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Thiamine and Pyridoxine loaded Vanillic acid Grafted Chitosan: A Functional Food ingredient to mitigate swimming induced Oxidative stress in Animal model

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Abstract

The study was conducted to evaluate the bio-functional properties of thiamine and pyridoxine-loaded vanillic acid grafted-chitosan (TPVGC) to perform as an oxidative stress mitigating agent. The antioxidant properties of TPVGC assessed in terms of DPPH and ferric reducing antioxidant power (FRAP) assay indicated that the bioactivities are mainly dose dependent. Further, the metabolic enzyme associated beneficial properties of TPVGC were assessed in an animal model through swimming induced stress. Animals were randomly distributed into four experimental groups and fed with TPVGC at 0, 0.8, 1.6 and 2.4% in the diet for a period of 45 days. During the experimental period, the animals were exposed to swimming exercise for 50 min daily. Average swimming-time and anti-fatigue activity revealed that the animals fed with graded level of TPVGC enhanced stamina and swimming activity. Control group fed without TPVGC were lethargic after 30 min of swimming. Metabolic responses were assessed in terms of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), catalase, superoxide dismutase (SOD) and acetylcholine esterase (AChE) activity. LDH, MDH, catalase and SOD activities of treatment group showed significant ($p<0.05$) reduction compared to control. However, a reverse trend was observed for AChE activity. This was further supported by the results of Principal Component Analysis (PCA) which indicates the major variables contributed to variation in case of individual tissue samples. Based on the results, it is inferred that the supplementation of TPVGC has stress mitigation role along with enhanced stamina during the swimming exercise. It is hence surmised that TPVGC can function as a potential nutraceutical due to its proven functional properties notably faster stress recovery and stamina boost, which has prospective applications in human healthcare/live-stock segments in the contemporary global scenario.

Keywords: Antioxidant properties; Swimming exercise; Stress; Thiamine; Pyridoxine; Chitosan and Stamina

Introduction

Stress is an unavoidable process in the life cycle of organisms. The prevailing environmental conditions may cause the bio-physiological stress to an animal or a human being. During the bio-physiological process, certain biochemical and metabolic changes are bound to happen and may cause several disorders and even lead to physical and mental diseases (Xiao et al., 2014). Stress may be acute or chronic, generally, animal tries to adapt and survive in acute stress response, whereas chronic stress response may lead to heavy physiological and metabolic changes, which results in diseases (VanItallie 2002). Swimming is one of the best chronic stress creation models in animals, which disturb the biochemical homeostasis. Swimming stress in the animal activates reactive oxygen species (ROS) production as an additional product during the normal process of tissue respiration. Therefore, regular exposure of the animal to adverse condition may lead to oxidative damage (Stoliar & Lushchak 2012). Oxidative stress is a condition, where

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enhanced production of ROS level in the system leads to disturbances in the cellular metabolism and its regulatory mechanism (Lushchak, 2011). To overcome the oxidative stress, animal or human body tries to produce anti-oxidative enzymes (glutathione peroxidases, catalase and SOD) and reactive oxygen species scavengers (Singh et al., 2010).

Generally, during exercise process the level of antioxidants may get depleted and therefore, leaving the body vulnerable for oxidative damage (Bentley et al., 2014). Researchers across the globe have investigated the potential effect of antioxidant supplementation on exercise induced stress. The oxidative stress and inflammation can be reduced by dietary supplementation of individual or mix of antioxidants (Bentley et al., 2014; Murer et al., 2014). In addition, majority of earlier studies have also reported that dietary supplementation of antioxidants showed stress mitigation role against the oxidative stress induced during exercise. Antioxidants supplementation also improves the endurance of exercise performance (Bentley et al., 2014; Bloomer 2007).

Chitosan is a natural amino polysaccharide, known for its unique properties like biodegradable, biocompatible, bio-functional, non-toxic, antioxidant and antimicrobial activity (Tejpal et al., 2017). Due to its biological importance, chitosan finds its applications in the field of agriculture, pharmaceutical, water treatment and cosmetics (Kong et al., 2010). In addition, the bio-functional properties of chitosan can be improved by adopting grafting technology. Researchers across the globe have reported that the grafting of phenolic acids to chitosan had enhanced several bioactivities *viz.,* antioxidant, antimicrobial and anti-diabetic (Casettari et al., 2012).

The inclusion of water soluble vitamins in diet is reported to have significant biological importance. Thiamine and pyridoxine play a crucial role in metabolism, neural function, immune function and synthesis of the neurotransmitters etc (Tejpal et al., 2017). Nevertheless, water soluble vitamins are unstable when they are exposed to unfavourable conditions like light, oxygen, temperature etc. Microencapsulation of water soluble vitamins could potentially solve the instability issues (Tejpal et al., 2017). However, there are no published reports available related to the effect of thiamine and pyridoxine-loaded vanillic acid grafted-chitosan (TPVGC) on stress mitigation and endurance of exercise performance against the swimming induced oxidative stress. Stress mitigation/ anti-stress products are an important component of nutraceutical industry. The global nutraceutical business is ever-growing, estimated at $149.5 billion in 2011, while the Indian nutraceuticals market spans at $1 billion, by means of a faster CAGR of 18% (Debjit et al., 2013). With this background, the study was conducted to evaluate the antioxidant and metabolic enzyme associated beneficial properties of thiamine pyridoxine loaded vanillic acid grafted-chitosan (TPVGC) to prove its role as a stress mitigating agent using an animal model.

Material and Methods

Thiamine and pyridoxine-loaded vanillic acid grafted-chitosan was synthesized as reported in our previous publication (Tejpal et al., 2017). DPPH free radical scavenging activity of thiamine and pyridoxine-loaded vanillic acid grafted-chitosan was evaluated as per the method described by Yen & Wu (1999). Solutions of TPVGC were prepared by dissolving them in double distilled water at 0.05, 0.1, 0.15, 0.20 and 0.25 mg mL^{-1} concentration. Accurately 1.5 mL of TPVGC sample was added into 1.5 mL of 0.1 mM DPPH in 99.50% ethanol, solution was mixed thoroughly and placed under the dark condition for 30 min at room temperature. The radical scavenging activity was measured at 517 nm in a double beam spectrophotometer (UV–VIS-1601 spectrophotometer, Shimadzu). Double distilled water and ethanol mixture was used as a control. DPPH radical scavenging activity was calculated using the following equation-

$$\text{DPPH free radical scavenging activity (\%)} = 1 - \frac{\text{Abs sample}}{\text{Abs control}} \times 100$$

The ferric reducing capacity of TPVGC was assessed as described by Oyaiza (1986). One mL of sample (0.05, 0.1, 0.15, 0.20 & 0.25 mg mL^{-1}) was carefully mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, followed by 30 min incubation at 50°C. To the reaction mixture 2.5 mL of 10% trichloroacetic acid was added. Then, 2.5 mL of distilled water was added to 2.5 mL of solution mixture. At the end, final reaction mixture was incubated for 10 min before recording the absorbance at 700 nm using

spectrophotometer (UV-VIS-1601 spectrophotometer, Shimadzu, Japan). Results are expressed as absorbance at 700 nm.

Wistar strain albino rats, weighing 180 to 200 g (Male) were chosen for the study. The animals were kept in cages (polypropylene), and maintained hygienically throughout the experimental period. During the acclimatization period animals were maintained at standard environmental conditions (temperature 28 ± 2°C; humidity, 60–70%; 12 h light/dark cycle) and fed with standard diet (M/s Sai Foods, Bangalore, India) and water *ad libitum*. After seven days of acclimatization period, twenty-four animals were randomly distributed among four experimental groups. Each group was fed with the diet, supplemented with 0 (control), 0.8, 1.6 and 2.4% of TPVGC. Hence, total four experimental groups *viz.,* control male (feed + 0% TPVGC); T_1M (feed + 0.8% TPVGC); T_2M (feed + 0.6% TPVGC); T_3M (feed + 2.4% TPVGC). The study was carried out as per the guidelines and recommendation of the Animal Ethics Committee of the Central Institute of Fisheries Technology, Cochin. During experimental period the animals were exposed to swimming stress over a period of 45 days (50 min/day). The anti-fatigue activity was carried out by following the procedure explained by Gupta et al., (2015) with slight modification. The swimming exercise was conducted in a glass tank (60 x 30 x 30 cm) filled with water. Animals were exposed to swimming exercise over a period of 45 days (50 mins/day). The Average swimming time was calculated on the weekly basis and used for the estimation of anti-fatigue activity using the following equation:

$$\text{Anti} - \text{fatigue Activity (\%)} = \frac{STT - STC}{STC} \times 100$$

Where,

STT- swimming time of treatment groups (min);

STC- swimming time of control group (min)

After completion of the feeding trials, experimental animals were sacrificed after anesthetizing using chloroform. For enzyme assay, tissue homogenates were prepared separately using muscle, kidney, liver and brain. For which, the tissues were homogenized in 0.25 M chilled sucrose solution using a homogenizer, followed by centrifugation at 5000 g at 4°C for 10 min. After centrifugation, the supernatants were collected and stored at -20°C.

Lactate and malate dehydrogenase activities were measured by the change in optical density (OD) at 340 nm for 3 min using the method of Wroblewski & Ladue (1955) and Ochoa (1955), respectively. Superoxide dismutase (SOD) activity was estimated as detailed by Misra & Fridovich (1972). Catalase activity was evaluated according to the method described by Claiborne (1985). AChE activity was measured by as per the method detailed by Augustinsson (1957). Tissue protein content was estimated according to Lowry et al. (1951).

The data pertaining to the diet efficiency and endurance in animals was analysed by employing ANOVA. Prior to the comparison of means the data was subjected to tests of normality and homogeneity of variances. Kruskal-Wallis rank sum test and Dunn's multiple comparison test were employed to data which were not normal. Regression analysis was performed to analyse the relationship between the duration of swimming (in minutes) and time interval in the growth period (weeks) of animals fed with the different diet formulations. Regression analysis was also done to analyse the influence of time interval of growth on anti-fatigue activity. Principal Component Analysis (PCA) was performed on the set of variables indicating enzyme activities recorded from muscle, liver, kidney and brain tissues of animals exposed to swimming to identify possible relationship between diet and enzyme activity. All analyses were performed using the R package (version 3.5.3).

Results and Discussion

In the present study the antioxidant and biofunctional role (mitigation of swimming induced oxidative stress) of TPVGC through dietary supplementation in animal model using Wistar strain rats was evaluated. DPPH activity and FRAP of thiamine and pyridoxine-loaded vanillic acid grafted-chitosan was assessed at various concentration and the results are given in Table 1. Both free radical scavenging and ferric reducing antioxidant power assays found to be significant ($p<0.05$) in dose dependent manner. Highest free radical scavenging activity and FRAP was recorded at the concentration of 0.25 mg mL^{-1}. Antioxidant properties of TPVGC were found to be significantly higher at the higher concentration. The present study is in support with the findings of Trung & Bao (2015) who stated that the antioxidant properties of chitosan is in a dose dependent manner. Chitosan possesses antioxidant

properties due to the free radical scavenging action through the nitrogen on C-2 position. Similar to chitosan, vanillic acid also found to have the antioxidant properties in dose dependent manner and higher DPPH and FRAP activities have been reported at higher doses of vanillic acid (Chou et al., 2010). Chatterjee et al. (2015) reported improved antioxidant properties of phenolic acid grafted chitosan such as gallic acid-chitosan, ferulic acid-chitosan, vanillic acid-chitosan and coumaric acid-chitosan conjugates. The DPPH activity of the TPVGC clearly indicated that combination of thiamine, pyridoxine and vanillic acid grafted-chitosan have the ability to transfer electrons and could effectively terminate the free radical-induced chain reaction. Similar to DPPH, FRAP is often used to measure the ability of antioxidative compounds to reduce the ferric ions to ferrous form. TPVGC with higher reducing power have better abilities to donate the electrons. The present finding is in accordance with the published reports which have shown improved reducing power for phenolic acid grafted chitosan conjugates (Eom et al., 2012; Lee et al., 2014).

During the experimental period of 45 days, all the animals were exposed to swimming exercise for 50 min/day. The average swimming time and anti-fatigue activity (%) showed significant ($p<0.05$) difference among the experimental groups (Fig. 1 & Fig. 2). Treatment groups fed with graded level of TPVGC depicted an increase in the average swimming time of rats compared to control group. Further, a regression model fitted to average swimming time against experimental week revealed that the TPVGC in feed was a significant factor which influenced the average swimming time. T1, T2 and T3 diet fed group could effectively increase the

Table 1. DPPH free radical-scavenging (DPPH) and Ferric reducing antioxidant power (FRAP) activity of TPVGC

	Concentration				
Activity	0.05 mg ml^{-1}	0.10 mg ml^{-1}	0.15 mg ml^{-1}	0.20 mg ml^{-1}	0.25 mg ml^{-1}
DPPH	57.23 ± 0.57^{a}	73.07 ± 1.85^{b}	81.64 ± 0.14^{c}	87.69 ± 0.24^{d}	91.06 ± 0.47^{e}
FRAP	0.11 ± 0.01^{a}	0.18 ± 0.01^{b}	$0.24\ ^{a}\pm0.01^{c}$	0.34 ± 0.01^{d}	0.42 ± 0.01^{e}

Values are expressed in mean ± SE with different superscript (a, b, c, d) in each row indicate significant difference ($p<0.05$)

Table 2. Regression Fit for swimming time data

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	28.2222	0.6728	41.945	< 2e-16 ***
trtT1	4.8333	0.6344	7.619	1.65e-10 ***
trtT2	6.8333	0.6344	10.772	6.35e-16 ***
trtT3	8.4444	0.6344	13.312	< 2e-16 ***
as.factor (week) 2	2.9167	0.7769	3.754	0.000382 ***
as.factor (week) 3	7.4167	0.7769	9.546	7.43e-14 ***
as.factor (week) 4	9.0000	0.7769	11.584	< 2e-16 ***
as.factor (week) 5	11.8333	0.7769	15.231	< 2e-16 ***
as.factor (week) 6	12.1667	0.7769	15.660	< 2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 1.903 on 63 degrees of freedom
Multiple R-squared: 0.9045, Adjusted R-squared: 0.8923
F-statistic: 74.54 on 8 and 63 DF, p-value: < 2.2e-16

swimming time by 4.83, 6.83 and 8.44 units respectively. The intercept and slopes of regression fit was significant at 1% level and the R^2 was 0.89. Furthermore, the regression analysis revealed that as the weeks progressed there was an increase of 2.91, 7.41, 9.00, 11.83 and 12.16 units in average swimming time ($p<0.01$). The regression fit (Table 2) clearly indicates that swimming time per se is significantly ($p<0.01$) influenced by the TPVGC in diet and duration of feeding (in weeks). Similarly, anti-fatigue activity of the animal fed with TPVGC showed elevation from the 1st week to 6th week of swimming exercise in the treatment groups (Fig. 2). The animals could pick up an increase of 6.62 units and 7.73 units ($p<0.05$) during the 5th and 6th week of feeding, respectively. The regression model fit was significant at 1% with $R^2 = 0.56$. The regression fit (Table 3) revealed that

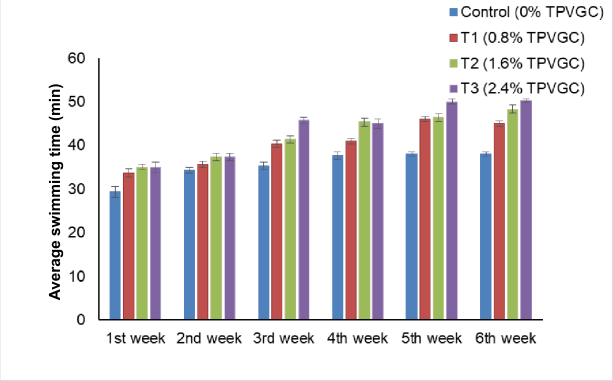

Fig. 1. Influence of dietary supplementation of thiamine and pyridoxine-loaded vanillic acid-grafted chitosan on average swimming time (min) of albino rats exposed to swimming exercise.

Values are expressed as mean ± SE (n= 6)

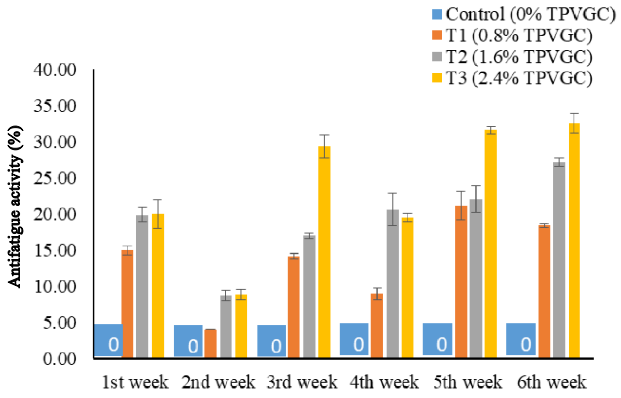

Fig. 2. Influence of dietary supplementation of thiamine and pyridoxine-loaded vanillic acid-grafted chitosan on % antifatigue activity of albino rats exposed to swimming exercise.

Values are expressed as mean± SE (n= 6)

the treatments and progressing weeks could effectively influence the anti-fatigue activity in animals at 1% level of significance. Results from the present investigation indicated that dietary supplementation of TPVGC at graded level has enhanced the average swimming time and anti-fatigue activity compared to control group.

Lactate dehydrogenase and malate dehydrogenase activities in the muscle, liver, kidney and brain tissue of rats exposed to swimming exercise showed significant ($p<0.05$) difference between control and treatment groups and are presented in Table 4. A clear trend of decreasing LDH and MDH activities were observed in the treatment group fed with graded level of dietary TPVGC. Highest LDH activity was recorded in the brain followed by muscle, liver and kidney. However, higher MDH activity was recorded in the muscle followed by brain, liver and kidney. Haffor & Alhazza (2007) reported that any change in the glycolytic and mitochondrial enzymes affects the LDH activity. Higher MDH activity indicates higher energy demand in animal and it also shows the cellular injury (Kawai & Hosaki, 1990). A higher LDH and MDH activities were observed in the control group due to swimming exercise, whereas a decreasing trend was observed in the treatment groups. It is postulated that the dietary supplementation of TPVGC in the diet could efficiently maintain the homeostasis of metabolic enzymes as reported by several authors (Anand, 2005; Alttas & Haffor, 2010; Gupta et al., 2015; Vinothiya & Ashokkumar, 2017; Yavari et al., 2015).

SOD and catalase activities in muscles, liver, kidney and brain tissues of rats exposed to swimming exercise are shown in Table 5. SOD and catalase activities in all tissues showed significant ($p<0.05$) difference between control and experimental groups. Control group has recorded higher SOD and catalase activities in all tissues, whereas, treatment group fed with increase in the level of the dietary TPVGC showed gradual decrease in the enzyme activities. Among the tissues, relatively higher SOD activity was recorded in the kidney followed by liver, brain and muscle in control group. However, higher catalase activity was observed in liver followed by kidney, muscle and brain in control group.

Generally, animals have wide range of antioxidant mechanism that includes both enzymatic and non-

enzymatic defensive system (Singh et al., 2010). In the present study, the dietary supplementation of thiamine and pyridoxine-loaded vanillic acid-grafted chitosan in the treatment group showed decreasing SOD and catalase activity which may be due to the anti-oxidative effect of TPVGC. Thiamine and pyridoxine are known to play key roles in maintaining the metabolic homeostasis of the animal, whereas, vaniilic acid grafted-chitosan is known for the antioxidant properties. It is hypothesized that the TPVGC, being an antioxidant helps to reduce the oxidative stress and hence regulated the SOD and catalase activity in the treatment groups. Similar results have been reported by several authors (Akhtar et al., 2010; Singh et al., 2010; Tejpal et al., 2017).

AChE activity in brain tissue showed significant ($p<0.05$) difference between the control and treatment group (Fig. 3). Control group showed lower AChE activity compared to the treatment groups. Acetylcholine act as a neurotransmitter and plays a

Table 3. Regression Fit for Anti-fatigue activity data.

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	13.088	2.424	5.399	2.28e-06 ***
TrtT2	5.618	2.100	2.676	0.01029 *
TrtT3	10.015	2.100	4.770	1.89e-05 ***
as.factor (week) 2	-11.065	2.969	-3.727	0.00053 ***
as.factor (week) 3	1.863	2.969	0.627	0.53346
as.factor (week) 4	-1.940	2.969	-0.653	0.51672
as.factor (week) 5	6.620	2.969	2.230	0.03069 *
as.factor (week) 6	7.736	2.969	2.605	0.01232 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 6.299 on 46 degrees of freedom
Multiple R-squared: 0.6211,Adjusted R-squared: 0.5635
F-statistic: 10.77 on 7 and 46 DF, p-value: 5.995e-08

Table 4. Effect of dietary supplementation of TPVGC on LDH and MDH activities in muscle, liver, kidney and brain tissue of albino rats exposed to swimming stress.

Parameters	Tissue	Treatments			
		Control (0% TPVGC)	T_1 (0.8% TPVGC)	T_2 (1.6% TPVGC)	T_3 (2.4% TPVGC)
LDH	Muscle	$1.78^{b} \pm 0.09$	$1.45^{b} \pm 0.17$	$1.00^{a} \pm 0.05$	$0.75^{a} \pm 0.07$
	Liver	$0.98^{b} \pm 0.04$	$0.75^{a} \pm 0.06$	$0.74^{a} \pm 0.04$	$0.63^{a} \pm 0.02$
	Kidney	$0.79^{b} \pm 0.04$	$0.62^{a} \pm 0.03$	$0.56^{a} \pm 0.05$	$0.53^{a} \pm 0.01$
	Brain	$1.81^{b} \pm 0.13$	$1.78^{b} \pm 0.10$	$1.65^{ab} \pm 0.02$	$1.43^{a} \pm 0.12$
MDH	Muscle	$120.70^{c} \pm 0.34$	$108.46^{b} \pm 2.55$	$95.65^{a} \pm 4.43$	$89.32^{a} \pm 1.91$
	Liver	$30.14^{c} \pm 1.86$	$22.59^{b} \pm 1.94$	$21.63^{b} \pm 1.36$	$14.06^{a} \pm 0.62$
	Kidney	$23.44^{c} \pm 3.25$	$16.64^{b} \pm 0.30$	$10.05^{a} \pm 0.35$	$9.36^{a} \pm 1.81$
	Brain	$109.33^{c} \pm 4.32$	$84.09^{b} \pm 2.73$	$72.71^{ab} \pm 0.49$	$67.23^{a} \pm 3.42$

Values are expressed as mean± SE with different superscript (a, b & c) in the row indicate significant difference ($p<0.05$) among the control and treatment groups.

Units: nanomoles of pyruvate utilized/mg protein/min (LDH); nanomoles of Oxaloacetate utilized/mg protein/min (MDH)

Table 5. Effect of dietary supplementation of TPVGC on SOD and Catalase activities in muscle, liver, kidney and brain tissue of albino rats exposed to swimming stress

Parameters		Treatments			
	Tissue	Control (0% TPVGC)	T_1 (0.8% TPVGC)	T_2 (1.6% TPVGC)	T_3 (2.4% TPVGC)
SOD	Muscle	$46.84^{b} \pm 1.11$	$40.88^{a} \pm 1.04$	$40.36^{a} \pm 1.55$	$37.85^{a} \pm 1.15$
	Liver	$124.72^{b} \pm 0.62$	$123.38^{b} \pm 3.99$	$118.67^{ab} \pm 0.75$	$115.35^{a} \pm 0.56$
	Kidney	$126.19^{b} \pm 4.21$	$106.55^{a} \pm 1.75$	$106.00^{a} \pm 1.36$	$102.54^{a} \pm 1.34$
	Brain	$60.59^{a} \pm 5.90$	$54.28^{a} \pm 0.52$	$52.10^{a} \pm 2.75$	$50.34^{a} \pm 2.24$
Catalase	Muscle	$13.12^{c} \pm 1.77$	$8.54^{b} \pm 0.74$	$6.40^{ab} \pm 0.55$	$4.08^{a} \pm 0.50$
	Liver	$64.88^{c} \pm 1.75$	$56.02^{b} \pm 1.09$	$53.64^{b} \pm 2.33$	$46.13^{a} \pm 0.87$
	Kidney	$53.23^{b} \pm 2.04$	$52.95^{b} \pm 0.90$	$43.85^{a} \pm 0.75$	$40.51^{a} \pm 0.83$
	Brain	$9.28^{c} \pm 0.34$	$7.84^{bc} \pm 0.47$	$6.54^{ab} \pm 1.09$	$5.05^{a} \pm 0.26$

Values are expressed as mean ± SE with different superscript (a, b & c) in the row indicate significant difference ($p<0.05$) between the control and treatment groups.

Units: 50% inhibition of epinephrine auto oxidation mg^{-1} protein min^{-1} (SOD) and m mol H_2O_2 decomposed min^{-1} mg^{-1} protein at 37°C (Catalase).

vital role in the central nervous system. Acetylcholinesterase help in metabolizing the acetylcholine into acetyl CoA and choline. This process helps to prevent the accumulation of acetylcholine in the synaptic buds. On the other hand, accumulation of acetylcholine affects the energy metabolism of nervous system and communication of nerve impulses, in addition this may cause behavioral changes in the animal (Adamson, 1977; Das et al., 2001; Tejpal et al., 2017). Present study reveals that exposure to swimming exercise lowers the AChE activity in control group which indicate higher level of accumulation of acetylcholine in the brain tissue of rats. Excessive accumulation of acetylcholine in brain is reported to be neurotoxic (Soreq & Seidman 2011). Dietary supplementation of TPVGC in the treatment group showed higher AChE activity of brain tissue.

PCA analysis was used to explore the overall relationship between the dietary supplementation of TPVGC and the enzyme activities (LDH, MDH, SOD, Catalase (CAT) and AChE) in muscle, liver, kidney and brain tissues. Prior to factor reduction the Kaiser-Meyer-Olkin statistics was computed to test sampling adequacy and Bartlett's test of sphericity conducted to check redundancy between variables and it was ensured that the data sets are suitable for PCA. The PC plots pertaining to the PCA of enzyme data pertaining to muscle, kidney, liver and brain is given in the Fig. 4. Principal component analysis of enzyme data pertaining to muscle revealed that the 1st two PCs explained 96% of the variability in the data (Fig. 4A). The PC plot clearly separated the samples according to the treatments. MDH, LDH & SOD influenced the swimming exercise in similar fashion and were positively correlated, whereas the catalase activity (CAT) was influenced the behaviour in opposite manner and negatively correlated. AChE was negatively correlated with all the other variables.

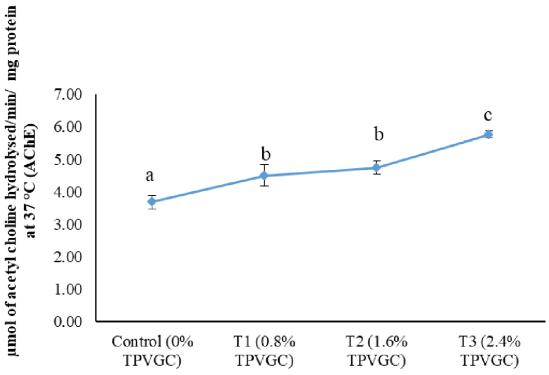

Fig. 3. Impact of dietary supplementation of TPVGC on AChE in brain tissue of albino rats exposed to swimming stress

Values are expressed as mean±SE with different superscript (a, b & c) in the figure indicate significant difference ($p<0.05$) among the control and treatment groups

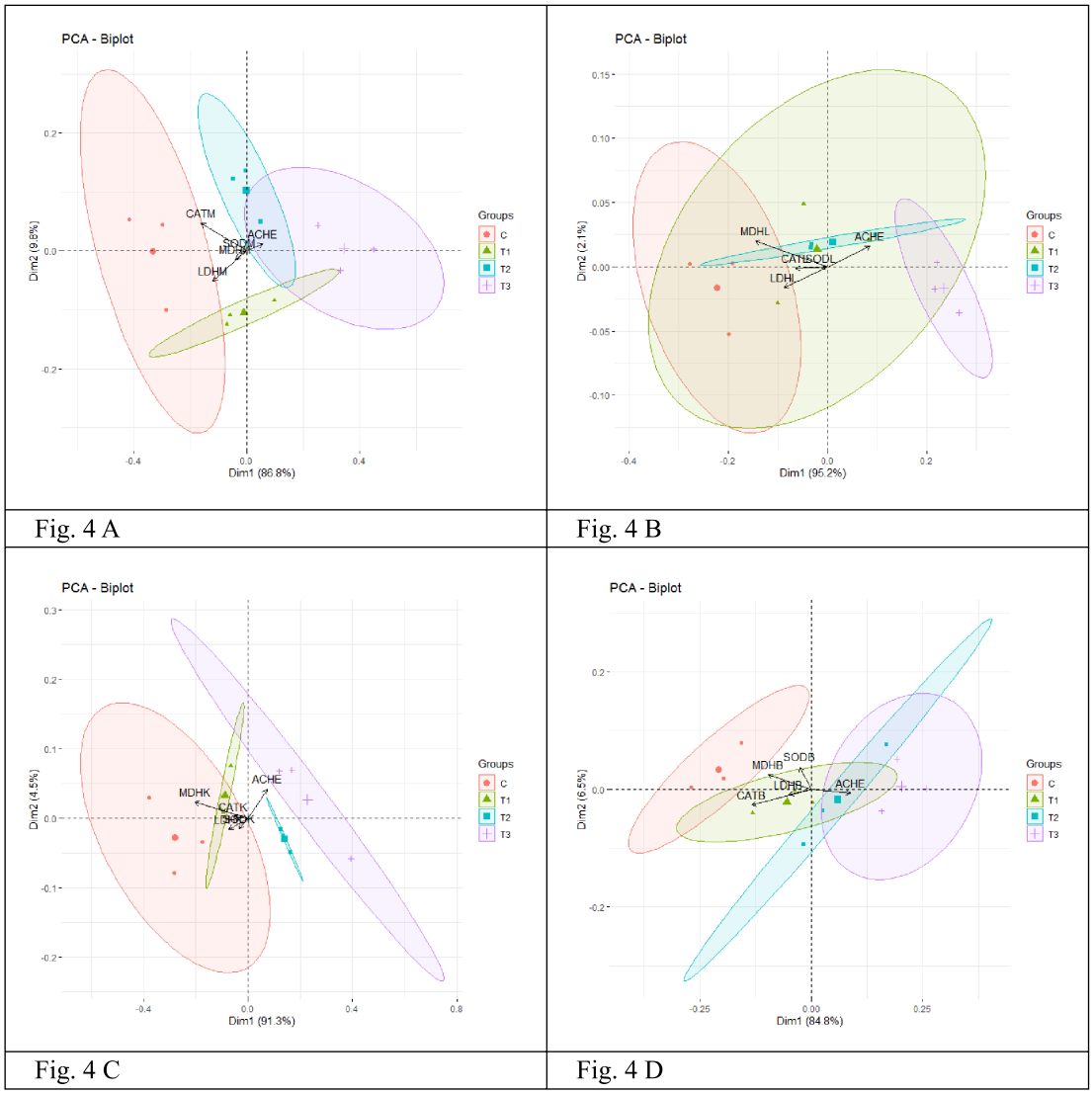

Fig. 4. Principal Component Analysis (PCA) of enzyme data. 4A. Depicts the influence on TPVGC in diet on enzyme activity in muscle tissue; 4B. Depicts the influence on TPVGC in diet on enzyme activity in liver tissue; 4C. Depicts the influence on TPVGC in diet on enzyme activity in kidney tissue and 4D. Depicts the influence on TPVGC in diet on enzyme activity in brain tissue

The PCA of enzyme data pertaining to liver tissues revealed that 97% of variation in data could be explained by the first 2 PCs (Fig. 4B). The PC plot clearly separated the samples of T3 and Control indicating the action of enzymes on these parts are highly significantly different. The variables CAT, SOD and LDH were correlated positively with each other exhibiting the same type of influence on the liver tissues. Whereas LDH and AChE were not correlated and MDH was negatively correlated. The PCA of enzyme data pertaining to kidney tissues revealed that 95% of the variation in the data could be explained by the 1st 2 principal components (Fig. 4C). The variables CAT, LDH and SOD were positively correlated and influence the action of enzymes in the same way, and the variables MDH & AChE were negatively correlated with these variables. The PCA of enzyme data pertaining to brain tissues of the animals revealed that the first 2 PCs could explain 91% variation in the enzyme data (Fig. 4D). LDH-brain and CAT-brain exhibited positive correlation whereas, AChE was negatively correlated with these variables. The variables MDH and SOD influenced the activity in brain in a

significantly different manner compared to LDH and CAT. SOD was not correlated with LDH and CAT.

The novel composite of thiamine, pyridoxine and chitosan grafted with vanillic acid has proven to exhibit synergistic effects against stress and fatigue in experimental rats, which has immense applications as nutraceuticals for livestock production sector, aquaculture and also for human healthcare products. The dietary supplementation of TPVGC showed increased anti-fatigue activity in dose dependent manner when exposed to swimming stress. The higher level of bio-beneficial effect against swimming induced stress was recorded in the animal fed at 2.4% TPVGC as revealed by the activity of LDH, MDH, SOD, catalase and AChE. From the results, it can be concluded that TPVGC can be used as a potential nutraceutical or as a functional food ingredient.

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