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LETTERS

Mechanistic insight into the antistress potential of eugenol: Modulation of the monoaminergic system in chronic unpredictable stress zebrafish model

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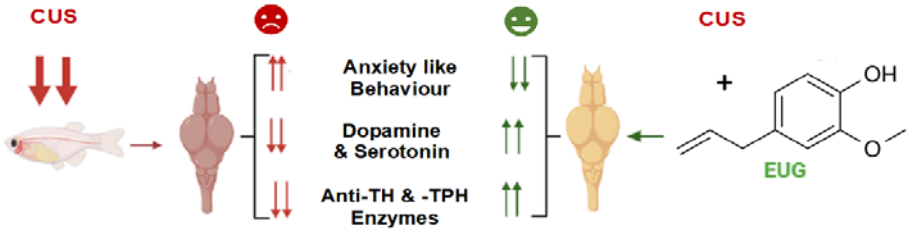
Article

ABSTRACT



The unwanted side effects associated with existing therapeutics against anxiety disorder warrants further preclinical investigations to discover new drug candidates. Herein, we report the anti-anxiety effect of Eugenol (EUG), a phytoconstituent, in chronic unpredictable stressed (CUS) model of zebrafish. The zebrafish were exposed to mild stress and the anxiety-like behaviour was quantified using the light-dark test and novel tank tests. It was observed that the stress induced a significant decline in number of entries and the time spent in the light compartment, increased latency to move into the top and spending less time therein and increased freezing duration, which indicated the anxiety in the fish. Interestingly, EUG treatment showed a reversal of behavioural alterations caused by CUS, and suggested the potential therapeutic effect. The reduced concentrations of stress related neurotransmitter monoamines, dopamine (DA) and serotonin (SE) were observed in CUS model during analysis using HPLC coupled with PDA detector. However, significantly increased levels of DA and SE were noted upon EUG treatment. On the other hand, immunohistochemistry studies on brain tissue of zebrafish revealed that the decreased activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) enzymes in stressed conditions were significantly increased upon EUG treatment. These results indicated the neuroprotective potential or regulatory role of EUG in stress induced alterations. The finding underscores the importance of exploring natural compounds like EUG for their therapeutic potential against neurochemical modifications caused by stress.

Keywords: Zebrafish, Chronic Unpredictable Stress, Eugenol, Dopamine, Serotonin, Neuroprotection.

INTRODUCTION

The stress, anxiety, and depression are complex conditions that affect human from the perspective of both physical and mental well-being.[1] These neurological disorders are just not limited to suffering individuals, but also pose significant societal challenges.[2] Their effects permeate workplaces, communities, and societies at large, while disturbing social dynamics, reducing productivity, and placing a heavy burden on healthcare system.[2] The individuals suffering from stress are frequently prescribed to chemical medications such as benzodiazepines like quetiapine, amitriptyline, tricyclics, venlafaxine, and selective serotonin reuptake inhibitors like citalopram, escitalopram, paroxetine, fluoxetine, and sertraline. These medications pose numerous side effects such as memory loss, agitation, nausea, sedation, disturbed sleep, muscle relaxation and the risk of dependence.[3] Many such medications are crucial in the treatment of the severe mental illnesses, but are found not to be always beneficial and rather lead to side effects and tolerance if taken for an extended period of time as complained by patients.[4] Consequently, there's a growing interest in exploring alternative therapeutics which can cope with said challenges. The medicinal herbs and phytochemicals have been represented as antidepressant and central nervous system (CNS) improving agents in numerous studies and suggested to have potential therapeutic benefits.[5] The phytoconstituents, such as phenolic compounds, flavonoids and carotenoids exhibit neuroprotective properties. Interestingly, these compounds have been reported to not only delay the progression of neurological diseases but also said to have preventive characteristics.[6] Therefore, plant derived biomolecules have appeared as potential drugs which may offer improved efficacy and fewer adverse effects in managing stress.[7]

One such aromatic phytoconstituent is Eugenol (EUG, $C_{10}H_{12}O_2$) (Fig 1) which belongs to the group of plant phenols.[8]

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The EUG is abundantly present in clove oil, nutmeg, cinnamon, and bay leaves. It is frequently employed as a local anesthetic, analgesic, and anti-inflammatory agent.[9] According to reports, ischemia and amyloid-beta peptide induced excitotoxicity were prevented by EUG treatment in neuronal cells.[10,11] Moreover, neuroprotective effects of EUG in hippocampus were derived from its capacity to stimulate brain derived neurotrophic factors. The EUG has also been reported to possess anti-depression, anti-epileptic and anxiolytic activities.[12,13]

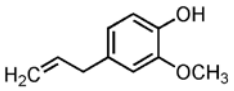


Figure 1. Chemical structure of eugenol (EUG).

Herein, this particular study reports the anti-anxiety effect of EUG in zebrafish (*Danio rerio*) chosen as a relevant animal model while focusing on stress related behavioural impairments and the associated neurochemical changes. The utilization of *Danio rerio* as an animal model has recently gained significant attention, particularly for studying the human neurological disorders. This organism shares similarities in neurotransmitter systems and genomics (60 to 80%) with mammals and therefore stands highly relevant for exploring various aspects of neurological functions and disorders.[14] The zebrafish were first subjected to chronic unpredictable stress (CUS) and fed with EUG to observe alteration in behavioural impairments. Furthermore, the role of neurotransmitters, dopamine (DA) and serotonin (SE) was explored in mediating the observed behavioural changes as both DA and SE are reported to be essential for regulating the mood, stress response, and overall mental well-being.[15] Additionally, the activities of associated hydroxylase enzymes, tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) were also assessed to substantiate the observations. Concisely, this study sheds light on the anti-stress potential of EUG in zebrafish based upon the examination of behavioral and neurochemical alterations.

MATERIALS AND METHODS

2.1 Materials

The Eugenol, Dopamine, Serotonin were procured from Sigma Aldrich. Perchloric acid (HClO4, analytical reagent grade with 60% purity) and acetonitrile were obtained from Fisher Scientific. Immunohistochemical staining kit, VECTASTAIN ABC KIT in conjunction with ImmEdge pen and Bloxall blocking solution were provided by VECTOR Laboratories, Inc. Primary antibodies, (anti-TH and anti-TPH), secondary antibodies and ProLong™ Gold Antifade Mountant were procured from Molecular Probes-Invitrogen.

2.2 Ethical note

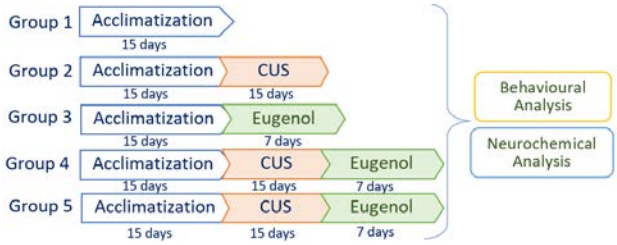
The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi provided the ethical clearance (38/99/CPCSEA), after approval of the Institutional Animal Ethics Committee (IAEC).

2.3 Animals and housing

The six months old and 2-3 cm long adult female and male zebrafish were procured from the animal facility, and kept in an experimental room with constant aeration, feeding with commercial flakes (three times a day), and temperature conditions of 28 ± 2 ºC with the 14/10 hours of light/dark cycle to help them acclimatize to the conditions.[16]

2.4 Treatment

The zebrafish were randomly distributed into five Groups, 1-Control; 2-CUS; 3-EUG; 4-CUS+EUG, and 5-CUS+ Aripiprazole (APZ) (Scheme 1). The CUS procedure (15 consecutive days) involved exposure to varied mild stressors twice a day. On the day of experiment, the fish were chosen at random, meticulously netted out of the tank, and put in a tank holding one liter of water containing EUG (5 mg/L) for 45 min for a period of one week.[17] For positive control, we used APZ (0.556 ng/L for 15 min).[18]



Scheme 1: Schematic representation of experimental design.

2.5 Chronic Unpredictable Stressors (CUS)

The zebrafish were exposed to various chronic stressors over a period of 15-days. The fish experienced two stressors per day as detailed in Table 1 below.[19]

Table 1: Schedule of Chronic Unpredictable Stressors.

Days	Types of Stressor
Day 1	Heat Stress (33 °C, 30 min) Cold Stress (23 °C, 30 min)
Day 2	Low water level (2 min) Overcrowding (10-12 fish in 150 ml water, 60 min)
Day 3	Tank Change (Fish were moved six times in a row from one tank to another). Cold Stress (23 °C, 30 min)
Day 4	Net Chasing (8 min) Heat Stress (33 °C, 30 min)
Day 5	Social isolation (60 min) Net Chasing (8 min)
Day 6	Over Crowding (10-12 fish in 150 ml water, 60 min) Low water level (2 min)
Day 7	Social isolation (60 min) Overcrowding (10-12 fish in 150 ml water, 60 min)
Day 8	Cold stress (23 °C, 30 min). Tank change (Fish were moved six times in a row from one tank to another)
Day 9	Low water level (2 min) Heat stress (33 °C, 30 min)
Day 10	Tank Change (Fish were moved six times in a row from one tank to another). Net Chasing (8 min)
Day 11	Social isolation (60 min) Overcrowding (10-12 fish in 150 ml water, 60 min)
Day 12	Social isolation (60 min) Cold Stress (23°C, 30 min)
Day 13	Low water level (2 min) Heat stress (33° C, 30 min)
Day 14	Low water level (2 min). Tank change (Fish were moved six times in a row from one tank to another)
Day 15	Net Chasing (8 min) Social isolation (60 min)

2.6 Light-dark test

The Light-dark test was performed to study anxiety and anti-anxiety behaviour as reported by Maximino et al., 2010.[20] The experimental tank was partitioned using opaque acrylic into three equal-sized compartments measuring, 25×25×30 cm. The water depth was kept at 20 cm. The grey tape was used to cover the walls of center compartment and floor, creating a neutral area wherever fish may decide to enter whether into dark or light compartment. The floor and walls of dark compartment were covered by black coloured tape. The floor and walls of light compartment were taped white. There was a rectangular door (5x7 cm) on each compartment, allowing unrestricted entries. One fish was placed in the center compartment and allowed to freely move between the compartments for 10 min. A camera was fixed on ceiling to record the behaviour of fish. One third of the tank water was swapped after every experiment. The videos were analyzed by using Behavioural Observation Research Interactive Software, or BORIS.[21]

Average number of compartment changes, latency to enter the light compartment, entries into the light and dark compartments, time spent in each of the three compartments (dark, neutral, and light) were among the variables measured.[22]

2.7 Novel Tank Test

In the novel tank test, a black coloured plastic tape was applied to cover three sides of the test tank (measuring 24×20×17 cm) having water level up to 14 cm from bottom. The behavioural movement was captured on camera directed at the exposed side of the tank. Each fish was allowed for 5 min to explore the tank. Prior to testing the behaviour of new fish, one-third water was replaced with fresh water. With the aid of BORIS, videos were examined (Friard and Gamba, 2016).[21] The 3x3 cm grid overlay on the computer screen allowed for the visual division of the test tank into top, middle and bottom zones. Upon the initial release, every fish descended to the bottom of the tank. When a fish touched the bottom, data collection started (approximately 5 sec). The variables studied were average latency to enter the top (sec), time spent in the top (sec) and freezing duration (sec).[22]

2.8 Quantification of neurochemicals by HPLC- PDA detector

The DA and SE were dissolved in 0.2 M $HClO_4$ solution to prepare their standard stock solutions. These standard stock solutions were stored in dark at 4 °C. The brain of zebrafish was carefully dissected and the tissues were weighed. Afterwards, the tissues were homogenized in ice-cold 0.2 M $HClO_4$ solution (10 µL/mg tissue), and centrifuged at 4 °C for 20 min (12,000 g). The supernatant was filtered and stored at -80 °C until analysis. The determination of components was carried out by High Performance Liquid Chromatography (HPLC, Agilent Infinity II) coupled with PDA detector. The chromatographic separation was performed using a C18 column (150 mm x 2.0 mm I.D.; 3 µm). The mobile phase consisted of $HClO_4$ (5mM) solution having 5% acetonitrile. The flow rate was maintained at 0.25 mL/min, and the separation temperature was kept at 30°C.[23]

2.9 Immunohistochemistry

The zebrafish brain was fixed in the 10% neutral buffered formalin and subsequently processed into paraffin blocks for tissue sectioning. Following this, deparaffinization and rehydration were conducted via serial dilutions of xylene and ethanol. The antigens were retrieved by heating in sodium citrate buffer (10 mM; pH 6.2) for 20-30 min at 95°C. Immunohistochemical staining was performed following the directions available with the VECTASTAIN ABC KIT. The tissues were incubated with primary antibodies (Anti-TH and Anti-TPH) diluted in buffer (1:200) at 4 °C Overnight. Following this, samples were incubated in biotinylated secondary antibodies for an hour and then incubated in VECTASTAIN ABC Reagent for half an hour. The chromogenic detection involved diaminobenzidine (DAB) and hematoxylin was used as counterstain. The negative controls were processed without primary antibody incubation. The stained sections underwent examination under a microscope (EVOS XL core) at 10x magnification and image analysis was carried out using ImageJ software. The ATM score (Average Threshold Measure) was calculated using a standard formula based on pixel intensity in the DAB channel.[24,25]

2.10 Statistical Analysis

The experimental data obtained were expressed as mean ± SEM. The ANOVA, one-way variance analysis, was used to analyse results obtained from different experimental groups of zebrafish. The Tukey's honestly significant difference (HSD) post hoc test was applied to make comparisons between experimental groups. The significance levels were set as; significant if $P<0.05$ and highly significant if $P<0.01$.

RESULTS AND DISCUSSION

The available medications for anxiety and depression have been associated with serious side effects, and therefore exploring alternative options becomes imperative.[4] The use of nanoplatforms and natural or herbal therapeutic interventions have proved to be potential medication to treat the neurological ailments.[26,27] The plant based active biomolecules such as aglycones, protopanaxadiol, protopanaxatriol, baicalein, curcumin, catechin, quercetin, resveratrol and wogonin have been reported to possess neuroprotective effects.[26] Therefore, this study focuses on examining the anti-anxiety potential of one such well known phytoconstituent, *i.e.*, Eugenol (EUG). The EUG is a naturally occurring aromatic phenolic biomolecule and commonly found in clove oil, nutmeg, cinnamon, and bay leaves. It has found widespread applications in pharmaceuticals, dentistry, food flavoring, agriculture, and cosmeceuticals.[28] The reported literature also highlights the pharmacological activities of EUG, including anti-inflammatory, anti-oxidant, analgesic, and anti-microbial properties. The EUG holds great promise for enhancing human health and has been recognized safe by the World Health Organization (WHO).[28] In this study, the evaluation of anti-anxiety potential of EUG was planned to be performed in CUS model of zebrafish. The CUS model of the zebrafish serves well to simulate the unpredictable stress and study the anxiety and depression like disorders in humans.[29] In a typical procedure, first the CUS model was developed while exposing zebrafish to two different mild stressors for 15 days as described in Table 1. All experimental groups including those fed

with EUG were analyzed for behavioural changes and associated neurochemical modifications as per study plan shown in Scheme 1. The alterations in fish behaviour due to induced stress and subsequent EUG treatment were studied by light-dark test and novel tank test. The light-dark test is a validated test for studying anxiety in zebrafish, that gives cue about the behavioural response of zebrafish in terms of their preference to enter dark or light compartments. The greater activity of zebrafish in the light region of the tank is associated with anti-anxiety or normal behaviour, whereas activity in the dark region of the tank indicates anxiety like disorders.[20] The results of this test revealed a significant reduction in the average number of compartment changes and decreased latency to enter into the light compartment in Group 2 (CUS) when compared to Group 1 (control), which indicated stress behaviour of zebrafish (Fig 2A and 2B). In contrast, Group 4 (CUS+EUG) exhibited a significant increase in compartment changes (Fig 2A) and latency to enter the light compartment (Fig 2B) when compared to Group 2, which was indicative of anti-stress behaviour induced by EUG feeding.

The stressed fish (Group 2) showed a highly significant decrease in number of entries (Fig 2C) and time spent in the light compartment (Fig 2D) when compared to Group 1. Notably, Group 4 exhibited a highly significant increase in number of entries and time spent in the light compartment as compared to stressed fish. The observation regarding behavioural response of Group 4 was also comparable with untreated control (Group 1) and aripiprazole (positive control) treated stressed fish of Group 5 (CUS+APZ) which suggested that EUG treatment could reduce anxiety like behaviour. No differences were observed among all experimental groups for the time spent in the neutral compartment (Fig 2D).

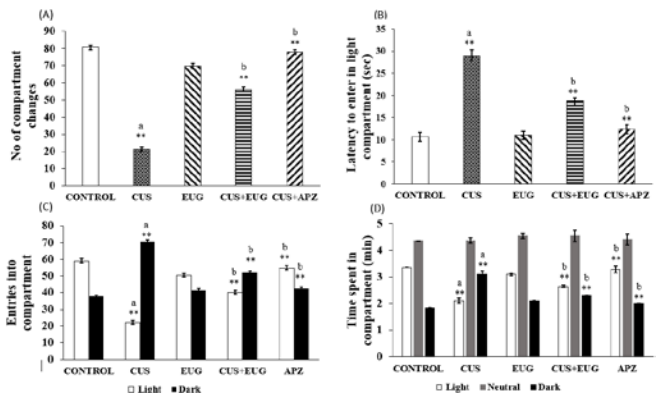


Figure 2. The effect of Eugenol treatment on compartment changes (A), latency to enter in light compartment (B), entries (C) and time spent (D) into light and dark compartment by zebrafish. Data is represented as Mean ± S.E. [a]As compared with controls (Group 1); [b]As compared with stressed fish (Group 2). The levels of significance are indicated as **P*<0.05 and ***P*<0.01. (CUS; Chronic Unpredictable Stress, EUG; Eugenol and APZ; Aripiprazole).

On the other hand, in novel tank test, chronic stressed fish (Group 2) exhibited a highly significant increase in the latency to enter the top, freezing duration, and a significant decline in the time spent on top when compared to non-stressed controls (Group 1), which was indicative of anxiety in the zebrafish (Fig 3A-3C). Conversely, the treatment of EUG (Group 4) resulted in a significant reduction in the latency to enter the top and freezing duration, along with a decline in the time spent on top when compared to stressed fish (Group 2) (Fig 3A-3C). These observations in Group 4 were also notably comparable to those observed in Groups 1 and 5 which again suggested the restoration of the normal behaviour (anti-anxiety effect) of zebrafish upon EUG treatment.

These results are of crucial importance and are in line with the fact that herbal bioactive molecules and plant extracts have potential neuroprotective effects as reported by different scientific groups based on the studies in stressed zebrafish and other animal models. The plant based active biomolecule, baicalein (a flavone isolated from *Scutellaria baicalensis Georgi*) has been reported to ameliorate anxiety like behaviour caused by stress in zebrafish model.[30] Similarly, nicotine was also reported to have anxiolytic potential in a study performed in the zebrafish.[31] A preclinical study conducted by Nachammai et al., also revealed anti-anxiety properties of the silibinin and naringenin (plant derived flavonoids) in the zebrafish model.[32] The acute administration of the hydroethanolic leaves extract of *Spondias mombin* gave rise to anxiolytic and antidepressant effects in zebrafish.[33] The extract of *Polygonum minus* leaves was also found beneficial in restoration of behavioural changes in CUS model of zebrafish.[34] Likewise, *Benincasa hispida* was reported to reduce the effects of acute stress in zebrafish and proved to be a potential neuroprotectant against stress and anxiety related mental disorders.[35] Recently, a study conducted using andrographolide containing extract of *Andrographis paniculate* in CUS zebrafish highlighted its role in alleviating depressive behaviour.[36]

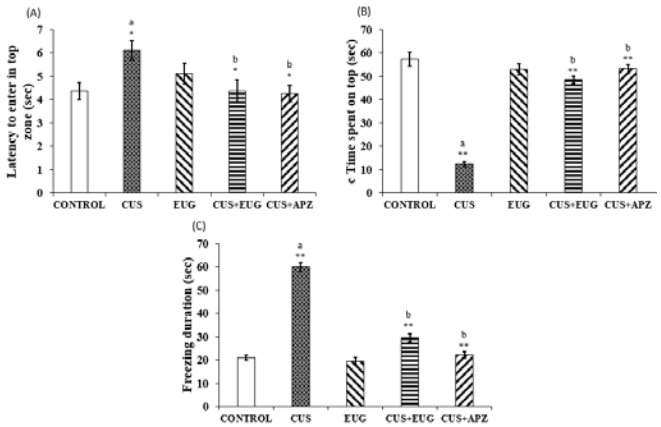


Figure 3. The effect of Eugenol treatment on zebrafish behaviour regarding latency to enter (A) and time spent in top zone (B) as well as freezing duration (C). Data is represented as Mean ± S.E. [a]As compared with controls (Group 1); [b]As compared with stressed fish (Group 2). The levels of significance are indicated as **P*< 0.05 and ***P* <0.01. (CUS; Chronic Unpredictable Stress, EUG; Eugenol and APZ; Aripiprazole).

The stress induced behavioural changes have been reported to result in altered levels of neurotransmitters and dysregulated hormone levels.[37] In this context, the decreased levels of brain

neurotransmitters, such as dopamine (DA) and serotonin (SE) have been correlated with induced stress in zebrafish.[38] Therefore, to comprehend the mechanism of neuroprotective potential of EUG in CUS model of zebrafish, we studied the concentrations of DA and SE in brain tissues of different experimental groups and analyzed for statistical significance by ANOVA. The stressed fish (Group 2) was found to have significantly decreased levels of DA and SE in comparison with the control group (Group 1) (Fig 4).

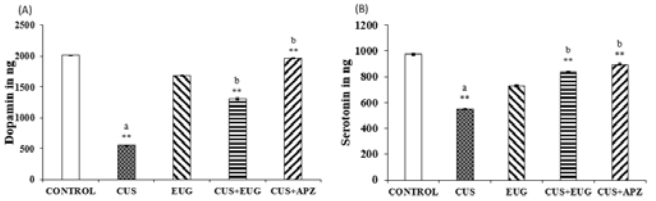


Figure 4. The changes in levels of dopamine (A) and serotonin (B) in different experimental groups of zebrafish. Results are represented as mean ± S.E. (*n*=9; each group). [a]As compared with controls (Group 1); [b]As compared with stressed fish (Group 2). The levels of significance are indicated as **P*< 0.05 and ***P* <0.01. (CUS; Chronic Unpredictable Stress, EUG; Eugenol and APZ; Aripiprazole).

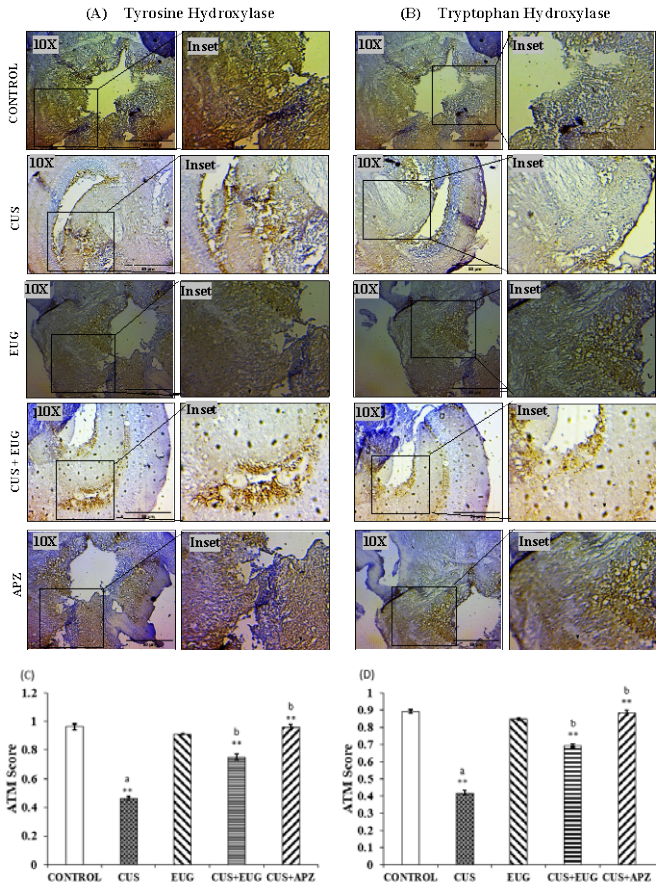


Figure 5. Immunohistochemistry analysis for the detection of TH (A) and TPH (B) levels in formalin-fixed paraffin-embedded serial sections of harvested brain tissues from different experimental groups of zebrafish. Scale bar, 50 µm. The quantitative ATM scores for the expression of TH (C) and TPH (D) are expressed as mean ± S.E. [a]As compared with controls (Group 1); [b]As compared with stressed fish (Group 2). The levels of significance are indicated as *P< 0.05 and **P <0.01. (CUS; Chronic Unpredictable Stress, EUG; Eugenol and APZ; Aripiprazole).

The observations were consistent with results reported by Fulchar et al. (2017)[39] wherein chronic unpredictable mild stress resulted in substantial decline in levels of DA and SE in zebrafish brain. On the contrary, EUG fed stressed fish in Group 4, exhibited a highly significant recovery in DA and SE levels when compared to stressed fish (Group 2). The glucosyl hesperidine showed anxiolytic effects by restoring the levels of DA and SE in zebrafish model of anxiety.[40] The costunolide active component of Anshen Buxin Six Pills (a traditional Mongolian medicine composed of six herbs) was reported to improve serotonin levels in stressed fish.[41] It has been demonstrated that flavonoids, namely silibinin and naringenin, possessed high affinity towards dopamine and serotonin receptors, which helped in restoration of the behavioural alterations in the acute stress model of zebrafish, suggesting anxiolytic properties of these phytoconstituents.[32] The restoration of both DA and SE to normal levels was suggestive of potential role of EUG as an anti-stress agent.

The *de novo* synthesis of neurotransmitters DA and SE in the brain is dependent upon tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), enzymes respectively.[42-44] Therefore, the levels of TH and TPH were also examined in the zebrafish brain by immunohistochemistry (IHC) (Fig 5A-5D) to validate the neuroprotective effect of EUG. The anti-TH and anti-TPH antibodies staining of brain tissue samples from different groups clearly showed the low levels of both the enzymes in stressed zebrafish (Group 2) as evidenced from light staining of tissues. Conversely, relatively high levels of both enzymes were evidenced by deep staining of tissues for Group 4 (CUS+EUG) which was comparable to Group 1 (control), Group 3 (EUG) and Group 5 (CUS+APZ) indicating restoration to normal levels (Fig 5A and 5B). The IHC images were further analyzed while calculating the average threshold measure (ATM) score to quantify pixel-based tissue staining. The ATM score is a single multiplicative measure, which includes both the amount and intensity of staining. It was found that chronic unpredictable stress (Group 2) caused a significant decline in ATM score which represented low levels of TH and TPH enzymes (Fig 5C and 5D) when compared with the control Group 1. However, the levels of TH and TPH enzymes based on ATM score were found to be increased in EUG fed stressed fish (Group 4) which were comparable to Group 1, Group 3 and Group 5. These results clearly suggested the neuroprotective characteristic of EUG wherein dysregulated dopaminergic and serotonergic pathways under stress conditions could be restored by EUG treatment. This study also sheds light on importance and applicability of zebrafish model for studying stress induced alterations in related neurotransmitter levels and associated enzymes.[45] Summarily, the results presented herein, underscore the importance of investigating natural compounds like EUG for their therapeutic potential in alleviating stress-induced neurochemical changes. However, future studies may be required to thoroughly evaluate the molecular mechanisms behind neuroprotective role of EUG for stress management.

CONCLUSION

This study focuses on evaluation of anti-stress potential of Eugenol (EUG) in chronic unpredictable stress (CUS) model of zebrafish (*Danio rerio*). The choice of zebrafish as an animal model in this study underscores its relevance and applicability in advancing our knowledge of stress-related neurobiology and potential therapeutic interventions. The EUG treatment in stressed zebrafish has been reported herein to reverse behavioural alterations and biochemical changes suggesting its potential therapeutic benefits. The light-dark and novel tank test revealed that EUG feeding to stressed zebrafish could reverse the stress induced behavioural changes. The low levels of neurotransmitters (dopamine; DA and serotonin; SE) and their associated enzymes (tyrosine hydroxylase; TH and tryptophan hydroxylase; TPH) in CUS model could be recovered to normal levels upon EUG treatment as examined by HPLC and immunohistochemistry analysis of fish brain samples. All these results highlighted the potential role of EUG in neuroprotection under stress conditions. However, further research may be required to elucidate the molecular mechanisms underlying anti-anxiety effects of EUG and explore its potential in stress management and neuroprotection. This study not only contributes to enhance our understanding of stress-related behaviours in zebrafish but also adds to provide a translational perspective of phytoconstituents. The findings may have implications for understanding stress responses in other vertebrates including humans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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