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Formulation of sweet flag oil (*Acorus calamus*) nanoemulsion by spontaneous emulsification method for the management of *Sitophilus oryzae*

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Abstract
Sweet flag (*Acorus calamus* L.) O/W nanoemulsion was formulated by the spontaneous emulsification method. The emulsion was formulated using sweet flag oil (6%), non-ionic surfactant with Hydrophile-Lipophile Balance (HLB) value-10.0 and cyclohexane as solvent. The emulsion was formulated with the different ration of oil and surfactant, 1:0.5, 1:1, 1:1.5 and 1:2. The minimum droplet size 17.9 nm was recorded in 1:2 (v/v) of oil and surfactant. The droplet diameter decreased with an increase in the surfactant concentration. The scanning electron microscope (SEM) image revealed that droplets were spherical in shape. The developed formulations were evaluated for insecticidal action against *Sitophilus oryzae.* The insecticidal action increased with an increase in surfactant concentration and dose. The median lethal concentration (LC_{50}) value was 0.51% for 1:2 ratio formulation while it was 2.35% for 1:0.5 ratio formulation. The LC_{50} value decreased with decrease in droplet size and increase in surfactant concentration.

Keywords: *Acorus calamus,* insecticide, nanoemulsion, *sitophilus oryzae,* spontaneous emulsification

Introduction
A wide range of insect pest attacks stored products with the commonest among them being beetles, weevils and moths (Obeng-Ofori *et al.*, 1998) [12]. In cereals, *S. oryzae* the common rice weevil is one of the most important storage pests, which causes severe damage to raw cereals throughout the world (Thomas *et al.*, 2002) [25]. It is mostly cosmopolitan in nature and causes severe losses in rice, maize, barley, wheat and other crop (Singh *et al.*, 1980) [23]. It causes enormous losses up to cent per cent in stored cereals in India and other countries (Karan *et al.*, 1974) [7]. A larva of *S. oryzae* consumes 14mg grain /day and its adult consumes about 0.4 mg grain/day (Golebiowska, 1969) [5].
Acorus calamus L. (Arales: Araceae) is mainly used as a medicinal plant Indian traditional medicine from ancient time. The *A. calamus* extract possess high contact toxicity against stored-product pests (Kim *et al.*, 2003) [8]. The β asarone was the main bioactive compound responsible for insecticidal activity of *A. calamus* (Yao *et al.*, 2008) [26] The rhizomes have been found to act on insects in many ways as insecticide (El-Nahal *et al.*, 1989) [2], ovicide (Risha *et al.*, 1990) [19]; oviposition deterrent (Rahman & Schmidt, 1999) [16], antifeedant (Reddy & Reddy, 2000) [17] and repellent (Pierce & Schmidt, 1993) [15].
Recently, nanoemulsion was used as important delivery system of insecticide against many insect pests. The nanoemulsion of *A. calamus* oil could be a promising solution for management of stored grain pest. There is no report on the *A. calamus* nanoemulsion formulation. The present study aims to develop nanoemulsion of *A. calamus* nanoemulsion by spontaneous emulsification method and to evaluate the insecticidal action against stored product pest.

Materials and Methods
Mass culturing of rice weevil, *S. oryzae*
The experiment was carried out at the Department of Agricultural Entomology, Agricultural College and Research Institute, Coimbatore during 2018-2019. The *S. oryzae* was cultured in plastic jars of 600 ml capacity (12 x 8 cm) on wheat grains that were sterilized in hot air oven at 100 °C for one hour. The paper strip was provided for the purpose of movement of the adult

insects. The culture was maintained at a room temperature of 30 ± 5 ºC and 70 ± 5% RH throughout the period of study.

Extraction of *A. calamus*

The 100g of *A. calamus* rhizome powder was subjected to soxhlet's extractor with 500 ml of hexane for 8h. The obtained extract was concentrated by rotary vacuum evaporator at 40-60 °C.

Spontaneous emulsification of *A. calamus*

The emulsion was developed by *A. calamus* oil (6%), blend of two non-ionic surfactant polyoxyethylene sorbitan monooleate and sorbtian monooleate with HLB value-10.0 and cyclohexane. The different ratio of oil and surfactant, 1: 0.5, 1:1, 1:1.5 and 1:2 was magnetically stirring at 700 rpm for 2 hours. The cyclohexane was then added to the homogenized organic phase.

Characterization of the emulsion

Droplet size and size distribution

The droplet diameter was measured with practical size analyser (Horiba Scientific Nanopartica SZ- 100). The formulations were diluted with water to decrease the multiple scattering effects.

Zeta potential measurements

The zeta potential of the developed formulations was studied by using Horiba Scientific Nanoparticle SZ- 100 analyser.

Scanning electron microscope (SEM)

The morphology of 1:2 ratio of *A. calamus* oil and surfactant formulation was analyzed using SEM (FEI quanta 250). Before analyzing the sample was subjected to gold – sputter coating. The surface of the sample was analyzed at different magnifications.

Transmission electron microscope (TEM)

A drop of 1:2 ratio of *A. calamus* oil and surfactant formulation was placed on a copper grid and allowed for drying. The micrograph of nanoemulsion was acquired using a transmission electron microscope (FEI Technai).

pH

The pH was checked by immersing the electrode of pH meter into the undiluted formulations. The pH values were taken at room temperature. The measurement was replicated thrice.

Viscosity

The experiment was conducted at a temperature of 25 ºC. The viscosity of O/W emulsion system will be high compared to W/O emulsion type. This measurement will help in validation of emulsion type.

Thermodynamic stability

The developed formulations were subjected to centrifugation at 12, 000 rpm for 30 minutes. Then formulations were observed for any phase separation.

The Thermodynamic stability was also analysed by freeze thaw cycle, the formulations were kept in a deep freezer at -20 ºC for 24h and room temperature for 24h. This cycle was repeated for a trice.

Heating and cooling cycle were performed by subjecting the formulations to 6 cycles of alternate heating and cooling by keeping the samples at 25 ºC and 4 ºC for 48h.

Bioassays

Twenty newly adult rice weevil was used as test insects**.** The experiments were carried out at 30±5ºC in a dark room, and 65±5% R.H. The 20g of wheat grains with 12.0% moisture content were taken in a plastic jar and the newly developed *A. calamus* emulsions at various concentrations *viz*., 0.2, 0.4, 0.6, 0.8, and 1.0.% w/v was added to the grains and shaken thoroughly. Untreated treated wheat grain was maintained as a control. The mortality was observed at 24, and 48 h after treatment. Five replications were maintained for each treatment.

$$\text{Mortality \%} = \frac{\text{Number of insects dead}}{\text{Total number of insects released}} \times 100$$

Data analysis

The percentages of insect mortality and standard error were calculated for each concentration of different emulsions. The significance of the difference was analysed using two- way analysis of variance (ANOVA). Results with a probability value (*P*) of less than 0.05 were considered to be statistically significant**.** The LC_{50} was determined using probit analysis at 95% confidence level ($P < 0.05$).

Result and Discussion

Droplet size and size distribution

The minimum droplet size of 17.9 nm was recorded in the formulation consist of 1:3 ratio and maximum size 51.2 nm was observed in 1: 0.5 ratio of oil and surfactant (Table1). Ostertag *et al.* (2012) [13] reported that good nanoemulsion had droplets size between 20-200 nm. The particle size decreases with an increase in the surfactant concentration which is because of a reduction in interfacial free energy and also acts as a mechanical barrier against coalescence (Reiss, 1975) [18]. This finding was in accordance with Kale and Allen (1989) [6], who reported that addition of surfactant to emulsion will result in small droplet size. The polydispersity of the droplet size was 0.18, 0.29 and 0.40 for the formulations with 1: 1, 1:1.5 and 1:2 ratios of oil and surfactant respectively. The nano range of droplet size is also because of the lower values of the polydispersity index (Shinoda & Saito, 1969) [21].

Table 1: Characterization of *A. calamus* nanoemulsion formulations

Ratio (oil: Surfactant)	Droplet size	Polydispersity index	Zeta Potential	Viscosity (cP)	pH
1:0.5	51.2 nm	12.0	-25.4 mV	0.92	6.54
1:1	33.4 nm	0.18	-51.4 mV	0.92	6.66
1:1.5	31.5 nm	0.29	-54.4 mV	1.38	6.79
1:2	17.9 nm	0.40	-54.0 mV	3.22	6.88

Scanning electron microscope (SEM)

The morphology of 1:3 ratio formulation analysed in SEM with 50,000 magnification. The image revealed the spherical shape of the nanoemulsion and it also confirms the nano range of the droplet size (Fig.1). The SEM image of neem oil nanoemulsion was an approximately spherical shape was reported by Anjali *et al.* (2012) [1].

Transmission electron microscopy (TEM)

The internal feature of the nanoemulsion (1:3) was given in Fig. The diameter was within 50 nm. The TEM micrograph recorded the spherical shape of the nanoemulsion and also confirms the nano size of the droplet diameter. Ghosh *et al.* (2013) [4] have reported TEM image of cinnamon nanoemulsion was spherical shape.

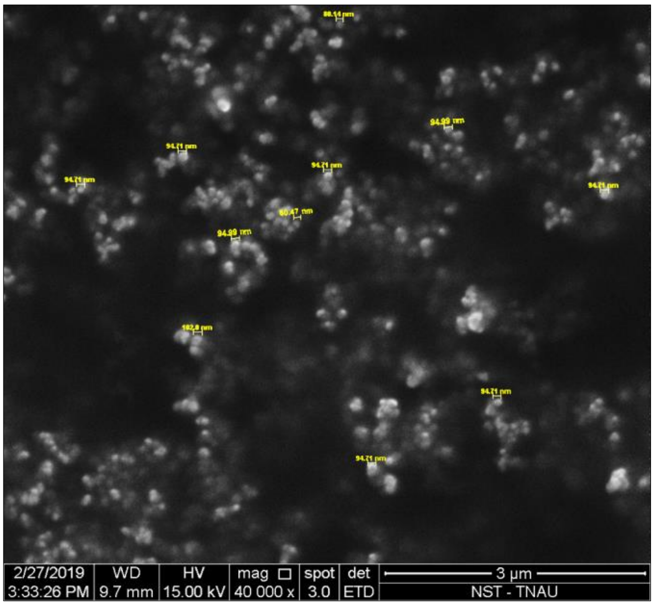

Fig 1: SEM image of *A. calamus* nanoemulsion 1:2 formulation

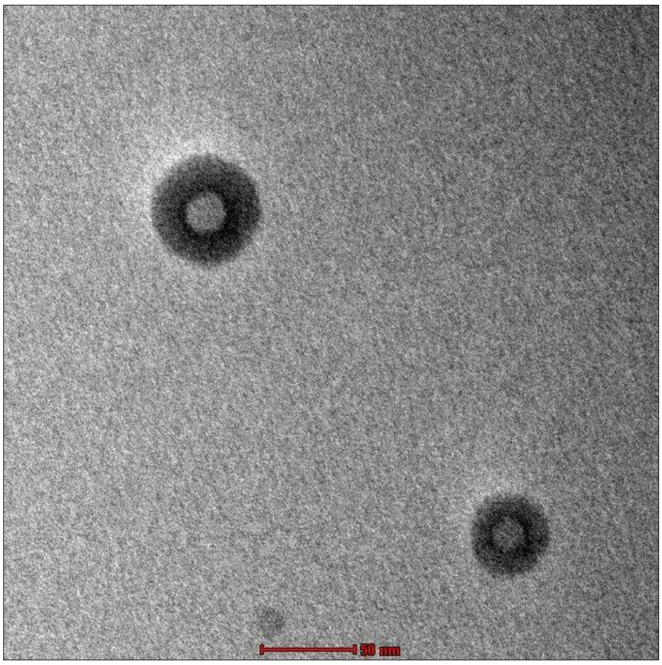

Fig 2: TEM image of *A. calamus* nanoemulsion 1:2 formulation

Viscosity

The viscosity increased with an increase in surfactant concentration (Table 1). The 1: 0.5 and 1: 1 formulation recorded minimum viscosity (0.92) while other formulation 1:1.5 and 1:2 showed 1.38 and 3.22 respectively. The result was in accordance with El Eini *et al.* (1976), who reported the addition of non- ionic surfactant has resulted in an increase in the viscosity of the emulsion.

pH

The pH of the formulations increases with increase in the surfactant concentration (Table 1). The 1: 0.5, 1:1, 1:1.5 and 1:2 nanoformulations of *A. calamus* showed pH values of 6.54, 6.66, 6.79 and 6.88.

Zeta potential measurement

The stability of the formulation was confirmed by measuring the zeta potential. The lowest zeta potential of -25.4 mV was recorded in 1:0.5 whereas other formulations showed more than -50 mV. The very low droplet charge result in repulsive force between the negatively charged droplets and prevented the emulsion form coalescence and flocculation (Stachurski & MichaŁek, 1996) [24]. The droplets are more negatively charged because of the hydroxyl ion adsorbed at the O/W interface and ethylene oxide group present in tween 80 creates more negative charges (Liu *et al.*, 2006) [9].

Stability test of the nanoemulsion

The phase separation was recorded only in 1: 0.5 ratio formulations while the other formulations were found more thermodynamically stable. The stability, increased with increase in the surfactant concentration. The thermal stability and kinetic stability differentiate the nanoemulsion from macro-scale emulsion. The thermal stability of the nanoemulsion differentiates it from emulsions with kinetic stability and eventually phase separation (K. Shinoda & Kunieda, 1983) [22].

Insecticidal action of nanoemulsion

The *A. calamus* nanoemulsion exhibited dose dependent insecticidal action on *S. oryzae*. A positive correlation between the surfactant concentration of the formulation and the insecticidal action was observed. The highest insect mortality of 89.00% was recorded in wheat grains treated with 1% v/w of 1: 2 ratio nanoemulsion, While with same concentration 1:1.5, 1:1 and 1:0.5 ratio formulation has recorded 77.00, 40.00 and 13.00% insect mortality (Table 2). Similar results were observed by Kim *et al.* (2003) [8] who reported that over 90 per cent mortality of adults of *S. oryzae* at 3 or 4 days after treatment was achieved using methanol extracts of *A. calamus* var *angustatus* rhizome and *A. gramineus* rhizome. The acetylcholinesterase (AchE) inhibitory potential of *A.calamus* essential oil and β-asarone, the major phytoconstituent present in the *in vitro* was earlier demonstrated by Mukherjee *et al. (2007)* [11]. (2007). Song *et al.* (2008) also reported that β-asarone at a low dosage (67.5 mg/kg) showed a marked induction effect on AChE activity in *R. dominica*. (Park *et al.*, 2003) [14] (*Z*)-asarone caused 70.0 per cent and 90.0 per cent mortality against *S. oryzae* adults at 0.064 and 0.255 mg/cm^2 at 4 days after treatment, respectively, with 100 per cent mortality on 7 days after treatment.

Table 2: Mortality of *S. oryzae* adult exposed to different sweet flag nanoemulsion formulation

Concentration	Mortality (%)			
	1:0.5	1: 1	1:1.5	1:2
0.2%	0.00±00	2.00±0.22	7.00±0.22	12.00±0.22
0.4%	1.00±0.18	11.00±0.18	22.00±0.22	37.00±0.22
0.6%	3.00±0.22	22.00±0.22	36.00±0.18	51.00±0.18
0.8%	6.00±0.18	29.00±0.18	57.00±0.22	71.00±0.18
1%	13.00±0.22	40.00±0.18	77.00±0.22	89.00±0.18
control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Values are the mean of five values (*n* = mean ± SE)				

The surfactant concentration and droplet size played a major role in increasing the insecticidal action of the formulation. The particle size of nanoemulsion was in nano- range, it had a high surface area, solubility, and mobility (Sasson *et al.*, 2007) [20]. It resulted in fast penetration into the insect cuticle and enhanced insecticidal activity (Margulis-Goshen & Magdassi, 2013) [10]. The formulation with higher concentration of surfactant has recorded the highest insecticidal action. The medial lethal concentration (LC_{50})

was minimum only 0.51% v/ w for 1:2 ratio formulation whereas 2.35, 1.25 and 0.67% v/w for 1:0.5, 1:1 and 1:1.5 ratio respectively (Table 3). The LC $_{50}$ vale decrease with increase in the surfactant concentration (fig 3).

Table 3: Median lethal concentration (LC_{50}) of *A. calamus* nanoemulsion to *S. oryza*

Formulations	χ^2 Value	Regression equation	$LC_{50(\%)}$	Fiducial limits	
				Lower	Higher
1: 0.5	0.06	3.1417x + 3.8302	2.35	0.70	9.12
1: 1	0.08	2.5009x + 4.7502	1.25	0.65	2.08
1: 1.5	1.14	3.137x + 5.5317	0.67	0.55	0.83
1: 2	1.24	3.1722x + 5.9269	0.51	0.41	0.62

*All lines are significantly good fit at $P \leq 0.05$

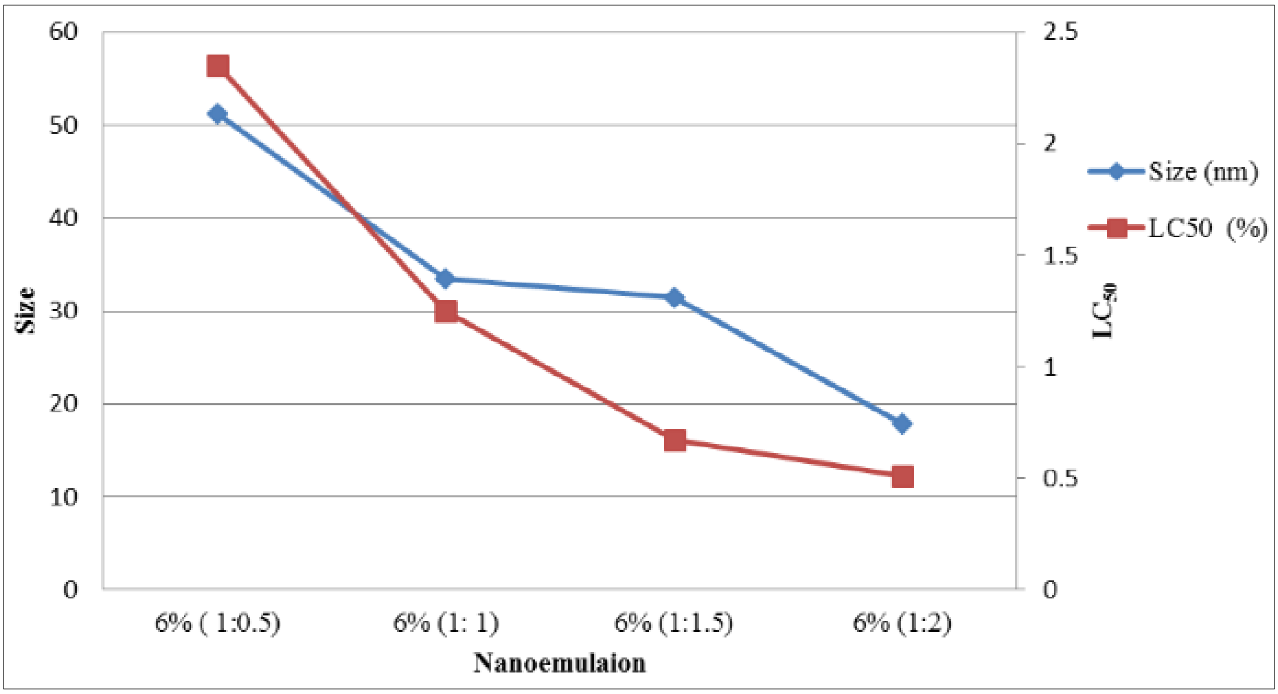

Fig 3: A plot of droplet size and LC_{50} against different ratios of *A. calamus* nanoemulsion

Conclusion

The present finding illustrates significant effect of surfactant concentration in droplet size, stability and insecticidal action. This is the first report of *A. calamus* nanoemulsion developed by spontaneous emulsification method. The formulated nanoemulsion has shown the potential insecticidal action against stored product pest. It could be used in protecting the seed material for upcoming season.

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