Evaluation of Anxiolytic Effect of Trans-Ferulic Acid in Zebrafish

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Abstract

Background: Anxiety disorders are on the rise and current anti-anxiety drugs like benzodiazepines and SSRIs (selective serotonin reuptake inhibitors) use are associated with lots of adverse drug reactions including tolerance, suicidal tendency especially on chronic use. Thus, there is a need for identification of newer anti-anxiety drugs. Zebrafish recently gained attraction as an animal model. The present study aimed to evaluate the anxiolytic effect of trans-ferulic acid in zebrafish.

Methods: This study was conducted in zebra fish in the animal house of our institute. Animals were divided into 7 groups, 10 fish in each group with the sample size of 350. Anxiolytic effect of trans ferulic acid was studied using five tests i.e., Novel tank test, Light dark preference test, Acute stress test, Mirror biting test and Social preference test. Study was initiated after approval of Institutional Animal Ethics Committee (IAEC), and the experiment was done as per The Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines and Good Laboratory practice.

Results: Our study showed that in novel tank test, zebra fishes treated with fluoxetine, clonazepam and trans-ferulic at doses of 150mg/L ,300mg/L and 450mg/L (274.9 ± 26.5 vs 124.3 ± 7.11 vs 131.1 ± 16.37 vs 138 ± 19.66 vs 170.3 ± 21.55) spent more time (sec) in upper zone compared to those fishes with distilled water (42.7 ± 8.6) or ethanol (45.9 ± 5.73).

In light dark preference test, zebra fishes treated with clonazepam **(**208.8 ± 27.72), fluoxetine (186.9 ± 37.09), and trans-ferulic acid 450mg/L (200.7 ± 20.51) spent more time in the light compartment compared to the distilled water (47.9 ± 16.52) or ethanol (95.2 ± 13.06). Also, zebra fishes treated with trans-ferulic acid at doses of 150mg/L and 300 mg/L (149.9 ± 14.66 vs 164.9 ± 18.96) spent more time(sec) in light compartment compared to those treated with distilled water (47.9 ± 16.52).

In acute stress test similar to novel tank test, Zebrafishes treated with Fluoxetine (305.30 ± 19.06), clonazepam (144.20 ± 26.43) and trans-ferulic acid 150mg/L, 300mg/L and 450mgL (114.40 ± 9.04 vs 153.20±35.37 vs 238.00 ± 34.90) spent more time (sec) in the upper zone compared to distilled water (60.80±7.44) or ethanol (55.70 ± 16.09).

In mirror biting test, Zebrafishes treated clonazepam (12.30±3.66), trans-ferulic acid 300mg/L (12.30 ± 1.87) and 450mg/L (9.00 ± 1.70) showed less number of mirror contacts compared to the distilled water (40.90 ± 13.89)

In social preference test zebra fish treated with trans-ferulic acid 300mg/L (27.50 ± 5.47) showed more number of entries in the empty arm compared to negative control (11.20 ± 3.26), vehicle control (9.70 ± 1.77), clonazepam (12.70 ± 2.31) and fluoxetine (4.5 ± 1.26).

Keywords**:** Anxiety, benzodiazepines, Zebrafish, compartment, preference.

Introduction:

Anxiety disorders belong to a group of mental disorders. It causes suffering for both patients and their families. (1) Selective serotonin reuptake inhibitors (SSRI), serotonin norepinephrine reuptake inhibitors (SNRI) and benzodiazepines are the

primary treatment measures for anxiety disorders. increased incidence of adverse effects and reduced efficacy with anti-anxiety drugs, there is a need to develop newer antianxiety drugs.[2]

Trans- ferulic acid (4 – hydroxy -3 methoxy cinnamic acid) is the isomer of ferulic acid. It is the polyphenolic compounds found in foods such as tomatoes, sweet corn and rice bran. Trans – ferulic acid is also found to have anxiolytic activity.[3] Trans ferulic acid tends to show similar mechanism like the diazepine group of drugs by binding to the alpha subunits.[4] Zebrafish (4- hydroxy -3methoxy cinnamic acid) is an emerging model system in behaviour neuroscience. [5]

In several studies anxiolytic animal models like acute stress test, light dark test and acute stress test used for anxiolytic activity.[6,7] In this study authors also studied anxiolytic activity using mirror biting and social preference test.

The present study is aimed to evaluate the anxiolytic effect of Trans-ferulic acid in zebrafish, which could increase the pool of drugs available for management of anxiety disorders in the future. Thus the authors evaluated anxiolytic effect of trans-ferulic acid in Zebrafish.

Materials and Method

Study setting : Ethical approval was obtained from Institutional Animal Ethics Committee (IAEC/SMVMCH/032/2022), Puducherry. The study was conducted in the animal house in the department of pharmacology, Sri Manakula Vinayagar Medical College and hospital (SMVMCH), Puducherry. The study design was Experimental animal study. Duration of the study was 1 year 6 months September 2022 to December 2023. The care and maintenance of the animal were done as per committee for the Purpose of Control ad Supervision of Experiments on Animal (CPCSEA) guidelines. Experiment was conducted based on Good Laboratory Practise (GLP).

Chemical and Drugs :

The following chemical and drugs were used in the study were Absolute ethyl alcohol 5.2mg (Euro life healthcare, Tamil Nadu), Clonazepam 0.75mg – (Oxra Sun Pharma, Mumbai), Fluoxetine 10mg (Oxra Sun Pharma, Mumbai), and Trans-Ferulic Acid 5 g – Sigma Aldrich, Mumbai

Sample size: Sample size was estimated to be 350 based on the previous study .[8] For evaluation of anxiolytic activity of trans-ferulic acid, the zebrafishes were divided into 7 groups (N=10) for each of the 5 test (Novel tank test, Light dak preference test, Acute stress test, Mirror biting test, Social preference test).

Analysis

Data was entered in Microsoft Exel. Data was summarized as mean standard error of mean). Analysis was done using SPSS version 24.0. $P<0.05$ was considerably significant

Methods

Preparation of Trans-acid solution

Ferulic acid solutions prepared by dissolving 0.2 g in 2900 microlitre absolute ethyl alcohol and concentration of 150mg/L,300mg/Land 450mg/L were obtained by dilution in water.

Grouping of fishes

Group	Drug	Number of fishes					Total
		Novel tank test	Light/dark preference test	Acute stress test	Mirror biting test	Social preference test	
1	Negative control (Distilled water)	10	10	10	10	10	50
2	Ethanol (Vehicle	10	10	10	10	10	50

	control) 6.6µl						
3	Clonazepam 0.75mg/L	10	10	10	10	10	50
4	Fluoxetine 10mg/L	10	10	10	10	10	50
5	Trans ferulic acid 150mg/L	10	10	10	10	10	50
6	Trans-Ferulic acid 300 mg/L	10	10	10	10	10	50
7	Trans–ferulic acid 450 mg/ L	10	10	10	10	10	50

1.Novel tank test [9,10]

Fish was separately moved to the tank, divided into three equal horizontal zones. The tank was filled with water upto 15cm. After introducing each fish, video was recorded for 6 minutes. The behaviour and locomotion were measure by the following parameters such as Time spent in the upper zone, time spent in the lower zone and number of transitions between the zones. Anxiolytic activity increase the time spent in the upper zone and decreases the time spent in the lower zone of the tank.

2.Light /Dark test [11,12]

The glass tank was divided into two equal parts, one with black background, sides and cover (dark side) and another with white background and sides but without cover, allowing the light input (light side). Movable slider was placed between the light and dark compartment. The fish was placed in the tank and after the acclimatisation of about five minutes the gate was opened , Filmed for 6 mins. The following parameters were observed the latency for the first entrance in the dark compartment, the time spent in the light compartment and the number of crosses between the compartment

3. Acute stress test

The fishes were treated with the corresponding drugs for 10 minutes and were chased for the next 2 minutes with a net before moving to the novel tank where they were recorded for 6minutes. The behavioural parameters were accessed as described for novel tank test.

4.Mirror Biting test

Novel tank apparatus was set up with the mirror inside, attached to the inner side wall of the tank. A line was drawn on the tank with the marker 0.5cm from the mirror, to represent the zone of contacting the mirror. Another line 2.5 cm from the first line (based on an average adult fish length) to represent the Zone of approach the mirror. Each fish was placed in the novel tank and immediately video was recorded for 6 minutes. The following parameters were noted latency to first approach the mirror, latency to first contact the mirror, number of mirrors approaches by the fish, number of mirror contacts by the fish , mirror biting frequency and mirror biting duration.

5.Social preference test[13,14]

Experimental fish were pre exposed to a drug or control for 20 minutes. Control or drug exposed Zebrafish (n=10) in each group was introduced individually to the central zone of the apparatus, temporarily separated (by transparent sliding dividing doors) from the two arms of the corridor. Following the initial 30 sec acclimation, the two sliding dividers were gently lifted and Zebrafish were released to explore the apparatus for 6 minutes.

Video recording was done for 6 minutes period and the following parameters were assessed number of fish entries in the central arm, number of fish entries in the conspecific arm, number of fish entries in the empty arm, time spent in the central arm, time spent in the conspecific arm

Results:

Table 1 Shows that Clonazepam 0.75mg/L and Fluoxetine10mg/L, and Trans-ferulic acid in ascending doses 150mg/L, 300mg/L, 450mg/L reduced the time spent in the lower zone and significantly increased time spent in the upper zo when compared to the Negative control (Distilled water) and Vehicle control (Ethanol). Number of transitions between the zones were reduced in Fluoxetine and Trans-ferulic acid 450mg/L group compared to the negative control (distilled water) and vehicle control (ethanol)

Table 1: Evaluation of anxiolytic effect of trans-ferulic acid in zebrafish using Novel tank test (n= 70)

Groups	Parameters	Mean ± Standard error of mean		
		Time spent in the upper zone of the tank (sec)	Time spent in the lower zone of the tank (sec)	Number of transitions be the4 Zones
I	Negative control water	42.7±8.6	198.1±18.73	27.4±2.08
II	Vehicle control Ethanol (6.6µL)	45.9±5.73	232.7±16.38	16.5±1.25
III	Clonazepam (0.75mg/L)	124.3±7.11$^{*\#}$	115.7±16.36$^{*\#}$	34.4±5.59$^{\#}$
IV	Fluoxetine (10mg/L)	274.9±26.5$^{*\#¥}$	17.2±5.78$^{*\#¥}$	8.6±3.49$^{*¥@}$
V	Trans-ferulic acid (150mg/L)	131.1±16.37$^{*\#Ω}$	89.3±9.95$^{*\#Ω}$	22.4±3.51
VI	Trans-ferulic acid (300mg/L)	138±19.66$^{*\#Ω}$	85.6±14.15$^{*\#Ω}$	29.4±5.06^{Ω}
VII	Trans-ferulic acid (450mg/L)	170.3±21.55$^{*\#Ω}$	48.5±15.33$^{*\#¥}$	13.4±1.38$^{¥@}$

* p<0.05 -compared to Negative control, $^{\#}$ p<0.05-compared to Vehicle control, $^{¥}$p<0.05-compared to Clonazepam, ^{Ω} p<0.05-compared to Fluoxetine, $^{@}$ p<0.05-compared to Trans-ferulic acid 300 mg/L

Table 2 Shows that Clonazepam 0.75mg/L and Fluoxetine 10mg/L, and Trans-ferulic acid treated zebrafishes in ascending doses 150mg/L, 300mg/L and 450mg/L reduced the time spent in the dark compartment and significantly increases the time spent in the light compartment, when compared to the Negative control (Distilled water) and Vehicle control (Ethanol) . Number of transitions between the compartments were reduced in Trans ferulic acid 450mg/L group compared to negative control (distilled water) and vehicle control (ethanol) group.

Table 2 : Evaluation of anxiolytic effect of trans-ferulic acid in zebrafish using light dark preference test (n=70)

Groups	Parameters	Mean ± Standard error mean		
		The latency for the first entrance in the dark compartment	Time spent in the light side (sec)	Number of crosses between the compartment
I	Negative control Distilled water	1.3±0.50	47.9± 16.52	8.2±1.66
II	Vehicle control Ethanol(6.6µL)	0.9±0.18	95.2±13.06	3.3±1.13
III	Clonazepam(0.75mg/L)	29.4±7.41	208.8±27.72$^{*\#}$	7.1±1.39
IV	Fluoxetine(10mg/L)	10.7±5.29	186.9±37.09$^{*\#}$	5.3±1.84
V	Trans-ferulic acid (150mg/L)	36.4±20.01	149.9±14.66*	10.8±2.33
VI	Trans-ferulic acid (300mg/L)	30±16.26	164.9±18.96*	10.7±1.57$^{\#}$
VII	Trans-ferulic acid (450mg/L)	16±10.09	200.7±20.51$^{*\#}$	3.3±0.52$^{\#\$@}$

*p<0.05-compared to negative control , $^{\#}$p<0.05-compared to vehicle control , $^{\$}$p<0.05-compared to Trans-ferulic acid 150 ,$^{@}$p<0.05-compared to Trans-ferulic acid 300

Table 3 shows that Clonazepam 0.75mg/L and Fluoxetine10mg/L, and Trans-ferulic acid in ascending doses 150mg/L, 300mg/L and 450mg/L treated fishes significantly decreased the time spent in the lower zone and significantly increases the time spent in the upper zone compared to the Negative control (Distilled water) and Vehicle control (Ethanol). Trans ferulic acid 450mg/L group shows reduced number of transitions when compared to the negative control (distilled Water) and vehicle control (ethanol)

Table 3 : Evaluation of anxiolytic effect of Trans-ferulic acid in zebrafish using Acute stress test (n=70)

Groups	Parameters	Mean ± Standard error of mean		
		Time spent in the upper zone of the tank (sec)	Time spent in the lower zone of the tank (sec)	Number of transitions between the zones
I	Negative control Distilled water	60.80±7.44	192.70±20.51	20.70±3.00

II	**Vehicle control Ethanol (6.6µL)**	55.70±16.09	219.80±31.53	16.30±4.53
III	**Clonazepam(0.75mg/L)**	144.20±26.43	141.60±17.81	25.40±7.47
IV	**Fluoxetine(10mg/L)**	305.30±19.06$^{*\#¥}$	12.80±6.70$^{*\#¥}$	5.70±2.45
V	**Trans-ferulic acid (150mg/L)**	114.40±9.04$^{\Omega}$	117.30±12.61$^{\#\Omega}$	30.50±5.53
VI	**Trans-ferulic (300mg/L)**	153.20±35.37$^{\Omega}$	125.70±31.24$^{\#\Omega}$	38.10±16.96
VII	**Trans-ferulic acid (450 mg/L)**	238.00±34.90$^{*\#\$}$	30.70±10.46$^{*\#¥@}$	8.10±3.29

*p<0.05-compared to Negative control , $^{\#}$p<0.05-compared to Vehicle control , $^{¥}$p<0.05- compared to Clonazepam ,$^{\Omega}$P<0.05- compared to Fluoxetine,$^{\$}$p<0.05-compared to Trans-ferulic acid 150, $^{@}$p<0.05-compared to Trans-ferulic acid 300

Table 4 shows that Clonazepam 0.75mg/L, Trans ferulic acid 300mg/L and 450mg/L showed less number of mirror contact compared to negative control (distilled water). For all other parameters, there was no statistical difference between the groups.

Table 4 : Evaluation of anxiolytic effect of trans-ferulic acid in zebrafish using Mirror biting test (n=70)

Groups	Parameters	Latency to first contact the mirror	Latency to first contact the mirror	Number of mirror approaches by the fish	Number of mirror contact by the fish	Mirror biting frequency	Mirror biting duration
I	**Negative control water**	20.30± 4	23.20± 3.63	3.90± 2.13	40.90± 13.89	173.80± 21.55	176.50± 20.59
II	**Vehicle control ethanol (6.6µL)**	19.40± 2.37	20.30± 2.40	1.80± 0.49	9.50± 1.46*	125.40± 26.32	157.30± 30.71
III	**Clonazepam (0.75mg/L)**	13.90± 4.43	15.60± 4.15	5.40± 2.20	23.30± 3.66*	93.00± 16.50	104.50± 18.11
IV	**Fluoxetine (10mg/L)**	21.40± 5.70	22.30± 5.61	1.70± 0.73	17.60± 6.64	152.90± 45.4	148.70± 40.53
V	**TFA**	16.70±	17.70±	3.50±	17.70±	133.0±	149.80±

	150mg/L	4.71	4.71	2.42	3.30	32.53	27
VI	**TFA 300mg/L**	12.50±3.06	12.00± 3.12	3.90± 1.03	12.80± 1.87*	104.40± 23.45	116.90± 16.45
VII	**TFA 450mg/L**	14.50± 5.16	19.20± 6.31	2.60± 1.76	9.00± 1.70*	163.80± 26.10	162.50± 26.17

TFA- Trans ferulic acid

*p<0.05-compared to Negative control

Table 5 shows that Zebra fishes treated with Trans ferulic acid 300mg/L showed more number of entries in the empty arm (refer figure 23) compared to Negative control, vehicle control, Clonazepam and fluoxetine. However, there was no statistical difference among the various groups with regard to time spent in the empty arm .

Table :5 Evaluation of anxiolytic effect of Zebrafish using Social Preference Test (n=70)

Groups	**Parameters**	**Mean± Standard error of mean**					
		Number of fish entries in the central arm	**Number of fish entries in the conspecific arm**	**Number of fish entries in the empty arm**	**Time spent in the central arm**	**Time spent in the conspecific arm**	**Time spent in the empty arm**
I	**Negative control (Distilled water)**	11.50± 2.97	15± 3.15	11.20± 3.26	7± 4.50	165.6± 22.66	22± 7.44
II	**Vehicle control (Ethanol)**	10.60± 1.58	11.30± 1.89	9.70± 1.77	6.50± 3.33	169.5± 22.16	21.70± 4.19
III	**Clonazepam (0.75mg/L)**	17.50± 2.32	19.20± 2.80	12.70± 2.31	10.20± 3.64	106.0± 18.89	41.30± 10.29
IV	**Fluoxetine (10mg/L)**	7.7± 1.03	8± 1.33	4.5± 1.26	40.5± 16.27*#	146.4± 35.43	58.4± 24.23
V	**Trans-ferulic acid (150mg/L)**	19.90± 1.84	21.10± 2.37	18± 3.30	4.50± 0.81 Ω	91.30± 27.04	34.2± 5.77
VI	**Trans-ferulic acid (300mg/L)**	0± 7.48*#Ω	31.70± 7.66*#Ω	27.50± 5.47*#¥Ω	6± 2.90 Ω	50.90± 13.66*#	57.40± 13.22
VII	**Trans-ferulic acid (450mg/L)**	12.30± 2.18@	15.40± 2.31	11.70± 2.58@	10± 7.85	133.1± 30.63	26.30± 9.39

Discussion

In Novel Tank Test, Trans ferulic acid treated zebrafishes showed increased time spent in the upper zone and decreased time spent in the lower zone of the tank. This response was also seen with our positive controls Clonazepam and Fluoxetine. Our study also showed the anxiolytic activity of trans ferulic acid showed dose-dependent effects. No other researchers have done studies for anxiolytic effect of trans ferulic acid in Zebrafish using Novel tank test and Acute stress test yet. However, research has been done on evaluation of anxiolytic activity of Trans ferulic acid in small laboratory animals like mice/rat using open field test and hole board test .[(16)]

In our study, in light dark test Trans ferulic acid treated zebrafishes showed increased time spent in the light side and decreased time spent in the dark side compartment of the tank., several studies have been conducted for evaluating anxiolytic activity of Trans ferulic acid using light dark test on small laboratory animals like mice/rat. In novel tank test and Acute stress test we found that Zebrafishes in the control group fluoxetine had statistically increased time spent in the upper zone. But in light dark test Clonazepam group was found to have more significant anxiolytic activity by increased time spent in the light compartment

In mirror biting test, we did not find any significant difference between the groups. In Social preference test Trans ferulic acid at dose of 300mg/L had more number of entries in the empty arm compared to the control groups. Evaluation of anxiolytic activity of trans ferulic acid using theses behavioural models have not been done by other researchers yet.

Strength of our study is that we used Zebrafish models which are cost effective models especially suited for the evaluation of drugs of brain disorders, genetic and behavioural studies.

Limitation of our study is that exploration of molecular mechanism underlying anxiolytic activity of trans ferulic acid was not done.

Conclusion

Trans ferulic acid 150mg/L, 300mg/L and 450mg/L was found to have anxiolytic activity in Zebrafish animal model.

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