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Research Article
Effect of *Cymbopogon citratus* Essential Oil (EO) on Handling Stress in Giant Freshwater Prawn (*Macrobrachium rosenbergii*)

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Abstract

Background and Objective: Effects of *Cymbopogon citratus* essential oil (EO) was tested on minimizing handling stress in *Macrobrachium rosenbergii* through the evaluation of their metabolite responses [glucose, lactate, glycogen, protein, Lactate Dehydrogenase (LDH), Malate Dehydrogenase (MDH), Acetylcholinesterase (AChE) and Alanine Aminotransferase (ALT)]. This study aimed to investigate the efficacy of *C. citratus* extract in the anaesthetization of *M. rosenbergii*. **Materials and Methods:** Three treatments including control, prawn exposed to stress alone (T_1) and prawn exposed to stress in the presence of *C. citratus* EO (T_2) were tested. A *C. citratus* EO at 500 µL L^{-1} had been determined in a previous study and was selected as the critical dose to be applied as an anesthetic agent. Handling stress was induced into prawns by netting, at 2 min interval for 30 min and their hemolymph were collected to determine the metabolite responses. **Results:** The increase of glucose, lactate and LDH of *M. rosenbergii* when exposed to handling stress alone (T_1) in comparison to T_2 (stress with anesthetic *C. citratus* EO) were identified. Further, a low glycogen level in parallel with low AChE activity was observed which indicates the involvement of secondary metabolites to cope with the energy demand in T_1 over T_2. **Conclusion:** This study indicates the efficiency of *C. citratus* EO to reduce stress during handling in *M. rosenbergii*.

Key words: Anaesthetic, *Cymbopogon citratus*, lemongrass, *Macrobrachium rosenbergii*, metabolite responses, secondary metabolites, stress handling

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anesthetic is the calmness-causing agents which are commonly applied during capture, handling, sorting and transporting of fishes and shellfishes to minimize stress[1]. In crustaceans, the increase of Crustacean Hyperglycemic Hormone (CHH) due to stress environment resulting in changes in metabolic enzymes and physiological performances of the animals[2,3]. Several chemicals have been applied to mitigate the anesthetic effects in prawns including tricaine methanesulfonate (MS222), however, it was not effective in crustaceans and produce side effects which make the prawns unsafe for human consumption[4]. Salin[5] has reported the efficient use of cold-anaesthetization using chilled sawdust to minimize stress in *M. rosenbergii* but only for a short-transportation period.

Cymbopogon citratus is usually called lemongrass and comes from a family of Graminaceae. It can be found in tropical and subtropical countries including Malaysia, Indo-China, Sri Lanka, Africa and South Africa[6]. Citral and geraniol are commonly reported as major compounds in *C. citratus* compound and possess several biological activities[7-9]. Interestingly, *C. citratus* has been found can act as a relaxant and antidepressant in forced swimming rats[10]. Besides, Blanco[11] had reported the use of *C. citratus* leaves in treating nervous excitement. Based on those mentioned above, it was then hypothesized that *C. citratus* extract may exhibit anesthetic effects on stressed *M. rosenbergii*. Thus, a study was conducted to investigate the efficacy of *C. citratus* extract in anaesthetization of *M. rosenbergii*.

MATERIALS AND METHODS

Preparation of the experimental animals: This study was carried out in the Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, Malaysia from 2014-2018. Adult *M. rosenbergii* with an average weight of 18-23 g were used in this study without regard to gender. Prawns procured from a local farm in Jelebu, Negeri Sembilan were brought in an aerated fiberglass tank (1000 L) at ambient water temperature. During this period, prawns were fed with commercial pellets by Cargill Sdn. Bhd., twice per day for 15 days before use in experiments.

Plant collection and extraction: A *Cymbopogon citratus* leaves were collected from a local garden in Kuala Terengganu and dried in an oven (25°C) for 3-5 days. Dried leaves were powdered using a heavy-duty blender (Waring, USA) and extracted by a hydro-distillation method as according to Kulkarni[12], with slight modifications. The crude essential oil of *C. citratus* was concentrated in a rotary evaporator (Buchi, Flawil, Switzerland) into 1 mL and stored in an amber bottle at 4°C until further use.

Preparation of *C. citratus* essential oil anesthetic: A *Cymbopogon citratus* essential oil was diluted in ethanol in the ratio of 1:10. A stock solution was made up by diluting 20 mL of essential oil in 200 mL ethanol. An appropriate volume of stock solution was added into a freshwater aquarium to obtain the concentrations needed. In this study, 500 μL L^{-1} *C. citratus* essential oil was determined previously as a minimum concentration to cause induction in *M. rosenbrgii*. Hence, 500 μL L^{-1} *C. citratus* essential oil was prepared and used in this stress analysis to determine the changes in hemolymph content after stress. Sole ethanol was used as a control in this study.

Metabolic responses of *M. rosenbergii* to handling in presence of *C. citratus* essential oil as anesthetic: Three different groups of prawns were performed in separate 15 L aquarium contained 10 L of freshwater. Six prawns were used in each treatment with five replicates each. The stress test was designed according to Saydmohammed and Pal[13], with slight modifications. In the T_1 group, test prawns were exposed to handling stress without anesthetic. In the T_2 group, prawns were exposed to handling stress with the presence of anesthetic at 500 μL L^{-1}, whilst in the control group, prawns were maintained without handling stress and no presence of anesthetic in the aquarium. Handling stress in T_1 and T_2 groups were created by disturbing the prawns by netting for 2 min interval in 30 min. Prawns were sampled after 30 min and their hemolymph were collected separately for analysis.

Hemolymph preparation: Hemolymph was collected from each prawn through the ventral sinus, placed in a centrifuge tube containing anticoagulant solution (0.114 M trisodium citrate and 1 M sodium chloride at pH 7.45) and centrifuged at 5000 g for 15 min. After separation, the supernatant was stored at -80°C until further biochemical analysis. All procedures were performed in the ice-cold condition (4°C).

Hemolymph biochemical analysis: The concentration of glucose, lactate and glycogen contents were determined using standard kits (Abnova, Taiwan), according to the manufacturer's protocols. The activities of LDH, ALT and AChE

were also determined using commercial assay kits from a similar manufacturer (Abnova, Taiwan). The MDH activities were determined using another commercial kit from a different manufacturer (Sigma Aldrich, USA). The protein content was determined using a method derived by Bradford[14]. A 100 µL hemolymph was diluted with 900 µL distilled water in a clean test tube. Then, 1 mL of Bradford reagent was added and color development was observed in 10 min. Another clear test tube was filled with bovine serum albumin (BSA) (Difco™, USA) standard at a similar amount of Bradford reagent (Difco™, USA). The absorbance of each mixture was measured using a UV/Vis Spectrophotometer (Thermo Fisher Scientific, USA) at 595 nm wavelength and the amount of total protein in the hemolymph sample was calculated based on the plotted calibration curve graph.

Statistical analysis: The hemolymph chemistry parameters were homogenous (Levene's test) and normally distributed (Kolmogorov-Smirnov test). Therefore, a one-way ANOVA test was performed and $p<0.05$ was considered as significant. All tests were performed using MINITAB 16.0 software.

RESULTS AND DISCUSSION

In this present study, data of glucose, lactate and glycogen and protein concentration in *M. rosenbergii* hemolymph after 30 min handling stress with and without *C. citratus* EO was presented in Fig. 1-4. A significantly higher glucose concentration with a simultaneously lower glycogen level was observed in the stressed group without *C. citratus* EO (T_1) as compared to T_2 and control. Similarly, an increase in lactate concentration, whilst stagnant in protein level was also found in the T_1 group as compared to T_2 and control group.

Meanwhile, data on different enzyme activities of *M. rosenbergii* after handling stress for 30 min with and without *C. citratus* EO was tabulated in Table 1. A significantly lower LDH activity was found in the stressed-treated group (T_2) as compared to the non-treated stress group (T_1). In contrast, MDH activity was found to increase in the stressed-treated group (T_2) as compared to the control and T_1 group, however, its increment was not significant. An increase of ALT activity was found in the stress-treated group (T_2) as compared to T_1 and control. Further, AChE activity was decreased in the stressed group (T_1) and found to increase in the stressed-treated group (T_2), with no significant difference.

Exposure to types of stress in captive animals will affect their physiological performance and immune-competence, which in turn reduced their current market value[15]. In crustaceans, stressful conditions will induce the regulation of

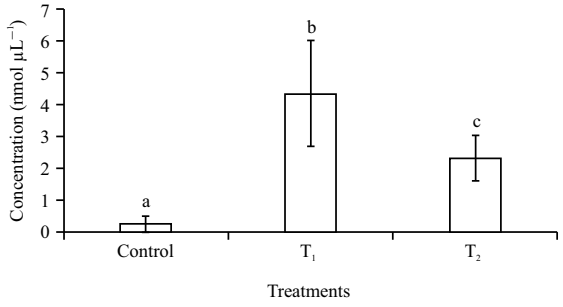

Fig. 1: Glucose content of *M. rosenbergii* hemolymph in response to handling stress

Different small letters (a, b, c) indicate significant differences amongst different treatments, T_1: Stressed group, T_2: Stressed group in the presence of *C. citratus* EO, $p<0.05$ was considered significant

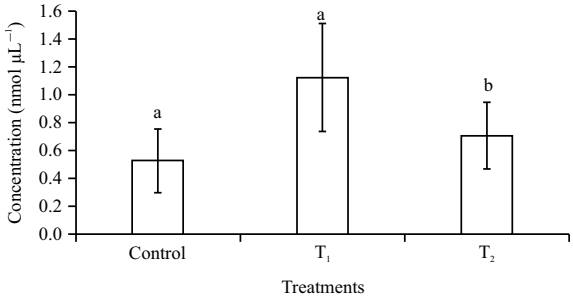

Fig. 2: Lactate content of *M. rosenbergii* hemolymph in response to handling stress

Different small letters (a, b) indicate significant differences amongst different treatments, T_1: Stressed group, T_2: Stressed group in the presence of *C. citratus* EO, $p<0.05$ was considered significant

Table 1: Enzymes of *M. rosenbergii* hemolymph in response to handling stress

Activity	Control	Stressed+Ethanol (T_1)	Stressed+*C. citratus* EO (T_2)
LDH	10.33 ± 2.23^b	16.79 ± 4.03^a	10.38 ± 3.40^b
MDH	6.17 ± 1.51^a	5.86 ± 0.70^a	6.70 ± 0.79^a
AChE	7.58 ± 0.70^a	7.45 ± 0.74^a	7.56 ± 1.00^a
ALT	3.96 ± 1.85^a	4.51 ± 1.84^a	4.96 ± 1.98^a

LDH: Lactate dehydrogenase, MDH: Malate dehydrogenase, AChE: Acetylcholinesterase, ALT: Alanine aminotransferase, LDH: µm mg^{-1}, MDH: Protein min^{-1}, AChE: mg protein min^{-1} at 37°C, ALT: Nanomoles of pyruvate formed mg^{-1} protein min^{-1} at 37°C, different superscripts in the same row (a, b) indicate significant differences amongst different treatments, $p<0.05$ was considered significant

Crustacean Hyperglycemic Hormones (CHH), elevate the glucose level thus results in gross changes in animal physiological performance[2,16]. Despite the vastness of techniques applied to mitigate stress in crustaceans[5,17], one in demand currently is the use of natural anesthetic comes from plant herbs[13,18,19].

This current study reported the potential use of *C. citratus* EO as stress minimizing agent in *M. rosenbergii*. A 500 µL L^{-1} of *C. citratus* EO was used to anesthetize *M. rosenbergii* in

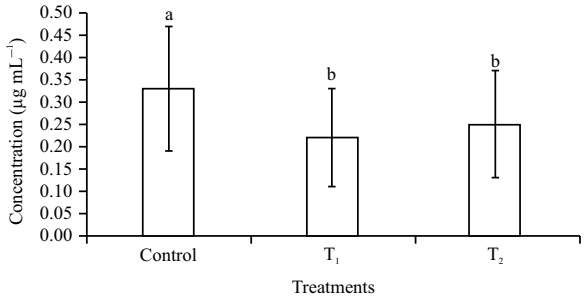

Fig. 3: Glycogen content of *M. rosenbergii* hemolymph in response to handling stress

Different small letters (a, b) indicate significant differences amongst different treatments, T_1: Stressed group, T_2: Stressed group in the presence of *C. citratus* EO, $p<0.05$ was considered significant

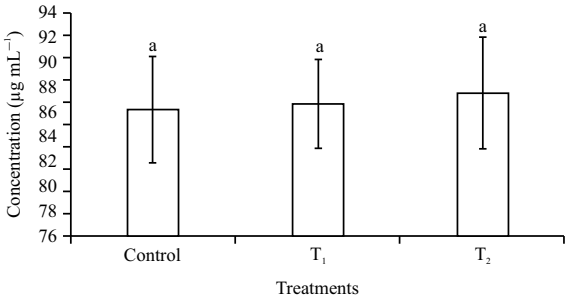

Fig. 4: Protein content of *M. rosenbergii* hemolymph in response to handling stress

Similar small letters (a) indicate no significant differences amongst different treatments, T_1: Stressed group, T_2: Stressed group in the presence of *C. citratus* EO, $p<0.05$ was considered significant

this study, which had been previously determined able to promote anesthetic effect to *M. rosenbergii*, with a high survival rate (92%). A significant increase of glucose level with a concomitant decrease of glycogen level ($p<0.05$) in stressed prawn alone (T_1), over stress-treated prawn with anesthetic *C. citratus* EO (T_2), respectively has been shown in Fig. 1-3. The high level of glucose in the T_1 group was likely maintained by utilizing the stored glycogen in the hemolymph due to stress handling. The finding was similar to Stentiford *et al.*[20], where they observed the utilization of glucose by the glycogenolysis process in Norway lobsters when exposed to parasites. Glycogenolysis is a process of breaking down the glycogen molecule into glucose[21]. Interestingly, glucose and glycogen levels showed an inverse trend in the T_2 group over T_1 which indicates the efficiency of *C. citratus* EO as a stress-relieving agent by providing calmness to the stressed prawns. As been reported by Vale *et al.*[22], *C. citratus* EO is commonly high in citral compound which is capable to produce anxiolytic, sedative and motor relaxant effects in mice. Nevertheless, the glucose level in T_2 was depicted higher than the control group, 2.34 ± 0.71 and 0.27 ± 0.25 nmol μL^{-1}, respectively. This situation might be due to the addition of *C. citratus* EO itself which exhibits stressor effects to the prawn, thus induce the stress response mechanisms[23]. Readman *et al.*[24] had stated the aversive effects of plant essential oils in aquatic fish even applied at low concentrations.

Further, the lactate level in the T_2 group was decreased significantly over T_1, 1.13 ± 0.39 and 0.71 ± 0.24 nmol μL^{-1}, respectively. The reduction might be due to the less stress experienced by the prawn in the T_2 group with the presence of *C. citratus* EO as compared to the stress-alone group (T_1). The formation of lactate is when the glycolysis process is performed under the anaerobic condition to maintain adequate energy during stress condition[25]. The decrease of the lactate level was parallel to the decreasing of LDH activity in T_2. This LDH enzyme is important in converting pyruvate into a lactate molecule particularly when rapid energy is needed during several stressors such as handling stress and environmental stress[26]. Ribas *et al.*[27], had reported the ability of 1000 mg L^{-1} clove oil to anesthetize seawater Senegal sole (*Solea senegalensis*) which resulted in a decrease of glucose and lactate hemolymph.

MDH is involved in aerobic metabolisms and plays an important role in metabolite exchange[26]. However, results show no significant difference in MDH activity in all treatment groups (Table 1). Findings were contradicted with Saydmohammed and Pal[13] which reported the increase of MDH activity in *M. rosenbergii* due to stress handling. However, results might vary as the sample used are also different. Readman *et al.*[24] also clarify that the results of the essential oil itself may acts differently in different procedures and animals used. Hemolymph protein in the present study was more or less similar among all treatment groups (Fig. 4), which revealed the protein was not affected by handling stress or anesthesia effects from *C. citratus* EO. The result was supported by Nagahama *et al.*[29], where the plasma protein in silver catfish (*Rhamdia quelen*) anesthetized with both *Hesperozygis ringens* and *Lippia alba* EO were not change. Typically the unaffected protein in hemolymph resulted in no changes in ALT activities represents no protein metabolism involved during treatment.

Acetylcholine is considered as an inhibitory transmitter in crustaceans which are stored and released from synaptic vesicles[30]. In the present study, AChE activity was found to be inhibited in the T_1 group, over control and T_2 (Table 1), which results in an increased level of acetylcholine at postsynaptic ends[31]. This enzyme inhibition in synaptic buds during handling was reported previously to cause erratic behavior in rats[32]. The addition of *C. citratus* EO in stressed prawn

increased AChE activity in the T_2 group as compared to T_1 which indicates the ability of *C. citratus* EO to ameliorate the chlorogenic effect due to stress handling in *M. rosenbergii*.

It seems that *C. citratus* essential oil used in this study can provide a relaxation effect to stressed prawns as depicted in their metabolites hemolymph. However, the hematological analysis also would be an advantage if could be determined, which important to support the finding, become our limitation in this study. Furthermore, the determination of their mode of actions also is crucial to provide a thorough understanding of the anesthetic mechanisms in crustaceans.

CONCLUSION

In conclusion, results indicate the potentiality of *C. citratus* essential oil as an anesthetic agent by minimizing handling stress in *M. rosenbergii*. However, the dosage during application may vary among species and the size of animals used needs to be further investigated.

SIGNIFICANCE STATEMENT

This study discovers the metabolite's responses in prawn hemolymph after been exposed to stress handling with the presence of *C. citratus* essential oil as an anesthetic agent. Findings would be beneficial for other researchers and also people in aquaculture industries to uncover the potentiality of *C. citratus* essential oil for further development as the demand for natural anesthetic is at high stake currently. Thus, a new natural anesthetic from other plant sources also can be developed and possibly with the rise of any other plant anesthetics or combination of them, may be arrived at.

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REFERENCES

1. Hoseini, S.M., H. Rajabiesterabadi and R. Tarkhani, 2015. Anaesthetic efficacy of eugenol on iridescent shark, *Pangasius hypophthalmus* (Sauvage, 1878) in different size classes. Aquacult. Res., 46: 405-412.
2. Thompson, I., A. White, T.C. Fletcher, D.F. Houlihan and C.J. Secombes, 1993. The effect of stress on the immune response of Atlantic salmon *Salmo salar* L. fed diets containing different amounts of vitamin C. Aquaculture, 114: 1-18.
3. Jorgensen, E.H., M.M. Vijayan, N. Aluru and A.G. Maulu, 2002. Fasting modifies aroclor 1254 impact on plasma cortisol, glucose and lactate responses to a handling disturbance in Arctic charr. Comp. Biochem. Phys. C, 132: 235-245.
4. da Cunha, M.A., F.M.C. de Barros, L. de Oliveira Garcia, A.P. de Lima Veeck and B.M. Heinzmann *et al*., 2010. Essential oil of *Lippia alba*: A new anesthetic for silver catfish, *Rhamdia quelen* Aquaculture, 306: 403-406.
5. Salin, K.R., 2005. Live transportation of *Macrobrachium rosenbergii* (De Man) in chilled sawdust. Aquac. Res., 36: 300-310.
6. Bertea, C.M. and M.E. Maffei, 2009. The Genus *Cymbopogon*: Botany including anatomy, physiology, biochemistry and molecular biology. In: Essential oil bearing plants: The genus Cymbopogon, Anand Akhila, CRC Press, United States, ISBN: 9780849378584, Pages:262.
7. Aiemsaard, J., S. Aiumlamai, C. Aromdee, S. Taweechaisupapong and W. Khunkitti, 2011. The effect of lemongrass oil and its major components on clinical isolate mastitis pathogens and their mechanisms of action on *Staphylococcus aureus* DMST 4745. Res. Vet. Sci., 91: e31-e37.
8. Costa, C.A.R.A., D.O. Kohn, V.M. Lima, A.C. Gargano, J.C. Florio and M. Costa, 2011. The GABAergic system contributes to the anxiolytic-like effect of the essential oil from *Cymbopogon citratus* (lemongrass). J. Ethnopharmacol., 137: 828-836.
9. Pereira, R.P., R. Fachinetto, A. da Souza Prestes, R.L. Puntel and G.N.S. da Silva *et al*., 2009. Antioxidant effects of different extracts from *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*. Neurochem. Res., 34: 973-983.
10. Komori, T., R. Fujiwara, M. Tanida, J. Nomura and M.M. Yokoyama, 1995. Effects of citrus fragrance on immune function and depressive states. Neuroimmunomodula., 12: 174-180.
11. Blanco, M.M., C.A.R.A. Costa, A.O. Freire, J.G. Santos Jr. and M. Costa, 2009. Neurobehavioral effect of essential oil of *Cymbopogon citratus* in mice. Phytomedicine, 16: 265-270.
12. Kulkarni, R.N., K. Baskaran and S. Ramesh, 2003. Five cycles of recurrent selection for increased essential oil content in East Indian Lemongrass: Response to selection and effects on heritabilities of traits and inter trait correlations. Plant Breed., 122: 131-135.
13. Saydmohammed, M. and A.K. Pal, 2009. Anesthetic effect of eugenol and menthol on handling stress in *Macrobrachium rosenbergii*. Aquaculture, 298: 162-167.
14. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

15. EL-Khaldi, A.T.F., 2010. Effect of different stress factors on some physiological parameters of Nile tilapia (*Oreochromis niloticus*). Saudi J. Biol. Sci., 17: 241-246.
16. Davidson, G.W., P.S. Davie, G. Young and R.T. Fowler, 2007. Physiological responses of rainbow trout *Oncorhynchus mykiss* to crowding and anesthesia with AQUI-S?. J. World Aquacult. Soc., 31: 105-114.
17. Coyle, S.D., S. Dasgupta, J.H. Tidwell, T. Beavers, L.A. Bright and D.K. Yasharian, 2005. Comparative efficacy of anaesthetics for the freshwater prawn *Macrobrachium rosenbergii*. J. World Aquacult. Soc., 36: 282-290.
18. Manush, S.M., A.K. Pal, T. Das and S.C. Mukherjee, 2005. Dietary high protein and vitamin C mitigate stress due to chelate claw ablation in *Macrobrachium rosenbergii* males. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol., 142: 10-18.
19. Parodi, T.V., M.A. Cunha., C.G. Heldwin, D.M. Souza and A.C. Martins *et al*., 2012. The anaesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol., 155: 462-468.
20. Stentiford, G.D., E.S. Chang, S.A. Chang and D.M. Neil, 2001. Carbohydrate dynamics and the crustacean hyperglycemic hormone (CHH): Effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). Gen. Comp. Endocrinol., 121: 13-22.
21. Kuo, C.M. and Y.H. Yang, 1999. Hyperglycemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*. J. Comp. Physiol. B, 169: 49-54.
22. Vale, T.G., E.C. Furtado, J.G. Santos and G.S.B. Viana, 2002. Central effects of citral, mycrene and limonene, constituents of essential oil chemotypes from *Lippia alba* (Mill.) N.E. Brown. Phytomedicine, 9: 709-714.
23. Souza, A.S.L., A.C. Peret, M. Hamoy, R.A.L. Souza, M.F. Torres and L.A.L. Barbas, 2019. Propofol and essential oil of *Nepeta cataria* induce anaesthesia and marked mycorelaxation in tambaqui *Colossoma macrpamum*: Implications on cardiorespiratory responses. Aquaculture, 500: 160-169.
24. Readman, G.D., S.F. Owen, J.C. Murrell and T.G. Knowles, 2013. Do fish perceive anaesthetics as aversive? PLoS ONE, Vol. 8. 10.1371/journal.pone.0073773.
25. Ridgeway, I.D., A.C. Taylor, R.J.A. Atkinson, G.D. Stentiford, E.S. Chang, S.A. Chang and D.M. Neil, 2006. Morbidity and mortality in Norway lobster, *Nephrops norvegicus* physiological, immunological and pathological effects of aerial exposure. J. Exp. Mar. Biol. Ecol., 328: 251-264.
26. Diamantino, T.C., E. Almeida, A.M.V.M. Soares and L. Guilhermino, 2001. Lactate dehydrogenase activity: An effective parameter in aquatic toxicity tests. Chemosphere, 45: 553-560.
27. Ribas, L., R. Flos, L. Reig, S. MacKenzei, B.A. Barton and L. Tort, 2007. Comparison of methods for anaesthesizing Senegal sole (*Solea senegalensis*) before slaughter: Stress responses and final product quality. Aquaculture, 269: 250-258.
28. Toni, C., A.G. Becker, L.N. Simoes, C.G. Pinheiro and L.L. Silva *et al*., 2013. Fish anaesthesia: Effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). Fish Physiol. Biochem., 40: 701-714.
29. Nagahama, T., K.R. Weiss and I. Kupfermann, 1993. Effects of cerebral neuron C-PR on body postural muscles associated with a food-induced arousal state in Aplysia. J. Neurophysiol., 70: 1231-1243.
30. Patel, N.M. and N. Dewaswala-Bhopalwala, 2020. Parasympathomimetic Medications. StatPearls Publisher, United States of America,.
31. Nilsson, O.G., P. Kalen, E. Rosengren and A. Björklund, 1990. Acetylcholine release in the rat hippocampus as studied by microdialysis is dependent on axonal impulse flow and increases during behavioural activation. Neuroscience, 36: 325-338.