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Research Article

Pycocyanin: A virulence factor of *Pseudomonas aeruginosa* in the disruption of brain homeostasis regulation in gold fish *Carassius auratus*

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Abstract

Recent reports reported that the oral and gut microorganisms are responsible for the regulation of the brain homeostasis mechanism. This brain homeostasis mechanism is affected by the colonization of non-periodontic microorganisms in the oral cavity and the gut compared to periodontic pathogens. Among the non-periodontic microorganisms, *Pseudomonas aeruginosa* is one of the gram-negative bacilli that play a major role in the development of cognitive impairment through the production of a secondary metabolite called pycocyanin.The present study aimed to test the effect of pycocyanin on the development of cognitive impairment for the first time with the help of a comparative two-staged behavioral analysis: non-infusive behavioral studies (NBS) and infusive behavioral studies (IBS) of goldfish *Carassius auratus*. Non-infusive behavioral experimental groups (BEGs) used in the NBS mimicked the condition of a healthy state and infusive behavioral experimental groups (BEGs) either received isolated metabolites and microbial cultures of the day – 3, 4, 5, and 6 in the form of oral infusions. Effect of metabolite/ microbial culture in the infusive study groups was proved by comparing the behavioral scores of non-infusive groups. Employed two-staged behavioral analysis proved that cognitive impairment induction (75-79%) was higher in the metabolite oral infusions compared to the microbial oral infusions in the behavioral study groups. Obtained results showed that induction of cognitive impairment resulted from reactive oxygen species (ROS) production and neuroinflammation was high in the brain regions due to the transportation of administrated metabolite from the gut to the brain in its purest form compared to the microbial oral infusions.

Keywords: Cognition, Neuroinflammation, Oral-Gut-Brain axis, *Pseudomonas aeruginosa*, Pycocyanin

INTRODUCTION

Pseudomonas aeruginosa is one of the gram-negative, non-motile, and opportunistic bacilli identified as one of the important non-periodontal pathogen that plays a major role in the development of cognitive dysfunctions through oral/gut dysbiosis in a host system (Diggle and Whiteley, 2020; Qin *et al.,* 2022; Mukilan, 2023; Mukilan *et al.,* 2024b). In a healthy state, native oral and gut microflora play a major role in developing cognitive functions through regulating the oral-gut-brain (OGB) axis via the blood-brain barrier (BBB). Other than the development of cognitive functions, it also plays a major role in the regulation of the hypothalamic-pituitary axis (HPA) by limiting the production of cortisol (Appleton, 2018; Bowland and Weyrich, 2022; Kumar *et al.,* 2024). However, these regulated conditions were disrupted by diseased conditions or dysbiosed oral and gut microflora states. This dysbiosis may happen due to pathogenic colonization, lifestyle changes, smoking, food hygiene, etc. (Kilian *et al.,* 2016; Sedghi *et al.,* 2021; Cicchinelli *et al.,* 2023). Initially, the formation of dysbiosis begins in the oral cavity (OC) due to poor oral hygiene, which may result in the aberration of beneficial flora present in the OC. Initially, it shows symptoms like dental cavities, tooth decay (dental caries), tooth loss, gum disease, and oral infectious diseases. These symptoms are developed by the colonization and proliferation of periodontal and non-periodontal pathogens in the different regions of the OC for a shorter or longer period (Radaic and Kapila, 2021; Georges *et al.,* 2022; Pisano, 2023; Ribero and Paster, 2023). Both of these pathogens result in the development of gut dysbiosis through its transmission to the gut. Compared to periodontal pathogens, non-periodontal pathogens are simultaneously responsible for developing mixed systemic diseases/disorders. Among other non-periodontal pathogens, *P. aeruginosa* was reported as one of the im-

portant nosocomial infectious agents in the medical field (Radaic and Kapila, 2021; Hou *et al.,* 2022; Abdulkareem *et al.,* 2023; Stefano *et al.,* 2023). It can able to acquire a drug resistance mechanism (DRM) within a short time through the intake of antibiotics/drugs during the treatment process. The development of DRM results in the suppression of immune and neuronal activities of a host system through the production of various primary and secondary metabolites (Langford *et al.,* 2020; Krockow *et al.,* 2023; Muteeb *et al.,* 2023; Pitchaikani *et al.,* 2024).

Recent reports show that pycocyanin is more prominent among the primary and secondary metabolites produced by *P. aeruginosa*. Produced pycocyanin was identified as a redox-active compound with two benzene rings and a heterocyclic structure in the center (Rashid *et al.,* 2022; Shouman *et al.,* 2023; Mudaliar and Prasad, 2024). The stated redox property altered the equilibrium within a biological system. The colonization and proliferation of *P. aeruginosa* in the OC and the gut may result in inflammation by producing pycocyanin (Moradali *et al.,* 2017; Qin *et al.,* 2022). Formed inflammation reduces the native microflora of the oral cavity and the gut. This reduction results in the colonization and proliferation of non-periodontal pathogens, which results in the formation of oral dysbiosis (OD) in the OC (Nie *et al.,* 2023; Santacroce *et al.,* 2023; Kuraji *et al.,* 2024). Formed OD may transmit higher levels of non-periodontal pathogens into the gut through the oral passage. Transmitted non-periodontal pathogens further proliferate in the gut and cause inflammation in the BBB (Liu *et al.,* 2021; Visentin *et al.,* 2023; Zhou *et al.,* 2023; Li *et al.,* 2024). Later on, produced pycocyanin crosses BBB and reaches the brain to reduce the neurotransmitter production in the presynaptic neuron. Reduced neurotransmitter production results in the development of cognitive impairment. Other than induction of cognitive impairment, higher level of pycocyanin results in the induction of oxidative stress (OS) through the reversal of redox activity in the brain tissues (Abdelaziz *et al.,* 2023; Jabłońska *et al.,* 2023; Mudaliar and Prasad, 2024). Induced OS further results in the development of neuroinflammatory reactions in the brain neurons. Prolonged neuroinflammatory reactions result in the development of neurodegenerative disorders in the host system (Tanaka *et al.,* 2020; Chidambaram *et al.,* 2022). Based on the literature survey, the present study was designed to study the role of pycocyanin in cognitive memory formation in goldfish *Carassius auratus* through the oral administration of metabolite-producing *P. aeruginosa*, and isolated pycocyanin metabolites. The initial attempt was made to show the effect of pure and mixed forms of metabolites in the development of cognitive impairment.

MATERIALS AND METHODS

Experimental fishes

Experimental adult naïve goldfish *Carassius auratus* (n = 60, body length: 6.5 – 7.5 cm, body weight: 6 – 14 g; both male and female) were purchased from a commercial aquarium located at P.N. Palayam, Coimbatore – 641 044, Tamil Nadu, India. Purchased fishes were transported to a laboratory aquarium (LA) in a stress-free environment with ambient temperature. Once reaching the LA, fishes were examined for the non-microbial patches in the skin and separated as behavioral experimental groups (BEGs) (n = 6/group). After group categorization, fish were housed in two home tanks for habituation. Home tanks serve as a local aquarium for maintaining experimental fishes (EF) throughout the study. The designed local aquarium consisted of 42 X 30 X 21 inches (length X breadth X height) sized glass rectangular tanks with a light and dark cycle of 14:10 hours, and continuous aeration throughout the day at a temperature of 20 ± 2 ºC. All EF were given commercial dry food pellets thrice a day to meet their energy needs. Aquarium water level and its quality were continuously monitored to maintain the needed dissolved oxygen content (DOC) within the aquarium. Aquarium water was changed on alternative days or whenever needed during the studies to maintain.

Ethical approval

The experimental study design, setup, and protocols followed Sri Ramakrishna Institutions (SRI) institutional animal care guidelines, Coimbatore, Tamil Nadu.

Behavioral experimental setup

The designed behavioral experimental setup (BES) consisted of three different cabinets for performing reward-based learning paradigms during non-infusive behavioral studies (NBS) and infusive behavioral studies (IBS). Among the three different cabinets, two cabinets were designated as the right cabinet (RC) and the left cabinet (LC). Designated RC and LC acted as a positive and negative reward chamber with two different color cues (blue and red). Both of these cabinets were located at the opposite ends of the BES, and a central cabinet (CC) was present between them. CC has a size of 30 X 30 X 21 inches and RC, & LC has a size of 6 X 30 X 21 inches (length X breadth X height). Developed BES was used for performing NBS and IBS in their respective timeline.

Behavioral experimental groups

Experimental fishes were designated into five different behavioral experimental groups (BEGs). Formulated BEGs include BEG - 1, BEG - 2, BEG - 3, BEG - 4, and BEG - 5. In NBS, each BEG (n = 6/group) was allowed

to perform NBS training and testing phases. After completion of NBS, IBS was carried out in a two-phased manner. In the first phase of IBS, BEGs of 2, 3, 4, and 5 received oral administration of culture from days 3 – 5 and in the second phase, isolated extracts from days 3 - 5 were orally administered to the BEGs of 2, 3, 4, and 5.

Non-infusive behavioral studies

Non-infusive behavioral studies (NBS) were carried out with the help of four different stages of behavioral analysis. Four different stages of behavioral analysis include habituation (days 1 - 4), exploration (days 5 - 7), training (days 8 - 10), and testing (days 14 - 16). Seventy hours of interval time gap was provided between the training and testing phases for the memory consolidation process. Behavioral responses of BEGs were observed based on the time spent in LC, CC, and RC during the training and testing phases.

Predator exposure test

After completion of NBS training, EF was allowed to perform a predator exposure test to test the effect of the reward-based learning paradigm on the development of fear memory in BEGs. A predator exposure test (PET) performed after NBS training was used to identify the amount of stress that affects EA's learning abilities. Like the post-PET training, it was also performed after testing to prove the absence of stress formation in the NBS. PET was performed in an experimental chamber (42 X 30 X 21 inches) with three different zones (14 X 10 X 21 inches/each), like complete fear zone (CF), mid fear zone (MF), and no fear zone (NF). *Pseudotropheus demasoni* (cichlid fish) was placed in a separate chamber in the NF and used as a predator.

Microbial culture acquaintance and its purity confirmation

Pseudomonas aeruginosa used in the present study was availed from PSG–IMSR, Coimbatore, Tamil Nadu. The acquired microorganism was quadrant streaked on cetrimide agar for its purity confirmation and species identification. Streaked plates were incubated at 37 ºC for 24 hours for their colony formation. Formed individual colonies were used to prepare cultures from day 1 - 9. Aroused cultures were used to prepare an oral infusion mixture and isolate metabolite (pycocyanin) needed for the infusive behavioral studies (IBS).

Preparation of extract

Fresh cultures (72, 96, 120, and 144 hours) of *P. aeruginosa* were used to isolate extract from the aroused cultures. The color change was initially confirmed in the nutrient broth medium from yellow to green. Confirmed bacterial culture was used to isolate metabolite with the help of centrifugation and phase separation methods. The centrifugation process was mainly used to pellet out the bacterial cells present in the culture. After centrifugation, the supernatant was collected and mixed with an equal amount of chloroform. After the addition of chloroform, the complete mixture was vortexed for 60 seconds and incubated at 4 °C for 60 minutes. After 60 minutes, the mixture was centrifuged at 12000 rpm for 20 minutes for the phase separation. The separated chloroform phase was collected in a new tube and mixed with an equal volume of 0.2 N hydrochloric acid. Gentle mixing of mixture results in the formation of a red color ring-like structure. Further, the pH of the isolated extract metabolite was neutralized with 0.2 N NaOH. Aroused cultures and extracted metabolites were taken at a ratio of 50:50 along with phosphate buffer saline (PBS) to prepare culture oral infusive and extract oral infusive mixtures.

Infusive behavioral studies

Infusive behavioral studies (IBS) were carried out in a two-phased manner with the help of training (days 24 - 26) and testing (days 30 - 32) phases after oral administration of culture and extract. Seventy two hours of interval time gap was provided after oral administration of culture and extracts on day 20 for the settlement of infused culture/extract in the gut. In the first phase of IBS, BEGs – 2, 3, 4, and 5 received oral infusions of culture from days 3 – 6 and were allowed to perform training and testing phases. Behavioral responses were calculated based on the time spent in LC, CC, and RC during the training, and testing phases. In the second phase, BEGs – 2, 3, 4, and 5 received oral infusions of isolated extract from days 3 – 6 to test the effect of the pure form of metabolite on the development of cognitive impairment. In both phases, BEG - 1 was used as a non-infusive control to validate the level of cognitive impairment developed in the infusive BEGs.

Open field test

Following the training and testing phases of IBS, an open field test (OFT) was performed to study the effect of culture oral infusions (COI), and extract oral infusions (EOI) on the development of anxiety-like behavior in the infusive BEGs. OFT was performed in a glass rectangular tank with a length, breadth, and height of 42 X 30 X 21 inches. The bottom of the tank was separated into equal-sized individual boxes of 10 X 5 cm in size. All BEGs were allowed to perform OFT after IBS training and testing in both the phases (first and second phases) between days 27-29, and 33-35.

Pictorial representation

Behavioral mean and standard error values of NBS (training, testing, PET) and IBS (two phases - training,

testing, OFT) were used to prepare the bar diagram using the Microsoft Excel Program.

RESULTS

The present study used two different approaches, i.e. microbiological techniques and behavioral analysis to prove the effect of secondary metabolite (pycocyanin) in the induction of cognitive impairment in a serene habituated environment. The formation of cognitive impairment was validated by a comparative two-step behavioral analysis (non-infusive and infusive behavioral analysis).

Identification of pycocyanin production time interval by culture-dependent analysis

Initially, the obtained culture was quadrant streaked in a nutrient agar plate to confirm the purity of the culture. After purity confirmation, individual colonies were used as inoculums to prepare day-wise simple and quadrant-streaked plates from days 1–9. After inoculum streaking, all plates were incubated at 37 ºC for their respective time intervals (Days 1-9). Representative photographs were taken between days 1-9 by placing simple and quadrant plates together in a single window. Obtained results showed that no metabolite production happened till 24 hours (Days 1, and 2), initial synthesis of metabolite production was started from day 2. After day 2, there was a gradual increase in metabolite production till 120 hours (Day – 5). A higher level of metabolite production (OD value – 1.38) was observed at day – 5, which gradually started to reduce from day - 6 onwards till day 9. Obtained results show the metabolite (pycocyanin) production between days 2 – 6 compared to days 1, 7, 8, and 9 (Fig. 1).

To validate the plate metabolite production results, the confirmatory nutrient broth culture method (CNBCM) was used. In CNBCM, an equal amount of nutrient broth (3 ml/tube) was taken in nine sterile test tubes. Later, the dispersed medium was inoculated with the individual colonies of *P. aeruginosa* and incubated at 37 ºC in an incubator for the specified time intervals from zero minute to 216 hours. Results were recorded and photographed at their respective time intervals 24, 48, 72, 96, 120, 144, 168, 192, and 216 hours (Days 1-9).

Results of CNBCM proved that initial metabolite production was started on day 2 and peaked on day 5. It also proved that metabolite production started to decline after day 6 (Fig. 2). Followed by CNBCM, metabolite was isolated from the cultures of days 1 – 9 with the help of a modified extraction method. The hydrogen ion concentration (pH) of the extracted metabolite was neutralized with the help of 0.2 N NaOH. The pH Neutralized results in the red color ring-like appearance in the extract, which shows the presence of the desired metabolite (pycocyanin) in the isolated extract. Isolated extracts and culture tubes were used to show the metabolite production from days 2 - 6. Obtained results also validated the pycocyanin production between days 2 – 6 (Fig. 3). Isolated days 2 - 6 extracts were used in infusive behavioral studies (IBS) .

Role of laboratory habituated stress-free environment in the development of cognitive memory formation

At first, cognitive memory formation was tested with the help of non-infusive behavioral studies (NBS). Employed NBS used a reward-based learning paradigm to evaluate behavioral responses during the training and testing phases in this initial behavioral analysis. NBS used red and blue colors placed in negative and positive chambers to identify learning abilities and information retrieval based on a food reward. Initially, all behavioral experimental groups (BEGs) [BEGs – 1, 2, 3, 4, and 5] were allowed to expose the experimental setup (ES) with the color cues available in both of the feeding chamber (FCs) during days 8 - 10. Behavioral responses of the non-infusive behavioral training phase showed that a habituated stress-free environment played a major role in gradually developing all BEGs' learning abilities during the consecutive days. From the training responses of all BEGs, it is proved that all experimental fishes learned about the provided stimuli with the help of a food reward in an activity-dependent manner during the training days (Fig. 4A). Later on, formed memory was tested in the ES after 3 days of the interval during the NBS testing phase (days 14-16). Behavioral responses of the NBS testing phase showed that information retrieval was high in all BEGs, which showed that the memory consolidation process was properly formed in the hippocampal brain regions. Obtained NBS testing scores showed higher level responses occurred in all BEGs compared to the NBS training scores.

The outcome of the NBS testing phase also showed that memory retrieval happened in a lesser amount of time compared to the time taken for grasping new information by the different brain regions (Fig. 4B). Finally, a comparative analysis of NBS training and testing scores showed the development of stronger cognitive memory formation in the ES with the help of a reward-based learning paradigm. During the NBS training phase, the amount of time taken by the EF was high due to the presence of new stimuli in an assimilated environment; as a result, the number of correct choices were low compared to the NBS testing scores. It also proved that experimental fishes took longer to grasp new information provided in the ES. A comparison of NBS testing scores with training scores proved that retrieval of

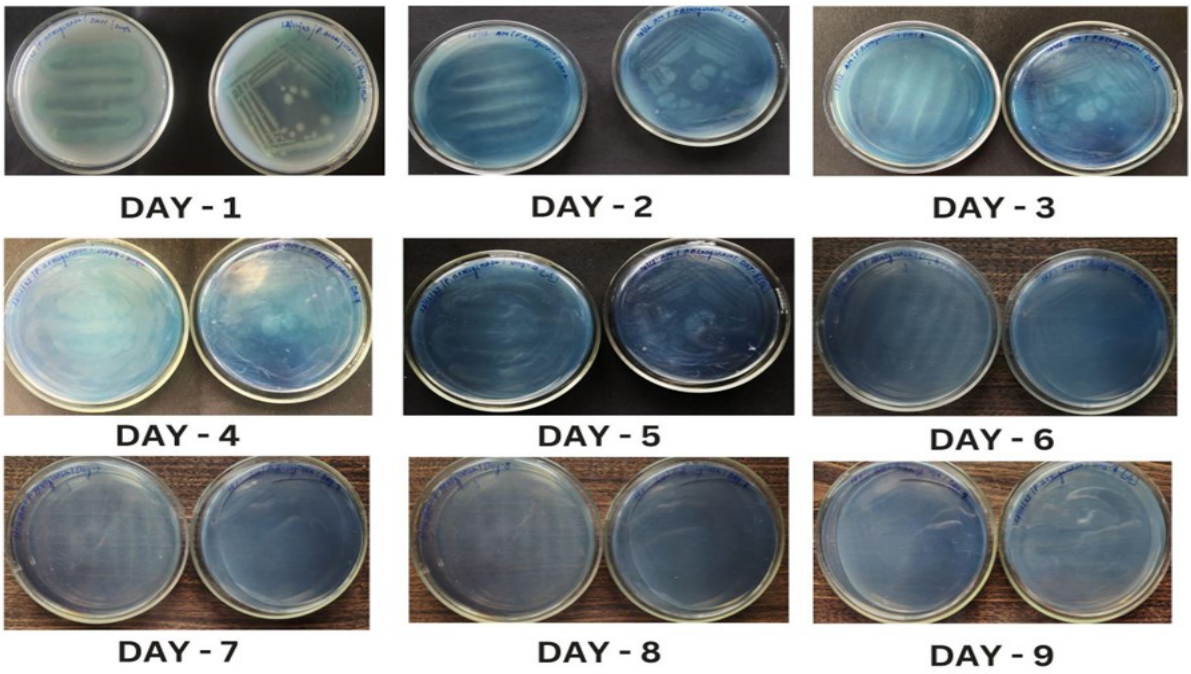

Fig. 1. *Representative plate picture showing the simple and quadrant streaked nutrient agar plates of days 1-9. Pycocyanin metabolite production was observed between days 2 – 6 compared to days – 1, 7, 8, and 9*

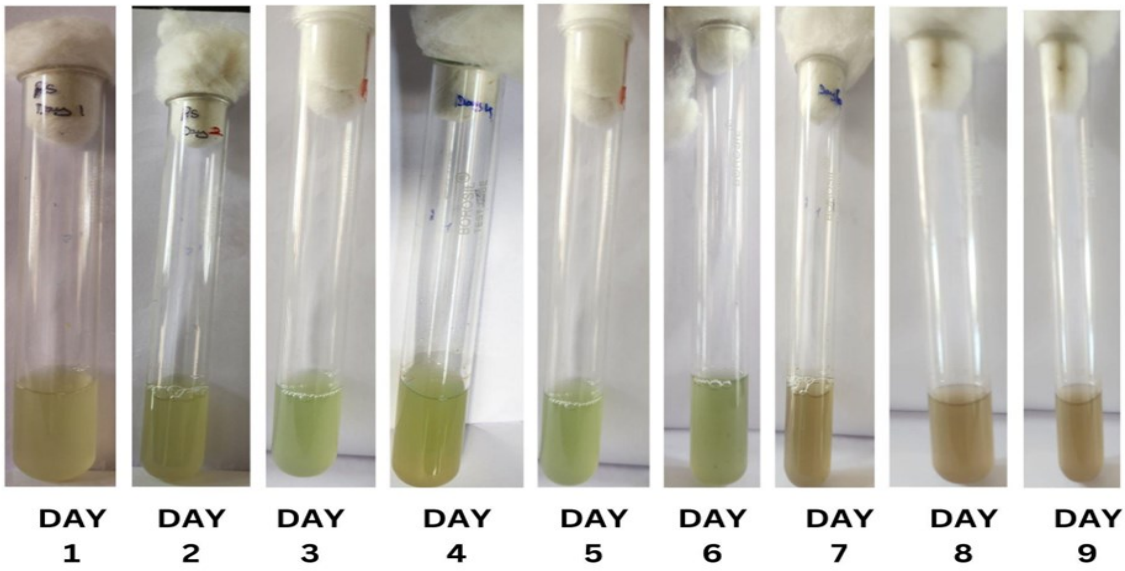

Fig. 2. *Confirmatory nutrient broth cultures showing the production of pycocyanin metabolite in the medium between days 2 – 6*

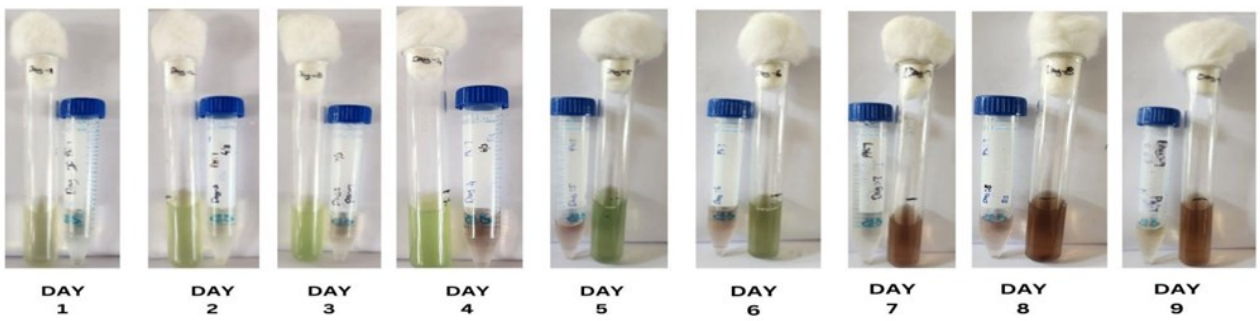

Fig. 3. *Showing the extracted metabolite along with the day-wise culture in an individual panel*

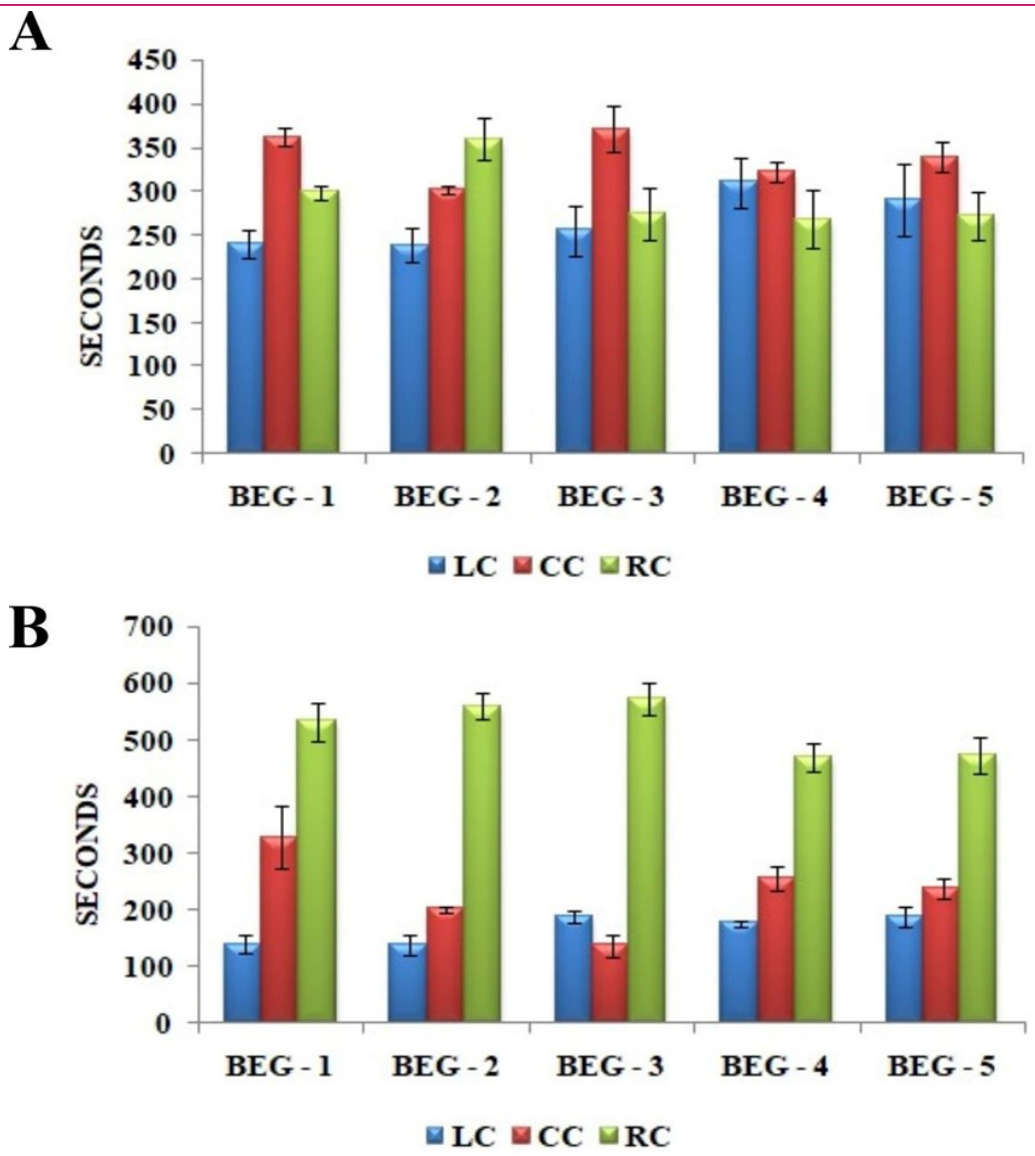

Fig. 4. *Behavioral responses of the non-infusive behavioral training (A) and testing (B) phases showed that habituated stress-free environment played a major role in the development of cognitive processes like learning and memory formation (BEG – behavioral experimental group, LC – left chamber, CC – central chamber, RC – right chamber)*

learned information was high due to the memory consolidation process occurring in the hippocampus. Proper memory consolidation also showed higher correct responses in a short period (Fig. 5).

At the end of the NBS training and testing phases, PET was performed to identify the development of fear memory formation. All BEGs were exposed to their natural predator (*P. demasoni*) in BES during the PET. The predator was placed in a separate chamber inside the NF zone in BES. After completion of the NBS training and testing phases, PET was performed between days 11-13 & 17-19 to test the amount of stress developed in the BEGs. In both phases, all BEGs spent more amount of time in the NF zone compared to the MF and CF zones, which showed the absence of stress in the EA (Fig. 6). Comparative analysis of PET scores (after training and testing) showed that NBS training and testing never stimulate stress development in the form of fear memory against its predator in a stress-free environment. However, it also showed that PET scores collected after testing had a high amount of time spent in the NF zone, which validates the absence of fear memory formation compared to the PET scores of post-training phase (Fig. 7).

Impact of pycocyanin oral infusions on the induction of cognitive memory decline

After completion of NBS, infusive behavioral studies (IBS) were carried out in two different stages between days 24-35. Following NBS, BEGs (BEGs – 2, 3, 4, and 5) received oral infusions of culture and extracts of days - 2, 3, 4, and 5. On day 20, aroused culture and isolated extracts of day – 2, 3, 4, and 5 were orally administrated into the respective groups with the help of

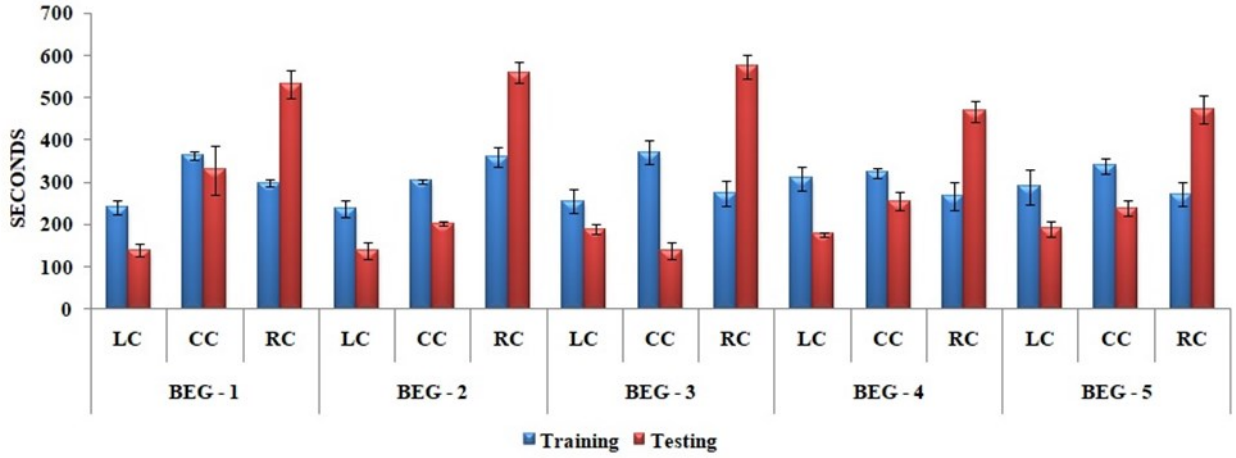

Fig. 5. *Comparative analysis of non-infusive training and testing scores showed the fish information acquaintance through learning abilities and its memory retrieval in a habituated stress-free environment (BEG – behavioral experimental group, LC – left chamber, CC – central chamber, RC – right chamber)*

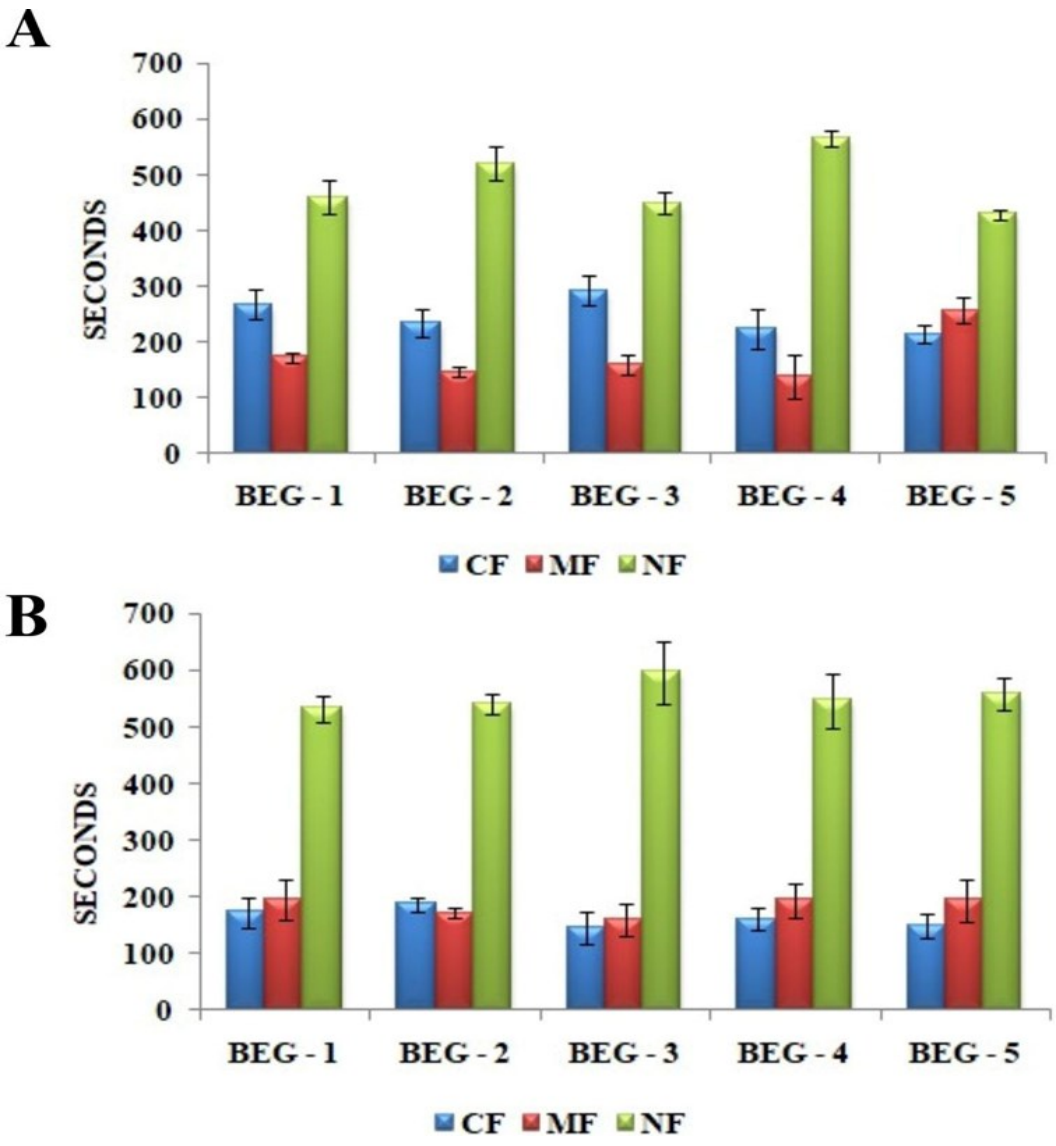

Fig. 6. *Behavioral scores of the predator exposure test showed non-infusive behavioral training (A) and testing phases (B) not involved in the causation of fear memory formation in a habituated stress-free environment (BEG – behavioral experimental group, CF – complete fear zone, MF – mid fear zone, NF – no fear zone)*

an oral gauge. After oral administration of cultures and extracts, respective administrated groups were provided with 72 hours of settlement time between days 21 - 23. IBS training and testing phases were carried out during 24 - 26 and 30 - 32 days. Culture-infused BEGs and extract-infused BEGs were trained in BES for a period of three consecutive days (Days 24 -26) with the same paradigm used for the NBS. Obtained behavioral training scores of culture-infused BEGs showed lower to higher levels of cognitive learning impairment in the infused groups (BEGs – 2, 3, 4, and 5).

Among the four infused groups, BEGs - 4, which received day-5 culture oral infusions (COI) showed a higher level of cognitive learning impairment compared to the other three infused groups. The level of cognitive impairment in BEG – 4 was validated by comparing the number of correct responses with the non-infusive group (BEG -1) (Fig. 8A). Followed by COI training, extract oral infusive (EOI) training was carried out to validate the effect of isolated metabolite on the learning abilities of infused BEGs on the consecutive training days. The outcome of the EOI training scores showed that metabolite infusions had less impact on the learning impairment compared to the BEG – 1 (Fig. 8A). IBS testing responses of the COI and EOI infused BEGs (BEGs – 2, 3, 4, and 5) showed that extract infusions induced a higher level of cognitive impairment compared to culture infusions. The formed cognitive impairment was high in the EOI due to the presence of metabolite alone in the infusions (Fig. 8B). Comparative analysis of COI and EOI training scores showed that fishes learning abilities were impaired in the extract-infused groups compared to the culture-infused groups. Compared to training scores, testing scores of EOI groups showed a higher level of cognitive decline than the COI groups. Formed cognitive decline was demonstrated by a reduction in the number of correct choices in the BES (Fig. 9 A & B). The outcome of the IBS proved that in isolated pure form, pycocyanin induces a higher level of cognitive impairment compared to culture infusions which are present in mixed form (Fig. 10).

To validate the relationship between cognitive memory impairment and anxiety-like behavior (AB) development, an open field test (OFT) was performed after the completion of COI and EOI training and testing phases between days 27 – 29, and 33-35. OFT scores of post (COI and EOI) training showed higher level AB development in the culture and extract-infused BEGs (BEGs – 2, 3, 4, and 5) compared to a non-infusive control (BEG – 1). Development of AB was proved by the amount of time spent in the outer (TSO) chamber compared to time spent in the inner chamber (TSI) (Fig. 11). Followed by the completion of the IBS post-testing OFT was performed to correlate the formation of cognitive impairment with the development of AB in the infused group compared to the non-infusive group. OFT scores of IBS post-testing showed that higher levels of AB may have an interrelationship with cognitive memory impairment (Fig. 12). The outcome of the post-training and testing OFT responses also showed that AB development had a greater impact on the learning abilities and its information retrieval in the culture and extract-infused BEGs compared to non-infusive control (BEG - 1). Comparative analysis of post-infusive training and testing OFT scores showed that in isolated/ pure form pyocyanin induces a higher level of AB by hindering the learning abilities and memory consolidation process in the brain regions. It was observed that COI and EOI BEGs spent more time in TSI than in TSO, which is vice versa in the non-infusive group (BEG – 1) (Fig. 13).

DISCUSSION

The present study showed the role of *P. aeruginosa's* virulence factor pycocyanin in the development of cognitive impairment through reduced brain plasticity

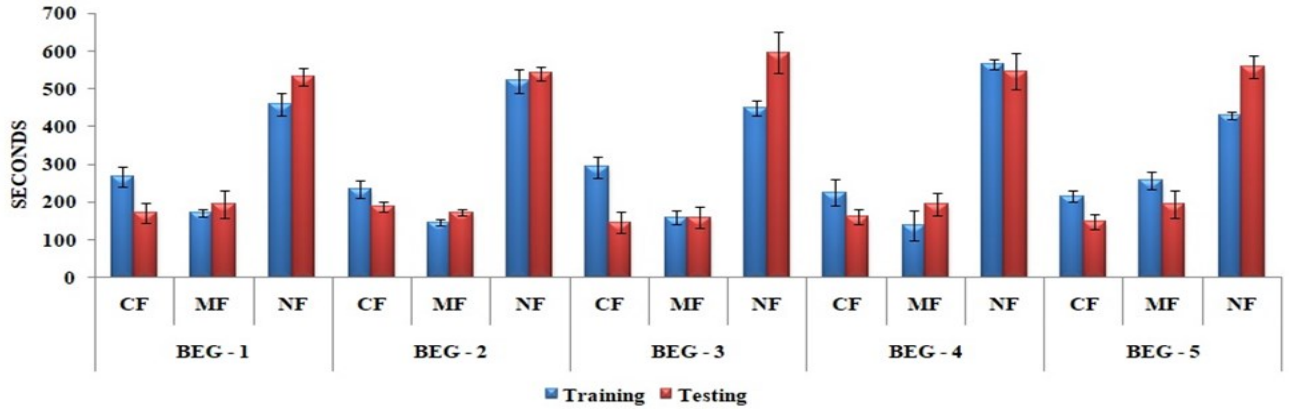

Fig. 7. *Comparative analysis of predator exposure test showed that non-infusive training and testing never stimulate the formation of fear memory against the predator in a stress-free environment (BEG – behavioral experimental group, CF – complete fear zone, MF – mid fear zone, NF – no fear zone)*

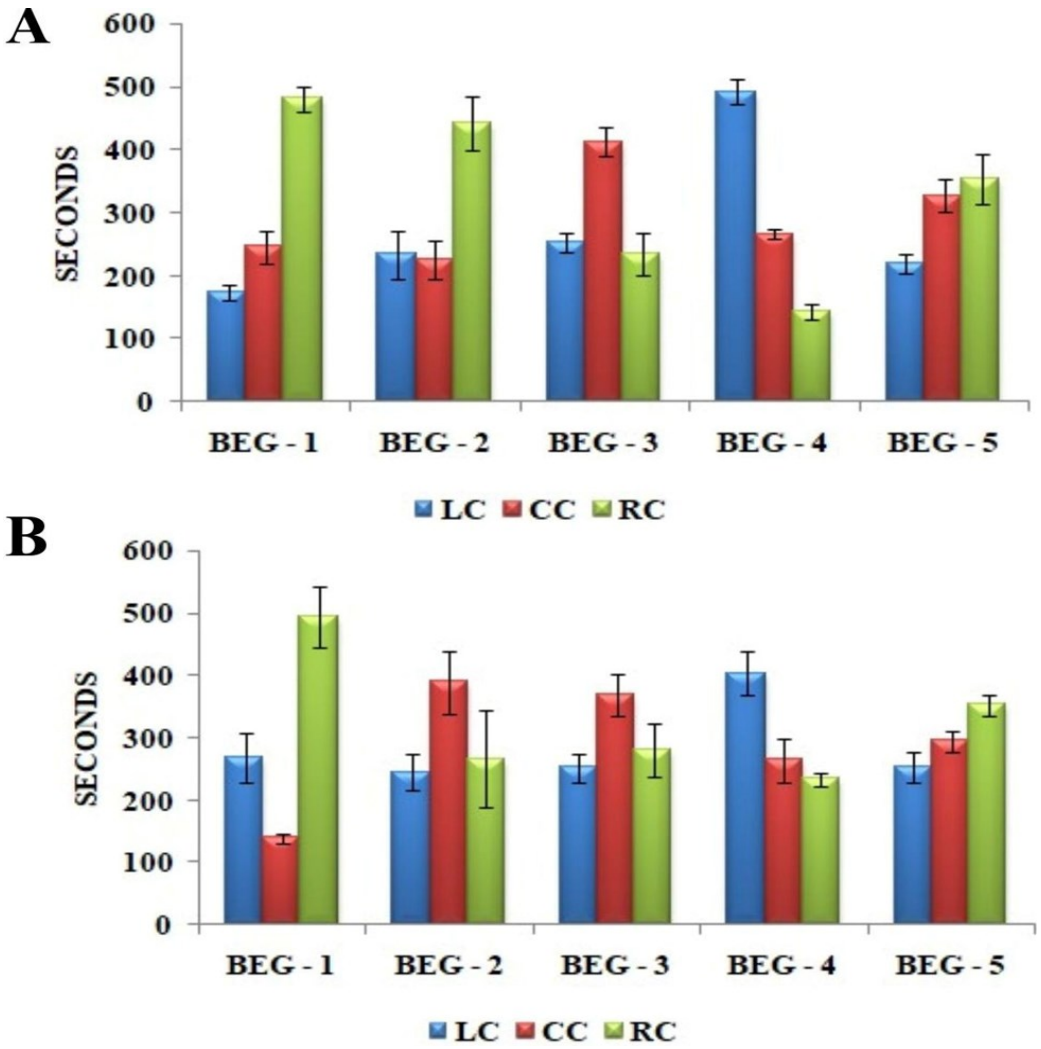

Fig. 8. *Behavioral training scores of the culture (A) and extract (B) infused behavioral experimental groups (BEGs) [BEGs – 2, 3, 4, and 5] showed that extract infusions had a slightly greater impact on the development of cognitive dysfunction compared to culture infusions. Formed cognitive decline was validated with the help of a non-infusive control (BEG – 1) in both the training phases (A & B) [BEG – behavioral experimental group, LC – left chamber, CC – central chamber, RC – right chamber]*

changes (Fig. 13). In healthy conditions, the development of cognitive functions like learning and memory formation was dependent on the brain synaptic plasticity changes occur in the brain regions like the olfactory bulb (OB), amygdala (A), and hippocampus (H) (Mukilan *et al.,* 2018a; Ahnaou*et al.,* 2020; Marzola *et al.,* 2023; Mukilan *et al.,* 2024a). In the above-stated brain regions, OB is primarily involved in the acquaintance of exposed stimuli and identifies the stimuli as exposed or unexposed within milliseconds. Exposure to new stimuli results in memory formation in the H through the A gating via the wired neuronal connections starting from OB (Mukilan *et al.,* 2018a; Jiang *et al.,* 2024). Once the organism is exposed to the new stimuli induces neuronal plasticity changes (NPCs) in the brain (Kourosh-Arami *et al.,* 2021; Mukilan, 2023).

In healthy conditions, activity-dependent changes in the presynaptic and postsynaptic neurons result in the structural modifications of brain neuronal circuits. At the initial stage, exposure to a new stimulus results in the formation of neurotransmitters (brain signaling molecules) from the presynaptic neurons (Avchalumov and Mandayam, 2021; Stahl *et al.,* 2022; Mukilan, 2023). Existing presynaptic neurons depend on the gut neurotransmitter precursor molecules (GNPM) for the synthesis of neurotransmitters like serotonin (5-HT), Acetylcholine (Ach), Norepinephrine (NE), and epinephrine (E) (Ganesh *et al.,* 2010; Chen *et al.,* 2021; Dicks, 2022). Among others, 5-HT plays a major role in initiating and developing cognitive learning and memory formation. This 5-HT is produced from an amino acid called tryptophan. Entered tryptophan is further converted into 5-hydroxytryptophan with the help of tryptophan hydroxylase (TH), which catalyzes the synthesis of 5-HT precursors in the enterochromaffin (EC) cells. These EC cells are the sub-cells of enteric endocrine

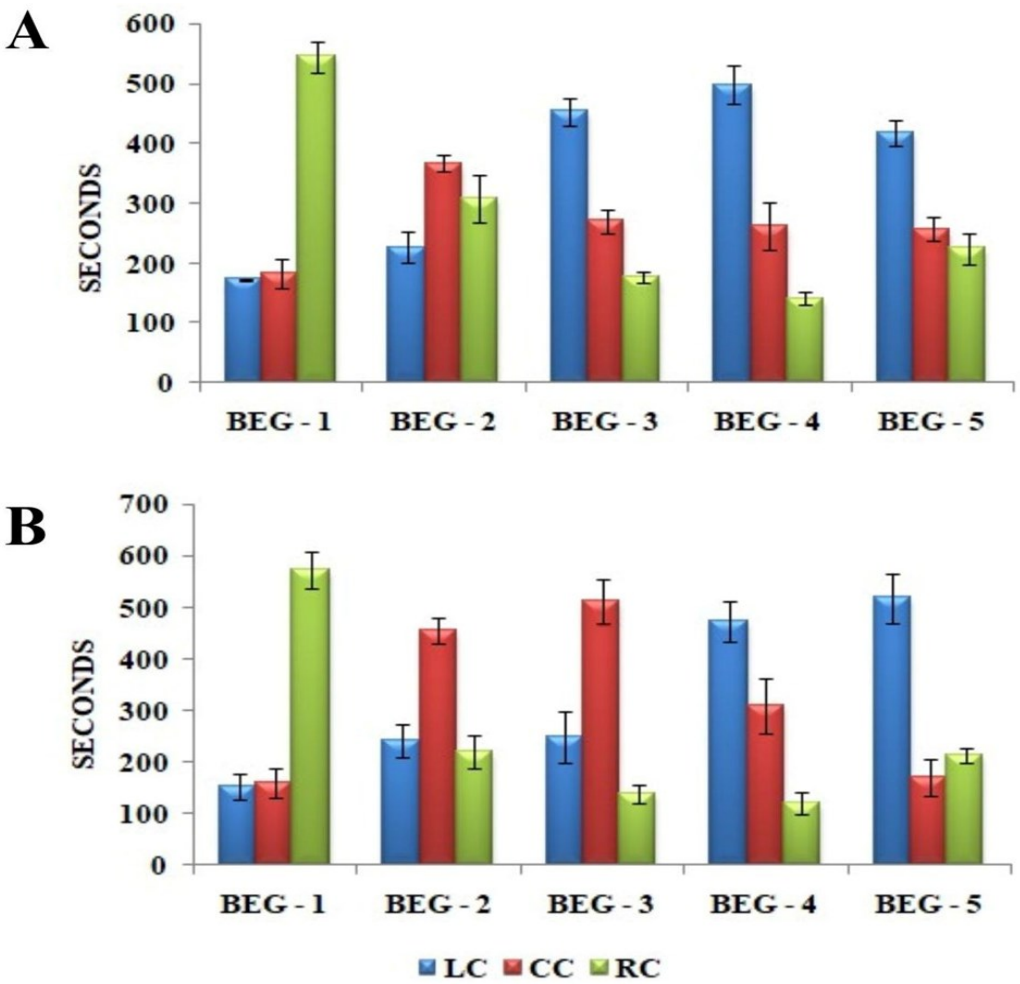

Fig. 9. *Behavioral testing responses of the culture (A) and extract-infused (B) behavioral experimental groups (BEGs) [BEGs – 2, 3, 4, and 5] showed that extract infusions induced a higher level of cognitive impairment compared to culture infusions. The cognitive impairment was high in the extract oral infusions (EOI) due to the presence of metabolite alone in the infusions (BEG – behavioral experimental group, LC – left chamber, CC – central chamber, RC – right chamber)*

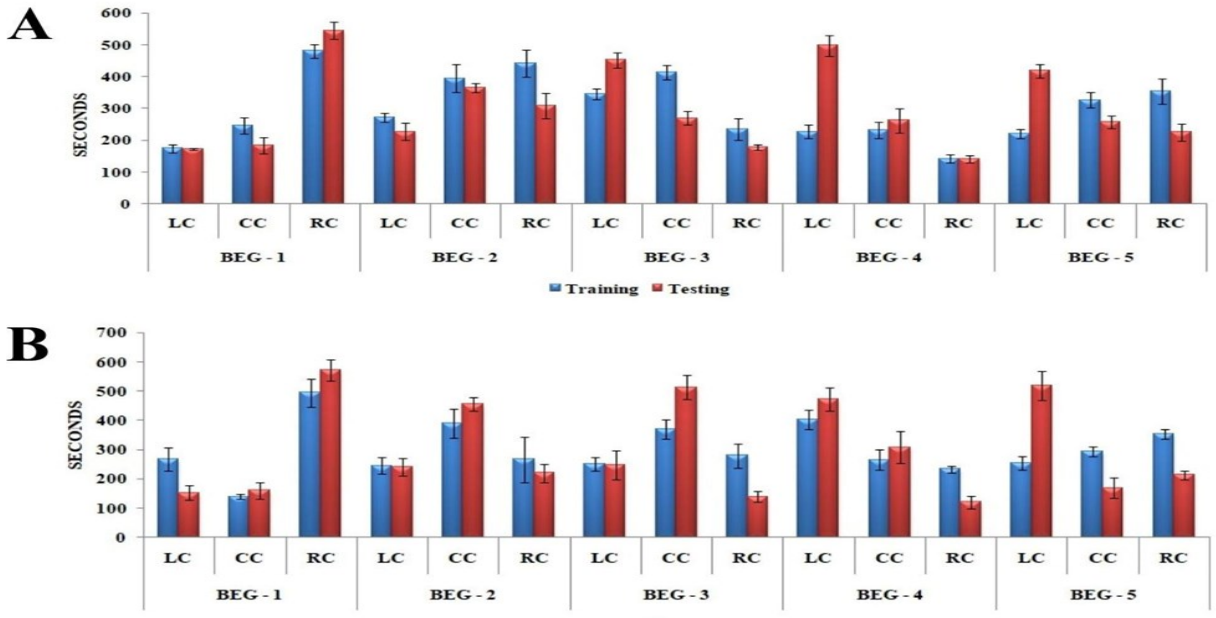

Fig. 10. *Comparative analysis of post-infusive training and testing scores showed that in isolated/pure form pycocyanin induces a higher level of cognitive impairment compared to infusions in mixed form (culture infusions) (BEG – behavioral experimental group, LC – left chamber, CC – central chamber, RC – right chamber)*

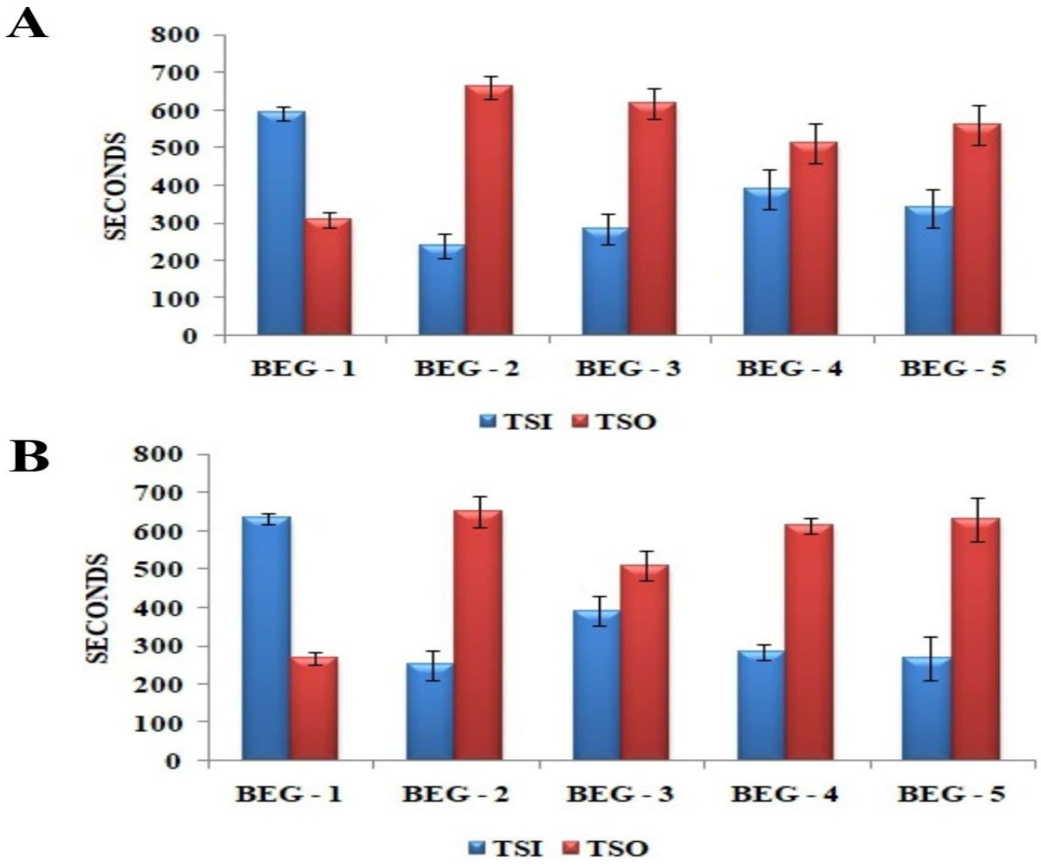

Fig. 11. *Behavioral training scores of open field test showed a lesser level of anxiety-like behavior development in the culture (A) and extract-infused (B) behavioral experimental groups (BEGs) [BEGs – 2, 3, 4, and 5] compared to a non-infusive control (BEG – 1) (BEG – behavioral experimental group, TSO - time spent in the outer compartment, TSI - time spent in the inner compartment)*

(EE) cells present in the gastrointestinal (GI) tract (Ganesh *et al.,* 2012; Höglund *et al.,* 2019; Maffei, 2020). The presence of EC cells makes the GI tract a rich source of 5-HT precursors. Later on, 5-HT precursors were transferred to the brain with the help of BBB via the enteric nervous system (ENS). Interrelationship with the oral cavity, gut, and brain forms the OGB axis (Liu *et al.,* 2021; Hu *et al.,* 2023; Sasso *et al.,* 2023).

The formed OGB axis also plays a major role in regulating the HPA axis through reduced cortisol synthesis within the host. Once reaching the brain, transmitted 5-HT precursor molecules may result in the synthesis of serotonin (5-hydroxytryptamine – 5 - HT) in the presynaptic neurons. Formed 5-HT is further released into the synaptic cleft (SC) for its binding with the specific 5-HT receptors present on the surface of postsynaptic neurons (Mukilan *et al.,* 2015; Mukilan *et al.,* 2018b; Thangaleela *et al.,* 2018; Mukilan, 2022).

Later on, excess neurotransmitter (5-HT) present on the SC was reuptake by the presynaptic neuron with the help of monoamine oxidase and it is further converted into 5-hydroxyindole acetaldehyde. Formed 5-hydroxyindole acetaldehyde was later converted into 5-hydroxindole acetic acid (5-HIAA) with the help of the aldehyde dehydrogenase (AH) enzyme. The presynaptic neurons later used formed 5-HIAA to synthesise 5-HT during the later stages (Redelinghuys, 2020; Liu *et al.,* 2021; Hu *et al.,* 2023). Bounded neurotransmitters further result in the initiation of calcium influx inside the postsynaptic neurons. Induction of calcium influx results in the activation and phosphorylation of neuronal signaling molecules like adenyl cyclase (AC), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and cAMP response element binding protein (CREB) (Ganesh *et al.,* 2012; Mukilan *et al.,* 2018a; Mukilan, 2023; Lisek *et al.,* 2024).

Phosphorylation of CREB results in the induction of immediate early gene cascades which results in the expression of immediate early genes like *Egr-1, C-fos,* and *C-jun*. Activated IEGs result in the upregulation of postsynaptic proteins like postsynaptic density protein – 95 (PSD-95), calcium/calmodulin stimulated protein kinase II (CaMKII) which results in the formation of proper cognitive memory formation within the hippocampal brain regions (Ganesh *et al.,* 2012; Mukilan *et al.,* 2018b; Rajan, 2021; Chen *et al.,* 2022; Mukilan *et al.,* 2024b). The outcome of the present study showed that *P. aeruginosa's* virulence factor pycocyanin, played a major role in the development of cognitive decline through the aberration of oral and gut microbio-

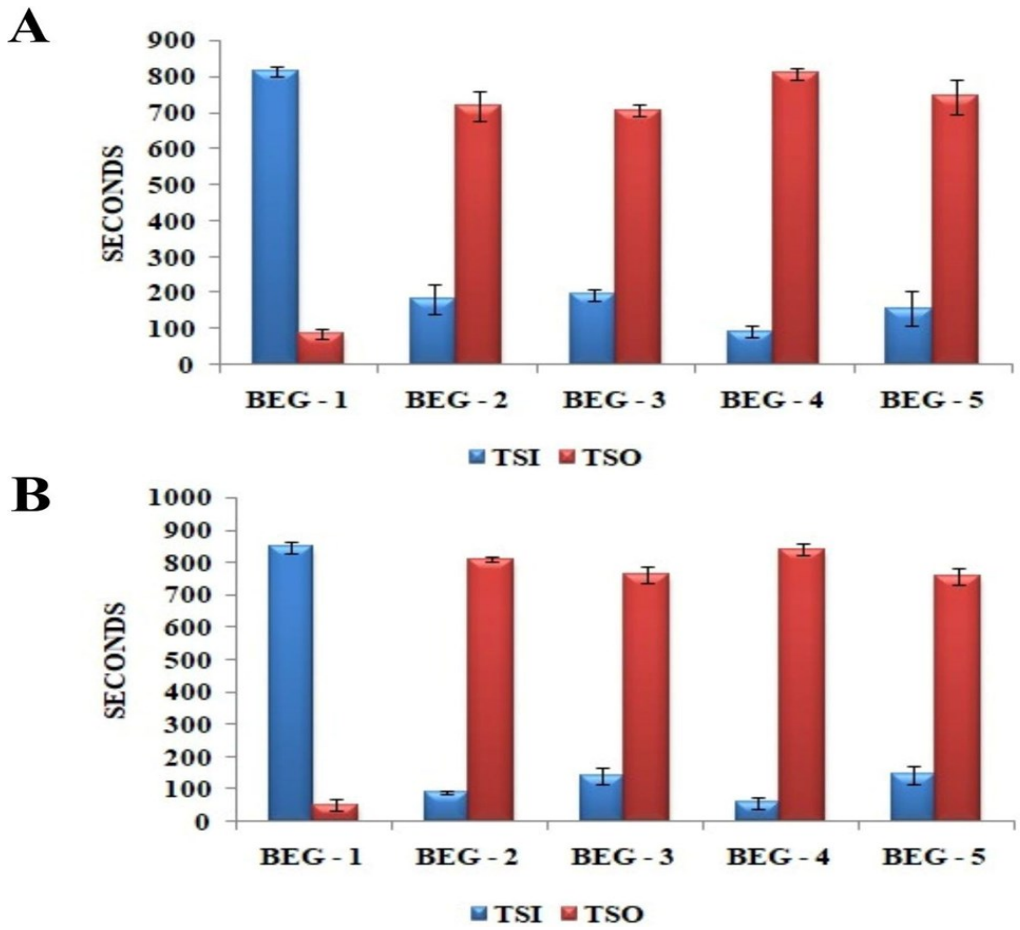

Fig. 12. *Behavioral testing responses of open field test showed a higher level of anxiety-like behavior development in the extract-infused behavioral experimental groups (BEGs) [BEGs – 2, 3, 4, and 5] compared to a non-infusive control (BEG – 1). However, culture infused behavioral experimental groups (BEGs) [BEGs – 2, 3, 4, and 5] showed a slightly higher amount of time spent in the inner chamber compared to the extract-infused groups (BEG – behavioral experimental group, TSO - time spent in the outer compartment, TSI - time spent in the inner compartment)*

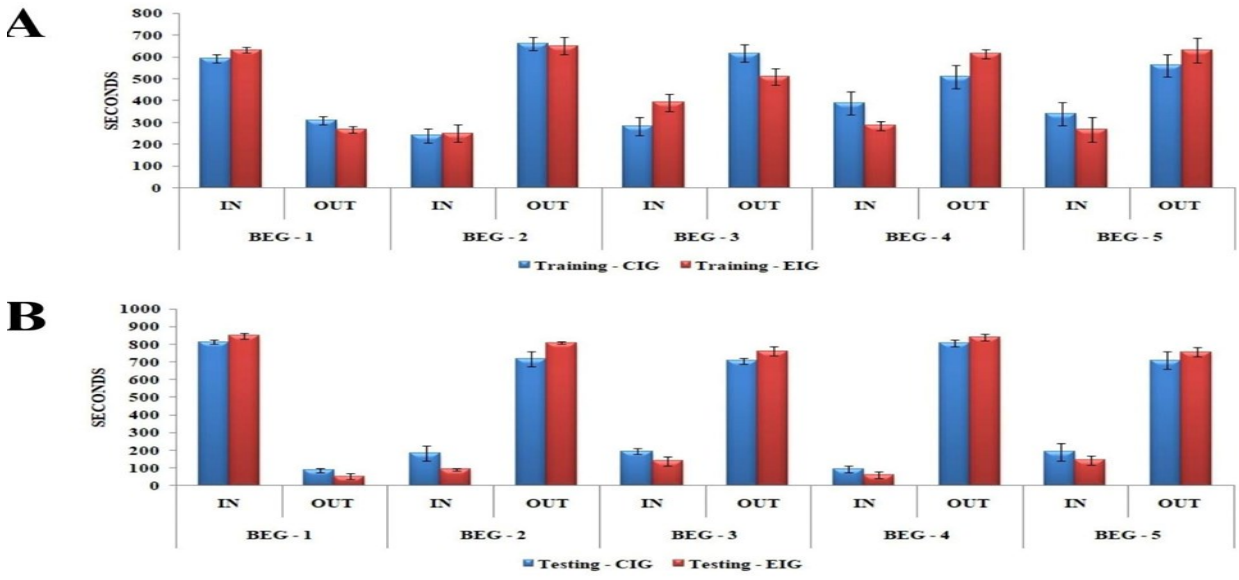

Fig. 13. *Comparative analysis of open field post-infusive training and testing scores showed that in isolated/pure form pyocyanin induces a higher level of anxiety-like behavior. Development of anxiety-like behavior was observed amount of time spent in the outer compartment (TSO) compared to the time spent in the inner compartment (TSI). (BEG – behavioral experimental group, CIG – culture infused group, EIG - extract infused group)*

ta by inducing oral/gut dysbiosis. Induced dysbiosis results in the reduced transportation of GNPM from the gut to the brain. Reduced transportation of GNPM may result in impaired neurotransmitter synthesis/production within the brain regions. This impaired state later on results in the formation of memory decline due to hindrances in the brain's neuronal signaling pathways.

Conclusion

The outcome of the present study proved that pycocyanin (a secondary metabolite) isolated from *P. aeruginosa* played a major role in the development of cognitive memory impairment. The metabolite (pycocyanin) was isolated from the aroused cultures of days 3, 4, 5, and 6 followed by metabolite isolation, and isolated metabolite was orally administrated to the infusive groups to study the direct effect of metabolite transmission from the gut to the brain. Transmitted pycocyanin results in the development of ROS, which results in localized inflammatory reactions in the brain centres that are responsible for cognitive learning and memory formation. Formed brain neuroinflammation further reduced fish learning abilities during infusive behavioral studies. Reduced learning abilities directly affects the development of cognitive decline through the dysregulation of the HPA axis via the production of cortisol. Produced cortisol resulted in the development of anxiety-like behavior in the infusive groups compared to non-infusive groups. Comparative two-staged behavioral analysis proved that metabolite oral infusions greatly impacted the development of cognitive memory loss compared to culture oral infusions. Other than the development of cognitive decline, metabolite infusions also play a major role in the formation of stress characteristics like anxiety behavior in the open field test. Overall results proved that pycocyanin a secondary metabolite of *P. aeruginosa* was possibly involved in the induction of cognitive memory decline through the oral-gut-brain axis. It also stated that poor oral hygiene and oral/gut dysbiosis played a major role in the development of cognitive decline during the later stages of life. Thus, the present research work opens up the unavoidable role of non-periodontal bacterial metabolites in the causation of cognitive memory dysfunctions.

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Conflict of interest

The author declare that he has no conflict of interest.

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