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Larvicidal activity of castor oil Nanoemulsion against malaria vector *Anopheles culicifacies*

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Abstract
Malaria is a vector borne disease, which is caused by *Plasmodium* parasite that is transmitted to humans through the bite of the *Anopheles* mosquito leading to transmission of the disease in countries of Southeast Asia including India. The problem of persistent toxicity along with the growing incidence of insect resistance, has led to the shift from synthetic to green pesticide. Castor oil (*Ricinus communis*) based nanoemulsion has been developed for large field breeding mosquitoes like *Anopheles culicifacies*. Castor oil nanoemulsion was formulated in different ratios comprising of castor oil, surfactants and water by High Pressure Homogenizer Mixer. The ratio of castor oil (10%) and surfactants (18%) was found to be stable. The castor oil nanoemulsion was characterized by dynamic light scattering and the PDI (polydispersity index) of the nanoemulsion was found to be 0.02. Castor oil nanoemulsion was screened for its efficacy against *An. culicifacies* using WHO protocol and compared with the bulk emulsion having droplet size of 118µm. The 24 h LC_{50} for castor oil nanoemulsion and castor bulk emulsion was found to be 3.4 and 52.31ppm, respectively. A botanical nanoformulation has been developed which is biodegradable, environment and user friendly.

Keywords: Nanoemulsion, *Anopheles culicifacies,* LC_{50}, Bulk Emulsion

1. Introduction
Mosquitoes are responsible for the transmission of various pathogen-borne diseases like malaria, filariasis, chikungunya, dengue and encephalitis that have resulted in morbidity and mortality around the world. Malaria is a vector borne disease caused by *Plasmodium* parasite transmitted to humans through the bite of the *Anopheles* species leading to epidemic diseases in countries of the Southeast Asia including India. Among the *Anopheles* species *An. culicifacies* is the primary malaria vector in India, being responsible for about 60–70% of malaria transmission in the country [1]. It is confined to the rural plain and semi urban areas of the country. Due to development of pesticide resistance in the mosquitoes, vector control becomes an important tool in the control of diseases. Vector population management at the larval stage is more convenient and becomes important in lieu of pest and drug resistance. Larval control can be executed by spraying the larvicides at the larval habitats [2]. Spraying of hazardous formulations to control vector adult increases the risk of environmental and human health damage. The toxicity problem, along with the growing incidence of insect resistance, has changed the focus from the development of synthetic to the development of natural and novel insecticides [3]. In that effort, some green botanical pesticides have already been reported [4]. Green pesticides are biodegederable [5] have high specific activities towards the target pests and nontoxic to nontarget pests. Main components of green pesticides are plant extracts and essential oils. Many plant extracts and essential oils have shown good larvicidal activity against the mosquitoes [5, 6]. These compounds are biodegradable and more sustainable options compared to synthetic pesticides. Thus there is a keen interest in the development of biodegradable ecofriendly larvicides.
Ricinus communis (Euphorbiaceae) is one such plant that is found commonly grown in India and has not been explored much as a larvicide against mosquitoes. There are various reports on the insecticidal activity of castor plant against different pests. Castor oil have been reported as ovicides against mosquitoes *An. stephensi*, *Culex fatigans* and *Aedes aegypti* [7].
In the present study a castor oil based nanoemulsion is formulated and compared with the bulk

emulsion for its efficacy against larvae of *An. culcifacies*. The formulation was made from the castor oil extracted from seed of *R. communis*. The seeds of *R. communis* are economical, readily available and biodegradable. Use of nanopesticides prepared by such materials would be a good alternative strategy for controlling pest populations and reducing the toxic effect of bulk formulations on the environment [8]. This is the first report on castor oil nano emulsions for mosquito population management. We have screened the nanoemulsion with a droplet size 114nm for its efficacy against third instar larvae of *An. culicifacies* using WHO 2005 protocol and compared it with the castor oil bulk emulsion having droplet size of 118µm [9].

2. Materials and Methods

2.1 Materials

Tween-80 and Span -20 propylene glycol were purchased from SD Fine Chemicals. Double distilled water was used for the preparation of nanoemulsion.

2.2 Mosquito larvae

Laboratory reared larvae of *An. culicifacies* were obtained from National Institute of Malaria Research Insectary. The larvae were maintained in the standard condition of 25 $\pm$ 2° C and were kept in dechlorinated tap water in an enamel bowl. Larvae were fed on dog biscuit and yeast powder in the (3:1) ratio.

2.3 Extraction of oil

Fruits of *R. communis* were collected and were shade dried. Seeds were removed from the fruits then about 100g of the seeds were crushed and subjected to Soxhlet extraction in 1.5-2 L of hexane for 8 hours. The solvent was removed using rotatory evaporator. The oil obtained was stored at 4°C till further analysis was done.

2.4 Preparation of nanoemulsions

The oil-in-water nanoemulsions were formulated using castor oil, a blend of surfactants (Tween 80 and Span-20) along with distilled water (Table 1). The concentration of castor oil (10%, v/v) and propylene glycol was fixed for all the formulations. Initially, coarse emulsion was prepared by adding distilled water to organic phase containing oil and surfactants in different ratios i.eF1, F2, F3, F4and F5, v/v (Table 1) using a magnetic stirrer, which was then subjected to ultrasonic emulsification using a 20kHz Sonicator (Ultrasonics, USA) with a power output of 750W. Energy input was given through sonotrode containing a piezoelectric crystal with a probe diameter of 13mm. Sonicator probe generates disruptive forces that reduce the droplet diameter converting coarse emulsion to nanoemulsion. The nanoemulsions were characterized and investigated for its stability. Bulk emulsion was also formulated using castor oil (10%), water- 75%, Tween-80, 15%. The general composition of formulations is given in (Table I).

2.5 Characterization of nanoemulsion

2.5.1 Accelerated Temperature Studies

To prove the stability of formulated nanoemulsions. The nanoemulsions were stored at 40°C and room temperature for one month. (Table I)

2.5.2 Droplet Size Distribution & PDI (Polydispersity Index):

The average particle size of the castor oil naoemulsion and bulk emusion and PDI were measured at room temperature by dynamic light scattering using a Nano-ZS Zetasizer (Malvern, UK).

2.6 GCMS analysis

FAME study of the castor oil

The fatty acid methyl esters (FAME) were prepared according to the modified method [10]. Around 0.05 g of oil was dissolved in 1.8 ml of petroleum ether and to this 0.2 ml of sodium methylate was added and vortexed so that the two layers were separated and 1µL of the upper layer containing the FAME was injected on a 30 m capillary column (0.25 mm i.d × 0.25 µm film), of Shimadzu GCMS QP2010 74707. GCMS temperature was programmed with initial oven temperature of 70 °C (hold time 5 min), which was increased at the rate of 10 °C/min to 300 °C (hold time 5min) and sample injection temperature was 260 °C, with a split ratio of 10.

2.7. Larvicidal activity of castor oil nanoemulsion against *An. culicifacies*

The castor oil nanoemulsion was screened for its larvicidal activity against third instar larvae of *An. culicifacies*. The third instar larvae of mosquitoes were treated with different concentrations of nanoemulsion, following the standard larval susceptibility test method [9]. Twenty larvae of *An. culicifacies* were placed in each of the five 250 ml containers containing the different concentrations of nanoemulsion i.e. 2,4,6,8, and 10ppm. Distilled water without any pesticide and another set-up containing water, surfactants and propylene glycol were maintained as control. Each test was performed in three replicates. Bulk emulsion was also screened for its efficacy against *An. culicifacies* (10, 20, 40, 80, 160, 320 ppm). The larval mortality was observed after 24hours. The moribund larvae were counted as dead.

2.8 Statistical analysis

The percentages of larval mortality and standard deviations were calculated for each concentration of the nanoemulsion. Lethal concentration (LC_{50} and LC_{90}) was determined at the 95% confidence level using probit analysis. Statistical analysis was carried out using SPSS software.

3. Results & Discussion

Based on stability study the F5 nanoemulsion was found to be stable with an average diameter of 114nm and their size distribution is shown in Fig:1. The polydispersity index (PI) of the nanoemulsions was 0.2 (Fig1), which indicated low polydispersity and thus provide long-term stability to the nanoemulsions. While the other formuations were not stable because of phase separation.

3.1 Fatty acid analysis of castor oil: These results show that there are three main groups of fatty acids: the poly-unsaturated omega-6,9 such as the linoleic acid, the mono-unsaturated omega-9 such as the ricinoleic, oleic, and 11-eicosenoic acids and the saturated one such as the palmitic and stearic acids, docosanoic, nonadecanoic acid and tetracosanoic acid methyl ester. This study reports the presence of docosanoic and tetracosanoic acid in the castor oil. GCMS analyses revealed that the ricinoleic acid was the major product in the oil composition, since its corresponding methyl ester presents the higher concentration (38%) followed by oleic and linoleic methyl esters in lower concentrations i.e. 21.42% and 19.09% respectively(as shown in Fig:2 and Table 2). Others like the palmitic acid, stearic acids, docosanoic, nonadecanoic acid, tetracosanoic acid methyl ester were also identified in low concentrations. Our results are in accord with others GC studies of castor oil which report that the ricinoleic acid is the major constituent [11]. Our results are also in good agreement with the findings that with the ricinoleic acid there are others fatty acids such as linoleic, oleic, stearic, and palmitic acids identified in very low concentrations [12]. Comparing to other studies, our study for the first time shows the presence of docosanoic and tetracosanoic acid in the castor oil.

3.2 Larvicidal activity of castor oil nanoemulsion against *An.culicifacies*

The castor oil nanoemulsion F5 was screened for its efficacy against *An. culicifacies*. At a concentration of 10ppm, 100% mortality results were obtained within 24h of treatment. While no mortality was observed in the control samples. While in the case of bulk emulsion 100% mortality was observed at 320ppm. This difference in efficacy could be attributed to the small size of the droplet of nanoemulsion (114nm), the smaller size results in the increase in surface area, that enhances the penetration of nanopesticides into the larvae and improves its efficacy. The results, show that the nanoemulsion formulation of castor oil is more effective with LC_{50}: 3.4ppm, LC_{90}: 8.9ppm than the bulk emulsion LC_{50}:52.31ppm, and LC_{90}: 242.28 ppm (Fig; 3and Table: 3). The nanometric pesticides have been reported to have increased efficacy [13]. Nano formulations have been reported to be safe for the environment [14]. Nanoemulsion of the castor oil is found to be rich in ricinoleic acid which confers unique properties. Ricinoleic acid ester in castor oil has been reported to control microparasites and ectoparasite [15, 16, 17], also inhibits oocyte maturation in the ticks [18] There are various reports on the insecticidal activity of the castor oil. It is reported to have shown efficacy against *Callosobruchus chinensis* [19], and *Acanthoscelides obtectus* [20]. Insecticidal activity of the aqueous extracts of *R. communis* leaves have been reported against larvae of various mosquitoes species *Culex pipiens* (Linné), *Aedes caspius* (Pallas), *Culiseta longiareolata* (Aitken) and *Anopheles maculipennis* (Meigen) [21]. Castor oil had been reported to have insecticidal activity against *Zabrotes subfasciatus* [22]. The insecticidal activity of the seed and leaf extract of *R. communis* have been studied against *Spodoptera frugiperda* and it was found that castor oil and ricinine are the major ingredients of the *R. communis* responsible for the insecticidal activity of seed [23]. The leaf methanol extract of *R. communis*, chloroform extracts of *Anisomeles malabarica* and *Gloriosa superba* to have the acaricidal and insecticidal activity against *Haemaphysalis bispinosa* and *Hippobosca maculate* [24]. Castor oil inhibits oocyte maturation in the ticks [25]. The effect of different treatments of *R. communis* plant extract (20%) and oil emulsion (5% and 10%) on mortality and oviposition behaviour of *Plutella xylostella* have been studied in laboratory and field cage experiments and found that 10% oil emulsion resulted in 100% mortality in both ingestion and contact toxicity tests [26]. In the present study, castor oil nanoemulsion resulted in 100% mortality of *An. culicifacies* larvae at 10ppm. High mortality of the larvae could be attributed to the presence of ricinoleic esters in the castor oil. Nanoemulsions are the new trend in the pesticide formulation. The size of droplet ranges from100-600nm [27]. Nanoemulsions have various advantages over emulsions like stability, long shelf life [28], low viscosity and transparency make them an attractive system for many industrial applications, such as drug delivery cosmetics and in pesticide delivery [29]. Neem oil based nanoemulsion has shown good efficacy as compared to synthetic pesticides [30]. This is the first time that a nanoemulsion formulation using castor oil with a small droplet size (Z-average diameter) of 114nm has been reported as an effective larvicide against *An. culicifacies*

Table 1: Composition of the formulations and their stability

Ingredients %w/w(gram)	F1	F2	F3	F4	F5
Castor oil	10	10	10	10	10
Propylene Glycol	5	5	5	5	5
Tween-80	4	4	6	9	14
Span-20	3	6	5	5	4
Distilled Water	78	75	74	71	67
Initial physical appearance	White emulsion	White emulsion	Translucent	Clear transparent	Clear transparent
Physical appearance after one month at R.T	Phase separation	Phase separation	No phase separation	No phase separation	No phase separation
Physical appearance (ATS) after one month	Phase separation	Phase separation	Phase separation	Phase separation	No Phase separation

Table 2: Fatty acids identified in castor oil

Peak	RT(Retention time) minutes	% Area	Name of the Compound
1	18.85	8.03	Palmitic acid methyl ester
2	21.26	0.21	Margaric acid methyl ester
3	22.87	19.09	Linoleic acid methyl ester
4	23.04	21.42	Oleic acid methyl ester
6	23.64	9.31	Stearic acid methyl ester
7	25.27	0.30	Cis-10-nonadecanoic acid, methyl ester

8	27.29	38.25	Ricinoleic acid methyl ester
9	27.42	2.62	Cis-11-eicosenoic acid, methyl ester
10	27.98	0.47	Nonadecanoic acid, methyl ester
11	31.26	0.53	Myristoleic, methyl ester, (Z)-
12	32.00	0.12	Docosanoic acid, methyl ester
13	35.74	0.12	Tetracosanoic acid, methyl ester

Table 3: Bioassay of the castor bulk and nanoemulsion against third instar larvae of *Anopheles culicifacies*

Concentration	% Mortality	LC_{50} (LCL- UCL)	LC_{90} (LCL-UCL) Regression Chi- Square(Df) Equation
Nanoemulsion			
1	26.3 ± 1.5 3.4 (2.0-4.6)	8.9(7.2-12.3)	y=23.131+7.341X 3.11(3)
2	43.3 ± 1.5		
4	53.3 ± 1.5		
8	76.3 ±0.6		
10	100 ± 0.0		
Bulk emulsion			
10	10± 0.8 52.31(37.52-72.77)	242.28(153.35-530.92)	y=23.36+0.2642X 2.29(4)
20	23 ± 1.6		
40	41 ± 2.4		
80	58 ± 2.4		
160	74.6 ± 2.6		
320	100 ± 0.0		
Control	Nil		

Where LCL and UCL are upper and lower fiducial limits at 95% confidence

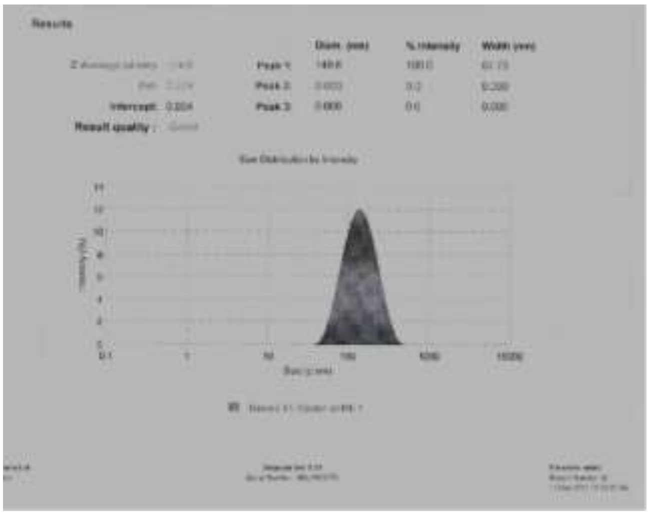

Fig 1: Particle size distribution by DLS

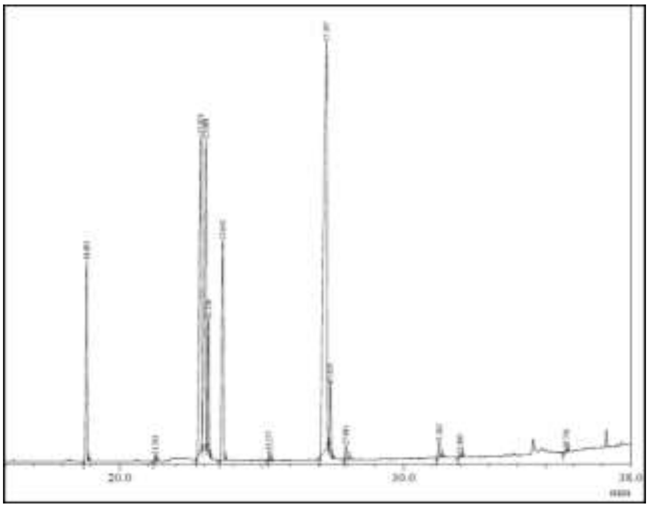

Fig 2: Chromatogram of castor oil

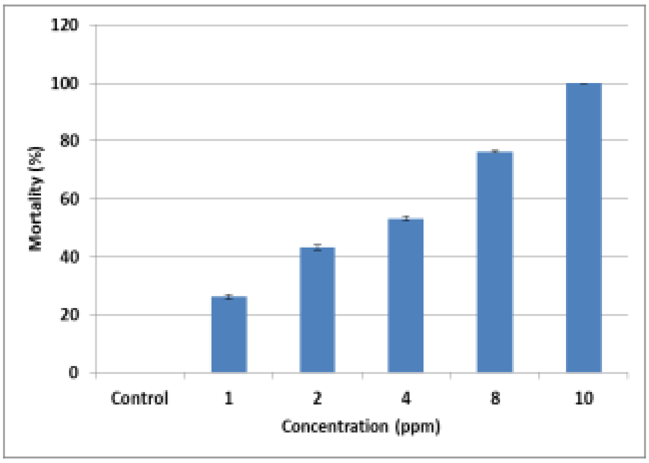

Fig 3: Percent mortality of *An. culicifacies* larvae within 24 hrs of treatment with castor oil nanoemulsion

Conclusion

Castor oil being plant-based is ecofriendly and has natural pesticidal action. The nanoemulsion formulation of castor oil ensures higher efficacy as a larvicidal agent against *An. culicifacies* when compared to its bulk or ordinary emulsion. From this study, it can be concluded that castor oil nanoemulsion can be used as a safe and effective alternative for controlling vector-borne diseases caused by mosquito larvae and may be recommended for field application.

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Conflict of Interests

The authors declare no conflict of interest.

Submission Deceleration and Verification

This work is not under consideration for publication elsewhere and publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Ethical Statement, Involvement of Human/ Animal in the study

Not Applicable

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