International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 02-03-2021, Accepted: 03-04-2020, Published: 03-05-2021
Volume 6, Issue 3, 2021, Page No. 05-10

Formulation, characterization, and evaluation of the insecticidal activity of thymus *vulgaris* oil nanoemulsion against the agricultural pest *Aulacophora foveicollis* (Red pumpkin beetle) (Chrysomelidae: Coleoptera)

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Abstract
Natural products such as plant essential oils are formulated as nanoemulsion and widely used in the application of insect pest control. *Thymus vulgaris*, an essential oil has many biological properties naturally. In the present study, using the GC-MS instrumentation the major chemical components of the thyme oil were identified. The chemical component, thymol was identified with the highest peak area of 15.65%. The O/W nanoemulsion was then formulated using the following components such as oil, surfactant, acetone and distilled water by ultrasonication method. The formulated nanoemulsion was investigated for the stability study and the stable nanoemulsion was characterized by DLS method with the mean droplet diameter of 12.81nm, PDI was 0.13 and zeta potential +59.4 mV values, everything together proves to have a good stability of the formulated emulsion and the mean pH value were also found to be 5.43 using pH meter. The nanoemulsion and the bulk emulsion were screened against the adult beetle, *Aulacophora foveicollis* to assess the insecticidal activity at different concentrations. The nanoemulsion has shown the higher insecticidal activity when compared to the bulk emulsion with an effective LC_{50} value 73.3% in nanoemulsion and 29.9% in bulk emulsion respectively. Therefore, from our study it could be concluded that nanoemulsion may be used as an effective agent against the agricultural pest *Aulacophora foveicollis* and also alternative for synthetic chemicals for sustainability of environment.

Keywords: *Aulacophora foveicollis*, insecticidal activity, nanoemulsion, plant oil, *Thymus vulgaris*

Introduction
In the past few years, several studies have focused on the potential use of essential oil applications in biological control of different insect pests. The essential oils are more rapidly degraded in the environment than synthetic compounds (Pillmore, 1993) [18]. Since, essential oils are natural products (Baser, 2010) [4] and mainly consist of secondary metabolites such as alkaloids, terpenoids, flavonoids, phenol, polyphenols, glycosides and tannins (Ge and Ding, 1996) [12]. Formulating such essential oils into nano-emulsion method can be used, to decrease the quantity of essential oils required (Qian, 2012) [19] and it is also a recent favorable method to improve botanical insecticide characteristics and effectiveness for commercial usage (Anjali *et al.*, 2012) [2]. The genus *Thymus*, member of the Lamiaceae family, consist of about 400 species and many more subspecies (De Martino 2009). Among them the most widely used species is *Thymus vulgaris*, which is generally known as the common or garden thyme. Investigations were done based on the chemical composition and biological properties of the *Thymus vulgaris* essential oil (Rassooli, 2004, Baydar, 2004 and Kabouche, 2005) [20, 5, 13].
The aim of our study was first to identify the chemical components of thyme oil, then formulate, *Thymus vulgaris* essential oil into nanoemulsion using ultrasonication method and to assess the insecticidal activity of the *Thymus vulgaris* oil nanoemulsion against the serious agricultural pest of cucurbit plants, *Aulacophora foveicollis*. The adult beetles are polyphagous and most destructive in nature (Doharey, 1983) [10].

Materials and Methods
Materials
The materials used for the study were thyme essential oil, Tween 80 as surfactant, acetone as solvent and distilled water.

Insect collection and rearing
Insects were collected early morning from the cucurbitaceous plants by hand picking method in the vicinity of Madras Christian College campus. Insects were reared under the laboratory condition and fresh leaves collected from the cucurbit plants were provided to them every day early in the morning.

GC-MS analysis
GC-MS analysis was performed by using JEOL, GC MATE II (GC Model), quadruple double focusing detector. One microliter of the extract was injected into the split less mode in an injection port of the GC column. The inlet temperature has been set at 220 °C for about 1 min then 10 °C min^{-1}. Total run time was set up to 60 min. Helium gas was used as the carrier gas at the constant flow rate of 1.0 mL/min. The interface temperature (GC to MS) was set to be at 250 °C.
MS was set in the scam mode. MS quad temperature was about 250 °C. Ions were obtained by an electron ionization mode. Molecular ions (mass range) were monitored for the identification which was set 50-600 m/z. peak area represents the relative percentage of the constituents present in the sample.

Preparation of nanoemulsion

The O/W nanoemulsion type was utilized, to prepare the nanoemulsion using thyme oil, surfactant Tween 80, solvent acetone and distilled water. The concentration of thyme oil (10%, v/v) and acetone (6%, v/v) was fixed for all the formulations. The coarse emulsion was prepared by adding distilled water to organic phase containing oil and surfactant in different ratios using a magnetic stirrer, which was then subjected to ultrasonication method. The formulated nanoemulsion was investigated for its stability and characterized by different techniques. Bulk emulsion was prepared using the Thyme oil (10%, v/v), Tween 80 (15%, v/v), acetone (6%, v/v), and distilled water (69%, v/v).

Characterization of nanoemulsion

Thermodynamic stability study: By different stress study, the stability of the emulsion could be identified (Shafiq *et al*., 2007) [23].

1. Centrifugation: The formulated emulsions were centrifuged at 1500 rpm for 30 min. After centrifugation, the emulsions were observed for any phase separation.
2. Heating-cooling cycle: The formulated emulsion was kept at alternate temperature at 40 °C and 4 °C for 48 hours and it was performed in triplets.
3. Freeze-thaw stress: The formulated emulsion was kept at the alternate temperature at -21 °C and 25 °C for 48 hours and this experiment was also triplicated.

Finally, the stable emulsion was examined for the characterization parameters.

pH measurement

The pH value of the nanoemulsion was measured using the pH meter at room temperature. The measurement was carried out in triplicates.

Droplet size distribution, PDI & Zeta potential

The droplet size distribution, polydispersity index (PDI) and zeta potential of thyme oil nanoemulsion formulation was determined by dynamic light scattering using a Nanotrac Wave II; Make- Microtrac Inc, (USA). Prior to the measurement studies, the emulsion was diluted with double distilled water (1:100) and the Samples were equilibrated at 25 °C for 1 min. The measurements were performed and the average droplet size was expressed in mean diameter. The surface charge of the nanoemulsion droplets was determined by measuring the electrophoretic mobility at 25 °C and the values of zeta potential were expressed in mV.

Bioassay

The prepared nanoemulsion and bulk emulsion using thyme essential oil were screened for its efficiency under the lab condition against the adult beetles, *Aulacophora foveicollis* using the leaf dipping method at different concentrations such as 10, 20, 30 & 40% respectively. The control experiment was treated with acetone. Each treatment consists of 10 beetles and freshly treated and untreated leaves were provided every 24 hours. The treatment was carried out for about 96 hours and mortality was recorded after every 24 hours. Each concentration was triplicated and mortality percentage was calculated.

Statistical analysis

The data were subjected to mean, standard deviation and the values of LC_{50} were calculated by using the probit analysis method (Chi, 2009) [7]. The significant difference between the treatment was analysed with one-way ANOVA ($P<0.05$) using the software SPSS, version 24.

Results and Discussion

The chemical composition of *Thymus vulgaris* oil was evaluated by using GC-MS method. *Thymus vulgaris* showed the presence of 15 chemical composition among them the major compounds were found to be thymol with the high peak area of 15.65%, followed by Carvacrol with 14.08%, Linalool with 12.08% and so on (Table 1 & Fig 1). The compounds found in our study were already reported to have a strong biological activity such as antibacterial (Sagdic and Ozkan, 2003) [21], antifungal (Sokmen *et al*., 2004) [27, 24], antioxidant (Tepe *et al*., 2005 and Marija Boskovic *et al*., 2019) [27, 14] and insecticidal activity (Szczepanik *et al*., 2012) [26]. The chemical compounds identified in this study were almost in line with the data of Baranauskiene *et al*., (2003) [3] and Boruga *et al*., (2014) [6]. After the stability study of all the formulated nanoemulsion, the stable nanoemulsion were examined for characterization. The stable nanoemulsion consisted of the mean droplet diameter of 12.81nm with 0.13 PDI value (Fig 2). Dordevic *et al.,* 2015 [11], has reported the particle size around 160 nm and PDI less than 0.15 of risperidone nanoemulsion. Currently, Samah *et al.,* 2021 [22], has also reported the mean droplet diameter of *Thymus vulgaris* nanoemulsion was about 52 nm with PDI value 0.15. In our study, the thyme oil nanoemulsion provides less particle size and PDI value when compared with others, which indicated the good stability and homogeneity of the nanoemulsion.

Zeta potential of above ± 25 mV proves to be the good stability of the formulated nanoemulsion. In our study, the zeta potential was found to be +59.4 mV, it indicates the good stability of the *Thymus vulgaris* nanoemulsion. Furthermore, Dordevic *et al* has also obtained zeta potential of -50 mV for risperidone nanoemulsion.

The pH value also indicated the stability of the nanoemulsion and the pH mean value recorded in our study is 5.43, which is lower than the pH value of rice bran oil nanoemulsion by Daniela S Bernardi *et al.,* 2011 [8] and also in agreement with the pH value of acetazolamide nanoemulsion by Morsi *et al*., 2014 which indicated the non-irritant for skin and eye.

Table 1 Components of Thyme oil

S. No	R. Time	Name of The Compound	Molecular Formula	Area %
1	14.73	β-Myrcene	$C_{10}H_{16}$	2.7
2	17.6	Borneol	$C_{10}H_{18}O$	7.45
3	20.33	Hexadecenoic acid, methyl ester	$C_{17}H_{32}O_2$	3.97
4	24.33	Thymol	$C_{10}H_{14}O$	15.65
5	24.85	Camphor	$C_{10}H_{16}O$	5.8
6	25.25	α- Caryophyllene	$C_{15}H_{24}$	6.55
7	25.67	Carvacrol	$C_{10}H_{14}O$	14.08
8	25.98	Camphene	$C_{10}H_{16}$	3.01
9	26.32	α-Phellandrene	$C_{10}H_{16}$	5.06
10	26.97	Linalool	$C_{10}H_{18}O$	12.08
11	27.28	4-Thujanol	$C_{10}H_{18}O$	4.06
12	28.15	D-Limonene	$C_{10}H_{16}$	3.01
13	29.15	Octadecanoic acid, methyl ester	$C_{19}H_{35}O_4$	2.79
14	29.88	α -Pinene	$C_{10}H_{16}$	2.77
15	30.85	γ-terpinene	$C_{10}H_{16}$	2.02

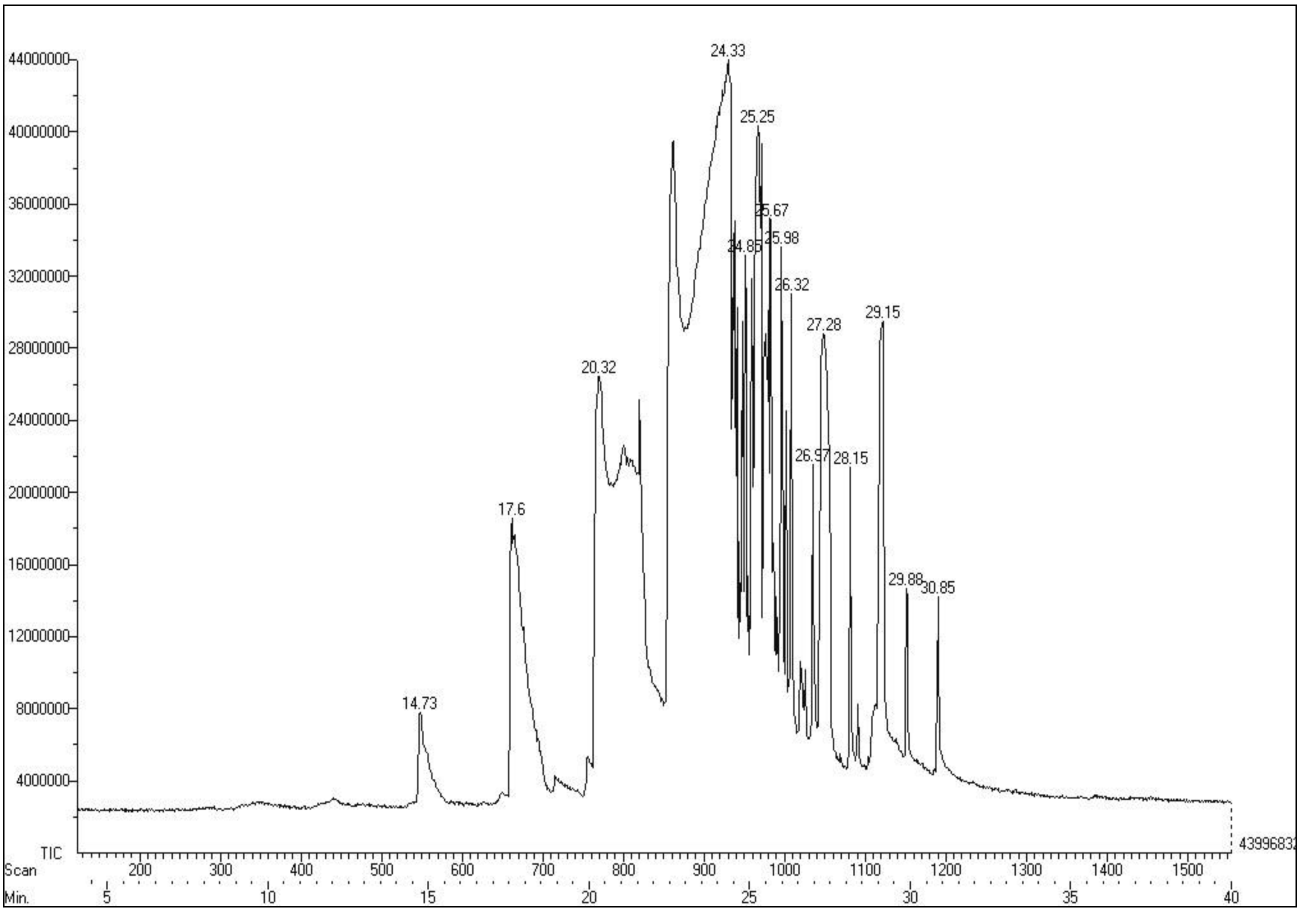

Fig 1: Chromatogram of *Thymus vulgaris* oil

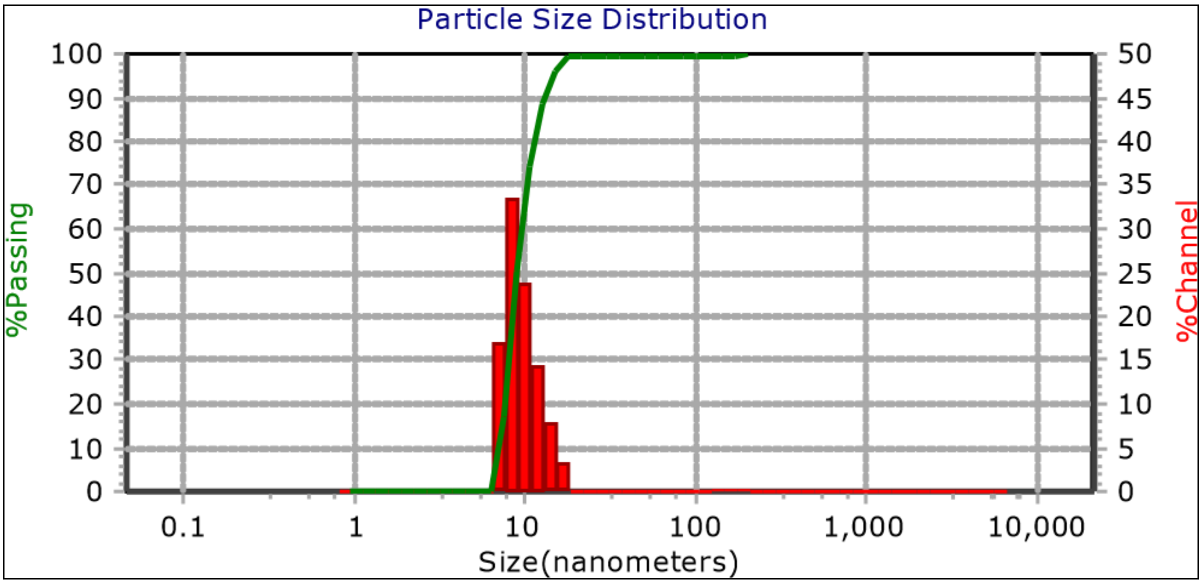

Fig 2 Particle size distribution by DLS

Efficacy of *Thymus vulgaris* nanoemulsion

The bioassay of the nanoemulsion formulation of *Thymus vulgaris* demonstrated toxicity effects on adult beetles, *Aulacophora foveicollis*. In our study, the nanoemulsion and the bulk emulsion of thyme oil at different concentrations were treated against the agricultural pest, *Aulacophora foveicollis*. Among the treated concentrations, 40% concentration has showed the highest mortality percentage with 73.3% in nanoemulsion and 29.9% in bulk emulsion respectively and no mortality was observed in the control samples. It has been observed that the calculated LC_{50} value of thyme nanoemulsion formulation is more effective with 26.30% than the LC_{50} value of the bulk emulsion with 89.12% respectively (Table 2 & Fig 3) and the one-way ANOVA also indicates a high significant difference among the 40% concentration of two different emulsions (df= 1, MS= 28.167, F= 42.250, p<0.003). The small size of the droplet helps to act efficiently on the cuticle of insects and it also helps to protect the secondary metabolites and guard in controlled release of bioactive ingredients on insects (Nuruzzaman *et al.*, 2016) [17]. The particle size of the nanopermethrin against the larvae of *Culex quinquefasciatus* was studied by Anjali *et al.*, 2011 [1]. The results obtained in the present work is compatible with Sugumar *et al.*, 2014, were the nanoemulsion of eucalyptus oil is highly effective towards the *Culex quinquefasciatus* larvae, were compared to the bulk-emulsion. The small particle size of nanoemulsion also simplify the more contact of pest (Massoud *et al.*, 2018) [15]. Overall, from our study *Thymus vulgaris* oil nanoemulsion may be used as an effective insecticidal activity against the agricultural pest, *Aulacophora foveicollis*.

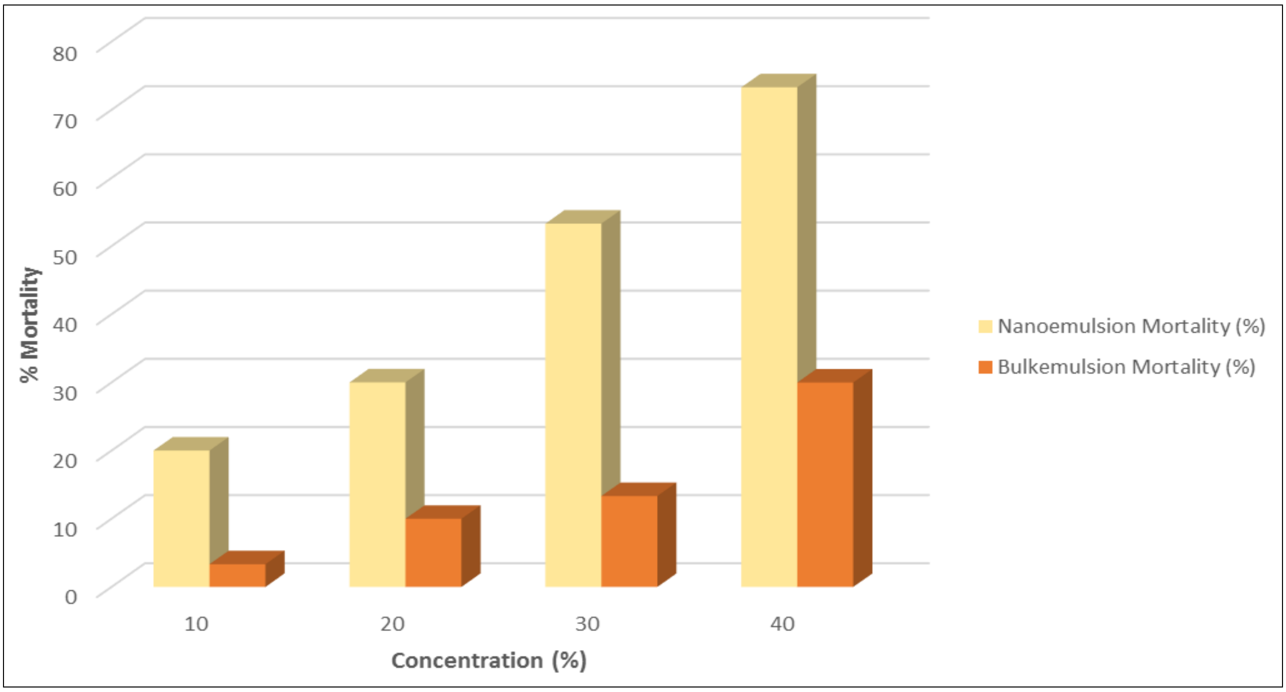

Fig 3: Comparison of Nanoemulsion and Bulk emulsion of *Thymus vulgaris* oil against the adult beetles, *Aulacophora foveicollis*.

Table 2: Bioassay of *Thymus vulgaris* oil, nano emulsion and bulk emulsion against the adult beetle, *Aulacophora foveicollis.*

Bioassay	Concentrations (V/V) (%)	24 Hrs (in %)	48 Hrs (in %)	72 Hrs (in %)	96 Hrs (in %)	Total Mortality (in %)	LC_{50} (v/v) (%)	R^2
Nanoemulsion	10%	0.00	0.00	3.33	16.67	20.00	26.30	0.9774
	20%	0.00	6.67	6.67	16.67	30.01		
	30%	13.33	6.67	13.33	20.00	53.33		
	40%	16.67	13.33	16.67	26.67	73.34		
Bulkemulsion	10%	0.00	0.00	0.00	3.33	3.33	89.12	0.9
	20%	0.00	3.33	3.33	3.33	9.99		
	30%	6.67	3.33	3.33	0.00	13.33		
	40%	3.33	13.33	0.00	13.33	29.99		

Conclusion

In our study, the chemical components of the *Thymus vulgaris* oil was identified and then the prepared nanoemulsion of *Thymus vulgaris* ensures the higher efficacy as an insecticidal activity against the agricultural pest *Aulacophora foveicollis* than compared to its bulk emulsion. Therefore, *Thymus vulgaris* nanoemulsion is effective against the agricultural pest and is found to be safe for environment than synthetic pesticides.

Acknowledgment

The authors wish to thank the Department of Zoology, Madras Christian College for enabling us to conduct the Research and the University of Madras. We also thank Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Madras (IITM), Chennai, for GC-MS analysis and “Vels Institute of Science, Technology & Advance studies (VISTAS), Chennai for DLS.

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