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Assessment of green solvents and extraction methods for biopesticide preparation from neem *Azadirachta indica* leaves against Oriental fruit fly *Bactrocera dorsalis* (Hendel)

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Abstract: This study was conducted to search for green technology that can extract metabolites from neem leaves for use in the development of botanical pesticide against *Bactrocera dorsalis* (Hendel) (Diptera:Tephritidae). Rice wine, rice wash, vinegar and distilled water were used as solvents and hot infusion, maceration, hot continuous reflux (Soxhlet), and fermentation were the methods employed. The different leaf extracts prepared by green technology were evaluated for their potentials as pesticide against *B. dorsalis*. Vinegar extract via Soxhlet extraction (V-S) for eight (8) h registered to have the highest mortality but not significantly different from vinegar - fermentation (V-F), rice wash - Soxhlet (RWa-S), vinegar - maceration (V-M), distilled water - fermentation (DW-F), and rice wash - fermentation (RWa-F) extracts. Phythochemicals present in the extracts are affected by the solvent-extraction interaction. Among the sixteen solvent-extraction interactions, the use of rice wash and fermentation is the most economical method in extracting the extracting the active components of neem leaves against *B. dorsalis*. Rice wash is a waste that can be utilized in the development of a biopesticide from neem leaves for pest management of *B. dorsalis*. This is the first report that rice wash is used as extracting solvent in green synthesis.

Keywords: rice wash; vinegar; rice wine; Soxhlet; fermentation

1. Introduction

The fruit fly species *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) has been throughout one of the most serious economic pests affecting agricultural fruits in India, East Asia, and the Pacific [1]. These oriental fruit flies deposit their eggs into fruit and vegetables, and subsequently the flesh are consumed by developing larvae [2]. The fruit flies' invasive action has caused major economic losses in horticultural crops production, and over 90 plants were found out to be affected [3]. Mango, banana, papaya, citrus, and guava are among the country's most important commercial crops attacked. As these fruits are of valuable importance in both domestic and international markets, control and management of this pest is necessary to prevent significant losses in fruit production.

The use of chemical pesticides, a traditional control measure, impose hazard by exposure to a variety of sources in which pesticide residues are found such as food and water; garden, and lawn use; farm use; and occupational exposures [4]. Contact to the active chemical components of pesticides such as organophosphates and carbamates can cause cardiovascular and respiratory diseases, skin cancer, hearing loss, and amputations [5]. Due to this reason, it is necessary to develop other methods with concern to environmental and public health risk. Biological and physical control methods such as soil covering nets [6], hymenopteran parasitic control [7], Eco-Trap mass control [8], and hybrid biological and chemical control techniques [9] have been applied. The use of spray baits containing protein hydrolysates [10], fruit-mimicking sticky traps (Ladd traps) [11] and natural product-based pesticides [12] were also considered as alternatives. Moreover, the use of nanopesticides for pest management has emerged due to the increasing popularity of researches in the field of nanotechnology for agricultural applications [13].

Biopesticides are used to control pests, pathogens, and weeds by a variety of mechanisms [14]. They inhibit the growth, feeding, development or reproduction of a pest or an insect pathogen. Plant extracts were the earliest recorded biopesticides and it was in the early 17th century that the plant extract like nicotine is used to control the plum beetle. The main groups of active compounds of plant-derived insecticides comprise an array of several compounds such as phenylpropanoids and phenolics, terpenoids and steroids, alkaloids and nitrogenated compounds. Botanical extracts are termed as Insect Growth Regulators (IGRs). Due to the wide array of phytochemicals in these botanical extracts, pests have a decreased chance to develop resistance [15]. The use of biopesticides in Integrated Pest Management (IPM) became popular [16].

In the past few years, importance of neem (*Azadirachta indica* A. Juss) in agriculture [17], toiletry, pharmaceutical and medicinal uses [18, 19] has grown considerably because of its numerous bioactive ingredients. Among the biological properties are antiallergenic, antidermatic, antifeedant, antiviral, antifungal, anti-inflammatory, antipyorrhoeic, antiscabic, insecticidal, larvicidal, antiimplantation, nematicidal, and spermatocidal. In India, neem biopesticides were effective against pests in stored grains like rice, wheat, corn, legumes, potato, tomato, etc. [20]. The leaf water extract of neem was also reported to possess insecticidal properties against mosquitos, *Aegis aegypti* and *Culex quinquefasciatus* [21]. The abovementioned activities can be accounted to the alkaloids, flavonoids, saponins, phenols, phytate, tannins, glycoside, steroids, triterpenoids seed, bark and root of the plant [22, 23]. The essential oil of leaves extracted with 1:1 v/v of distilled water and acetone contain major compounds of β-elemene, γ-elemene, germacrene D, caryophyllene and bicyclogermacrene [24].

Neem affects insects which suck up plant juices and those which chew plant parts. Azadirachtin, as the active ingredient, serves as a growth regulator, feeding deterrent and oviposition deterrent. It acts as growth regulator by reducing the level of the ecdysone, a hormone which disrupts the molting process of the insects. The larvae do not develop into adult [25]. Azadirachtin was found to be a mixture of seven isometric compounds Azadirachtin-A to -G with Azadirachtin-A as the dominant and Azadirachtin-E as the most effective growth regulator. Some natural additives such as garlic (*Allium sativum*) and hot pepper (*Capsicum frutescens*) exhibit synergistic effect on the neem product [26].

The active components of botanicals, the secondary metabolites, depend on the way how they are extracted and the solvents used to separate them from one another. Numerous studies have been conducted and still being conducted to search for the best technique and solvent that can extract the metabolites [27]. Among these techniques are the use of liquid, steam and supercritical extractions. The conventional methods like hot infusion, maceration, decoction, Soxhlet extraction, etc., are being modified and pretreatment of samples like microwave and ultrasound processes were adopted [28, 29]. Soxhlet is a conventional extraction method which is still considered as one of the reference methods in comparing the success of newly developed methodology [30]. The use of rice wine, rice wash, and vinegar in the extraction of phytochemicals has not been reported. This study aimed at using green solvents and extraction methods in the preparation of biopesticide for *B. dorsalis.*

2. Materials and Methods

2.1 Plant Materials Collection and Preparation and Extraction Methods

The leaves of the *A. indica* plants were collected along the research area of Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. Branches were collected early in the morning. The samples were then washed thoroughly with tap water and were hanged for five days under the shade. The clean leaves were then detached from the braches and were ground in a blender and stored in a tightly covered container until further use.

Four different methods of extractions were done. These are Soxhlet or hot continuous extraction, hot infusion, fermentation and maceration. Soxhlet method was done as follows: the ground plant material (15 g) was loaded into the cellulose thimble which was then placed inside the Soxhlet extractor. The solvent was heated using the heating mantle and the solvent began to evaporate, moving through the apparatus to the condenser. The condensate then dripped into the reservoir containing the thimble. When the level of solvent reached the siphon it poured back into the flask and the cycle begins again. The process runs for a total of 8 hours.

Hot infusion was done by adding 15 g of previously weighed ground leaves into 150 mL boiling solvent. The mixture was set aside for 15 minutes. The extract was then filtered using Whatman No. 42 filter paper and the filtrate was placed in amber bottle and stored in the refrigerator until used for analysis.

Fermentation method was carried out by soaking 15 g of the plant material into 150 mL of the solvent. The container was covered with aluminum foil and stored inside the locker. The mixture was set aside for ten days. After which the mixture was then filtered and the filtrate was placed in amber bottle and stored in the refrigerator until used for analysis.

Maceration was done by placing 15 g of the plant material in 150 mL of the solvent. The mixture was then set aside for one hour, then filtered and the filtrate was placed in amber bottle and stored in the refrigerator until used for analysis.

2.3 Extracting Solvents

Four green solvents were used in this experiment. These are rice wine, rice wash, vinegar, and distilled water. The rice wine was produced at Panupdupan, Lamut, Ifugao, Philippines. The rice wash was prepared by washing 100 g of bungkitan glutinous rice with 100 mL of distilled water. The rice washing was then drained into a beaker. Another 100 mL of distilled water was added to the glutinous rice, washed and the second washing was then added to the first washing. The combined rice wash was then filtered to remove the suspended solids. The vinegar, called sukang "basi" was prepared by fermentation of sugar cane. The other solvent was distilled water.

2.4 Trapping of Male Fruit Flies

The procedure recommended by Chang *et al.* [12] was followed in this experiment with some modifications.

2.5 Mortality test

The insecticidal action of the plant extracts prepared using different solvents and extraction methods were evaluated in improvised olfactometer. The olfactometer was a transparent plastic food container measuring 4.5 in top diameter, 3.5 in bottom diameter, and 4 in high. The cover was cut forming a 2-in diameter hole and was replaced with organza fabric to prevent the fruit flies from suffocation during the test. Male fruit flies were introduced into the olfactometer using a transparent tube. A cotton ball was wet with ten drops of the extract and placed at the top center of the container 5-10 minutes after the fruit flies were introduced. The container was then covered and mortality were observed after 3, 6, 12, 18, 22 and 24 h. Effect of the pure solvents on mortality of fruit flies was also determined.

Percent mortality was computed using Abbot's formula, that is by subtracting the number of dead flies in the control from the number of dead fruit flies as affected by the treatment and dividing the difference by the total number of flies inside the plastic container. Lethal concentration of the extracts (the one with the highest mortality at 24 hours and the extract which was prepared the most economical way) was computed using the Probit formula.

2.6 Data Analysis

The percentage mortality of adult fruit flies were compared per plant extraction method and extraction solvents using Analysis of Variance and Duncan Multiple Range Test.

3. Results

3.1 Effect of Method of Extraction

Mortality of fruit fly treated with the leaf extracts of the different extracts prepared by four extraction methods and four extracting solvents were recorded and presented in Figure 1. The insecticidal experiments were replicated thrice. Observations were done at 3, 6, 12, 18, 22 and 24 h to determine which of the extraction methods and solvents would be the best in extracting the insecticidal properties of *A. indica* against fruit fly, *Bactrocera dorsalis* (Hendel).

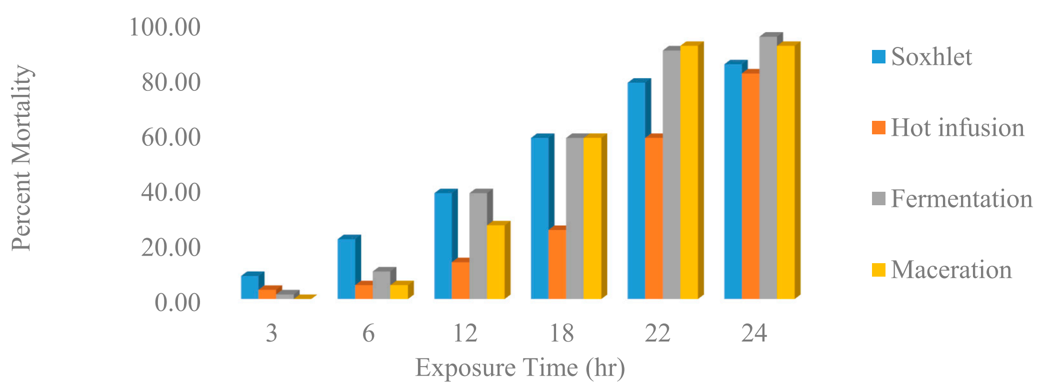


Figure 1. Effect of methods of extraction on the anti-fruit fly properties of neem leaves extract.

It was observed that Soxhlet extraction showed higher mortality at three (3) and six (6) h of exposure irrespective of what solvent was used (Figure 1 and Appendix A). This effect can be due to the higher amount of bioactive components extracted in this method. The total phenolic content and antioxidant activity of *Quercus infectoria* Galls was reported to be higher in Soxhlet technique than supercritical carbon dioxide extraction [27]. Similar study on the total phenolic and anti-radical capacity of extracts from *Pinus radiata* bark showed that total phenols and tannin content was highest in Soxhlet technique compared to microwave-assisted and ultrasound-assisted extractions [32]. Soxhlet extraction causes a more rapid rupture of cells facilitating the release of their contents [29]. This may explain why the neem leaves extract via Soxhlet extraction showed mortality against fruit flies at six (6) h which is higher than those from hot infusion, maceration and fermentation. At 12, 18 and 22 h exposure, on the other hand, hot infusion gave the lowest mortality, while Soxhlet, fermentation and maceration gave no significant differences. At 24 h, the extraction method has no significant effect on the mortality.

3.2 Effect of Solvent on the percent mortality

The effect of green solvents used in the extraction of the insecticidal properties of neem leaves is shown in Figure 2 and Appendix B. Mortality rate was observed to be comparable at 3 and 6 h exposure of the fruit flies to the different extracts. At 12, 18, 22 and 24 h, rice wash, vinegar and distilled water gave no significant differences on the mortality, though it can be observed in the graph that vinegar gave the highest percent mortality from 12-24 h exposure. Rice wine gave the lowest mortality from 3-24 h. This can be attributed to the volatiles from rice wine such as ethanol and acetic acid. Fruit flies are attracted to some volatile wine and vinegar compounds. The fruit flies which did not die immediately has thrived with the wine components.

In contrast to rice wine, vinegar which also has undergone fermentation and might contain some volatile compounds that attract fruit flies, was able to register the highest mortality. Vinegar contains polyphenols that have antioxidant properties [33]. The polyphenols in vinegar in addition to the bioactive components extracted from the neem leaves might have the synergistic effect on the anti-fruit fly activities giving the highest mortality after 22 and 24 h.

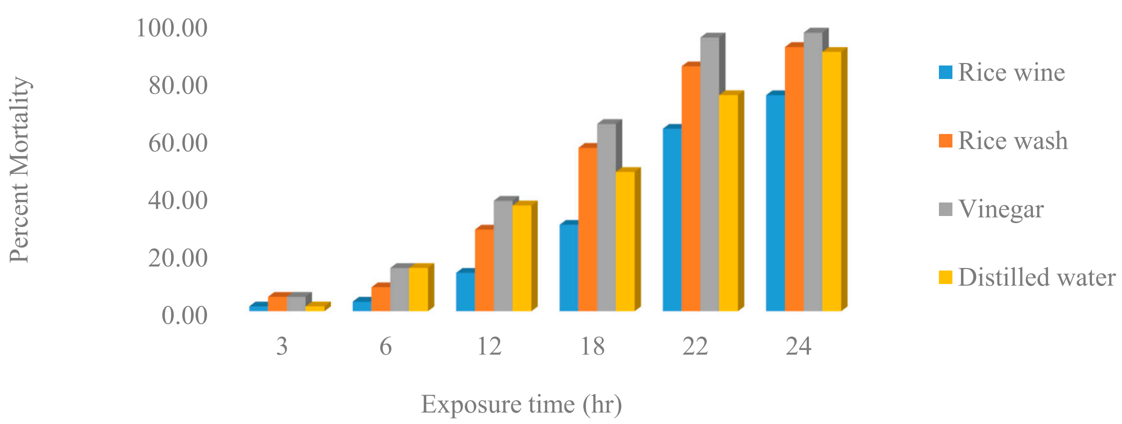


Figure 2. Effect of green solvents on the anti-fruit fly properties of neem leaves extract.

The effect of rice wash as solvent cannot be disregarded in this study. It is not significantly different with the vinegar and distilled water from 12 – 24 h exposure time. In fact, it is higher than the mortality of the distilled water extract. The use of rice wash in the extraction of phytochemicals is not being practiced by the green scientists.

3.3 Interaction of Solvent and Extraction Method

Analysis of Variance of the interaction between solvent and extraction method showed significant differences at 5% level (Table 1). There were sixteen *extraction x solvent* combinations in this study. Soxhlet-vinegar (S-V) gave the highest mortality rate against *Bactrocera dorsalis* (Hendel) but not significantly different from fermentation-vinegar (F-V), Soxhlet-rice wash (S-RWa), maceration-vinegar (M-V), fermentation-distilled water (F-D), fermentation-rice wash (F-RWa), and Soxhlet-distilled water (S-D). In terms of utilization of materials, fermentation using rice wash is more economical. Rice wash is a waste material and does not have value at all. Fermentation is done without the use of electricity. The rice wash and the organisms in the neem leaves will ferment even without the addition of commercial yeast.

Table 1. Effect of extraction method and solvent interaction on the percent mortality of *B. dorsalis*.

Extraction Method	Mean % Mortality (Extraction x Solvent Interaction)*			
	Rice Wine (RW)	Rice Wash (RWa)	Vinegar (V)	Distilled Water (D)
Soxhlet (S)	27.78^{g}	54.44^{ab}	61.11^{a}	50.00^{abcd}
Hot infusion (HI)	15.56^{h}	37.78^{defg}	40.00^{cdefg}	31.11^{fg}
Fermentation (F)	35.56^{efg}	51.11^{abcd}	56.67^{ab}	52.22^{abc}
Maceration (M)	45.56^{bcde}	40.00^{cdefg}	52.22^{abc}	44.44^{bcdef}

* - Means with the same letter superscript are not significantly different at 5% level using Duncan's Test.

3.4 Phytochemical Analysis of the Different Extracts

The phytochemicals present in the different extracts are affected by the solvent-extraction interaction (Table 2). Coumarins are reported to be present in the uncrushed twigs of neem [34]. The fresh matured leaves, on the other hand, contains saponins (high concentration), tannins and glycosides (moderately high), while alkaloids, terpenes and flavonoids (low) [35]. The different extracts have different components, which means that the combination of extracting solvent and method affect the secondary metabolites that can be extracted.

Table 2. Phytochemicals present in the different extracts.

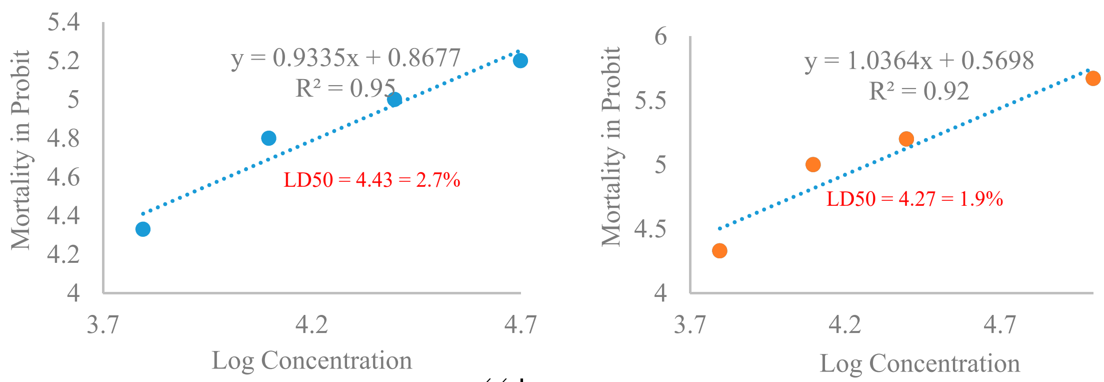
	Alkaloids	Coumarins	Cardiac Glycosides	Flavonoids	Saponins	Tannins	Terpenoids
Soxhlet							
Rice Wine	+	+	+	+	+	-	+
Rice Wash	-	+	-	+	+	+	+
Vinegar	-	+	-	+	-	-	+
D. Water	-	+	+	+	-	+	+
Hot Infusion							

Rice Wine	+	+	+	+	+	-	+
Rice Wash	-	+	-	+	-	-	+
Vinegar	-	+	+	+	+	-	+
D. Water	-	+	+	+	+	+	+
Maceration							
Rice Wine	+	+	+	+	+	-	+
Rice Wash	+	+	-	+	-	+	+
Vinegar	-	+	+	+	+	-	+
D. Water	-	+	+	+	+	-	+
Fermentation							
Rice Wine	+	+	+	+	+	-	+
Rice Wash	+	-	+	-	+	-	+
Vinegar	-	+	-	+	+	-	+
D. Water	-	+	+	-	-	+	+

Legend: + indicates the presence of the phytochemical and - means absent.

3.5 Lethal Concentration of the Extracts (with high mortality rates)

The lethal concentrations of the fermentation – rice wash extract (F-RWa) (Figure 3a) and Soxhlet – vinegar (S-V) extract (Figure 3b) were calculated using Probit analysis. This was done to compare the lethality of the two extracts, S-V being the one with the highest interaction effect and F-RWa being the most economical among the sixteen *extraction x solvent* methods defined in this study. The lethal dose for F-RWa was computed to be 2.7% v/v of the extract while 1.9% v/v for the S-V extract. This shows that neem extracts prepared by fermentation using rice wash and Soxhlet extraction using vinegar are effective insecticides against *B. dorsalis*.



(a) (b)

Figure 3. Graphical presentation of the lethal dose for (a) F-RWa and (b) S-V extracts.

4. Discussion

Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

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5. Conclusion

In conclusion, green extraction methods and green solvents are effective in extracting the insecticidal components of neem leaves. Fermentation – rice wash and Soxhlet – vinegar extractions are effective extraction – solvent combinations in extracting the insecticidal properties of neem against fruit flies, *B. dorsalis*. However, extraction via fermentation using rice wash as solvent is more economical than using Soxhlet method and vinegar as solvent. Soxhlet employs continuous heating for eight hours where electricity and water are being consumed. While fermentation do not use electricity and water during the process and rice wash is a waste. The latter is a new technology which can be used in the formulation of biopesticide from neem leaves against *B. dorsalis*. This is the first report that rice wash is used as extracting solvent in green synthesis.

Supplementary Materials: None

Author Contributions:

This research article is a work of three authors. Conceptualization, Danila Paragas, Kathlia Cruz and Elaida Fiegalan; Data curation, Danila Paragas; Investigation, Danila Paragas; Methodology, Danila Paragas, Kathlia Cruz and Elaida Fiegalan; Resources, Danila Paragas, Kathlia Cruz and Elaida Fiegalan; Supervision, Kathlia Cruz and Elaida Fiegalan; Validation, Danila Paragas, Kathlia Cruz and Elaida Fiegalan; Visualization, Danila Paragas and Kathlia Cruz; Writing – original draft, Danila Paragas; Writing – review & editing, Danila Paragas and Kathlia Cruz.

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Appendix A Effect of method of extraction on percent mortality of *Bactrocera dorsalis* (Hendel).

Method of Extraction	Percent Mortality at Different Exposure Time (hour)*					
	3	6	12	18	22	24
Soxhlet	8.33^{a}	21.67^{a}	38.33^{a}	58.33^{a}	78.33^{ab}	85.00^{a}
Hot Infusion	3.33^{ab}	5.00^{b}	13.33^{b}	25.00^{b}	58.33^{b}	81.67^{a}
Fermentation	1.67^{ab}	10.00^{b}	38.33^{a}	58.33^{a}	90.00^{a}	95.00^{a}
Maceration	0.00^{b}	5.00^{b}	26.67^{ab}	58.33^{a}	91.67^{a}	91.67^{a}

*-Means with the same letter superscript in a column are not significantly different at 5% level using DMRT.

Appendix B. Effect of solvent on the percent mortality of *Bactrocera dorsalis* (Hendel).

Solvent	Percent Mortality at Different Exposure Time (hour)*					
	3	6	12	18	22	24
Rice Wine	1.67	3.33	13.33^{b}	30.00^{b}	63.33^{b}	75.00^{b}
Rice Wash	5.00	8.33	28.33^{ab}	56.67^{a}	85.00^{ab}	91.67^{a}
Vinegar	5.00	15.00	38.33^{a}	65.00^{a}	95.00^{a}	96.67^{a}
Distilled Water	1.67	15.00	36.67^{a}	48.33^{ab}	75.00^{ab}	90.00^{ab}

*-Means with the same letter superscript in a column are not significantly different at 5% level using DMRT.