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Original Article

Synergistic Antifungal Activity of Magnoliae Cortex and Syzyii Flos against *Candida albicans*[1]

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ABSTRACT

Candida albicans is a dermal fungus of the human body that is known to cause oral candidiasis, vaginal candidiasis, and bloodstream infections in immunocompromised people or in certain environmental conditions. As cases of strains resistant to antifungal agents in *C. albicans* have been reported, studies using plant materials as safe antifungal agents are being actively conducted. In this study, a total of 17 edible plant extracts showed antifungal activity against *C. albicans* as a result of evaluating a 280-plant extract library using paper disk diffusion method. Among them, the four extracts with the strongest antifungal activity (Cinnamomi Cortex, Cinnamomi Ramulus, Magnoliae Cortex, and Syzygii Flos) were selected and evaluated for synergistic antifungal activity against *C. albicans*. The combination of Magnoliae Cortex and Syzygii Flos showed a synergistic activity. The antifungal activity was evaluated based on the concentrations of magnolol and eugenol, the respective components of Magnoliae Cortex and Syzygii Flos. Magnolol and eugenol showed synergistic antifungal activities at the concentration ratio of 1:25 – 1:61. The antifungal activity of these two compounds contributes 28 to 48% to the synergistic antifungal activity of the combination of Magnoliae Cortex and Syzygii Flos extract. In this study, we propose that a combination of Magnoliae Cortex and Syzygii Flos can effectively inhibit the growth of *C. albicans* and that magnolol and eugenol are the responsible inhibitory compounds.

Keywords: *candida albicans*, magnoliae cortex, syzygii flos, antifungal activity, synergy, magnolol, eugenol

1. INTRODUCTION

Candida albicans is a member of normal microbial flora on the human skin (Hedderwick and Kauffman, 1997), but can cause candidiasis (Kumamoto and Vinces, 2005). Candidiasis can cause oral (Epstein, 1990), genital (Donders *et al.*, 2010; Sobel, 2007), and skin infections (Bodey and Luna, 1974) in healthy people, especially in immunocompromised people (Edmond *et al.*, 1999; Jarvis *et al.*, 1991). Candidiasis is the second leading cause of death by infections in hospitals (Sagué *et al.*, 1993).

Antifungal agents used to treat candidiasis belong to the azole family, such as fluconazole, intraconazole, and voriconazole, as well as the polyene family, such as amphotricin B (Garcia-Cuesta *et al.*, 2014). Fluconazole is the most commonly prescribed antifungal agent for *C. albicans* infections (Pfaller *et al.*, 2010), while amphotericin B is used commonly for the treatment of many fungal infections (Sugar *et al.*, 1995). However,

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strains resistant to fluconazole and itraconazole have been reported. (Xiang *et al.*, 2013; Zhao *et al.*, 2013), while amphotricin B has been reported to exhibit renal toxicity (Fanos and Cataldi, 2000).

Therefore, new alternative agents with excellent antifungal activity and less side effects are required. However, due to structural and functional similarities between fungal and human cells, it is difficult to develop an antifungal agent without side effects (Blaszczyk *et al.*, 2000). Previous studies have shown the antifungal activity of extracts of *Cinnamomum parthenoxylon* (Adfa *et al.*, 2020) and *Abies holophylla* (Kim *et al.*, 2016). Antifungal activity compounds have also been reported in *Pestalotiopsis theae* and *Curvularia* sp. isolated from woods (Hidaya *et al.*, 2019). Edible plants are recognized to be less toxic. Many edible plants have been reported for their antifungal activities against *C. albicans* (Bais *et al.*, 2013; de Toledo *et al.*, 2011; Duarte *et al.*, 2005; Vaijayanthimala *et al.*, 2000).

Magnoliae Cortex is the stem bark of *Magnolia ovobata* Thunb. It has been reported to exhibit anti-inflammatory (Lee *et al.*, 2005), anti-microbial (Ho *et al.*, 2001; Sakaue *et al.*, 2016), and antioxidant activity (Shen *et al.*, 2010). Syzygii Flos, known as clove, is the flower bud of *Syzygium aromaticum*. Clove is used as a spice in many countries and has been used to remove bad breath for a long time (Bhowmik *et al.*, 2012). Clove has been reported to have various medical effects, such as antioxidant (Lee and Shibamoto, 2001; Shobana and Akhilender Naidu, 2000), anti-cancer (Liu *et al.*, 2014), and anti-stress effect (Singh *et al.*, 2009). Clove is also known to be effective in killing bacteria (Cai, 1996; Lee and Son, 2006) and fungi (Silva *et al.*, 2017).

Many studies have been conducted to reduce the amount of antifungal agents used through synergistic activities by mixing plant compounds. The addition of plant antifungal compounds was known for many benefits such as antifungal efficacy against a wide range of fungi, increased safety, reduced toxicity, and reduced antifungal resistance. The synergistic effect of the combination of two substances is useful and effective because it can increase antifungal activities (Mukherjee *et al.*, 2005). Another benefit of synergistic combination is the complementary action, because each compounds can have different growth inhibitory mechanisms which complement each other (Wagner and Ulrich-Merzenich, 2009).

In this study, the synergistic antifungal activity of Magnoliae Cortex and Syzyii Flos against *C. albicans* was demonstrated and confirmed that their major components, magnolol and eugenol, respectively, contribute to this synergistic antifungal activity.

2. MATERIALS and METHODS

2.1. Plants extracts, strain, and medium

Plants and their extracts library were prepared in the previous study (Ham and Kim, 2018). *Candida albicans* KCTC 7965 was purchased from Korean Collection for Type Cultures in Korea Research Institute of Bioscience and Biotechnology (Jeongeup, Korea) and stored at −80℃ in a mixture with 15% glycerol. YPD medium was made with 1% yeast extract, 2% peptone, and 2% dextrose. YPD plate was made with YPD medium and 1.5% agar. SDA plate was made with 3% Sabouraud Dextrose Broth (catalog number: BD 238230, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and 1.5% agar. RPMI-1640 medium was made with 1.04% RPMI-1640 medium (catalog number: R6504, Merck KGaA, Darmstadt, Germany) and 0.165 M 3-(N-morpholino)propanesulfonic acid as a buffer. The analytical standards, eugenol (catalog number: E51791) and magnolol (catalog number: D3971), were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO, United States) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively.

2.2. Measurement of growth inhibition zone by plant extracts

Each extract of 50 mg was dissolved in 1 mL of methanol and incubated at 50℃ for 5 min. After vortexing, the dissolved extract sample was centrifuged at 13,000 rpm for 10 min. The transparent supernatant was used for further experiments. The supernatant with 20 μL was loaded 5 times onto paper disc (Catalog number: 49005040, Advantec MFS, Inc., Dublin, CA, USA) and dried. As a control, 20 μL of methanol, the solvent in which the extract was dissolved, was loaded 5 times and dried.

C. albicans stored at -80℃ were inoculated on YPD plates and incubated at 26℃ for 2 days. A single colony was incubated in 5 mL of YPD medium and incubated at 26℃ and 250 rpm for 1 day. Absorbance of the pre-cultured cells was measured and diluted with YPD medium to make the concentration of the initial cells (Abs_{600} = 1). After the diluted cells with 0.1 mL was spread on YPD plate, a paper disc with an extract was placed in the center of the plates and incubated at 26℃ for 1 days. After incubation, it was judged that an extract having a growth inhibition zone more than 1 mm around the paper had an antifungal activity. The experiments were repeated 3 times per extract and the average value showing the diameter of the growth-inhibiting zone with the standard deviation was calculated.

2.3. Measurement of the growth inhibition of *C. albicans* in YPD medium

After the transparent sample solution was prepared by the method described in the previous paragraph, it was diluted according to the test concentration and 50 μL of the diluted sample was mixed in 5 mL of YPD medium. The control YPD medium was treated with 50 μL of methanol. The pre-cultured *C. albicans* was inoculated to have an Abs_{600} value of 0.05 and then cultured at 26℃ and 250 rpm for 18 hours. The cell concentration was measured with a spectrophotometer (Optizen 2120UV, Mecasys Co., Ltd., Daejeon, Korea) at an absorbance of 600 nm. The relative growth was calculated by comparing the cell growth to the control.

2.4. Evaluation of synergistic growth inhibition in a liquid culture

In order to evaluate the synergistic effect of the selected extracts, sample concentrations that inhibited the growth of *C. albicans* by 20% were determined. The determined concentrations for extracts are presented in the result section. Extracts at the indicated concentrations were prepared by mixing 50 μL of one extract and 50 μL of the other extract in 5 mL of YPD medium. The control group was treated with 100 μL of 99.5% methanol, the solvent in which the extract was dissolved. Three different control cultures were used: culture with 100 μL of methanol; culture with 50 μL of the first extract; and culture with 50 μL of the other extract. The pre-cultured *C. albicans* was inoculated to have an Abs_{600} value of 0.05 and then cultured at 26℃ and 250 rpm for 18 hours. The cell concentration of the culture medium was measured with a spectrophotometer (Optizen 2120UV). Relative growth was calculated and compared to the cell growth of the control.

2.5. Evaluation of synergistic growth inhibition by checker board method

To measure the antifungal activity on *C. albicans*, CLSI M27-A2 broth microdilution methods (NCCLS, 2002) using checkerboard combinations was employed. *C. albicans* stored at -80℃ were streaked on SDA plates and incubated at 35℃ for 1 day. A single colony was suspended in 0.145 mol/L saline and vortexed for 15 seconds. The suspended colony was measured

by absorbance at a wavelength of 530 nm with a spectrophotometer (Optizen 2120UV) and adjusted with saline to 0.5 McFarland turbidity in order to prepare a *C. albicans* suspension. The inoculated concentration of *C. albicans* in the culture for measuring the antifungal activity was $1 \times 10^3 \sim 5 \times 10^3$ CFU/mL. Both tested extracts were dissolved separately in RPMI 1640 medium. Two dissolved extracts were mixed in 50 μL each. As a control, 50 μL of each sample and 50 μL of RPMI 1640 medium were used. After inoculation, cells were incubated at 35℃ for 2 days. The sunk cells were suspended by pipetting and cell turbidity of wells was measured at 530 nm with a microplate reader (Synergy LX, BioTek Instruments, Inc., Winooski, VT, USA).

Growth inhibition (GI %) was calculated by the equation stated below. The cell density was measured by absorbance at a wavelength of 530 nm with a spectrophotometer.

GI (%) = (Cell density of control − Cell density of sample) / Cell density of control × 100

Compared with the antifungal activity as GI % of each treatment, it was judged that there was a synergistic antifungal activity when the antifungal activity of the mixture were 2 times or more than individual two treatments (Chusri *et al.*, 2014).

2.6. Quantitative analysis of eugenol and magnolol by high-performance liquid chromatography

High-performance liquid chromatography used for the analysis of eugenol and magnolol was composed with vacuum degasser & mixer (catalog number: SDV40A), gradient column oven (catalog number: CTS30), auto-sampler (catalog number: YL9150 Alias), solvent delivery pump (catalog number: SP930D), and UV / Vis detector with dual wavelength (catalog number: YL9120) from YL Instruments Co., Ltd. (Anyang, Korea).

YMC-Triart C18 (catalog number: TA12S05-2546WT, YMC Korea Co., Ltd, Seongnam, Korea) was used for the analytic column. All samples were filtered with SEPARA syringeless filter vial (catalog number: MV32ANPPV002FC01, GVS North America, Inc., Sanford, ME, USA) prior to analysis. Results were analyzed with Autochro-3000 software (version 2.0.0) program from YL Instruments Co., Ltd.

The operating conditions for the analysis of eugenol were the same as those of a previous study (Seal, 2016). The mobile phase was acetonitrile (solvent A) and 1% (v/v) acetic acid (solvent B). The solvent ratio (solvent A : solvent B) was 10:90 at 0 minute, 40:60 at 28 minute, 60:40 at 39 minute, 90:10 at 50 minute, 10:90 at 55 minute, and 10:90 at 65 minute. The wavelength for eugenol analysis was 272 nm, speed of the mobile phase was 0.7 mL/min, column temperature was 28℃, and analysis time was 65 minutes.

The conditions for the magnolol analysis were the same as those of a previous study (Kim and Kim, 2004). The mobile phase for magnolol was acetonitrile and 2% (v/v) acetic acid, at the ration of 50:50. The analysis conditions for magnolol were 289 nm of wavelength for detection, 1 mL/min for speed of the mobile phase, 26℃ for column temperature, and 60 minutes for analysis time.

3. RESULTS and DISCUSSION

3.1. Antifungal activity of four plant extracts

Antifungal activity against *C. albicans* was measured by disk diffusion test using a methanol extract library of 280 edible plants listed in the Korean Pharmacopoeia or Herbal/Korean Traditional Products from National Institute of Food and Drug Safety Evaluation. The growth inhibition activity of 17 se-

lected plant extracts is shown in Table 1. The size of the clear zone was 34.8 mm by Cinnamomi Cortex, 19.8 mm by Cinnamomi Ramulus, 19.8 mm by Magnoliae Cortex, and 16.8 mm by Syzygii Flos. The antifungal effect according to the concentration was observed in four plant extracts, with a diameter of ≥ 15 mm in the inhibition area (Fig. 1). Three plant extracts, Cinnamomi Cortex (Fig. 1A), Magnoliae Cortex (Fig. 1C), and Syzygii Flos (Fig. 1D), showed ≥ 78% growth inhibitory activity against *C. albicans* at a concentration of 0.5 g/L. Cinnamomi Cortex and Cinnamomi Ramulus is a bark and young branch, respectively, of *Cinnamomum cassia* Presl. However, the growth inhibitory effect of Cinnamomi Ramulus extract on *C. albicans* was much less than that of Cinnamomi Cortex extract. Cinnamomi Cortex extract showed no cell growth at 0.5 g/L. *trans*-Cinnamaldehyde as an antifungal compound from Cinnamomi Cortex inhibited chitin synthases, the cell wall synthesizing enzymes of *Saccharomyces cerevisiae*, in vitro (Bang *et al.*, 2000a). Cinnamaldehyde was reported to be an antifungal chemical in Cinnamomi Ramulus (Wan *et al.*, 2017). Magnolol and honokiol from Magnoliae Cortex inhibited the growth of seven pathogenic fungi (Chen *et al.*, 2019) and magnolol had a synergistic antifungal activity with azoles against *C. albicans* (Sun *et al.*, 2015). Eugenol from Syzygii Flos synergistically inhibited the growth of *C. albicans* with nystatin (Silva *et al.*, 2017). A chemical-genetic profile analysis suggested that eugenol interfered with two permeases for the transport of aromatic and branched chain amino acids in the cytoplasmic membrane of *S. cerevisiae* (Darvishi *et al.*, 2013).

Table 1. Antifungal activity of 17 plant extracts on the growth of *C. albicans*. The diameter of growth inhibited zones is an average and standard deviation of three samples

Plant used for extracts	Diameter of growth inhibited zone (mm)
Control	0.0 ± 0.0
Acori Gramineri Rhizoma	7.0 ± 0.0
Amomi Tsao-ko Fructus	8.0 ± 0.0
Angelicae Tenuissimae Radix	8.8 ± 0.4
Cinnamomi Cortex	34.8 ± 2.4
Cinnamomi Ramulus	19.8 ± 1.7
Cnidii Rhizoma	9.0 ± 0.0
Cocculi Radix	9.3 ± 1.4
Flower of *Rosa multiflora*	8.2 ± 0.4
Impatientis Semen	14.8 ± 0.4
Magnoliae Cortex	19.8 ± 1.5
Moutan Cortex Radicis	12.2 ± 0.8
Phellodendri Cortex	7.3 ± 0.3
Polygalae Radix	9.3 ± 0.5
Rhizome of *Kaempferia galangal*	7.3 ± 0.5
Sanguisorbae Radix	8.0 ± 0.0
Scutellariae Radix	7.5 ± 0.0
Syzygii Flos	16.8 ± 1.0l

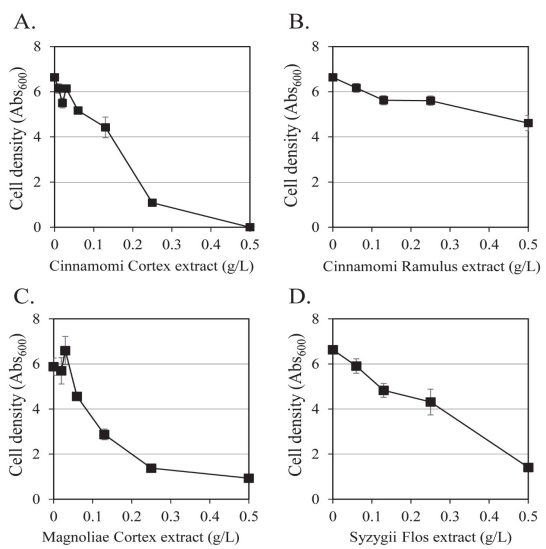

Fig. 1. Antifungal activity against *C. albicans* according to the concentration of the selected four plant extracts, (A) Cinnamomi Cortex extract, (B) Cinnamomi Ramulus extract, (C) Magnoliae Cortex extract, and (D) Syzygii Flos extract. The values are average of three independent experiments.

3.2. Synergistic antifungal activity of Magnoliae Cortex and Syzygii Flos extract

Synergistic growth inhibitory effect on *C. albicans* was tested using a mixture of two extracts among the four tested extracts in Fig. 1 (Fig. 2). The test concentration of the four selected extracts in Fig. 2 were determined to have GI 20% activity from Fig. 1, with 0.06 g/L for Cinnamomi Cortex, 0.3 g/L for Cinnamomi Ramulus, 0.06 g/L for Magnoliae Cortex, and 0.12 g/L for Syzygii Flos. Inhibitory activity against the growth of *C. albicans* was measured for six combinations of four selected plant extracts. Although the five combinations showed just the sum of their respective inhibitory effects, the combination of Magnoliae Cortex extract and Syzygii Flos extract showed ≥ GI 60%. The inhibitory activity above the sum of their respective inhibitory effects suggest a synergistic effect by mixing the two tested extracts.

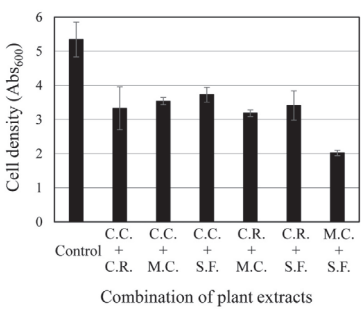

Fig. 2. Antifungal activity of combination of two antifungal plant extracts against *C. albicans*. C.C.: Cinnamomi Cortex extract (0.06 g/L); C.R.: Cinnamomi Ramulus extract (0.3 g/L); M.C.: Magnoliae Cortex extract (0.06 g/L); S.F.: Syzygii Flos extract (0.12 g/L). The values are average of three independent experiments.

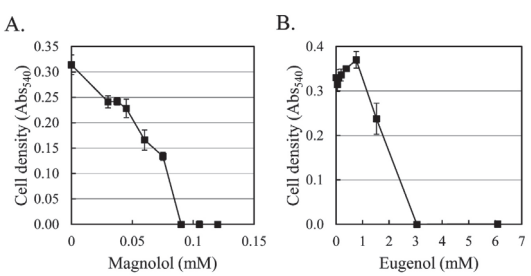

Fig. 3. Antifungal activity of magnolol and eugenol against *C. albicans*. The values are average of six independent experiments.

3.3. Growth inhibition of *C. albicans* by magnolol and eugenol

Magnolol is known as an antifungal compound in Magnoliae Cortex against *C. albicans* (Chen *et al.*, 2019; Sun *et al.*, 2015) and eugenol is an antifungal compound in Syzygii Flos extract (Darvishi *et al.*, 2013). Using a high-performance liquid chromatography analysis, the Magnoliae Cortex extract contained 6.44% magnolol, with a retention time of about 41.1 min, while Syzygii Flos extract contained 56.68% eugenol, with a retention time of about 49.8 min.

Both magnolol and eugenol, the assay standard compounds, inhibited the growth of *C. albicans* (Fig. 3). The growth inhibitory effect was shown in proportion to the concentration of magnolol and *C. albicans* growth was completely suppressed by magnolol in concentrations ≥ 0.09 mM (24 mg/L) (Fig. 3A). This result is consistent with the inhibitory concentration of magnolol on the biofilm formation of *C. albicans* in a previous study (Zhou *et al.*, 2017).

Eugenol did not inhibit the growth of *C. albicans* until 0.76 mM (0.125 g/L), but showed complete inhibition at concentrations above 3.05 mM (0.5 g/L) (Fig. 3B). This result is twice the value of 0.25 g/L in planktonic growth of *C. albicans* (Tampieri *et al.*, 2005) and is consistent with the minimal inhibitory concentration of 0.5 g/L for sessile *C. albicans* in a previous study (He *et al.*, 2007). The main component of Syzygii Flos is eugenol (Pinto *et al.*, 2009) and the antifungal activity of eugenol on fungi other than *C. albicans* have been reported in previous studies (Chami *et al.*, 2004; Cheng *et al.*, 2008; de Oliveira

Pereira *et al.*, 2013). The antifungal activity of magnolol against *C. albicans* was about 34 times stronger than that of eugenol.

3.4. Synergistic inhibition of magnolol and eugenol on growth of *C. albicans*

To test the synergistic growth inhibition of magnolol and eugenol, a checker board experiment was performed at various concentrations of magnolol (0–24 mg/L) and eugenol (0–0.3 g/L). The synergistic concentration range was determined to have more than twice the inhibitory activity of all the results of the individual compounds. The synergistic growth inhibitory concentration range was 8–24 mg/L for magnolol and 0.25–0.3 g/L for eugenol. The concentration ratio of magnolol and eugenol for the synergistic inhibitory activity was 1:25–1:61.

Considering the content of magnolol in Magnoliae Cortex and eugenol in Syzygii Flos extract, the antifungal activity on *C. albicans* by combination of magnolol and eugenol was estimated to contribute 28–48% of the antifungal activity by combination of Magnoliae Cortex extract and Syzygii Flos extract. This estimated value suggests that there are additional compounds with antifungal activity in addition to magnolol and eugenol in the Magnoliae Cortex and Syzygii Flos extract, respectively. In a previous study, it was reported that honokiol and obovatol, in addition to magnolol, were chemical components present in Magnoliae Cortex (Shen *et al.*, 2009). Honokiol had an antifungal activity on *C. albicans* (Bang *et al.*, 2000b). Obovatols inhibited the cell wall synthesis of *S. cerevisiae* (Hwang *et al.*, 2002). In addition to eugenol, Syzygii Flos had phenolic compounds (Shan *et al.*, 2005) and these phenolic compounds, including caffeic acid (Ma and Ma, 2015), ferulic acid (Shi *et al.*, 2016), elagic acid (Li *et al.*, 2015), and salicylic acids (Amborabé *et al.*, 2002), showed antifungal activities.

With the recent increase in reports of azole resistance of *C. albicans* (Whaley *et al.*, 2017), it has become very important to discover and develop new antifungal compounds, as well as reduce the use of antifungal agents by mixing antifungal agents. Moreover, synergistic antifungal activities had been reported in previous studies, such as oil from *Ocimum basilicum* var. Maria Bonita with fluconazole (Cardoso *et al.*, 2016), ethanolic extract of the leaves of *Ocimium gratisimum* L. with ketoconazole and nystatin (Nweze and Eze, 2009), *Melaleuca alternifolia*, *Origanum vulgare*, and *Pelargonium graveolens* essential oils with amphotericin B (Rosato *et al.*, 2008), and thionin-like peptide from *Capsicum annuum* fruits with fluconazole (Taveira *et al.*, 2016). In this study, the synergistic action between the extracts of Magnoliae Cortex and extract of Syzygii Flos was suggested. In this study, we proposed an antifungal treatment that utilizes increased antifungal activity while minimizing the amount of use by combining only plant extracts without using antifungal compounds.

Table 2. The growth inhibition of *C. albicans* by combination of eugenol and magnolol. The synergistic growth inhibition is indicated in bold. Growth inhibition was calculated by comparing the cell density (Abs_{595}) at the indicated eugenol and magnolol concentrations to the cell density without eugenol and magnolol (Abs_{595}). The statistical analysis was performed by one-way ANOVA using Tukey method. Values that differ from the control at a 95% confidence level are marked with a star on top of symbols

		Eugenol (mg/L)			
		0	125	250	300
Magnolol (mg/L)	0	0.0%	0.0%	12.1%	14.2%
	4	2.9%	2.1%	28.5%	31.1%
	8	20.9%	15.0%	**44.7%***	**45.6%***
	10	21.1%	25.6%	**50.9%***	**55.8%***
	12	20.7%	26.9%	**53.8%***	**59.7%***
	16	35.3%	41.6%	**71.4%***	**77.9%***
	24	54.4%	60.4%	100.0%	100.0%

4. CONCLUSION

As agents to inhibit the growth of *C. albicans*, which causes candidiasis, 17 edible plant extracts were identified. Among the four extracts with a high antifungal activity, a combination of Magnoliae Cortex and Syzyii Flos, whose antifungal effect was increased synergistically, is proposed as an agent for effectively eliminating *C. albicans*. The antifungal activity of this combination is explained by magnolol and eugenol partially and the presence of additional antifungal compounds was suggested. The results of this study propose a new safe method of using plant extracts for the treatment of candidiasis.

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