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RESEARCH PAPER

Formulation of Salicylic Acid Nanoemulsion with its Insecticidal and Antimicrobial Activities

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A B S T R A C T:

Pesticides play a vital role in modern agriculture and have a direct effect on yield gain. As a result, new formulations to combat agricultural pests and diseases are developed consistently. Thus, new agro-based formulations whose active ingredients are made up of inert materials have been emphasized. A new nanopesticide formulation from salicylic acid as a main active ingredient that can be applied against a range of plant pests and pathogens including fungi, bacteria and insect pests. The new product also contained oleic acid, Tween 80, and distilled water using a sonicator. The nanoemulsion was tested for its insecticidal effect against two stored insect pests (Sitophilus zeamais and Oryzaephilus surinamenses), four fungi (Alternaria solani, Fusarium oxysporum, Diplodia sp., and Bipolaris sp.)for antifungal activity and four bacteria (Ralstonia sp., Xanthomonas sp., Bacillus subtillis, and Pseudomonas fluorescence) for antibacterial activity.

The results displayed that the newly synthesized product had a significant impact on the insect pests utilized in the experiment and the variation in percentage mortality in accordance with the insect species used in the experiment has been observed. Hence, *Oryzaephilus surinamensis* was more susceptible than *Sitophilus zeamais* during the fourth week of exposure time mortality (86.66 and 70%) respectively. In addition, the prepared nanoemulsion was effective against *A. solani*, and *F. oxysporum* with inhibition zone (23.45 and 19.66) respectively. However, the product didn't exhibit any toxicity against the tested bacteria.

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1. INTRODUCTION

Conventional chemical pesticides have been employed on a large scale especially in developing countries in their efforts to minimize insects and disease infection in order to increase crop yield and preserve adequate food. However, controversy exists over the global dependence on such agents, given their excessive misuse, volatility, long-distance transport, adverse health effects on consumers and eventual environmental contamination (Jaacks et al., 2022). For decreasing the indiscriminate use of conventional pesticides to be in line with safe environmental applications the use of nanotechnology (i.e. nanopesticides) has been incorporated into the process and is in the initial stage of development (Chhipa, 2019).

The smart delivery of nanopesticides can afford controlled release kinetics, while powerfully improving permeability, stability, and solubility. Further, it can enhance pest-control efficiency over extended periods by stopping the premature degradation of active ingredients which is essential to reduce the dosage to overcome pesticide loss (Kumar et al., 2019). Due to their tiny size and large surface area, nanoparticle formulations of pesticides have been proposed to create a better spatial distribution of pesticides on leaf surfaces, and leading to increase efficacy on treated crops.

Post-harvest infestation whether disease or insect pests consider a serious problem that causes economic damage and deteriorates the quality of food products (Upadhyay and Ahmad, 2011). In the past, the majority of insect control programs inside food commodities were heavily dependent

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upon chemical pesticides, but in recent years the number of insecticidal compounds that can be used to control insect pests inside food commodities has been severely reduced. New regulatory requirements for current insecticides, consumer preferences for reduced chemical use, and the high costs of developing and registering new replacement insecticides have all contributed to this decline (Arthur and Phillips, 2003).

Therefore, due to a dearth of research on the utilization of salicylic acid as a nanoemulsion this study was performed to find a suitable solution to assist more practical use of safe natural and efficient pesticides. Hence, a new product was synthesized with a unique efficiency to combat insect pests and plant pathogenic fungi and bacteria.

2.MATERIALS AND METHODS

2.1Nanoemulsion Preparation

Nanoemulsion was prepared following the procedure used by Begum et al. (2019) with some modification. 3% of Salicylic acid (Biochem company, France) was used along with the excipients including oleic acid, Tween 80 and distilled water. Forty milliliters of Tween 80 were measured in a 250 ml flask then three grams of Salicylic acid was added. Ten milliliters of Oleic acid were measured and placed in a 100 ml glass bottle, then 47 ml of the distilled water was added. All three containers were placed in an ultrasonic water bath (70ºC) till salicylic acid dissolved to leave a pure solution. When all three containers reached an equal temperature (70ºC), salicylic acid dissolved in Tween 80 was added to oleic acid in the 100 ml glass bottle. Then followed by the gradual addition of distilled water to this mixture along with occasional shaking using a vortex until a cloudy nanoemulsion was formed. The nanoemulsion was then cooled to room temperature and kept between 4ºC to 10ºC.

2.2Characterization of the Nanoemulsion

2.2.1 Assessment on insects

The new-formulated pesticide was assessed on two common stored insect pests, *Oryzaephilus surinamensis* and *Sitophilus zeamais*. To achieve this, a serial dilution of the pesticide was prepared which were 2500, 1250 and 625 ppm. Rice (5 grams) infested with O. *surinamensis* was placed in petri plates at a rate of 10 adults per plate and smashed chickpea infested with S. *zeamais* was also placed in separate plates containing 10 adults per plate in a completely randomized design (CRD) in three replicates. Then each insect pest separately, were sprayed with prepared concentration at a rate of 0.5 ml/plate, using a 50 ml spray bottle. Distilled water was sprayed as an untreated control. The plates were then incubated at (25^{o}C) and examined weekly for the mortality rates according to the following formula:

Mortality = No. of dead insects/ Total No. of insects * 100

2.2.2 Assessment on fungi

The new pesticide formulation was evaluated on 4 plant pathogenic fungi which were *Alternaria solani*, *Fusarium oxysporum*, *Diplodia sp.* and *Bipolaris sp.* which have previously been identified and kept in the fungal bank in the department of Plant Protection college of Agriculturl Engineering Science university of Salahaddin. From each fungus, three plates of fresh cultures were prepared by spreading 100 µL of spore suspension from each fungus and letting it grow for 7-10 days to cover the surface of the plates in an incubator. Various concentrations of two-fold dilution from the newly formulated Nanoemulsion (10000, 5000, 2500, 1250 and 625 ppm) were prepared and then each concentration was applied to all 4 types of fungi using the disc diffusion method with three replicates along with the untreated control which was distilled water only. The discs were soaked into each pesticide concentration and then placed at the center of the plate (Cooper and Dobson, 2007). The plates were incubated at 25^{o}C in an incubator for one week then the inhibition zones were measured using the digital caliber vertically and horizontally and then the averages were calculated.

2.2.3 Assessment on bacteria

The produced nanoemulsion was evaluated against some common plant pathogenic bacteria which included *Ralstonia sp.*, *Xanthomonas sp.*, *Pseudomonas fluorescens* and *Bacillus subtilis*. Bacterial suspension from each bacteria was prepared from bacterial cultures grown on nutrient agar (NA) by adding 10 mL of sterilized distilled water (SDW) to the petri dish surfaces, then spread by a sterilized spreader to give a bacterial suspension. 100 µL of each bacterial suspension was taken with the aid of a micropipette and spread over the surface of previously prepared nutrient agar plates. The five concentrations of nanoemulsion were prepared (10000, 5000, 2500, 1250, and 625) ppm. Each concentration was applied for all types of bacteria using the disc diffusion method by Drago et al. (1999) along with the untreated control which was included plates inoculated with bacteria only. The

experiment was a complete randomized design with three replicates.

The discs were soaked into the pesticide and placed at the center of the plate containing the bacterial suspension. The plates were incubated at 25^{o}C for one week then the inhibition zone was measured and calculated. Data were analyzed using analysis of variance (ANOVA) and for comparisons, multiple range tests (P=0.05) were made using StatGraphics Centurion software.

2.2.4 Stability

To examine the pesticide stability, phase diagrams were constructed on a w/w basis to represent the points at which transparent, one-phase systems were found. The Sample that retained a transparent one-phase appearance after four weeks was taken to indicate the presence of a nanoemulsion following the method used by Flanagan et al. (2006). A tube of nanoemulsion was stored under room temperature 25±3^{o}C for four weeks and observed for any change in its physical appearance and then photographed weekly to observe if it kept its one-phase transparent appearance.

2.2.5 Thermostability

To test the thermostability of the nanoemulsion, the formulation was stored at incubator (25°C) for up to six months and then kept at 54°C for 14 days in an oven. The changes in the sample's physical appearance were visually observed. When the temperature of the nanoemulsion system is increased, the solubility of the non-surfactant decreases. The cloud point of the nanoemulsion can be defined as the temperature at which the transparent nanoemulsion solution becomes cloudy, i.e. from one phase to two phases or three phases (Chen and Tao, 2005).

2.2.6 Particle Size

The measurement of the globule size was performed by the mean of Dynamic Light Scattering & Zeta potential analyzer (Model: SZ-100z), (Horiba Jobin Jyovin HORIBA SZ100 Z Product, Japan). The light source was solid state laser diode, 532nm, 10mW, and the detectors were 3 Photomultipliers tubes, (2 for size rear/side PMT), 1 for Zeta measurements (Forward PMT)· The dispersion medium viscosity of the sample was 0.893 mPa·s (millipascal second) and temperature of the holder was 25.1^{o}C. Before measuring the particle size, all the impurities were removed by rinsing the containers with acetone thoroughly. The sample was loaded into a disposable cuvette (standard volume 1.2ml), which was also rinsed with acetone and air-dried in a fume hood previously, by the use of a disposable syringe holding microfilter. Filtering one drop per second of the sample into the inclined cuvette by 45^{o} to prevent the formation of the bubble then the filled cuvette was covered carefully with its lid and put on the sample holder to read the information and take the data.

2.2.7 Surface Tension

The surface tension of the nanoemulsion was measured using a Krüss BP100 bubble-pressure tensiometer (Krüss Company, Germany) at 25 ± 0.1 °C. Before the measurements, the instrument was calibrated with deionized water (72.0 ± 0.2 mN/m). The measurements were always carried out with effective surface ages ranging from 10 to 100000 ms. The contact angle for the diluted nanoemulsion (2ml of deionized water was added to 1ml of the pesticide) on the hydrophobic substrate was measured with a DAS-100 drop shape analyzer (Krüss Company, Germany) at 25 ± 1 °C where the environmental humidity was kept constantly at 50 ± 5%. Paraffin film was chosen as a solid substrate. According to previous research, the contact angle of deionized water on paraffin film is around 106 °, 29 so. The deionized water was used to check the instrument prior to the measurements.

2.2.8 Shelf Life

The nanoemulsion pesticide was tested for its shelf life (directly after the formulation was made), 1, 2, 3, 4, 5, 6, 7 and 8 months following the method used by Rashid (2016). The plant pathogenic fungus, *A. solani* was used to test the efficiency of the product. To achieve this, three petri dishes of fresh cultures were prepared by spreading 100 µL of the fungus spore suspension and letting it grow for 7-10 days to cover the surface of the petri dishes. The pesticide applied on the fungus using the disc diffusion method. The discs were soaked into the pesticide and then placed at the center of the petri dish. The petri dishes were incubated at 25^{o}C for one week then the inhibition zones were measured using the digital caliber vertically and horizontally and then the averages were calculated.

3 Results

3.1 Assessment on Insects

The laboratory test conducted using various concentrations of a new nanoemulsion pesticide exhibited a significant effect on the mortality of

Sitophilus zeamais adult stage (F(3,47) =73.55, P <0.001). The highest concentration 2500 ppm, with a 58.33% mortality rate was not significantly different from 1250 ppm with a 46.67% mortality rate, while the control treatment attained the lowest mortality 3.33% (Figure 1a).

Percentage mortality showed significant variation in accordance with the time adults were subjected to the pesticides (F(3,47) = 120.58., P <0.001, Figure 1b). Thus, the highest and lowest mortality (70 and 0%) was recorded during the fourth and first week of exposure respectively.

Likewise, the synthetic nanopesticide concentration (F(3,47) =63.61, P <0.001) significantly affected *Oryzaephilus surinamensis* adult mortality. The most efficient dose was 2500 ppm which recorded 78.33% , followed by 1250 ppm and 625 ppm which recorded (65 and 58.33%) mortality respectively (Figure 2a).

Moreover, mortality is significantly influenced by exposure time (F(3,47) =60, P <0.001). As a result, fewer individuals survived when subjected for a longer period of time to the nanoemulsion. The highest mortality was obtained at 86.67% after four weeks whereas the lowest mortality was recorded after one week of treatment 30.00% (Figure 2b).

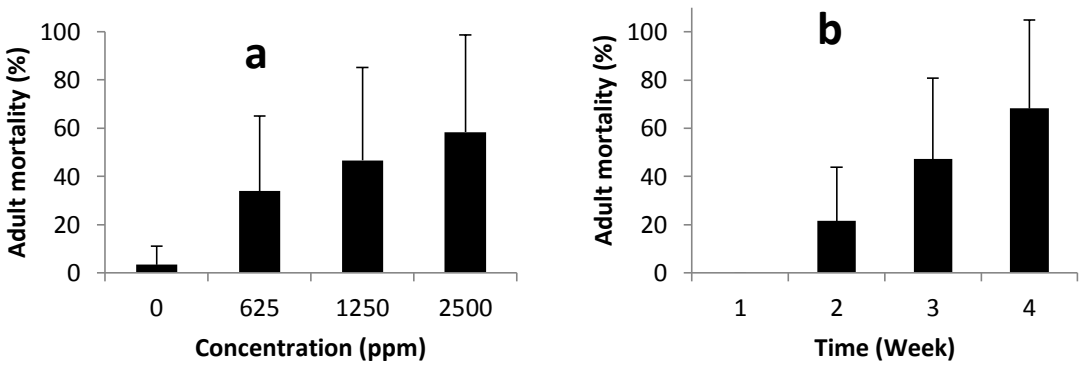

Fig 1: Adult mortality of *Sitophilus zeamais* according to (a. concentration of the product) and (b. time). (Error bars represent Standard Deviation)

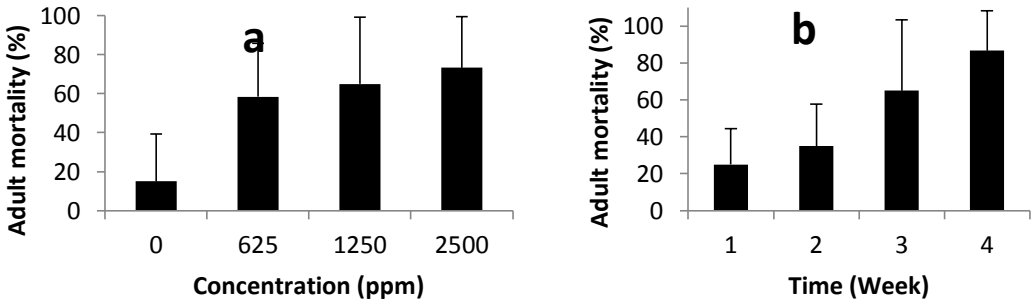

Fig 2: Adult mortality of *Oryzaephilus surinamensis* according to (a. concentration of the product) and (b. time). (Error bars represent Standard Deviation)

Insect species examined on the basis of LC_{50} values

The toxicity of the nanoemulsion pesticide was examined on the adult stage of both *Sitophilus zeamais* and *Oryzaephilus surinamensis* inside Petri dishes and the result revealed that the saw-toothed grain beetle was more susceptible than the granary weevil with an average LC_{50} of 1012.18 during the experiment time. Additionally, there was a strong correlation between the length of exposure and changes in LC_{50} values and thus, minimum values (305.43 and 32.74) were attained in the last week of treatment for *S. zeamais and O. surinamensis* respectively (Table 1).

Table 1: The LC_{50} values (ppm) of the product under different exposure times.

Stored product insect type	Time (weeks)	LC $_{50}$ (ppm)	Average
S. zeamais	1	5799.64	
	2	2094.28	
	3	1139.43	
	4	305.43	2334.70
O. surinamensis	1	1878.51	
	2	1662.85	
	3	474.63	
	4	32.74	1012.18

3.2 Assessment on Fungi

The results of fungal assessment study showed that the new formulated nanoemulsion suppressed the growth of *Fusarium oxysporum* ($F(5,17) = 126.7$., $P < 0.001$) and *Alternaria solani* ($F(5,17) = 27.85$ $P < 0.001$) significantly. At the concentration of 10000 ppm, the pesticide produced the highest inhibition zone against *A. solani* (28.6mm). The results also showed that the concentrations of 10000, 5000, and 2500 ppm inhibited the growth of both tested fungi,however, at the concentrations of 1250, 625, no inhibition zones were noticed (Figure 3a and b).

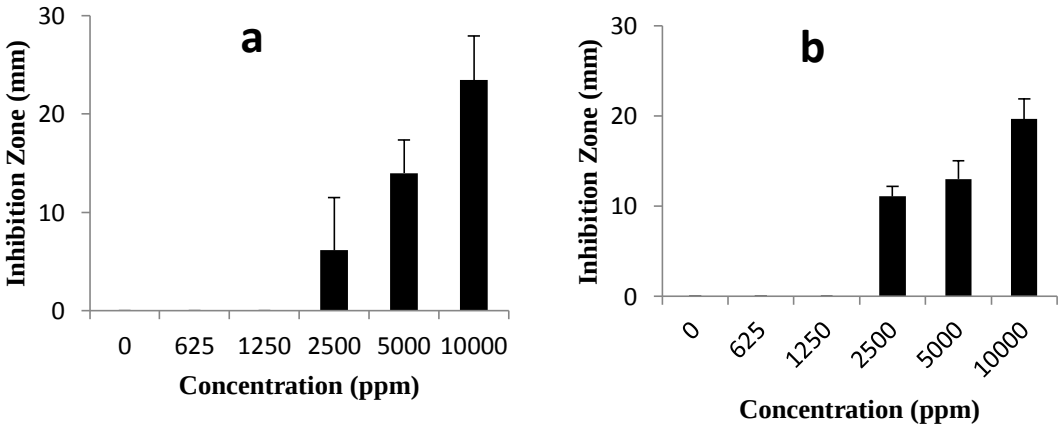

Fig 3: Inhibition zone of the pesticide against (a. *Alternaria solani*) and (b. *Fusarium oxysporum*) (Error bars represent Standard Deviation)

3.3 Assessment on bacteria

3.4 The results indicated that there was no inhibition impact of the nanoemulsion detected on all tested bacteria. **Stability**

After storing a tube of the pesticide for four weeks and observing its appearance weekly, the result showed that the sample kept its one-phase appearance and no separation had happened throughout the four weeks of storage.

3.5 Thermostability

The results showed that the nanoemulsion was stable for three months of storing at room temperature (25^{o}C) (Figure 4a), but after that, it started to become cloudy (Figure 4b) and the separation of the emulsion occurred in the last month (Figure 4d). And after exposing for high temperature (54^{o}C) for two weeks, the clustering was detected.

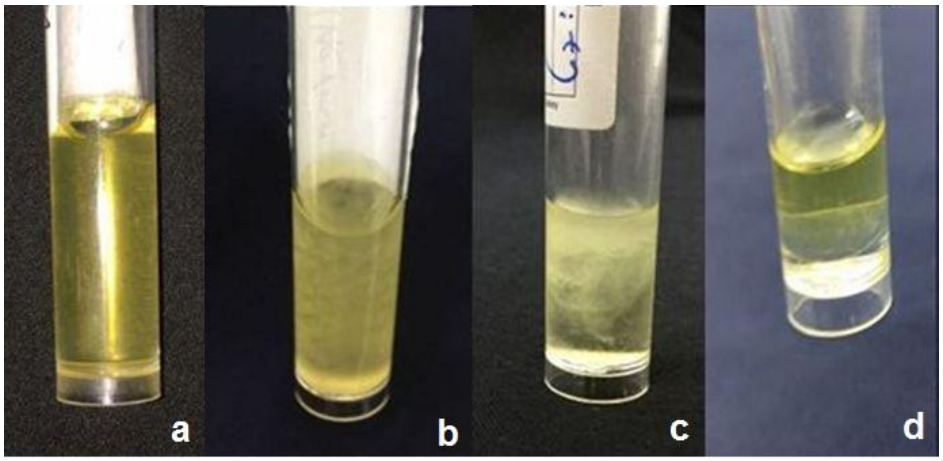

Fig 4: Thermostability of the nanoemulsion (a. first day of the formulation, b. after four months, c. five months and d. six months of storing at 25^{o}C)

3.6 Particle Size

The particle size analysis of the formulation was shown in (figure 5). The mean globule size was (92.9 nm).

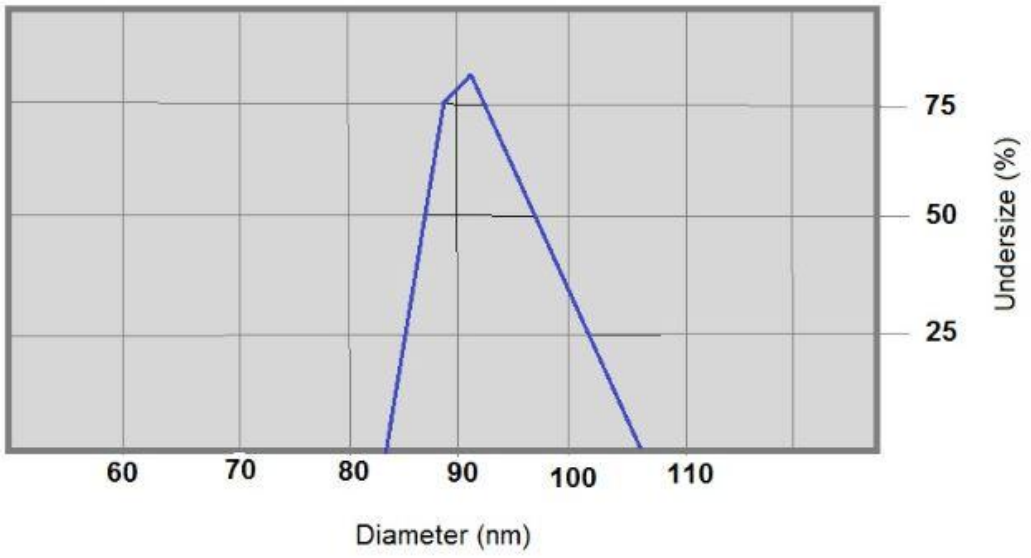

Fig 5: A typical particle size distribution by DLS of the formulated nanoemulsion.

3.7 Surface Tension

The surface tension at room temperature 25±3^{o}C was significantly lower than the control (water) sample which had 72 mN/m, while the surface tension of the product was 31.97 mN/m (Milli-Newtons per meter).

3.8 Shelf Life

The results of measuring shelf life against the fungal pathogen *A. solani* showed that the inhibition zone had the largest size right after formulating (30.8 mm), then the inhibition zone declined gradually at each month of the test until reaching the lowest degree (10.9mm) at the 8th month which was the last test of measuring shelf life (Figure 6).

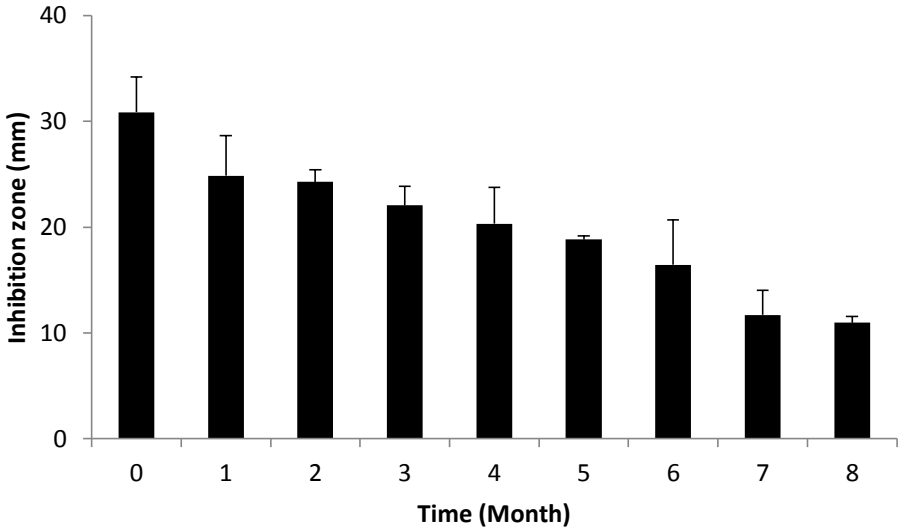

Fig 6: Shelf life of the pesticide against *Alternaria solani* throughout 8 months. (Error bars represent Standard Deviation)

Discussion

The nanoemulsion was chosen as the delivery system for use as an antibacterial, antifungal, and insecticidal formulation. In this work, the bioactivity of salicylic acid nanoemulsion against *Sitophilus zeamais* and *Oryzaephilus surinamensis* was investigated to provide an innovative strategy for the management of insect pests attacking stored products. Previous research by Duarte et al. (2015) suggested that the nanoemulsion contains 5% (w/w) of Rosemary (*Rosmarius officinalis*) essential oil, 5% of polysorbate 20 and 90 mL of water can be considered as promising insecticidal agents.

Antifungal activities of the product were determined by the inhibition zone as shown in the (figure 3), which showed high efficacy against 2 of tested fungi out of 4, which were *Alternaria solani* (with 23.44 mm inhibition zone) and *Fusarium oxysporum* (with 19.66 mm inhibition zone). Similar results were observed by Sangpueak et al. (2021) they also used Salicylic acid as a fungicide, and they concluded that the most efficacy for induction was obtained at concentrations of 200 ppm of SA compared to a conventional fungicide. They also added that SA can reduce disease severity by 33.3% compared with the control. The current results are also in line with that of Jendoubi et al. (2015)'s work the researchers observed that applying of SA at 200 µM in hydroponic tomato plants infected by Fusarium wilt minimizes the disease from 70% to 9% only.

The formulated pesticide remained stable for four weeks at room temperature (25±3^{o}C) and kept its transparent appearance and no separations were noticed. The formulation was also stable at 25 ◦C for three months, however separation of the phases occurred in the fourth month and after exposure to high temperature (54^{o}C) agglomeration occurred. In the same manner in stability studies on nanoemulsion Singh and Vingkar (2008) observed that the product remains stable for three months at 25 - 30°C. This indicated that by increasing the temperature, the kinetic motion of molecules was increased to force the surfactants to be loosely adsorbed on the oil-water interface, thus increasing the probability of collision and coalescence, therefore destabilizing the emulsions system (Chen and Tao, 2005).

Based on earlier researches by (Sonneville-Aubrun et al., 2004, Lett, 2016), were considered the emulsion with an average droplet diameter (Z) lower than 300 nm is a nanoemulsion. Herein, our nanoemulsion showed Z-average size of 92.9 nm which is in the range of nanoemulsion formulation size.

The result of surface tension displayed 31.97mN/m which is in accordance with the results stated by Zainuddin et al. (2019) who tested two formulated nanoemulsions and found the tension values of 38.62 mN m and 37.32 mN m^{-1}.

The test of shelf life was carried out over eight months, and showed the highest inhibition zone at the start of the test (30.8mm), and remained active at the eighth month showing an inhibition zone of

10.9mm. In a determination of shelf life, high inhibition zones of *Xanthomonas vesicatoria* and *Ralstonia solanacearum* were observed with values of 22.3 to 21.7 mm and 20.3 to 18.3 mm respectively (Rashid, 2016). However, it is noticed that the antibacterial activity of the nanoemulsion formulations was almost similar from the start of the test to the end on *X. vesicatoria.*

4. CONCLUSIONS

The new pesticide formulation demonstrated a significant impact against the two species of fungi (*Alternaria solani* and *Fusarium oxysporum*) and the stored insects (*Oryzaephilus surinamenses* and *Sitophilus zeamais*). However, the product did not show any activities against the species of bacteria. Therefore, it can be conclude that the new formulation of pesticide can be used as a fungicide and an insecticide to solve a range of agricultural pest problems. Further, the formulated nanoemulsion has a small particle size that exhibits its efficacy with good stability when stored at room temperature and remained active for eight months which indicates a decent shelf life.

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