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Indian Cobra (Naja naja) venom improves cognitive behaviour in Drosophila melanogaster

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Abstract
The purpose of our research is to assess the anti-stress response of *Naja naja* venom and combinations of caffeic acid and para coumaric acid on the behavioral changes in *Drosophila melanogaster* under starvation for 36 h. Wild type flies were divided into eight groups and correspondingly, eight groups of starved flies (36h) were also maintained: (i) control and groups treated with – (ii) *Naja naja* venom (NNV), (iii) caffeic acid (CA), (iv) *p*-coumaric acid (PCA), (v) NNV+CA, (vi) NNV+PCA, (vii) CA+PCA, and (viii) NNV+CA+PCA. Cognitive behaviour (phototaxis, smell chemotaxis, taste chemotaxis, hygrotaxis, thermotaxis, and negative geotaxis) and status of redox homeostasis in flies were assessed in all groups of flies. Control flies showed normal cognitive behaviours and these behaviours are reversed in starved groups of flies. A tendency towards normalcy has been noticed in NNV/photochemical treatment. Although, the administered doses of NNV and combinations of CA, PCA could normalize cognitive behaviour and reduce oxidative stress the effects are maximum with NNV+CA+PCA treatment indicating that pharmacologically active small peptides present in snake venom (sarafotoxin, disintegrin, Kunitz-type inhibitor, natriuretic peptide, crotamine etc.) along with phytochemicals could evoke maximum preventive effect.

Keywords: naja naja venom, caffeic acid, para coumaric acid, drosophila melanogaster

Introduction
The fruit fly, *Drosophila melanogaster* is a highly useful controllable model organism to study the behavioural/physiological/biochemical/ molecular mechanisms due to the biologically and physiologically preserved pathways between the fly and humans [1]. *D. melanogaster* is the model organism used to research disciplines that range from basic genetics to human diseases. The fly genome is 60 percent homologous to humans, who are less redundant and further about 75 percent of the genes, are responsible for human diseases are homologous in the fly also [2]. *Drosophila* has been used for over a decade to investigate cognition or intellectual disability, which has provided a large amount of disease-relevant information [3]. In the fruit fly, food deprivation is known to trigger aggressive food quest, sugar behavior and a decrease in gustatory responses [4]. Snake venom is made up of active biological compounds such as proteins and pharmacologically active small peptides (sarafotoxin, disintegrin, Kunitz-type inhibitor, natriuretic peptide, crotamine etc.) [5]. Several snake proteins are known to interact with components of the human haemostatic system to evoke toxic effects [6]. Further, the diverse molecular mechanisms of snake venom mediating systemic effects on the muscular, nervous and circulatory systems have been reported [7]. Latest studies on venom activity have shown promising results for snake venom toxins against numerous experimental pathophysiological conditions such as arthritis, leukemia, neural damage, stroke, Parkinson's disease and Alzheimer's disease [8, 9]. Snake venom could play preventive roles in the associated behaviour deficiencies in neurodegenerative disorders [10].
The bioactive natural products generated by the plants have been shown to have distinctive pharmacological properties [11]. Compounds such as flavones, condensed tannins, phenolic acids, sulphated phenolic acid and lignin are obviously seen in sea grass such as *Halodule uninervis* than the terrestrial plants. Phenol and phenyl propanoid derivatives consisting of *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid and vanillic acid are rich in *H. uninervis* [12]. It has been documented in several studies that caffeic acid has several other pharmacological properties, such as anti-apoptotic, anti-inflammatory and antioxidant activities [13, 14]. A phenolic derivative, *p*-coumaric acid (PCA), belongs to the hydroxycinnamic acid family. In plants, several mushrooms and in *H. uninervis*, PCA has been detected and it is known to possess potent antioxidant properties [15]. PCA is known to reduce oxidative stress, inhibits genotoxicity and exerts neuroprotection [16]. The current investigation is an attempt to investigate the comparative effects of anti-stress activities of *Naja naja* venom (NNV), caffeic acid (CA), and *p*-coumaric acid (PCA) and their combinations in response to starvation in *D. melanogaster*.

Materials and Methods

Chemicals

Caffeic acid, *p*-coumaric acid, benzaldehyde, yeast tablets, agar, nepagin, propionic acid, phosphate buffered saline, trichloroacetic acid, acetic acid, guanidine hydrochloride, thiobarbituric acid, n-butanol, dichromate, cumene hydroperoxide, ethylenediaminetetraacetic acid, hydrogen peroxide, glutathione reductase, Tris-hydrochloride, nicotinamide adenine dinucleotide phosphate, glutathione, and sucrose were purchased from Sigma-Aldrich, Bangalore, Karnataka, India. Wild type (WT) of *D. melanogaster* was obtained from the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, Telangana, India.

Venom source

With permission from the Tamil Nadu Forest Department, *Naja naja* venom was acquired from the Irula Snake Catchers Co-operative Society, Mamallapuram, Tamil Nadu, India. For experiments, venom was weighed and diluted in distilled water according to the need.

Venom and Phytochemical Treatment

In the current study, the wild type of *D. melanogaster* (Canton S) was used and the flies were maintained at 22-25 ºC and grown on a diet/nutrient medium (one medium unit containing 360ml of distilled water, 2.5g of agar, 17g of maize powder, 15g of sucrose, 6g of yeast tablets, 1g of nepagin, and 1ml of propionic acid) under 12:12 (L:D) condition (lights on-06:00 and lights off-18:00) [17]. The flies were split into two major groups (fed and starved groups) with eight sub groups each (group I - WT control, group II - NNV, group III - CA, group IV - PCA, group V- NNV + CA, group VI- NNV + PCA, group VII- CA + PCA, group VIII- NNV + CA + PCA) (NNV – 0.1%; CA and PCA – 0.5% administered through 100 ml of media). The concentration has been selected based on previous observations [18]. Behavioural studies were carried out in both fed and starved groups. All the assays were performed in triplicate. Adult flies (n=30) from all groups were collected and fly homogenate was prepared using 0.1M sodium phosphate buffer (pH-7.3) and centrifuged (3500 rpm for 5 minutes) at 8 ºC; supernatant was collected and used for further analysis.

Phototaxis: response to light

The apparatus consists of a test tube (1.5 × 12 cm) connected by a cello tape with another test tube and divided into three equal compartments. Initially, the set-up containing approximately 30 flies plugged with cotton was left separately for 30 minutes in a dark room. Then, a light source (Philips, India) that acted as an attractor for the flies was placed at 1 metre. The flies were then softly pounded down to the position of the flies and kept in the bottom of the vial. The set-up was kept in a horizontal position to the light source. The light was then turned on and a timer began, and the flies were counted at 1 minute in the compartments [19].

Smell chemotaxis: response to volatile chemicals

As previously mentioned, the smell chemotaxis assay was performed [20]. Benzaldehyde (1 ml; 100 mM) was soaked in the cotton plug in the test tube (1.5 × 12 cm) which was divided into three equal compartments. Th flies were softly tapped to the bottom, a timer began, and flies were counted in each compartment at 1 minute.

Taste chemotaxis: response to non-volatile chemicals

Taste chemotaxis was performed [21]. The cotton plug of the test tube (1.5 × 12 cm) was soaked with 1 ml of 100 mM sucrose. The test tube was divided into three equal compartments. About 30 flies were placed into the test tube and were gently pounded into the bottom of the test tube. A timer was then started and then flies at each compartment were counted at 1 minute.

Hygrotaxis: response to humidity

Hygrotaxis assay was performed as mentioned previously [22]. A vial (10×2 cm) was filled with 1 ml of distilled water, covered with parafilm and was kept overnight. After about 12 hours, another vial of same size with about 30 flies was taken. After removing parafilm and water from the first vial, two vials related to a help of a transparent tape. The connected vials were compartmentalized into three equal zones (I, II and III and compartment I moisturized zone). Timer was started and a fly in each compartment was counted at 1 minute.

Thermotaxis: response to temperature

Two vials (10×2 cm) were used in the study. One vial was heated to a temperature of 45º and 4º C were instantly connected to a vial by means of transparent tape comprising of 30 flies [23]. The connected vials were compartmentalized into three equal zones (I, II and III – compartment III heated zone). At 1 minute the numbers of flies were counted.

Negative geotaxis: response to gravity

About 30 flies are positioned in a vertical glass column (10cm X 2 cm) sealed at one end with cotton. After a short recovery period of five minutes, flies were softly tapped to the bottom of the column [23]. The number of flies beyond the distance of 9 cm was counted at 1 minute.

Results

Statistical analysis showed that in behavioural studies NNV+PCA+CA showed more significance than NNV and control groups. Similar results were noticed in starved group (Fig. 1-7 & Table 1). In phototaxis assay, about 93% flies in NNV+PCA+CA group showed attraction towards light whereas the same treatment in starved condition (36 h) showed that 80% of flies are attracted toward light (Fig. 1 & Table 1). A negative response was shown by the fed and starved groups in smell chemotaxis (response to benzaldehyde). Both fed and starved groups retracted away from the smell of benzaldehyde. Highly significant activity was shown by NNV+PCA+CA groups, about 91% flies retracted to the third compartment in fed NNV+PCA+CA group and about 75% flies retracted to the third compartment in starved NNV+PCA+CA group (Fig. 2 & Table 1). In Taste chemotaxis (response to sucrose), both fed and starved groups attracted towards sucrose rich region. Highly significant activity was shown by NNV+PCA+CA groups, about 90% flies attracted towards sucrose in fed NNV+PCA+CA group and about 80% flies attracted towards sucrose in starved NNV+PCA+CA group (Fig. 3 & Table 1).

A positive response was shown by the fed and starved groups in hygrotaxis for both fed and starved groups attracted towards first compartment which was pre incubated for humid condition. Highly significant activity was shown by NNV+PCA+CA groups, about 91% flies attracted towards humidity in fed NNV+PCA+CA group and about 83% flies attracted towards humidity in starved NNV+PCA+CA group (Fig. 4 & Table 1). Thermotaxis was carried out in high and low temperature, in which the flies showed retraction from high and low temperature, proving that it is stable at humid / 23ºC. Highly significant activity was shown by NNV+PCA +CA groups, about 92% flies retracted to the first compartment away from the third compartment which was pre-heated to 45ºC in fed NNV+PCA+CA group and about 76% flies retracted to the first compartment away from the third compartment which was pre-cooled to 4ºC in fed NNV+PCA+CA group (Fig. 5 & Table 1). A similar result was shown by the starved groups also. Highly significant activity was shown by NNV+PCA+CA groups, about 92% flies retracted to the first compartment away from the third compartment which was pre-heated to 45ºC in starved NNV+PCA+CA group and about 70% flies retracted to the first compartment away from the third compartment which was pre-cooled to 4ºC in starved NNV+PCA+CA group (Fig. 6 & Table 1). In negative geotaxis, the percentage of flies escaped beyond 9cm was noted in both the fed and starved groups. Both the groups showed a positive result. Highly significant activity was shown by NNV+PCA+CA groups, about 88% flies escaped beyond 9cm in fed NNV+PCA+CA group and about 82% flies lies escaped beyond 9cm in starved NNV+PCA+CA group (Fig. 7 & Table 1).

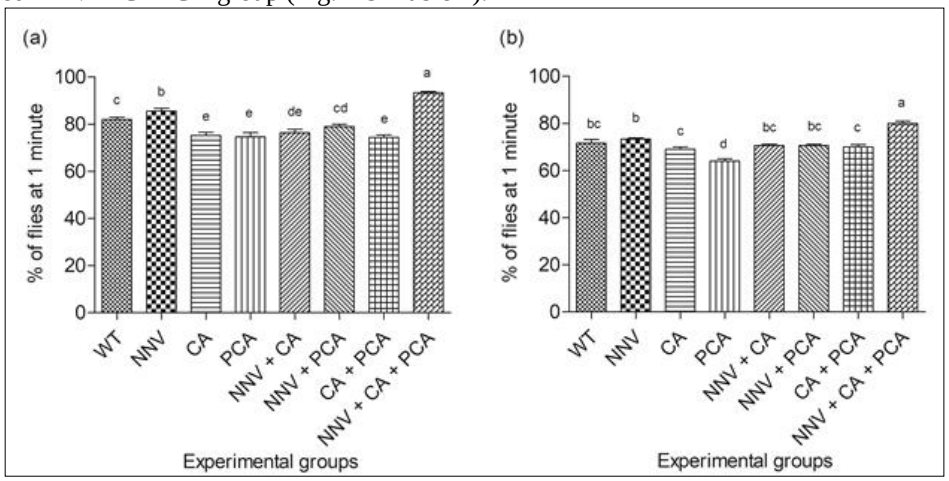

Fig 1: Phototaxis

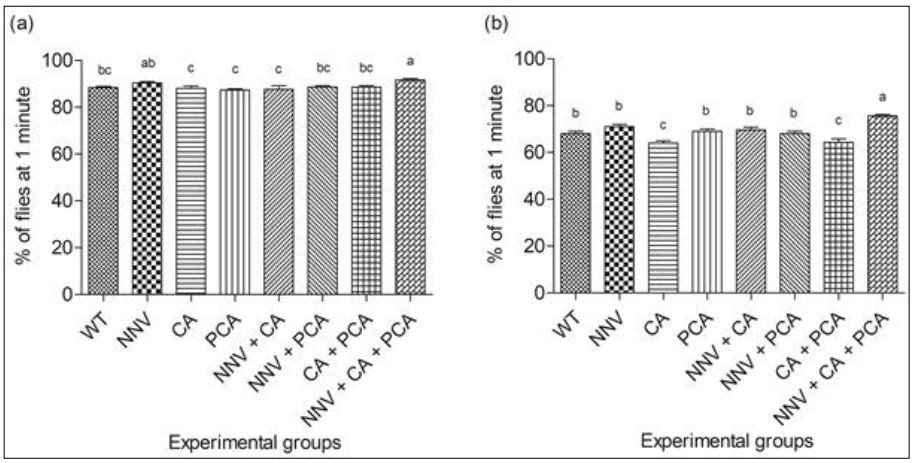

Fig 2: Smell chemotaxis

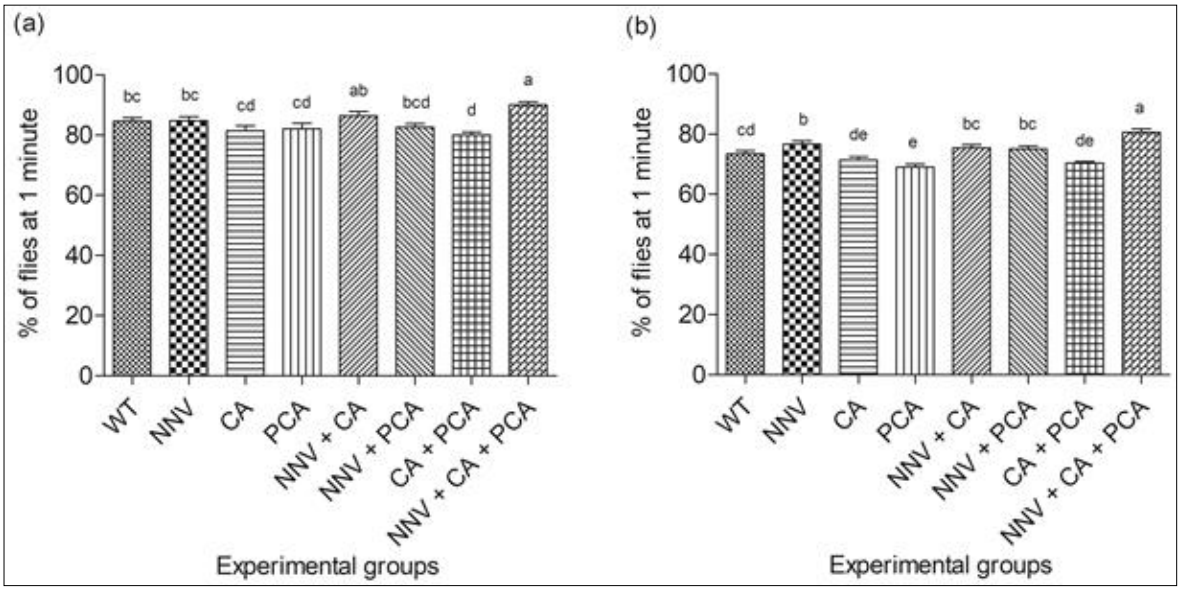

Fig 3: Taste chemotaxis

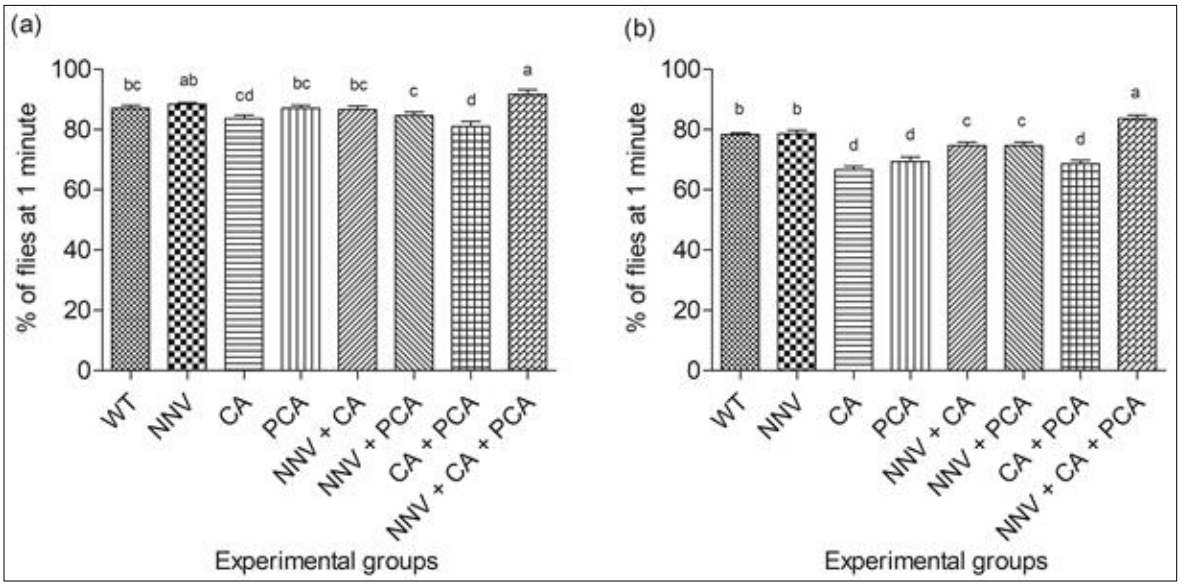

Fig 4: Hygrotaxis

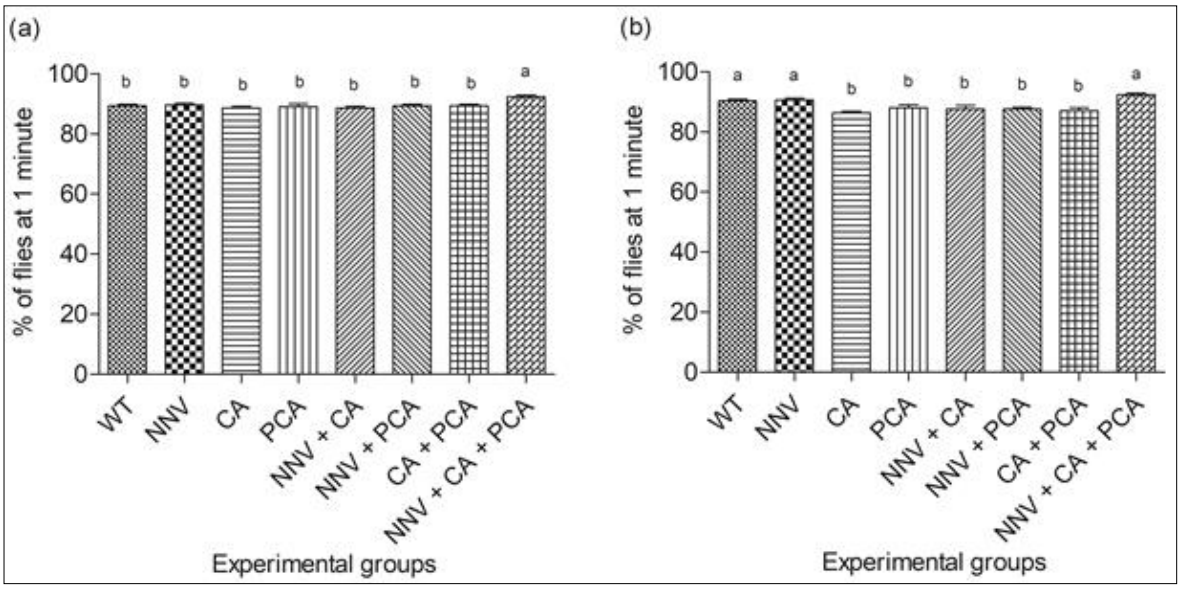

Fig 5: Thermotaxis (High temperature)

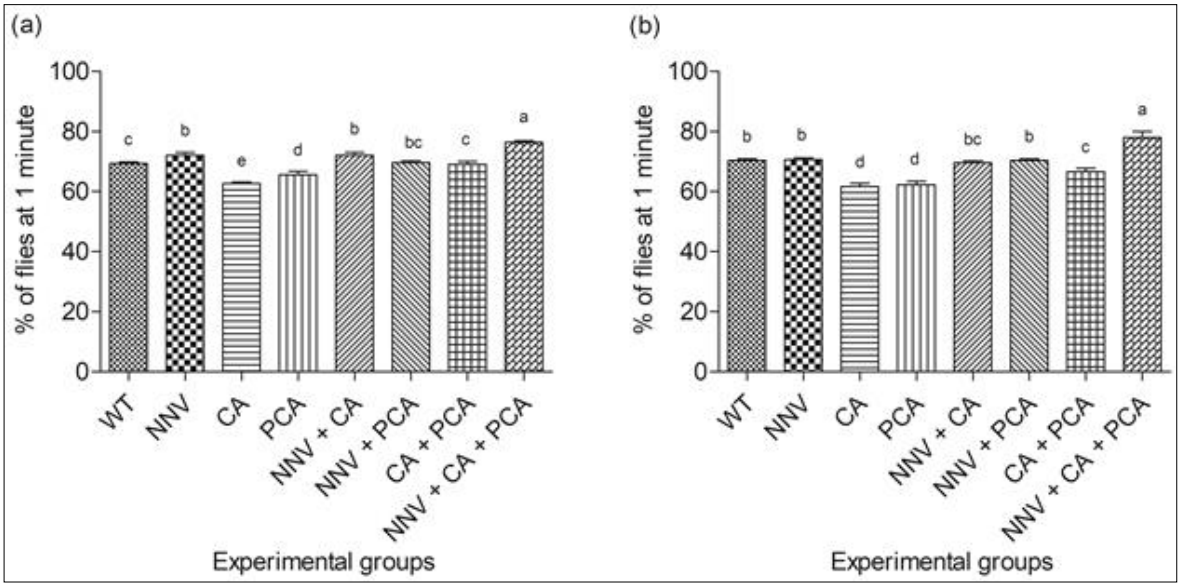

Fig 6: Thermotaxis (Low temperature)

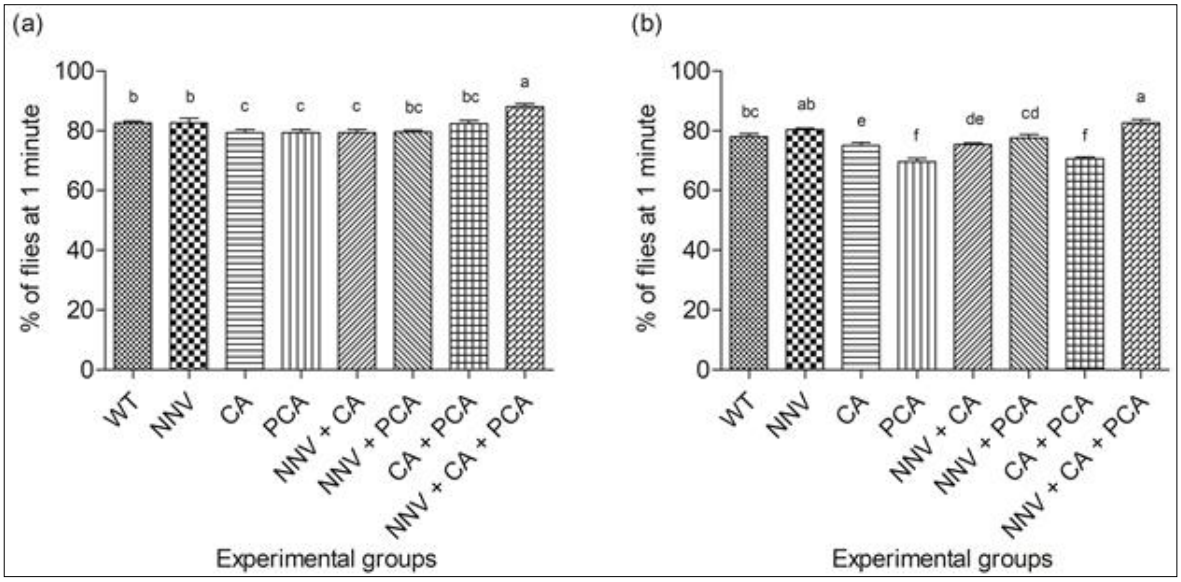

Fig 7: Negative geotaxis

Table 1: Statistical analysis of behavioural studies

Parameters	Experimental groups	Mean ± SD		Grouping		DF		F-value		P-value	
Fed and Starved	Fed and starved	Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved
Phototaxis	WT	82.000 ± 1.000	71.667 ± 1.528	c	bc	7	7	87.17	65.87	P<0.005	P<0.005
	NNV	85.667 ± 1.155	73.333 ± 0.577	b	b	7	7	87.17	65.87	P<0.001	P<0.001
	CA	75.222 ± 1.347	69.000 ± 1.000	e	c	7	7	87.17	65.87	P<0.01	P<0.01
	PCA	74.670 ± 1.760	64.000 ± 1.000	e	d	7	7	87.17	65.87	P<0.01	P<0.01
	NNV + CA	76.333 ± 1.528	70.667 ± 0.577	de	bc	7	7	87.17	65.87	P<0.001	P<0.001
	NNV + PCA	79.000 ± 1.000	70.667 ± 0.577	cd	bc	7	7	87.17	65.87	P<0.001	P<0.001
	CA + PCA	74.333 ± 1.155	70.000 ± 1.000	e	c	7	7	87.17	65.87	P<0.01	P<0.01
	NNV + CA +	93.333	80.000 ±	a	a	7	7	87.17	65.87	P=0.000	P=0.000

	PCA	± 0.577	1.000								
Smell chemotaxis	WT	88.667 ± 0.577	68.000 ± 1.000	bc	b	7	7	9.57	36.95	P<0.001	P<0.001
	NNV	90.333 ± 0.577	71.000 ± 1.000	ab	b	7	7	9.57	36.95	P=0.000	P<0.001
	CA	88.000 ± 0.577	64.000 ± 1.000	c	c	7	7	9.57	36.95	P<0.001	P<0.005
	PCA	87.333 ± 0.577	69.000 ± 1.000	c	b	7	7	9.57	36.95	P<0.001	P<0.001
	NNV + CA	87.667 ± 1.528	69.667 ± 1.155	c	b	7	7	9.57	36.95	P<0.001	P<0.001
	NNV + PCA	88.667 ± 0.577	68.000 ± 1.000	bc	b	7	7	9.57	36.95	P<0.001	P<0.001
	CA + PCA	88.667 ± 0.577	64.333 ± 1.528	bc	c	7	7	9.57	36.95	P<0.001	P<0.001
	NNV + CA + PCA	91.667 ± 0.577	75.667 ± 0.577	a	a	7	7	9.57	36.95	P=0.000	P=0.000
Taste chemotaxis	WT	84.667 ± 1.155	73.333 ± 1.155	bc	cd	7	7	15.97	38.22	P<0.001	P<0.005
	NNV	84.778 ± 1.347	76.667 ± 1.155	bc	b	7	7	15.97	38.22	P<0.001	P<0.001
	CA	81.444 ± 1.711	71.333 ± 1.155	cd	de	7	7	15.97	38.22	P<0.005	P<0.005
	PCA	82.110 ± 1.840	69.000 ± 1.000	cd	e	7	7	15.97	38.22	P<0.005	P<0.01
	NNV + CA	86.333 ± 1.528	75.333 ± 1.155	ab	bc	7	7	15.97	38.22	P<0.001	P<0.001
	NNV + PCA	82.667 ± 1.155	75.000 ± 1.000	bcd	bc	7	7	15.97	38.22	P<0.001	P<0.001
	CA + PCA	80.000 ± 1.000	70.333 ± 0.577	d	de	7	7	15.97	38.22	P<0.005	P<0.01
	NNV + CA + PCA	90.000 ± 1.000	80.667 ± 1.155	a	a	7	7	15.97	38.22	P<0.001	P<0.001
Hygrotaxis	WT	87.000 ± 1.000	78.333 ± 0.577	bc	b	7	7	21.08	76.5	P<0.001	P<0.001
	NNV	88.333 ± 0.577	78.667 ± 1.155	ab	b	7	7	21.08	76.5	P<0.001	P<0.001
	CA	83.667 ± 1.155	66.667 ± 1.155	cd	d	7	7	21.08	76.5	P<0.001	P<0.01
	PCA	87.000 ± 1.000	69.333 ± 1.528	bc	d	7	7	21.08	76.5	P<0.001	P<0.01
	NNV + CA	86.667 ± 1.155	74.667 ± 1.155	bc	c	7	7	21.08	76.5	P<0.001	P<0.001
	NNV + PCA	84.667 ± 1.155	74.667 ± 1.155	c	c	7	7	21.08	76.5	P<0.001	P<0.001
	CA + PCA	81.000 ± 1.730	68.667 ± 1.155	d	d	7	7	21.08	76.5	P<0.001	P<0.01
	NNV + CA + PCA	91.667 ± 1.528	83.667 ± 1.155	a	a	7	7	21.08	76.5	P=0.000	P<0.001
Thermotaxis (high temperature)	WT	89.333 ± 0.577	90.333 ± 0.577	b	a	7	7	10.04	21.14	P<0.001	P<0.001
	NNV	89.667 ± 0.577	90.667 ± 0.577	b	a	7	7	10.04	21.14	P<0.001	P<0.001
	CA	88.667 ± 0.577	86.333 ± 0.577	b	b	7	7	10.04	21.14	P<0.001	P<0.001
	PCA	89.000 ± 1.000	88.000 ± 1.000	b	b	7	7	10.04	21.14	P<0.001	P<0.001
	NNV + CA	88.667 ± 0.577	87.667 ± 1.155	b	b	7	7	10.04	21.14	P<0.001	P<0.001
	NNV + PCA	89.333 ± 0.577	87.667 ± 0.577	b	b	7	7	10.04	21.14	P<0.001	P<0.001
	CA + PCA	89.333 ± 0.577	87.000 ± 1.000	b	b	7	7	10.04	21.14	P<0.001	P<0.001

	NNV + CA + PCA	92.333 ± 0.577	92.333 ± 0.577	a	a	7	7	10.04	21.14	P=0.000	P=0.000
Thermotaxis (low temperature)	WT	69.333 ± 0.577	70.333 ± 0.577	c	b	7	7	73.11	70.08	P<0.005	P<0.001
	NNV	72.000 ± 1.000	70.667 ± 0.577	b	b	7	7	73.11	70.08	P<0.001	P<0.001
	CA	62.667 ± 0.577	61.667 ± 1.155	e	d	7	7	73.11	70.08	P<0.01	P<0.01
	PCA	65.667 ± 1.155	62.333 ± 1.155	d	d	7	7	73.11	70.08	P<0.01	P<0.01
	NNV + CA	72.000 ± 1.000	69. 667 ± 0.577	b	bc	7	7	73.11	70.08	P<0.001	P<0.005
	NNV + PCA	69.667 ± 0.577	70.333 ± 0.577	bc	b	7	7	73.11	70.08	P<0.005	P<0.001
	CA + PCA	69.000 ± 1.000	66.667 ± 1.155	c	c	7	7	73.11	70.08	P<0.005	P<0.01
	NNV + CA + PCA	76.333 ± 0.577	78.000 ± 2.000	a	a	7	7	73.11	70.08	P<0.001	P<0.001
Negative geotaxis	WT	82.667 ± 0.577	78.000 ± 1.000	b	b	7	7	23.1	68.46	P<0.001	P<0.001
	NNV	82.667 ± 1.528	80.333 ± 0.577	b	b	7	7	23.1	68.46	P<0.001	P<0.001
	CA	79.333 ± 1.155	75.000 ± 1.000	c	c	7	7	23.1	68.46	P<0.005	P<0.005
	PCA	79.333 ± 1.155	69.667 ± 1.155	c	c	7	7	23.1	68.46	P<0.005	P<0.01
	NNV + CA	79.333 ± 1.155	75.333 ± 0.577	c	c	7	7	23.1	68.46	P<0.005	P<0.005
	NNV + PCA	79.667 ± 0.577	77.667 ± 1.155	bc	bc	7	7	23.1	68.46	P<0.005	P<0.001
	CA + PCA	82.333 ± 1.155	70.667 ± 0.577	bc	bc	7	7	23.1	68.46	P<0.001	P<0.01
	NNV + CA + PCA	88.000 ± 1.000	82.667 ± 1.155	a	a	7	7	23.1	68.46	P=0.000	P=0.000

Discussion

Our study showed the average phototaxis rather than fast and slow phototaxis (Fig. 1 & Table 1). Previous studies indicated that fruit flies were first gently pounded down, so that rapid phototaxis could be the initial response; and after a few minutes the response slowed down, which could be due to slow phototaxis [24]. Smell chemotaxis reaction to volatile chemicals was assessed by the behavioral response of fruit flies. Flies in a test tube travelled quicker from the repellent mounted on a Q-tip at the open end and out of a cotton swab earlier in the assay [25]. We used the same way in the present and the result was shown (Fig. 2 & Table 1) that we were able to find a faster assay for volatile attractants. Another research indicated that the smell chemotaxis - olfaction test is based on a single bound fly turning towards a higher chemical concentration [26]. Flies were fed with various chemicals or food sources, each colored with a different color, and then the ingested color was calculated to determine the amount of tastant consumed by the flies [27]. Another research showed that several repulsive or attractive tastants were tested using the proboscis extension reflex [28]. In our research, we showed that the method used to test the sucrose and water response was shown in (Fig. 3 & Table 1). The response to water in our findings is different from previous studies.

The response of our sample to high humidity was essentially like previous studies (Fig. 4 & Table 1). The temperature response of flies was previously calculated by placing them in the middle between high temperature in one tube and low temperature in another, as for humidity above, and by providing them with a linear thermal gradient [29]. Our findings showed that, by measuring the movement of the flies in a thermal gradient, flies avoid heat and cold were described in (Fig. 5, 6 & Table 1). The negative geotaxis response to gravity was previously tested by using a vertical counter current apparatus and a vertical maze [30]. Recent research has shown that negative geotaxis and fly movement in both male and female flies fed with Spectracide® solution have decreased significantly [31]. In seeking us, the comparable results of previous studies were seen in (Fig. 7 & Table 1). In conclusion, our study demonstrated that, various behavioral studies in *D. melanogaster* by time dependent manner (1 minute) for all parameters. The effect of NNV, CA, PCA, and their combinations might be can induced oxidative stress, and its protective effects via free radicals scavenging can restore the cellular redox status and reduce stress in *D. melanogaster*. According to the light-dark cycle, living organisms perform their roles. As a dynamic interplay of multiple processes involved in producing open rhythms of multiple biological functions, the data produced in *D. melanogaster* can also be extended to humans by multiple proteins varying their expression in *D. melanogaster* and humans.

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Author's contribution
Sheeja S Rajan carried out all the experiments, examined the data and wrote the manuscript's preliminary draft. The principal investigator who planned the experiment and contributed to the manuscript was Perumal Subramanian.

Conflicts of interest
The authors announce that no conflicts of interest exist.

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