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Ameliorative Role of *Plectranthus esculentus* on 4-Vinylcyclohexene Monoepoxide-Induced Oxidative Stress in *Drosophila melanogaster*

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Abstract: *Plectranthus esculentus* is a plant used to relieve pain and digestive disorders. 4-Vinylcyclohexene 1,2-monoepoxide (VCM) is a toxic metabolite of 4-vinylcyclohexene. The lifespan effects of leaf varieties of *P. esculentus* fractions (vat-bebot, MV1) and 2 (vat-riyon, MV2) and their ameliorative effects on VCM-induced oxidative stress were evaluated in *D. melanogaster*. Flies were treated with vehicle (ethanol), Gallic Acid (0.1 mM), MV1 (100 mg/10 diet), MV2 (200 mg/10 diet), VCM (100 μM), (VCM+Gallic Acid), (VCM+MV1) and (VCM+MV2) in the diets for 5 days. The results indicated that MV1 and MV2 reduced the lifespan of flies without affecting the survival of flies after 7 days of treatment. *P. esculentus* restored VCM-induced depletion of total thiol and inhibition of glutathione S-transferase, acetylcholinesterase, and catalase activities ($p < 0.05$). Our results demonstrated that short term consumption of *P. esculentus* is beneficial and that MV1 offered better ameliorative effects than MV2.

Keywords: *Plectranthus esculentus;* Gallic acid; 4-vinylcyclohexene 1,2-monoepoxide; Antioxidants; Oxidative Stress.

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1. Introduction

Plectranthus esculentus is a widely used African perennial dicotyledonous shrub that can grow up to the height of 2 m [1,2]. It is a pleasant-smelling herb possessing soft hairy leaves and four-angled and hairy succulent stems [2]. In Nigeria, it is commonly grown in Plateau and Kaduna states because of its edible tubers [3]. It is used for the treatment of digestive disorders, stomach aches, and the relief of pain [4]. It is also claimed that *P. esculentus* is used to treat cancer [5,6]. The plant also possesses anti-diabetic property [6,7].

4-Vinylcyclohexene-1,2-monoepoxide (VCM) is an ovotoxic metabolite of 4-vinylcyclohexene (VCH). Exposure to VCM has been reported to lead to germ cell destruction in female mice and rats. Furthermore, VCM has been reported to induce oxidative stress by

altering cellular redox homeostasis and also deplete the activities of antioxidant enzymes such as catalase and Glutathione S-transferase, thereby resulting in excessive production of free radicals and the resultant oxidative stress [8].

Antioxidants prevent oxidative stress by terminating oxidative chain reactions by removing free radical intermediates, thereby inhibiting the progression of other oxidative reactions [9,10]. Furthermore, antioxidants have been reported to be useful in the treatment and management of oxidative stress-related diseases [11-13]. Indeed, several medicinal plants have been shown to possess antioxidant properties due to their polyphenolic content [14,15]. For instance, gallic acid is a phenolic compound with proven antioxidant properties [16].

Drosophila melanogaster is an arthropod belonging to the family Drosophilidae; it is a dipteran, i.e., a two-winged insect [8]. Over the years, *D. melanogaster* has gained increasing popularity in experimental biology. Experiments have shown that many of the genes that are important for fly development are critical for all animal development, including humans. Although the morphology of the fly differs distinctly from that of humans, many underlying building blocks and processes are conserved through evolution [17].

Moreover, several antioxidant defense systems present in humans are also found in flies. This has made it reasonable to use *Drosophila* for the study of antioxidant effects of natural products and oxidative stress induced by environmental toxins. *D. melanogaster* possesses enzymes such as superoxide dismutase, catalase, glutathione reductase, and glutathione S-transferase (GST) [18]. Thus, flies are being used to study different diseases in which oxidative stress is implicated. Here, we used VCM to induce oxidative stress and sought to understand if *P. esculentus* would ameliorate its toxic effects using *D. melanogaster* as a model.

2. Materials and Methods

2.1. Chemicals.

Gallic acid, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetylcholine iodide, 5’5’-dithiobis(2-nitrobenzoic acid (DTNB), were purchased from Sigma Chemical Company, St Louis, USA.

2.2. Preparation of fractions.

Two varieties of fresh leaves from *P. esculentus*, locally referred to as ‘vat-bebot’ and ‘vat-riyom’, here symbolized as variety 1, and variety 2 respectively, were taken Heipang farms, Plateau State, Nigeria. They were authenticated by C.D. Gadu, National Root Crop Research Institute, Vom, Plateau State, Nigeria. Voucher specimens of the two varieties were deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Jos. Voucher numbers: UJ/PCG/HSP/17L01c (‘vat-bebot’, variety 1) and UJ/PCG/HSP/17L01b (‘vat-riyom’, variety 2). The leaves were properly washed, sliced, chopped, air-dried, and powdered. The powdered leaves were macerated individually with solvents based on the order of increasing polarity: hexane, ethyl acetate, methanol, and water. The aqueous extracts were then lyophilized, while the other fractions were concentrated *in vacuo* and deposited in a desiccator until used. After *in vitro* antioxidant activity [19], methanol fractions of varieties 1 and 2 (MV1 and MV2) were the most active. Thus, they were used for the present study.

2.3. Drosophila melanogaster stock and culture.

Both genders of *Drosophila melanogaster* (Harwich strain 1-3 days old) used for the study. They were reared and maintained in *Drosophila* Laboratory, Biochemistry Department, University of Ibadan, Nigeria on cornmeal medium mixed with brewer's yeast (1% w/v), agar-agar (1% w/v), and nipagin (preservative, 0.08% v/w) at temperature (23±2°C) under 12 h dark/light cycle.

2.4. Treatment of flies and preparation of samples.

In order to determine the appropriate doses of MV1 and MV2 to be used, longevity and 7 days survival assays were carried out using 1- to 3- days old *D. melanogaster* (both genders). The flies were divided into different groups, each containing five replicates/groups (30 flies/vial), treated with MV1 (50, 100, and 200 mg/10 g Diet) and MV2 (25, 50, 100, and 200 mg/10 g Diet). Daily mortality was recorded and used to determine the lifespan and 7 days survival rate. Thereafter, we selected 100 mg/10 g diet of MV1 and 200 mg/10 g diet of MV2 to ameliorate the toxic effects of VCM (100 mM) after 5 days of treatment. Afterward, flies were anesthetized in ice, weighed, homogenized in 0.1 M phosphate buffer (pH 7.4, a ratio of 1 mg:10 µL), and centrifuged at 4000*g* for 10 minutes at 4°C in a refrigerated centrifuge (Thermo fisher Sorvall Legend Micro 17R (Fresco)). Thereafter, the supernatants obtained were transferred into new labeled Eppendorf tubes and used for the determination of total protein, total thiol (T-SH), as well as catalase, glutathione S-transferase, and acetylcholinesterase activities. We chose 5 days as the treatment period since, at this period, there was no significant mortality of flies compared with control. The VCM dose of 100 µM and a gallic acid dose of 0.1 mM were selected based on previous studies.

2.5. Assessment of biochemical parameters.

2.5.1. Total protein determination.

The Total protein content of the samples was determined using the Bradford method [20]. The reaction mixture contained 75 µl of distilled water, 25 µl of the sample, and 600 µl of Bradford reagent. Bovine Serum Albumin (BSA) was used as a standard protein in plotting the standard calibration curve. The absorbance was read at 595 nm.

2.5.2. Total thiol (T-SH) determination.

The Total thiol content of the samples was determined using the method of Ellman [21]. The reaction mixture contained 510 µl of 0.1 M phosphate buffer, 25 µl of sample 35 µl of distilled water, and 30 µl of DTNB. Absorbance was read at 412 nm after 30 minutes of incubation. The calibration curve was plotted using GSH as standard.

2.5.3. Determination of Glutathione S-transferase (GST) activity.

The GST activity was determined using the method outlined by Habig and Jakoby [22]. The reaction mixture contained 510 µl of solution A (20 ml of 2.5 mM Ethylenediaminetetraacetic acid (EDTA) containing 0.25 M phosphate buffer, pH 7.0, 10.5 ml of distilled water and 500 µl of 0.1 M GSH, 60 µl of a sample, and 30 µl of 25 mM CDNB. Change in absorbance was read at 340 nm for 2 minutes, 10 seconds interval.

2.5.4. Determination of acetylcholinesterase activity.

The Acetylcholinesterase activity was determined according to the method of Ellman et al. [23]. The reaction mixture contained 285 µl of distilled water, 180 µl of 0.1 M phosphate buffer, pH 7.4, 60 µl of DTNB, 15 µl of a sample, and 60 µl of acetylcholine. Change in absorbance was monitored at 423 nm for 3 minutes, 15 seconds interval.

2.5.5. Determination of catalase activity.

Catalase activity was determined according to the method outlined by Aebi [24]. The reaction mixture contained 590 µl of 19 mM Hydrogen peroxide (H_2O_2) and 10 µl of the sample. The change in absorbance corresponding to the rate of clearance of H_2O_2 was monitored at 240 nm for 2 minutes, 10 seconds interval.

2.5.6. Determination of negative geotaxis and rate of emergence of offspring.

The locomotor performance was investigated using the negative geotaxis assay [8,25]. The emergence rate of *D. melanogaster* offspring after exposure to VCM, MV1, and MV2 were carried out as previously described [26].

2.5.7. Statistical analysis.

Statistical analysis was carried out using Graph Pad prism 7. Data were expressed as Mean±Standard deviation. Statistically significant differences were determined using One Way ANOVA with $p < 0.05$ taken to be significant.

3. Results and Discussion

The effect of *Plectranthus esculentus* on the lifespan of *D. melanogaster* is depicted in Fig. 1. The MV1 (50, 100, and 200 mg/10 g diet) decreased the lifespan of flies by 3.8, 24.1, and 24.1%, respectively (Fig. 1A). In addition, MV2 (25, 50, 100 and 200 mg/10 g diet) decreased longevity of flies by 16.1, 17.9, 17.9 and 14.3% respectively (Fig. 1B).

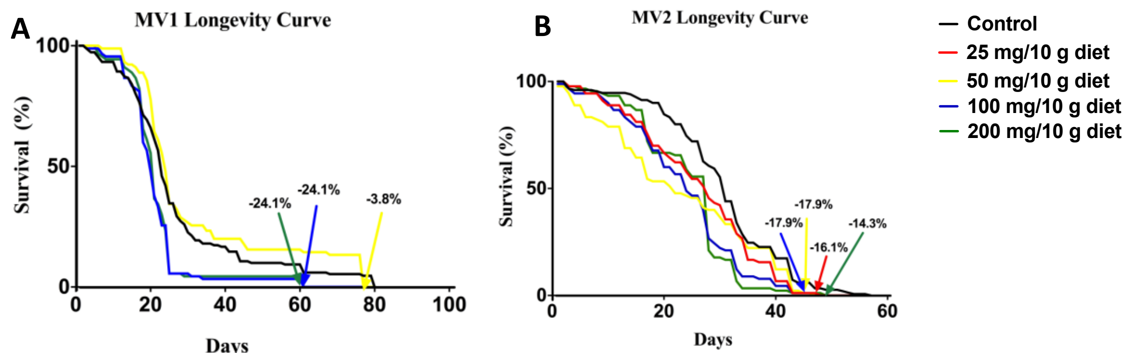


Figure 1. Effects of *Plectranthus esculentus* on the longevity of *D. melanogaster*. (A): Longevity curve of *D. melanogaster* treated with MV1 longevity; (B): Longevity curve of *D. melanogaster* treated with MV2. 30 flies/vial, n=3. MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*).

The 7 days survival assay indicated no significant mortality of flies in each of the MV1- and MV2 -treated flies compared with control (Fig. 2).

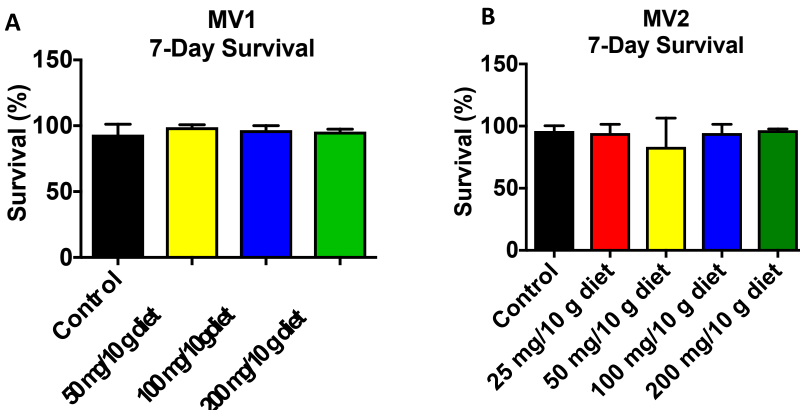


Figure 2. Effects of *Plectranthus esculentus* on the survival rate of *D. melanogaster*. (A): Survival rate of *D. melanogaster* exposed to MV1(0, 50, 100, and 200 mg/10 g Diet) for 7 days; (B) Survival rate of *D. melanogaster* treated with MV2 (0, 25, 50, 100 and 200 mg/10 g Diet) for 7 days. Data are expressed as Mean $\pm$ Standard deviation with no significant difference compared with control, $p > 0.05$, 30 flies/vial, n=3.MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*).

The effects of *P. esculentus* on VCM-induced behavioral deficit and inhibition of acetylcholinesterase activity are shown in Fig. 3. MV1 restored VCM-induced reduction of negative geotaxis and inhibition of acetylcholinesterase in flies treated for 5 days ($p < 0.05$).

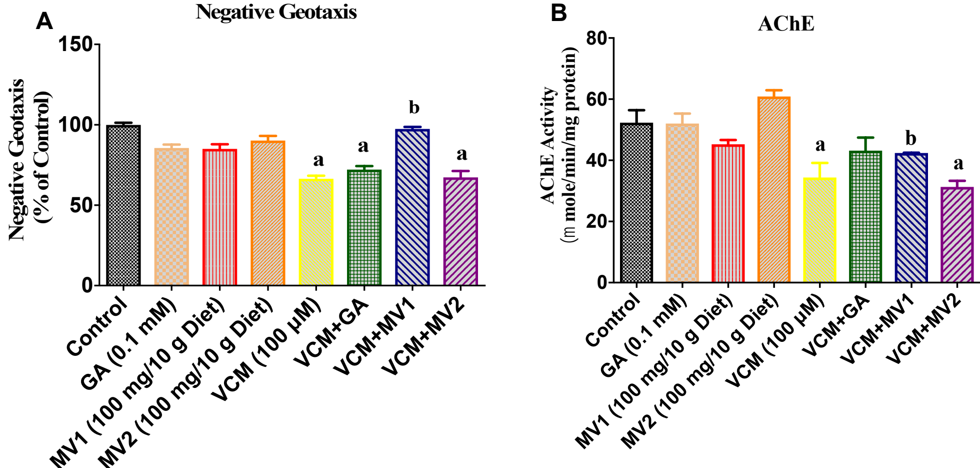


Figure 3. Effects of *P. esculentus and* VCM on negative geotaxis and acetylcholinesterase activity of *D. melanogaster*. (A): Effects of MV1 (100 mg/10 g Diet)and MV2 (100 mg/10 g Diet) on VCM-induced behavioral deficit; (B)Effects of MV1 (100 mg/10 g Diet) and MV2 (100 mg/10 g Diet) on VCM-induced inhibition of AChE activity in *D. melanogaster* after treatment for 5 days. Data are expressed as Mean $\pm$ Standard deviation. 'indicates significant difference compared with control. 'b' indicates a significant difference compared with VCM-treated flies, $p < 0.05$, 30 flies/vial, n=3. MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*); VCM: 4-Vinylcyclohexene 1,2-monoepoxide.

The effects of *Plectranthus esculentus* on total thiol content and glutathione S-transferase as well as catalase activity in VCM-induced toxicity, are shown in Figs. 4 and 5, respectively. Flies treated with VCM caused significant depletion of total thiol content (Fig. 4A) and inhibition of GST (Fig. 4 B) and catalase (Fig 5) activities ($p < 0.05$). However, the co-treatment of flies with MV1 restored VCM-induced reduction of T-SH and GST activity.

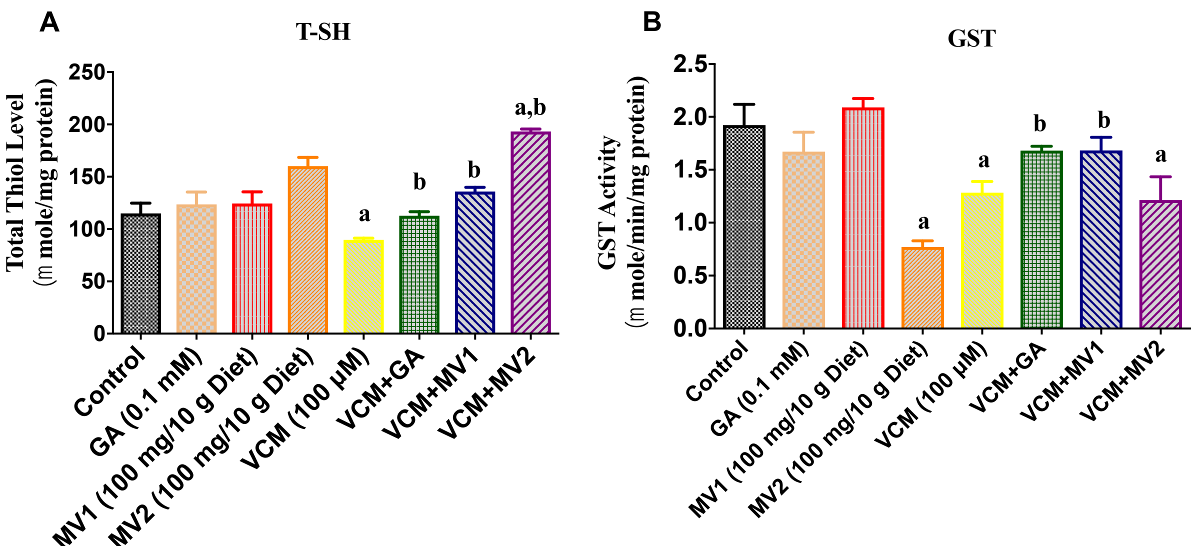


Figure 4. Effects of *P. esculentus and* VCM on T-SH level and GST activity of *D. melanogaster*. (A): Effects of MV1 (100 mg/10 g Diet) and MV2 (100 mg/10 g Diet) on VCM-induced depletion of T-SH level; (B) Effects of MV1 (100 mg/10 g Diet) and MV2 (100 mg/10 g Diet) on VCM-induced inhibition of GST activity in *D. melanogaster* after treatment for 5 days. Data are expressed as Mean ± Standard deviation. 'a' indicates significant difference compared with control. 'b' indicates significant difference compared with VCM-treated flies at $p < 0.05$, 30 flies/vial, n=3. MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*); VCM: 4-Vinylcyclohexene 1,2-monoepoxide; T-SH: Total thiol; GST: Glutathione S-transferase.

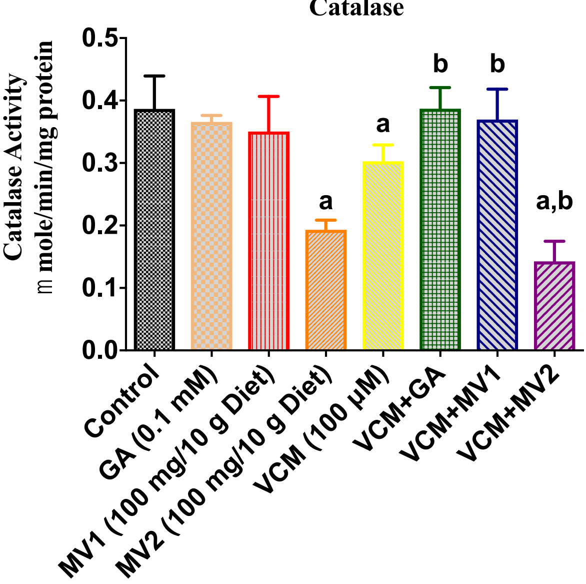


Figure 5. Effects of *P. esculentus and* VCM on T-SH level and catalase activity of *D. melanogaster*. Effects of MV1 (100 mg/10 g Diet) and MV2 (100 mg/10 g Diet) on VCM-induced inhibition of catalase activity in *D. melanogaster* after treatment for 5 days. Data are expressed as Mean ± Standard deviation. 'a' indicates significant difference compared with control. 'b' indicates a significant difference compared with VCM-treated flies at $p < 0.05$, 30 flies/vial, n=3. MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*); VCM: 4-Vinylcyclohexene 1,2-monoepoxide.

The emergence rate of flies after treatment with VCM and *P. esculentus* was depicted in Fig. 6. VCM significantly reduced offspring emergence rate after 5 days of treatment. However, MV1 and MV2 improved offspring emergence but not up to the threshold of the control flies. Gallic acid, used as a standard drug, offered better protection than MV1 and MV2 in this respect.

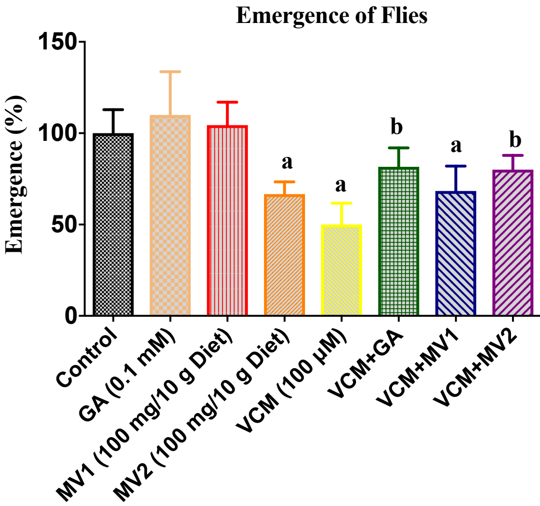


Figure 6. Effects of *P. esculentus and* VCM on the Emergence rate of *D. melanogaster*. Effects of MV1 (100 mg/10 g Diet) and MV2 (100 mg/10 g Diet) on VCM-induced reduction emergence rate of *D. melanogaster* after treatment for 5 days. Data are expressed as Mean ± Standard deviation. 'a' indicates significant difference compared with control. 'b' indicates a significant difference compared with VCM-treated flies at $p < 0.05$, 30 flies/vial, n=3. MV1: methanol fraction of 'vat-bebot' variety of *P. esculentus*); MV2 (methanol fraction of 'vat-riyom' variety of *P. esculentus*); VCM: 4-Vinylcyclohexene 1,2-monoepoxide.

Oxidative stress can occur when oxidants overwhelm the antioxidant defense system in living organisms [27-29]. The balance can be restored through the use of extracellular antioxidants, which are able to boost the antioxidant defense. In this context, synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and trolox are being used [28]. However, there are restrictions currently on their usage due to their reported carcinogenic effects [28]. There is, therefore, an ongoing search for medicinal plants rich in antioxidants with little to no side-effects in the management of human diseases. Here, we investigated the ameliorative role of two leaves fractions of *P. esculentus* on VCM-induced oxidative damage in a non-target organism, *D. melanogaster.* Indeed, VCM, a metabolite formed by the epoxidation of 4-vinylcyclohexene in the liver by P450 isoform (CYP 2A) [8], induced oxidative stress in *D. melanogaster* via the disruption of the redox balance in the flies.

A balanced redox state is essential for normal physiological function and cellular metabolism (30,31). The total thiol status is considered a major plasma antioxidant *in vivo* [32]. In biological systems, protein thiols are most sensitive to fluctuations in the cellular redox state (32-34). Glutathione is the major intracellular protein thiol, and it is principally in the reduced form while excellular proteins, on the other hand, are predominantly S-cysteinylated, and it is mainly in the disulfide form [32]. The ameliorative effect of all the samples, including the standard gallic acid on total thiols level of the flies challenged with VCM is indicative of its antioxidative properties as it has been reported by Solakan et al. (35) that a reduced thiol concentration increases when oxidative stress occurs.

Glutathione S-transferases (GSTs) are a superfamily of multifunctional enzymes involved in the cellular detoxification of several xenobiotics [33,36,37]. GSTs carry out their detoxification function by catalyzing the nucleophilic attack of glutathione on the electrophilic centers of the substrates, and thus play a very important role against carcinogens, therapeutic drugs, and various types of cellular oxidative damage [36-40]. Independent administration of the standard gallic acid led to an activity that was almost the same as that of the VCM-treated group. Possibly, the administration of this standard antioxidant compound in healthy flies led

to a decrease in the synthesis or uptake of the enzyme in an attempt to maintain the total antioxidant potential of the cell according to [36]

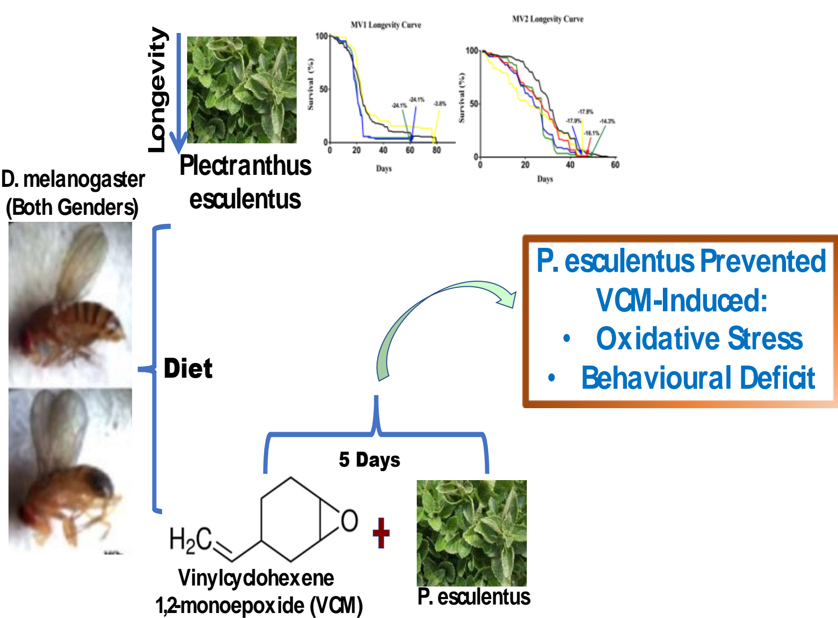
The neurotransmitter acetylcholine (AChE) is secreted by the postganglionic cholinergic neurons, and it allows for the transmission of nerve impulses across the synapse [41]. AChE, therefore, terminates synaptic transmission, thereby preventing continuous transmission at nerve endings; hence it is essential for the normal functioning of the central and peripheral nervous system [41-44]. Flies co-administered with VCM and MV1 had a significant increase in AChE activity compared with the VCM-treated group, thus depicting MV1's ability to successfully restore AChE activity.

Negative geotaxis assay in *Drosophila* is based on the fly's natural behavior of moving opposite to the gravitational vector [45]. This assay is used to measure behavioral changes in *Drosophila* in response to genetic or environmental factors, including exposure to toxic substances, aging, and neurodegenerative disorders (46-48). In this study, MV1 ameliorated VCM-induced behavioral deficit in the flies by restoring the flies' climbing ability to rates comparable to control.

Oxidative stress has been implicated in male and female infertility (49-51) as free radicals can affect the embryo or sperm in its microenvironment (52). The reduction in the eclosion rate of the flies treated with VCM is indicative of this effect. It can, therefore, be concluded that oxidative stress can greatly affect the reproductive cycle negatively [53-56]. However, MV1 and MV2, as well as the standard gallic acid, ameliorated VCM-induced decrease in the eclosion rate, thus demonstrating that its antioxidative properties have the potential to improve reproduction.

4. Conclusions

Overall, VCM-induced toxicity was via oxidative stress and behavioral deficit in *D. melanogaster*. However, *P. esculentus,* especially the methanol extract of 'bebot' leaves (MV1) prevented VCM-induced toxicity (Scheme 1). Our finding also revealed that MV2 showed minimal ameliorative effects on VCM-induced toxicity compared with MV1.



Scheme 1. Ameliorative mechanism of *P. esculentus* against VCM-induced toxicity in *D. melanogaster*.

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Conflicts of Interest

The authors declare no conflict of interest.

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