Original Research Article

Phytochemical Constituents and Toxicity of the Ethanol Extract of *Ricinus communis* (L.) in *Drosophila melanogaster*

ABSTRACT

The study aimed to evaluate the toxic ability of the ethanol extract of *Ricinus communis* (L.) in *Drosophila melanogaster* fruit fly model. The toxicity was determined through different criteria, including the ability to cause harmful effects on second instar larvae, reproduction, growth and development, and the movement ability of fruit flies. The results showed that the ethanol extract of *Ricinus communis* expressed its high toxicity against 2nd instar larvae of *Drosophila melanogaste* with the LD_{50} value of 64.63 mg/mL. In addition, *Ricinus communis* extract reduced the growth rate, reproduction and decreased the movement ability of *Drosophila melanogaster*. The total flavonoid and polyphenol content of the ethanol extract of *Ricinus communis* were 338.26 mgQE/g extract and 160.43 mgGAE/g extract, respectively. These findings contribute to confirming the toxic properties of ethanol extract of *Ricinus communis* and their potential use in preventing and controlling pest.

Keywords: Drosophila melanogaster, flavonoid, polyphenol, *Ricinus communis*, toxicity

1. INTRODUCTION

Botanical pesticides are effective in pests controlling and management. They are inexpensive and easily biodegraded. In addition, botanical pesticides have various modes of action due to the phytochemical composition in different plants. They are known to be available with low toxicity to non-target organisms (Rizvi *et al*., 2016; Rizvi *et al*., 2018).The Mekong Delta is famous for its diverse and abundant plant source. Many species of plants with toxic effects and insect antagonism have been used by many Vietnamese people for many years. *Ricinus communis* (L.) (*R. communis*) is commonly known as a species of flowering plant in the family Euphorbiaceae, which grows wild and is grown in many tropical regions. The ethanol extract of *R. communis* leaves has been shown to have hepatoprotective effects in rats (Gupta *et al.,* 2006). *R. communis* seed pod showed its effects on the central nervous system in rats at low doses. Antihistamine and anti-inflammatory properties were found in the ethanol extract of *R. communis* root bark. It also inhibited the respiratory chain reaction of mitochondria. The leaves, roots, and seed oils of *R. communis* have medicinal potential, including treating inflammation, liver disorders, hypoglycemia, and laxatives (Zarai *et al.,* 2012).

Fruit fly, with its scientific name of *Drosophila melanogaster* (*D. melanogaster*), is well-known as a model organism in toxicological studies and in testing pesticide activity (Rodrigues *et al.*, 2021). Although *D. melanogaster* is not considered an agricultural pest as it does not damage crops on a large scale, it still affects various fruits, mainly guava and bananas. Therefore, it is necessary to study the toxicity of *R. communis* extract in a fruit fly model to demonstrate its potential role in insect pest control and management.

2. MATERIAL AND METHODS

2.1. Experimental materials

Experimental materials: Leaf and stem of *R. communis* collected in Can Tho city was taken to remove the damaged parts. It was then washed, chopped, and dried before grinding into powder.

Experimental subjects: Wild fruit fly *D. melanogaster* strain Canton S (CS) was supplied from the Biofunctional Chemistry laboratory (Kyoto Institute of Technology, Japan). *Chemicals:* Ethanol 96^{o}, distilled water, Pertox, gallic acid, quercetin, Folin-Ciocalteu, $AlCl_3$, $NaNO_2$, NaOH (China) and propionic acid and sodium benzoate (India) and some other chemicals.

2.2. Methods

2.2.1. Sample extraction

Preparation of ethanol extract: After milling, the sample was placed in a cloth bag and soaked in sufficient ethanol (96°). After 24 hours for 3 times of soaking, the solution in the soaking vessel was filtered through the filter paper to remove the powder residue. It was then evaporated to recover the solvent. The extract was taken for solvent evaporation to obtain the ethanol extract.

2.2.2. Qualification and quantification of chemical composition

Qualification of natural compounds: The chemical compositions of *R. communis* extract, including alkaloids, flavonoids, phenolics, saponins, and tannins, were determined by qualitative methods of the natural compounds (Phung, 2007).

Quantification of total polyphenol content: The polyphenol content was determined by the method of Singleton (1999) with some corrections. The reaction mixture consisting of 250 µL of extract, 250 µL of water, and 250 µL of Follin-Ciocalteu reagent was mixed. Then, 250 µL of Na_2CO_3 10% was added and incubated for 30 min at $40^{o}C$ in a thermostat. The spectral absorbance of the reaction mixture was measured at 765 nm. Gallic acid was used as a positive control to create the standard curve equation. The

total polyphenol content of each extract was calculated using the standard curve equation of gallic acid, and the results were expressed in milligrams of gallic acid equivalent (GAE) per gram of the extract (mg GAE/g extract).

Quantification of total flavonoid content: Total flavonoid content was determined by the $AlCl_3$ colorimetric method of Bag *et al.* (2015) with some corrections. The reaction mixture consisting of 200 µL of extract or standard at the investigated concentration was mixed in 200 µL of distilled water to react with 40 µL of $NaNO_2$ 5% and shaken well, after that kept stand for 5 min. After 5 min, continue to add 40 µL $AlCl_3$ 10% to the mixture and shake well. The reaction mixture was incubated for 6 min. 400 µL of NaOH 1 M and distilled water were added to the mixture to make up a volume of 1 mL. The reaction mixture was measured absorbance spectrophotometrically at 510 nm. Quercetin was used as a positive control. The total flavonoid content in the extract was determined based on the standard curve equation of quercetin and the results were expressed in milligrams of quercetin equivalent (QE) per gram of the extract (mg QE/g extract)

2.2.3. Experimental methods on fruit flies

Investigation of the toxic ability on the second instar larvae: The effect of *R. communis* extract on the mortality of fruit fly larvae was investigated according to the method of Riaz *et al*. (2018) with some corrections. In this experiment, the second instar larvae of fruit flies were used to determine the toxicity potential of *R. communis* extracts. The composition of feed medium in the treatment was supplemented with the extract at different concentrations, including 30, 60, 90, 120, and 150 mg/mL of feed. The pesticide Pertox was used as a positive control. The investigated concentration was based on the research method of Marcus and Fiumera (2016). 40 second instar larvae were selected to put in each feed vial. Each treatment was repeated 5 times (5 vials). The monitoring criteria in this experiment included the percentage of larvae that died after 7 days of survey, the lethal concentration of 50% (LD_{50}), and the total number of flies that emerged after 10 days of monitoring.

Investigation on the growth and development of fruit flies: The effect of *R. communis* extracts on the growth and development of fruit flies was identified based on the research method of Chowański *et al.* (2018) with some corrections. 6 males and 4 females newly emerged within 2 days and have not yet mated were selected to mate for 24 hours. The parent flies were then removed, whereas the eggs were kept so that they could grow in the test medium. The results recorded included the number of larvae pupating, weight of 3^{rd} instar larvae and pupae, total number of flies hatched, % of flies

with different phenotypes present in the treatments. The adult flies in this survey were designated as the "P" generation.

Investigation on fertility: The effect of *R. communis* extract on fertility was determined based on the method of Ferdenache *et al.* (2019) with some corrections. 4 female and 6 male flies in the "P" generation were mated for 24 hours. The results recorded in the F1 generation included the number of larvae pupating and the total number of flies hatched.

Investigation on the movement ability: The identification of mobility of fruit flies was based on the method of Valéria *et al.* (2014) with some corrections. 20 male flies of the "P" generation were selected to anesthetize with CO_2 and transferred them to plastic tubes marked with a 6 cm line from the bottom of the test tube. After 30 minutes, the flies were completely awake and acclimatized to the conditions in the test tubes. Tap the tubes of the treatments simultaneously so that the flies completely fall to the bottom of the tubes. Recorded the number of flies moving over the preset 6 cm line in 10 seconds. Each treatment was repeated 5 times.

2.3. Statistical analysis of data

Experimental data are averaged by Excel 2013. One-way analysis of variance (One-way ANOVA) and Tukey's test at 5% significance level to compare the data collected between treatments using the Minitab software of version 16.

3. RESULTS AND DISCUSSION

3.1. Qualification and quantitation of chemical composition

3.1.1. Qualitative result

The qualitative result showed that the chemical composition of *R. communis* extracts had the presence of biologically active compounds. The result is expressed in Table 1.

Table 1. Qualitative result of natural compounds present in *R. Communis* extracts

Extract	Alkaloid	Flavonoid	Tannin	Saponin	Phenolic
R. communis	+	+	+	-	+

Note: (+): present; (-): absent

The result of Table 1 shows that the chemical composition of *R. communis* contains biologically active compounds such as alkaloids, phenolics, flavonoids, and tannins. The result is completely consistent with previous studies demonstrating the role of these

groups of compounds in antagonizing insects. Secondary defensive metabolites could be stored as inactive or produced in response to insect or bacterial attacks. Bioactive compounds such as phenolics, alkaloids, benzoxazinoides, cyanogenic glucosides, glucosinolates, and terpenoids have been shown to be effective for insect pest management (Fürstenberg-Hägg *et al.*, 2013). In the study of Riaz *et al.* (2018) on the toxicity, chemical composition, and enzyme inhibition of weed species towards *D. melanogaster*, the research result on chemical composition analysis reported that the weed species such as *Euphorbia prostrata, Parthenium hysterophorus, Fumaria indica, Chenopodium murale,* and *Azadirachta indica* with insect resistance (*D. melanogaster*) containing compounds such as flavonoids, saponins, tannins, steroids, cardiac glycosides, alkaloids, anthraquinones, and terpenoids. This larvicidal activity was due to biologically active substances such as alkaloids, tannins, lignin, saponins, gallic acid, flavones, and kaempferol that were detected in a previous study. Thus, *R. communis* extract contains groups of compounds with biological activities of insect resistance that have been studied and proven in previous studies.

3.1.2. Quantitative result

Flavonoids and polyphenols are two groups of compounds with many potential biological activities used to control and manage insect pests (Tlak and Dar, 2021). Therefore, quantification of total flavonoids and total polyphenols is important to assess the insect resistance of *R. communis*. The content of polyphenols and flavonoids present in the extract of *R. communis* was determined based on the linear regression equation of the standard substance of gallic acid ($y = 0.0778x + 0.0255$, $R^2 = 0.9975$) and quercetin ($y = 0.0046x + 0.0218$, $R^2 = 0.9832$). The quantitative result recorded that *R. communis* extract contained a 160.43 mgGAE/g polyphenol compound and flavonoids of 338.26 mgQE/g extract. Recently, a study has identified that the extract from *Zea mays* is rich in polyphenol and negatively affects the growth, development, and adult body characteristics in *Manduca sexta* L., a specialized insect pest in the family Solanaceae (Tayal *et al.*, 2020). Consistent with these results, many documents demonstrated that different groups of polyphenols collectively protected most plant species against a variety of insect pests. For instance, chlorogenic acid in *Dendranthema grandiflora* (Ramat.) protected the effect against *Frankliniella mysidentalis*; pisatin (flavonoid) against *Acyrthosiphon pisum* Harris; and ferulic acid in rice against *Nilaparvata lugens* Stal (Leiss *et al.*, 2009; Morkunas *et al.*, 2015; Yang *et al.*, 2017). Harborne and Williams (2000) also proved that flavonoids keep an important function in the protection of plants against plant-feeding insects and herbivores. Therefore, it can be assumed that polyphenols and flavonoids play an important role in resistance to insect pests.

3.2. Experimental results on the fruit fly model

3.2.1. Investigation results of toxicity on the second stage of fruit fly larvae

The experiment used *R. communis* extract at different concentration, including 30, 60, 90, 120, and 150 mg/mL. The control treatment used standard feed without extract. The results are shown in Figure 1.

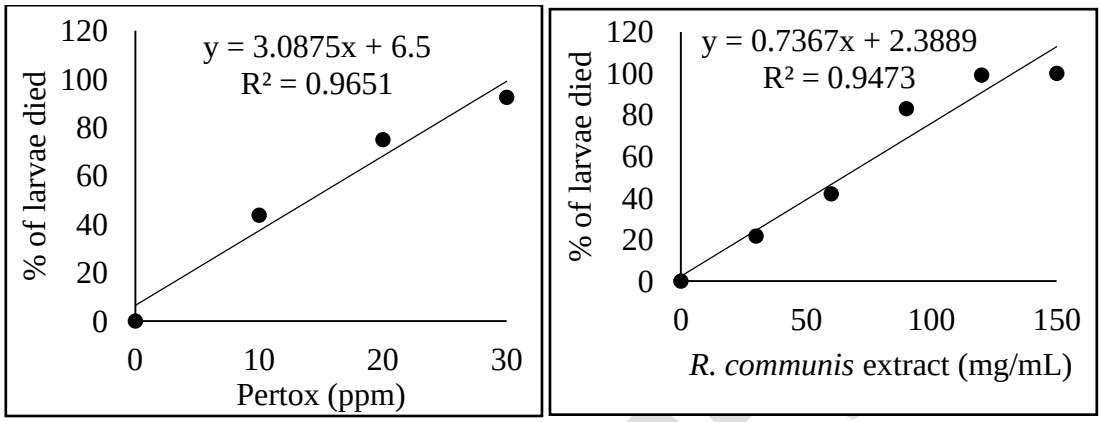

Figure 1. Percentage of larvae died corresponding to each concentration of Pertox and *R. communis* extract.

In the condition of adding chemical insecticide Pertox, with the same number of larvae in each treatment, after 7 days of testing, the results showed that at a concentration of 30 ppm Pertox insecticide added to the feed, the highest percentage of dead larvae was 92.5%, LD_{50} value of 13.15 ppm ($y = 3.0875x + 6.5$; $R^2 = 0.9651$). This result proved that the insecticide Pertox was highly effective in causing death to fruit fly larvae (Figure 1).

In the addition of *R. communis* extract, the percentage of dead larvae was statistically significantly different from the control at all concentrations. The 50% lethal value (LD_{50}) of *R. communis* extract was determined through a linear regression equation ($y = 0.7367x + 2.3889$; $R^2 = 0.9473$) with LD_{50} value of 64.63 mg/mL. Notably, at the high concentration of 150 mg/mL, the extract had the highest toxic effect, with the number of larvae dying after 7 days of investigating up to 100% (Figure 1). The findings showed that *R. communis* extract strongly affected mortality in the second instar larvae of *D. melanogaster* fruit fly. Previous studies have demonstrated that *R. communis* leaf extract was highly effective in killing the third instar larvae of *Aedes albopictus* with LC_{50} and LC_{90} values of 149.58 ppm and 268.93ppm, respectively (Waris *et al.*, 2020). In a study by Phowichit *et al.* (2008) with the aim of evaluating the insecticidal activity of the leaf extract of *Jatropha gossypifolia* (L.) - a species of plant in the Castoraceae family, against *S. litura* and the activity of detoxifying enzymes. *In vitro* bioassays have

shown that the treatment of the second instar larvae of *Spodoptera litura* by immersing in the extracts from the aged leaves of *Jatropha gossypifolia* (L.) at concentrations of 3000 - 10000 ppm has been found to have significant toxicity with an LC_{50} value of 6.56 mg/mL at 24 h post exposure. Simultaneously, *S. litura* larvae that survived after treatment showed a significant reduction in carboxylesterase and glutathione-s-transferase activities. This extract showed the potent insecticidal activity and ability to act as an alternative insecticide against *Spodoptera litura*. An other study of Jaleel *et al.* (2020) were evaluated the repellency of four botanicals (*Seriphidium brevifolium, Piper nigrum, Azadirachta indica* and quercetin) in acetone dilutions against the *B. dorsalis* and *B.* correcta on mangoes. The result showed that the number of visits after 24–48 h, oviposition punctures, and pupae made by both species were lower on the treated mangoes in comparison to untreated mangoes. S. *brevifolium, P. nigrum, A. indica* and quercetin have significantly reduced the visits, ovipositional punctures, and pupae of both species.

3.2.2. Investigation on the reproductive and developmental ability of fruit flies

Plants are sometimes used as pest control agents as growth regulators rather than direct toxic pesticides because they inhibit insect growth and development. (Pavela, 2008). Most plants act as feeding inhibitors in various respects, such as food repellents or feed inhibitors and other substances involved in the inhibition process of growth, egg production, and development. *R. communis* extract with a concentration of 20 mg/mL used to investigate of feed showed its ability to affect the growth and development of fruit flies. The results are recorded in Table 2.

Table 2. Survey results on the effect of extracts on the reproductive and developmental ability of fruit flies

Treatment	Growth and development				Reproduction	
	Number of pupae	Number of flies hatched	Time of emerging	% of flies having deformed phenotypes	Number of pupae	Number of flies hatched
Control	100.67^{a}	98.00^{a}	10	0.00^{b}	99.67^{a}	95.00^{a}
R. communis (20 mg/mL)	24.67^{b}	23.33^{b}	14	24.44^{a}	16.00^{b}	7.00^{bc}

Note: Means ± standard deviations with different letters in the same column represent statistically significant differences at the significance level of 5% using Tukey's test.

The study results in Table 2 shows that the *R. communis* extract is toxic and affects the growth and development of fruit fly larvae and pupae. The number of larvae pupated in the medium supplemented with extract was 4.08 times lower than that of the control treatment. Monitoring the number of flies hatched from larvae showed that the extract also affected this stage. The number of pupation larvae that were hatched with a low percentage reared in the medium supplemented with extract was 4.2 times lower than that of the control treatment. The life cycle of fruit flies in the treatments containing the extract was also different from that of the control. In the treatment with the ethanol extract of *R. communis*, the life cycle lasted for 14 days that was 4 days slower than the control of 10 days. Research by Chowański *et al.* (2018) on the insecticidal properties of *Solanum nigrum* and *Armoracia rusticana* extracts on the reproduction and development of *D. melanogaster*, the results also demonstrated that *Solanum nigrum* and *Armoracia rusticana* extracts both reduced the number of pupae and total number of flies emerged compared with the control treatment. In another study, the eggs of *Spodoptera ridgiperda* died at a rate of 97.7% after only one day of exposure to the extracts of *Lychnophora ericoides* and *Trichogonia velvetosa*. Therefore, only 2.3% of eggs hatched, a very low percentage to sustain a population could cause damage (Tavarez *et al.*, 2009).

In addition, the study also recorded different phenotypes in the "F1" generation of flies raised in the diet supplementing with the extract. The differential phenotypic ratio in the extract treatment accounted for 24.44% of the total number of flies that emerged (Table 2). The distinct phenotypes were mainly in the wings of fruit flies, causing their wings to stick together, reducing moving function (Figure 2). This result is consistent with the previous study by Sosa *et al.* (2018), which showed that pupae under treatment with *V. nebularum* extract had malformations in their thorax and abdomen, some larvae – pupae had incomplete molting; and some remnants had wing defects in the adult stage, resulting in an inability to mate.

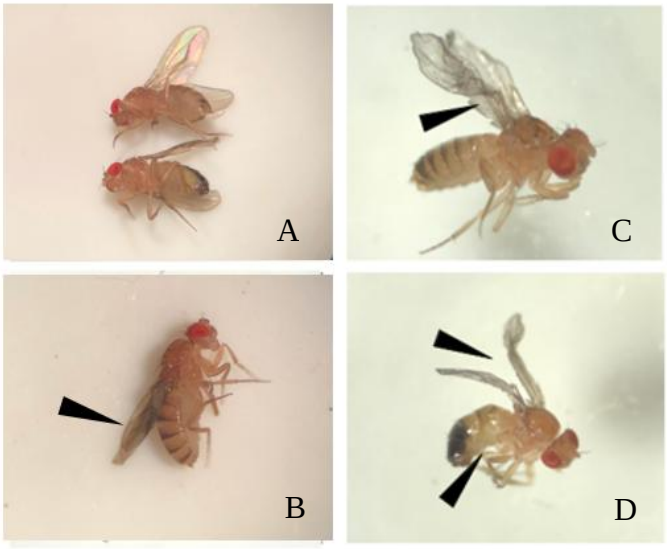

Figure 2. Normal and differential phenotypes of fruit fly

(A) normal wing (top) and deformed wing (bottom); (B), (C) deformed wing; (D) deformed abdomen and reduction in size

The weight of larvae and pupae is an indicator reflecting the growth and development of fruit flies. The effect of the extract was determined based on the weight criteria for larvae and pupae in the "P" generation.

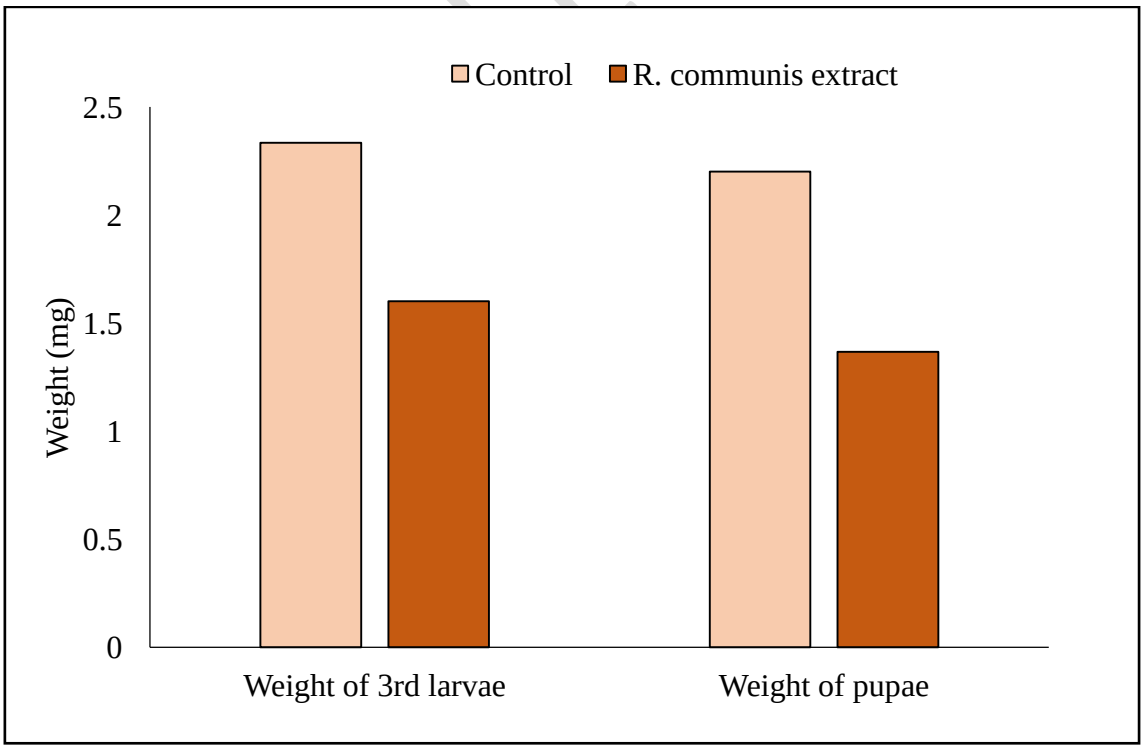

Figure 3. Weight of larvae and pupae

The survey results (Figure 3) showed that the weight of larvae in the treatment containing *R. communis* extract tended to be lighter than that of the control treatment. The weight of the larvae weighed after 7 days of the survey in the medium containing the extract was 1.60±0.10 mg, which decreased and was significantly different from the control at 2.33±0.15 mg. Similarly, the pupae weight in the extract medium obtained 1.37±0.06 mg that also decreased and had a statistically significant difference compared with the control at 2.2±0.1 mg. The extract had the ability to inhibit the feed digestion and absorption during the development of the larvae, causing weight loss, which is one of the causes of the poor development of fruit fly larvae, unable to pupate, and increased mortality (Vinuela *et al.*, 2000). The observed reduction in larval body weight was consistent with the previous finding in the study by Shu *et al.* (2018) on the effect of azadirachtin extracted from Neem tree; the development of *Spodoptera litura* F. also showed a decrease in larval size when treated with azadirachtin, besides that this larval weight also decreased 43.4% compared to the control. The pupal weight of *Manduca sexta* L. in the study of Tayal *et al.* (2020) also showed that they were lighter after feeding with the addition of Purple maize, suggesting that nutritional stress of the larvae had a negative effect on subsequent life stages.

The ethanol extract of *R. communis* used to investigate with a concentration of 20 mg/mL showed that the extract affected the reproduction of fruit flies. The extract reduced fertility, leading to a decrease of 6.23 times in the number of pupated larvae compared to the control treatment. The number of flies that emerged from pupae also confirmed the effect of the extract on this stage. The number of pupae that emerged was reduced by 0.56 times compared with the number of pupae. Ecdysteroids and juvenile hormone (JH) are very important hormones for the reproduction of *D. melanogaster*. The formation of reproductive cell of female fruit flies is stimulated under the influence of JH, leading to oocyte development (Toivonen and Partridge, 2009). Therefore, the reduction of fertility may be related to the extract's antagonistic activity on key reproductive hormones (JH/ecdysteroids). In *Anopheles stephensi*, treatment with Azadirachtin resulted in structural abnormalities of the ovary with a complete cessation of oocyteogenesis, spermatocyte formation, and impairment of vitelline shell formation, as well as follicle cell degeneration (Lucantoni, 2006). Moreover, Azadirachtin reduced the ability of successful mating in *D. melanogaster* flies and negatively affected the number and size of follicles and oocytes. All in all, it can be concluded that the extract can disrupt oocyte and spermatogenesis due to the affected ratio of ecdysone and JH.

3.2.3. Investigation results on the movement ability of fruit flies

The ability to inhibit the motility in fruit flies of *R. communis* extract was shown in Figure 4. The effect was determined based on the % number of flies moving above and below the marked line of 6 cm in each treatment.

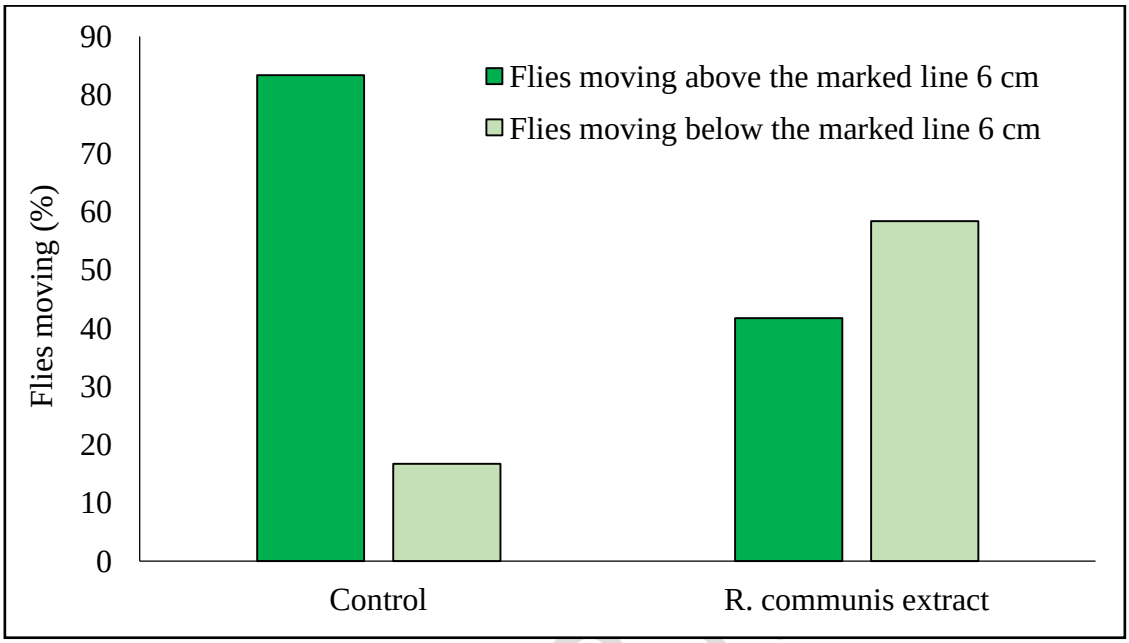

Figure 4. Comparison of the locomotion of fruit flies

Figure 4 showed that the extract was highly effective in inhibiting the movement activity in *Drosophila melanogaster* fruit flies. The number of flies moving over the 6 cm marked line in 10 seconds in the medium supplementing with the extract was 41.67% lower and significantly different from the control at 83.33%. The survey results were similar to the study of Valéria *et al.* (2014), demonstrating that the exposure of flies to 50 mg/mL of hydroalcoholic extract from the leaves of *D. furfuracea* (HEDF) for 7 days changed the movement activity of flies compared with the control. The treated flies remained mostly at the end of the column, which indicated a decrease in motility. Besides moving behavior parameters, the activity of acetylcholinesterase (AChE), and the enzyme involved in the response releasing neurotransmitter acetylcholine of the central nervous system of insects, has been shown to be inhibited at a concentration of 50 mg/mL of HEDF present in fly's food (Kim and Lee, 2013). The inhibitory activity of the enzyme acetylcholinesterase was reported to be due to pesticides (Menozzi *et al.*, 2004). In addition, it has also been described that acetylcholinesterase is the most sensitive enzyme affected by pesticides (Fremaux *et al.*, 2002). Moreover, inhibition of esterase activity in insects by plant products has been reported. Subsequently, significant reduction in acetylcholinesterase, total esterase (TE) and arylesterase (AE) activities, were also described in the 4th instar larvae of *T. granarium* treated during 80 h exposure to Phosphine (Falak and Shakoori, 2004). It is possible to affirm that the

R. communis extract can be correlated in inhibiting fruit flies' motility and acetylcholinesterase enzyme activity.

4. CONCLUSION

Ethanol extract of *R. communis* contains biologically active compounds such as flavonoids, polyphenols, alkaloids, tannins. *R. communis* extract is effectively toxic to the second instar larvae of fruit flies, affecting their fertility and development, especially limiting the mobility of fruit flies. Further studies are needed to demonstrate that the insecticidal effect is related to two hormones that affect the reproduction of fruit flies, including JH and Ecdysteroids.

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