DOI: http://doi.org/10.4038/agrieast.v16i2.112

Efficacy of extracts of the different plant parts of *Piper nigrum* against bruchid beetle *(Callosobruchus maculatus)* in stored cowpea

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Abstract

*The Cowpea (*Vigna unguiculata*) is an important legume crop because of its protein constituent. Though it is highly consumed by Sri Lankan, the production is restricted due to the insect pest infestation. Callosobruchus maculatus is the one among the insect pests causing severe damage to the cowpea seeds at field and storage. The management of C. maculatus using risk-free substances is inevitable for the steady supply of cowpea grains and to ensure the safety of consumers. Thus, a laboratory study aims to investigate the efficacy of different plant parts of Piper nigrum, namely seed, leaf, and stem against C. maculatus, by comparing with the control and the composition of secondary metabolites in such plant parts. Four treatments, viz., pepper seed methanol extract (10% w/v), pepper leaf methanol extract (10% w/v), pepper stem methanol extract (10% w/v), and methanol as control, were evaluated against C. maculatus. The effect of the extracts of the different plant parts of P. nigrum, in terms of percentage of mortality, fecundity, newly emerged adults of C. maculatus at its F1 generation, weight loss in cowpea seeds, number of damaged cowpea seeds, and the germination percentage of cowpea seeds were evaluated under laboratory condition at 28-30 °C and 75±5% RH. The phytochemical qualitative analysis and repellent effect were also performed for different pepper extraction. The results revealed that the per cent mortality was 100% in the cowpea seed lots treated with methanol extraction of pepper seed followed by pepper stem methanol extraction, whereas zero eggs were laid on the cowpea seeds treated with pepper seed methanol extract as the treatment recorded with 100% mortality at 48 hours of the experiment. The study also indicated zero adult emergences, damaged seeds, and weight loss in such treatment with maximum viability of cowpea seeds. Further, the highest repellency effect was also recorded in the cowpea seeds treated with methanol extraction of pepper seeds. Thus, methanol extraction of pepper seed was recommended as the best among the checked treatments. The phytochemical analysis confirmed the presence of alkaloids, glycosides, saponin, phenol, steroids, flavonoids, terpenoids, and tannins in the seed and stem of the pepper plants. Although similar secondary metabolites were present in the seed and stem of pepper plants, the methanol extract of pepper seed exhibited maximum efficacy against C. maculatus. Thus, further studies are vital to confirm the presence of different components in each group of secondary plant metabolites.*

Keywords: – *Callosobruchus maculatus*, Cowpea, Extract, Methanol, *Piper nigrum*

Introduction

Food scarcity is emerging as a massive problem in developing countries. Therefore, developing countries mainly tubers, and pulses for energy and protein, respectively (Kala and Mohan, 2010). Cowpea (*Vigna unguiculata*) is considered an alternative source of animal protein for millions of people

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Received: 10.06.2022 Accepted: 08.12.2022

worldwide. In Sri Lanka, the production and productivity of cowpea are affected by the lack of access to modern technologies such as improved varieties and accompanying crop and pest management practices, inputs such as fertilizers, and poor output market access (Kebede *et al*., 2020).

Pests are a significant problem both in the field and storehouses, and they cause losses of more than half of the expected yield (Sirohi and Tandon, 2011). Among them, *C. maculatus* causes heavy loss quantitatively and qualitatively to cowpea seeds during storage. The larval stages are considered the most destructive because they feed and develop inside the seeds. Therefore, they are not suitable for human and animal consumption and cannot be used as planting materials.

Various measures are employed to curb the menace of this pulse beetle. Among them, chemical control is the primary and most effective control of pulse beetle in storage. The continuous use of synthetic insecticides has raised the hazardous effect on the public and the environment due to its residual effect. The adverse effects of synthetic insecticides on human health, non-target organisms, and the environment, and the ability of the insect to develop resistance to some of the insecticides (Yusuf *et al*., 2006) and the cost usually associated with purchasing them are the main areas to concentrate much before choosing the synthetic insecticides. Thus, the need for eco-friendly and cheaper pest control methods is inevitable (Asawalam *et al*., 2008).

In recent times, considerable efforts have been directed at screening plants to develop new botanical insecticides as alternatives to synthetic insecticides. Plant from the family, Piperaceae, constituted a promising source of biologically active compounds with insecticidal activity.

MATERIALS AND METHODS

Raising of insect culture

The pure culture of *C. macuatus* was raised on cowpea seeds, variety Wijaya sterilized in the oven at 50°C for 4 hours and maintained under laboratory conditions, 28-30 °C and 75±5% RH. Five pairs of *C. maculatus* were introduced into the jar containing sterilized seeds. Then it was covered with a muslin cloth and fastened with a rubber band to get mass culture.

Preparation of plant extract

The leaves, stems, and seeds of *Piper nigrum* were obtained from the home garden at Kothmale, Sri Lanka and brought to the laboratory of the Department of Agricultural Biology, Faculty of Agriculture, Eastern University Sri Lanka. Collected materials were cleaned, washed, cut into small pieces, and dried for 3 to 4 days until they become crispy. Then dried materials were ground using an electric grinder, and powders of different plant part were kept separately in airtight containers.

Twenty-five grams from each powder were dissolved separately in 250ml methanol for 48 hours. Then they were filtered using a muslin cloth, and filtrates were kept in amber bottles separately in the refrigerator.

Application of treatments

Three seed lots, each having five hundred sterilized seeds, were taken to treat with 10 ml of each methanol extraction of seed, stem, and leaf of *P. nigrum* separately. Three plastic containers, each with differently treated seed lots, were shaken horizontally for

5 minutes to ensure the spread of extraction. Then the treated seed lots were allowed shade drying until the evaporation of methanol and leaving a film of extraction over the seeds. Another seed lot treated with methanol was kept as a control. Each treated and control seed lot was equally divided into five as one contained a hundred seeds and weighed to set five replications. Later, each sample consisting of 100 seeds was separately placed into plastic containers. Unsexed five pairs of newly emerged *C. maculatus* were introduced into each plastic container, covered with a muslin cloth, and fastened with a rubber band to disallow any insect's entry or exit.

All treatments were arranged in a Completely Randomized Design (CRD) and kept at ambient room temperature (30ºC±2ºC) in the laboratory. The effectiveness of these extracts as post-harvest seed protectants were determined and compared with an untreated check in terms of the different parameters viz., per cent mortality, fecundity, and newly emerged adult of *C. maculatus* at its F1 generation, weight loss, damaged seeds, and seed germination. All the adults were allowed to remain until their natural death under laboratory conditions.

Parameters measured

1. Percent Mortality

The per cent mortality was measured at 24 and 48 hrs after the introduction of adult bruchid beetle in each treatment using the following equation;

$$\text{Percent Mortality} = \frac{\text{No. of dead insects}}{\text{Total number of insects}} \times 100$$

2. Fecundity

The total number of eggs laid on the surface of cowpea seeds was counted two days intervals from one week after the introduction of adult bruchid beetles until the death of all adults in each treatment.

3. Newly emerged adults of *C. maculatus* at its F1 generation

The numbers of newly emerged adult beetles were counted daily from emergence to completion. After each reading the emerged adult beetles were removed to facilitate the next reading.

4. Percent weight loss

After the completion of the F1 generation of adult *C. maculatus*, the per cent weight loss in cowpea seeds was calculated with the following formula;

$$\text{Percent weight loss} = \left[\frac{\text{Initial weight of seed lot - Weight of seed lot after the emergence of F1 generation}}{\text{Initial weight of seed lot}}\right] \times 100$$

5. Percent damaged seeds of cowpea

After fering F1 generation of adult *C. maculatus*, the per cent damaged cowpea seeds in each treatment was evaluated using the following equation;

$$\text{Percent damaged seeds} = \frac{\text{Number of damaged seeds}}{\text{Total number of seeds}} \times 100$$

6. Percent seed germination

Randomly selected twenty-five cowpea seeds have been taken from the cowpea seed lot at the commencement of the study and from each treatment after the F1 generation to check the seed germination percentage. The selected seeds were soaked in distilled water overnight, and after draining off of water; the seeds were placed between the filter paper and kept inside the Petri dish for 24 hours to facilitate germination. The viability of seeds was evaluated by counting germination percentage using the equation listed below.

$$\text{Percent seed germination} = \left[\frac{\text{Number of seed germinated}}{\text{Number of seed taken for test}}\right] \times 100$$

Repellency effect of pepper

According to Murugan (2010), an experiment was conducted to find the repellency effect of different pepper extraction. A central plastic cup with a 10 cm diameter was connected around its circumference by four other plastic cups of 7 cm diameter using 15 cm length and 0.5 cm diameter plastic straws. Four sets of cowpea seeds, each with fifty seeds, were taken and thoroughly mixed with 5 ml of methanol extracts of seed, leaf, and stem of pepper and methanol (Control). The treated seed lot was kept in each cup of 7 cm diameter fixed peripheral to the center cup. The experiment was replicated five times. Twenty *C. maculatus* adults were carefully released into the center cups through its lids. The beetle's movement was noted at 15 minutes, 30 minutes, 1 hour, 2 hours, and 24 hours intervals and the per cent repellency was calculated using the following equation.

$$\text{Percent Repellency} = \left[\frac{\text{Adult beetle in control treatment - Adult beetle in treatment}}{\text{Adult beetle in control treatment + Adult beetle in treatment}}\right] \times 100$$

Plate 1. Experimental setup to find the repellency effect of pepper plant parts against *Callosobruchus maculatus*

Quantitative analysis of secondary phytochemicals

The presence of phytochemicals in methanol extracts of seeds, leaf, and stem of pepper were analyzed using standard procedures described by Talukdar *et al*., (2010).

Screening for Alkaloids (Mayer's Test)

2 ml of the extract was boiled with diluted hydrochloric acid and the mixture was filtered and to the filtrate a few drops of Mayer's reagent was added. A cream or white color precipitate produced immediately indicates positive results.

Screening for Reducing sugars

To 1ml of extract and1ml of Benedict's reagent was added. The mixture is heated on a boiling water bath for 2 minutes. Solution appeared green showing the presence of reducing sugar**.**

Screening of Phenol

To 1ml of the extract 3ml 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

Screening For glycosides (Keller Kilianin Test)

Five mililitre of extract was added with 2 ml of glacial acetic acid which was followed by the addition of few drops of ferric chloride solution and 1ml of concentrated Sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Screening for Terpanoids (Salkowski Test)

Five mililitre of extract was taken in a test tube and 2ml of chloroform was added to it followed by the addition of 3ml of concentrated sulphuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpanoids.

Screening for Flavonoids (Alkaline Reagent Test)

Two mililitre of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoid compound.

Screening for Saponins (Foam Test)

Two mililitre of extract was taken in a test tube and 6ml of distilled water was added to it. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Screening for Steroids

One mililitre of extract was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added by the sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicates the presence of steroids.

Screening for Tannins

One mililitre of the plant extract was taken in a dry clean test tube and 1ml of the distilled water is gently poured with 0.5ml of 5% $FeCl_3$. Brownish green or blue black coloration show positive results.

Screening for Anthraquinones

Three mililitre of plant extract was taken in a test tube and 3ml of benzene and 5ml of 10% Ammonia were added. Formation of pink, violet or red coloration in ammonical layer detect the presence of anthraquinones.

Data analysis

The significance of treatment effects on measured parameters was determined by analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, version 9.4. Treatment means were compared using Duncan Multiple Range Test (DMRT) at $\alpha = 0.05$.

Results and Discussion

Effect of pepper extracts on the adult *C. maculatus* mortality

The study indicated the significant differences between the treatments in causing adult bruchids mortality. A 100 % mortality was observed in the cowpea seeds treated with *P. nigrum* seed methanol extract 48 hours after treatment (Table 1), which was superior to the other extracts taken from the leaf and stem of *P. nigrum*. The methanol extract of *P. nigrum* stem showed efficacy next to seed with 56% of mortality 48 hours after treatment.

The studies conducted by Rao and Chakraborty, (1982) and Kossou, (1989) evidenced the uneven distribution of bioactive compounds in different plant parts. Accordingly, six times higher bioactive compounds were confirmed in leaves of *Nicotiana tabacum* than in root, stalk, or inflorescences and more azadirachtin in the neem kernel than in leaves and other tissues of the neem plant.

Islam *et al*., (2013) stated the highest adult mortality (83.0 %) by applying the black pepper seed at 1.00 g/kg followed by black pepper seed at 0.50 g/kg and black cumin seed at 1.00 g/kg.

Aslam *et al*. (2002) stated that the black pepper caused 100% mortality in *C. chinensis* after 3.75 days of treatment, which is par with the findings of the present study. Further, Swella and Mushobozy (2007) specified that, among the natural protectants evaluated, black pepper seed powder was superior in protecting cowpea seeds, equivalent to Actellic dust's efficacy.

Effect of pepper on the F1 generation of the adult *C. maculatus*

The methanol extract of pepper seed drastically reduced the survival of adult *C. maculatus* on cowpea seeds; thus, zero fecundity was observed (Table 1). Though substantial amounts of eggs (213.6 in cowpea treated with methanol extract of leaf and 152.8 in cowpea treated with methanol extract of the stem) were observed in cowpea treated with leaf and stem of pepper, the emergence of the F1 generation of *C. maculatus* was reduced by such application. However, a statistically equivalent percent reduction in the emergence of the F1 generation has been observed in cowpea treated with leaf and stem of pepper (Table 1). It clearly stated the harmful effects of pepper extracts on embryo development reduced the emergence of the F1 generation.

Shaalan *et al.*, (2005) reported that seeds mixed with oil extracts, and pepper powder reduced insect oviposition, egg hatchability, and postembryonic and progeny development. The present study evidenced the zero fecundity of *C. maculatus* on cowpea seeds treated with pepper seed methanol extraction.

Asawalam and Emosairue, (2006) revealed 100 % egg mortality in bruchids by applying *Piper guineense* seeds powder. Abdullah and Muhammad (2004); Ofuya and Dawodu, (2002) disclosed that powders of *Piper guineense* had pronounced effects on the fecundity of *C. maculatus* in terms of deterrence, which was comparable to Actellic dust.

The significant reduction in oviposition performance and emergence was explored due to the spices powder's toxic effect on the pulse beetle eggs (Chaubey, 2008; Miah, *et al*., 1993). Further mentioned that the active components of the spice might affect the physiology of the beetle.

Effect of pepper on the quality of cowpea seeds

Statistical analysis showed a significant difference among tested treatments concerning damaged cowpea seeds and weight loss. The result revealed that the highest seed damage and weight loss occurred in control, as the seed lot did not undergo special treatment. Moreover, the cowpea seeds treated with methanol extract of leaf and stem of pepper had low weight loss compared to the control, whereas none of damaged seeds and weight were observed in cowpea seeds treated with methanol extract of pepper seeds. This might be probably due to the higher insecticidal property of the pepper seed extract.

Upon hatching, the larvae chewed into seeds directly below the eggs. The lifecycle was completed into a single seed, and finally, the beetle came out from the seeds by making a circular emergence hole called a window. Feeding the larva inside the seeds caused the weight losses, whereas the fewer weight losses in treated seeds, due to the early adult mortality, less oviposition, and low adult emergence.

The seed germination test revealed a reduction in germination in all tested treatments compared to the initial. The cowpea seed lot taken for the experiment showed 92.8% of germination at the commencement. The germination percentage in cowpea seed treated with methanol extraction of pepper seeds exhibited 85.3 % (Table 1) germination whereas it was 41.3 and 36 % respectively in methanol extract of pepper stem and leaf. The findings Islam *et al.*, (2013) mentioned that the effect of different doses of black pepper, black cumin, garlic, and methi powder on seed weight loss by the attack of pulse beetle was statistically significant and showed that the seeds weight loss was inversely proportional to the doses of spice powder. The lowest weight loss (29.00 %) was found in gram seeds with black pepper powder at 1.00 g/kg. The highest weight loss was observed in the control treatment (77.10 %). This result was in agreement with the present study. Swella and clearly stated that the methanol extract of pepper seed protected the quality of cowpea seeds.

Mushobozy, (2007) stated that, among the natural protectants evaluated, black pepper seed powder was superior in protecting cowpea seeds. Its efficacy was similar to that provided by Actellic dust.

Botanical pesticides inhibit or disrupt insect feeding by rendering the treated materials unattractive or unpalatable. The insects remain on the treated material indefinitely and eventually starve to death, which is true in pepper extracts (Rajashekar *et al*., 2012).

Table 1: Mortality, fecundity, and numbers of adults at F1 generation of *C. maculatus*, weight loss, damaged seeds, and per cent reduction in cowpea seeds germination by the *Callosubruchus maculatus*

Treatment	Per cent mortality		Fecundity	Newly emerged adults in the F1 generation	Percentage of Emergence	Percent reduction in the emergence of new adults over untreated Control	Percent damaged seeds	Percent weight loss	Germination percentage
	24 hrs	48 hrs							
Pepper seed methanol extract	98 [a]	100[a]	0.0 [a]	0.0 [a]	0.0 [a]	100.0 [a]	0.0 [a]	00.0 [a]	85.3 [a]
Pepper leaf methanol extract	10 [c]	38[c]	213.6 [bc]	101.6 [c]	47.6 [b]	36.2 [b]	65.0 [b]	23.0 [c]	36.0 [b]
Pepper stem methanol extract	26 [b]	56[b]	152.8 [b]	64.8 [b]	42.4 [b]	43.1 [b]	56.4 [b]	14.9 [b]	41.3 [b]
Untreated Control	02 [c]	16[d]	297.6 [c]	222.0 [d]	74.6 [c]	-	81.6 [c]	38.6 [d]	9.3 [c]

*Values are the mean of five replications
Values are transformed into arcsine and square root transformations.
In each column, means with similar alphabets do not vary significantly at P=0.05 by DMRT.

Repellency effect of pepper

The repellency effect of pepper seed methanol extract was higher than other tested treatments in all observed time intervals (Table 2), and a hundred per cent was achieved in such treatment within 30 minutes of the experiment. The 50 % and 33.3 % repellency was observed in cowpea seeds treated with pepper stem and leaf methanol extract.

There are several studies on the repellency effect of botanicals against *C. maculatus*. Due to the repellency effect, insect pests will be kept away from their host and protect the crops with minimal impact on the ecosystem (Talukder *et al*., 2004; Talukder, 2006; Isman, 2006; Murukan, 2010).

Table 2: Repellence effect of pepper seed, leaf and stem treated with cowpea seeds against *Callosobruchus maculatus*

Treatments	15 Min after treatment	30 Min after treatment	1 hour after treatment	2 hours after treatment	24 hours after treatment
Pepper seed methanol extract	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Pepper leaf methanol extract	66.7^{b}	33.3^{c}	27.3^{c}	23.1^{c}	20.0^{c}
Pepper stem methanol extract	66.7^{b}	71.4^{b}	55.6^{b}	45.5^{b}	38.5^{b}
Control	0.0^{c}	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}

*Values are the mean of five replications
Values are transformed into arcsine transformations.
In each column, means with similar alphabets do not vary significantly at P=0.05 by DMRT.

Qualitative analysis of phytochemicals presents in different methanol extracts

The phytochemicals such as glycosides, saponin, phenol, steroids, flavonoids, terpenoids, and tannins were present in all tested plant parts of pepper (Table 3). The presence of alkaloids was only observed in the seed and stem of *Piper nigrum*. Anthraquinones were not found in *P. nigrum* leaf, stem, and seed methanol extract. Though similar secondary phytochemicals are present in the seed and stem of pepper, the present study showed the superiority of seed extract against *C. maculatus*. It stated the need for further studies to analyze the individual components in each of the tested secondary plant metabolites responsible for the insecticidal activity of methanol pepper seed extract.

According to Tiwari *et al.*, (2011), phytochemicals are bioactive chemicals of plant origin, and they are considered secondary metabolites synthesized by plants for self-defending purposes. They are naturally synthesized in all plant body parts: bark, leaves, stem, root, flower, fruits, and seeds. The presence of phytochemicals with insecticidal activity in the black pepper has been extensively studied. There is an increasing interest in introducing black pepper secondary metabolites for managing pest infestation (Jirovetza *et al*., 2002).

Table 3: Presence of secondary phytochemicals in the methanol extract of pepper seed, leaf and stem

Phytochemical	Pepper Seed methanol extract	Pepper Leaf methanol extract	Pepper Stem methanol extract
Alkaloids	+	-	+
Glycosides	+	+	+
Saponin	+	+	+
Phenol	+	+	+
Steroids	+	+	+
Flavonoids	+	+	+
Terpenoids	+	+	+
Tannins	+	+	+
Anthraquinones	-	-	-

+ present, - Absent

Conclusions

The present study revealed the superiority of *Piper nigrum* seed methanol extract in protecting cowpea seeds from *Callosobruchus maculatus* at storage with 100 % of mortality in 48 hours of application.

The qualitative analysis of the plant secondary metabolites showed the presence of glycosides, saponin, phenol, steroids, flavonoids, terpenoids, and tannins in the seed, stem, and leaves of the pepper plants. However, alkaloids were present in the stem and seed of pepper plants. Though similar secondary metabolites were present in the seed and stem of pepper plants, the methanol extract of pepper seed exhibited maximum efficacy against *Callosobruchus maculatus*. Thus, further studies are essential to confirm the presence of different components in each group of secondary plant metabolites.

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