OPTIMIZATION OF PIPERINE EXTRACTION FROM BLACK PEPPER (*PIPER NIGRUM*) USING DIFFERENT SOLVENTS FOR CONTROL OF BEDBUGS

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ABSTRACT

Synthetic insecticides are known to cause negative environmental impact, an outcome which has led to increased reaseach activity on natural bioresources as possible substitutes. The objective of this study was to determine the efficacy of piperine in controlling bedbugs. Black pepper (*Piper nigrum* L.) and all the reagents were obtained from commercial sources and used without further purification. The optimal conditions for piperine extraction and its efficacy as an insecticide were investigated. Piperine ($C_{17}H_{19}NO_3$) was extracted using Soxhlet extraction method and its properties determined. Ethanol (C_2H_5OH) gave the highest yield of piperine compared to dichloromethane (CH_2Cl_2). Due to low water-solubility of piperine, different solvent mixtures were used to improve solubility. Toxicity against bedbugs (*Cimex lectularius*) was carried out and the optimum concentration determined to be 1.4 g L^{-1} of piperine in an aliquot of ethanol/water (v/v 1:4). At this concentration, both mature and young bedbugs took approximately 300-minute post-exposure to die. The extract was also 100% effective in inhibiting hatching of bedbug eggs while 83.4 % the unsprayed eggs hatched within seven days. Piperine can, therefore, serve as an insecticide against bedbugs since it is effective on all developmental stages of the bugs.

Key words: Bedbug, inhibit, insecticide, piperine, toxicity.

INTRODUCTION

Bedbugs (*Cimex lectularius*) are small, oval, brownish insects that suck blood of animals or humans. Adult bedbugs have flat bodies, and are about the size of an apple seed but after feeding, however, their bodies swell and have a reddish color (Miller *et al.*, 2013). Young bedbugs are smaller, whitish-yellow in color; nearly invisible to naked eye if not fed. Although insecticides have been formulated to assist in the control of bedbugs, the insects have continuously developed resistance to some of the high environmental pollutants synthetic insecticides used (Dang *et al.*, 2017). Due to heavy bedbug infestations in army barracks in 1942, Dichlorodiphenyltrichloroethane (DDT) ($C_{14}H_9Cl_5$) was heavily used to control bedbugs (Potter, 2011). However, the DDT failure against *C. lectularius* was first reported in 1947 at a Naval Receiving Station in Pearl Harbor, Hawaii (Johnson, 1948). By the 1950s, bed bug resistance to DDT was widespread (Zhu, 2008; WHO, 1992;Busvine, 1958) and).

Black pepper (*Piper nigrum* L.) is an evergreen climbing plant that originated in the Western Ghats of India and its adoption subsequently spread to other countries (Thangaselvabal *et al.*, 2008). It is commonly grown in tropical regions like Brazil, Indonesia and India at temperatures ranging between 15 and 40 °C. The vine does well under humid conditions at altitudes of up to 1,500 m above sea level and rainfall of about 180 cm per year distributed throughout the year (Sivaraman *et al.*, 1999). The fruits of *Piper nigrum* are used to produce white and green peppers. To get black pepper, the unripe peppercorns are sun-dried causing the outer skin to turn black and wrinkle (Govindarajan, 1977). Fruits of *P. nigrum* has pungency and flavor characteristics which makes it an ideal spice and used for medicinal purpose ((Ahmad *et al.*, 2012; Leung, 1980).

The fruits of *P. nigrum* contain piperine alkaloid, pungent resin, volatile oil, piperidine and starch. Of all these alkaloids, literature review shows that piperine has the highest percentage in concentration (Mukherjee, 2008; Kokate *et al.*, 2010). Piperine, (2*E*,4*E*)-5-(benzo[*d*][1,3] dioxol-6-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one, is responsible for the sour taste of *P. nigrum*. Piperine has the methylenedioxyphenyl group which is responsible for killing of insects rather than repelling them (Duke, 2010;

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Trease, 1983).

Piperine (figure 1) has low solubility of approximately 40mg L^{-1} in water, but soluble in ethanol and other organic solvent . Chloroform is the best solvent for extraction of piperine although it is one of the carcinogens (Tripathi, 2017). This property formed the basis of piperine extraction from *P. nigrum* using ethanol (which is non-toxic to humans) in comparison to dichloromethane (an alternative to chloroform) in order to establish the solvent that extract more of the piperine. The objective of this study was to determine the efficacy of piperine in controlling bedbugs.

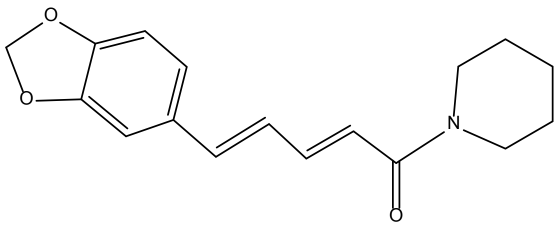

Figure 1. Structure of piperine.

MATERIALS AND METHODS

All solvents, reagents and black pepper were obtained from commercial sources and used without further purification. Bedbugs were obtained from hostels and some from highly bedbug infested homes. The pepper was dried and ground to fine powder and approximately 10 g was placed in a soxhlet thimble and then extracted using 100 mL of ethanol for 240 minutes. The solution obtained in the round bottom flask was then concentrated using a water bath maintained at at 90 °C during the concentration process. Approximatelt 10 mL 10% of alcoholic potassium hydroxide (KOH) was added to the residue with continuous stirring. The insoluble residue was then filtered and alcoholic solution concentrated at room temperature (20 °C). Some crystals were formed at the sides of round bottom flask, which were dissolved in hexane: ethanol mixture and the solution formed transferred into a vial and left to concentrate at room temperature to crystallize out piperine.

Experimental extraction of piperine

Approximately 10 g of black pepper powder were subjected to same extraction apparatus and similar duration of time to ensure uniformity in extraction procedure for comparison purposes. The experiments were done in triplicate. The ground pepper powder was placed in a soxhlet thimble and then extracted with dichloromethane (DCM) in a round bottom flask as described by Shingate *et al*. (2013). The procedure was repeated using ethanol as the extraction solvent. Determination of piperine properties was done according to the method described by Saha *et al*.(2013).

Bioassay

Toxicity of piperine was evaluated using bedbugs sourced from hostels within Dedan Kimathi University of Technology neighbourhood, stored in a well aerated transparent vial with a rug and covered with a cap. Bedbugs were kept at room temperature and all the tests were done under similar laboratory conditions to avoid sudden external environmental change (Romero *et al*., 2009). The vial was kept on an open shelf that was maintained at 25 °C to avoid suffocating insect. Mature bedbugs were classified as those with length above 2 mm, while those that attained 2 mm were considered as young bedbugs. Piperine was first dissolved in ethanol and further dissolved in water to obtain light-yellow solutions with different concentrations of 1.3, 1.5, 1.8, 2.1 and 2.5 g/L. Efficacy tests was carried out in 3 replicates in Randomized Complete Block Design (RCBD) - 5 × 2 factorial arrangement for both mature and young bedbugs in vials. To each test vial, a piece of cloth was first placed to simulate the practical conditions of application and avoid drowning the specimen. Different concentrations of piperine concentrations were prepared in ethanol/water (1:4) and ethanol/water (1:2) respectively. The prepared solutions were then sprayed to the specimen with the help of a spray bottle. Each concentration was applied to sub-sets of bedbugs (young and old) in triplicates. Response time for both groups of specimen to die were determined. The results were analyzed using Anova as per literature guidelines (Festing, 2014).

Test on bedbug eggs

On collecting the bedbugs for the purpose of this research, the specimens were kept in a vial with a rug and after a 7 days, observations were made on eggs..The bedbugs were separated from the rug, divided into two for further evaluation. The eggs were placed in separate vials for the purpose of determining their hatchability . One was sprayed with piperine dissolved in water/ethanol mixture (4:1) at optimum concentration determined from the bioassay experiments while the other one remained unsprayed as a control. The vials containing bedbug eggs were kept under similar conditions.

RESULTS AND DISCUSSION

Extraction of Piperine

There was a difference in piperine yield and extraction time for DCM and ethanol solvents. Ethanol took 3 to 4 hours for the solvent to clear compared to DCM which took two hours. The time difference could be attributed to the differences in boiling points of the solvents (39.6 and 78.4 °C for DCM and ethanol, respectively) since DCM requires less energy to complete one cycle than ethanol. For DCM, after the first 4 empties, the solvent in the thimble turned clear implying no piperine was left in the sample. The difference in cycles is attributed to piperine solubility in different solvents. Piperine is slightly soluble in water (40 mg/L, or 1 g/25 L (18° C) and more so in alcohol (1 g/15 mL), ether (1 g/36 mL) or chloroform (1 g/1.7 mL) (Harwood, 1989).

The extraction process for piperine using ethanol yielded 1.01 g (10.1%, w/w) while the extraction using DCM yielded 74 mg (7.42%, w/w) of piperine from the dried black pepper powder. This outcome was much desired as there are some healthy side effects associated with prolonged and long exposure to DCM (Rioux, 1988). Although DCM is a preferred substitute for chloroform, recent studies have shown that length exposure to this solvent has similar effect to that of chloroform (Schlosser *et al.*, 2015). Schlosser and co-workers pointed to the possibility of DCM being carcinogenic to humans based on animal studies that resulted in carcinogenicity of the liver and lungs in mice. Ethanol gave a better yield despite requiring more energy i.e. longer time to extract piperine from the ground *P. nigrum* fruits. Use of ethanol as a solvent is a better option considering the health implications of DCM. (Ahmed, 1995).

Bioassay

Piperine solubility in water was a major hindrance in this study and therefore a better solvent system to dissolve crystals was required. Due to this challenge, a specific mass of piperine was weighed first then dissolved in a known volume of ethanol. Piperine fully dissolved in ethanol. When a known volume of water was added to the mixture, piperine crashed out of solution. Consequently, piperine was first dissolved in ethanol and then added drop-wise into water with vigorous stirring to obtain concentrations of 1.3, 1.5, 1.8, 2.1 and 2.5 g/L in in water/ethanol mixtures of (4:1) and (2:1) respectively. Thus, prepared solutions were used within five days of preparation. Efficacy test was carried out on both mature and young bedbugs. The results obtained are presented in Figures 2 and 3.

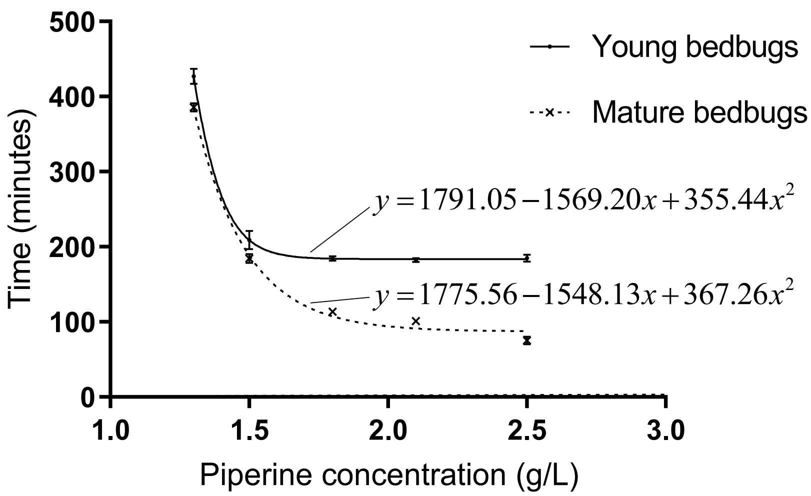

Figure 2. Time taken by young bedbugs (continuous curve) and mature bedbugs (broken curve) to die in different piperine concentrations in ethanol/water (1:4) mixture

It was clear from the bioassay results that mature bedbugs take a relatively shorter time to die as compared to young ones when sprayed with the same concentration of the solution. For example, at piperine concentration of 2.0 g L^{-1} in ethanol/water (1:4) mixture, mature bedbugs died after 100 minutes while young bedbugs died after 200 minutes (Figure 3). At concentration 1.4 g L^{-1}, both mature and young bedbugs took approximately 300-minute post-exposure to die. This was taken to be the optimum dosage for the extract. However, as concentration reduced, the time taken by both groups narrowed to a common time. The results indicated that the potency of the formulation is dependent on the piperine concentration for the ethanol/water (1:4) mixture. In other words, the cold ethanol/water (1:4) mixture alone behaved like water and was not sufficient to kill either of the bedbug groups at low concentrations ≤ 1 g L^{-1}. Cold water can only drown the bedbugs since they cannot swim. Nonetheless, hot water (over 45^{o} C) is known to kill bedbugs (Johnson, 1941).

Similarly, the bioassay results for piperine in ethanol/water (1:2) mixture for both mature and young bedbugs followed a comparable trend (Figure 3). For example, at piperine concentration of 2.0 g L^{-1}, it took mature bedbugs 50 minutes on average to die while young bedbugs took approximately 100 minutes. The results indicate that increasing the amount of ethanol by two-fold reduces the time it takes for piperine to kill the both young and mature bedbugs by half respectively. Although ethanol was used as a solvent, pure ethanol killed bedbugs at an average of 3.33 minutes for mature bedbugs and 9.33 minutes for young bedbugs, implying that ethanol can be used as an insecticide. Alcohol is known to kill insects through contact action (Sims and O'brien, 2011). However, economic factors were taken into consideration when deciding on the best solvent system for this purpose. Minimum amount of ethanol and least possible amount of piperine were used to determine the optimum concentration. In addition, ethanol is flammable and therefore cannot be considered as a safe option for bedbug control.

From the bioassay results above, using ethanol/water (1:2) was not economical due to high amount of ethanol required which can make the insecticide expensive. Therefore, a concentration of 1.4 g/ L of piperine in ethanol/water (1:4) was considered to be the optimum concentration for an insecticide formulation. Despite the fact that it takes longer time (≈ 300 minutes) to kill the bugs, the final result is satisfactory compared to other synthetic insecticides that work within days and may not have ability to destroy the eggs (AFPMB, 2019).

It is also worthy to note that control specimens for this study survived for extended periods of time. The mature

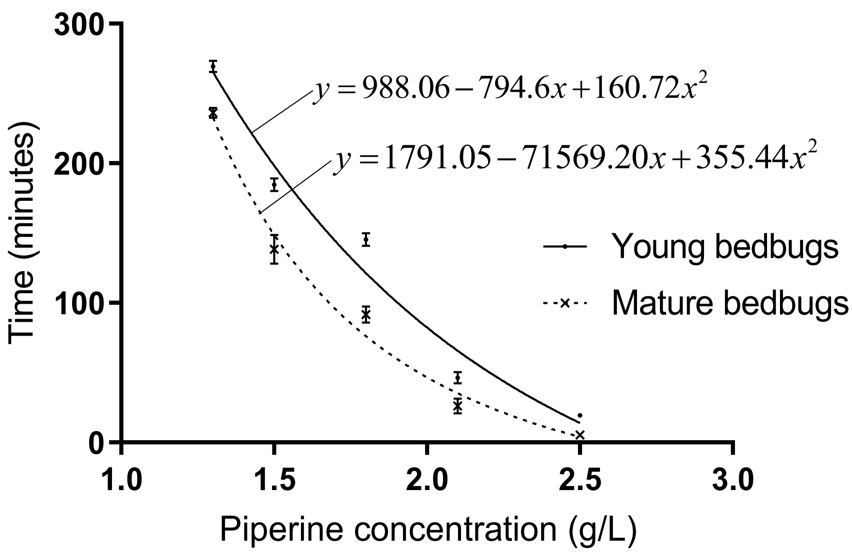

Figure 3. Time taken by young bedbugs (continuous curve) and mature bedbugs (broken curve) to die in different piperine concentrations in ethanol/water (1:2) mixture

control bedbugs stayed as long as 22 days while young ones survived up to 56 days without any supplements. This observation matched the earlier one which indicated that mature bedbugs died faster than the young ones on spraying them with insecticides.

Analysis of Variance (ANOVA) tables for the data obtained for piperine in ethanol/water (1:2) mixture for both mature and young bedbugs was performed to determine the significance of the relationship between the dependent and independent variables. The data set was picked for analysis since the response towards piperine concentrations by both mature and young bedbugs exhibited a similarity in trends. A low p-value (indicates that a significant relationship exists between dependent and independent variables. This was determined for the two sets of data represented by equations (mature bedbugs) and (young bedbugs) respectively. The results are given in Table I and Table II below.

Test on bedbug eggs

Out of 12 eggs that were in the vial sprayed with the piperine solution, none hatched. This indicated the ability of the insecticide to inhibit hatching of the bedbug eggs. This may have been caused by piperine and ethanol which acting as a thin film on the egg shell and therefore disrupting the hatching. The sprayed eggs turned from white to yellow lastly darkening in color which was an assurance that hatching would not take place. Nonetheless, of the seventeen eggs in the unsprayed vial, fourteen eggs hatched after seven days while three did not even after twenty days. This represents a viability rate of 83.4%. This is comparable to the data that was obtained (Miller, 2013).

CONCLUSION

Piperine was extracted from black pepper using DCM and ethanol in 7.42% and 10.1% yields, respectively. Piperine in water/ethanol (4:1) solvent mixture has potential to be used as an insecticide against bedbugs, although it may work better in a suitable surfactant or solvent system. In addition, young bedbugs take relatively longer time to die in comparison to mature bedbugs under similar conditions. This implies that there are differences in the mode of action of insecticide at different stages of their life cycle. On the other hand, the ability of piperine to inhibit the hatching of bedbug eggs is of significance since a single spray has the potential eliminate an entire generation of the bedbugs - it is effective against all stages. However,

TABLE I - ANOVA TABLE FOR MATURE BEDBUGS TREATED WITH PIPERINE IN ETHANOL/WATER (1:2) MIXTURE

	Degrees of Freedom	**Sum of squares**	**Squared sum of squares**	**F-value**	**p-value**
Regression	2	101485	50742	265.55	1.164e-10
Residuals	12	2293	191		
Total	14				

Table I shows ANOVA results for mature bedbugs. The low p-value (1.164e-10) indicated that there existed a significant relationship between dependent and independent variables. Therefore, it was concluded that there is a significant relationship between dependent and independent variables.

TABLE II - ANOVA TABLE FOR YOUNG BEDBUGS TREATED WITH PIPERINE IN ETHANOL/WATER (1:2) MIXTURE

	Degrees of Freedom	**Sum of squares**	**Squared sum of squares**	**F-value**	**p-value**
Regression	2	122147	61073	208.01	4.856e-10
Residuals	12	3523	294		
Total	14				

A similar analysis (Table II) was performed for data obtained for young bedbugs. A low p-value (4.856e-10) also led to a conclusion that a significant relationship existed between dependent and independent variables.

further research should be carried out to determine better solvents for piperine to fully work as an insecticide. Research should also target the possibility of including piperine in soap formulations that can be used for laundry work as a way of destroying bedbug eggs, larvae or mature bedbugs. Further work to establish the mode of action of piperine on bedbugs is also recommended.

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