

Research Article

Characterization and Synergistic Antifungal Evaluation of a Food Grade Microemulsion System against *Aspergillus flavus*

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Abstract: In this study, we aim to study the possibility of enhancing the antifungal application of microemulsions in food industry. The physicochemical properties of multi-component microemulsions containing Cinnamon Essential Oil (CEO), ethanol, Tween 80, water and lactic acid have been studied. Lactic acid showed positive effect on oil solubilization and the formation of U-type microemulsion. Four microemulsion formulations were selected and used for particle size distribution and viscosity measurements. The synergistic antifungal effects of CEO and lactic acid on *Aspergillus flavus* were studied by measurements of Fractional Inhibitory Concentration Index (FICI). Antifungal tests suggested that the microemulsions inhibited the viable fungal spores and the mycelium growth effectively as the Minimal Inhibitory Concentration (MIC) of the microemulsions ranged from 4 mg/mL to 11 mg/mL. The addition of lactic acid in CEO/ethanol/Tween 80/water system also greatly improved the antifungal activity of microemulsions. It was found that the CEO had synergistic antifungal activities in combination with lactic acid for the value of FICI was ranged from 0.30 to 0.35. This study shows the possibility of enhancing the antifungal application of microemulsions in food industry.

Keywords: Antifungal activity, cinnamon essential oil, lactic acid, microemulsion, synergistic effect

INTRODUCTION

Essential Oils (EOs), as natural extracts, have been often used as antifungal agents with good results for the reason that they have been considered as flavorings in Europe and categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration according to Rodriguez *et al.* (2007). Among them, Cinnamon Essential Oil (CEO) has been used for centuries to protect food from microbiological infection and exhibited a strong antifungal activity. Manso *et al.* (2013) found that the CEO shows strong antifungal effects on *Aspergillus flavus* by incorporating into food packaging materials as antimicrobial agent. Currently, the CEO has been used in various industries and considered to be one of the most important natural resources. However, the applications of CEO are limited to a certain extent by the hydrophobic due to the main component of the CEO is cinnamaldehyde which has a poor solubility in water. In addition, the volatile odor of CEO also restricts its application in the food industry.

Microemulsions are thermodynamically stable, transparent and isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactant molecules, typically in conjunction with a cosurfactant and have potential use as delivery systems for

substances which are normally of limited use due to their hydrophobicity, toxicity or inability to access the site of action according to Flanagan and Singh (2006). By the emulsification, the hydrophobic and volatile odor of the CEO could be improved. There are many papers related to microemulsions for antifungal use, but rarely concerned with CEO microemulsions. Zhang *et al.* (2008) reported that microemulsions with glycerol monolaurate as oil phase improves the formation of microemulsions and enhances the antifungal activities.

Lactic acid has been widely used for food preservation due to the lack of acute and chronic toxicity. There are few papers relevant to the synergistic effects between essential oils and lactic acid. Dimitrijević *et al.* (2007) reported that a small amount of lactic acid could significantly increase the antifungal activity of rosemary and thyme oils. Since CEO has been used as the oil phase of the microemulsion system, the addition of lactic acid may be a way to enhancing the natural antifungal effect of the oil phase.

Food grade microemulsions have attracted growing interest of researchers and shown great potential use on industry. However, the diversification of food systems and the limited choice of components lead to difficulties in the formulation of food grade microemulsions. We conducted this study aim to

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establish a microemulsion system of food-grade and evaluate the antifungal activities against *Aspergillus flavus*, which is a pathogen against plants, animals and humans.

The present study takes into consideration the evaluation of the physicochemical characterization and the synergistic antifungal effects against *Aspergillus flavus* of a food grade microemulsion system. The formulation consists of CEO, lactic acid, Tween 80, ethanol and water. It is found that the two antifungal agents (CEO and lactic acid) can be fully dissolved in the formulations and the established system exhibits a better antifungal effect on *Aspergillus flavus*.

MATERIALS AND METHODS

Materials:

Chemicals: Food grade CEO was purchased from Shanghai Yunfeng Fragrance and Flavor Co., Ltd. (Shanghai, China) and stored at 4°C in a refrigerator. Polyoxyethylene sorbitan mono-oleate (Tween 80), lactic acid and ethanol were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Microorganism: The fungal strain *Aspergillus flavus* CICC40375 was purchased from China Center of Industrial Culture Collection and preserved at the Department of Food Science and Engineering, Shanghai Institute of Technology. The strain was cultured on the PDA medium (Sinopharm Chemical Reagent Co., Ltd Shanghai, China) at 28°C.

The spore suspensions were prepared in the PDB medium (Sinopharm Chemical Reagent Co., Ltd Shanghai, China) to a concentration of 1×10^7 spores per ml. Spore population was counted by the haemocytometer.

Phase diagrams: The phase behavior of the compositions in the microemulsions was shown by the pseudo-ternary phase diagram, which was conducted at 25°C according to Zhang *et al.* (2008). Mixtures of ethanol and CEO (cosurfactant-oil phase) were placed in sealed tubes at precalculated weight ratios of oil phase to cosurfactant and maintained at 25°C in a water bath. The single-phase region can be recognized in phase diagrams by adding the aqueous phase (water and lactic acid) to the oil-cosurfactant phase dropwise. Each sample was fully stirred and examined after it was equilibrated at 25°C for at least 24 h. The samples that remained transparent and homogeneous were identified as microemulsion area in the phase diagram (Zhang *et al.*, 2009). The total monophasic area (A_T) was calculated according to the method described by Zhang *et al.* (2008).

Determination of viscosity: Viscosity was analyzed along dilution lines at 25°C by using a viscometer (model NDJ-5S), 25 mL sample was pre-sheared for 2 min and measured after 30s.

The experiment was replicated three times.

Particle size distribution determination: The microemulsion formulation samples were analyzed along dilution lines at 25°C by Malvern Zetasizer Nano S (Malvern Instruments Ltd., UK), which could provide unequaled sensitivity and versatility in the determination of particle size distributions.

Determination of Minimum Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration Index (FICI):

The individual and combined Minimal Inhibitory Concentrations (MICs) of CEO and lactic acid were determined in test tubes according to the method of broth dilution. Fifty microlitres of each dilution (four elected formulations, CEO and lactic acid) were added to 10^6 spores per ml of 5 ml PDB. The tubes were then incubated at 28°C by an incubator shaker at 220 rev min⁻¹. The MIC was recognized by the highest dilution, which was shown no visible growth.

The FIC (fractional inhibitory concentration) for each combination was transformed from the MICs according to Davidson and Parish (1989) and López-Malo *et al.* (2007) calculated by the following formulas:

$$FIC(\text{lactic}) = \frac{\text{lactic acid concentration in the mixture with inhibitory power}}{\text{MIC of lactic acid}}$$

$$FIC(\text{CEO}) = \frac{\text{CEO concentration in the mixture with inhibitory power}}{\text{MIC of CEO}}$$

The synergy effect of the mixture (CEO and lactic acid) was described by the FICI (Fractional Inhibitory Concentration Index), which can be transformed from the FIC by the following formula:

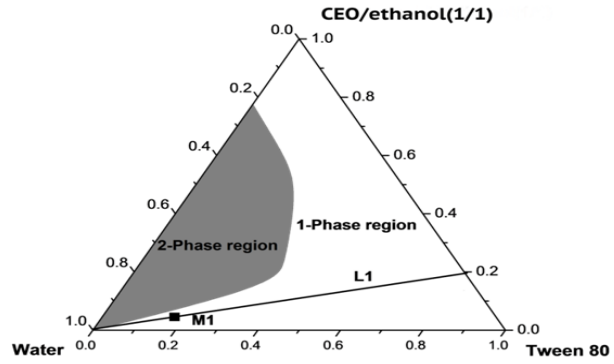
$$FICI = FIC(\text{lactic}) + FIC(\text{CEO})$$

The results were interpreted according to Timurkaynak *et al.* (2006) as follows:

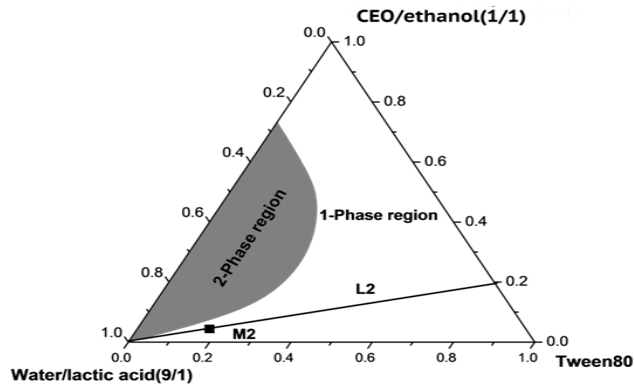
$FICI \leq 0.5$ synergistic, $0.5 < FICI < 1$ partially synergistic, $FICI = 1$ additive, $1 < FICI \leq 4$ indifferent and $FICI > 4$ antagonistic

RESULTS AND DISCUSSION

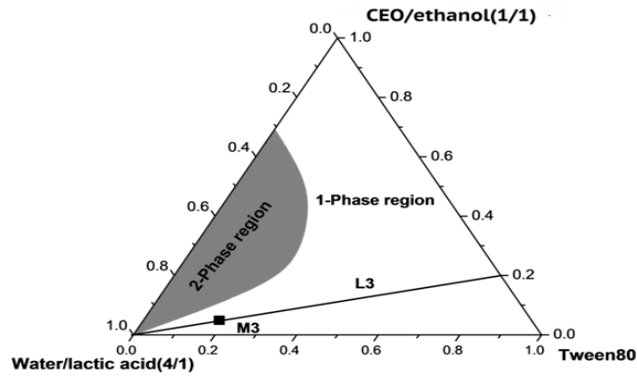
Phase diagrams: The phase behavior of lactic acid in the aqueous phase with different concentrations was presented in Fig. 1. The four-component-system phase diagram of CEO/Tween 80/water/ethanol was shown in Fig. 1a. It is obvious that a smaller single-phase region area [$A_T = 67.2\%$] was obtained compared with the five-component system of CEO/ethanol/Tween 80/lactic acid/water presented in Fig. 1b [$A_T = 73.8\%$], c [$A_T = 77.5\%$] and d [$A_T = 88.8\%$]. By the addition of lactic acid in aqueous phase with different concentrations, we can see obviously changes in the



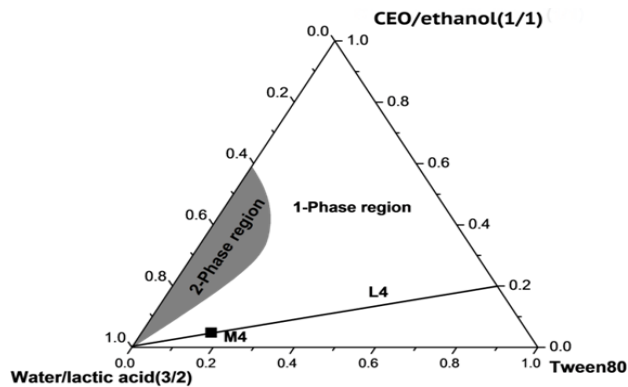
(a)



(b)



(c)



(d)

Fig. 1: Phase diagrams of the system water/lactic acid/cinnamonyl EO/ethanol/Tween 80 at 25°C with different weight ratios of lactic acid; (a): water; (b): water/lactic acid (9/1); (c): water/lactic acid (4/1); (d): water/lactic acid (3/2)

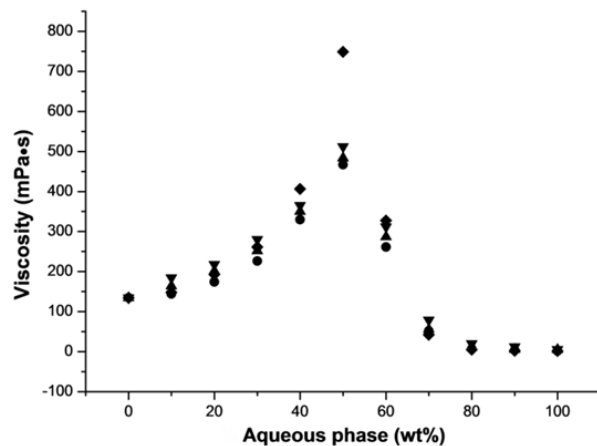


Fig. 2: Variation of the viscosity of four different lactic acid containing microemulsion formulations along dilution lines at 25°C. (water: ◆, water/lactic acid (9/1): ●, water/lactic acid (4/1): ▲, water/lactic acid (3/2): ▼)

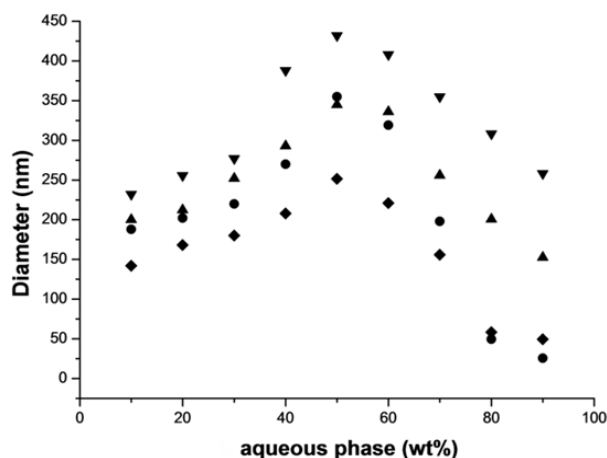


Fig. 3: Variation of particle size of four different lactic acid containing microemulsion formulations along dilution lines at 25°C. (water: ◆, water/lactic acid (9/1): ●, water/lactic acid (4/1): ▲, water/lactic acid (3/2): ▼)

parameter and A_T reached 100% when lactic acid/water = 1:1 was used. As we know, the increased oil content in microemulsions may enhance its antifungal activity, from that formulation viewpoint, four dilution lines (L1, 2, 3 and 4) with the same weight ratio of oil phase to surfactant and different lactic acid contents were determined from phase diagrams (Fig. 1) for the further study.

The results demonstrated that the addition of aqueous phase with different concentrations of lactic acid significantly improved the single-phase region area, which was a proof that the lactic acid could strengthen the formation of the microemulsion systems (Yagmur *et al.*, 2002). It was reported by Zhang *et al.* (2008) that the linear chain organic acids have a better capability to form the microemulsion systems than the nonlinear ones. The change caused by the addition of lactic acid in the phase behaviour can be explained by the theory of film properties. The isotropic phase area increases with the increase in the concentration of lactic acid, actually contributes to the combination of the

cosurfactant and surfactant film, which could reduce the degree of bending of the film.

Viscosity and particle size measurements: Variations of the viscosity and particle size of four different lactic acid containing microemulsion formulations along dilution lines are presented in Fig. 2 and 3 and the curves were all bell-shaped. For the four dilution lines L1, 2, 3 and 4, the viscosity (Fig. 2) reached a maximum when the amount of aqueous phase was around 50%. The viscosity decreased when the amount of aqueous phase continues to increase and gradually increased when the aqueous phase was at a low amount. Interestingly, the distribution of the particle size also has the same trend (Fig. 3). In addition, the viscosity of the four formulations increased with the increase of the lactic acid containing.

The results demonstrated that the changes in the microstructure of the microemulsion could be reflected by the trends of viscosity and particle size. The particle

Table 1: Minimal inhibitory concentrations (MICs) of different compositions against *Aspergillus flavus*

	Composition(w/w)	MICs (mg/mL)
ME1	Water/CEO/Ethanol/Tween80 = 40:1:1:8	11
ME2	Water/Lactic acid/CEO/Ethanol/Tween80 = 36:4:1:1:8	7
ME3	Water/Lactic acid/CEO/Ethanol/Tween80 = 32:8:1:1:8	5
ME4	Water/Lactic acid/CEO/Ethanol/Tween80 = 24:16:1:1:8	4
CEO		0.5
Lactic acid		8

Table 2: Inhibitory concentrations of essential oils and lactic acid in the formulations and fractional inhibitory concentration indexes (FICI) for *Aspergillus flavus*

	CEO (mg/mL)	Lactic acid (mg/mL)	FICI ^a
ME2	0.14	0.560	0.35
ME3	0.1	0.800	0.30
ME4	0.08	0.128	0.32

^a: FICI = MIC of drug in combination/MIC of drug alone; FICI ≤ 0.5: synergism; 0.5 < FICI < 1: partial synergism; FICI = 1: additive; 1 < FICI ≤ 4: indifferent; FICI > 4: antagoni

size and homogenization of the system were measured by Z-average and Particle Dispersion Index (PDI), the smaller of the PDI value, the more homogeneous of the system is. The PDI values are under 0.2 when the particle size ranged from 1nm to 100nm and the microemulsion systems maintain homogeneous and transplant. The viscosity of the microemulsion system increased with the increase of particle size, which is mainly because the changes of interaction force between the dispersed phases particles. The increase of the particle size led to the increase of the interaction force between the dispersed phase particles, with which the viscosity was increased. Lactic acid showed positive effect on oil solubilization and the formation of U-type microemulsion, however, the viscosity and particle size of the microemulsion systems were also be increased. Boned *et al.* (1994) also conducted a viscosity experiment confirmed that theory.

Antifungal effectiveness of microemulsions: Four microemulsion formulations with the same weight ratio of oil phase to surfactant and different lactic acid content were selected from the four dilution lines (Fig. 1a to d). The compositions are water/CEO/ethanol/Tween80 = 40:1:1:8 (ME1), water/lactic acid/CEO/ethanol/Tween80 = 36:4:1:1:8 (ME2), water/lactic acid/CEO/ethanol/Tween80 = 32:8:1:1:8 (ME3) and water/lactic acid/CEO/ ethanol/Tween80 = 24:16:1:1:8 (ME4), respectively. Among all the samples, no phase separation was observed after placing them in a centrifuge at 4000g for 15 min at 25°C. The same phenomenon was also observed after they were stored for one month at 28°C.

The MICs of formulations and their single components were showed in Table 1. The MICs of pure CEO and pure lactic acid are 0.5 mg/mL and 8 mg/mL.

In the formulations, the MICs of the CEO and lactic acid ranged from 0.08 mg/mL to 0.14 and 0.56 to 1.28 mg/mL, respectively. For the MEs, the value of MIC ranged from 4 mg/mL to 11 mg/mL. The MIC is considered as the minimum concentration of the antifungal substance, which is able to inhibit the visible growth of the fungi after one certain incubation period According to Andrews (2001). This method has been used and considered as a fundamental instrument for the determination of susceptibility of microorganism to antimicrobial agents. The results obtained from the determination of the MIC value relative to the synergism. When analyzing the FICI classification (Table 2) we verified synergism effects of the formulations when combined the CEO with the lactic acid according to the value of FICI was ranged from 0.30 to 0.35. For the formulations were observed, the MIC value for the combination between the CEO with the lactic acid is lower than when tested with the CEO and lactic acid alone. In other words, the combination is more effective.

The results indicated that the antifungal activity of CEO could be enhanced by the in combination with lactic acid. The antifungal activity of the lactic acid related to different modes of action with regard to the classical theory of “weak-acid preservative” according to Stratford *et al.* (2009). The synergistic effect between EOs and other antifungal agents has already been studied by Blaszyk and Holley (1998) and Knowles and Roller (2001). However, the mechanism of the synergistic effect has not yet been clearly studied. Most of the studies focused on the effects of the cellular membrane (Gutierrez *et al.*, 2010). The synergistic effect of lactic acid has been proposed to be by a number of actions, including membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and the accumulation of toxic anions (Brul and Coote, 1999).

CONCLUSION

In summary, the physicochemical characterizations of CEO/ethanol/Tween 80 /water/lactic acid microemulsion system has been studied and four microemulsion formulation ME1 (water/CEO/ethanol/Tween 80 = 40:1:1:8), ME2 (water/lactic acid/CEO/ethanol/Tween 80 = 36:4:1:1:8), ME3 (water/lactic acid/CEO/ethanol/Tween 80 = 32:8:1:1:8) and ME4 (water/lactic acid/CEO/ethanol/Tween 80 = 24:16:1:1:8) were selected according to the phase diagrams. Antifungal results against *Aspergillus flavus* showed that the elected formulations could inhibit the viable fungal spores and the mycelium growth effectively. The synergistic antifungal effect on *Aspergillus flavus* was found in the formulations of ME2, ME3 and ME4 with the combination of CEO and lactic acid.

This study showed the possibility of microemulsions for antifungal applications in food industry as a promising delivery carrier. Besides, this work also requires further studies to make a thorough inquiry on the effects of other combinations of EOs and lactic acid against pathogenic microorganisms at various levels, in order to enhance the antifungal application of natural resources in food industry.

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