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FORMULATION AND EVALUATION OF SOLVENT-FREE MICROEMULSIONS OF LEMONGRASS OIL AND CITRAL AS NATURAL ANTIFUNGAL AGENT AGAINST SOME PHYTOPATHOGENIC FUNGI

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Abstract

In the current study, lemongrass essential oil (LGO) and its main pure constituent citral were formulated in solvent-free microemulsions that based on water for formulation and delivery. The microemulsions were tested in vitro against two soil-borne and two air-borne sets of fungi that have deleterious effect on crops. These fungi include *Pestalotia longisetula, Fusarium oxysporum, Macrophomina phaseolina, Alternaria raphani*. Parameters related to chemical composition of LGO, surfactant optimization, microemulsions formulation, characterization and stability under challenged conditions were also evaluated. Results indicated that LGO composed mainly of neral, (35.5%), geranial, (47.8%) and myrecene (10.0%). Both LGO and citral microemulsions possessed in vitro antifungal activity against the tested fungi. This activity is concentration dependent and also relies on the response of each fungus toward LGO, citral and surfactant composition. All microemulsions had particle size ≤ 20 nm even without the use of co-surfactants. Microemulsions made of mixed surfactant are characterized by smaller particle size than those formulated using single surfactant. This study demonstrated the optimization of water-based microemulsions that carry essential oil nanoparticles and its potentials for application as natural plant antifungal agents.

Keywords : Lemongrass oil, surfactant optimization, microemulsion, nanoparticles, natural antifungal.

Introduction

Phytopathogenic fungi can lead to a serious economic loss which is valued to about 50 % of the produce due to decreased yield and diminished quality (Thippeswamy *et al.*, 2013). A detailed review regarding the massive quantity of crops loss due to plant pathogens (including fungi) was previously reported (Leadbeater, 2014). Fungal infestation can take place during crop growth in the filed or at a post-harvest stage during storage or marketing. Therefore, the use of fungicides is inescapable in the crop integrated management to reduce as much as possible the consequences of fungal damage. Common synthetic fungicides are available in the market and each of them is designed for specific type of fungi and crop (Leadbeater, 2014). Some synthetic fungicides are having a reduced risk status granted long time ago by US EPA for use on fruits (Adaskaveg *et al.*, 2004). However, awareness of the potential toxicity of synthetic fungicide (Gupta, 2018) along with the global call for safer and eco-friendly bioactive ingredients urges manufacturers to seek for natural alternatives, especially phytochemicals (Zhang *et al.*, 2018).

Antifungal evaluation of some natural phytochemicals from plant origin showed promising inhibitory activities against different plant pathogenic fungi (Marutescu *et al.*, 2017; Zaker, 2016). Essential oils (EOs) are among the volatile phytochemicals that showed antifungal activity against these types of fungi (Nazzaro *et al.*, 2017). Therefore, they are considered for potential application in post harvest treatment of different fruits against *Fusarium* spp. and other fungi-causing diseases (Vilaplana *et al.*, 2018; Kayode *et al.*, 2018; Sharma *et al.*, 2017; Thomidis and Filotheou, 2016). Lemongrass EO (LGO) is an example of these oils that can completely inhibit the mycelial growth of different phytopathogenic fungi that infect rice seeds (Naveenkumar *et al.*, 2017). The antifungal activity of other EOs was also demonstrated against *Aspergillus parasiticus* (Yooussef *et al.*, 2016) and *A*lternaria *alternata* in tomatoes (Chen *et al.*, 2014). EOs also showed fungicidal and fungistatic activity against citrus fruits (Simas *et al.*, 2017) and stored grain fungi belonging to *Fusarium spp*. (Kumar *et al.*, 2016) This group of fungi is known for production of mycotoxins in stored grains, which can harm humans (Masheshwar *et al.*, 2009). The antifungal activity of EOs is also reported in active packaging in which these oils are incorporated in the matrix of the packaging material during fabrication (Priyadarshi *et al.*, 2018; Van Long *et al.*, 2016). Vapors of EOs can also be used to inhibit the growth of some grains-attacking fungi (Bozik *et al.*, 2017). The mechanism of the antifungal activity of EO was fully illustrated elsewhere (Nazzaro *et al.*, 2017).

Based on the above mentioned, EOs are considered as candidates for application as natural crop antifungal agents. However, that will create another problem because EOs must be diluted and delivered in organic solvent vehicle before application due to their hydrophobic nature. Therefore, to shift to a whole integrated safer and eco-friendly phytofungicide formulations, EOs would better be emulsified in water to form water-based EO emulsions. These emulsions represent a promising trend towards "green" fungicides based on EOs as an active ingredients and water as a diluent and vehicle to substitute organic solvents. In accordance with this line, several EO emulsions having antifungal activity were formulated by adopting different emulsification approaches. These include macroemulsions (Ribes *et al.*, 2018; Gill *et al.*, 2016), nanoemulsions (Sharma *et al.*, 2018) and microemulsions (He *et al.*, 2016).

In accordance with that trend, the authors in the current study will investigate the possibility of formulation of lemongrass essential oil (LGO) and its main constituent citral in water-based microemulsion for potential application as plant antifungal agent. This EO was chosen based on its economic feasibility and reported activity against

phytopathogenic fungi (Ali *et al.*, 2015; Tzortzakis and Economakis 2007). Unlike a relevant study (Naveenkumar, et. al., 2017) which included organic solvent like cyclohexanone in their LGO macroemulsions, the current investigation aims to formulate 100% water-based microemulsions with particles size < 100 nm without using co-surfactants. Parameters related to surfactant optimization to reach the maximum solubilized load of LGO and pure citral will be studies. Then, the optimized microemulsions will be characterized and their physical stability under challenged conditions of freezing and heating will be evaluated. Finally, the *in-vitro* antifungal activity of the different microemulsions will be assessed against some phytopathogenic fungi known for their deleterious effect on crops.

Material and Method

Chemicals and fungal strains

Lemongrass essential oil (LGO) was obtained from the fresh herb of *Cymbopogon citratus* using steam distillation process. An industrial scale distillation unit located at the Horticultural Research Institute, Medicinal and Aromatic Plant Research Section, Kanater, Egypt, was used for that purpose. The sample of LGO was kept at - 4°C during storage till used within 1 week.

Pure citral standard sample (98%) was purchased from Sigma-Aldrich (St Louis, Missouri, USA). This compound exists in the form of mixture of two isomers namely: neral (*cis*) and geranial (*trans*). Tween 80 was purchased from Sigma-Aldrich Chemie GmbH, Riedstr, (Steinheim, Germany), Brij 35 was purchased from Loba chemie. PVT .Ltd. (Mumbi, India). Meanwhile, the other surfactants (Table 1) were kindly donated from Rhodia-Home, Personal Care &Industrial Ingredients, (Milano, Italy).

Isolates of four phytopathogenic fungi, including *Pestalotia longisetula, Fusarium oxysporum, Macrophomina phaseolina, Alternaria raphani* were isolated from different infected plants like strawberry and cucumber collected from Beheira and Qalyubia governorate, Egypt. The isolated fungi were purified out either by hyphal tip or single spore technique (Dhingera and Sinclair, 1985). The fungi were maintained during the experiments on potato dextrose agar (PDA, 200 g grated potato, 20 g dextrose, and 20 g agar) (PDA) medium at 25°C (Atlas, 1995).

Chemical analysis of LGO using gas chromatography

The major components that constituent LGO were revealed using GC analysis under the following analytical conditions, LGO (20 µL) was diluted in 1 mL diethyl ether in a glass vial. Then 2 µL of this mixture were injected (at a split ratio 10,1) into Agilent GC equipped with flame ionization detector (FID). A fused silica capillary column DB5 (30 m × 0.32 mm × 0.25 µm) was used to separate the different volatile components. The oven temperature was programmed from 50 °C to 220 °C at a rate of 3 °C/min. The injector and detector temperatures were 220 °C and 230 °C respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. The volatile oil constituents were reported as percent of the total peak areas after FID. All values were means of two injections. The available authentic samples were used to identify only the major components of the LGO which include citral (neral and geranial) and myrcene.

Table 1 : Types of surfactants tested for the formulation of LGO and citral microemulsions and their maximum solubilized loads

Surfactant code	Commercial name	Chemical name	Ionic charge	HLB value	Max. solubilized load (% v/w)*
S1	Arkopal N 100	Nonylphenol $(ethoxylate)_{10}$	Nonionic	13.2	1.0
S2	Soprophor CY/8	Tristyrylphenol $(ethoxylate)_{20}$	Nonionic	13.7	< 0.5
S3	Alkamuls 14/R	Castor oil $(ethoxylate)_{60}$	Nonionic	14.9	< 0.5
S4	Tween 80	Sorbitane monooleate $(ethoxylate)_{20}$	Nonionic	15	< 0.5
S5	Brij 35	Laurylether $(ethoxylate)_{23}$	Nonionic	16	< 0.5
S6	Soprophor FL	Polyarylphenol phosphate amine salt$(ethoxylate)_{40}$	Anionic	16	< 0.5
(Smix)**	Arkopal N 100 & Soprophor FL	As above	Anionic (- 19 mV)	14.6	2.5

* ml of LGO or citral / 100 g of 5% surfactant solution.
**Mixture of S1 and S6 at 1:1 weight ratio.

Optimization of the surfactant(s) system and formulation of microemulsions

Six different surfactants (S1-S6) and a surfactant mixture (Smix) (Table 1) were evaluated for their potentials to solubilize the maximum possible load of LGO and pure citral, (separately), in water-based microemulsion. The oil titration method (Gaysinsky *et al.*, 2008) was used to formulate all microemulsions without using organic solvents as co-surfactants. The procedure was first initiated by dissolving, separately, each of the surfactants (S1-S6 & Smix) into the appropriate amount of distilled water to end up with seven groups of surfactant solutions each contains 5 wt% surfactant. The solutions were then warmed to 40°C to facilitate surfactants dissolution in the aqueous phase. Then, different volumes of LGO and citral ranging from 0.5% up to 3% (ml LGO or citral / g surfactant solution, respectively) were titrated, separately, into each of the seven groups of the surfactant solutions using automatic pipette. The titration process was carried out at an increment of 0.5% oil phase between each concentration up to 3%. After the end of titration process, samples were vortexed for 1 minute then left at room temperature to equilibrate for 24h. The selection of the optimum surfactant(s) for formulation of microemulsions is based on the surfactant's ability to solubilize the highest possible load of LGO or citral in a transparent aqueous system with particle size < 100 nm.

Formulation of LGO and citral microemulsions using the optimized surfactant(s)

After optimizing the appropriate surfactant(s), LGO and citral were re-formulated in new microemulsions using the optimized surfactant(s) via the same oil titration method (Gaysinsky *et al.*, 2008). These optimized microemulsions will be subjected to a full characterization and stability studies followed by antifungal activity assessment.

Characterization of microemulsions

Appearance

Glass vials containing LGO and citral microemulsions were examined visually against bright light to check their transparency. The UV-vis absorption of the microemulsions were also evaluated spectrophotometrically using Shimadzu (UV-160 1PC spectrophotometer, Japan) at λ600 nm to establish a quantitative measure for the transparency of the samples.

Particle size analysis

The particle size of microemulsions was measured using the dynamic light scattering instrument Zetasizer (Nano-ZS model ZEN3600, Nanoseries, Malvern Instruments, UK) at a fixed angle of 173^{o}. Before measurement, all samples were filtered through 0.20 µm single use syringe filter unit (Minisart®, Sartorius Stadium Biotech GmbH Germany) to remove impurities. Each microemulsion sample was diluted before measurement with distilled water to only 0.05% to prevent multiple scattering. The measurements are based on the Brownian motion of the hydrated particles, thus it provides information on the hydrodynamic diameter (nm) of the microemulsion particles. Sizes quoted are the *z*-average mean of the microemulsion hydrodynamic diameter (nm) obtained from 6 measurements for each sample (2 replicate x 3 measurements each).

The refractive indices (RI) used for measurements were 1.33 for the continuous phase (water). RI for the different dispersed phases are, 1.4870 for single surfactant (S1), 1.5025 for mixed surfactant (S1/S6), 1.4896 for citral or LGO microemulsion using single surfactant and 1.5023 for citrl or LGO microemulsion using mixed surf,.

Thermal stability of microemulsions during storage

This evaluation was carried out according to Collaborative International Pesticides Analytical Council (CIPAC 2000) standard test methods. Accordingly, microemulsion samples were stored at zero °C for 7 days, while another set of the same microemulsions were stored at 54 ± 2 °C for 14 days.

The stability criteria after storage under the previous extreme conditions are that the volume of the separated layer (if any) from the microemulsions shall not be more than 0.3 ml (JMPS FAO/WHO pesticides specifications, 2016). Persistence of initial transparent appearance of each microemulsion after storage is also taken as a criterion for assessing the microemulsion stability.

In vitro antifungal activity assay

The antifungal activity of LGO and citral microemulsions was tested against *Pestalotia longisetula, Fusarium oxysporum, Macrophomina phaseolina, Alternaria raphani* and by using poison food technique (Singh *et al.*, 2008). Different concentrations of the prepared microemulions (0.00, 31.25, 62.5, 125, 250 and 500 ppm) were added to sterilized PDA media before solidification (lightly above the solidification temperature of agar), and shaken thoroughly. The media were then poured into a set of three glass Petri dishes (9 cm in diameter) and three replicates under aseptic conditions in a laminar flow chamber with filter (Labconco Corporation, Kansas City, MO, USA). After partial solidification of the media in the plate, one mycelial 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 at 25 ± 2°C until the fungal growth in the control dishes was almost completed. Percentage of inhibition was calculated due to the treatments against control, using the following formula adopted from reference (Cakir *et al.*, 2004) as follows:

$$\% \text{ inhibition} = (C\text{-}T\ /C) \times 100 \quad ....(1)$$

Where:

C, the average of three replicates of hyphal extension (mm) of control.

T, the average of three replicates of hyphal extension (mm) of plates treated with the different microemulsions.

Therefore, the higher the % inhibition, the more profound is the antifungal activity.

Determination of EC_{50} value

The EC_{50} value (concentration causing 50% reduction in mycelial growth was estimated for LGO and citral microemulsions by using probit analysis (Proban) Computer Program of the probity of the tested fungus percentage inhibition vs. logs of the concentrations (ppm) of the prepared formulation.

Statistical analyses

Data collected from chemical analysis and also from the particle size analysis of the LGO and citral microemulsions were mean of two replicates ± S.D. On the other hand, data collected from antifungal activity of LGO and citral microemulsions were analyzed by MSTAT-C program. The means differences between the antifungal activity of LGO and citral were compared by the least significant difference test (LSD) at 5% level of significance.

Results and Discussion

Chemical composition of LGO

In the current study lemongrass essential oil (LGO) and its individual major constituent citral were formulated in water-based microemulsion and evaluated as natural antifungal agents. The study is started by determining the chemical composition of LGO especially its inherent content of citral.

Gas chromatographic (GC) analysis revealed that LGO has a high percentage of citral (83.3±0.5%) represented by its two isomers namely; *cis*-citral (neral, 35.5%) and *trans*-citral (geranial, 47.8%). Citral is thought to be the biological active constituent of LGO due to its reported antimicrobial properties (Lu *et al.*, 2018; Shi *et al.*, 2016; Leite *et al.*, 2014). Beside this compound, LGO also contain myrcene (10.0 ±0.2%) along with some other minor compounds. No reports were found about the antifungal activity of myrcene

which may cause citral to be the prime antifungal agent in LGO. However, the presence of myrcene beside citral may lead to synergistic antifungal effect but that is only speculations which was not proven or reported yet.

After the chemical composition of LGO was revealed, a process of systematic selection of the appropriate surfactant system that can lead to formation of water-based LGO and citral microemulsions was conducted as discussed in the following section.

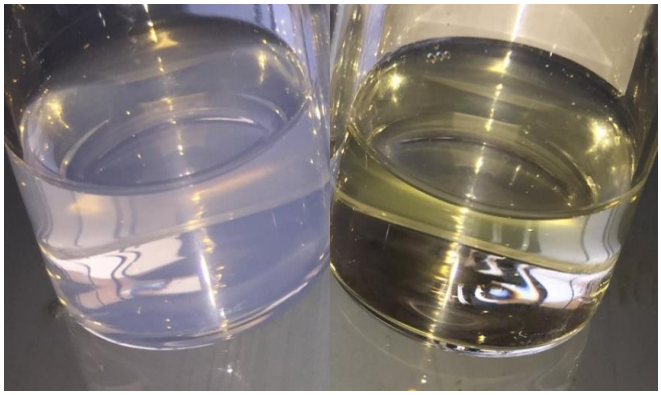
Optimization of the surfactant system and formulation of microemulsions

Microemulsification technique was adopted in the current study due to its simplicity and the possibility for scaling up to industrial level without needing costly machinery. In addition microemulsions are characterized by ultra-fine droplet size (< 100 nm) and thermodynamic stability which means long shelf life. Microemulsions are formed when the interfacial tension between the oil and water phases become ultra-low. That can be fulfilled by using certain amounts of the appropriate surfactant(s) and co-surfactants which together can form aggregates in water called micelles. The emulsification takes place in the domain of these micelles in a process known as solubilization (Prince, 1977). As a result of this process, a transparent isotropic and thermodynamically stable water-based microemulsion is formed. In order to facilitate and increase the solubilized amount of LGO or citral in the microemulsions, the surfactant system must be optimized qualitatively and quantitatively to achieve maximum solubilization. In order to accomplish that goal, six different surfactants including nonionic and anionic members (Table 1) were evaluated in the current study for their capacity at 5.0wt% in water to solubilize the maximum mount of LGO and citral. These surfactants were chosen based on their common use in agrochemical formulation practice.

Results in Table 1 showed that Arkopal N100 (S1) is the only surfactant that was able to solubilize a maximum pay load of 1.0 % (v/w) of each of LGO and pure citral in microemulsion. That makes the ratio of the mass of the surfactant (g) to the volume of solubilized oil (ml) equals 5/1, respectively. Using such a relatively high surfactant concentration is common in microemulsion formulation in order to attain the ultra-low interfacial tension necessary to induce microemulsion formation (Prince, 1977). Even higher surfactant/oil ratio as 6/1 was previously adopted to formulate cassia EO microemulsion for controlling the growth of the pathogenic fungus *G. citri-aurantii* (Xu *et al.*, 2012). Moreover, that previous study, used organic solvents like ethanol as co-surfactant to facilitate the formation of microemulsion. That is considered as a drawback in the pursuit of production of eco-friendly phytofungicide. Therefore, in the current investigation we avoided the application of any organic solvent and replaced it by using the optimized surfactant (S1) which was selected among the others (Table 1) as indicted previously.

From the convenience point of view it is better to formulate a concentrated antifungal stock having high content of the active ingredient which can be diluted with water as appropriate prior to application. To fulfill that purpose in our investigation, the payload of LGO and citral must be increase over 1.0% at the same surfactant concentration (5%) without losing the microemulsion characteristics of the formula.

Therefore, in a trial to formulate more concentrated LGO and citral microemulsions without organic solvent as co-surfactant, the optimized surfactant (S1) was coupled with another anionic surfactant (S6) at 1:1 weight ratio to obtain a new surfactant mix (Smix, Table 1). This trial was based on previous knowledge which indicated that mixing two surfactants of different polarities can increase the oil solubilization capacity of the new surfactant mix compared to each individual one (Kunieda *et al.*, 1995). Result from that trial revealed that the new surfactant mix (Smix) was able to increase the maximum solubilized load of LGO and pure citral in both microemulsions to 2.5% (v/w) using the same original concentration (5.0%) of the surfactant (Figure 1). Based on that result, the new surfactant/oil ratio of the microemulsion made of surfactant mixture becomes 5/2.5 (i.e. 2/1) instead of 5/1 as in the case of microemulsions made of single surfactant. That means more LGO and pure citral can be incorporated in the microemulsion stock at the same surfactant concentration, which finally represent an economic feasibility.



1% LGO microemulsion (S1), abs. 0.044 | 2.5% LGO microemulsion (Smix), abs. 0.007

1% citral microemulsion (S1), abs. 0.056 | 2.5% citral microemulsion (Smix), abs. 0.022

Fig. 1 : Appearance and UV/vis ($\lambda_{600\ nm}$) absorption values of the different concentrations of LGO and citral microemulsions formulated using single surfactant (left) and mixed surfactants (right).

It worth indicating that, it would be possible for us to increase the solubilized load of LGO or citral more than 2.5% in the microemulsion stock by using co-surfactants like short chain alcohols (ethanol, isopropanol) or by increasing the surfactant concentration over 5.0%. However, that strategy was relinquished as we aimed at solvent-free antifungal formulas with the least possible amount of surfactants.

After discussing the selection and optimization of the appropriate surfactant system for microemulsion formation it becomes necessary to reveal the parameters that were used for the characterization of these microemulsions. Therefore evaluation of the appearance and particle size will be discussed in the following section.

Characterization of microemulsions

Appearance

The appearance of the colloidal systems is one of the initial attributes that can characterize microemulsions and distinguish them from traditional macroemulsions (Prince, 1977). Due to their ultra-fine particle size which is < 100 nm microemulsions do not scatter the incident light so they appear translucent or transparent. Figure 1 showed the appearance of the formulated microemulsions using a single surfactant (S1) and mixed surfactants (Smix), respectively. From the figure it is evident that use of single surfactant led to formation of bluish translucent microemulsion having the maximum load of 1.0% (v/w) of LGO or citral. On the other hand microemulsions formulated using mixed surfactants (Smix) looked more clear and transparent even at higher load of LGO and citral as 2.5% (v/w). That indicates a smaller particle size and more efficient solubilization under the effect of the mixed surfactants. The yellow color of the microemulsion made of the mixed surfactants is due to the inherent yellow color of the second-coupled anionic surfactant (S6).

The UV (vis) absorption values corresponding to all microemulsions was about 0.0 which emphasis the visual observation and ensures the absence of large oil particles and formation of microemulsion.

Particle size

Microemulsion characterization must also involve particle size analysis to confirm the ultra-fine size (< 100 nm). Figure 2 showed the particle size distribution of the formulated microemulsions using single and mixed surfactants. The sizing process started firstly by assessing the particle size of the empty micelles which are aggregates of surfactant monomers that assemble in water before titration of the oil phase. Figure 2(a) indicated that the mean particle size of the empty micelles formulated using single surfactant was 10.5±0.06 nm with a monomodal size distribution pattern and polydispersibility index (PDI, 0.16). On the other hand the empty micelles made of mixed surfactants had lower mean diameter (6.5±0.06 nm) with bimodal size distribution and PDI (0.25).

Titration of LGO into each of the corresponding micellar solutions made of single and mixed surfactants led to the formation of LGO microemulsions having average particle size 17.3±0.13 nm (for single) and 8.9±0.2 nm (for mixed) surfactants, respectively (Figure 2b). The increase of particle size of empty micelles (Figure 2a) after addition of LGO (Figure 2b) is an evidence of micelles swelling to accommodate the solubilized load of LGO. The degree of swelling reached in microemulsions and consequently the increase in particle size can only be obtained with suitable surfactant or surfactant mixture. That justifies the difference of particle size between LGO microemulsion formulated using single surfactant (17.3 nm) and mixed surfactant (8.9 nm). This come in accordance with previous study (Chu and Pilrma, 1989) which indicated that mixing an anionic and nonionic surfactants leads to much smaller emulsion particle size than using single surfactant along.

In addition the second surfactant in the mixture can act as a co-surfactant that helps in reducing the interfacial tension leading to smaller particle size.

Similarly, titration of pure citral into its corresponding empty micellar solutions made of single and mixed surfactants led to the formation of citral microemulsions having average size 20±0.4 nm (single) and 5.8±0.01 nm (mixed) surfactants, respectively (Figure 2 c). The same previous comment on the relation between particles size and surfactant system composition which was used in case LGO can also be applied on citral microemulsions.

One should take into consideration that the nature of the oil phase (whole LGO vs pure citral) is another factor that can contribute to the difference in particle size of their microemulsions. Citral is just a single component (consist of *cis* and *trans* isomers) that interacts with the surfactant micelles in a defined mode. On the other hand, the whole LGO is a multi-component complex oil phase that interacts differently with the surfactant micelles. That led to the observed difference in particle size between LGO and citral microemulsions.

Stability of the microemulsions under challenging conditions

An antifungal microemulsion formula should be physical stable under challenging conditions such as wide range of temperature variation. That will make the formula practically applicable in different geographical regions where radical change in temperature could be encountered. Therefore LGO and citral microemulsions formulated using single and mixed surfactants were challenged with extreme ranges of temperatures ranging from 0°C for 7 days to 54°C for 14 days, as the specifications demand (CIPAC, 2000). The stability criteria after storage under these conditions are that, the volume of the separated oil layer (if any) from the microemulsions shall not be more than 0.3 ml (JMPS FAO/WHO pesticides specifications, 2016).

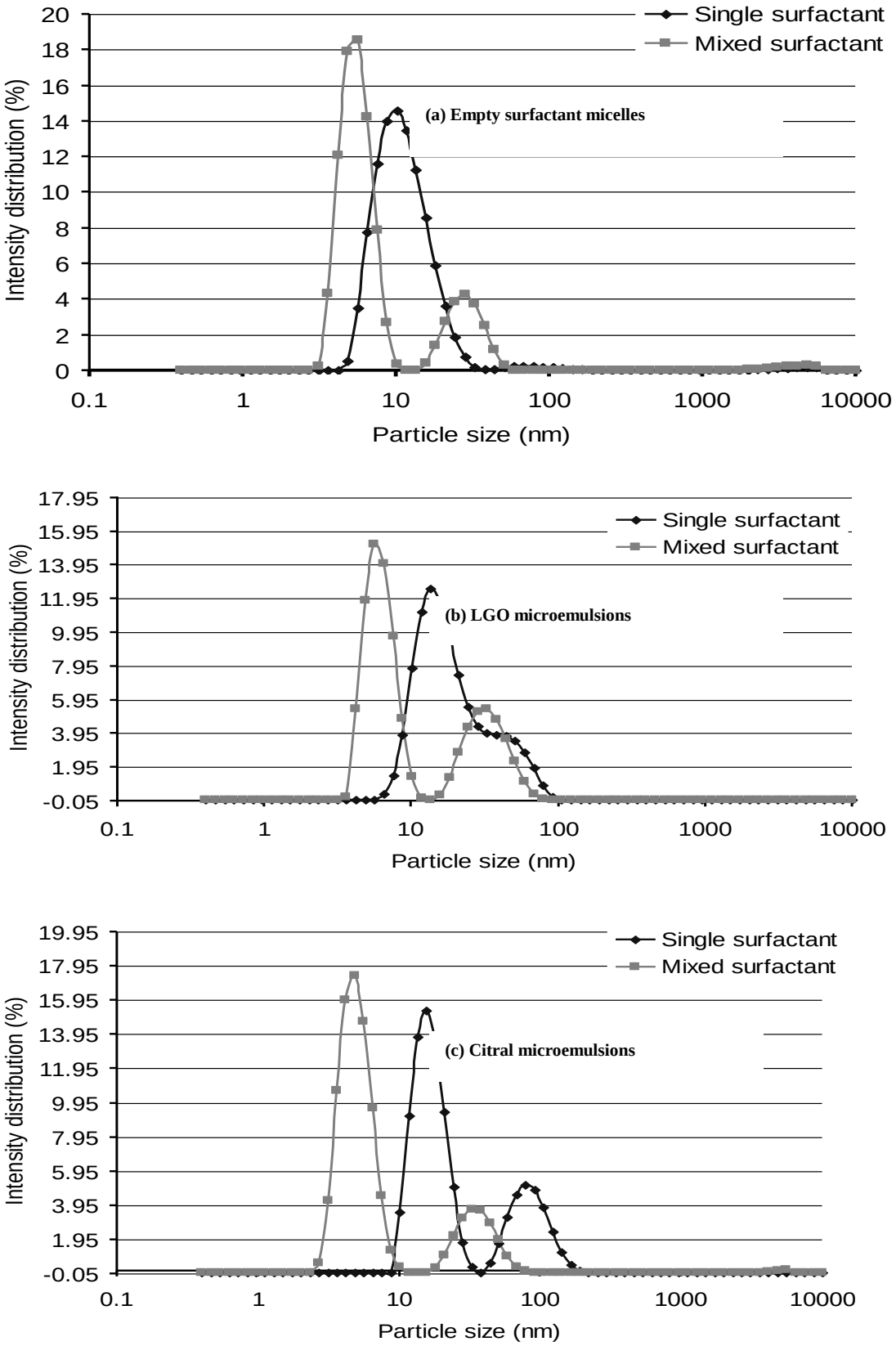


Fig. 2 : Particle size distribution of empty surfactant micelles (a), LGO microemulsion (b) and citral microemulsion (c)

Storage under freezing conditions

Result obtained from that evaluation showed that LGO and citral microemulsions formulated using single or mixed surfactants solidified and became frozen after storage at 0°C for 7 days. Upon gentle thawing of the microemulsions by leaving at room temperature for a while, they return back into their original liquid state with no trace of oil separation from all microemulsions, which came in accordance with the specification demand. However, the appearance of LGO and citral microemulsions made of single surfactant changed from bluish translucent before storage into cloudy after storage. That means losing of the microemulsion characteristic and conversion into traditional macroemulsion where particle size becomes > 100 nm. Fortunately, this

process was found to be reversible which means that these cloudy macroemulsions restored their original bluish translucent appearance and hence their initial microemulsion characteristics after setting for a while at room temperature. It took 40 minutes for LGO and 110 min for citral microemulsions at room temperature to restore their translucent appearance.

On the other hand, LGO and citral microemulsions made of mixed surfactants maintained their transparent appearance and microemulsion characteristics and did not lose them after thawing at the end of the freeze storage period.

Storage under extreme thermal conditions

Result obtained from that evaluation showed that storage of microemulsions at 54°C for 14 days had no effect on microemulsions formulated using mixed surfactants. There was no oil separation and the appearance of LGO and citral microemulsions was transparent after the thermal storage as they were before.

Similarly, microemulsions of LGO and citral formulated using single surfactant showed no oil separation, however, the samples turned cloudy indicating lose of the microemulsion characteristics. This process was also found to be reversible as the case of freeze storage. It took about 20 minutes at room temperature after the of end thermal storage period for microemulsions made of single surfactant to restore their original bluish translucent appearance.

From these results it can be inferred that single surfactant lead to the formation of temperature sensitive microemulsions, while mixed surfactants render their corresponding microemulsions to become more thermally stable. The temperature sensitivity observed in the current investigation is a common phenomenon among microemulsions formulated using nonionic surfactants, such as (S1) (Kunieda and Shinoda, 1982). It frequently occurs at the maximum solubilized load of the oil phase (Shinoda and Friberg, 1986) as the case of 1.0% LGO and citral in our investigation. That is due to the breakdown of hydrogen bonds between the surfactant head group and water which decrease the hydrophilicity of the surfactant (i.e. lower the HLB) leading to microemulsion destabilization and transformation into cloudy macroemulsions.

On the other hand miroemulsions made of surfactant mixture are more temperature tolerant. That is because the hydrophilicity of the second anionic surfactants (S6) in the mixture increases with increasing temperature (Sottmann, 2009). Therefore when nonionic (S1) and anionic (S6) surfactants are mixed together, their mixture can compensate for the decrease in hydrophilicity of (S1) when temperature increases. Therefore, an appropriate mixture of these two surfactants allows the preparation of temperature tolerant microemulsions (Aramaki *et al.*, 1997; Kahlweit and Strey, 1988) which was practically proved in our investigation.

Determination of the temperature tolerance range of microemulsions made of single surfactant

As demonstrated previously, LGO and citral microemulsions made of single surfactant showed temperature sensitivity. Therefore, a second thermal evaluation was performed in the current investigation in order to determine a defined temperature range through which LGO and pure citral microemulsions made of single surfactant can persist and maintain their appearance as microemulsions. That attempt was fulfilled by visual observation of these microemulsions during slow and gradual cooling and heating cycles above and below the formulation temperature. Results of this evaluation showed that at the formulation temperature (25°C), the microemulsions made of single surfactant had bluish florescent and translucent appearance (Figure 3, sample a). Upon increasing the temperature gradually, the intensity of that bluish color increase until reaching 29°C -30°C at which the microemulsions completely lost their bluish appearance and became cloudy dispersion indicating the loss of the microemulsion characteristics (Figure 3, sample b). On the other hand, if the temperature is decreased gradually below 25°C, the microemulsions made of single surfactant becomes crystal clear and totally transparent (Figure 3, sample c), indicating the retention of the microemulsion character. Based on that result, the optimum range of temperature tolerance of LGO and citral formulated using single surfactant is preferably 25°C or below.

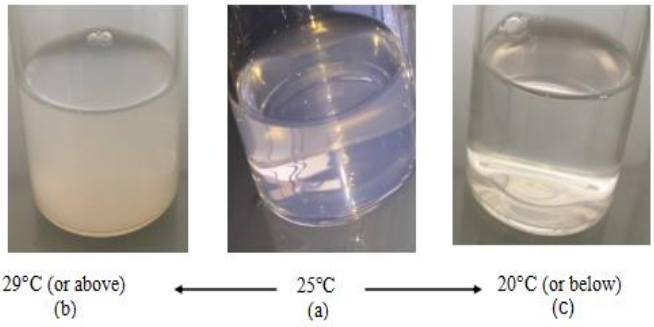


Fig 3: Effect of temperature on the appearance of 1wt% LGO microemulsions formulated using single surfactant.

Note: the same photos and temperature ranges are also applied to citral microemulsions formulated using the same single surfactant under the same conditions.

Antifungal activity of LGO and citral microemulsions

The previously optimized and characterized LGO and citral microemulsions are subjected to antifungal activity evaluation against some phytopathogenic fungi. Results of this evaluation is shown in Table 2-4.

From Table 2 it is clear that both LGO and pure citral microemulsions formulated using mixed surfactants (Smix) possessed antifungal activity against the tested fungi. The extent of this activity depends on the concentration of LGO and citral and also on the response of each fungus toward both oils. For instance, at 500 ppm citral microemulsion showed complete inhibition (100%) of the growth of *P. longisetula* while LGO showed only (85%) inhibition. This fungus is an airborne plant pathogen which can cause Pestalotia leaf spot which initially infect the leaf then spread to the fruit causing rot of different fruits like strawberries (Mouden et al 2014; Rodrigues *et al.*, 2014; Embaby, 2007).

On the other hand, LGO microemulsion at 500 ppm was able to completely inhibit (100%) the growth of *F. oxysporum* (Table 2), while citral microemulsion was less active (71.1%) at the same concentration. *F. oxysporum* is a soil borne fungus that can cause fusarium wilt in wide variety of vegetables and fruits such as root, stem and crown rots,

head blight, and scab on crops (Saremi and Saremi, 2013). It comprises over 100 strains each one of them is specific to certain host. That indicates the large monetary loss that can be caused by this fungus and the potentials of LGO microemulsion as a natural fungicide against this group of fungi. Parallel to our trend (24) a water-based nanoemulsion composed of a mixture of LGO and clove EO at 1:1 weight ratio was formulated. They found that the nanoemulsion was able to control the tomato fusarium wilt and also protect the seed and seedlings with no phytotoxicity.

LGO microemulsion also showed high antifungal activity against *M. phaseolina* at 500 ppm (Table 2). The growth inhibition was (92.2%) while citral microemulsion was able to inhibit only (72.67%) of the fungal growth. *M. phaseolina* is a soil borne pathogenic fungus which can cause several diseases to plant root and shoot systems. That includes charcoal rot (Hemmati *et al.*, 2018), crown and root rot (Avilés *et al.*, 2008), beside other types of blights and wilts.

Table 2 also indicates that LGO microemulsion at 500 ppm can completely inhibit (100%) the growth of *A. raphani*. This inhibition was higher than that of citral microemulsion which showed only (81.1%) inhibition. Different synthetic fungicides are commonly used to control *A. raphani* (Shoaib *et al.*, 2017).

Table 2 : Antifungal activity of LGO and citral microemulsions formulated using mixed surfactants against some plant pathogenenic fungi.

Fungal strain	Microemulsion	Concentration of LGO and citral in the microemulsion (ppm)					
		0.00	31.25	62.50	125	250	500
		Inhibition (%)					
P. longisetula	LGO	0.00	68.88	73.33	76.66	80.78	85.22
	Citral	0.00	73.33	75.56	78.89	88.15	100.0
F. oxysporum	LGO	0.00	39.33	46.0	49.33	59.66	100.0
	Citral	0.00	38.88	50.44	54.44	67.78	71.11
M. phaseolina	LGO	0.00	16	30.44	73.78	81.88	92.22
	Citral	0.00	0.0	11.56	54.44	66.66	72.67
A. *raphani*	LGO	0.00	51.11	56.66	61.11	78.55	100.0
	Citral	0.00	54.88	57.77	65.22	72.22	81.11

L.S.D For interaction (substance X concentration):
P. longisetula = 1.51
F. oxysporum = 5.09
M. phaseolina = 1.71
A. raphani = 0.85

However, to our knowledge there are no reports so far which evaluated essential oil microemulsions for controlling that species of *Alternaria*. It worth to indicate that *Alternaria* blight has been reported to cause up to 57% of crop loss and 46.38% of seed yield loss (Shoaib *et al.*, 2017). That shows the high potentials of using LGO microemulsion as natural antifungal agent for growth control of *A. raphani.*

Table 3 shows the antifungal activity both LGO and citral microemulsions formulated using single surfactant. However, before discussing the results in Table 3 it is important to recall the temperature sensitivity of such microemulsions (Figure 3, sample b) and also the protocol of evaluating the antifungal evaluation (Materials & Methods section). According to that protocol, the different concentrations of microemulsions were mixed with the PDA media while the later was still in liquid form. That means the temperature of the media must be above 32-40°C which is the agar solidification temperature. Therefore, one can predict that the microemulsions made of single surfactant will exceed their temperature sensitivity range and consequently will lose their micro-structure and transfer into cloudy macroemulsion (Figure 3, sample b). So it is possible that the growth inhibition effect reported in Table 3 described the antifungal activity of LGO and citral macroemulsions rather than microemulsions. Despite this possibility, the authors decided to report the fungal growth inhibition under the previously mentioned conditions just for comparison with the activity of microemulsions made of mixed surfactants (Table 2).

From Table 3 it is clear that both LGO and citral microemulsions at 500 ppm can completely inhibit (100%) the growth of *P. longisetula*. It was reported that the severity of the disease caused by *P. longisetula* increase at temperature lower than 25°C (Embaby, 2007). That makes LGO and citral microemulsions formulated using single surfactant appropriate for controlling *P. longisetula* at cold storage places of crops like at coolers during post harvest treatment.

On the other hand LGO microemulsion was more effective than citral in the growth inhibition of *F. oxysporum*, *M. phaseolina and A. raphani* at the same microemulsion concentration (500 ppm). Table 3 also shows that *F. oxysporum* was more resistant than the other tested fungi toward both microemulsions even at the highest concentration.

Table 3 : Antifungal activity of LGO and citral microemulsions formulated using single surfactants against some plant pathogenic fungi.

Fungal strain	Microemulsion	Concentration of LGO and citral in the microemulsion (ppm)					
		0.00	31.25	62.50	125	250	500
		Inhibition (%)					
P. longisetula	LGO	0.00	72.22	75.56	80.44	86.33	100
	Citral	0.00	76.66	83.33	84.44	86.66	100
F. oxysporum	LGO	0.00	51.11	53.33	55.55	76	85.55

	Citral	0.00	55.55	57.78	60.78	65.22	70.78
M. phaseolina	LGO	0.00	76.66	78.88	81.11	84.11	91.88
	Citral	0.00	37.78	46.33	68.55	71.89	78.22
A. *raphani*	LGO	0.00	64.11	67.77	70	80	100
	Citral	0.00	55.55	60.77	68.22	71.88	76.66

L.S.D For interaction (substance X concentration):
P. longisetula = 2.47
F. oxysporum = 1.92
M. phaseolina = 5.23
A. raphani = 1.26

The antifungal activity of LGO is mainly originated from its main constituent citral. Gas chromatographic analysis of LGO in the current study revealed that citral represents ~83% of the composition of that oil. Therefore most of the antifungal activity reported in the current work originates from citral. The mechanism of action of this compound was previously investigated against different fungi. For instance, in case of human pathogenic fungi like *Candida albicans*, the inhibition activity of citral was accomplished via alteration of the morphology of that organism (Leite *et al.*, 2014). Those investigators also concluded that the activity does not include either the cell wall or complexation with the fungal sterol (ergosterol) of the cell wall. On the other hand in phytopathogenic fungi citral can inhibit mycelia growth through cell membrane disruption and consequent loss of cellular components as in the case of *Geotrichum citri-aurantii* (Zhou *et al.*, 2014). Citral mechanism of action may also link with the damage of the cell organelles and the cell membrane as revealed in the case of *Trichophyton mentagrophytes* (Park *et al.*, 2009). Another study showed that citral perform it action by targeting the fungal plasma membrane by decreasing its lipid levels via suppression of ergosterol biosynthesis in the cells as the case with *Penicillium italicum* (Tao *et al.*, 2014).

From these investigations one can indicate that citral is a multi-target antifungal agent that can exert its antifungal activity by different mechanisms, all of which can contribute to the inhibition of fungal growth.

Table 4 showed the EC_{50} of LGO and citral microemulsion formulated using single and mixed surfactants. Surprisingly, EC_{50} of single surfactant formulas was less than (in most fungi) that of the mixed surfactant microemulsions indicating higher antifungal activity by using single surfactant. We assume that the interaction between the oil phases with the mixed surfactant is higher than that with single surfactant due to the proven high solubilization capacity of mixed surfactant as we described previously in surfactant optimization section.

Table 4 : EC_{50} values of LGO and citral microemulsion formulated using single and mixed surfactants.

Fungal strain	Microemulsion	EC_{50} (ppm)	
		Single surfactant	Mixed surfactant
P. longisetula	LGO	9.24	4.63
	Citral	3.39	6.25
F. oxysporum	LGO	47.76	78.12
	Citral	14.75	103.65
M. phaseolina	LGO	1.23	94.80
	Citral	62.45	170.62
A. raphani	LGO	19.98	43.01
	Citral	16.15	25.23

As a result, the oil phases (LGO and citral) will be tightly held to, or entrapped within the domain of the micelles made of mixed surfactants. That will make the free fraction of LGO or citral which is available to interact with the fungal hyphae be less in case of microemulsions made of mixed surfactant. That can lead to less antifungal activity compared with single surfactant microemulsions.

Conclusion

LGO and citral microemulsions have promising activities as natural antifungal agents against some air-borne and soil-borne plant pathogenic fungi. Formulation of these microemulsions using mixed surfactants can solubilize more of these oils in solvent-free and water-based delivery system and can also lead to more temperature tolerant formula than using single surfactant. On the other hand microemulsion prepared using single surfactant has more antifungal activity than those formulated using mixed surfactant. Therefore single surfactant microemulsions are recommended for application in cold storage regions as in coolers for post harvest crop protection. On the other hand the mixed surfactant microemulsions can be applied at variable temperature environments. The described solvent-free LGO and citral microemulsions can be scaled up for future field evaluation due to their simple preparation and ease of application.

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