

Original Research Article

Eco-friendly oil-in-water emulsion formulation of eucalyptus oil against some important phytopathogenic fungi

Abstract

Antifungal activity of eucalyptus oil against some important phytopathogenic fungi and development of an effective and stable oil water emulsion formulation of agricultural use were demonstrated in this study. Stability studies were prolonged at different conditions to predict the stability of the prepared formulation. Different parameters, namely, pH, viscosity, flash point, density, surface tension, effect of centrifugation, persistent foaming and emulsion stability were determined during stability studies. The activity of the oil itself and its developed formulation oil in water emulsion (EW) was tested *in vitro* with the poised food technique. The results demonstrate that the prepared formulation showed the better storage stability. Also, had good antifungal activity as a mycelia growth inhibition against all tested phytopathogenic fungi in compared with active substance of eucalyptus oil.

Keywords: Oil-in-water, emulsion, formulation, eucalyptus oil, antifungal activity, phytopatogenic fungi.

1. Introduction

Phytopathogenic fungi are one of the major economic problems of crop production. Apart from their potentiality to cause yield losses and fruit decay, many of them represent a very serious risk for consumers because of their production of dangerous mycotoxins (Nanasombat and Wimuttigosol, 2011). For many years, a variety of different synthetic fungicides have been extensively used as antifungal agents to inhibit the growth of phytopathogenic fungi. However, the widespread use of fungicides has significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food, and threat to human health and environment in addition; there is a risk of pathogenic microorganisms developing resistance (Barnard et al., 1997; Brent and Hollomon, 1998). In an attempt to reduce the use synthetic fungicides, many researchers have concentrated attention on the use of safe and biodegradable alternatives as

natural fungicides to replace synthetic chemicals, for protecting crops from various plant diseases (Copping and Menn, 2000; Costa et al., 2000; Ujvary, 2002; Paranagama et al., 2003).

One such alternative is the use of natural plant protectants with pesticidal activity, as well as they tend to have low mammalian toxicity, less environmental effects and wide public acceptance essential oils. Essential oils (EOs) gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional uses (Sawamura, 2000; Ormancey et al., 2001) and commonly used as natural preservatives and fragrances in cosmetic products. More recently, thanks mainly to their antimicrobial properties, new applications as food preservatives (Burt, 2004), growth promoters in livestock (Calsamiglia et al., 2007), natural pesticides in organic agriculture (El-Shafei et al., 2010; Aloui et al., 2014) and insecticides (Phillips et al., 2010) are emerging. Eos typically are volatile and they rapidly evaporate from surfaces. An additional important problem in practical application of essential oil in agriculture is a lack of persistent efficacy in field (Chen et al., 2013). It is thus desirable to formulate them, in its effective concentration, in a way that allows minimizing the evaporation and protecting the oil, from high temperature, oxidation and UV light, at the same time. Besides, such formulations should allow for a selective release and for the increase of the shelf life of the oil. For this purpose, oil-in water emulsions (O/W) are one of the preferred formulations of EOs.

Oil-in-water emulsions are now receiving considerable attention because of the need to reduce or eliminate volatile organic solvents for safer handling. Because they are water based, So, the aim of the present study was to prepare an environmentally friendly formulation oil-in-water emulsion (EW) of eucalyptus oil and check shelf life stability by measuring number of physical parameters before and after storage tests to be sure that the formulation was stable and evaluate *in vitro* antifungal activity of the stable formulation against some important phytopathogenic fungi.

2. Materials and Methods

2.1. Chemicals.

Eucalyptus oil was purchased from Kato Aromatic Company for Flavors, Fragrances, Coloring Extracts and Essential Oils, Giza, Egypt, Non-ionic surfactant; Ethoxylated Castor oil HLB 14.9 (ALKAMULS 14/R) was kindly supplied by Rhodia Home, Personal Care and

industrial Ingredients, Milano, Italy. Propylene glycol, sodium hydroxide were purchased from ADWIC; El Nasr Pharmaceutical Chemical Co., Egypt, calcium carbonate was purchased from Sigma- Aldrich Chemie GmbH Steinheim, Germany, magnesium oxide and methyl red were purchased from Qualikems Fine Chemicals. PVT. Ltd. India. Ammonia Solution was purchased from Prolabo. Agar and Dextrose used in the biological activity test are products of El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt. Streptomycin Antibiotic is a product of El Nil Co. for Pharm. and Chem. Ind, Cairo, Egypt. Water used in all preparations obtained from Water distiller LABCONCO water PROT.M PS LABCONCO Corporation, KANSAS City, Missouri 64132-USA.

2.2. Fungal species used.

Five phytopathogenic fungi were used in this study; *Colletotrichum dematium*, *Drechslera hawaiiensis*, *Humicola Fuscoatra*, *Phoma spp* and *Nigrospora sphaerica* were provided by Fungicides, Bactericides and Nematicides Department, Central Agricultural Pesticides Laboratory (CAPL), Dokki, Giza, Egypt.

2.3. Emulsion Preparation

The oil-in-water emulsion formulation was prepared by incorporating the oil phase into the water phase; eucalyptus oil (20.00 wt %) was mixed together with the surfactant by stirring (using a magnetic stirrer with hot plate “Terrey Pines Scientific”, USA). The water phase was prepared with deionized water, preservative and propylene glycol. Finally, the oil phase was added into the water phase under high shear mixing.

2.4. Physicochemical characterization.

2.4.1. Visual Inspection

The concentrated oil water emulsion formulation was observed for color, homogeneity, emulsion stability or separation during storage.

2.4.2. Emulsion stability and re-emulsification (MT 36.3)

The formulation, when diluted at $30 \pm 2^\circ\text{C}$ (Notes 6 & 7) with CIPAC Standard Waters A and D. (MT 18).

2.4.3. Storage stability

Stability tests at elevated temperatures are designed to increase the rate of chemical degradation or physical change of a product. Accelerated testing is performed at elevated temperatures in an attempt to obtain information on the shelf life of a product in a relatively short time. Accelerated testing involves extrapolations from higher to lower temperatures and from shorter to longer storage periods. The FAO Pesticide Specifications recommend testing of the relevant product parameters before and after storage at $54^{\circ}\text{C} \pm 2$ for 14 days (MT 46.3). Also, liquid formulations (oil water emulsion) may be adversely affected by storage at low temperature. Storage at low temperature may result in crystallization of active constituent, significant changes in viscosity or separation of formulation. The liquid formulations should also be tested at $0 \pm 2^{\circ}\text{C}$ or lower for 7 days. (MT 39.3).

2.4.4. Mean particle size distribution.

The mean particle size of oil-in-water emulsion of eucalyptus was determined by light scattering, using Zetasizer Ver.6.20 (Malvern Instruments Ltd., Worcestershire, England) Sample analysis was carried out after sample preparation. The concentrated emulsion was diluted with deionized water (1:100) to avoid multiple scattering effects, and placed directly into a metal jar that circulated the sample through the measuring-glass cuvette.

2.4.5. Freeze -Thaw Cycles

Freeze-thaw cycles are a method of putting stress on the formulations to stimulate the conditions that are encountered in warehouse storage. Test tubes filled with the prepared formulations and hermitically closed were vertically stored for 12h in freezer at -20°C , and then for 12h at room temperature $25^{\circ}\text{C} \pm 2$. Emulsion was exposed to four such cycles and observed for any physical changes like creaming, coalescence and phase separation. The formulation is considered "stable" if there is no substantial separation after four cycles.

2.4.6. Centrifugation test

The centrifugation is a relatively simple method and allows one to accumulate a large set of data for a relatively short period of time. Laboratory Centrifuge REMI Centrifuge REMI Equipments Bombay-India- R32A.4000002 was used to determine the stability of the emulsions

against gravity. Each sample was centrifuged for 10 min at 5000 rpm in 10 ml graduated plastic test tube, to monitor whether phase separation occurred. The formulation was centrifuged at 25°C.

2.4.7. Persistent foam

Persistent foam is a measure the amount of foam likely to be present in a spray tank or other application equipment following dilution of the product with water. Specified amount of the prepared formulation is added to CIPAC standard waters A and D (95ml) in the measuring cylinder and made up to the mark. The cylinder is stoppered and inverted 30 times. Stand the cylinder on the bench and left undisturbed for the specified time. The volume of foam was noted. [CIPAC MT 47.2].

2.4.8. pH Measurement.

pH value of prepared formulation was measured by using a pH Meter “Jenway Instruments pH 3510 pH meter. It was recalibrated before testing. [CIPAC MT 75.3].

2.4.9. Surface Tension.

Surface tension of the prepared formulation was measured using “Sigma 700” by du Noüy Ring, a platinum/iridium ring. The instrument recalibrated before testing, the sample measured should be clean, homogenous and free from any bubbles and has a stable surface.

2.4.10. Density measurement.

Density of the prepared formulation was measured using digital density meter model DDM 2910 by touch screen. Rudolph Research Analytical, USA.

2.4.11. Flash point.

Flash point is an important property of the formulations, because it is used in various ways to control the conditions under which substances are considered potentially dangerous as fire hazards are stored, used and transported. Measurement of flash point of prepared formulations was carried out by tag open-cup method by Koehler instrument company, INC, USA. The flash point was recorded as the temperature at the thermometer when a flash appeared. [CIPAC MT 12].

2.4.12. Viscosity Measurement.

The apparent viscosity of emulsion play an important role for determining the stability in terms of creaming and coalescence as settling of oil droplet depend on viscosity of the medium. Viscosity of the prepared formulation was measured at different shear rates, without dilution, using "Brookfield DV II⁺ PRO" digital Viscometer. (Brookfield, USA). UL rotational adaptor. The temperature was kept at 25°C during the measurement by water bath TC-502. USA and each reading was taken after equilibrium of the sample. The flow curves of all prepared formulations were obtained by directly reading the viscosity (mPas) and shear rate (s⁻¹) from the viscometer. [ASTM, 2010].

2.5. Biological activity.

In vitro antifungal activity of eucalyptus oil and prepared stable oil water emulsion formulation was performed with the poisoned food technique (Singh *et al.*, 2008). in which we measure the inhibitory zone diameter using potato dextrose agar (PDA) media (200 g grated potato, 20 g dextrose, 15 g agar) (Atlas, 1995). Different concentrations of prepared formulation were used in antifungal activity test against five fungus species viz. *Colletotrichum dematium*, *Drechslera hawaiiensis*, *Humicola Fuscoatra*, *Phoma spp* and *Nigrospora sphaerica*. The Erlenmeyer flasks containing media were sterilized in an autoclave at a pressure of 15 lb/sq inch and temperature 121°C for 15 min. The different concentrations was added to sterilized media, cooled to 30-35°C, and shaken thoroughly. To avoid bacterial contamination, Streptomycin was added to the media before pouring into petri dishes (9 cm diameter). The media were then poured into a set of three petri dishes (three replicates) under aseptic conditions in a laminar flow chamber with filter (Labconco Corporation, Kansas City, Missouri 6432). After partial solidification of the media in the plate, a disc (4 mm diameter) of the fungal species was cut from 1-week-old culture with the help of a cork borer and inoculated to the center of the poured plates of treatments and control sets. The plates were sealed with Parafilm and then incubated (NÜVE San. Mlztirvetic A.S Ankara, Turkey) at 25 ± 2°C until the fungal growth in the control dishes was almost completed. (Agarwal *et al.*, 2001).

2.6. Statistical analysis.

The concentration of the prepared oil water emulsion formulation that inhibiting the fungi mycelium growth by 50% (EC₅₀) values were determined by the linear regression (LPd line

Computer Program) of the probit of the tested fungus percentage inhibition vs. logs the concentrations (ppm) of the tested formulation.

3. Results and Discussion

3.1. Formulation characteristics.

The formulations of agrochemicals cover a wide range of systems that are prepared to suit a specific application. The main purpose of any agrochemical formulation is to make handling and application of the active ingredient as easy as possible. An important function of the formulation is to optimize the biological efficacy. This is achieved in most cases by controlling the physical characteristics of the formulation. An important criterion for any agrochemical is its safety, both to the crop and to the agrochemical workers. (Knowles, 2008).

Stability of Emulsion.

It is known that emulsions are thermodynamically unstable systems which exist in the metastable state (Sjoblom, 2006). Emulsion stability refers to the ability of an emulsion to resist changes in its properties over time: the more stable the emulsion, the more slowly its properties change (McClements, 2005). The instability of an emulsion is often a consequence of two different physical processes: increasing particle size linked with coalescence or flocculation, and migration of particles leading to creaming or sedimentation (Palazolo et al., 2005 and Sjoblom, 2006).

The stability of oil water emulsion formulation can be predicted by measuring some physical parameters before and after accelerated tests. Storage at 0°C and 54°C has been used to control physical and chemical stability. It has been generally accepted that two weeks at 54°C represent 2 years in normal conditions. There is no evidence which indicate that a product has a satisfactory shelf life (of at least 2 years) in the different temperature zones. The test thus provides a useful guide for performance after storage in warm or continental temperature climates. However, it is not quite sure that the product which passes these tests will be satisfactory in field conditions. (Gašić et al., 2012).

A preliminary study on the emulsion stability of concentrated oil water emulsion of **eucalyptus oil** showed that the phase separation was the major problem when oil water emulsion was diluted

with standard water (CIPAC A & D) at a ratio of 5: 95 (v/v) emulsion: water. The prepared formulation produced a white emulsion when diluted and no separation or sedimentation was observed in either cases (CIPAC A or D) or no change in color or appearance through the storage period. (7 days at $0^{\circ}\text{C} \pm 2$ and 14 days at $54 \pm 2^{\circ}\text{C}$). Also, the stabilities of the product on initial emulsification and re-emulsification were good. Such signs are good preliminary indication of physical stability.

Mean Particle Size

It is known that droplet size is one of the most important factors governing its stability in emulsions, and reduction of droplet size usually leads to formation of stable emulsions. Usually finer droplets give better long term stability in the formulation provided they can be prevented from flocculating, coalescing or growing by Ostwald ripening (Knowles, 2005). Also, the changes in droplet size as a prediction of time become more important to the stability of the emulsion, because the o/w emulsion possess the stability against sedimentation or creaming. The value of particle size was slightly differed in storage formulation than the fresh prepared formulation. The particle size of formulation ranged from $1.20\text{ }\mu\text{m}$ to $1.28\mu\text{m}$. The Droplet size of EW formulations should be below 2.00 microns. (Knowles, 2008).

Centrifugation Test

The centrifugation test is based on the principle of using centrifugal force to separate two or more substances of varied densities, such as two different liquids or a liquid and a solid, and is a useful tool for assessing and predicting the shelf life of emulsions (Khan et al., 2010). No phase separation after centrifugation was seen in oil-in-water emulsion samples kept at different storage conditions. This was presumably due to the proper homogenization speed during emulsion formulation which might have prevented the breakage of the formulations during testing (Abdurahman and Rosli, 2006).

Freeze -Thaw Cycles

On freezing of an o/w emulsion the following physiochemical processes that promote emulsion destabilization, may occur: (i) ice crystals form which results in the oil droplets being forced closer together; (ii) as a result of the ice formation there may be insufficient free water to maintain the emulsifier molecules at the surface of the oil droplets fully hydrated; (iii) ice

crystallization leads to an increase in the concentration of soluble constituents in the non-frozen aqueous phase surrounding oil droplets (freeze concentration), any increase in ion concentration would screen electrostatic charge repulsion forces between the oil droplets; (iv) ice crystals formed on freezing may penetrate and disrupt the interfacial membrane surrounding the oil droplets leading to coalescence of the droplets on thawing; (v) the fat phase within the droplets may solidify and protruding fat crystals may penetrate and disrupt the interfacial membrane surrounding the oil droplets leading to partial coalescence of the oil droplets at low temperatures and full coalescence on thawing (McClements, 2004). The prepared formulation is stable and no substantial separation after four cycles.

pH Test

The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles (Issa et al., 2000). The formulation exhibited acidic pH value. The pH values of the prepared formulations were in range (4.58-4.61), indicating that the formulations having acidic character implying that it will have good biological activity. (Molin and Hirase, 2004).

Surface Tension

The prepared formulation having the surface tension range (30.13-30.62 mN/m). Lower surface tension is a desirable characteristic for most agricultural sprays because it facilitates the spreading of droplets upon impaction on leaves or other target surfaces, to increase the surface active area and improves penetration and uptake of the product into the plant. (Giardino et al., 2006).

Flash Point

The flash point is a measure of the tendency of a sample to form flammable mixtures with air in controlled laboratory conditions and is parameter for storage and handling consideration of flammable materials (Encinar et al., 2005). The liquid formulations must obtained in this study, was much higher than the prescribed minimum limit of 24.5°C (Kumar and Parmar, 2000). The prepared formulation in the all storage condition having high value of flash point more than 70°C and this is quite safe.

Viscosity

The viscosity of a fluid is the property that determines the resistance offered to a shearing force under laminar flow conditions, e.g. resistance to slow stirring, or to flow through a capillary or narrow channel (APVMA, 2005). The prepared formulation of oil water emulsion formulation, showed shear-thinning behavior. The shear thinning fluid viscosity is a function of the shear rate, with higher shear causing lower viscosity. (Zhong et al., 2013). Fig (1).

Density

The variation of density was 0.9788-1.01896 g/cm³.

Persistent Foam

Persistent foam is a measure of the amount of foam likely to be present in a spray tank or other application equipment following dilution of the product with water. Results are given in Table 2. The volume of foam from the samples in CIPAC standard waters A and D is low and passed through the recommended rat of foam.

Table 1. Physicochemical properties of oil-in-water emulsion formulation of eucalyptus oil before and after storage.

Time		Fresh formulation	After 7 days	After 14 days	Freeze-thaw
Temperature		Room temp.	0°C	54°C	4 Cycles
pH value (1%)		4.61	4.58	4.59	4.60
Surface tension (mN/m)		30.13	30.40	30.39	30.62
Density(g/cm ³)		0.9788	0.9991	1.0189	1.0007
Flash point (°C)		Over 70°C	Over 70°C	Over 70°C	Over 70°C
Particle Size Distribution (Mean Diameter)		1.28µm	1.25 µm	1.23µm	1.20 µm
Stability of Emulsion and Reemulsification	0.5 h	0/0	0/0	0/0	0/0
	1 h	0/0	0/0	0/0	0/0
	2h	0/0	0/0	0/0	0/0
	24h	0/0	0/0	0/0	0/0
	REE	0/0	0/0	0/0	0/0

*REE: Reemulsification after 24.5 h

Table 2. Volume of persistence foam (cm³) observed in oil-in-water emulsion formulation of eucalyptus oil before and after storage test in CIPAC Water A and D.

Sample Code	CIPAC Water A			CIPAC Water D		
	1 min	5 min	12 min	1 min	5 min	12 min
Fresh Formulation	5	4	3	4	3	3
After 7 days 0 °C	6	5	4	5	4	3
After 14 days 54 °C	5	5	4	5	4	3
Freeze-thaw cycles	6	5	4	4	3	4

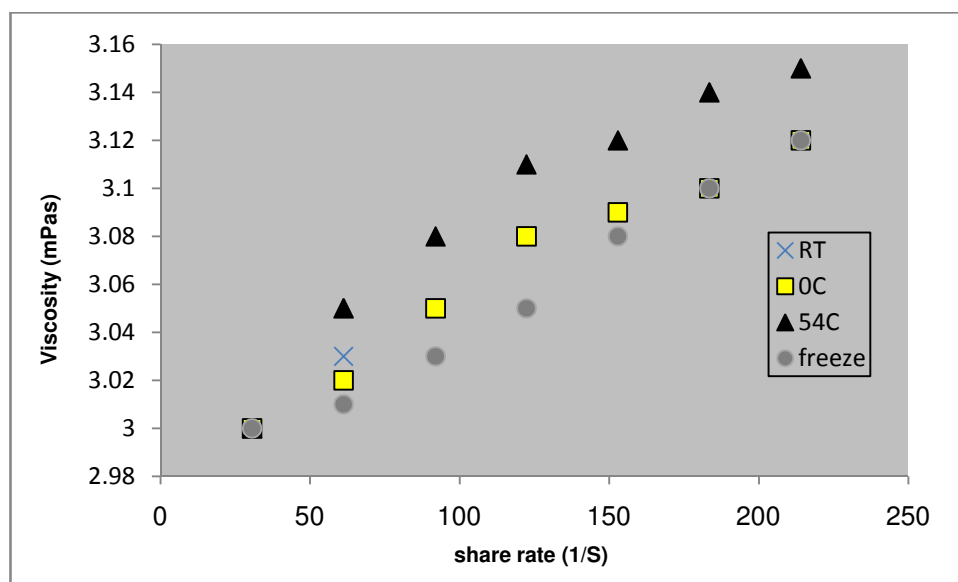


Figure 1. Rheological Properties of oil-in-water emulsion formulation of eucalyptus oil before and after storage.

3.2. Formulation activity.

3.2.1. Effect of eucalyptus oil and its oil water emulsion formulation on the linear growth of the tested phytopathogenic fungi.

Fungal infections cause significant loss in many economic crops. Crop losses are estimated to be about 14% worldwide (Agrios 2005). Chemical control may be available to effectively and extensively reduce the effects of most fungal diseases but field application of these chemical

fungicides may not always be desirable. Excessive and improper use of these fungicides presents a danger to the health of humans, animals, and the environment. In recent years, interests have been generated in the development of safer antifungal agents from natural plant products such as, essential oils to control fungal diseases. Many essential oils have been reported to have antifungal activities with no side effects on humans and animals (Tabassum and Vidyasagar, 2013). Previous *in vitro* and *in vivo* investigations suggested that the essential oils could be used as effective antifungal agents against many phytopathogenic fungi (Lee et al., 2007; El-Mohamedy et al., 2013).

The fungicidal activity of eucalyptus oil and its prepared oil in water emulsion formulation against five important phytopathogenic fungi; *Colletotrichum dematium*, *Drechslera hawaiiensis*, *Humicola Fuscoatra*, *Phoma spp* and *Nigrospora sphaerica*. We have calculated growth percentage inhibition due to treatment against control, using the following formula (Cakir et al., 2004).

$$\text{Percentage inhibition} = \left(\frac{C - T}{C} \right) \times 100$$

where C is the average of three replicates of hyphal extension (mm) of control, and T is the average of three replicates of hyphal extension (mm) of plates treated with tested materials.

The *in vitro* antifungal activity of prepared formulation and the corresponding active material by PF techniques are reported in Table (3 and 4) as a minimum effective concentration of 50 % of mycelial growth (EC₅₀) with the corresponding 95% Confidence limits. The results indicated that the percentage inhibition of mycelial growth increases with increasing concentration of eucalyptus oil suggesting that this essential oil inhibited the growth of all tested phytopathogenic fungi in a dose dependent manner. The prepared formulation demonstrated strong mycelial growth inhibition in all tested phytopathogenic fungi; the prepared formulation more effective than the corresponding active material regarding the value of EC₅₀ in the case of all examined fungi. The lower the value of EC₅₀ is the higher the efficacy of the tested materials in the test under consideration. The EC₅₀ of active substance on the tested phytopathogenic fungi; *Colletotrichum dematium*, *Drechslera hawaiiensis*, *Humicola Fuscoatra*, *Phoma spp* and *Nigrospora sphaerica* were 5025.36, 4440.60, 4625.6, 5277.4 and 6115.79 ppm , respectively.

Whereas, the EC₅₀ of prepared oil water emulsion formulation on the tested phytopathogenic fungi were 196.37, 287.58, 353.00, 312.1 and 490.30 ppm, respectively.

Table 3. Effect of different concentrations of active substance of eucalyptus oil against different phytopathogenic fungi

Tested Fungi	% inhibition at different concentrations (ppm)						EC ₅₀	95 % Confidence limit	
	2000	4000	6000	8000	10000	12000		Lower	Upper
<i>Colletotrichum dematium</i>	20.84	42.83	56.21	65.90	77.79	87.85	5025.3	4238.6	5814.7
<i>Drechslera hawaiiensis</i>	15.21	44.65	65.09	77.58	85.21	89.98	4440.6	2703.6	5979.9
<i>Humicola Fuscoatra</i>	14.47	27.71	62.88	75.56	83.50	88.58	4625.6	2741.2	6333.5
<i>Phoma spp</i>	8.34	34.65	57.25	72.33	81.83	87.91	5277.4	3201.7	7338.6
<i>Nigrospora sphaerica</i>	2.38	22.59	48.65	68.29	80.82	88.39	6115.7	2982.0	9438.9

Table 4. Effect of different concentrations of oil water emulsion formulations of eucalyptus oil against different phytopathogenic fungi

Tested Fungi	% inhibition at different concentrations (ppm)					EC ₅₀	95 % Confidence limit	
	62.5	125	250	500	1000		Lower	Upper
<i>Colletotrichum dematium</i>	7.78	28.77	61.77	87.68	97.82	196.37	159.7	239.4
<i>Drechslera hawaiiensis</i>	0.17	3.45	24.05	65.88	93.61	287.58	237.6	664.4
<i>Humicola Fuscoatra</i>	1.05	8.32	32.28	67.87	91.74	353.00	171.6	849.7
<i>Phoma spp</i>	0.44	6.78	35.87	77.88	97.12	312.1	239.1	409.4
<i>Nigrospora sphaerica</i>	2.08	8.82	25.26	50.77	75.96	490.30	352.5	769.7

4. Conclusion

Keeping in mind that the oil water emulsion formulation is free from any solvent except water, in addition to dilution tolerance, and using of environmentally friendly oils. Emulsion formulation was prepared. Characterization of emulsion for its pH, surface tension, viscosity, flash point, density, surface tension analysis indicated a stable formulation, which was safe to handle and transport. Further, stability of emulsion was established at various process

parameters (temperature, centrifugation and free-thaw properties). The formulation had good antifungal activity as a mycelia growth inhibition against all tested phytopathogenic fungi in compared with active substance of eucalyptus oil. The presented results are initial findings and evaluation of the prepared formulation should be continued in the field to determine its efficacy, and to estimate the economic aspects of its use and compare the activity with commercial fungicide.

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