catalase increased significantly among treatments. Tan *et al., (*2018) found an increase in SOD and catalase enzymes in hybrid grouper incorporated with dietary ginkgo biloba leaf extract. Paray *et al.*, (2020) showed an increase in SOD and catalase in *C. carpio* incorporated with dietary oak. In the present study, also both these enzymes were high in treatment groups compared to control group, showing a better health status of these fishes.

Histological examination is considered an important tool to monitor fish health (Raskovic *et al.*, 2013). The intestine and liver are the most important organs involved in the digestion and absorption of nutrients from food, and so, analysing these organs are considered necessary (Takashima and Hibiya, 1982). Liver is considered as the main gland associated with digestive system in fish, therefore the histological changes in liver can

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indicate effects of feed components (Raskovic *et al*.,2011). In the present study hepatocyte diameter and size of nucleus were the parameters checked. The result showed an increase in hepatocyte diameter of liver among treatments when compared to control. The size of the nucleus remained the same and showed no difference in size and also showed an increase in the intensity of staining. Histological analysis of digestive system is also considered a good indicator of the nutritional status of fish (Caballero *et al*., 2003). A similar study was done by Jasim *et al*., (2016) in common carp to know the effect of using fish bio silage instead of fish meal. They also found that the size of the hepatocytes increased in treatment fishes, which showed high growth rate. So, they concluded it as the result of fat reserves. The increase in the length of intestinal villi was also reported in the same study, which was suggested to be one of the reasons for increase in the growth, due to increase in surface area for absorption of nutrients.

Thus, the results obtained in the present experiment reveals that *P.guajava* leaf extract can act as a growth promoter, antimicrobial and antistress agent and also improved immune responses significantly. Further research to use compounds from leaf extract for developing feed additive as an alternative to antibiotics in aquaculture is highly beneficial.

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