Research Article

Effects of Crude Extracts of *Areca catechu* L. on Locomotory Behaviour in *Drosophila melanogaster*

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ABSTRACT

Background: Areca nut (*Areca catechu* L.) is a plant of immense economic value in South Asian countries, and has a traditional and aesthetic value in many parts of India. Areca nut has a variety of secondary metabolites such as alkaloids, tannins, flavonoids, and triterpenes. Several conflicting reports have argued about the safety of Areca nut consumption by people across the globe. **Materials and Methods:** Methanolic extract of the areca nut was fed to *Drosophila melanogaster* and its effects were investigated by studying their locomotory behaviour. **Results:** The RING assay and larval crawling assay have been used to study the flies' locomotory behaviour, and the results were substantially influenced by the various doses of areca nut extract, with the flies appearing lethargic in their movement at higher concentrations. **Discussion:** Flies reared on a medium containing various concentrations of Areca nut methanolic extracts exhibited variations in their locomotory behaviour. This finding is significant because research conducted by many researchers on Drosophila melanogaster using other substances such as ethanol and caffeine yielded comparable results. Further studies are warranted in order to extrapolate the findings to analyze and understand similar anomalies in higher organisms like humans.

Keywords: *Areca catechu* L, Arecoline, Drosophila melanogaster, RING Assay.

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INTRODUCTION

Areca nut seed is derived from the *Areca catechu* L plant, which belongs to the family of palm trees. It is one of the important commercial crops in South Asian countries.[1] It is widely cultivated in India, China, Malaysia, Indonesia, and other countries. The effectiveness of the Phyto-chemical constituents in natural products greatly depends on the polarity of solvents. These solvents in turn are used to extract the secondary metabolites from the areca nut seeds. Some of the commonly used solvents include Methanol, Ethanol, Acetone, Chloroform, and diethyl ether.[2] Processed and unprocessed areca nuts are consumed in many places across the world and they can also be used in combination with tobacco, in the form of Pan Masala and Gutkha.[3] Areca catechu L is grouped as one of the psychoactive substances that are used as a social drug.[4] It is known as Adake in Kannada and Supari in Hindi. The chemical composition of areca nut includes carbohydrates, fats, proteins, crude fibres, polyphenols, alkaloids, tannins, and so on.[5] People who normally consume areca nut for a long time get habituated and many of them are not aware of how harmful these compounds are. Studies conducted on children and adults suggest that there is an increase in health risks and the severity caused by the prolonged consumption of areca nut and their products.[6,7]

Drosophila melanogaster has been a highly valuable model organism for researching locomotory behaviour, and investigating the biological rhythm of locomotion in these flies has yielded a lot of information. The internal Circadian clock aids organisms in adapting to their surroundings. Insects and mammals have a lot

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in common when it comes to their circadian rhythms. These clocks are physically and functionally linked to the optic system, which aids in synchronizing ambient light-dark cycles and controls a variety of endocrine, autonomic, and behavioural activities.[8,9] clock (CLK), cycle (CYC), period (PER), and timeless are key transcription factors that govern circadian rhythm in insects and mammals (TIM). cryptochrome (CRY1 and CRY2) genes replace timeless genes in animals. Of all these factors, PER is the most characterized one. It was isolated from several dipteran and lepidopteran insects.[10-13] There are also studies on caffeine and its effect on locomotory behaviour in animals. Acute caffeine consumption increases locomotory activity in rats, but chronic administration of the same shows a decrease in locomotion.[14,15] Other workers also showed that in the case of Drosophila melanogaster, the use of caffeine increases locomotory activity in flies and decreases the length of sleep.[16]

MATERIALS AND METHODS

Preparation of *Areca catechu* L. Extract

The Areca catechu L. (Thirthahalli variety) was procured from a farm in Thirthahalli village, Shimoga district of Karnataka. Dried seeds of areca nut were used in the extraction process. The extraction involved Soxhlet apparatus and important organic solvents such as Acetone, Chloroform, Diethyl Ether, Ethanol, and Methanol. Out of all the extracts prepared methanolic extract was preferred as the results were promising.[17] The Soxhlet extraction method was preferred in this work as it is an affordable method used in laboratories and a reliable option. The extraction was carried out for 15 cycles, once the solvent in the thimble turned colourless, the reaction was stopped and the sample was placed in a hot air oven. After three days, the sample was taken out and the crystals of *Areca catechu* L. were collected and stored.[18]

Drosophila melanogaster culturing and maintenance

Drosophila melanogaster, Oregon K strain, was obtained from the Drosophila Stock Centre, Department of Zoology, Mysore University. The flies were subcultured using the standard Wheat cream agar media. They were maintained at a constant temperature of 25±10°C. The composition of the media is as follows

For 1000 ml of Water (1l) – Rava (Semolina) 100g, Jaggery 100g, Agar-Agar 10g, and Propionic acid 7.5 ml.[19]

Isolation of Virgin flies

The experiment utilized *Drosophila melanogaster* flies that had been cultured in a laboratory environment. In a new culture medium, two females and three males of Drosophila were allowed to mate. The flies were removed from the bottles once the larvae were spotted, and the bottles were examined for the emergence of adult flies. Because the adults are sexually immature for a few hours after emergence, they were referred to as virgin flies. Males and females were separated and used for the experiment during this interval.[20]

Experimental setup of the control and test group of flies

Once the virgin flies were isolated, and sufficient numbers of flies were available for the experimental work, they were added to the culture media containing different concentrations of methanolic extract of areca nut (0.1%, 0.5%, and 1%) and sub-cultured. A control group was also maintained to compare the results. Three generations of flies were studied and the observations were recorded. These flies were used in studying the locomotory behaviour of *Drosophila melanogaster* flies.

Larval Crawling Assay

Drosophila larvae exhibit locomotory activity by moving about on the media or the culture bottle walls. To observe the movements of larvae in this experiment, we used agar media in a Petri plate and a graph sheet. Agar-agar with a concentration of 1.5 percent was made and put onto Petri plates, where it solidified. On the Petri plates, Drosophila larvae from the control and test groups were free to wander about. The larvae's movement in one minute was recorded, with movement in both horizontal and vertical directions taken into account. The exact movement displayed by the larvae was observed with precision when the Petri plates were put on a graph sheet, and the data were collected for study.[21,22]

RING Assay

Adult Drosophila flies respond to the stimuli in several ways. *Drosophila melanogaster* flies display locomotory behaviour, which allows them to move around in the wild as well as in lab conditions. These flies are usually found wandering around in the wilderness hunting for food, mates, and other things. However, in the laboratory, movement is limited to the culture bottle, and most of the time it occurs away from the medium. This is a classic case of negative geotaxis in operation. The goal of the present experiment was to investigate this negative geotaxis behaviour. To further understand

this behaviour, the RING (Rapid Iterative Negative Geotaxis) assay was used.[23] Flies were put on the test tube stand after being transferred to transparent graduated tubes. They are given about 10-15 min to acclimate (This time varies from place to place depending on the species and strain of the flies used). There were 6 flies in each tube, and five tubes were placed on the stand. The stand was tapped four times before the flies were permitted to fly up for the fifth time, and the recording was done with a mobile camera. The recording was stopped exactly after 5 sec, and the process was repeated 10 times with a 2-min break between each round. Image processing software was used to analyze the photos, and the data was recorded for study.[24] 5 different sets were used in this experiment to avoid any discrepancies with the result and the results were recorded using a high-resolution mobile camera and processed to obtain the data. The data were analyzed using Graph pad prism, where Mean, Standard deviation, and Error were studied. One-way Anova was used for the comparison.

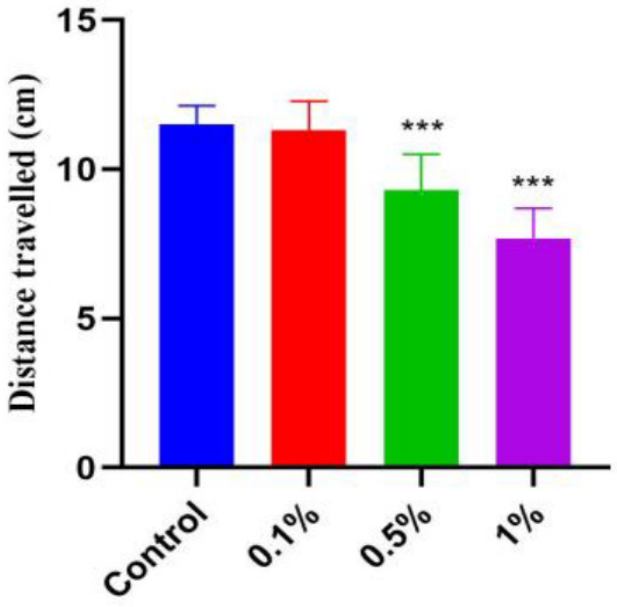

Figure 1b: Comparison of RING assay results between Control group and Test group (The results are based on the movement of the flies in the assay tube and are measured in cm).

RESULTS AND DISCUSSION

Methanolic extract of Areca catechu extract was prepared using the Soxhlet method. The extract was then used to treat Drosophila melanogaster flies. The flies treated with the extract were used in the experiment and as mentioned in the methodology above, they were used in the larval crawling assay and RING assay. Control flies and test groups (0.1%, 0.5%, and 1%) were maintained in triplicates to avoid any loss of data or errors in recording the data.

RING Assay

The flies that were grown in the standard wheat cream agar media were allowed to move in the culture bottle and they travelled an average distance of 11.51 cm^2. Whereas the flies from the test groups were found to be lethargic in 0.5% and 1% concentration of the extract and travelled less than the flies from the control group and 0.1% group. Flies were grown in 0.1%, 0.5%, and 1% concentrations of methanolic extract travelled the average distances of 11.32 cm, 9.31 cm, and 7.68 cm respectively (Figure 1 a).

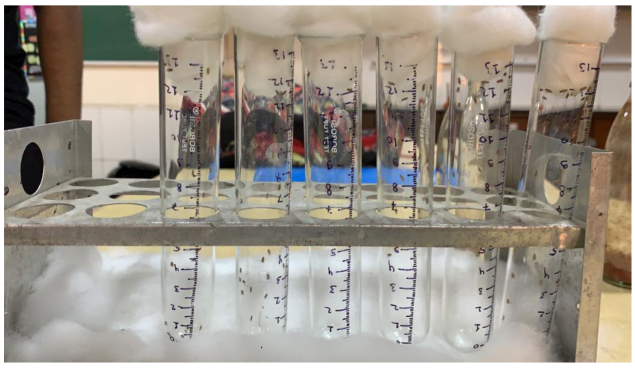

Figure 1a: *Drosophila melanogaster* flies placed in tubes for RING assay.

The graphs above indicate that there was a significant reduction in the movement of flies in 0.5% and 1% concentration media as compared to control and 0.1% concentration media (Figure 1 b). The *p*-value of the unpaired *t*-Test was 0.366 between Control and 0.1% concentration and therefore it didn't show any significant difference. Whereas for 0.5%, the *p*-value was <0.001 and represented as *** in the graph. For 1% concentration, the *p*-value was found to be less than that of 0.5% and it is also represented as *** in the graph. The difference between the means (SEM) of Control and 0.1% was -0.1900 ± 0.2085, between Control and 0.5% it was -2.200 ± 0.2452 and for 1% it was -3.830 ± 0.2156 (Table 1).

Larval Crawling Assay

The third instar larvae of Drosophila melanogaster were selected for the assay as they move about freely away from the media. It's easy to track its movement as they are big enough to be measured accurately. The effect of the methanolic extract of *Areca catechu* L. was studied in the flies grown in 0.1%, 0.5%, and 1% concentrations. In the initial part of the study, a graduation from 0.1% to 5% concentrations of the extracts was added during the

Table 1: Distance Travelled by 30 Drosophila melanogaster flies in Control, 0.1%, 0.5%, and 1% concentrations of media containing methanolic extract of *Areca catechu* L.

Fly Number	Control Data	0.1% Data	0.5% Data	1% Data
F1	12.2	12.5	10.9	9
F2	12.6	12.4	9.1	9.7
F3	12.5	12.4	9.5	9.7
F4	11.9	11.9	10	8.2
F5	11.7	10.7	10.2	8
F6	11.1	12.5	10.3	7.7
F7	10.6	12.3	7.5	6.9
F8	10.6	12.2	7.7	7.5
F9	11.6	11.2	8.2	7.6
F10	10.9	10.8	9.3	7.7
F11	11.3	11.6	8.6	6.8
F12	11.6	10.8	8.5	7.1
F13	11.1	11.2	8.5	7.6
F14	11.3	11.1	10.3	8
F15	11.7	12.4	8	7.4
F16	11.5	12.2	8.3	7
F17	11.5	10.9	8.9	8
F18	11.4	10.4	9	8
F19	12.2	11.3	10	8.8
F20	11.2	12	9.2	7.9
F21	12.3	11.4	9.5	7
F22	10.7	9.1	9.2	7.5
F23	11	11.5	9.3	7.2
F24	11.3	11.3	10	7.5
F25	11.4	8.3	10.9	9.7
F26	12.6	11.5	6.4	6.3
F27	12.5	10.4	10	8.4
F28	11.3	11.2	11.2	5.8
F29	11.3	11	12	6.4

preparation of the culture media. The flies transferred to media containing more than 1% concentration did not survive or the growth was inconsistent. Therefore, concentration was scaled down to 0.1%, 0.5% and 1%. The following graphs highlight the movement shown by Drosophila larvae on the Petri plate with agar media on it. The difference in larval movement among the four groups was analyzed using an unpaired student *t*-Test. The *p*-value was <0.01 for all the three test groups when they were compared to the control group. Therefore, they are represented as *** in the graph. The difference between Mean (SEM) of Control and 0.1% was -0.1300 ± 0.02236, between control and 0.5% it was -0.2100 ± 0.02076 and for 1% it was -0.3000 ± 0.02213 (Figure 2).

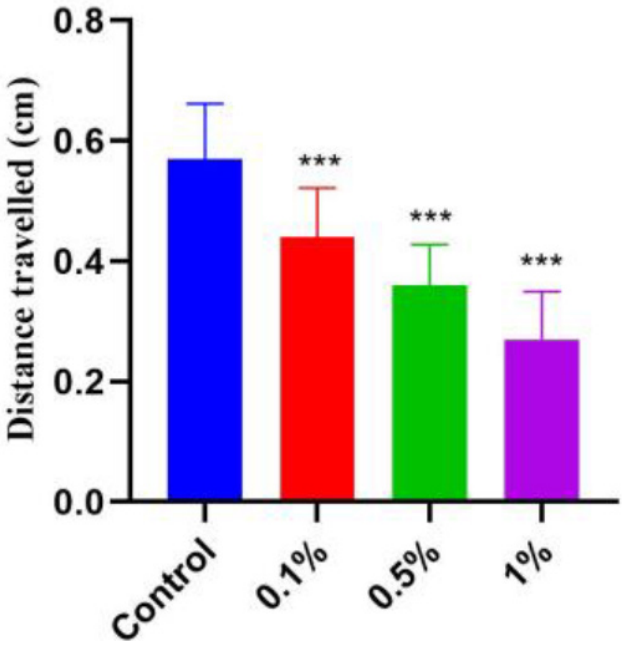

Figure 2: Comparison of the distance travelled by larvae on the agar plate in Control, 0.1%, 0.5%, and 1% groups (Distance is measured in cm).

This analysis shows that there is a significant difference between the control and test groups in their locomotory behaviour. With the increase in the concentration of the methanolic extract of *Areca catechu* L, the movement of the larvae drastically decreased. This trend is consistent with the RING assay as shown above (Table 2).

DISCUSSION

Areca catechu L is a tropical palm plant belonging to the Arecaceae family. In traditional medicine, it has been used to treat a variety of ailments. Its capacity to kill parasitic organisms that cause diseases in people is one of its most notable features. In various regions of the world, the areca nut seeds are consumed as a masticatory material.[25] Areca nut seeds are also known to be one of the most important psychoactive substances. In this study, we aimed to discover more about the effects of areca nut seed extract on animal locomotion.

Drosophila is an important model for studying developmental and behavioural characteristics. Many studies on Drosophila flies have revealed substantial differences in their locomotory and sexual behaviour when they were exposed to three different concentrations of the compounds. According to a study done by Seggio *et al.*, alcohol exposure during the larval stages has a significant impact on Drosophila melanogaster locomotory behaviour.[26] Wu *et al.*, in their work on Drosophila, explained the effect of caffeine

Table 2: Distance travelled by Drosophila melanogaster larvae in Control, 0.1%, 0.5%, and 1% concentrations of media containing methanolic extract of *Areca catechu* L.

Larval Number	Control in cm	0.1% in cm	0.5% in cm	1% in cm
L1	0.5	0.5	0.4	0.3
L2	0.5	0.5	0.4	0.3
L3	0.6	0.4	0.4	0.3
L4	0.5	0.4	0.4	0.4
L5	0.6	0.4	0.3	0.4
L6	0.6	0.6	0.4	0.2
L7	0.6	0.4	0.3	0.4
L8	0.5	0.5	0.5	0.3
L9	0.6	0.4	0.4	0.3
L10	0.7	0.5	0.4	0.4
L11	0.5	0.4	0.4	0.3
L12	0.5	0.5	0.2	0.3
L13	0.5	0.5	0.4	0.3
L14	0.6	0.4	0.3	0.3
L15	0.6	0.5	0.4	0.3
L16	0.6	0.6	0.4	0.2
L17	0.6	0.5	0.4	0.3
L18	0.7	0.4	0.3	0.2
L19	0.7	0.4	0.4	0.3
L20	0.6	0.5	0.4	0.2
L21	0.4	0.4	0.4	0.2
L22	0.6	0.5	0.3	0.3
L23	0.5	0.4	0.3	0.3
L24	0.6	0.4	0.4	0.2
L25	0.7	0.5	0.3	0.2
L26	0.5	0.3	0.4	0.1
L27	0.6	0.2	0.4	0.2
L28	0.3	0.4	0.3	0.1
L29	0.6	0.4	0.3	0.2
L30	0.7	0.4	0.2	0.3

on their sleep.[27] In the RING assay, flies cultured in media containing 0.1%, 0.5%, and 1% of areca nut extract exhibited decreased movement and as shown in the graph above, the *p*-value was found to be 0.366 for 0.1% and <0.01 for 0.5% and 1% concentration. The flies in the control and test groups were compared, and the results demonstrate that as the concentration of the methanolic extract of areca nut was increased, the fly movement decreased. The distance travelled by the flies in 5 sec was calculated. The results of the larval crawling assay were also similar to that of the RING assay. The Drosophila larvae that were grown in 1% concentration of methanolic extract of areca nut moved slowly and erratically. All the three test groups showed decreased movement and the *p*-value was found to be <0.01. These results suggest that when the compound was added to the culture media in different concentrations, initially there was no significant difference observed between the control and the test group. But when the flies were cultured in the media containing different concentrations of the compound for 3-4 generations, the result showed considerable variation in their locomotory behaviour.

CONCLUSION

When cultured in regular wheat cream agar media at 25±1°C, Drosophila melanogaster has a normal sleep-wake cycle or exhibits a 12-hr light/ 12-hr dark cycle. Flies reared in a medium containing various concentrations of Areca nut extract exhibited variations in their circadian rhythm, which resulted in aberrant movement in both larvae and adults. Drosophila locomotion was substantially reduced after treatment with a methanolic extract of areca nut. This finding is significant because research conducted by many researchers on Drosophila melanogaster using other substances such as ethanol and caffeine yielded comparable results. Further studies can help us understand human conditions caused due to consumption of areca nut alone or in combination with other substances.

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CONFLICT OF INTEREST

The current work doesn't have any conflict of interest at the time of writing this manuscript.

ABBREVIATIONS

Hr: Hour; **Min:** Minute; **Sec:** Second; **Cm:** centimeter; **SEM:** Standard Error of Mean; **RING:** Rapid Iterative Negative Geotaxis; **ANOVA:** Analysis of Variance; **CLK:** Clock; **CYC:** Cycle; **PER:** Period; **TIM:** Time; **CRY:** Cryptochrome; **L:** Liter; **G:** Gram; **Ml:** Milliliter.

SUMMARY

Areca catechu L. is an important economic crop in South Asian countries. The effect of areca nut on oral health is a much-debated topic world over. Till date there haven't been any published reports on the effects of methanolic extract of areca nut on *Drosophila melanogaster* locomotory behaviour. The current study emphasizes on how *Drosophila melanogaster* flies react when they are treated with different concentrations of the methanolic extract of areca nut. Significant deviations in the locomotory behaviour were observed in the present study. Further studies are warranted for analysing the same in higher organisms.

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