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Formulation and Evaluation of collegial effect of Volatile Oil and Flavonoid Nanoemulsion for its Anti-pest and Anti-fungal Properties

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Abstract: We have formulated Volatile oil, Flavonoid and Collegial combination of both based nanoemulsion with antipest and antifungal properties. It is stabilized and particle size characterized by using Malvern Zeta sizer has been tested using and found the size in the range of 10.0 to 25.0 nm. It was further evaluated in field for antioxidant, IGR, insect antifeedant, antifungal, and nematicidal activity.

Introduction:

Nanoemulsion is a type of formulation with uniform and extremely small droplet sizes typically in the range between 10 and 100 nm [3].As these have finer droplet sizes, they possess optical transparency, good stability to gravitational separation and droplet aggregation, as well as increased bio-efficacy against conventional microemulsion (diameter >200 nm).[4,5].It has been observed that nanoemulsions also require lower concentration of surfactants, typically 5-15% as compared to conventional microemulsion that needs at least 20% of the surfactants. We can also avoid the use of a number of organic solvents and adjuvants, which are considered to be harmful to the environment; so, they can be used in foods, beverages, agrochemicals and pharmaceuticals [6]. Nanoemulsions are thermodynamically unstable systems so high energy input is required for their preparation. Generally, two main approaches are used for the preparation of nanoemulsions: High-energy methods include extreme shear stirring, high-pressure homogenizers, ultrasound generator and microfludizer, which uses high mechanical energy to achieve the blending and crushing of oil and water phase [3]. Due to the high cost, high energy methods are not preferred. And low-energy emulsification methods, based on the spontaneous formation of nanoemulsions by taking benefits of the internal chemical energy of the system, are energy-efficient and involve phase transition taking place during this emulsification process. Many low-energy methods such as spontaneous phase inversion

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temperature (PIT), spontaneous emulsification and phase inversion composition (PIC) methods have been proposed to formulate nanoemulsions, which depends on specific changes in temperature at constant composition and specific changes in composition at a constant temperature [7].

The growing demand for natural products has intensified in the past few decades as these are extensively used as biologically active compounds and are being considered as an alternative strategy for sustainable insect pest management in agriculture. Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by the plant as secondary metabolites. In nature, essential oils seem to be an important agent and play an important role in the protection of the plants as antibacterial, antiviral, antifungal, insecticides etc.

Volatile (Allium sativum) belongs to the Alliaceae family and has 1-2% (dry basis) essential oil. Volatile is a commonly consumed food ingredient that is derived from a widely cultivated plant, Allium sativum. It is a strongly scented culinary herb believed to be native to the high plains of Western Central Asia and cultivated throughout the world [8]. Volatile oil is the product of the steam distillation of volatile and it is also edible-though regarded as unpalatable.

The oil is often obtained by steam distillation [9]. The Sulphur containing constitutes diallyl disulfide and diallyl trisulfide are the predominant constituent of the oil, with diallyl disulfide comprising up to 60% of the oil by weight [8]. It is known to have medicinal properties such as lowering blood cholesterol, anti-inflammatory and anticancer activity and also antibacterial, antifungal and antiviral and antioxidant properties. Volatile is one of many plants that discovered to create secondary metabolites as a defense to pests and disease damage [10].

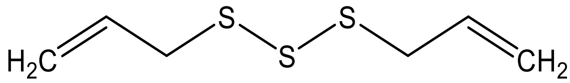

Fig. 1 Structural Formula of Volatile Oil

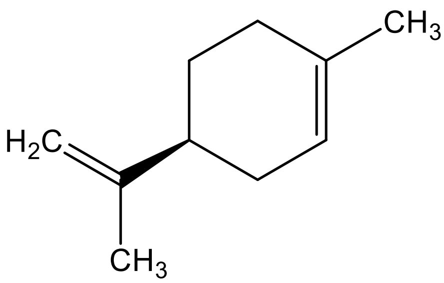

Fig. 2 Structural Formula of Flavonoid

Flavonoid is a common naturally occurring compound with a citrus scent and lemon-like flavor, and occurs in citrus and certain fruits, vegetables and spices. This is chemically a

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monoterpene that exhibit toxicity against several insects [11]. It is used as both an active and inert ingredient in pesticides products, and as an ingredient in food products, soaps and perfumes. As an active ingredient, it is used as an insecticide, insect repellent and animals (dogs and cats) repellent and it is also used as a solvent or fragrance in pesticide formulations. It is also commonly used in products such as certain food, soaps and perfumes. FDA considers Flavonoid to be Generally Recognized as Safe as a food additive when used as a synthetic flavoring substance and adjuvants.

Though, research on nanoemulsion is growing, but nanoemulsion based on a plant extract oils less exploited, due to poor solubility in water. This issue can be solved by encapsulating these oils in oil-in-water emulsions or nanoemulsion [12].

The present work envisages developing nanoemulsions based on Volatile oil and Flavonoid using low-energy spontaneous emulsification technique, which to the best of our knowledge, is the first report of its kind. In this report nanoemulsion is developed by mixed surfactants (both anionic and non-anionic like calcium dodecylbenzene sulfonate, Tristrylphenol ethoxylate sulfate and castor oil ethoxylate 40 moles) using volatile oil as oil phase and activities.

It has been observed that the mixed surfactants perform better for various applications, as compared to single pure surfactants [13]. Therefore, we used the mixture of non-ionic Octylphenol ethoxylate (PO-10), Castor oil ethoxylate 40 moles (Stepantex CO-40), EO-PO copolymer, Tween 80 and anionic calcium dodecylbenzene sulfonate (CABS), Tristrylphenol ethoxylate sulfate (Soprophor series), to prepare nanoemulsion formulation.

Volatile oil is regarded as a botanical insecticide for its great stability and antipest, antifungal and other activities, its structural formula is shown in Fig. 1 but its wider application in pest control due to its water insolubility. The low-energy method has been used in our work which involves constant temperature but different compositions (using different ratio of different anionic and non-ionic surfactants) during emulsification by adding water to an oil-surfactant mixture to obtain the oil-in-water (o/w) nanoemulsion [14]. To obtain the best emulsification conditions we investigated the effects of the mass ratio of OP-10 and CABS, the mass ratio of oil and the mixed surfactant, stirring rate, the addition rate of water and the emulsification temperature on the mean droplet size of the nanoemulsion. The emulsion stability at ambient and long term stability at 54^0C for 2 weeks was studied to check the shelf life and stability of

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emulsion and PH effect and also the insecticidal activity was further studied. The main objective of this work is to obtain the stable emulsification condition for the preparation of transparent stable nanoemulsion of Volatile oil and Flavonoid via the PIC method and to evaluate the assessment of the shelf life of this nanoformulation.

Experimental procedure:

Materials

Volatile oil and Flavonoid were purchased from local market. Octylphenol ethoxylate (PO-10), Castor oil ethoxylate 40 moles (Stepantex CO-40), EO-PO copolymer, Tween 80 and anionic calcium dodecylbenzene sulfonate (CABS), Tristrylphenol ethoxylate sulfate (Soprophor series) were received from Solvay, Croda, Stephan and Core Chemicals. All the chemicals used are of analytical grade. The Stirrer, Overhead stirrer (IKA Model Euro-ST 20D S000) and distilled water, centrifuge, PH Meter, beaker, measuring cylinder, Particle size Analyzer (Malvern), laminar flow, Mettler Toledo weighing balance were used for the preparation of nanoemulsion and further studies.

Preparation of Primary nanoemulsion of Volatile oil (GNE)

The primary nanoemulsion was formulated using a spontaneous emulsification process [15]. The surfactants and the Volatile oil were mixed in different ratios keeping Volatile oil as 5% and slowly added into aqueous phase drop wise followed by stirring (500 rpm) for 2 hours at room temperature. Different proportion of surfactants mixed with 5% Volatile oil and water (15:5:5, 10:10:5, 5:15:5,3:12:5) were standardized.

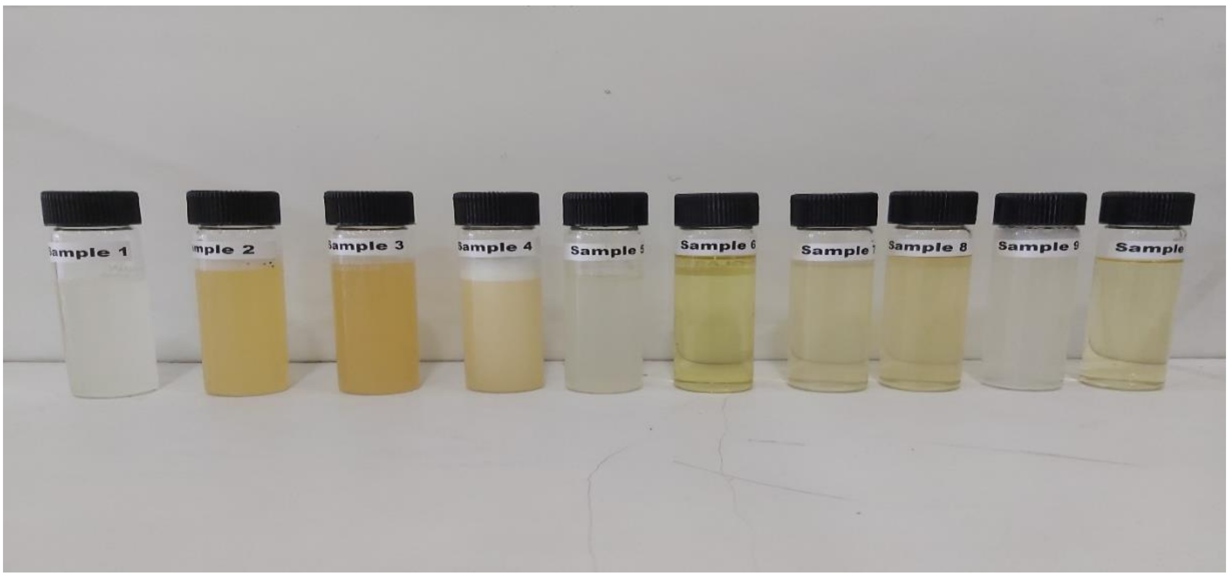

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Fig. 3 Volatile oil nanoemulsion with different percentage of surfactants

Preparation of Primary nanoemulsion of Flavonoid (DLNE)

In the same way, nanoemulsion of Flavonoid was formulated using surfactants mix and water with 5% Flavonoid (15:5:5, 10:10:5, 5:15:5,3:12:5,3:15:5) were standardized.

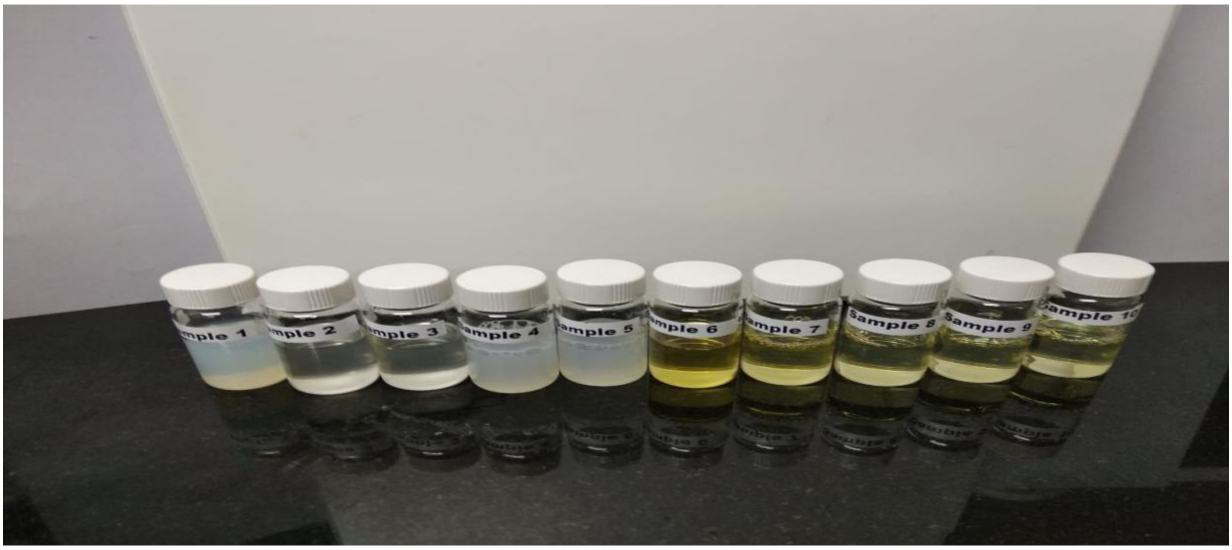

Fig. 4 Flavonoid nanoemulsion with different percentage of surfactants

Preparation of Primary nanoemulsion of Flavonoid with Volatile oil

Standardized primary emulsion of Flavonoid, surfactant mix and water (10:10:5) was used to prepare Nanoemulsion with a 5% concentration of Volatile oil by a similar procedure of spontaneous emulsification. Volatile oil was added to the standardized primary nanoemulsion of Flavonoid drop wise and stirred using IKA overhead stirrer at 500 rpm for 2 hours at room temperature.

Determination of particle size of nanoemulsions

Particle size analyzer (Malvern) is based on Dynamic Light Scattering (DLS) which helps to detect the fluctuation of the scattering intensity due to the Brownian motion of a particle in suspension or emulsion. DLS measurement was done 25^{0}C. The sample solution was prepared in distilled water and 5 ml of the sample solution was taken into a glass vial.

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Table 1

Average droplet size (nm) of Volatile Oil Nanoemulsion (GNE)

Nanoemulsion	GNE1	GNE 2	GNE 3	GNE 4	GNE 5	GNE 6	GNE 7	GNE 8	GNE 9	GNE10
Composition	CABS-10+FFT 40-5+5 Volatile oil+water	CABS-15+FFT 40-5+5 Volatile oil+water	CABS-20+FFT 40-5+5 Volatile oil+water	Soprophor FLK-15+FFT 40-5+5 Volatile oil+water	Soprophor BSU-15+FFT 40-5+5 Volatile oil+water	Soprophor 4D384-10+FFT 40-10+5 Volatile oil+water	Soprophor 4D384-5+FFT 40-15+5 Volatile oil+water	Soprophor 4D384-3+FFT 40-12+5 Volatile oil+water	Soprophor 4D384-3+FFT 40-15+5 Volatile oil+water	Soprophor 4D384-3+FFT 40-17+5 Volatile oil+water
Size (nm)	25.15±3.5	25.05±3.0	25.25±3.5	24.95±3.2	25.15±3.5	23.39±2.5	23.15±3.4	24.82±3.5	25.15±4.5	29.25±4.5

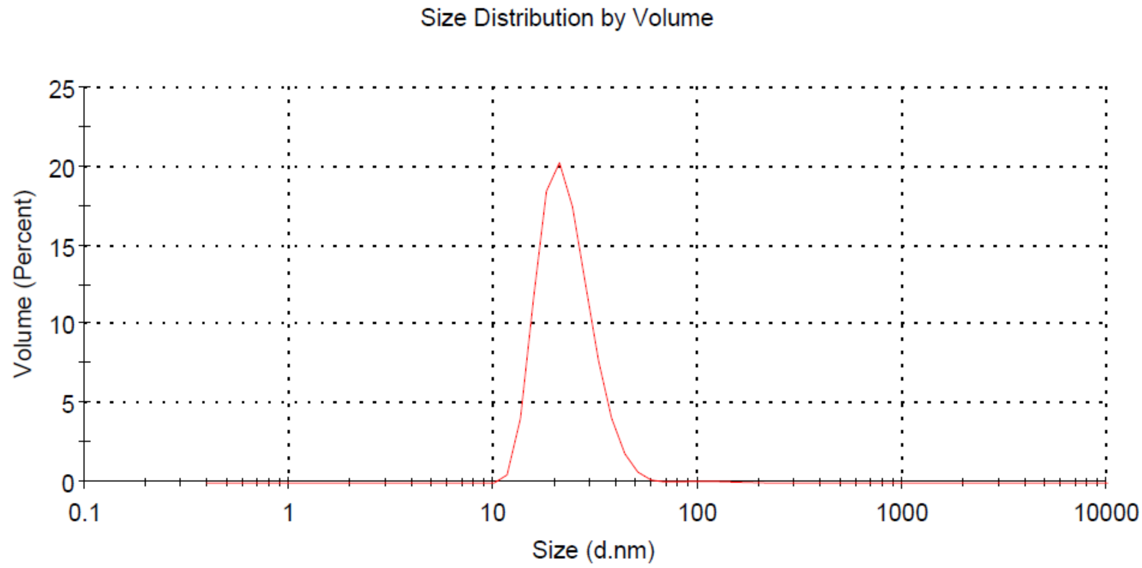

Fig. 5 Droplet size of GNE 6

Table 2

Average droplet size (nm) of D Limonene Nanoemulsion (DLNE)

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Nanoemulsion	DLNE1	DLNE2	DLNE3	DLNE4	DLNE5	DLNE6	DLNE7	DLNE8	DLNE9	DLNE10
Composition	CABS-10+FFT 40-5+5 Flavonoid +water	CABS-15+FFT 40-5+5 Flavonoid +water	CABS-20+FFT 40-5+5 Flavonoid +water	Sopraphor FLK-15+FFT 40-5+5 Flavonoid +water	Sopraphor BSU-15+FFT 40-5+5 Flavonoid +water	Sopraphor 4D384-10+FFT 40-10+5 Flavonoid +water	Sopraphor 4D384-5+FFT 40-15+5 Flavonoid +water	Sopraphor 4D384-3+FFT 40-12+5 Flavonoid +water	Sopraphor 4D384-3+FFT 40-15+5 Flavonoid +water	Soprophor 4D384-3+FFT 40-17+5 Flavonoid +water
Size (nm)	10.99±3.5	9.83±3.0	11.94±3.5	11.05±3.2	10.38±3.5	11.71±2.5	10.63±3.4	10.83±3.5	12.54±4.5	12.07±4.5

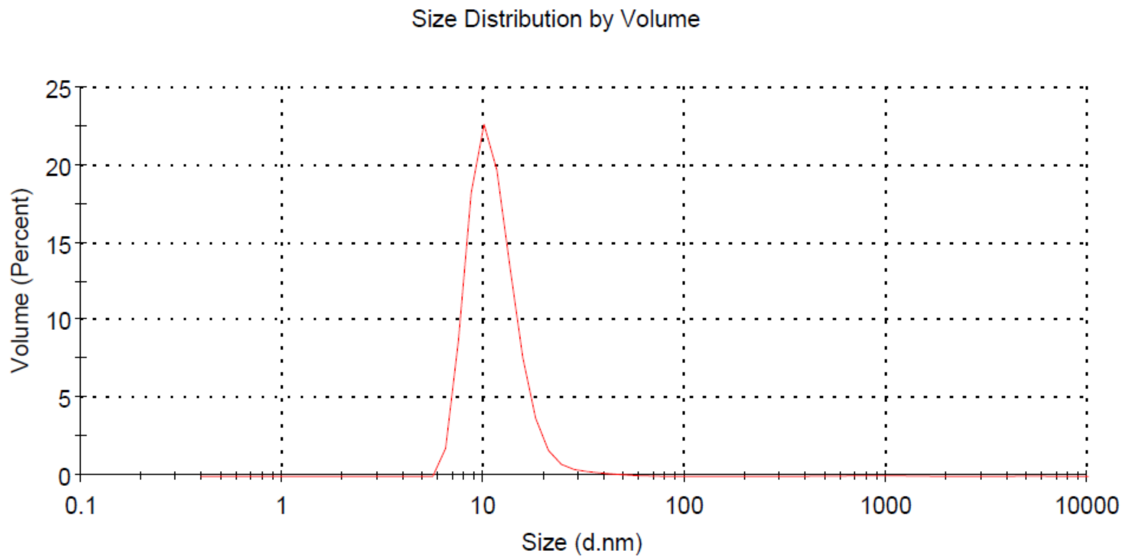

Fig. 6 Droplet size of DLNE

Table 3

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Average droplet size (nm) of D Limonene & Volatile oil Nanoemulsion (DLGNE)

Nanoemulsion	DLGNE1	DLGNE2	DLGNE3	DLGNE4	DLGNE5	DLGNE6	DLGNE7	DLGNE8	DLGNE9	DLGNE10
Composition	CABS-10+FFT 40-5+2.5 Flavonoid+2.5 Volatile oil +water	CABS-15+FFT 40-5+2.5 Flavonoid+2.5 Volatile oil +water	CABS-20+FFT 40-5+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor FLK-15+FFT 40-5+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor BSU-15+FFT 40-5+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor 4D384-10+FFT 40-10+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor 4D384-5+FFT 40-15+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor 4D384-3+FFT 40-12+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor 4D384-3+FFT 40-15+2.5 Flavonoid+2.5 Volatile oil +water	Soprophor 4D384-3+FFT 40-17+2.5 Flavonoid+2.5 Volatile oil +water
Size (nm)	10.99±3.5	9.83±3.0	11.94±3.5	11.05±3.2	10.38±3.5	11.71±2.5	10.63±3.4	10.83±3.5	12.54±4.5	12.07±4.5

Determination of morphology of nanoemulsions

The morphology of the Volatile oil nanoemulsion and Flavonoid nanoemulsion were studied by Transmission Electron Microscope (TEM). In this analysis, a drop of nanoemulsions was placed on a carbon-coated copper grid of 400 mesh size and allowed to dry in vacuum [16], washed with distilled water and stained with a negative stain (2% uranyl acetate). The extra water was dried using filter paper gently and then viewed under TEM at different magnification levels.

Determination of PH of nanoemulsions

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The PH of Volatile oil and Flavonoid nanoemulsions was determined using PH meter at 25 ± 1^0C.

Table 4

No.	**Volatile Oil Nanoemulsion (GNE)**	**pH**	**No.**	**Flavonoid Nanoemulsion (DLNE)**	**pH**
1	GNE 1	5.3	1	DLNE 1	5.6
2	GNE 2	5.3	2	DLNE 2	5.8
3	GNE 3	5.5	3	DLNE 3	5.7
4	GNE 4	5.5	4	DLNE 4	5.6
5	GNE 5	5.6	5	DLNE 5	5.7
6	GNE 6	6.1	6	DLNE 6	6.9
7	GNE 7	6.1	7	DLNE 7	6.8
8	GNE 8	6.2	8	DLNE 8	6.8
9	GNE 9	6.2	9	DLNE 9	6.9
10	GNE 10	5.9	10	DLNE 10	6.8

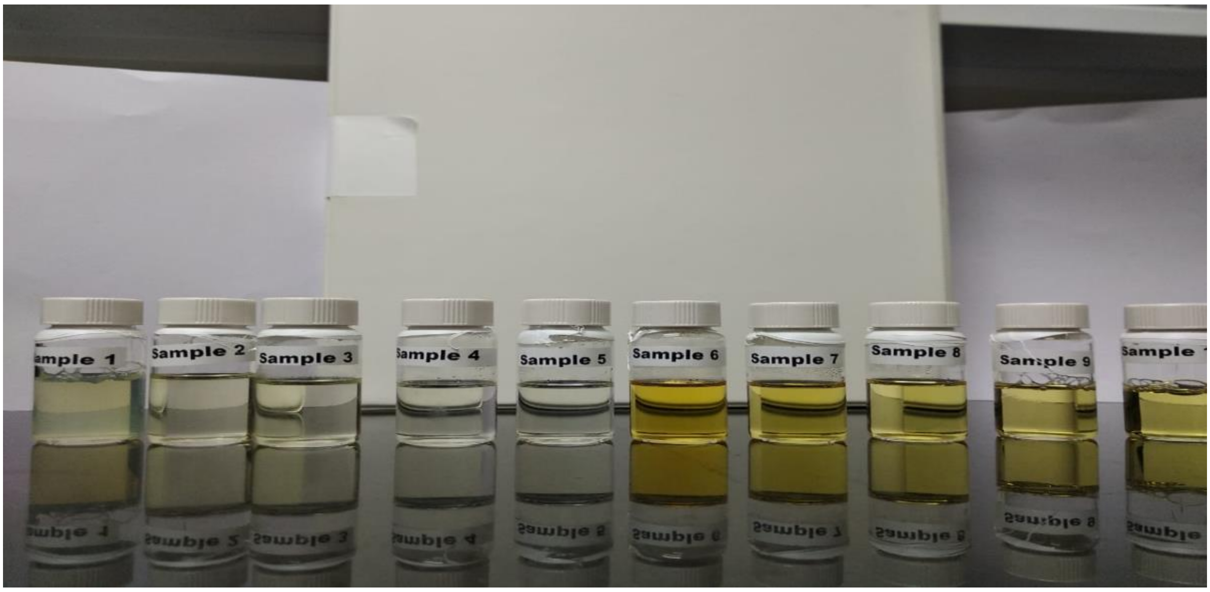

Fig. 7 Stability of nanoemulsion

Determination of stability of nanoemulsions

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The stability of Volatile oil and Flavonoid nanoemulsion were checked by centrifuging the nanoemulsion at 3500 rpm for 30 minutes [17] and also studied at 54^{0}C for 2 weeks.

Results and discussions:

Preparation of primary emulsion and nanoemulsion

Volatile oil, CABS, castor oil ethoxylate 40 moles and Soprophor 4D384 and distilled water were used as raw materials for the preparation of primary oil-in-water (O/W) emulsions. FFT-40 surfactant has less impact of PH and ionic strength. Similarly, Flavonoid oil, castor oil ethoxylate 40 moles (FFT 40) and Soprophor 4D384 and distilled water were used for the preparation of (O/W) primary emulsion. After studying different combination of raw materials, it has been observed that the best ratio of oil, surfactants and distilled water is 10:10:5. The increase in the surfactant to Volatile oil or Flavonoid oil results a decrease in droplet size (Fig. 3 & 4). Combination of these 3 emulsifiers gives better stability and got required particle size.

Droplet size of Volatile oil and Flavonoid Oil nanoemulsions

The droplet size of Volatile oil nanoemulsion is shown in (Table 1) and (Fig.3). The average droplet size of Volatile oil nanoemulsion with different percentage of surfactants ranged from 23.39± 2.5 nm to 29.25± 4.5 nm (Fig. 5). The prepared nanoemulsions with 5% Volatile oil, 10% Soprophor 4D384 and FFT-40 10% (GNE 6) and showed the minimum droplet size and were found to be 23.39± 2.5 nm. It has been observed that the increase in surfactant concentration in nanoemulsion resulted in decreasing the droplet size and turbidity. The emulsion turbidity is a function of concentration and the size of particle [18].

The droplet size of Flavonoid oil nanoemulsion is shown in (Table 2) and (Fig.4). The average droplet size of Flavonoid oil nanoemulsion with different percentage of surfactants ranged from 9.83± 3.0 nm to 12.54± 4.5 nm (Fig. 6). The prepared nanoemulsions with 5% Flavonoid oil, 10% Soprophor 4D384 and FFT-40 10% (DLNE 6) and 5% Flavonoid oil, 5% Soprophor 4D384 and FFT-40 15% (DLNE 7) and showed the minimum droplet size and were found to be 11.71± 2.5 nm and 10.63± 3.4 nm.

Stability of nanoemulsions

The stability of nanoemulsion depends on the physicochemical characteristics of its ingredients including surfactants, oil phase and water phase [19]. This study was carried out by

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centrifuging the nanoemulsions (Volatile oil and Flavonoid) at 3500 rpm for 30 min [17]. Results showed that the nanoemulsions were physically stable at room temperature and after stability study at 54^{0}C for 14 days (Fig. 3,4 and 7). The stability of nanoemulsions droplets of the oil was due to the surfactant which reduced the interfacial free-energy providing the mechanical barrier to calescence [20].

pH of nanoemulsions

PH have an important role in the stability of the emulsion system [19]. The PH of Volatile oil and Flavonoid oil's nanoemulsions measured given in the Table 4. The PH value of the Volatile oil nanoemulsion ranged from 5.3 to 6.2 and that of the Flavonoid nanoemulsions from 5.6 to 6.9. No relation has been observed for the PH change with increasing concentration of surfactants in nanoemulsions.

In this study, we report nanoemulsion of volatile oil and Flavonoid using mixed surfactants (non-ionic and anionic) through the low-energy emulsification method. On the basis of Malvern Zeta sizer, the nanoemulsions were obtained in the average size range of 10 nm for Volatile oil and 25.01 nm for Flavonoid. The nanoemulsion was further evaluated *in vitro* for antioxidant, IGR, insect antifeedant, antifungal, and nematicidal activity.

Conclusions:

The optimized nanoemulsion showed significant better IGR, insect antifeedant, antifungal and nematicidal activity without loss of antioxidant potential in comparison to volatile oil. This system has the potential for agriculture application.

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