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# Synergistic action of propolis with levodopa in the management of Parkinsonism in *Drosophila melanogaster*

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**Abstract:**

**Background:** The Phosphatase and tensin-induced putative kinase 1 ($PINK1^{B9}$) mutant for *Drosophila melanogaster* is a key tool that has been used in assessing the pathology of Parkinsonism and its possible remedy. This research was targeted toward determining the effects of ethanolic extract of propolis, with levodopa therapy in the management of Parkinsonism.

**Method:** The $PINK1^{B9}$ flies were divided into groups and fed with the different treatment doses of ethanoic extract of propolis. The treatment groups were subjected to 21 days of administration of propolis and the levodopa at different doses after which percentage climbing index, antioxidant activity and lifespan studies were done.

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**Results:** Propolis alone improved motor activity, antioxidant and lifespan in *Drosophila melanogaster* than in PINK1 flies. Propolis in combination with levodopa significantly (P<0.05) improved physiological parameters at higher than lower concentrations in Parkinsonism *Drosophila melanogaster* demonstrating its importance in managing side effects associated with levodopa.
**Conclusion:** Propolis is a novel candidate as an alternative and integrative medicinal option to use in the management of Parkinsonism in both animals and humans at higher concentrations.

**Keywords:** aging, antioxidant activity, catalase, climbing index, Drosophila melanogaster, Levodopa induced dyskinesia, lifespan, oxidative stress, Parkinson's disease, Parkinsonism, PINK1$^{B9}$, propolis

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## Introduction

Parkinson's disease (PD) is a common disorder affecting 1% of the elderly above 65 years, characterized by the loss of dopaminergic neurons in substantia nigra [1]. Common symptoms are uncontrolled movements such as akinesia, bradykinesia, resting tremor and rigidity. These symptoms are both in humans [2, 3] and in animal models of PD [4, 5]. PTEN-induced putative kinase 1 (PINK1) is responsible for the maintenance of mitochondria structure and function [6–8]. Loss of PINK1 gene in *Drosophila* culminates in a phenotype expressing Parkinsonism, with mitochondrial destruction occurring before muscle degeneration, as well as the destruction of dopaminergic neurons [3]. The *Drosophila melanogaster* PINK1$^{B9}$ mutant model portrays several of these features of PD [3, 9, 10] and has been used to study neuronal dysfunction and neurodegeneration. PINK1$^{B9}$ model provides quite enormous information regarding the pathogenesis of PD and mitochondrial dysfunction [11].

Replacement of dopamine with Levodopa is a cornerstone in treatment for PD [12, 13]. In the basal ganglia, this levodopa is converted to dopamine by aromatic L-amino acid decarboxylase [14]. However, the long-term levodopa therapy (5–10 years from onset of therapy) is often complicated by significantly disabling motor fluctuations and dyskinesia, called levodopa-induced dyskinesia (LID), thus negating its beneficial effects [13]. This is a major disadvantage to levodopa therapy. In the conversion of levodopa to dopamine, the metabolism of dopamine, unlike other neurotransmitters, leads to the yield of dopamine quinones and ROS [15, 16]. Once established, these LIDs are presented with difficulty in treatment and therefore efforts should be made to prevent them [17]. Besides the clinical effects on the patient, LID can affect the quality of life increasing the cost of healthcare and shortening the average lifespan of individuals [18].

Propolis is a resinous material obtained by bees from buds of plant sources near their hives [19]. Propolis is used by bees as a sealant in the hives [20]. Propolis contains over 300 compounds including volatile organic compounds, flavonoid aglycones, phenolic acids, and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, amino acids [21]. Many natural remedies are employed in the treatment of neurodegenerative disorders [2, 22] but none has been employed as a treatment for LIDs. Propolis extracts were reported to be neuroprotective [23]. Propolis also has broad-spectrum biological and pharmacological properties, such as antioxidant [23], anti-inflammatory [24], antiproliferative [25], anti-cardiovascular diseases, antidiabetic [25] and hepatoprotective activities [26]. Therefore, the aim of this study was to determine if propolis would be a viable candidate for the treatment of LIDs and associated symptoms.

## Materials and methods

This study **was** approved and consented by Institutional Research and Ethics Committee of Kampala International University and the Animal research committee.

### Drosophila stocks

Wild type (W$^{1118}$) and PTEN-induced putative kinase 1 (PINK1$^{B9}$) mutant *Drosophila melanogaster* flies were obtained from the Bloomington Stock Center as previously described [27–31]. These flies that were obtained had the red eye balancer, meaning they appeared phenotypically normal but were genetically modified to express parkinsonian symptoms. However, the balancer allowed them to appear normal because the flies that expressed parkinsonian symptoms had greatly impaired functions and this would affect their transportation.

## Drosophila culture and crosses

Before the experimentation, the pupae were allowed to emerge from the vials which arrived and flies that expressed the red-eye balancer were selected out using a monocular microscope present in the Institute of Biomedical research Laboratory Kampala International University. These were flies which would express the $PINK1^{B9}$ gene and expressed symptoms of Parkinsonism. These flies were then crossed and larvae were allowed to emerge after which grouping was done.

### Drosophila sampling

The number of flies used in each experimental group was seventeen (17) and experiments were conducted in triplicates, thus 17 × 3 = 51 flies per group. This sample size was obtained by the use of the power analysis method as done by the G-power software version 3.1 and the flies were divided into groups as shown in Table 1. The total number of flies used in the study was 51 × 6 groups x 4 experiments = 1224 flies.

**Table 1:** Different group allocations used in the experiment, the abbreviations representing them and the rationale of drug and extract administration to their food.

| Group label | Drosophila spp. | Treatment | Number of flies in each experiment |
|---|---|---|---|
| WT (Negative control) | $w^{1118}$ | No treatment | 17 × 3 repeats = 51 |
| $PINK1^{B9}$ (Positive control) | $PINK1^{B9}$ | No treatment | 17 × 3 = 51 |
| $PINK1^{B9}$/lev (Comparative control) | $PINK1^{B9}$ | Levodopa 250 mg/kg | 17 × 3 = 51 |
| $PINK1^{B9}$/pro | $PINK1^{B9}$ | Propolis 500 mg/mL | 17 × 3 = 51 |
| $PINK1^{B9}$/lev/$pro^{250mg/mL}$ | $PINK1^{B9}$ | Propolis 250 mg/mL and levodopa 250 mg/kg of food | 17 × 3 = 51 |
| $PINK1^{B9}$/lev/$pro^{500mg/mL}$ | $PINK1^{B9}$ | Propolis 500 mg/mL and levodopa 250 mg/kg of food | 17 × 3 = 51 |

## Negative geotaxis

Twenty-one (21) days after experimental exposure, negative geotaxis was conducted using standard protocols [30]. In brief, a vertical distance of 8 cm above the bottom surface was marked by drawing a circle around the entire circumference of the vial and a group of ten flies was transferred into the lower vial carefully preventing the escape of any fly [30]. The number of flies which crossed the 8-cm mark by 10 s after were recorded for 3 consecutive repeats, allowing 1 min rest period between intervals. The number of flies used in this experiment was 51 × 6 groups = 306 flies.

## Antioxidant activity assessment

The molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color [32]. The antioxidant potential by free radical scavenging of propolis, the change in optical density of DPPH radicals was measured by collecting 0.2 mL of Drosophila melanogaster supernatant after feeding on experimental diets and diluting the solution with 2 mL of DPPH solution (0.5 mM). After 30 min in the dark, the absorbance was measured at 517 nm [33]. The number of flies used in this experiment was 51 × 6 groups = 306 flies and the percentage of the DPPH radical scavenging is calculated using the equation as given below:

% inhibition of DPPH radical = $([A_{br} - A_{ar}]/A_{br})$ x 100; Where $A_{br}$ is the absorbance before reaction and $A_{ar}$ is the absorbance after the reaction has taken place.

### Catalase activity determination assay

This was determined as previously described [30]. Approximately 200 mL of hydrogen peroxide 95% was obtained then added to Triton ×100 buffer 200 mL. These were mixed slowly and they were added to 200 mL of homogenized fly tissue. The oxygen bubbles produced by enzyme and trapped by the triton X-100 buffer appeared as foam and was measured by height from a standard curve in the form Absorbance = 0.056 catalase activity + 0.1, $R^2$ = 0.9604 was generated. The number of flies used in this experiment was 51 × 6 groups = 306 flies.

### Life span studies

The Drosophila was kept in groups of eight per vial in two vials one containing male and the other containing female flies as earlier stated. Upon commencement of the lifespan experimentation, virgin flies were allowed to emerge from the pupae and transferred into fresh vials of food. The flies in each vial were counted every 3 days interval and live flies were transferred to new vials while the dead flies were recorded. Flies of the same genealogy were used, to minimize differences arising from genetic backgrounds [30, 31]. The number of flies used in this experiment was 51 × 6 groups = 306 flies.

### Statistical analysis

This was done using Graph Pad Prism Version 6 and information was presented as figures and a Table. ANOVA test was done and Tukey's test was used to determine sources of variation and significant differences (P<0.05) were indicated with different super- scripts i. e. letters a, b, c. The lifespan data were analyzed using Kaplan–Meier survival analysis and Mantel-Cox was performed on the survival curves, with significance being reported when P<0.05.

## Results

### Combination of propolis and levodopa improved the climbing activity in motor impaired PINK1$^{B9}$/lev *Drosophila melanogaster*

The study showed that treatment with propolis prevented a decline in motor function significantly and improved the climbing activity in *Drosophila melanogaster*. When compared to the untreated groups, there was a significant increase in the climbing index (Table 2). This improvement was dose-dependent with the group that received 500 mg/mL of propolis (PINK1$^{B9}$/lev/pro$^{500mg/ml}$) having a significant improvement in climbing activity compared to the group that received 250 mg/mL (PINK1$^{B9}$/lev/pro$^{250mg/ml}$) as shown in Figure 1.

**Table 2:** Comparisons on climbing activity, catalase and DPPH activity in *Drosophila melanogaster.*

| Tukey's multiple comparisons test | Climbing Index | Catalase activity | DPPH activity | Lifespan |
|---|---|---|---|---|
| | Adjusted P values | | | |
| WT vs. PINK1$^{B9}$ | <0.0001 | 0.2619 | 0.2234 | Log-rank (Mantel-Cox) test for curve comparisons $X^2$ (5)=18.52, P=0.0001 |
| WT vs. PINK1$^{B9}$/lev | <0.0001 | 0.0015 | 0.006 | |
| WT vs. PINK1$^{B9}$/prop | <0.0001 | <0.0001 | 0.8636 | |
| WT vs. PINK1$^{B9}$/lev/pro$^{250mg/mL}$ | <0.0001 | 0.7027 | 0.0828 | |
| WT vs. PINK1$^{B9}$/lev/pro$^{500mg/mL}$ | <0.0001 | 0.0158 | 0.9993 | |
| PINK1$^{B9}$ vs. PINK1$^{B9}$/lev | 0.2362 | 0.0308 | 0.1635 | |
| PINK1$^{B9}$ vs. PINK1$^{B9}$/prop | >0.9999 | <0.0001 | 0.0368 | |
| PINK1$^{B9}$ vs. PINK1$^{B9}$/lev/pro$^{250mg/mL}$ | >0.9999 | 0.0257 | 0.9859 | |
| PINK1$^{B9}$ vs. PINK1$^{B9}$/lev/pro$^{500mg/mL}$ | 0.0005 | 0.0004 | 0.1343 | |
| PINK1$^{B9}$/lev vs. PINK1$^{B9}$/prop | 0.1711 | <0.0001 | 0.001 | |
| PINK1$^{B9}$/lev vs. PINK1$^{B9}$/lev/pro$^{250mg/mL}$ | 0.3195 | 0.0002 | 0.6314 | |

| | | | |
|---|---|---|---|
| PINK1$^{B9}$/lev vs. PINK1$^{B9}$/lev/pro$^{500mg/mL}$ | <0.0001 | <0.0001 | 0.0035 |
| PINK1$^{B9}$/prop vs. PINK1$^{B9}$/lev/pro$^{250mg/mL}$ | 0.9976 | <0.0001 | 0.0126 |
| PINK1$^{B9}$/prop vs. PINK1$^{B9}$/lev/pro$^{500mg/mL}$ | 0.0006 | 0.0004 | 0.9647 |
| PINK1$^{B9}$/lev/pro$^{250mg/mL}$ vs. PINK1$^{B9}$/lev/pro$^{500mg/mL}$ | 0.0003 | 0.1711 | 0.0475 |

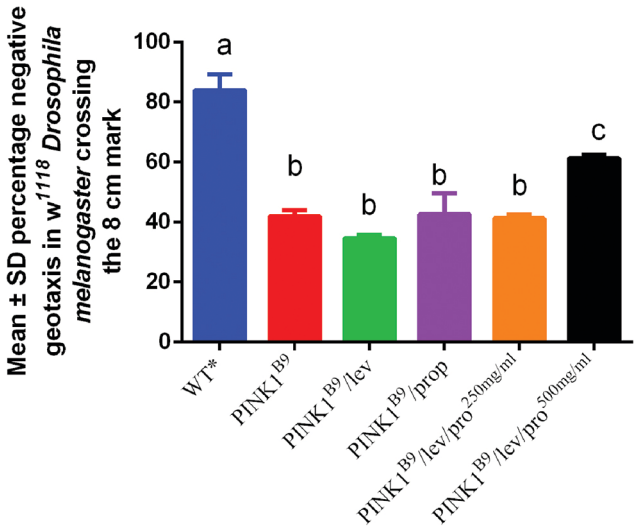


**Figure 1:** Propolis extract protects PINK1$^{B9}$ *Drosophila melanogaster* flies from motor impairments caused by the 21 days of levodopa therapy.
**Key:** Experimental groups WT = wildtype (W1118), PINK1 = mutant drosophila with parkinsonism while pro = propolis. Significant differences are represented by different superscripts a, b, c.

## Synergistic action of propolis and levodopa on DPPH and hydrogen peroxide scavenging activity in *Drosophila melanogaster*

WT flies (W$^{1118*}$) showed the highest levels on catalase in comparison to the mutant flies without any treatment (Figure 2(a)). PINK1$^{B9}$ flies under levodopa for 21 days showed significantly lower catalase activity compared to untreated PINK1$^{B9}$ flies demonstrating the oxidative effects of levodopa therapy alone. In addition, a combination of propolis with levodopa was associated with significant (P<0.05) dose-dependent increase in catalase activity and the effective concentration identified was a combination of levodopa and propolis at 500 mg/mL (Table 2). Furthermore, hydrogen peroxide scavenging activity by DPHH was highest in the 500 mg/mL levodopa-propolis combination in comparison to the 250 mg/mL demonstrating that effects are dose dependent. There were no significant differences between propolis administered alone and the effects of the combined therapy at 500 mg/mL as well as the wild type (Figure 2(b) and Table 2) showing that synergistic effects are due to propolis administration and these help restore tissue function as in the normal.

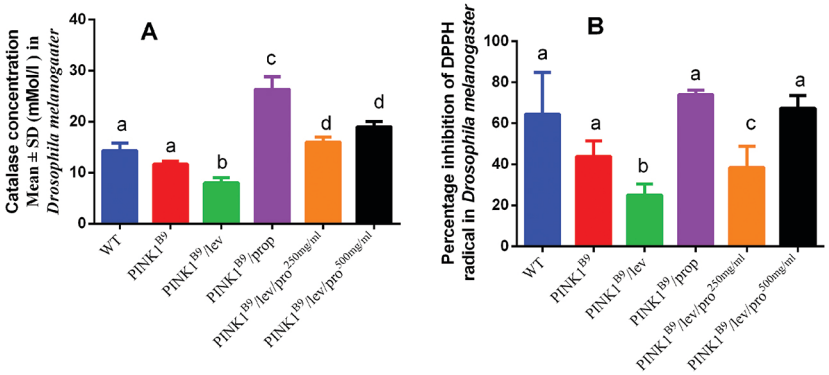

**Figure 2:** Effects of propolis and levodopa therapy on antioxidant activity in *Drosophila* tissue. (A); depicts the plotted means of catalase activity within the blended tissues of flies from all the 6 groups conducted by a method adapted from Iwase *et al.*, 2013; (B) depicts the plotted means of free radical scavenging activity within the tissues of flies from all the six groups using DPPH.
Experimental groups WT = wildtype (W1118), PINK1 = mutant drosophila with Parkinsonism while pro = propolis. Significant differences are represented by different superscripts a, b, c, d.

### Propolis improved the Lifespan in *Drosophila melanogaster*

The study showed that lifespan was highest in the wild-type flies i. e. flies free of Parkinsonism symptoms and PINK1$^{B9}$ flies had significantly shortest lifespan (Figure 3). Propolis alone extended the lifespan in Parkinson flies better than either combination; however 500 mg/mL was more effective in extending lifespan than 250 mg/mg combination of propolis and levodopa.

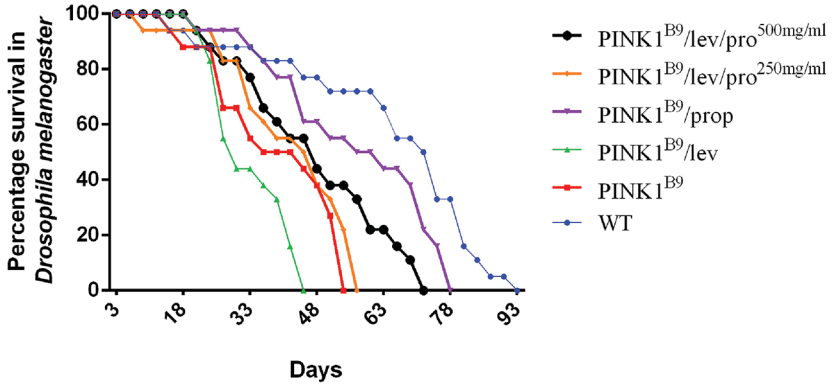


**Figure 3:** Lifespan performance in Drosophila melanogaster following propolis diet.

## Discussion

The study showed that the administration of ethanolic extract of propolis (500 mg/mL) with levodopa (250 mg/kg) not only significantly prevented the motor impairment which has been associated with the long-term use of levodopa but also improved motor function (Figure 1 and Table 2). This was important since Parkinson's disease which is associated with degeneration of dopaminergic receptors is associated with motor dysfunction demonstrating the relevance of the current study [1–5] especially for developing countries where chemotherapeutical options are often limited due to infrastructure challenges, leading to the promotion of alternative medicine. Since propolis contains phenolic hydroxyls [34–36], the observed physiological benefits in *Drosophila melanogaster* are associated with its phytochemical composition. In the study, evidence on the ability to use propolis to manage neurodegeneration following long-term use of levodopa is provided and this was in agreement with previous findings [35, 37]. Since the Parkinsonism phenotype used in this study of PINK1 is associated with mitochondrial function [34, 38], findings in this study offer an insight on the role of propolis in oxidative phosphorylation and hypothesizing the role of phenols in selectively modulating tissue respiration and this was in agreement with previous findings [39–41].

The study also showed that propolis in combination with levodopa effectively increased catalase and reduced concentrations of reactive oxygen species in *Drosophila melanogaster* (Figure 2(a)–(b)). Since levodopa metabolism has been associated with reactive oxygen species [15, 16], findings in the study offer a novel option for use in integrative medicine i. e. use of levodopa and propolis to minimize on the side effects of levodopa therapy. This is important since PD treatment primarily involves replacement of dopamine with levodopa [12, 13], findings in the study revalidate the therapeutical advantages of propolis, thus lower the therapeutical index and would reduce on the incidence of toxicities associated with high levodopa therapies. This would subsequently lead to decreased morbidities and mortalities once findings are translated to humans [18]. In addition, propolis alone is presented as a novel candidate to guide drug discovery due to its superior advantages than the combined therapy at low concentrations (Figure 2 and Table 2). This is important since Parkinson disease is associated with neural damage following excessive accumulation of reactive oxygen species [42–44].

A low survival rate was characterized with all flies having Parkinsonism (Figure 3) due to defective coordination of the skeletomuscular system, leading to decreased ability to look for food, starvation and death (Figure

1). These findings are in agreement with several studies in *Drosophila melanogaster*, demonstrating the importance of an efficient neuromuscular connection to improve on survival [30, 31]. These findings are in agreement with epidemiological studies in humans with Parkinsonism who have been associated with a lower lifespan than healthy individuals [45]. In addition, an extension in lifespan was observed in propolis alone than in either combination with levopodopa at either 250 or 500 mg/mL (Figure 3) due to its higher antioxidant activity (Figure 2). These findings are in agreement with previous findings in which flavones from propolis were associated with an increase in lifespan in *Drosophila melanogaster* [46]. A strong antioxidant activity due to activity of DPPH and catalase especially at high concentrations of propolis (Figure 2(b) and Table 2) offers a rationale for its mechanistic potential. Furthermore, using a combined therapy was found to be more effective at higher concentrations showing that the synergistic associated with propolis are dose dependent. This is because propolis is a natural product rich in several organic and inorganic compounds such as phenols and flavonoids [19, 21]. In this study, protective effects associated with propolis therapy in the management of Parkinsonism is in agreement with previous studies which have shown this to be related to the flavonoid content in propolis [46], thus validating it as a candidate for chemotherapeutical development. An increase in antioxidant activity has already been associated with an increase in lifespan in *Drosophila melanogaster* [30] demonstrating the reliability of the current findings.

## Conclusion

Propolis in combination with levodopa reduces the toxic effects associated with levodopa side effects in Parkinsonism thus validating it as a candidate for therapeutical development. The use of propolis alone in the management of Parkinsonism was associated with superior performance than in combination with the current conventional therapy. Since experiments in this study were only conducted in triplicates, a follow up study focusing on changes in inflammatory markers, secondary messengers and neurotransmitter activity would offer further insights to guide therapy better since these were not investigated in the current study due to infrastructure limitations.

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