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Extraction of Pyrethrins from *Chrysanthemum cinerariifolium* petals, its application as a Bioinsecticide and study of its effects on Rice Weevil *Sitophilus oryzae*

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Abstract

The Rice Weevil *Sitophilus oryzae* is one of the most destructive primary storage product pests worldwide and is known for attacking stored grains such as wheat, corn, oats, and rice. A wide variety of agricultural chemicals are being used as pesticides but sole reliance on these chemical-based insect-killers is not safe and sufficient. These are now being replaced by organic or bio-pesticides which are biodegradable, have less toxicity to humans, and provide slow but effective insect repulsion. Thus, a pyrethrin-based bio-insecticide emulsion was prepared by solvent extraction method using locally available *Chrysanthemum cinerariifolium* flower petals. Its biological activity against the rice weevil *Sitophilus oryzae* was then observed. HPLC analyses were done to validate the presence of pyrethrin in the extract. The bio-insecticide was sprayed on the rice weevils in concentrations of 20%, 40% and 60%. It was observed that pyrethrin extract in solvent mixture i.e. petroleum ether, acetone and ethanol (1:1:1) proved much more effective than the extract containing petroleum ether as the sole solvent. Telsta® (Clothanidin), a synthetic chemical pesticide was set as a control group and was observed to be more potent as it killed 95% of the rice weevils on direct contact. The death percentage from petroleum ether method was 90%, at concentrations 20%,40% and 60%, while in solvent mixture method, death percentage was 90% at conc. 20% and 100% at conc. 40% and 60% respectively after 48 hours for both methods. It was concluded that natural biopesticides are almost equally potent against rice weevils as the chemical pesticides, but they require longer exposure time (24-48h) for their effectiveness.

Keywords: HPLC, Storage product pests, Bioinsecticide effectiveness, Natural pest control, Pyrethrin extraction.

Introduction

Every year almost 20-40% of the world's grain crop is lost during storage and much of this loss is attributed to insect pest attack (Finegold, 2019). Storage product pests reach grain storage from standing field crops to various processes involving grain collection and transport. More than one thousand insect species have been attributed to damaging stored grains across the world, but a few species are listed as extremely damaging. Such invasive insect pests not only target agricultural products by damaging plant stems and roots, chewing leaves, draining plant juices but also deteriorate their nutritional value by accelerating the decaying process (Ram Ujagir & Byrne, 2009; Srivastava & Subramanian, 2016).
The Rice Weevil (*Sitophilus oryzae*) is one of the most destructive primary storage product pests worldwide. According to research by (Mehta, Kumar, & CS, 2021) the

rice weevil is a cosmopolitan and is known to be one of most disastrous insect-pests causing approximately 10-65% of damage under moderate storage whereas under prolonged storage it accounts for 80% of damage to stored grains such as wheat, rye, corn, oats, sorghum, barley, cashew nuts, buckwheat, and rice (Koehler, 1999). The rice weevil (as shown in Figure 1) is notorious for not only reducing grain yield but also deteriorating the quality of grain left undestroyed by eating out the embryonic regions thereby decreasing their protein content and lowering chances of seed germination. Its damage range is greater than other types of weevils because it possesses the ability of flight and causes almost complete destruction of grains as its larvae develop within grain kernels (Jackson, 2015). In a study by (Khan, Haider, & Khan, 2022), it was revealed that strains of *Sitophilus oryzae* obtained from Punjab, Pakistan, have a significant resistance to many insecticides having active ingredients chlorpyrifos-methyl, permethrin and pirimiphos-methyl. As such their control becomes a necessity (PennStateUniversity, 2023; Rayees Ahmad, 2022).

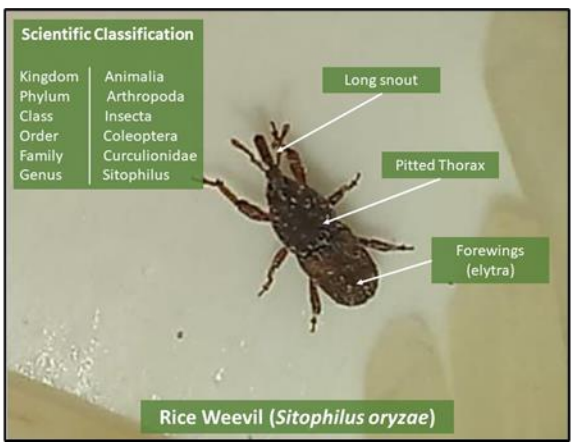

Figure 1. Classification and morphology of Rice Weevil, *Sitophilus oryzae*.

A variety of chemical pesticides are currently being used as fumigants to eliminate such pests. Although methyl bromide and phosphine are permitted for fumigation under Chemical Control Techniques by FDA/WHO, they are still very toxic and pose considerable risks including residual accumulation, acute or chronic toxicity to mammals, numerous human health hazards and chances of pest re-infestation. Likewise, a wide variety of agricultural chemicals often known as 'Miracle weapons' are largely being used as pesticides but sole reliance on these chemical-based insect-killers is not safe and sufficient (Mehta *et al.*, 2021; Penn State University, 2023). Multiple complications including pest resistance, residual hazards, control failures and numerous health risks such as ADHD, Alzheimer's disease, respiratory distress, headaches, skin irritation, severe intoxication and even cancer in humans, may arise. Therefore, chemical pesticides are now being replaced by organic or biopesticides which are biodegradable, have less toxicity to humans, and provide slow but effective insect repulsion (Amusat, Okewole, Rafiu, & Amusat; Proctor, 1994).

According to the National Pesticide Information Center, pyrethrin is a naturally occurring compound which is toxic to insects and has been modified into pyrethroids to make it more potent (U.S. Environmental Protection Agency, 2018). *Chrysanthemum* flowers are found to be rich in Pyrethrin I and Pyrethrin II by (Shawkat, Khazaal, & Majeed, 2011) and have been proven toxic to flour beetle *Tibolium castanum*, a pest of stored flour grains. The extract of *Chrysanthemum cinerariifolium*, is found to be rich in two compounds Pyrethrin I and Pyrethrin II along with other compounds (Grdiša *et al.*, 2013) which are toxic to most insect types (Chrysanthemum cinerariifolium, 2009).

Thus, the purpose of this study is to create a pyrethrin-based biopesticide emulsion from *Chrysanthemum cinerariifolium* (shown in Figure 2) flower petals that works efficiently against common insect pests like *Sitophilus oryza*e and prove its efficacy when rivaled against chemical pesticides.

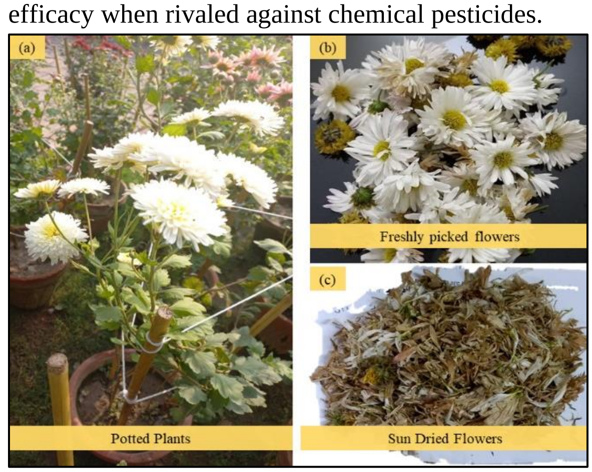

Figure 2.(a) Potted plant, (b) Fresh flowers and (c) Sun-dried flower petals of *Chrysanthemum cinerariifolium.*

MATERIALS AND METHODS

Material Collection

In August 2021, Rice Weevils (*Sitophilus oryzae*) were collected from a local rice shop in Lahore. They were stored in a glass jar with a perforated lid, while super kernel white rice was provided as food source.

This weevil population was raised in two batches for successive generations until January 2022.
In December 2021, the first batch of *Chrysanthemum cinerariifolium* flowers (Common Name: Dalmatian Pellitory) was collected from a nursery and sun-dried for 6 days, weighing 55 g. The second batch was also collected in January 2022, sun-dried for 3-4 days, crushed with a grinder, and weighed 53 g.

Preparation of Different Extracts

Extract 1

For the first extract, petroleum ether was used as a solvent to extract pyrethrin from *Chrysanthemum cinerrarifolium* petals. 25 grams of petals were soaked in 200 mL of petroleum ether for 3 days. The yellow-colored filtrate was obtained after filtration. Methanol 80% was added to separate the yellow layer from the rest. The separated yellow layer was subjected to rotary evaporation at 150 rpm and 30 °C for 7 to 10 minutes, resulting in a pyrethrin solution. Different concentrations (20%, 40%, and 60%) of this solution were prepared, and the extract was used for biological testing on rice weevils (Phenix, 2007).

Extract 2

As for the second extract, a mixture of three solvents (100 mL each of petroleum ether, ethanol, and acetone) was used to extract pyrethrin from *Chrysanthemum cinerrarifolium* petals. After soaking the petals for 3 days, the mixture was filtered to obtain a greenish-yellow filtrate. Methanol (80%) was added, resulting in a cloudy solution and an exothermic reaction was observed. Rotary evaporation at 140 rpm and 50 °C for 20 minutes yielded extracted pyrethrin solution, which was further purified by ultracentrifugation. Diluted concentrations (20%, 40%, and 60%) of pyrethrin were prepared using extracts for biological testing on rice weevils. The effectiveness of pyrethrins in killing the weevils was assessed by comparing the results of both extract 1 and 2. The Heidolph Hei-VAP Rotary Evaporator with Dry Ice Condenser Glassware and Vacuum Pump was used in this

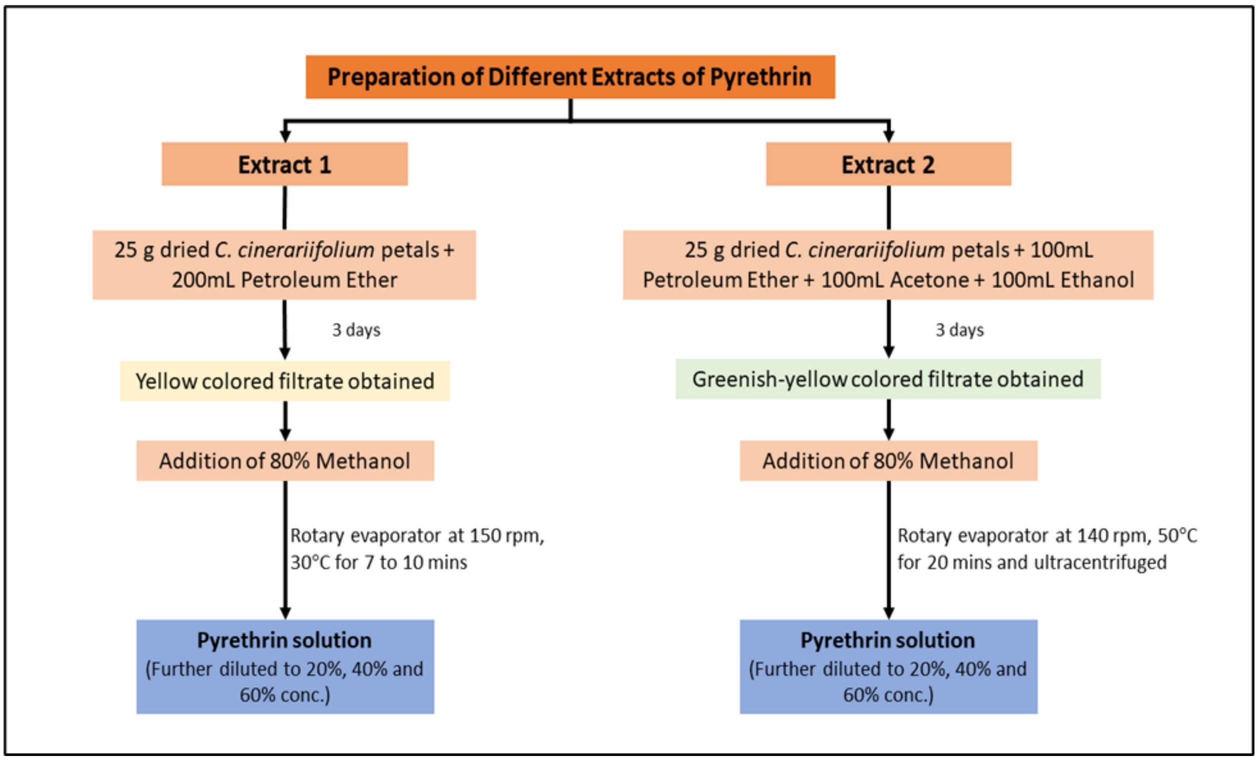

Figure 3. Preparation of Extracts of Pyrethrin from Chrysanthemum cinerariifolium petals.

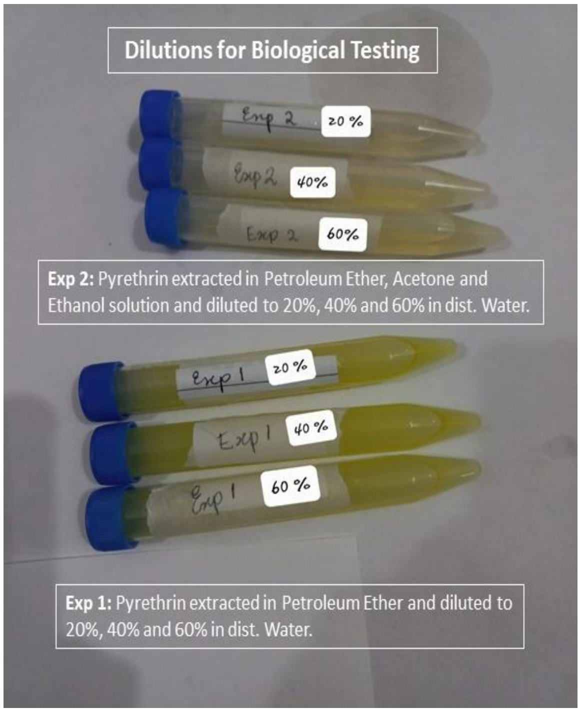

Figure 4. Dilutions of 20%, 40% and 60% pyrethrin.

experiment to evaporate solvents (petroleum ether, acetone and ethanol) and purify the pyrethrin sample. To prevent pyrethrin degradation, temperature and rotation speeds were adjusted according to their boiling points, avoiding temperatures above 65 °C. Reverse phase liquid chromatography **(**HPLC) was used to validate the presence of pyrethrin in the sample. HPLC works based on variance in affinity using two phases: mobile phase (liquid) and stationary phase (polar). A 250 mm × 4.6 mm ID C18 (ODS) column was used with Acetonitrile: H2O 80:20 (v/v) as the mobile phase at a flow rate of 1 ml/min. The volume injection was 20 µL, and the detection wavelength was set at 350 nm with a column temperature of 30 °C (Kim *et al*., 2021; Ott *et al*., 2000).

Biological Testing

Biological testing was performed on rice weevils (*Sitophilus oryzae*) kept in a well-ventilated container with white rice as their food source. Two extracts were prepared for pyrethrin at concentrations of 20%, 40%, and 60% (as shown in Figure 4). The mortality rates of the weevils were observed over a week using the test cage method according to ASTM guidelines, and the solutions were stored in a refrigerator. HPLC was used for quality detection of pyrethrin with a C18 (ODS) column at a temperature of 30°C (Essig & Zhao, 2001). The mobile phase consisted of acetonitrile and water at a flow rate of 1mL/min, with a volume injection of 20 microliters. The effect of pyrethrin on the rice weevils was assessed after every 12 hours by spraying them with the prepared concentrations (20%, 40%, and 60%) using both extracts 1 and 2. The number of dead and alive weevils was counted to determine the potency of each pyrethrin solution (Jantan & Zaki, 1998).

Bioassay method to test the toxicity of insecticide

The bioassay procedure as stated in (Paramasivam & Selvi, 2017) was followed for determining toxicity of the pyrethrin based insecticide. The insects were divided randomly into six groups and one of the groups was used as the control group which was

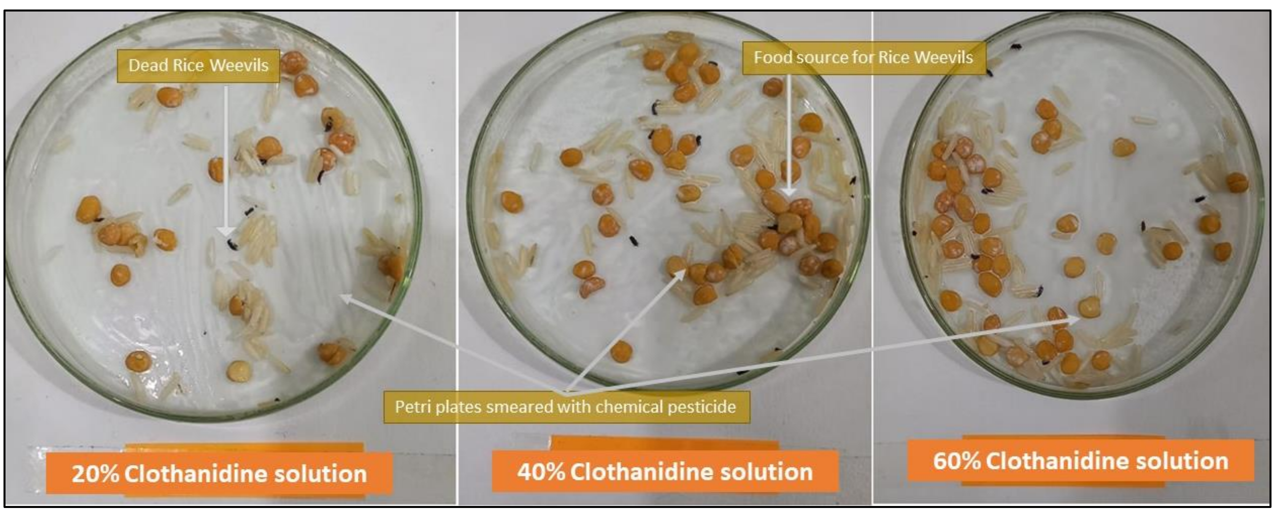

Figure 05. Chemical Testing by spraying chemical pesticide, Telsta® Clothianidin, in concentrations of 20%, 40% and 60% on Rice Weevil.

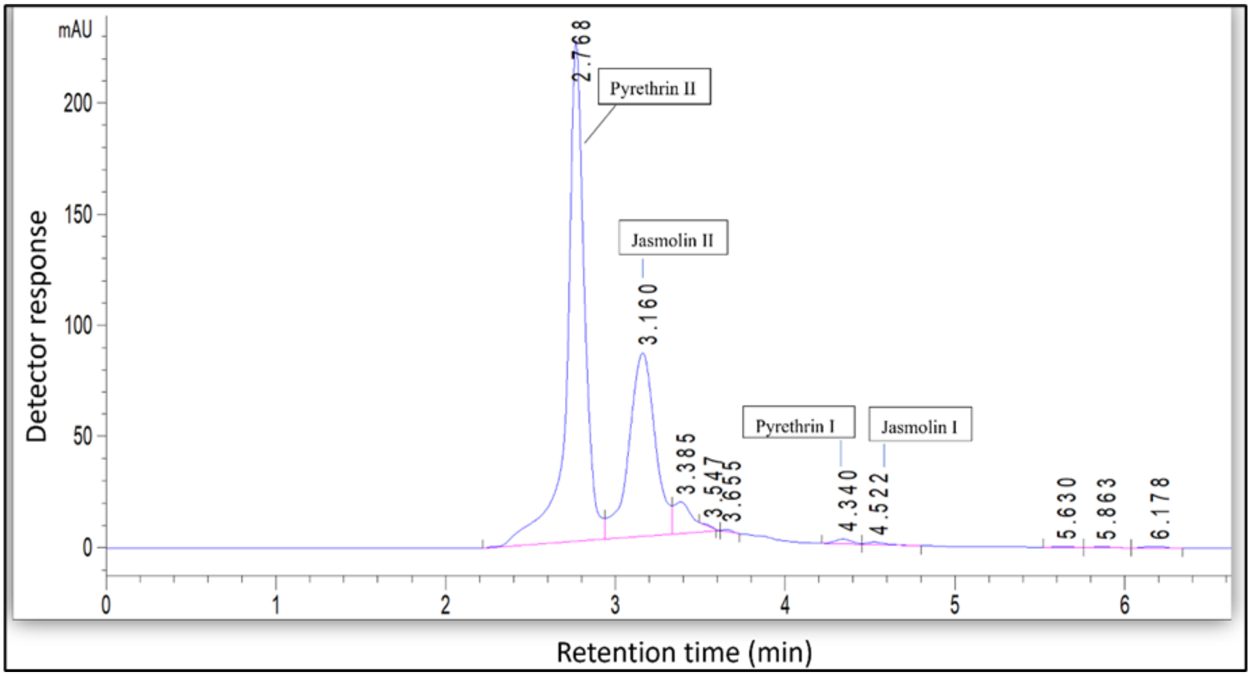

Figure 6. HPLC Analysis of pyrethrin extract from Chrysanthemum cinerariifolium petals

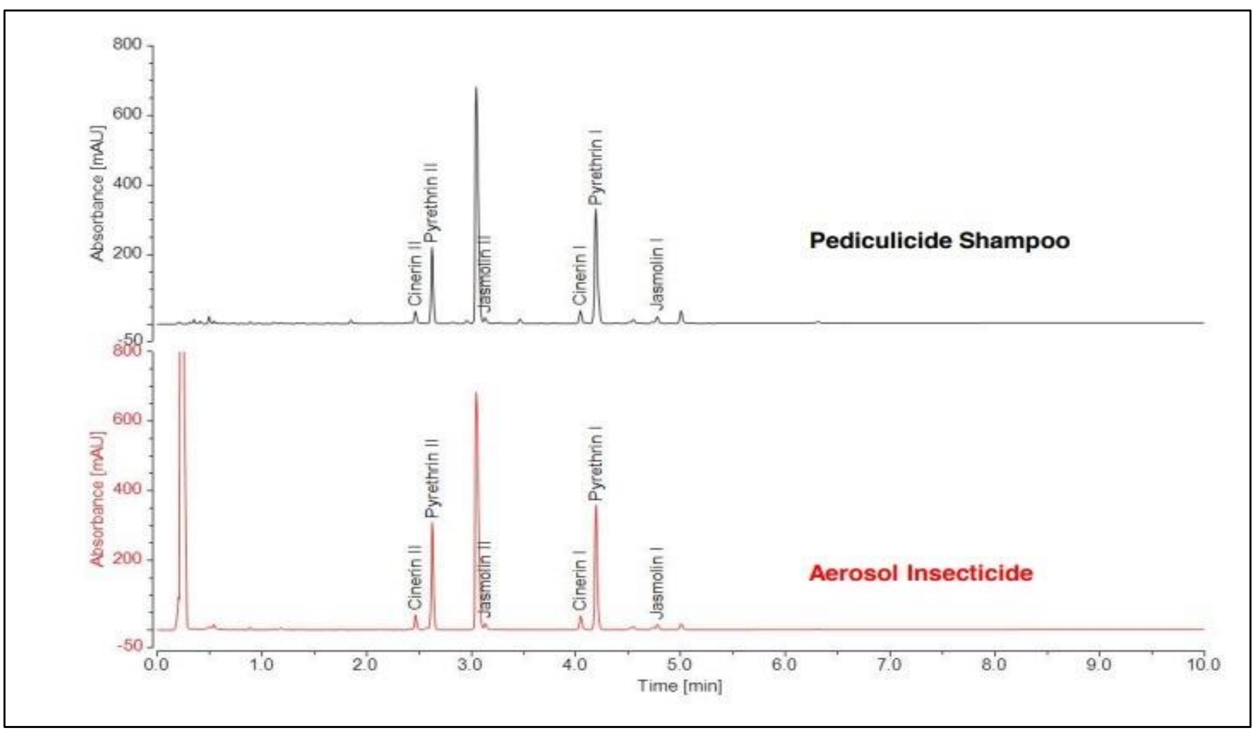

Figure 7. Validation of the presence of pyrethrin in *Chrysanthemum cinerrarifolium* by comparsion with literature. Source: (Thomas et al., 2016)

treated with solvent petroleum ether only. The other five groups were treated with pyrethrin concentrations 20%, 40% and 60%. The insects were exposed to different pyrethrin concentrations while being kept in the containers with food supply. A timed procedure followed to record the mortality percentage was calibrated after every 24 hours and was repeated for 3 days, according to the procedure used by (Jantan & Zaki, 1998).

Chemical Testing

For chemical testing, the synthetic pesticide Telsta® with Clothianidin as its active ingredient was used as a control for comparison. Three sets of ten rice weevils were taken, and three dilutions (20%, 40%, and 60%) of Telsta® were prepared (as shown in Figure 5). The activity of the weevils was noted every 12 hours after being sprayed with the solution. Both the chemical and biological testing results were compiled to compare the efficacy, strength, and effectiveness of the synthetic pesticide Telsta® with the bioinsecticide pyrethrin.

RESULTS

First extract that include petroleum ether as a solvent gives a cloudy yellow extract solution after filtration and rotary evaporation,due to its cloudiness, the extract was subjected to ultracentrifugation but still could not be clarified any further. As HPLC

Table 1.Results of Biological Testing using prepared pyrethrin insecticide on Rice Weevils.

Concentration		After 12 hours	After 24 hours	After 36 hours	After48 hours
Extract 1	**20%**	4 dead	6 dead	7 dead	9 dead
	40%	6 dead	7 dead	7 dead	9 dead
	60%	6 dead	7 dead	8 dead	9 dead
Extract 2	**20%**	5 dead	7 dead	9 dead	9 dead
	40%	7 dead	7 dead	10 dead	10 dead
	60%	7 dead	8 dead	10 dead	10 dead

Apparatus can only utilize a clear solution, therefore, this extract was not nominated for HPLC. Due to the nonconformity of HPLC for extract 1 solvent, it was not undertaken for HPLC analyses but was used later for biological testing. The second extractshowed clear yellow solution after filtration and rotary evaporation. This extract was subjected to HPLC as it was polar and hence, miscible with acetonitrile. The HPLC was performed on a set wavelength of 264nm Two prominent peaks are obtained for Pyrethrin II and Jasmolin II (a compound of pyrethrin), while smaller peaks indicate compounds Pyrethrin I, Cinerin I and II, and Jasmolin I, as shown in Figure 6. All these compounds are collectively potent towards the insecticide effect of pyrethrin.

The highest area (636.64 mAU*min) is obtained for pyrethrin II (Table 1) which is the most dominant compound hence it is the active compound responsible for insecticide action.

Comparison of HPLC Peaks with Literature

As pure pyrethrin could not be sourced locally due to import restrictions on chemicals, the presence of pyrethrin in the *Chrysanthemum cinerariifolium* extract was confirmed by comparison of the above obtained HPLC peaks with those from the study of (Thomas, Glinski, Wong, Acworth, & Mohindra, 2016). Both sets of peaks matched for pyrethrin I, pyrethrin II, jasmolin I and II, and cinerin I and II, confirming the accuracy and presence of required compound in extract. The prepared bioinsecticide's graph also aligned with literature, confirming the presence of pyrethrin.

Biological Testing of Pyrethrin on rice weevils

In biological testing, the rice weevils were sprayed with the pyrethrin extract dilutions (20%, 40% and 60%). It was observed that the activity of rice weevils was decreased on initial spraying which can be attributed to the immediate presence of liquid droplets which was perceived by the rice weevils as a distraction. It was noted after a certain time interval that most of the insects, on ingesting the pyrethrin present in the spray, had died. As the spray acts directly on the nervous system on touch or ingestion thus paralyzing them, ultimately resulting in their death. The rice weevils were observed after every 12 hours and noted the effect for 48 hours in total, as shown in Table 1. In conclusion, it is clear from the table that extract 1 pyrethrin kills rice weevils substantially more effectively than extract 2 pyrethrin.

The toxicity interactions of an insecticide with a biological system such as rice weevils in this study are determined by the dosage concentrations. This toxicity value is expressed by lethal dose LD_{50} i.e. dose per unit weight lethal to 50% of the population of the organism.

A small batch population of 10 rice weevils was used in duplicates for precise measurements and each

Table 2. Results of Chemical Testing using Telsta® Clothianidin on Rice Weevils

Concentrations		After 12 hours	After 24 hours	After 36 hours	After48 hours
Synthetic pesticide	**20%**	10 dead	10 dead	10 dead	10 dead
	40%	10 dead	10 dead	10 dead	10 dead
	60%	10 dead	10 dead	10 dead	10 dead

batch was a random sample of the population. According to (Paramasivam & Selvi, 2017) insecticide toxicity is usually determined by utilizing a dose-response association. After spraying the insects with appropriate concentrations of pyrethrin, a regular 24h interval was kept for observation for 3 days (i.e., 48 h). The mortality percentage was calculated by referring to Table 1, as follows:

For Extract 1:

$$Mortality\ \% = \frac{\text{No. of insects dead on exposure to extract after 48h}}{\text{Total Number of insects}} \times 100$$

For Extract 2:

$$Mortality\ \% = \frac{\text{No. of insects dead exposed to extract after 48h}}{\text{Total Number of insects}} \times 100$$

Thus, extract 1 with only petroleum ether as solvent kills 90% of insects while extract 2 with petroleum ether, acetone and ethanol gives 100% mortality rate.

Testing of chemical insecticide on rice weevil

Chemical Testing was done by using aerosol solution of liquid Telsta ® Clothianidin on rice weevils and was observed for 48 hours, by checking the activity of rice weevil after every 12 hours. Telsta ® is a chemical pesticide with the same mode of action as pyrethrin for being a non-systemic pesticide which works on the nervous system of insects. Therefore, on immediate contact, the insects were paralyzed right away and died within half an hour. However, to keep equal intervals in both chemical and biological testing, a 24-hour interval was kept for chemical testing observations. The result of chemical testing was hundred percent cause faster killing of insects.

DISCUSSION

According to Environmental Protection Agency (EPA) all pesticides must be reviewed prior to sales and distribution to ensure environmental safety (Helen M. Andrews, 2018). As such pre-advised mechanical methods from IPM (Integrated Pest Management) and Bt (a selective microbial insecticide) are now being replaced with naturally occurring sources like pyrethrum. Extracted from *Chrysanthemum* species, pyrethrum (dried, powdered flower heads) is generally safe for mammals but is poisonous enough to kill both pests and beneficial insects without discrimination.

Average pyrethrin content in wild populations of Dalmatia (Croatia) flower petals is 0.89% ranging from 0.75 to 1.04% (Ban, Sladonja, Luki, Luki, & Gani, 2010). Therefore, on analysis of extraction methods, solvent mixture showed better potency as a solvent by producing a more concentrated solution of pyrethrin II.The quality of pyrethrins I and II was further validated by comparison with graphs from literature such as "Determination of Pyrethrins in Pyrethrum Oil Extracts by UHPLC with Charged Aerosol Detection (thermofisher.com)" whereby an HPLC analysis between a Pediculicide (lice-killing) shampoo containing pyrethrin and an aerosol insecticide containing pure pyrethrin and associating compounds was concluded. Thence reported pyrethrin graphs were compared with those obtained for pyrethrin I and II along with accessory compounds i.e., jasmolin I and II and cinerin I and II in this study and were found to coincide correctly. After verification, the resultant extract was tested for efficiency against rice weevil (*Sitophilus oryzae*). Conventional chemical techniques used against rice weevil include fumigation with organo-phosphorous compounds such as fenitrothion and pirimiphos-methyl (Trematerra & Throne, 2012).

Another aim of the study was to evaluate the potency of naturally extracted pyrethrins against rice weevil as it is a competent insect which requires potent insecticides for complete mortality as compared to other common pests like *Tribolium castaneum*. *T. castaneum* is a prevalent pest, often used as a model organism due to its synanthropic nature, short life cycle and small size (Choe, 2019). However, the choice of rice weevils (*S. oryzae)*, which is far more destructive and responsible for eating out the germ portion of the crop, abridges this gap in research.

Biological tests on rice weevils triggered slow paralysis, gradually resulting in the death of insects. Both solvent extracts yielded 99% mortality results. An additional mortality factor was also considered for a previous batch of weevils unexposed to any pesticide but found dead. This death was attributed to fungal growth in the container leading to 95% mortality rate of weevils. The observed infestation showed signs of Metarhizium which adheres to the outermost cuticular wax of the host by hydrophobic proteins and then penetrates it, hence killing the host (St. Leger & Wang, 2020).

The rice weevils were also treated with chemical insecticide Telsta ®. Its active ingredient is Clothanidin, formulated at 20 SC (suspension concentrate, having solid active ingredient dispersed in water). As permitted by FMC Pakistan, recommended dosage of 200ml/Acre is advised for use specifically against dusky cotton bug (SHAD, 2019). Telsta ® forms a thick white liquid over the sprayed surface and kills rice weevils on immediate contact. Pyrethrin has an advantage leaving no residues and thus can be washed off easily. It has a half-life of 11.8 hours in water and 12.9 hours on soil and also has less tendency to penetrate soil below 15 centimetres or to become vapours in the air (Bond, 2014). Bioinsecticides obtained from

*Chrysanthemum cinerariifolium*are also more cost-effective as it is readily available in winter, requires low-cost solvents and one-time HPLC analysis for the detection in every batch.

CONCLUSION

Considering the global stored crop losses due to attack of pests and increased awareness regarding the health and environmental hazards of chemical pesticides, the demand of organic and biodegradable pesticides has increased manifold. This research explored an effective method of pyrethrin extraction, preparation, and administration to target pests, rice weevils (*Sitophilus oryzae*). Pyrethrin emulsion was effective against rice weevils as it gives high killing percentage (90-100%) in limited amount of time. Moreover, the effectiveness of pyrethrin-based biopesticides against storage grain insect-pests and the comparison of its effects with chemical pesticide indicated that chemical pesticides are more potent but pose various hazards in terms of pesticide resistance and residues that spoil the quality of stored crops as compared to the pyrethrin that comes from natural sources.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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Author contribution

Fatima Saeed and Fatima Jawed contributed equally in conceiving the idea, writing the draft and carrying out all experimental work. Shahnaz Choudhry, supervised the work.

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