



# *In Vitro* Evaluation of Nanoemulsions of Curcumin, Piperine and Tualang Honey as Antifungal Agents for *Candida* Species

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## Abstract

**Introduction:** Vulvovaginal candidiasis (VVC) has received enormous attention, not only due to its negative influences on women's life but also because of the escalating trend of fungal resistance towards current antifungal drugs. In recent decades, researchers have been focusing on the development of natural products as antifungal agents due to their low side effects compared to standard antifungal drugs. In this study, the antifungal activities of curcumin, piperine, and tualang honey (TH) in single, combination, and combined nanoemulsions were evaluated.

**Materials and Methods:** The nanoemulsions were prepared by dissolving curcumin, piperine and TH in the nanoemulsions base which was prepared by mixing Capryol, Tween 80/Kolliphor RN40 and Transcutol HP. Well diffusion methods were used and the zone of inhibitions were compared to standard drug, fluconazole.

**Results:** The antifungal activity of these natural products alone were low and not all combinations were significant. Moreover, both curcumin and piperine are known to have low bioavailability that might limit its fungicidal efficiency. Hence, nanoemulsions of curcumin, piperine and honey were then developed in this study. The nanoemulsions of three natural compounds have possessed favorable antifungal activity (more than 80%) against the wide range of *Candida* spp. Particularly, *Candida albicans* was more susceptible to this nanoemulsions compared to other species tested and some of them were the most resistance to fluconazole.

**Conclusions:** In concise, this study showed evidence in support of the therapeutic use of nanoemulsions of curcumin, piperine and TH in antifungal infections.

**Keywords:** Nanoemulsions, Curcumin, Piperine, Tualang Honey, Vulvovaginal Candidiasis

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## Introduction

Vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis (inflammation of vaginal) after bacterial vaginosis and trichomoniasis.<sup>1</sup> Approximately, 75% of women worldwide experience a single episode of VVC and 50% of them have at least a second episode in one year. Moreover, up to 10% of all women of the reproductive age group will be influenced by more than 4 episodes of this infection, which is known as recurrent infections.<sup>1,2</sup> Women with VVC are often presented with vaginal symptoms such as discharge ('cheese-like' material or watery secretion), odor, itching, irritation or burning which negatively affects the quality of the life in women.<sup>3</sup> *C. albicans* is the principal causative species in VVC,<sup>4,5</sup> but non-*albicans Candida* (NAC) species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. guilliermondii* are also very common in causing VVC.<sup>6,7</sup> Azole antifungal agents such as fluconazole, clotrimazole,

miconazole, itraconazole are reported to be effective in treating VVC in a Bayesian network meta-analysis.<sup>8</sup> However, several studies have demonstrated that these azole antifungal agents are in fact not effective against the *Candida* species causing VVC.<sup>9,10</sup> This has urged the discovery of alternative antifungal agents in VVC.

The use of natural products from plants as potential antifungal agents are promising, as they have been proven to be able to inhibit the synthesis of fungal cell wall, sphingolipids and protein.<sup>11</sup> A novel strategy is to find specific inhibitors of virulence factors, that infective ability of the pathogen is silenced without killing it and natural selection of drug resistant mutants can be avoided. Molecules of plant origin can be served as a good source to novel molecules and targets for anti-candidal therapy. Based on several reported studies, curcumin,<sup>12,13</sup> honey<sup>14,15</sup> and piperine<sup>16</sup> have shown to exhibit antifungal properties. These compounds have shown

to inhibit the growth of *Candida* species when administrated alone or singularly but whether they have a combinatory effect or not has not been reported so far.<sup>17</sup>

Tualang honey (TH), which is also known as *Apis dorsata* is produced from rock bee. They construct hives on Tualang trees (*Kompassia excelsa*) that is primarily located in the North-Eastern tropical rain forest region in Malaysia.<sup>18</sup> Most types of honey produce hydrogen peroxide and gluconic acid due to glucose oxidization by glucose oxidase. The produced hydrogen peroxide can significantly contribute to the antimicrobial activity of honey,<sup>19</sup> and the concentrations similarly differ between honey due to the geographical origins.<sup>20</sup> Besides that, stingless honeybees are eusocial insects distinguished to five diverse genera, i.e. *Lestrimelitta*, *Meliponula*, *Melipona*, *Trigona* and *Dectylurina*, which play an imperative role in pollination.<sup>21</sup> These genera likewise produce propolis, a resinous mixture which honeybees (*Trigona thoracica*) derive from sap flowers, tree buds, or other botanical sources, then added with salivary enzymes, beeswax and other bee metabolism compounds. Honeybees utilize propolis to secure the hive by sealing cracks and openings, making the interior wall smooth and repair combs allowing the hive entrance easier to guard.<sup>22</sup>

Another natural product of interest, curcumin (diferuloyl methane) is a yellow lipid-soluble polyphenolic dietary compound originated in the rhizome of turmeric (*Curcuma longa*). It is commonly used in cooking and as coloring agents in food.<sup>23</sup> Traditionally, turmeric is used to treat diseases such as wound infections, rheumatoid arthritis, jaundice, eye infections, liver and dental diseases, digestive disorders for instance ulcers, acidity, indigestion, dyspepsia, flatulence and lessen the hashish hallucinatory effects of other psychotropic drugs.<sup>13</sup> Extensive pharmacological properties of curcumin such as anti-inflammatory, anticancer, antioxidative antimicrobial and hepatoprotective properties have been meticulously documented.<sup>24</sup> The antifungal mechanism of curcumin is not fully understood, but it has been proposed that its fungicidal mechanism could be involved in chitin accumulation on the outer layer of the cell wall.<sup>25</sup>

On the other hand, black pepper (*Piper nigrum* L.) belongs to the Piperaceae family, which is commonly harvested for its fruit. During the olden days, black pepper was used as a preservative and seasoning, where it is known as peppercorns in dried state or “King of Spices” in South India. Pepper is utilized mostly in curry recipes and is incorporated into the remedies in Ayurvedic and other conventional therapeutic systems. It is used in folk medicine for treating leprosy, antiseptic diuretic, carminative, aphrodisiac, rheumatoid arthritis, cough, melanoderma and peripheral neuropathy due to the polyphenolic compounds like tannins, volatile compounds and phenols.<sup>26</sup> Furthermore, the pepper spicy tang is due to piperamides, which are the pungent bioactive alkaloids found within the fruit's skin and seeds. It has been studied that, volatile oils from pepper leaves (humulene, limonene,  $\beta$ -, $\alpha$ -pinene) and pepper berries (elemol, bisabolene,  $\alpha$ -cubebene,  $\alpha$ -bisabolol,  $\alpha$ -guaiene) were able to inhibit the growth of *Aspergillus niger*, *Candida albicans*, *Saccharomyces cerevisiae* and *Trichoderma* spp.<sup>27</sup>

Curcumin and piperine are known to have low bioavailability, which has hindered its development as effective antifungal drugs.<sup>28,29</sup> Several attempts have been made to enhance the efficacy of curcumin such as the incorporation of liposome,<sup>30</sup> dendrosomal,<sup>31</sup> and nanotechnology such as nanoparticles.<sup>32</sup> Among different nanotechnology, nanoemulsions are described as colloidal dispersions fabricated from water, oil and surfactant with droplets radius of between 10 nm and 50 nm.<sup>33</sup> They can be produced from high-energy methods such as high-pressure homogenization, microfluidization and sonication, and low-energy methods that rely on spontaneous formation of droplets between oil and water phases.<sup>34</sup> Nanoemulsions can overcome the drawback of drugs or compounds with poor aqueous solubility and low bioavailability.<sup>35</sup> Nanoemulsions have been incorporated in curcumin to increase the bioavailability and stability.<sup>36-38</sup> Considering the therapeutic uses of curcumin, piperine and honey in traditional medicine, the present study aimed to study the antifungal effects of these natural products in single as well as in combination on *Candida* strains with the incorporation of nanoemulsions technology.

## Materials and Methods

### Natural Compounds

Curcumin, piperine and fluconazole were purchased from Sigma-Aldrich Corporation, USA. All the used chemicals were of analytical grade. Wild-type honey commonly known as TH was harvested from *lebah madu* or honeybee (*Apis Dorsata*) from Mount Ledang forest, Johor Bahru, Malaysia.

### Test Sample Preparation

#### Preparation of Test Stock Solutions

The used compounds were prepared in 100% Dimethyl sulfoxide (DMSO) at the concentrations of 0.5 mg/mL and 1 mg/mL. Subsequently, these compounds were prepared in combinations of two at various ratios ranging from 1 mg/mL to 10 mg/mL.

#### Preparation of Nanoemulsions

The nanoemulsions were prepared by dissolving curcumin, piperine and TH in the nanoemulsions base. The nanoemulsions base was prepared by mixing Capryol, Tween 80/Kolliphor RN40 and Transcutol HP. Heating the nanoemulsions over water bath at 70°C was required to fully dissolve the compounds. The stock solutions were then diluted to 1 mg/mL, 3 mg/mL and 5 mg/mL using distilled water.

### Anti-fungal Assay

#### Microbial Inoculum Standardization

Eight strains of *Candida* were used in this study. Six species of *Candida* were used, namely *C. albicans* (ATCC 10231, ATCC 14053, and ATCC 90028), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 14243), *C. kefyr* (ATCC 2512), *C. rugosa* (ATCC 10571) and *C. parapsilosis* (ATCC 22019). These strains were grown on Sabouraud dextrose agar (SDA) plates at  $\pm 37^\circ\text{C}$  and adjusted to 0.5 McFarland standard to obtain an inoculum size of  $1 \times 10^6$   $\mu\text{L}$  per mL. The antifungal assay was performed

according to the guidelines in CLSI Document M44 (2018).

### Agar Well Diffusion

Lawn cultures of the *Candida* species were prepared on an SDA plate using a sterile glass spreader. Four wells sized 12 mm in diameter were made into the agar and 100 µL of the test compounds (1 mg/mL, 3 mg/mL, 5 mg/mL) were added into each well. These plates were incubated for a 24-hour period at ±36°C, under aerobic condition. At the end of the incubation period, the diameter of inhibition zones was measured in mm. Fluconazole (1 mg/mL, 3 mg/mL and 5 mg/mL) and 100% DMSO were used as positive and negative controls, respectively.<sup>39</sup> The assays were carried out in triplicates and in three independent experiments. The antifungal activity was calculated using the following formula, where fluconazole acted as the positive control in this study.

$$\frac{\text{Zone of inhibition of compound}}{\text{Zone of inhibition of positive control}} \times 100\%$$

### Statistical Analysis

The mean values were expressed as the mean ± standard deviation (SD) and were analyzed using two-way ANOVA and Tukey's multiple comparison test using GraphPad Prism 8. All tests were performed with a confidence level of 95%. The *P* value of <0.05 was considered as significant.

## Results

### Susceptibility of ATCC Strains to Fluconazole

Fluconazole was used as the positive control in this study. From the well diffusion assay, the zone of inhibition measured for *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 14243) and *C. kefyr* (ATCC 2512) was the largest, which suggested highest susceptibility towards fluconazole. On the other hand, the zone of inhibition measured for *C. glabrata* (ATCC 2001) and one of the *C. albicans* strain (ATCC 10231) was the smallest. This could suggest the lowest susceptibility of these strains towards fluconazole. All the used strains were susceptible to fluconazole (Table 1).

### Antifungal Activities of Single Compound

Prior to assessing the therapeutic effects of natural compounds in combination, the antifungal properties of each compound (honey, curcumin and piperine) at 1 mg/mL was compared to fluconazole at 1 mg/mL (Table 1). Curcumin, piperine and TH showed the least antifungal activity against *C. parapsilosis* (ATCC 22019). However, curcumin had the highest antifungal activity against *C. glabrata* (ATCC 2001), while piperine and TH were effective against *C. albicans* (ATCC 14053) and *C. krusei* (ATCC 14243) respectively.

### Antifungal Activities of Combined Compounds

The combinations were composed of one compound at a fixed ratio and another compound at varying ratio as followed (1) curcumin and piperine, (2) piperine and curcumin, (3) honey and piperine, (4) piperine and honey, (5) honey and curcumin and (6) curcumin and honey. Table 2 showed the antifungal activity of significant pairs. Combinations of curcumin with piperine at varying ratio were able to inhibit the growth of all *Candida* strains except ratio pair of 1:2. However, combinations of piperine with curcumin were able to inhibit *C. albicans* (ATCC 14053) and *C. krusei* (ATCC 14243) with the antifungal activity 100% more than fluconazole at ratio as low as 1:2. A combination of piperine and TH was also able to inhibit *Candida* spp. at a ratio of 1:2 but not all ratios were significant. The least effective combination was TH with curcumin, where only ratio 1:2 and 1:10 were significant and only effective against *C. kefyr*, *C. albicans* and *C. rugosa*.

### Antifungal Activities of Nanoemulsions

Since the combination of curcumin and piperine at the ratio of 1 to 4 can inhibit the growth of all *Candida* strains, we used the same ratio to develop the nanoemulsion compounds contained curcumin, piperine and TH. Eight combinations of nanoemulsions (Table 3) were developed and each combinations were diluted to 1 mg/mL. For well diffusion assay, concentration of nanoemulsions at 1mg/mL did not show any antifungal activities. Hence, the concentration of nanoemulsions increased to 3 mg/mL compared to the zone of inhibition with fluconazole at 3 mg/mL (Table 4).

**Table 1.** Antifungal Activities of Each Natural Compounds Compared to Fluconazole

Candida Species	ATCC	Antifungal Activities as compared to Fluconazole (%)			
		Fluconazole (1 mg/mL)	Curcumin (1 mg/mL)	Piperine (1 mg/mL)	Tualang Honey, TH (1 mg/mL)
<i>C. parapsilosis</i>	22019	2.40 (S)	14.80	13.92	34.39
<i>C. albicans</i>	14053	2.20 (S)	95.71	118.57	104.48
<i>C. rugosa</i>	10571	2.00 (S)	40.97	37.73	42.19
<i>C. krusei</i>	14243	2.40 (S)	100.00	83.33	106.35
<i>C. glabrata</i>	2001	1.70 (S)	133.75	100.00	66.25
<i>C. albicans</i>	90028	2.00 (S)	100.00	100.00	95.71
<i>C. kefyr</i>	2512	2.40 (S)	56.63	77.57	90.29
<i>C. albicans</i>	10231	1.70 (S)	128.33	93.63	64.60

\**C. albicans* (ATCC 10231, ATCC 14053, and ATCC 90028), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 14243), *C. kefyr* (ATCC 2512), *C. rugosa* (ATCC 10571) and *C. parapsilosis* (ATCC 22019).

(S) indicates susceptible to fluconazole.

\*Blue denoted more than 100% antifungal activities as compared to fluconazole.

\*Orange denotes less than 50% antifungal activities as compared to fluconazole.

**Table 2.** Antifungal Activities of Curcumin, Piperine and TH

ATCC	Antifungal Activities as compared to Fluconazole (%)									
	1:2	1:4	1:6	1:8	1:10	1:2	1:4	1:6	1:8	1:10
	Curcumin:Piperine					Piperine:Curcumin				
22019	-	81.31	84.11	84.11	86.92	-	40.19	68.22	-	-
14053	-	78.75	75.00	78.75	58.75	125.00	-	-	87.50	83.75
10571	-	100.00	61.67	128.33	111.67	-	105.00	71.67	128.33	78.33
14243	-	78.33	71.67	100.00	78.33	100.00	-	166.67	100.00	95.00
2001	-	72.99	89.78	75.18	80.29	-	-	67.88	-	-
90028	-	70.00	66.36	66.36	54.55	-	-	54.55	79.09	57.27
2512	-	90.00	124.29	81.42	114.29	-	152.86	52.86	100.00	71.43
10231	-	64.17	58.33	64.17	55.83	39.17	47.50	50.00	44.17	64.17
Tualang Honey (TH):Piperine					Piperine:Tualang Honey (TH)					
22019	-	58.88	93.46	-	-	-	-	121.50	84.11	-
14053	83.75	-	-	91.25	87.50	62.50	-	-	78.75	83.75
10571	-	88.33	138.33	105.00	111.67	-	161.67	78.33	105.00	66.67
14243	128.33	-	100.00	78.33	78.33	-	105.00	-	111.67	121.67
2001	53.28	41.61	78.10	78.10	72.99	-	-	67.88	-	-
90028	-	-	66.36	72.73	45.45	-	-	75.45	84.55	75.45
2512	142.86	171.43	85.17	100.00	114.29	-	71.43	81.43	67.14	90.00
10231	-	72.50	-	47.50	85.83	66.67	64.17	55.83	77.50	58.33
Tualang Honey (TH):Curcumin					Curcumin:Tualang Honey (TH)					
22019	-	-	-	-	-	-	53.27	-	124.30	112.15
14053	-	-	-	-	96.25	-	-	-	108.75	-
10571	-	-	-	-	71.67	-	-	155.00	-	183.33
14243	-	-	-	-	-	-	-	-	-	188.33
2001	-	-	-	-	-	-	65.69	63.58	89.78	65.69
90028	-	-	-	-	-	-	-	63.64	54.55	79.09
2512	128.57	-	-	-	75.71	-	-	-	124.29	-
10231	-	-	-	-	-	-	50.00	72.50	83.33	94.17

Note. *C. albicans* (ATCC 10231, ATCC 14053, and ATCC 90028), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 14243), *C. kefyr* (ATCC 2512), *C. rugosa* (ATCC 10571) and *C. parapsilosis* (ATCC 22019).

Blank (-) denotes insignificant combinations of two natural compounds.

Each combination was significant to each other at  $P < 0.0001$  when analyze using two-way ANOVA. When comparing to fluconazole, certain nanoemulsions have antifungal activities more than 100% compared to fluconazole, while some were less than 50%.

In order to increase the antifungal efficacy, we attempted to increase the concentration to each nanoemulsion compound to 5 mg/mL and tested on all *Candida* strains. All eight nanoemulsions were significant to each other at  $P < 0.0001$  when analyzed using two-way ANOVA. The antifungal activity of all nanoemulsion compounds was also enhanced

**Table 3.** The Combination of Compounds in Nanoemulsions in Ratio

Nanoemulsions	Curcumin	Piperine	Honey
A	1	1	1
B	1	1	4
C	1	4	1
D	4	1	1
E	4	4	1
F	4	4	4
G	1	4	4
H	4	1	4

to 50% and above when compared to fluconazole at 5 mg/mL (Table 5). Out of eight combinations, the antifungal activity of nanoemulsion A showed more than 100% against half of the tested *Candida* spp.

The antifungal activity of nanoemulsion 1 was then compared with combination of and single compounds (Table 4). Those with the highest antifungal activity were selected as the effective compound or combinations against each *Candida* spp. The single compound of curcumin was effective against *C. glabrata* (ATCC 2001) and *C. albicans* (ATCC 10231). On the other hand, a combination of piperine with curcumin and curcumin with TH were effective against *C. kefyr* (ATCC 2001) and *C. krusei* (ATCC 14243) respectively. *C. parapsilosis* (ATCC 22019) and *C. rugosa* (ATCC 10571) were susceptible to the combination of piperine with TH. Besides that, nanoemulsion with curcumin, piperine and TH were only antifungal against *C. albicans* (ATCC 15053 and ATCC 90028).

## Discussion

### Susceptibility of ATCC Strains Towards Fluconazole

Fluconazole belongs to fluorine-substituted, bis-triazole antifungal agents. It acts by binding to fungal cytochrome

**Table 4.** Antifungal Activity of Nanoemulsions Compared to Fluconazole

ATCC	A	B	C	D	E	F	G	H
	Antifungal Activities of Compound Nanoemulsions (3 mg/mL) Compared to Fluconazole (3 mg/mL) (%)							
22019	79.17	69.44	65.28	61.11	73.61	59.78	65.28	58.33
14053	127.45	92.16	111.76	92.16	90.20	82.35	88.24	94.12
10571	75.00	62.50	66.67	50.00	45.83	43.06	48.61	48.61
14232	88.33	66.67	78.33	71.67	70.00	53.33	68.33	60.00
2001	66.67	63.89	63.89	59.72	45.83	47.22	54.17	51.39
90028	129.41	103.92	105.88	105.88	100.00	86.27	101.96	80.39
2512	69.70	60.61	57.58	60.61	54.55	48.48	48.48	53.03
10231	86.67	81.68	81.68	76.67	65.00	61.67	71.67	70.00
Antifungal Activities of Compound Nanoemulsions (5 mg/mL) Compared to Fluconazole (5 mg/mL) (%)								
22019	87.50	61.11	68.06	76.39	66.67	50.00	65.28	54.17
14053	125.49	109.80	100.00	107.84	113.73	86.27	98.04	86.27
10571	81.94	66.67	66.67	70.83	61.11	61.11	62.50	55.56
14243	105.00	78.33	91.67	93.33	86.67	78.33	71.67	66.67
2001	84.72	79.17	81.94	81.94	87.50	58.33	73.61	62.50
90028	109.80	92.16	105.88	98.04	98.04	92.16	94.12	84.31
2512	89.39	65.15	71.21	69.70	69.70	54.55	68.18	57.58
10231	113.33	71.67	90.00	78.33	81.67	61.67	85.00	75.00

*C. albicans* (ATCC 10231, ATCC 14053, and ATCC 90028), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 14243), *C. kefyr* (ATCC 2512), *C. rugosa* (ATCC 10571) and *C. parapsilosis* (ATCC 22019)

Blue denotes more than 100% of antifungal activity as compared to fluconazole.

Orange denotes less than 50% of antifungal activity as compared to fluconazole.

**Table 5.** The Antifungal Activity of All Compound, Combinations of Compounds and Nanoemulsion

ATCC	Antifungal Activity as compared to Fluconazole (%)									
	Single Compound			Combination of Two Compounds						Nanoemulsion A
	Curcumin	Piperine	TH	Curcumin: Piperine (1:4)	Piperine: Curcumin (1:4)	Curcumin: TH (1:4)	TH: Curcumin (1:10)	Piperin:TH (1:6)	TH: Piperine (1:4)	Curcumin + Piperine + TH (1:1:1)
22019	14.80	13.92	34.39	81.31	40.19	53.27	-	121.50	58.88	87.50
14053	95.71	118.57	104.48	78.75	125.00	108.75	96.25	62.50	83.75	125.49
10571	40.97	37.73	42.19	100.00	105.00	155.00	71.67	161.67	88.33	81.94
14243	100.00	83.33	106.35	78.33	100.00	188.33	-	105.00	128.33	105.00
2001	133.75	100.00	66.25	72.99	67.88	65.69	-	67.88	53.28	84.75
90028	100.00	100.00	95.71	70.00	54.55	63.64	-	75.45	66.36	109.80
2512	56.63	77.57	90.29	90.00	152.86	124.29	128.57	71.43	142.86	89.390
10231	128.33	93.63	64.60	64.17	39.17	50.00	-	66.67	72.50	113.33

*C. albicans* (ATCC 10231, ATCC 14053, and ATCC 90028), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 14243), *C. kefyr* (ATCC 2512), *C. rugosa* (ATCC 10571) and *C. parapsilosis* (ATCC 22019)

Blue denotes highest antifungal activity for each *Candida* spp. among all compounds.

Blank (-) denotes insignificant combinations.

TH, Tualang Honey.

P-450 to interrupt the conversion of lanosterol to ergosterol, which lead to a disrupt in fungal membranes and ultimately fungal cell death.<sup>40</sup> Fluconazole has been used as an antifungal agent for various fungal infections. A single dose of 150 mg of fluconazole was found to be effective, safe and well-tolerated for the treatment of vaginal candidiasis.<sup>41</sup> A meta-analysis was performed to compare the antifungal effectiveness of ten antifungal drugs including fluconazole, clotrimazole, miconazole, itraconazole, ketoconazole, econazole,

butoconazole, terbinafine and terconazole in treating VVC. Fluconazole was found to be the most effective among 10 antifungal drugs against VVC.<sup>8</sup> In this study, *C. parapsilosis*, *C. krusei* and *C. kefyr* showed the most susceptibility towards fluconazole (Table 1), whereas *C. glabrata* and *C. albicans* presented the highest resistance towards fluconazole. The higher resistance in *C. albicans* could be due to the mutation in *ERG11*, a protein that encodes enzyme 14- $\alpha$ -demethylase. The mutation may alter the protein structure that reduces



the binding affinity of fluconazole.<sup>42</sup> On the other hand, mutations in CgPDR1 was found in *C. glabrata* that resulted in over-expression of drug efflux pump in azole-resistant.<sup>42</sup> This could explain the less susceptibility of *C. glabrata* towards fluconazole.

### Antifungal Activities of Single Compounds

Curcumin, piperine and honey are natural products that are known to have several therapeutic effects including antifungal properties.<sup>43-45</sup> Indeed, all three natural compounds showed certain antifungal properties towards eight ATCC *Candida* strains tested in this study (Table 1), namely *C. glabrata* (ATCC 2001), *C. kefyr* (ATCC 2512), *C. parapsilosis* (ATCC 22019), *C. albicans* (ATCC 10231, ATCC 90028, ATCC 14053), *C. rugosa* (ATCC 10571) and *C. krusei* (ATCC 14243). After being compared to fluconazole, the administration of curcumin, piperine and honey alone were showed to be more effective than fluconazole in inhibiting the growth of *C. glabrata*, *C. albicans* and *C. krusei*. These results are in consistent with previous studies where curcumin could inhibit the growth of *C. albicans* (ATCC 10261), with a minimum inhibitory concentration (MIC) ranging from 250 µg/mL to 2000 µg/mL.<sup>46,47</sup> Curcumin along with its derivative, methyl cinnamaldehyde have portrayed antifungal activity towards a wide range of *Candida* sp. strains, where some of the species are impervious to the standard drugs used in treating infectious diseases.<sup>12</sup> The primary antifungal properties of curcumin can be due to the interaction of the hydroxyl groups with the fungal cell membrane, which consequently lead to damage in energy metabolism, genetic material synthesis, cell leakage and the modification of phospholipid and fatty acids profiles.<sup>48</sup> According to Rai et al<sup>49</sup> the antimicrobial mechanism of curcumin also involves the perturbing of FtsZ protofilaments GTPase activity that has an essential role in microbial cytokinesis. FtsZ shapes the filaments with repeating subunits arrangements from head-to-tail, where these filaments structure a ring along the longitudinal midpoint or septum of the cell known as the Z ring.<sup>50</sup> Thus, disrupting the FtsZ protofilaments GTPase activity can prevent the assembly of FtsZ within the Z ring and restrain cell proliferation that eventually lead to cell death.

The results of this study were consistent with Sayadi and colleagues' study,<sup>51</sup> where TH exhibited antifungal activity with MIC of 25% (v/v), particularly against *C. albicans*. The antifungal properties of TH can be due to the presence of high flavonoid and phenolic contents.<sup>20</sup> The phenolic acid content can affect the cytoplasmic membrane permeability, which steers the leakage of cellular contents such as proteins, nucleic acids and inorganic ions (potassium and phosphate) that ultimately lead to cell death.<sup>52</sup> Furthermore, other types of honey also possess antifungal activities across different countries. Koc et al.<sup>15</sup> demonstrated that four Turkish honeys can exhibit both fungicidal and fungistatic activities against *C. albicans* at a concentration of 1.25% (v/v). Lavender honey also showed an *in vitro* antifungal activity against *Cryptococcus neoformans*, *C. krusei* and *C. albicans* at a concentration of 10% (w/v).<sup>53</sup> Similarly, an *in vitro* study of Medihoney was demonstrated to examine the efficiency in inhibiting all 21

bacterial strains as well as yeasts, i.e. *C. neoformans*, and *C. albicans* with MIC of 1.8% to 10.8% (V/V) respectively.<sup>54</sup>

On the other hand, Ghoshal et al.<sup>55</sup> acknowledged that lactones, terpenoids and alkaloids are the main components responsible for antifungal activity. Hence, the presence of both terpenoids and alkaloids in the *Piper nigrum* leaf extract can exhibit antifungal properties that may serve as a reason for its customary use as a therapeutic plant. The antifungal properties of phenolic compounds found in Piperaceae act as a free radical scavenger. Polyphenolic compounds have an imperative role in stabilizing lipid oxidation, as it is associated with antioxidant activity. Therefore, the phenolic compounds could directly influence with an antioxidative action. The powder of *Piper nigrum* can be utilized as a natural remedy against *Candida* spp. especially *C. albicans* due to its abundance in phenolic and alkaloid content.

### Antifungal Activities of Combined Compound

Despite the comparable antifungal activities of single natural compounds against each *Candida* strain compared to fluconazole, *C. parapsilosis* were the least susceptible to these compounds. To improve the antifungal efficiency, we combined two natural compounds together and tested their antifungal activities (Table 3). A combination of curcumin with piperine at varying concentrations were consistently significant against all *Candida* strains, among all combinations. However, piperine combined with different ratios of curcumin were not significant against all *Candida* strains. These suggested that the synergistic effect of this combination could be owing to the increasing concentration of piperine.

Consistently, Sharma et al<sup>56</sup> showed that the use of curcumin alone provided insignificant antifungal activity in animal-modelled systemic fungal infection. However, administration of piperine together with curcumin can enhance the inhibition of the hepatic and intestinal glucuronidation and its efficacy, as well as similar antifungal potential to fluconazole. This was further supported by pharmacokinetic studies of curcumin and piperine where piperine, at all doses used in the study can enhance the serum concentration, extent of absorption and bioavailability of curcumin up to 2000% in both rats and humans with no adverse effects.<sup>57</sup>

The following combinations, TH + Piperine and Piperine + TH were able to exhibit antifungal activity against all the tested strains within the range of 1 mg/mL + 2 mg/mL to 1 mg/mL + 6 mg/mL. On the other hand, a combination of TH with curcumin at a varying ratio showed the least significant and only two ratio pairs (1:2 and 1:10) were susceptible to *C. rugosa*, *C. albicans* and *C. kefyr*. However, the combination of curcumin with TH at varying ratios were more significant. These suggested that enhancing the concentration of curcumin may exhibit certain inhibitory effects when combined with TH. There is limited research on the combinatory effects of both curcumin and honey. However, previous research showed that Manuka honey at 5%-20% and whey protein isolate (WPI) in 100-250 µg/mL can reduce the growth of *Streptococcus pyogenes*, *Shigella sonnei* and *Proteus vulgaris*, whereas combination of curcumin and WPI can

completely inhibit the growth of *Listeria monocytogenes* and *Staphylococcus aureus*.<sup>17</sup> In the same study, a combination of Manuka honey, WPI and curcumin can inhibit all the tested pathogen mentioned above, except *Bacillus subtilis*.<sup>17</sup> In addition, a combination of Algerian honey with natural compound, starch of Ginger showed a decrease in MIC value of honey from 53%-57% (vol/vol) to 51% (vol/vol) with ginger starch contributing an additive effect to the antifungal activity of honey.<sup>35</sup> These also suggested that honey can be combined with natural products to exhibit antifungal properties by varying the concentration of honey.

### Antifungal Activities of Nanoemulsion Compounds

Since the antifungal activities of TH and curcumin in combination were less effective, curcumin, piperine and honey were combined together. We incorporated nanoemulsions that contained all these three compounds. Nanoemulsion can be described as fine oil/water or water/oil dispersion that is stabilized by surfactant molecule with droplet size range from 20 to 600 nm. The main components of nanoemulsion are consisted of oil, emulsifying agents and an aqueous medium. Due to its small size and non-toxic or non-irritant nature, nanoemulsion has been widely used to improve the drug delivery system.<sup>58</sup> In this study, nanoemulsions were developed using Capryol 90, Kolliphor RH40, and Transcutol HP. Capryol 90 is used as the oil phase with the highest solubility.<sup>59</sup> Kolliphor RH40 can help reduce the emulsion droplet size,<sup>60</sup> while Transcutol HP acts as cosurfactant.<sup>61</sup>

All nanoemulsions, which were consisted of curcumin, piperine and TH, were able to inhibit the growth of all *Candida* strains and each nanoemulsions were significant to each other. Furthermore, the antifungal activity of nanoemulsions at 5 mg/mL was more than 50% when compared to fluconazole (Table 4). Certain combinations showed more than 100% of antifungal activities over fluconazole. This suggested that the antifungal efficiency of these nanoemulsions compounds was as effective as the standard drug. These results were in consistent with Abd-Elsalam and Khokhlove's study where nanoemulsions of eugenol oil demonstrated higher antifungal activity than the control without the addition of eugenol oil.<sup>62</sup> Nanoemulsion also can be incorporated into antifungal drug, Amphotericin B (AmB-NE) in which AmB-NE showed sustained release and higher flux rate that resulted in enhanced penetration of AmB-NE6 across the skin compared to Fungisome and Drug Solution (DS).<sup>63</sup> A similar technique has also been applied in essential oil of oregano to prevent fungal contamination in cheese production.<sup>64</sup>

### Antifungal Activity of Single, Combination and Nanoemulsion of Compounds

Out of eight nanoemulsion, nanoemulsion A at 5mg/mL showed antifungal activity of more than 100% against half of the *Candida* spp. when compared to fluconazole (Table 4). This suggested that nanoemulsion A could be the most affective antifungal agent compared to other nanoemulsions. Therefore, the antifungal activity of nanoemulsion A was compared both alone and in combinations of compounds (Table 5). In this study, the combination of piperine and

curcumin at 1:4 ratio was the most effective against *C. kefyr*, while *C. krusei* was the most susceptible to curcumin and TH at 1:10 ratio. On the other hand, piperine combined with TH showed the highest antifungal activity against *C. parapsilosis* and *C. rugosa* in the range of 1:4 to 1:6 ratio. Curcumin alone has the most fungicidal effect against *C. glabrata* and *C. albicans* (ATCC 10231).

However, nanoemulsion A with curcumin, piperine and TH at the lowest ratio, 1:1:1 exhibited antifungal activity of more than 80% against all *Candida* strains. Incorporation of nanoemulsion in antifungal drug, itraconazole<sup>65</sup> and sulconazole<sup>66</sup> have shown to be able to penetrate well into intestine membrane and skin. Since curcumin and piperine are known to have low bioavailability,<sup>67</sup> dissolving both compounds in nanoemulsion can increase their bioavailability to maximize their therapeutic effects. On the other hand, AL-Waili et al<sup>68</sup> demonstrated that adding honey to propolis has shown to reduce the MIC towards multi-drug resistance *Staphylococcus aureus*, *Escherichia coli* and *C. albicans* when compared to propolis alone. Collectively, the combination of curcumin and piperine with honey in nanoemulsion are promising in inhibiting fungal growth. Besides that, *C. albicans* (ATCC 14053 and ATCC 90028) were the most susceptible to this nanoemulsion, where these two strains were the most resistance to fluconazole in this study. These results again emphasized that nanoemulsion A could be develop as an alternative to fluconazole in treating fluconazole resistant-*Candida* spp.

### Conclusions

In concise, our results showed that incorporation of nanoemulsion in natural products such as curcumin, piperine and TH are able to exhibit favorable antifungal activities against *Candida* spp., especially fluconazole-resistance *C. albicans* strains. Further studies should be conducted to investigate the permeability and pharmacokinetics of nanoemulsion to reveal its true potential as antifungal agents and thus can be applied for the treatment of fungal infections.

### Authors' Contributions

ZXP, SV, ASAM, JKEY, JYT, SQC and NWHT conducted and collected the experiment data. SPS guided on the nanoemulsions development while PM supervised the entire study.

### Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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