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Research Article

Evaluation of Anti-Nociceptive Activity of *Annona squamosa* in Different Pain Models of Zebrafish

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Abstract

The present work was designed to evaluate antinociceptive activity of *Annona squamosa* in different pain models of zebrafish. Evaluation of anti-nociceptive activity of Annona squamosa was done in different pain models i.e. cinnamaldehyde and menthol induced nociception. A total of 90 zebra fish were taken and divided into 3 groups where each group except control (group 1) was divided into 4 subgroups i.e. A, B, C and D. The anti-nociceptive activity of *Annona squamosa* was determined by number of line crossing and behavioural response were also evaluated by novel tank diving test.

In cinnamaldehyde induced nociception, *Annona squamosa* significantly increased the number of line crossing from 71.3 ± 8.8 in cinnamaldehyde alone treated group to 102.1 ± 16.3 and 114.7 ± 18.5 in cinnamaldehyde + *Annona squamosa* @ 200 µg/ml treated group and cinnamaldehyde + *Annona squamosa* @ 400 µg/ml treated group, respectively. However, in menthol induced nociception, the effect of *Annona squamosa* was found to be non-significant.

The novel tank diving test revealed that *Annona squamosa* showed behavioural modification by significantly increasing the time spent in upper portion of tank, average entry duration as well as number of entries into upper portion of tank in comparision to cinnamaldehyde alone treated groups. However, *Annona squamosa* significantly decreased the other behavioural parameters like number of erratic movements, number of freezing bouts and freezing duration in comparision to cinnamaldehyde alone treated groups.

Keywords: *Annona squamosa*; Zebrafish; Antinociceptive Activity

Introduction

Pain is a distressing feeling often caused by intense or damaging stimuli. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage" [13]. Pain or nociception triggers a variety of physiological and behavioural responses to protect the organism against an aggression and usually results in a subjective experience or perception of pain in sentient beings [2]. Pain and nociception are evolutionary well conserved mechanisms with an important role for the survival of animals [6,10,11].

Annona squamosa, the plant of Annonaceae family, also known as custard apple is commonly found in deciduous forests, also cultivated in wild in various parts of India. It is mainly grown in gardens for its fruits and ornamental value. It is known as custard apple, sugar apple. The plant is reported to possess analgesic, anti-inflammatory, antipyretic, antiulcer, antiseptic and abortifacient activities [12].

The Zebrafish (*Danio rerio*) is a freshwater fish belonging to the Minnow family (Cyprinidae) of the order Cypriniformes. Native to South Asia, it is a popular aquarium fish, frequently sold under the trade name Zebra danio. The Zebrafish is an important and widely used vertebrate model organism in scientific research, for example in drug development, in particular pre-clinical development [16]. Additionally, zebrafish display a repertoire of inflammatory cells, mediators and receptors that are similar to those in mammals including humans [17].

The objective of the present investigation was to evaluate anti-nociceptive activity of *Annona squamosa* in different pain models of Zebrafish.

Material and Methods

Experimental material

Collection and processing of plant

Fresh leaves of *Annona squamosa* were procured from Department of Plant Physiology, Jawaharlal Nehru Krishi Vishwa Vidyalaya (J.N.K.V.V.), Jabalpur (M.P.). The collected leaves were dried in shade, powdered by using grinder and used for preparation of ethanolic extract as per the method described by Bernatoniene., *et al*. [3].

Preparation of ethanolic extract by soxhlet method

Crude extract was prepared by using ethanol (90 per cent). About 20 gm of the coarsely grounded plant leaves was taken in a thimble made up of whatman filter paper No. 1 and placed in soxhlet apparatus with 500 ml round bottom flask containing 400 ml solvent at a temperature 80 ± 5ºC. The extraction was allowed to continue for 12 hrs. The extracts were collected in petri plates and kept in water bath at 90 ºC for evaporating the extra solvent. The per cent yield was calculated. The extract was kept in air tight container at 4ºC for further studies.

Figure 1: Leaves of Annona squamosa.

Figure 2: Crude extract.

Experimental animals

The proposed work was conducted in adult Zebrafish (*Danio rerio*) of either sex. The healthy adult Zebrafish were procured from local commercial breeder and maintained in laboratory following CPCSEA guidelines. All fish were fed with fish pellets (Tetra bits complete, Optimum micro-pellet) ad-libitum during the study period.

Evaluation of anti-nociceptive activity of *Annona squamosa*

The anti-nociceptive activity of *Annona squamosa* was analyzed in different pain models of zebrafish. A total of 90 zebrafish were divided into three groups and each group except group 3, was divided into four subgroups- A, B, C and D. The experimental procedures were adopted as per the standard protocol [9].

Experimental design

Initially, zebrafish were anesthetized with iced water (4°C). The anaesthetized fish were then transferred to wet sponge and either extract of *Annona squamosa* or meloxicam were administered to respective group animals, 30 min before the administration of algogenic substances. Animals of all groups (except control) received algogenic substance such as cinnamaldehyde (0.33 µM) and menthol (0.006 per cent) through intramuscular route.

Group	Model	Sub groups	No. of zebra fish	Treatment
1.	Cinnamaldehyde induced nociception	A	10	Cinnamaldehyde (0.33 **µM**; 5.0 **µL**; i.m)
		B	10	Cinnamaldehyde (0.33 **µM**; 5.0 **µL**; i.m) + Ethanolic extract of *Annona squamosa* (200 **µg**/mL; 20 µL; i.p.)
		C	10	Cinnamaldehyde (0.33 **µM**; 5.0 **µL**; i.m) + Ethanolic extract of *Annona squamosa* (400 **µg**/mL; 20 µL; i.p.)
		D	10	Cinnamaldehyde (0.33 **µM**; 5.0 **µL**; i.m) + Meloxicam (0.2 mg/mL; 20 **µL**; i.p.)
2.	Menthol induced nociception	A	10	Menthol (0.006 per cent; 5.0 **µL**; i.m)
		B	10	Menthol (0.006 per cent; 5.0 **µL**; i.m) + Ethanolic extract of *Annona squamosa* (200 **µg**/L; 20 µL; i.p.)
		C	10	Menthol (0.006 per cent; 5.0 **µL**; i.m) + Ethanolic extract of *Annona squamosa* (400 **µg**/mL; 20 µL;i.p.)
		D	10	Menthol (0.006 per cent; 5.0 **µL**; i.m) + Meloxicam (0.2 mg/mL; 20 **µL**; i.p.)
3.	Control		10	Normal saline (20 **µL**; i.p.)

Table 1

Cinnamaldehyde induced nociception

Cinnamaldehyde (0.33 µM) dissolved in 1 per cent tween 80, injected in tail of the fish 30 minute after treatment with ethanolic extract of *Annona squamosa*. The anti-nociceptive activity of ethanolic extract of *Annona squamosa* was evaluated by calculating the number of line crossing by each fish during 0-5 minutes (after administration of cinnamaldehyde). Further, behavioural responses to noxious stimuli were monitored by novel tank diving test.

Menthol induced nociception

Menthol (0.006 per cent) dissolved in saline solution, injected in tail of the fish 30 minute after treatment with ethanolic extract of *Annona squamosa*. The anti-nociceptive activity of ethanolic extract of *Annona squamosa* was evaluated by individually calculating the number of line crossing by each fish during 0-10 minutes (after administration of menthol). Further, behavioural responses to noxious stimuli were monitored by novel tank diving test.

Parameters of the study

Number of line crossing

For behavioural analysis, after treatment and application of the algogenic agent, the animal was placed in a glass Petri dish (10 x 15 cm) and the nociceptive response was quantified in terms of locomotor activity or number of line crossings performed during a certain time, specific for each model. The anti-nociceptive activity was calculated individually during the analysis time of each nociception model as described by Ohnesorge., *et al.* [11].

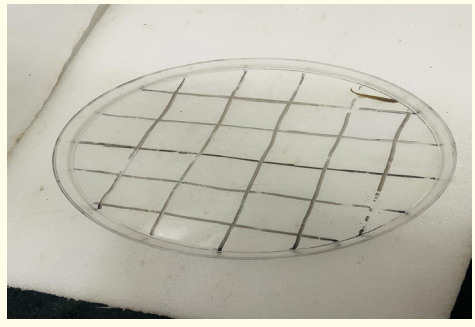

Figure 3: Petri-Dish for Number of Line Crossing.

Novel tank diving paradigm

The novel tank diving test is quick to implement (5-10 minutes) and sensitive to various pharmacological, genetic and environmental manipulations. The effectiveness of *Annona squamosa* was monitored via observation of behaviour in a novel tank and following movements were recorded as described by Collier., *et al.* [4]:

- Time spent in upper portion of tank
- Number of entries into upper portion of tank
- Average entry duration
- Number of erratic movements
- Number of freezing bouts
- Freezing duration

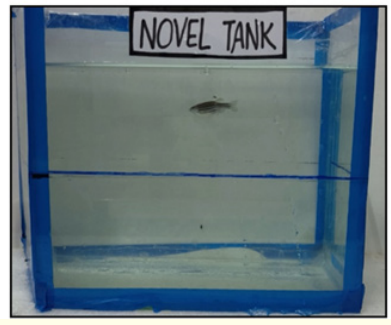

Figure 4: Novel Diving Tank.

Behavioural anti-nociceptive activity

The concentrations of the noxious and antagonistic agents, as well as the time of nociceptive action analysis were used based on the above parameters where two nociception models were considered: 0.33 µM cinnamaldehyde, 0.006 per cent menthol and meloxicam was taken as the standard nociceptive antagonist (cyclo-oxygenase inhibitor).

Result and Discussion

The present experiment was carried out to investigate the anti-nociceptive activity of *Annona squamosa* in zebrafish. Zebrafish behavioural assays are currently used for high throughput phenotyping and testing various psychotropic drugs. Zebrafish models are extensively employed in experimental neuroscience and behavioural research due to their easily quantifiable and robust physiological and behavioural phenotypes. The implementation of this novel animal model in neurobehavioral research has given researchers the new tools to model various psychopathologies and elucidate their pathological mechanisms and moreover the efficacy of *Annona squamosa* (Custard apple) as analgesic agent provides a better substitute for compounding of herbal medicines irrespective of allopathic drugs.

In the present research work, for monitoring the effectiveness of *Annona squamosa* through behavioural analysis of zebrafish, after treatment and application of the algogenic agent in various pain models, animals were investigated for the nociceptive response via various parameters in terms of locomotor activity or number of Line Crossings (LC) performed during a certain time, specific for each model [11] and Novel tank diving test which evaluates time spent in upper portion of tank, number of entries into upper portion of tank, average entry duration, number of erratic movements, number of freezing bouts and freezing duration as described by Collier., *et al.* [4].

Efficacy of ethanolic extract of *Annona squamosa* leaves on Cinnamaldehyde induced nociception

The effect of *Annona squamosa* on cinnamaldehyde induced nociception has been summarized in Table 1 and graphically depicted in Figure 5.

Cinnamaldehyde induced nociception model was categorized into four subgroups i.e. A, B, C and D. Fish of subgroup 'A' were treated with cinnamaldehyde (0.33 µM; 5.0 µl; I/M), subgroup 'B' with cinnamaldehyde (0.33 µM; 5.0 µl; I/M) + ethanolic extract of *Annona squamosa* (200 µg/ml; 20 µl; I/P), subgroup 'C' with cinnamaldehyde (0.33 µM; 5.0 µl; I/M) + ethanolic extract of *Annona squamosa* (400 µg/ml; 20 µl; I/P) and subgroup 'D' with cinnamaldehyde (0.33 µM; 5.0 µl; I/M) + meloxicam (0.2 mg/ml; 20 µl; I/P). The anti-nociceptive activity of ethanolic extract of *Annona squamosa* was evaluated by calculating the number of line crossing by each fish during 0-15 minutes (after administration of cinnamaldehyde). Further, behavioural responses to noxious stimuli were monitored by novel tank diving test.

Number of Line crossing by zebrafish

The mean values of number of line crossing by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 192.5 ± 18.4, 71.3 ± 8.8, 102.1 ± 16.3, 114.7 ± 18.5, 139.5 ± 15.2, respectively. Number of line crossing by zebrafish was significantly reduced in cinnamaldehyde alone treated group as compared to the control group. Ethanolic extract of *Annona squamosa* increased number of line crossing during 0-15 minute interval and the difference was significant as compared to cinnamaldehyde alone treated group.

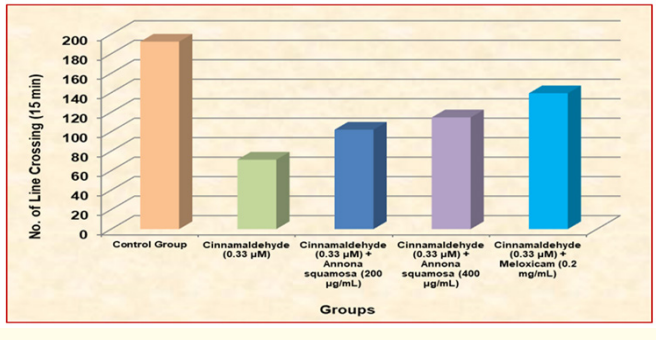

Figure 5: Effect of ethanolic extract of *Annona squamosa* leaves on number of line crossing by Zebrafish in cinnamaldehyde induced nociception.

Parameters	No. of Line Crossing (15 min)
Control Group	$192.5^{a} \pm 18.4$
Cinnamaldehyde (0.33 µM)	$71.3^{d} \pm 8.8$
Cinnamaldehyde (0.33 µM) + *Annona squamosa* (200 µg/mL)	$102.1^{c} \pm 16.3$
Cinnamaldehyde (0.33 µM) + *Annona squamosa* (400 µg/mL)	$114.7^{bc} \pm 18.5$
Cinnamaldehyde (0.33 µM) + Meloxicam (0.2 mg/mL)	$139.5^{b} \pm 15.2$

Table 2: Effect of ethanolic extract of *Annona squamosa* leaves on number of line crossing by zebrafish in Cinnamaldehyde induced nociception.

*Values are mean of ten observations

*Means bearing different superscript differ significantly ($p < 0.05$).

Novel tank diving paradigm

In present study, novel tank test was carried out to evaluate various behavioural responses to noxious stimuli for the duration of 0-10 minutes. The mean values of various behavioural parameters of zebrafish in cinnamaldehyde induced nociception have been presented in Table 2.

The mean values of time (sec) spent in upper portion by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 310.2 ± 30.7, 66.6 ± 12.8, 99.3 ± 9.1, 115.7 ± 19.0 and 135.5 ± 26.3, respectively. Time spent in upper portion of tank by zebrafish was significantly reduced in cinnamaldehyde alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* increased time spent in upper portion of tank during 0-10 minute interval and the difference was found significant as compared to cinnamaldehyde alone treated group. The mean values of time spent in upper portion of tank in *Annona squamosa* treated group were significant to meloxicam treated group and cinnamaldehyde alone treated group.

The mean values of number of entries into upper portion of tank by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 39.5 ± 6.8, 6.2 ± 2.7, 10.2 ± 4.6, 12.1 ± 3.8 and 15.07 ± 5.3, respectively. Number of entries into upper portion of tank by zebrafish was significantly reduced in cinnamaldehyde alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* increased number of entries into upper portion of tank during 0-10 minute interval and the difference was found to be significant as compared to cinnamaldehyde alone treated group.

The mean values of average entry duration (sec) by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 15.1 ± 4.2, 5.0 ± 2.1, 7.5 ± 2.8, 10.00 ± 4.7 and 12.0 ± 4.8, respectively. The mean values of average entry duration found significant among all treatments.

The mean values of number of erratic movements by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.59 ± 0.3, 15.07 ± 4.4, 8.8 ± 2.7, 2.05 ± 1.4 and 1.50 ± 0.9, respectively. Number of erratic movements by zebrafish was significantly increased in cinnamaldehyde alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* significantly reduced the number of erratic movements during 0-10 minute interval as compared to cinnamaldehyde alone treated group. The differences in mean values of number of erratic movements in *Annona squamosa* treated group were significant to meloxicam treated group and significant to control group.

The mean values of number of freezing bout by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.51 ± 0.1, 12.5 ± 4.6, 7.0 ± 3.1, 6.4 ± 2.1 and 3.0 ± 1.3, respectively. Number of freezing bout by zebrafish was significantly increased in cinnamaldehyde alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* reduced the number of freezing bout during 0-10 minute interval and the difference was significant as compared to cinnamaldehyde alone treated group.

The mean values of freezing duration (sec) by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.8 ± 0.3, 210.2 ± 22.1, 90.1 ± 8.1, 44.3 ± 9.3 and 19.6 ± 3.1, respectively. Freezing duration by zebrafish was significantly increased in cinnamaldehyde alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* significantly reduced the freezing duration during 0-10 minute interval as compared to cinnamaldehyde alone treated group. The differences in mean values

of number of freezing bout in *Annona squamosa* treated group were significant to control group as well as meloxicam treated group.

These findings indicate that cinnamaldehyde as algogenic substance reduced locomotor activity and produced abnormal behavioral responses. *Annona squamosa* alters the locomotor activity and behavioral responses in cinnamaldehyde induced nociception. These results indicate that *Annona squamosa* shows anti-nociceptive effect through TRPA1 channels.

Similarly, Sharma., *et al.* [14] reported the anti-nociceptive activity of *Bacopa monnieri* against cinnamaldehyde induced nociception which indicate the role of TRPA1 channels in its action. Kojima., *et al.* [7] also observed substantial expression of TRPA1 channels on enterochromaffin cells of the gastrointestinal tract and evaluated the effects of a selective TRPA1 agonist, on constipation-induced abdominal pain. Their findings indicated that the analgesic effect of the TRPA1 agonist was due to direct desensitization of TRPA1 channels.

Efficacy of ethanolic extract of *Annona squamosa* leaves on menthol induced nociception

The effect of *Annona squamosa* on menthol induced nociception has been summarized in Table 4 and graphically depicted in Figure 6.

Menthol induced nociception model was categorized into four subgroups i.e. A, B, C and D. Fish of subgroup 'A' were treated with menthol (0.006 per cent; 5.0 µl; I/M), subgroup 'B' with menthol (0.006 per cent; 5.0 µl; I/M) + ethanolic extract of *Annona squamosa* (200 µg/ml; 20 µl; I/P), subgroup 'C' with menthol (0.006 per cent; 5.0 µl; I/M) + ethanolic extract of *Annona squamosa* (400 µg/ml; 20 µl; I/P) and subgroup 'D' with menthol (0.006 per cent; 5.0 µl; I/M) + meloxicam (0.2 mg/ml; 20 µl; I/P). The anti-nociceptive activity of ethanolic extract of *Annona squamosa* was evaluated by calculating the number of line crossing by each fish during 0-15 minutes (after administration of menthol). Further, behavioural responses to noxious stimuli were monitored by novel tank diving test.

Number of Line crossing by zebrafish

Groups	Parameters (Novel tank)					
	Time spent in upper portion of tank (sec)	No. of entries into upper portion of tank	Average entry duration (sec)	No. of erratic movements	No. of freezing bouts	Freezing duration (sec)
Control Group	$310.2^{a} \pm 30.7$	$39.5^{a} \pm 6.8$	$15.1^{a} \pm 4.2$	$0.59^{c} \pm 0.3$	$0.51^{c} \pm 0.1$	$0.8^{d} \pm 0.3$
Cinnamaldehyde (0.33 µM)	$66.6^{d} \pm 12.8$	$6.2^{d} \pm 2.7$	$5.0^{d} \pm 2.1$	$15.07^{a} \pm 4.4$	$12.5^{a} \pm 4.6$	$210.2^{a} \pm 22.1$
Cinnamaldehyde (0.33 µM) + *Annona squamosa* (200 µg/mL)	$99.3^{c} \pm 9.1$	$10.2^{c} \pm 4.6$	$7.5^{cd} \pm 2.8$	$8.8^{b} \pm 2.7$	$7.0^{b} \pm 3.1$	$90.1^{b} \pm 8.1$
Cinnamaldehyde (0.33 µM) + *Annona squamosa* (400 µg/mL)	$115.7^{bc} \pm 19.0$	$12.1^{bc} \pm 3.8$	$10.00^{bc} \pm 4.7$	$2.05^{c} \pm 1.4$	$6.4^{b} \pm 2.1$	$44.3^{c} \pm 9.3$
Cinnamaldehyde (0.33 µM) +Meloxicam (0.2 mg/mL)	$135.5^{b} \pm 26.3$	$15.07^{b} \pm 5.3$	$12.0^{ab} \pm 4.8$	$1.50^{c} \pm 0.9$	$3.0^{c} \pm 1.3$	$19.6^{d} \pm 3.1$

Table 3: Effect of ethanolic extract of *Annona squamosa* leaves on behavioral responses zebrafish in Cinnamaldehyde induced nociception.

The mean values of number of line crossing by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 188.8 ± 19.6, 50.3 ± 9.6, 72.3 ± 6.5, 98.7 ± 12.1 and 120.3 ± 17.9, respectively. Number of line crossing by zebrafish was significantly reduced in menthol alone treated group as compared to the control group. Ethanolic extract of *Annona squamosa* increased number of line crossing during 0-15 minute interval but the difference was non-significant as compared to menthol alone treated group.

Novel tank diving paradigm

In present study, novel tank test was carried out to evaluate various behavioural parameters for the duration of 0-10 minutes. The mean values of various behavioural parameters of zebrafish in menthol induced nociception have been presented in Table.

The mean values of time (sec) spent in upper portion by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup

Parameters	No. of Line Crossing (Mean ± S.E.) (15 min)
Control Group	188.8^{a} ± 19.6
Menthol (0.006 %)	50.3^{c} ± 9.6
Menthol (0.006 %) + *Annona squamosa* (200 µg/mL)	72.3^{c} ± 6.5
Menthol (0.006 %) + *Annona squamosa* (400 µg/mL)	98.7^{b} ± 12.1
Menthol (0.006 %) + Meloxicam (0.2 mg/mL)	120.3^{b} ± 17.9

Table 4: Effect of ethanolic extract of *Annona squamosa* leaves on number of line crossing by zebrafish in Menthol induced nociception.

*Values are mean of ten observations

*Means bearing different superscript differ significantly ($p < 0.05$).

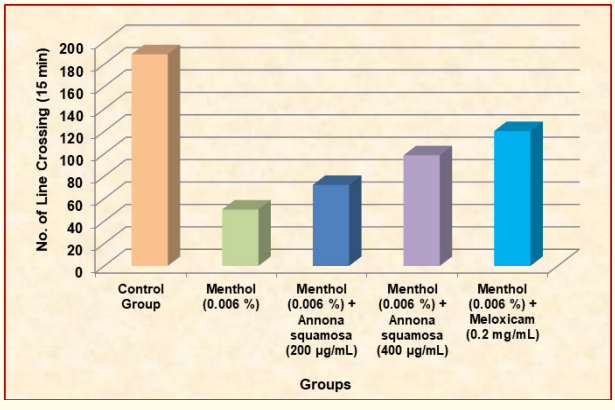

Figure 6: Effect of ethanolic extract of *Annona squamosa* leaves on number of line crossing by zebrafish in Menthol induced nociception.

D were 314.5 ± 31.5, 49.1 ± 6.1, 82.1 ± 6.5, 98.2 ± 17.1 and 113.6 ± 16.6, respectively. Time spent in upper portion of tank by zebrafish was significantly reduced in menthol alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* increased time spent in upper portion of tank during 0-10 minute interval but the difference was non-significant as compared to menthol alone treated group.

The mean values of number of entries into upper portion of tank by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 34.6 ± 5.6, 3.05 ± 1.2, 7.5 ± 1.8, 10.2 ± 2.7 and 12.06 ± 5.2, respectively. Number of entries into upper portion of tank by zebrafish was significantly reduced in menthol alone treated group as compared to control group. Ethanolic extract of *Annona squamosa* increased number of entries into upper portion of tank during 0-10 minute interval but the difference was non-significant as compared to menthol alone treated group.

The mean values of average entry duration (sec) by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 16.1 ± 2.8, 3.08 ± 1.6, 5.2 ± 1.6, 7.5 ± 2.8 and 10.0 ± 4.8, respectively. Mean values of average entry duration were non-significant among all treatments.

The mean values of number of erratic movements by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.60 ± 0.2, 17.0 ± 4.4, 10.0 ± 2.7, 3.0 ± 1.9 and 2.0 ± 1.1, respectively. Mean values of number of erratic movements were non-significant among all treatments.

The mean values of number of freezing bout by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.79 ± 0.4, 14.0 ± 5.2, 8.6 ± 3.1, 7.1 ± 2.9 and 3.7 ± 1.3, respectively. Mean values of number of freezing bout were non-significant among all treatments.

The mean values of freezing duration (sec) by zebrafish of control, subgroup A, subgroup B, subgroup C and subgroup D were 0.75 ± 0.6, 230.2 ± 19.9, 110.8 ± 16.8, 56.7 ± 8.7 and 24.8 ± 4.5, respectively. Mean values of freezing duration were non-significant among all treatments.

These findings indicate that menthol as algogenic substance reduced locomotor activity and produced abnormal behavioral responses. *Annona squamosa* did not alter the locomotor activity and behavioral responses in menthol induced nociception. These findings indicated that antinociceptive effect of *Annona squamosa* is independent of TRPM8 channels.

In contrast to findings of present study, Lashinger., *et al.* [8] showed that N-(3-aminopropyl)-2-{[(3-methylphenyl)methyl] oxy}-N-(2-thienylmethyl) benzamide hydrochloride (AMTB), a

TRPM8 antagonist, attenuated the volume-induced painful micturition response in rats and was useful in limiting nociception in painful-bladder syndrome. Soares., *et al.* [15] also found that oleanolic acid inhibit nociceptive response induced by menthol in zebrafish, suggesting that oleanolic acid modulates TRPM8 channels.

The adult zebrafish has been proposed as a promising alternate model in translational research especially in nociceptive study caused by different noxious stimuli [1,5,9]. In the present study, the therapeutic potential of ethanolic extract of *Annona squamosa* leaves was evaluated for anti-nociceptive effect in different chemically induced pain model of zebrafish.

Groups	Parameters (Novel tank)					
	Time spent in upper portion of tank (sec)	No. of entries into upper portion of tank	Average entry duration (sec)	No. of erratic movements	No. of freezing bouts	Freezing duration (sec)
Control Group	$314.5^{a} \pm 31.5$	$34.6^{a} \pm 5.6$	$16.1^{a} \pm 2.8$	$0.60^{c} \pm 0.2$	$0.79^{c} \pm 0.4$	$0.75^{d} \pm 0.6$
Menthol (0.006 %)	$49.1^{c} \pm 6.1$	$3.05^{c} \pm 1.2$	$3.08^{d} \pm 1.6$	$17.0^{a} \pm 4.4$	$14.0^{a} \pm 5.2$	$230.2^{a} \pm 19.9$
Menthol (0.006%) + *Annona squamosa* (200 µg/mL)	$82.1^{bc} \pm 6.5$	$7.5^{bc} \pm 1.8$	$5.2^{cd} \pm 1.6$	$10.0^{b} \pm 2.7$	$8.6^{b} \pm 3.1$	$110.8^{b} \pm 16.8$
Menthol (0.006%) + *Annona squamosa* (400 µg/mL)	$98.2^{b} \pm 17.1$	$10.2^{b} \pm 2.7$	$7.5^{bc} \pm 2.8$	$3.0^{c} \pm 1.9$	$7.1^{b} \pm 2.9$	$56.7^{c} \pm 8.7$
Menthol (0.006%) + Meloxicam (0.2 mg/mL)	$113.6^{b} \pm 16.6$	$12.06^{b} \pm 5.2$	$10.0^{b} \pm 4.8$	$2.0^{c} \pm 1.1$	$3.7^{c} \pm 1.3$	$24.8^{d} \pm 4.5$

Table 5: Effect of ethanolic extract of *Annona squamosa* leaves on behavioral responses of zebrafish in Menthol induced nociception.

*Values are Mean ± SE

*Values are mean of ten observations

*Means bearing different superscript differ significantly ($p < 0.05$).

Transient Receptor Potential (TRP) channels in sensory neurons play a significant role in the physiology of pain. Among TRP channels, subtype TRPA1, constitute one of the largest group of nociceptive ion channels. However, there is evidence suggesting that TRPM8 channels also known as the cold and menthol receptor 1 (CMR1) manipulation has a therapeutic benefit in pain [1]. Ethanolic extract of *Annona squamosa* leaves used in present research, produced anti-nociceptive effect against cinnamaldehyde induced nociception suggesting the role of TRPA1 channels, in anti-nociceptive effect of *Annona squamosa.* However, *Annona squamosa* did not prevent menthol induced nociceptive behaviours in zebrafish which indicate that the anti-nociceptive effect of *Annona squamosa* is independent of TRPM8 channels.

Summary

Zebrafish display an aberrant phenotypic behaviour following an algogenic agent acid injection, accompanied by other behavioural changes (e.g., reduced locomotion, increased freezing, and changes on vertical activity etc). The present experiment was carried out to investigate the antinociceptive activity of *Annona squamosa* in zebrafish.

A total of 90 zebrafish were taken and divided into 3 groups of different nociceptors (Cinnamaldehyde and Menthol) and these 3 groups were further sub-divided into 4 subgroups of each major group alongwith one control group. For monitoring the effectiveness of *Annona squamosa* through behavioural analysis of zebrafish, after treatment and application of the algogenic agent in various pain models, animals were investigated for the nociceptive response via various parameters in terms of locomotor activity or number of line crossings (LC) performed during a certain time, specific for each model and Novel tank diving test which evaluates time spent in upper portion of tank, number of entries into upper portion of tank, average entry duration, number of erratic movements, number of freezing bouts and freezing duration.

According to the observational record of our present research work, it was found that cinnamaldehyde and menthol administration offers an excellent opportunity for assessing nociception related behavioural phenotypes in zebrafish pain models via activation of TRPA1 and TRPA8 receptors. Our data showed the pharmacological potential of ethanolic extract of *Annona squamosa* leaves as

a nociception inhibitor.

Our findings suggest that the anti-nociceptive effect of this extract on acute pain seems to be modulated by TRP channels. These results encourage the continuation of the study aiming to isolate and characterize the active agent. Therefore, this study adds new scientific evidence and highlights the potential of the *Annona squamosa* leaves in the development of phytomedicines with analgesic properties.

Conclusion

- Ethanolic extract of *Annona squamosa* exert significant antinociceptive activity against cinnamaldehyde induced nociception which showed that *Annona squamosa* produce its anti-nociceptive effect through TRPA1 (Transient receptor potential ankyrin 1) receptors.
- Ethanolic extract of *Annona squamosa* did not alter the nociception induced by menthol. This indicated that the anti-

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