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The fungicidal activity of novel nanoemulsion ($X8W_{60}PC$) against clinically important yeast and filamentous fungi

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Abstract

Surfactant nanoemulsions are water in oil preparations that proved to have a broad spectrum biocidal activity against a variety of microorganisms including Gram-positive and Gram-negative bacteria, spores and enveloped viruses. These preparations are non-toxic to the skin, mucous membrane and gastrointestinal tissues at biocidal concentrations. In this study, 0.1% of the nanoemulsion designated $X8W_{60}PC$ has shown fungicidal activity against yeast including *Candida albicans* and *C. tropicalis* in 15 minutes. *C. tropicalis* was more sensitive than *C. albicans*, which required a longer time or a higher concentration of the nanoemulsion to achieve killing. Neutral to slightly alkaline pH was more effective in killing the yeast cells than acidic pH. Using the minimum inhibitory concentration assay, 0.08% of the nanoemulsion was inhibitory to *C. albicans*, and *parapsilosis* and filamentous fungi including *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Aspergillus fumigatus* and *Fusarium oxysporum*. None of the individual ingredients was as effective a fungicidal as the nanoemulsion at equivalent concentration. This shows that the nanoemulsion structure is an important factor in the anti-fungal activity. The $X8W_{60}PC$ has great potential as a topical anti-fungal agent and further investigation into the mechanism of fungicidal action is warranted.

Key words: nanoemulsion, fungicidal, topical treatment, yeast, filamentous fungi, disinfectant.

Introduction

There are over 50,000 species of fungi. Fewer than 300 have been implicated in human diseases, and fewer than a dozen cause about 90% of all fungus infections. However, infections caused by unusual fungi are often difficult to identify and manage. Since fungi are eukaryotes, as are human cells, they are more difficult to treat. They grow in the form of unicellular yeast or multicellular filamentous molds. Fungi are involved in the production of different forms of diseases, including allergies to fungal antigens, productions of toxins or direct invasion of hosts [1].

The search for effective anti-fungal topical agents has always been challenging due to drug side effects and acquired resistance. Many of the presently available anti-fungal drugs used in topical treatment are inhibitors of fungus metabolic pathways. While they do not produce adverse and side effects at prescribed concentrations, they are not especially effective [2, 3]. Some antifungal drugs such as azoles are responsible for emergence of resistant strains [4–7].

While many disinfectants kill fungi, they are not suitable for topical treatment [8, 9]. For example, aldehydes and phenols are used as surface disinfectants rather than topical antiseptics, because of their adverse effects on epithelial cells [10]. Quaternary ammonium compounds have limited application due to their low efficacy against fungi [11].

Given this, any non-toxic agent that has a rapid, broad-spectrum antifungal activity could be of great value. There is a continuing need to develop more effective, safe, topical antifungal treatments that will not induce resistance. Nanoemulsions are micellar lipid nanoparticles in a uniform population of droplets ranging in diameter from 400–800 nm. They possess encapsulating properties and can be tailored for a variety of uses [12–15]. Nanoemulsions can entrap and de-

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liver a wide variety of substances, including alcohols, non-polar solvents, detergents, solid particles, and aqueous materials. We have recently shown that the nanoemulsion stucture itself has extensive bactericidal and sporicidal activity [12, 16–18]. These components effectively inactivate enveloped viruses, including influenza types A and B, sendai, sindbis, vaccinia, and *Herpes simplex* [18–20]. Soybean oil nanodroplets, stabilized by detergent and solvent, selectively fuse with bacterial membrane or viral envelope, destabilizing lipids and initiating disruption of the pathogens [12]. Nanoemulsions are non-toxic to skin, mucous membranes, and gastrointestinal tissue at biocidal concentrations [17]. Primary dermal irritation tests were conducted in rabbits and no skin irritation or other clinical signs of toxicity were seen during the study (unpublished data).

In the present paper we report that a novel nanoemulsion, X8W$_{60}$PC, has an effective anti-fungal activity at very low concentrations. X8W$_{60}$PC consists of of oil, three non-ionic detergents, solvent, and water.

Materials and methods

Organisms and media

Candida albicans (ATCC #90028), *Candida tropicalis* (clinical isolates), *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Aspergillus fumigatus* were provided by Carl Young (Clinical Pathology Laboratory, University of Michigan, Ann Arbor, MI). *Candida parapsilosis* (ATCC #90875) and *Fusarium oxysporum* (ATCC #26225) were purchased from ATCC. Yeast was grown on BHI agar or BHI broth supplemented with 5% sucrose (BHI-suc) at 37 °C. In some experiments, we used BHI broth with different pH. The medium was prepared by adding either 1N sodium hydroxide or hydrochloric acid to obtain the desired pH. *M. gypseum*, *T. spp.*, and *A. fumigatus* were grown on BHI agar at RT. *F. oxysporum* was grown on PDA agar at RT.

Fungal suspensions

Twenty-four hours prior to susceptibility testing, *Candida* spp. cultures were established from single colonies and incubated in BHI-suc at 37 °C on a shaker. The cells were counted using a hemocytometer and diluted to approximately 2×10^7 CFU per ml in BHI-suc for use in susceptibility testing as described below. Filamentous fungi were cultured on solid media until extensive fungal growth occurred. The mycelial mats were covered with 5 ml per plate of physiological saline. The fungi from the two plates were scraped and resuspended in 30 ml of physiological saline in a flask containing glass beads. The crude suspension was agitated vigorously for 2 to 3 min to dislodge spores and hyphal cells from any aggregates. After agitation, any remaining fungal clumps were removed by filtration through a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). The turbid suspensions were then concentrated by centrifugation and resuspended in a final volume of 8 ml with sterile saline. Viability counts of each suspension were performed on the day of their preparation. CFU were determined by plating 10 μl samples of 10-fold serial dilutions on agar plates. All suspensions were stored at 4 °C until used in susceptibility testing.

X8W$_{60}$PC and its compounds

The X8W$_{60}$PC surfactant nanoemulsion was prepared in a two-step procedure. An oil phase was prepared by blending the following ingredients: 64% oil, 8% solvent, 8% detergent 1 (D1), 1% detergent 2 (D2), 0.7% detergent 3 (D3). The mixture was heated at 70 °C for 30 minutes [12]. The nanoemulsion was then formed by mixing with water (18.3%) using the Silverson L4RT Mixer (fine Emulsor Screen) for 3 minutes at 10,000 rpm. All detergents were purchased from Sigma Chemicals (St. Louis, MO). Solvent was obtained from Aldrich (Milwaukee, WI) and oil was obtained from Croda Inc. (Mill Hill, PA).

X8W$_{60}$PC susceptibility testing of yeast

One milliliter of *C. albicans* suspension (2×10^7 CFU per ml) was mixed with 1 ml of nanoemulsion or BHI medium (control) and incubated at 37 °C for different time intervals. After treatment, the yeast suspensions were centrifuged, supernatants were aspirated and cells were diluted in fresh BHI medium. Serial ten-fold dilutions were prepared and plated on BHI plates. Plates were incubated at 37 °C for 48 h before the colony forming units were counted.

Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) assays

Serial two-fold dilution of X8W$_{60}$PC, each of its ingredients or Nystatin (100 μl per well) in BHI-suc medium was prepared on a 96-well flat bottom plate.

One hundred microliters inoculum of *C. albicans* and *C. parapsilosis* (2×10^4 CFU/well), *M. gypseum* (420 CFU per well), *T. mentagrophytes* (100 CFU per well), *T. rubrum* (100 CFU per well), *A. fumigatus* (100 CFU per well) or *F. oxysrporum* (100 CFU per well) were added to the wells. The plates were incubated either at 37 °C (*C. albicans*), or at RT (*C. parapsilosis* and filamentous fungi) until confluent growth appeared at the control wells. Fungal growth was examined both microscopically and by reading turbidity at 595 nm or 750 nm for *C. parapsilosis* and *F. oxysporum* on an ELISA reader. To assess MFC, fungal cells treated as described above were harvested at different time periods, washed three times in sterile water, resuspended in 100 μl of BHI and plated on BHI or PDA (*F. oxysporum*) plates. Plates were incubated either at 37 °C (*C. albicans*) or RT (*C. parapsilosis*, and filamentous fungi) until colonial growth appeared.

Electron microscopy photographs

C. albicans at 2×10^4 CFU/100 μl were treated for 90 min with 100 μl of 2% nanoemulsion or incubated in BHI (control). Thermanox cover slips were pretreated with poly-L-lysine and 20 μl of each suspension was placed on the cover slips for one minute before draining the excess. These cover slips were then placed in fixative for 20 minutes (2.5% gluteraldehyde in phosphate buffer) and dehydrated in a grated series of ethanol. The cover slips were dried using the HMDS (hexamethyldisilazane) technique [21]. The cover slips were then mounted, sputter coated with gold, and examined using an AMARY 1000-B SEM (Bedford, Massachusetts). Images were recorded on Polaroid film.

Results

To determine whether surfactant nanoemulsions have anti-fungal properties, we examined the effect of $X8W_{60}PC$ on two clinically important yeast species: *C. albicans* and *C. tropicalis*. As shown in Figure 1, $X8W_{60}PC$ had strong fungicidal effect on both species. One percent $X8W_{60}PC$ reduced *C. albicans* CFU more than four logs and *C. tropicalis* CFU approximately six logs in 15 minutes. Since *C. albicans* appeared to be less susceptible to nanoemulsion treatment, we chose it to investigate the fungicidal kinetics of $X8W_{60}PC$. The organism was treated with two concentrations of $X8W_{60}PC$ for 30, 60 and 120 minutes.

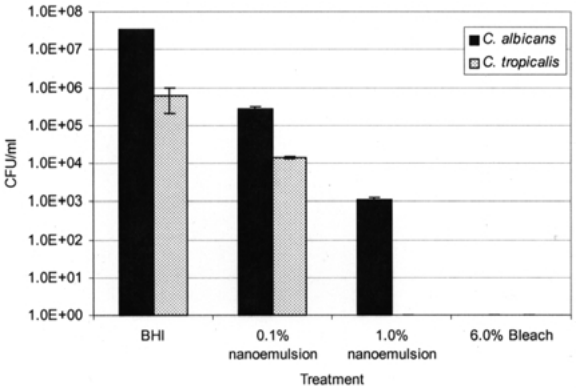

Figure 1. Susceptibility of *Candida spp* to $X8W_{60}PC$ nanoemulsion. Cells were either incubated in BHI medium (control) or treated with different concentrations of nanoemulsion or 6% bleach for 15 minutes at 37 °C. After treatment, cells were washed and plated on BHI plates to assess the number of CFU. The bars represent standard error.

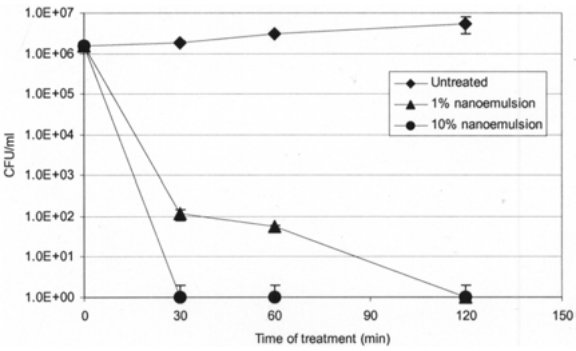

Figure 2. Kinetics of fungicidal activity of $X8W_{60}PC$ nanoemulsion on *Candida albicans*. Cells were either incubated in BHI medium (control) or treated with different concentrations of the nanoemulsion for variable periods of time at 37 °C. After treatment, cells were washed and plated on BHI plates to assess the number of CFU. The bars represent standard error.

Ten percent $X8W_{60}PC$ for 30 min or 1% $X8W_{60}PC$ treatment for 120 min resulted in six log reduction of *C. albicans* CFU (Figure 2).

In order to depict the cell damage, scanning electron microphotographs were taken. *Candida albicans* was treated for 90 min with 100 μl of 2% nanoemulsion, fixed and prepared for electron microscopy scanning. As shown on Figure 3B–D, yeast cells treated with nanoemulsion lost their round shape and integrity due to coalescence with droplets nanoemulsion surrounded the cells. Figure 3 A shows control yeast cells. Untreated cells do not reveal any changes in shape and integrity and undergo budding proliferation process.

To examine the pH variation on nanoemulsion anti-fungal activity, *C. albicans* was treated with

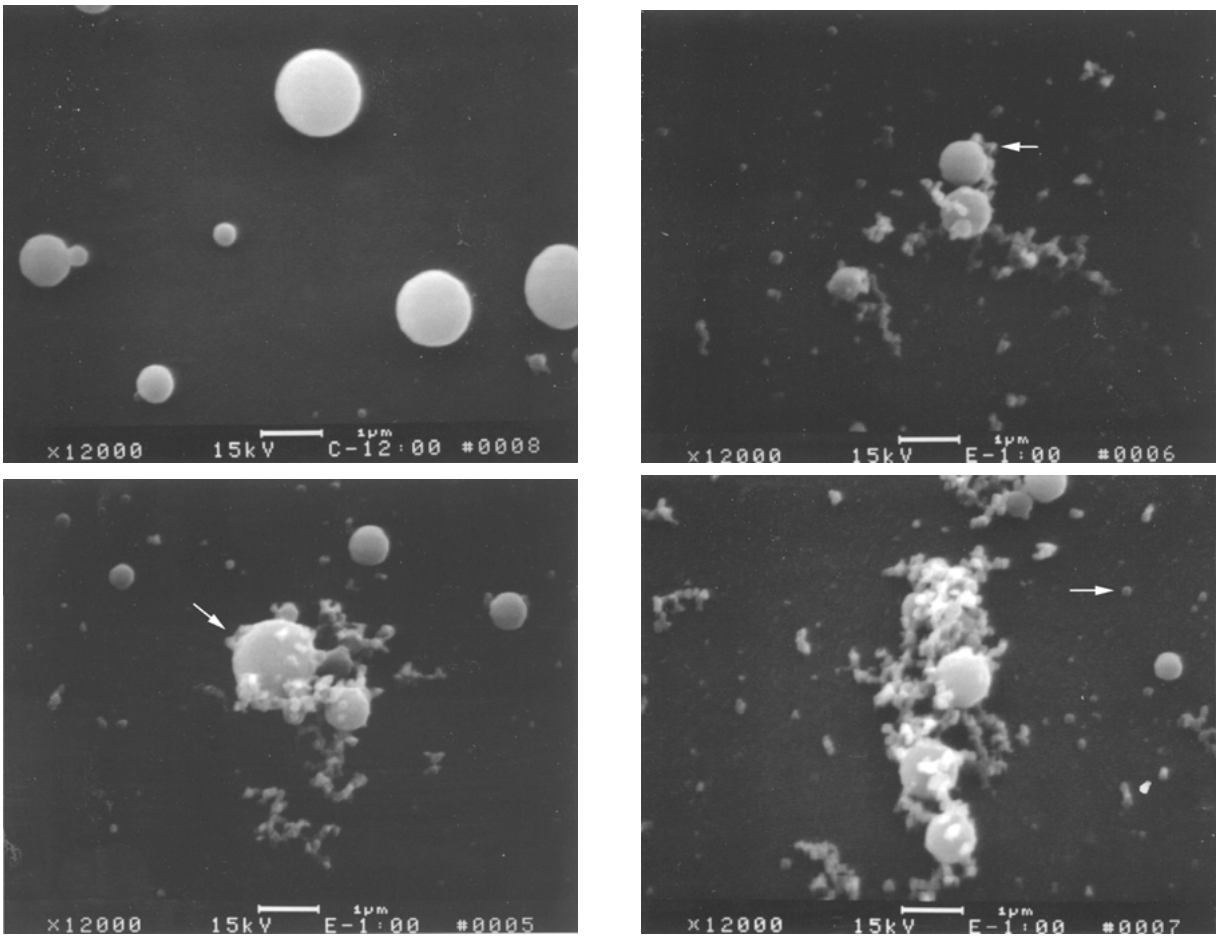

Figure 3. Electron microphotographs of *Candida albicans* treated with naoemulsion. Yeasts were either untreated or incubated with 1% $X8W_{60}PC$ for 90 minutes at room temperature and were subjected to electron microscopy staining procedures. A – untreated yeasts, B, C, and D – depict different phases of cell damage caused by nanoemulsion. Arrows indicate the droplets of nanoemulsion. Magnification = 12,000×.

1% nanoemulsion at different pH (range 3–9). The nanoemulsion showed the highest fungicidal activity at pH range 7–9 (Figure 4). At pH lower than 7, the nanoemulsion was only slightly effective. pH higher than 9 was itself detrimental to the yeast (data not shown).

Since some ingredients of $X8W_{60}PC$ have antimicrobial activity, we investigated the susceptibility of *C. albicans* to the individual ingredients at concentrations equivalent to their concentrations in the nanoemulsion. Solvent, D1 and D3 were completely ineffective against *C. albicans* in the concentrations found in 0.1% of nanoemulsion. Only D2 partially reduced yeast growth (Figure 5).

The efficacy of $X8W_{60}PC$ against yeast prompted us to determine the minimal inhibitory and fungicidal

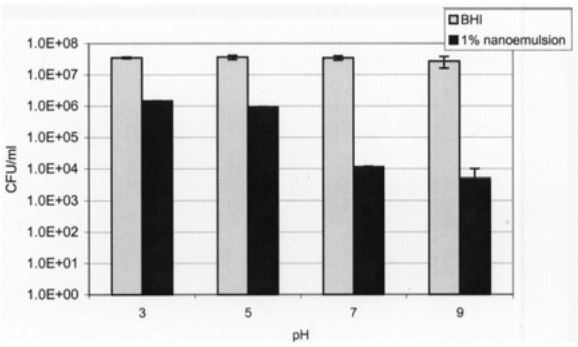

Figure 4. Effect of pH on susceptibility of *Candida albicans* to $X8W_{60}PC$ nanoemulsion. Cells were treated either with BHI medium or 1% nanoemulsion at different pH for 15 minutes at 37 °C. After treatment, cells were washed and plated on BHI plates to assess the number of CFU. The bars represent standard error.

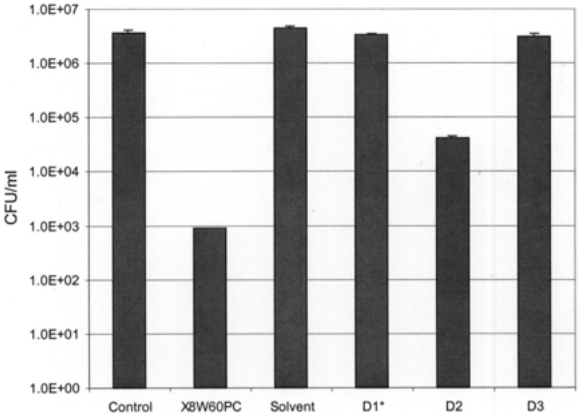

Figure 5. The susceptibility of *Candida albicans* to $X8W_{60}PC$ nanoemulsion or its individual active ingredients. Cells were treated with either 0.1% $X8W_{60}PC$ nanoemulsion or with individual ingredients at the equivalent concentrations for 15 minutes at 37 °C. After treatment, cells were washed and plated on BHI plates to assess the number of CFU. The bars represent standard error. * – D1, D2, and D3 depict three detergents used to prepare $X8W_{60}PC$.

Table 1. Minimal fungicidal concentration (MFC) of $X8W_{60}PC$ nanoemulsion and Nystatin on yeast and filamentous fungi.

Microorganism	$X8W_{60}PC$ (%)	Nystatin (units/ml)
Candida albicans	0.063	31.3
Candida parapsilosis	0.016	31.3
Microsporum gypseum	0.040	n/t*
Trichophyton rubrum	0.032	n/t
Trichophyton mentagrophytes	0.016	n/t
Aspergillus fumigatus	0.010	n/t
Fusarium oxysporum	0.032	62.5

* Not tested.

concentrations of the nanoemulsion to *C. albicans* and *parapsilosis*, *T. spp*., *M. gypseum*, *A. fumigatus*, and *F. oxysporum* (Figure 6 and Table 1). As a reference anti-fungal agent, Nystatin has been used in some experiments in parallel to nanoemuslion. Aspergillus fumigatus was the most susceptible, <0.01% nanoemulsion resulted in complete killing and *C. albicans* was the most resistant to the nanoemulsion fungicidal activity (Figure 6 and Table 1). Overall, all tested fungi were susceptible to the nanoemulsion at a concentration below 0.1%.

Discussion

There is an ongoing need for new anti-fungal agents to combat the continuous development of resistant yeast or filamentous fungi species due to inappropriate use of anti-fungal drugs in humans and animals [22–24]. Surfactant nanoemulsions are a treatment option. Nanoemulsions are novel water-in-oil formulations that are stabilized by the addition of small amounts of surfactant and solvent [12]. The water-immiscible, liquid phase is mixed into an aqueous phase by high stress mechanical extrusion, yielding a uniform population of droplets ranging in diameter from 400–800 nm. Due to their intrinsic features, nanoemulsions can be further diluted in aqueous solutions and stored at a broad range of temperature. Recently, surfactant nanoemulsions have been reported to have extensive bactericidal, sporicidal and virucidal effects [12, 16–20].

In this study, we showed that the surfactant nanoemulsion designated $X8W_{60}PC$ has fungicidal activity. At 1% concentration, $X8W_{60}PC$ reduced the number of *C. albicans* CFU by more than four logs within 15 minutes of treatment and two-hour treatment reduced the number of *C. albicans* CFU by six logs. Of note, Nystatin, which is presently used for topical treatment, needs to be applied for approximately four weeks to treat cutaneous candidiasis with a dose as high as 100, 000 units $gram^{-1}$ [25]. Moreover, the nanoemulsion is most effective at physiological pH, another good feature in its potential application as an anti-fungal drug for topical treatment.

Since some ingredients of $X8W_{60}PC$ are biocidal, we tested whether these ingredients alone, at concentrations equivalent to those in the nanoemulsion, have anti-fungal activity. D1, D3 and solvent were ineffective against yeast at equivalent concentrations. Ten to 1000 times higher concentrations of the individual ingredients were required to obtain a comparable fungicidal activity (data not shown). D2, at a concentration equivalent to that of the nanoemulsion, had some fungicidal activity; it reduced the number of CFU by approximately two logs (Figure 5). These data suggest that the fungicidal activity of $X8W_{60}PC$ is not due to its ingredients, but to the nanoemulsion structure.

Minimal Inhibitory Concentration assays further confirmed the anti-fungal activity of the nanoemulsion. There was a slight variation in susceptibility among the tested fungi. *C. albicans* appeared to be the least susceptible (Figure 6A) and *Candida parapsilosis* (Figure 6B) and *Trichophyton* spp. (Figure

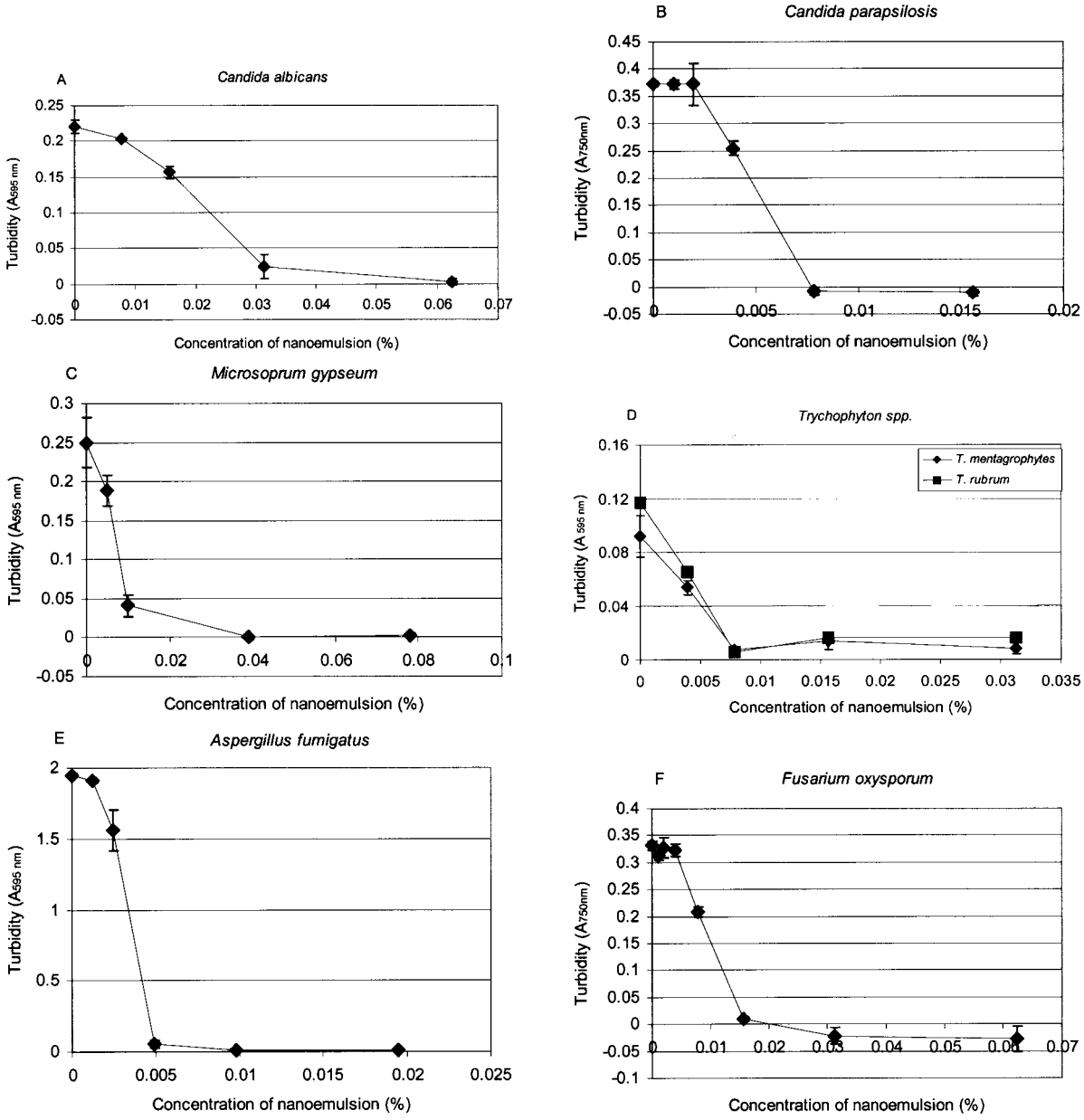

Figure 6. Minimal Inhibitory Concentrations of $X8W_{60}PC$ nanoemulsion on *C. albicans* (A) and *C. parapsilosis* (B), *Microsporum gypseum* (C), *Trichophyton spp.* (D), *Aspergillus fumigatus* (E), and *Fusarium oxysporum* (F). Two-fold dilution of $X8W_{60}PC$ nanoemulsion in BHI was mixed with a fixed number of CFU of fungi and incubated until fungal growth was observed. Turbidity was measured on an ELISA plate reader. The bars represent standard error.

6D) were most susceptible. Nevertheless, the variation in susceptibility was minimal, and the MIC concentration never exceeded 0.1% of nanoemulsion.

These *in vitro* data clearly show that $X8W_{60}PC$ has potential as a topical anti-fungal agent against yeast and filamentous fungi, however, several issues have yet to be addressed. First, in susceptibility testing, we used only seven fungal species from five different genera, which are representative of clinically important fungi. More species have to be included in future studies to better define the nanoemulsion anti-fungal spectrum. Secondly, although *in vitro* studies are a good indicator of anti-microbial activity, *in vivo* tests will be required to prove clinical efficacy. Thirdly, while

the surfactant nanoemulsion at biocidal concentrations is non-toxic in short term application to skin (unpublished data), mucous membranes, and gastrointestinal tract [17], its long term toxicity has never been tested. Therefore, chronic toxicity tests are essential. Finally, due to the rapid action of the nanoemulsion, it is unlikely that therapeutic concentrations could result in development of resistant strains, however, this cannot be excluded at this time. Currently, we are in the process of investigating all these issues.

In conclusion, since $X8W_{60}PC$ exhibits fungicidal activity on yeast and filamentous fungi, this nanoemulsion has potential as a topical treatment for a variety of mycoses.

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