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GEOTACTICAL AND NEUROCHEMICAL PHENOTYPES OF *Drosophila melanogaster* FOLLOWING *Nigella sativa* OIL EXPOSURE

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ABSTRACT

Drosophila melanogaster is a holometabolous frugivorous fly with neurobiological and neurogenetic modelling importance, owed to its small size, short life cycle, fast reproductive rate, low cost in maintenance and a small tetra-chromosomal genome. *Nigella sativa* (Black seed) is a widely researched medicinal plant considered by some sources as a miracle plant, capable of curing all diseases. Being the most abundant neurotransmitter in Drosophila, glutamate plays an important role in learning and memory, neuroexcitation, and also neuro-inhibition. This research investigated the impacts of *Nigella sativa* oil on the survival, glutamate levels and geotactical locomotion in Harwich strains of *Drosophila melanogaster*. These were executed using the survival assay, spectrophotometric glutamate assay and negative geotaxis assay, respectively. The flies were divided into control, lower dose and higher dose groups. The groups were exposed to *Nigella sativa* oil for five days at 0 mL, 0.1 mL and 0.6 mL *Nigella sativa* per mL of feed medium. The results showed a higher survival rate, glutamate level and negative geotactic ability for the flies exposed at lower dose, while the higher *Nigella sativa* dose recorded lesser values in the trio. This indicates that *Nigella sativa* administered at 0.6 mL/mL of feed may be lethal to the general survival and physiological functions of adult Drosophila. The lower dose however shows a high potential of maintaining and improving the geotactical/locomotive and neurochemical activities in the flies, as further studies are on to further identify the most therapeutic dose of *Nigella sativa* in *Drosophila melanogaster*, with a range suggested based on the findings of this research.

Key words: *Drosophila melanogaster, Nigella sativa. Survival, Glutamate, Negative geotaxis*

INTRODUCTION

There has always been need to develop animal models that accurately recapitulate human disorders in a bid to identifying alternative drugs for such disorders. This need has accorded more popularity to *Drosophila melanogaster* (common fruit fly) particularly for its low maintenance cost, fast reproductive rate, small size, short life cycle, and tetra-chromosomal genome. It is therefore considered widely as a valid genetic model for several human disorders including Alzheimer's, Parkinson's, Huntington's, and spinocerebellar ataxia, especially as over 65% of human disease-associated genes have a correlate (homologue) in Drosophila (Markstein 2019).

Nigella sativa (also referred to as Habbatus-sawdaa or black seed) is considered one of the most widely used medicinal plant across the world (Ahmad et al.

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2013). While therapeutic potentials may be ascribed to many other medicinal plants available in nature, *Nigella sativa* is considered one of the greatest forms of therapeutics in the Tibbun-nabawiyy system of Islamic medicine, to such extent that it was declared a "remedy for all diseases except death" (Al-Bukhari, 1997). It is historically notable for its anti-inflammatory, analgesic, anti-pyretic, anti-bacterial, and anti-neoplastic potentials as proven by modern science (Ali and Blunden 2003; Naz 2011).

Despite the extensive validations of biological activities and therapeutic potentials of *Nigella sativa* in humans and rodents (Naz 2011; Ahmad et al. 2013; Randhawa and Alenazi 2016), the phenotypes of Drosophila following *Nigella sativa* exposure has not been detailed in literature. Therefore, this study aimed at filling this gap in knowledge. Specifically, this research aimed at determining the effect of *Nigella sativa* oil on the locomotory/geotactical and neurochemical properties of *Drosophila* melanogaster through negative geotaxis and glutamate assays in the flies. The study also assessed the survival rates of the flies upon dietary exposure to *Nigella sativa* oil.

MATERIALS AND METHODS

Acquisition and Breeding of Flies

Male and female wild type *Drosophila melanogaster* (Harwich strains) were acquired from the Drug Metabolism and Molecular Toxicology Research Laboratory, Department of Biochemistry, University of Ibadan, Ibadan, Nigeria. The culture and feeding medium, which was largely corn-meal based, was hygienically adopted from protocols reported by Abolaji et al. (2015). The flies were maintained under a cool and regulated temperature (23 ± 3°C), 60% humidity and natural day/night cycles in the Neurophytotherapy Lab, Department of Anatomy, Olabisi Onabanjo University, Sagamu, Nigeria. Cold-pressed and purified *Nigella sativa* oil was procured from Hemani® International, Pakistan. This research was conducted with the ethical approval of the Anatomical Research Ethics Committee, Olabisi Onabanjo University, Sagamu, Nigeria, numbered BS/18/VII/23-013.

Experimental Design and Dosing

The research experimental design consisted of three groups, namely; control, low dose, and high dose. While the control group was maintained on the normal feed, the latter two groups were treated with 0.1 mL and 0.6 mL of *Nigella sativa* oil respectively per mL of feed, corresponding to 10% and 60% of the feed volume respectively. Each treatment vial contained 4 mL of dispensed feed. The feed for each group was prepared separately as described in Appendix I, while the respective volumes (or percentages) of *Nigella sativa* oil was added shortly after the cooked feed was done, before dispensing into the treatment vials. Each group consisted of four treatment vials each of which in turn had thirty flies of both genders as shown in Table 1, while the experiment lasted five days similar to Abolaji et al. (2015).

Negative Geotaxis Assay

In order to assay for the negative geotactical effects of dietary exposure of the flies to *Nigella sativa*, ten flies from each treatment vial across the groups were introduced on day-3 into the climbing apparatus made of calibrated vertical columns of high density polyethylene (length-15 cm; diameter, 2 cm) following anaesthesia on mild ice. One hour duration was allowed for the flies to completely recover from the anaesthetic exposure. A vertical distance of 8 cm above the bottom surface was marked with a circle around the circumferences of the column.

The flies were gently tapped to the bottom of the column by a gentle bang of the column on the assay platform, and the number of flies that passed the 8 cm mark by the 10th second after the tapping was recorded as a percentage of total flies per trial. The assay was repeated for the same group five times, allowing for 1 minute rest period between each vial. The score for each trial was recorded as average of three, while the score for each group was recorded as an average of the four vials that constitute the group.

Table 1: Experimental Design

Group	Regimen	No. of Vials/ Group	No of Flies/ Vial
Control	Media without *Nigella sativa* oil content	4	30
Lower Dose	0.1 mL *Nigella sativa* oil per mL of feed	4	30
Higher Dose	0.6 mL *Nigella sativa* oil per mL of feed	4	30

Glutamate Assay

After 6 days of treatment with *Nigella sativa*, oil the flies from respective vials were anaesthetised on mild ice and homogenized in 0.1 M phosphate buffer, pH 7.0 (1 mg: 4 µL), and centrifuged at 5000 rpm for 5 minutes. The supernatant was thereafter decanted into labelled Eppendorf tubes for the determination of glutamate levels through spectrophotometry. Flies which died earlier than the freshly anaesthetised ones were homogenised and also assayed for glutamate following the above procedure.

Survival Assay
The survival rates of the flies across the experimental duration was examined through the quantification of the flies that survived and died on each day of the research.

Statistical Analysis
Data were expressed as mean ± standard error of mean. The experimental and control groups were compared using 1- way analysis of variance and Bonferroni's multiple comparison test. Ninety-five percent confidence interval was employed in determining statistical significance of group differences.

RESULTS

The following are the results obtained from the research

Survival Rate
The following are the survival assay results:
Figure 1 illustrates the flies' survival rate from day 1 to day 5 of the experiment. A significant decline in the number of surviving experimental flies (low and high NS) commenced from the second day of administration, falling to as low as 35% and 12.5% for low and high dose flies respectively, while no decline was recorded in the control flies until the fourth day of administration (falling to 80%). Seventy-five percent (75%) of the control flies eventually survived until the last day of the research, while the low dose groups had only 12.5% of the flies surviving through the last day of the research duration. The high dose flies however had no surviving fly by the end of the fifth day.
By the end of the sixth day, following the experimental duration, metamorphoses was observed in the same vials from which the dead flies of the high dose group were collected yielding a total of 9 adult *Drosophila melanogaster* flies after the research.

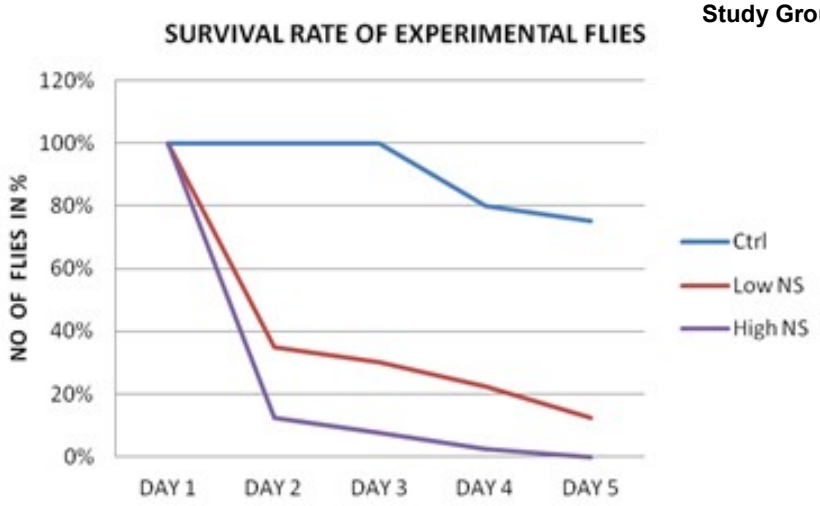

Figure 1: Curve Showing the Percentage Mean (±SEM) Survival Rates of the Flies

Negative Geotaxis Assay
The following are the negative geotaxis results:
Figure 2 shows the negative geotactical properties of the experimental and control flies across the groups. The control flies recorded the highest percentage of flies that crossed the denoted mark. The lower dose group (with 0.1 mL 10% *Nigella sativa* exposure per mL of feed) had the closest percentage of flies that crossed the mark to the control, with an insignificant difference of 3.3%. The higher dose group (with 0.6 mL *Nigella sativa* exposure per mof feed) however had a significantly lower percentage of negatively geotactic flies than both the lower dose and the control.

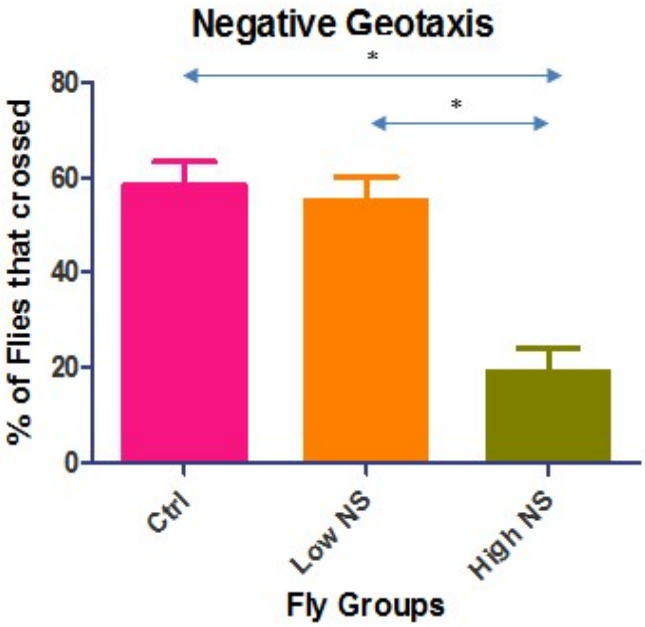

Figure 2 Graph Showing the Percentage Mean (±SEM) of the Flies that crossed During the Geotaxis across the Study Groups (* Significant at $p<0.05$)

Glutamate Assay
The following are the glutamate assay results:
The mean glutamate levels in the flies were assayed using spectrophotometry, and Figure 3 illustrates a significant decrease in glutamate level of the high dose group when compared with the control. The low dose however maintained an insignificantly lower glutamate level than the control and

insignificantly higher level than the high dose group. Upon further analysis, the mean glutamate levels in the freshly sacrificed flies were higher across the respective groups than the levels recorded in the previously dead flies though insignificantly.

DISCUSSION

The impact of *Nigella sativa* oil on the life span of Drosophila was investigated through the survival assay, and the result presented above showed that 0.4 mL of the oil per 4 mL of feed (10%) significantly reduced the life span of the flies by 87.5%, while the higher dose - 2.4 mL/mL of feed (60%) reduced the life span by a 100% within five days.

But despite the adverse results on the adult, the oil seemed not as lethal to the holometaboly of pre-adult stages of the flies as it was to the adults, for the *Nigella sativa*-containing media accommodated the metamorphosis of laid eggs into the various instars of larvae and subsequently adults. This may be explained by how able Drosophila larvae have been reported to move and survive in harsh conditions like hypoxia and anoxia, unlike adults which quickly get paralysed (Callier et al. 2015). The non-feeding third instar Drosophila larvae, among all stages, have also been reported as most resistant to gamma rays (Paithankar et al. 2017).

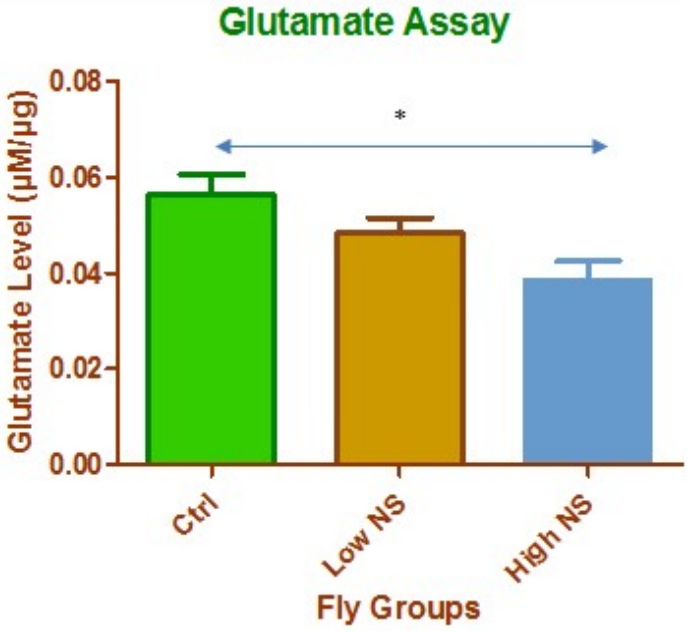

Figure 3 Graph showing the mean glutamate levels (±SEM) in the flies across the groups (* Significant at $p<0.05$)

As part of its innate escape response, appropriate vertical movement is a critical phenomenon for the survival of flying animals (Bae et al. 2016). The locomotory and climbing activity of the flies was thus assayed after five days of *Nigella sativa* exposure at the described doses using the negative geotaxis assay. Both experimental groups in this study showed *Nigella sativa* oil to effect a decrease in the percentage of negatively geotactic flies, with an inverse proportionality to the *Nigella sativa* dosage administered. A significantly impaired climbing behaviour was thus characterised to the high dose group compared to the control, but not the low dose group.

As negative geotactic ability has been shown to be sensitive to several factors including oxidative stress, age, and previous cold exposure (Linderman et al. 2012), high but not low dose of *Nigella sativa* has been shown through this study to adversely affect negative geotaxis in Drosophila.

Glutamate remains the most abundant excitatory neurotransmitter in Drosophila (Shin et al. 2018), while glutamatergic neurons are also highly abundant in the Drosophila central nervous system (Liu and Wilson 2013). Neuro-excitability was thus assayed in the flies through glutamate neurotransmitter assay, and *Nigella sativa* has been shown by this study to significantly decrease the glutamate level in the high but not the low dose group, suggesting lethality of the higher dose. The lower dose flies however maintained similar glutamate level with the control flies.

Neurotransmitters however may act on different spatial and temporal scales (Liu and Wilson, 2013) and glutamate similarly has been reported to vary in its activities within Drosophila. Zimmerman et al. (2017) reported that glutamate promote wakefulness by increasing the duration of wake bouts through its excitatory role at neuromuscular junctions in some neuronal groups, while in others, it promoted sleep. Liu and Wilson (2013) also reported glutamate with inhibitory activities similar to GABA's in the antennal lobe, while its extracellular accumulation has been suggested by (Ikeda et al. 1989) to cause neuronal cell damage.

The need for further studies and characterisations is thus emphasised to elucidate which exact roles of glutamate is being mediated by *Nigella sativa*, in order to accomplish a better understanding, and as well proffer evidenced hypotheses on the implications of its increase or decrease within the Drosophila homogenate.

This study, after observing the expected reduction in glutamate release following death of the Drosophila flies, further reveals that it take a while before the neurotransmitters are significantly lost in the dead flies. This was buttressed by the fact that glutamate level recorded in the flies which previously died before the fifth day was only 20% lesser than that recorded in those sacrificed at the end of the fifth day. It may thus be important to determine the progressive rate of neurotransmitter loss for the purpose of knowledge.

Conclusion

The impacts of *Nigella sativa* oil on the neurochemical, behavioural and survival phenotypes of *Drosophila melanogaster* are detailed above. The flies exposed to the higher dose of *Nigella sativa* showed the least level of negative geotactical locomotion, glutamate levels, and higher incidence of mortality in comparison with the control group. This suggests lethality of the higher dose to the general survival and physiological functions of Drosophila. But as the lower dose group showed milder and mostly insignificant differences from the control, this study indicates that *Nigella sativa* administered at low dose has a high potential of maintaining the locomotive and neuro-excitatory traits in *Drosophila melanogaster* if accurately administered.

The identification of the actual favourable and therapeutic dose of *Nigella sativa* oil in Drosophila flies thus remains a work in progress, and is hereby recommended within a range less than 10% of the feed medium for feed exposure, based on the findings reported above.

Conflict of Interest

None declared.

Acknowledgements

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Appendix I: Preparation of Treatment Media

The treatment media was prepared as follows:

1) 0.85 L of water was measured and divided into 0.70 L and 0.15 L.
2) The 0.70 L portion of water was boiled.
3) 52 g of corn meal and 30g of glucose were added to the 0.15 L portion of water.
4) 0.5 g of Nipagin was dissolved in a small amount of water.
5) 7.9 g of Agar (OXOID Agar Bacteriological) was added to the boiling water and continuously stirred.
6) 5 g of Yeast was dissolved in a little water.
7) After 10 min the corn meal and glucose mixture was added to the boiling mixture and continuously stirred.
8) After 10 min, the dissolved yeast was added.
9) The mixture was maintained on heat and stirred for 20-25 min.
10) The dissolved Nipagin was added to the heating mixture and stirred.
11) After five minutes, the medium was removed from the hot plate/gas and allowed to cool for a minute.
12) *Nigella sativa* oil was immediately added afterwards at 0.1 mL/mL of the feed for the lower dose media, and at 0.6 mL/mL of feed for the higher dose media. Nothing was added at this stage to the control media.
13) The media were thereafter dispensed into the respective treatment vials.
14) Each dispensed vial was covered with tissue paper for protection during cooling.

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