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Comparison of nanopellets formulation with phostoxin against five important pests of stored products

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Abstract
The resistance to synthetic pesticides and harmful effects of their accumulation in the environment has created the need for natural and non-persistent insecticides. The aim of this work was using controlled release with technology to solve the stability of essential oil in applying places with two formulations. In this research, Nanoemulsion of Eucalyptus essential oil based pellets were prepared by Dry Mixing Method and physical load of Nanoemulsion of Eucalyptus essential oil on PVA, which followed by pressing the mixture, using pellet maker apparatus, to form pellets. Insecticidal efficacy of produced pellets was investigated against adults (1-3 days old) of *Tribolium castaneum* (Herbst), *Callosobruchus maculatus* (F.), *Rhyzopertha dominica* (F.), *Oryzaephilus surinamensis* (L.) and *Sitophilus oryzae* (L.) under 28±2 ºC and darkness in laboratory condition. The mortality of two different formulations was recorded against mentioned pests and compared with phostoxin. The phostoxin showed 100% mortality after 24h in all studied pests other than *Rhyzopertha dominica* and Nano formulated pellet (formulation I) showed better mortality in compare with formulation II. The stability of pellet recorded in four month against *Sitophilus oryzae* phostoxin revealed 100% mortality in all months but the efficacy of formulation I decreased after second month with 27.2% and formulation II was effective with 71.2% mortality.

Keywords: Nanoeemultion, Eucalyptus, Controlled release, 1, 8-Cineole, PVA, pellet formulation, fumigant toxicity.

1. Introduction

The protection of stored products against insect pests has been a major problem from the development of agriculture. Control of this kind of insect relies heavily on the use of gaseous synthetic insecticides and fumigants, which has led to problems such as ozone depletion, environmental pollution, increasing costs of application, pest resurgence and resistance and hazard effects on non-target organisms in addition to direct toxicity to users [1, 2]. In many storage systems, fumigants such as methyl bromide (MeBr) and phosphine (PH3) are the most economical and convenient chemicals for managing stored-product insect pests, not only because of their ability to kill a broad spectrum of pests but also because of their easy penetration into the product while leaving minimal residues [3]. The wide use of MeBrand PH3 is being restricted owing to potential ozone-depleting properties and to increasing resistance in stored-product insects respectively [4]. In addition, there have been some arguments about the genotoxicity of phosphine [5]. In order to control this kind of species without disturbing the environment, natural products have been screened for potential sources of repellents and insecticides [6]; in fact, plant essential oils have traditionally been used to kill or repel insects [7], being considered as an alternative to stored-grain conventional pesticides because of their low toxicity to warm-blooded mammals and their high volatility [8] and are also bio-degradable [6]. Eucalyptus is a diverse genus of flowering trees and shrubs in the Myrtle family, Myrtaceae. The oil extracted from eucalyptus leaves possesses allelopathic property and prevents insects from attacking it, thereby, acting as a natural pesticide [9, 10]. In assays, such as, fumigation activity [11] and repellency [12], the efficiency of eucalyptus oil has been demonstrated [13]. Controlled release technology has emerged as an alternative approach with the promise to solve the problems accompanying the use of some agrochemicals, while avoiding possible side effects with others [14]. The overall aim of controlled release formulation consists of protecting the supply of the reagent and allowing its automatic delivery to the target at controlled rates to maintain its concentration within an optimum concentration over a long period of time.

Also controlled release technology could assist in improving protection for stored grains against insect and rodent pests [15]. Nanoemulsions are emulsions whose droplet size is uniform and extremely small with the size ranging from 20 to 200nm. The use of Nanopesticides would be a contemporary measure for the control of pests and reducing the toxic effect of synthetic bulk pesticides on the environment [16]. Poly (vinyl alcohol) (PVA) is a water soluble polymer based on petroleum resources with unique properties such as good transparency, lustre, antielectrostatic properties, chemical resistance and toughness [17]. It has also good gas barrier properties and good printability. Its water solubility, reactivity, and biodegradability make it a potentially useful material in biomedical, agricultural, and water treatment areas [18]. Because it is a biodegradable polymer and cheap to make Poly (vinyl alcohol) has been used as a polymer matrix for encapsulation of the pharmacological reactive agents [19]. In present study we loaded Nanoemultion of Eucalyptus essential oil, on Poly (vinyl alcohol), biodegradable polymer, and evaluated insecticidal efficacy of produced pellet formulation, against five stored product pests; we also calculated the stability of pellet in different time period.

2 Material and methods

2.1 Chemicals

These essential oil was purchased from Giah essence Industry Co, Ltd, Golestan Province, Iran Poly (vinyl alcohol) (Molar Weight: 72000 g/mol; hydrolysis mole: 98) was purchased from Merck (Darmstadt, Germany).

2.2 Insects

All culturing insects were obtained from laboratory cultures from Tehran University, stored product pests incubation room other than Rice weevil that collected from Rice store, located in the main market of Rasht, Iran. Cowpea beetle (*Callosobruchus maculatus* (F.)), Rust red flour beetle (*Tribolium castaneum* (Herbst)), lesser grain borer (*Rhyzopertha dominica* (F.)), saw-toothed grain beetle (*Oryzaephilus surinamensis* (L.)) and Rice weevil (*Sitophilus oryzae* (L.)) were reared on cowpea, flour mixed with yeast (5% W/W), wheat and grain at 12-13% RH, Respectively. All culturing media were purchased from a local market and kept in a freezer at -5 ºC for 48 h and then were used as culturing medium. Experimental procedures were carried out at 28±2ºC in a dark room, and 65±5% RH.

2.3 Preparation of Nanoemulsion

The oil-in-water Nanoemulsion was formulated using eucalyptus oil, non-ionic surfactant (tween 80) and water. The concentration of eucalyptus oil (3%, v/v) was fixed for formulation.

2.4 Pre-polymer preparation

One hundred and twenty grams of recrystallized urea and 225 mL of 37% formaldehyde solution were charged into a three neck flask, equipped with mechanical stirrer, reflux condenser and water bath. The pH of solution was adjusted to 8-8.3 by adding of NaOH solution. The solution was then heated to temperature of 70 °C. The reaction was run for 1 h, while its temperature was maintained at 70 °C. At the end of the reaction, this urea-formaldehyde pre-polymer resin was cooled and diluted with distilled water to be 500 mL of UF resin solution. The resin concentration was 0.42 g mL^{-1}.

2.5 Microencapsulation process

Microencapsulation process consists of 2 steps: Emulsion step and microcapsule shell formation step. In the emulsion step, UF resin was added with a certain amount of distilled water to make specified concentration of resin. Refined palm oil and UF resin solution were heated in water bath to 50-70 °C. As the temperature was reached, the mixture was stirred with ULTRA TURRAX homogenizer at the rotation speed of 15600 rpm for 10- 50 min. White milky viscous liquid was produced. After homogenizing process, the homogenizer was replaced with a helical type stirrer. In the step of microcapsule shell formation, the stirring speed was maintained around 100 rpm, in order to keep

2.6 Homogeneous phase

Very low stirring speed could result in coalescence of oil bubble (which leads to phase separation), but high speed stirring would break up microcapsule shell formation. Citric acid solution was then added to the liquid mixture, to adjust its pH to 3. This process was run for 3-6 h, while its temperature was maintained constant. At the end of the process, the liquid mixture was cooled by addition of ice and cold distilled water. After one h of cooling, microcapsules product appeared as white solid. Some solid product floated at the top of liquid and the other settled at the bottom. These two solid products were filtered, washed with distilled water and then dried at 40°C under vacuum condition. Dried top and bottom microcapsule products were weighed and analyzed its oil content. Certain amount of dried microcapsules was grounded and N-hexane was added to extract the palm oil from ground microcapsules. The remaining ground solid was then filtered, dried in an oven at 100 °C and finally weighed. The oil content can then be determined. A sample of microcapsule product to be measured its diameter was prepared by attaching a thin layer of microcapsule paste on a microscope slide glass. The microcapsules image was captured by optical microscope provided with digital camera. The resulting image (picture) was interpreted using Image Pro Plus software, in which the diameter of around 100 or more microcapsule bubbles (in one picture) could be measured [20].

2.7 Pellets preparation

PVA and Nanoeemultion of Eucalyptus essential oil mixture was prepared by Dry Mixing Method (DMM) in ice temperature on stirrer with 500 rpm for 4 h. Preparation of mixture was done in tight 100 ml balloons and mixing power provided with magnetic stirrer. Then prepared mixture was transferred to FTIR pellet maker apparatus (Thermo Nicolet part No. 0016-035, USA) and pellets were produced by hydraulic pressure under 100 Kg. Production of a pellet from prepared mixture was done in about 1 min. Average of ten 1 g pellets diameter and thickness were measured by calliper that were 1.3±0.1 and 0.93±0.01 cm, respectively. The pellet formulation I made by PVA without Nanoemulsion formulation of essential oil and pellet formulation II made by PVA with Nanoemulsion of essential oil.

2.8 Bioassays

Adults (1-3 days) were used as test insects. Experiments were carried out at 28±2ºC in a dark room, and 65±5% R.H. Mortality was assessed 24, 48 and 72 h after treatment. Three and five gram novel pellets were used as experimental traits and phostoxin pieces on this study were 0.25 and 0.3 g in weight. Barrels with 159 L volume and 20 L casks were used as fumigation chambers for Phostoxin and pellets, respectively. Fumigation chambers were sealed by parafilm and adhesive tape (5 cm diameter). Insects were located in small vials dangled from barrel and cask caps.

2.9 Data analysis
Mortality percentages were calculated by the Abbott correction formula for natural mortality in the untreated control (Abbott, 1925).

$$\text{Mortality (\%)} = \frac{\text{\% test mortality} - \text{\% control mortality}}{100 - \text{\% control mortality}} \times 100$$

Mortality data were altered in arcsin $\sqrt{x}$ then were analyzed using the SPSS 18.0 software. Mean comparisons were performed using one way analysis of variance (ANOVA). Tukey test at $\alpha = 0.01$ was used to determine the difference between the mean of mortalities. The factorial was used for stability test.

3 Results
3.1 Nanoemulsion formulation
A volume of eucalyptus oil (3%), Tween 80 and water was prepared using magnetic stirrer at 250rpm for 30min. The average diameter of 1:2 ratio of bulk emulsion was found to be 7224nm and PDI is 0.89. Also found to be unstable (phase separated) during thermodynamic stability study. This bulk emulsion was further subjected to ultrasonication method. The Nanoemulsion of 1:2 ratios was found stable after thermodynamic stabilization cycle. The emulsion showed no phase separation [21].

3.2 Mortality
Phostoxin. The mortality for phostoxin against *O. surinamensis* and *S. oryzae* was 100% in 0.3 g after 24 h, but for *C. maculatus* and *R. dominica* recorded 85.9 and 56.3 in 0.25 g mortality varied from 39.5% (*R. dominica*) to 94% (*Sitophilus oryzae*). The mortality in 48 h and the higher dose (0.3) for *R. dominica* was 92.1 and for the other was 100%.Three days after treatment (72 h) in all doses for all studied insect the mortality was 100%.

Pellet I. The lowest and highest mortality in 5 g after 24 h was 34.5% (*O. surinamensis*) and 48.3% (*C. maculatus)* respectively. *C. maculatus* and *R. dominica* showed 69.7, 71.9 and 65.9% respectively. The mortality in highest dose after 48 h was 47, 48.7, 51.3, 80 and 100 for *T. casteneum, O. surinamensis, S. oryzae, R. dominica* and *C. maculatus* respectively. The highest mortality after 72 h belongs to *R. dominica* and *C. maculatus* with 98.1 and 100% in 3 g and both of them had 100% mortality in 5 g dosage.

Pellet II. The lowest mortality in this formulation was 12.5% (*T. confusum*) in 3 g after 24 h and highest mortality was 100% for *C. maculatus* and *R. dominica* in 5 g after 72 h. The mortality after 72 h was 54.7, 62.9, 70.6, 73.3 and 88.7% for *T. casteneum, C. maculatus, S. oryzae, O. surinamensis* and *R. dominica* respectively in 3g dosage but in dosage 5g mortality was 100% for *C. maculatus* and *R. dominica* and 78, 79.5 and 81.4 for *T. casteneum, S. oryzae* and *O. surinamensis* respectively.

3.3 Stability
The mortality was recorded against *Sitophilus oryzae* that was 100% for phostoxin in all four month. Pellet I and Pellet II after 1 month showed 65.3 and 77.2% mortality respectively, and after four month the mortality was 27.2 and 78.4 for Pellet I and Pellet II respectively.

Table 1: Mortality percent± S.E. of different stored product beetles exposed to phostoxin 0.25 and 0.3 g pieces in 24, 48 and 72 h.

Insects	**Time**					
	24		**48**		**72**	
	0.25 g	**0.3 g**	**0.25 g**	**0.3 g**	**0.25 g**	**0.3 g**
Oryzaephilus surinamensis	79.9±3.4	100	100	100	100	100
Sitophilus oryzae	94±2.1	100	100	100	100	100
Tribolium casteneum	83±6.3	100	100	100	100	100
Callosobruchus maculatus	59.1±2.8	85.9±2.8	100	100	100	100
Rhyzopertha dominica	39.5±2.2	56.3±3.1	79.3±5.1	92.1±2.4	100	100

Table 2: Mortality percent± S.E. of different stored product beetles exposed to botanical pellet I 3 and 5 g in 24, 48 and 72 h

Insects	**Time**					
	24		**48**		**72**	
	3 g	**5 g**	**3 g**	**5 g**	**3 g**	**5 g**
Oryzaephilus surinamensis	21.3±2.6	34.5±3.1	32.1±4.4	48.7±5.8	53.2±5.4	69.7±1.9
Sitophilus oryzae	25.7±3.1	37.6±5.4	42.2±2.6	51.3±3.1	57.5±2.1	71.9±1.6
Tribolium casteneum	18.6±2.3	35±2.8	22±4.4	47±6.4	59.1±6.4	65.9±6.7
Callosobruchus maculatus	33.8±1.9	48.33±3.7	67.3±6.4	100	100	100
Rhyzopertha dominica	25.1±2.9	33.4±1.9	51.9±3.8	80.4±3.4	98.1±4.1	100

Table 3: Mortality percent± S.E. of different stored product beetles exposed to botanical pellet II 3 and 5 g in 24, 48 and 72 h

Insects	**Time**					
	24		**48**		**72**	
	3 g	**5 g**	**3 g**	**5 g**	**3 g**	**5 g**
Oryzaephilus surinamensis	15.7±3.3	25.3±2.5	26.7±1.9	59.3±2.4	73.3±6.6	81.4±5.1
Sitophilus oryzae	8.4±5.6	29.4±3.4	20.3±3.2	60.2±4.4	70.6±3.5	79.5±1.9
Tribolium casteneum	12.5±5.1	32.1±3.6	40.7±3.1	53.7±2.1	54.7±5.5	83.7±2.8
Callosobruchus maculatus	14.1±6.5	40.1±4.7	36.2±5.7	71.2±3.2	62.9±2.4	100
Rhyzopertha dominica	13.5±4.3	30.1±1.8	65.4±6.2	65.4±5.7	88.7±3.6	100

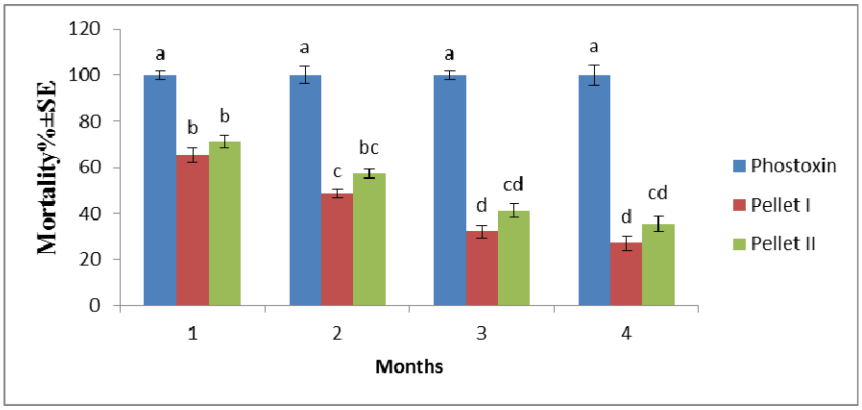

Fig 1: Stability test of two types of tablet nano formulation and phostoxin against *S. Oryzae.*

4. Discussion

Chemical insecticides such as phostoxin and methyl bromide have some disadvantages like genotoxicity, environmental pollution, resistance occurring in insect populations along with the risk of being misused too [4, 22, 23]. According to Montreal clean air act protocol, using mentioned chemical insecticides in storages is banned since 2005 in developed countries and since 2015 in developing countries like Iran.

The use of synthetic-based pesticides over the past few years has increased the resistance toward the stored products pests [24-26], thus, instilling the need for finding alternative ways to control this pests. The plant-based pesticides are in high demand, because, they are sustainable in the environment; they are safe and are also pleasant to use [27]. The plant based compounds have natural pesticide activity, because, they contain certain bioactive compounds that prevent them from being eaten by herbivores. Although, the main function of these compounds is defense against plant-eating insects, [28]. Eucalyptus oil is the oil distilled from the leaves of eucalyptus, a genus of the plant family Myrtaceae. Eucalyptus oil is among the essential oils that have been registered as an insect repellent by US Environmental Protection Agency (US EPA) [29]. The seed and leaf extract of eucalyptus oil contain compounds that are fumigant toxicity. The fumigant activity of Eucalyptus has been evaluated against similar insects [30-33]. Obtained results about susceptibility of experimental insects to Eucalyptus, confirm prior researchers findings [31, 34]. The steam distillation of aromatic plants and their derivatives yield essential oils that are rich in bioactive compounds, which forms non-toxic compounds when they undergo degradation. Since, they do not cause any adverse health effects; they are used as insect repellents and a safe and eco-friendly alternative to synthetic pesticides [35]. A problem that arises with the use of essential oils as pesticides is that their effect is short lasting [36]. To overcome this problem, essential oils are formulated into Nanoemulsions. It also has technological limitations, such as hydrophobicity, reactivity, and volatility of the bioactive molecules constituting the essential oils [37]. This problem may be overcome by formulating Essential oils into Nanoemulsion which is transparent and can be used in food and beverage products, thereby, decreasing the amount of Essential oils required [38]. The emulsions that have droplets which are very small with size ranging from 20 to 200nm are called Nanoemulsions. The method of preparation determines the stability of the formulated Nanoemulsion. The nanoemulsions are formed when the oil phase is dispersed in an aqueous phase and it is stabilized due to the presence of surfactant that forms a layer at the interface of the droplet, thus, separating the oil from aqueous phase owing to the stability of emulsion [38-40].

Application of controlled release formulations in agriculture and study on different polymers for use as pesticide delivery agents were performed by many researchers [41-44]. Petroleum derived polymers with biodegradable, non-pollutant residues in environment and non toxic characteristics, could be useful in pesticide delivery systems in agriculture [43]. However, application of natural polymers is more recommended [41, 42, 44]. The pellet size were varied in different researches. Hu *et al.* [41] used 8 cm diameter spheres in their work. Singh *et al.* [44] were made some beads (1.07-1.34 mm in size) and measured them by caliper. In present study 3 and 5 gram pellets thickness and diameter were measured by caliper and their average was 0.93±0.01 and 1.3 mm that were recorded as pellets size. In this study, pellets were produced and expressed fumigant toxicity to *Oryzaephilus surinamensis, Sitophilus oryzae, Tribolium confusum, Callosobruchus maculatus* and *Rhyzopertha dominica*. According to obtained results we propose that Nanoemulsion of essential oil could be produced in pellet formulation and these pellets could have sufficient potency for insect pests control. The insecticidal efficacy varied with insect species, dose of pellets, weight of pellets and exposure time. Physical loading of essential oils and their constituents on biodegradable polymers could be performed and their application in stores will be possible by this manner. Insecticidal efficacy of produced pellets should be evaluated against other stored products pests such as moths and mites in future studies. Also pellets could be produced by other biodegradable polymers such as starch and poly (vinyl acetate) as a cheaper compounds.

In present study fumigant toxicity of 5 gram pellets were tested against some stored products pest in 24, 48 and 72 h, that shown in table 1, 2 and 3. In case of phostoxin results indicates that *R. dominica* and *O. surinamensis* had lowest and highest resistance, respectively. In formulation I *Tribolium casteneum* and *Callosobruchus maculatus* had the lowest and highest mortality. A significant increase of adults mortality was observed in all cases with increasing doses (P<0.01). Fumigation containers volumes were varied (e.g. 250, 500, 710 and 9000 ml) between different works [31-34]. In this research we used 250 ml glass vials that have similarities to other fumigation vials in prior works [31, 34].

In the present study, phostoxin pieces were compared with natural origin pellets to show the novel pellets potential as insecticide in storage and Nano technology was used for eucalyptus essential oil formulation before pellet process. Natural pesticides have some benefits in compare with chemicals such as their low toxicity to human and other non-target organisms, having no harmful residue in environment and reducing the chance of misuse etc [7]. Plant essential oils are relatively new and promising alternatives for chemical insecticides now [7, 45]. Their use as fumigant agent in large scales encountered some problems such as low vapor pressure that could be solved using some formulations such as control released formulations [45]. The vapor pressure of eucalyptus is very low (< 1 mm Hg at 20 °C) while Phosphine (31, 920 mm Hg at 23 °C), Methyl bromide (1250 mm Hg at 20 °C) and Sulphuryl fluoride (12,087 mm Hg at 20 °C) have high vapor pressure. Evidently, compounds of plant origin can be used only for small-scale applications or for space treatment [45]. Controlled release formulations were applied in agriculture and studied using different polymers as pesticide delivery agents by many researchers [41-44]. Polymers with biodegradable, non-pollutant residues and nontoxic characteristics could be beneficial in pesticide delivery systems in agriculture [43]. However, application of natural polymers is more recommended [42, 44]. Some researchers used naturally origin polymers as barrier matrix [42, 44]. While other researchers used petrochemical polymers as barrier in their formulations [43]. Using natural polymers could reduce the risk of pollution and health problems. Despite of, chemical origin polymers have better binding potential and release efficiency [18, 45]. In current study we used pellet formulation with fumigant active ingredient for the first time. So, the pellet making chamber was completely sealed. Obviously, size and shape of formulation is related with the efficiency of release. Similar studies used different size of formulations from spheres with 8 cm [41] to beads with 1.07-1.34 mm [44] diameter. Smaller diameter with higher surface/volume relation could reduce the release time. Our results showed that control of stored product beetles using Phostoxin, was more effective than natural compounds. The residue and side effects of botanical materials and their derivatives are less than Phostoxin. Total benefits of natural compounds in addition to their potential insecticidal efficiency make their use justifiable. Overall, we are hopeful that similar studies will be done on new alternatives for chemical pesticides.

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