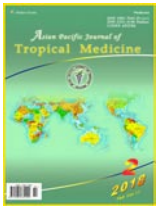

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In vitro fungistatic activity of 36 traditional oriental medicines and their synergistic effect against *Trichophyton rubrume*

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ABSTRACT

Objective: To investigate the fungistatic activity and synergistic effects of natural products and their constituents, including traditional oriental medicines (TOMs). **Methods:** Fungistatic activities of TOMs prepared by hot-water (115 ℃) or ethanol (70%; 40 ℃) extraction were determined by their minimum inhibitory concentration. To assess possible synergistic effects, minimum inhibitory concentrations of various combinations were evaluated. **Results:** By evaluating antifungal susceptibility of *Trichophyton rubrum*, which is a major causative fungus for several types of dermatophytosis, we confirmed that ethanol extracts were more active than hot-water extracts in 25 of the 36 TOMs, suggesting that the constituents with high hydrophobicity tend to contribute significantly to fungistatic activity. We selected four TOMs with high fungistatic activity, including Aucklandiae radix, Gentianae macrophyllae radix, Scutellariae radix, and Galla rhois, and their synergistic effects were investigated through the combination studies between TOMs or TOM-conventional drug terbinafine. In combinations between four TOMs, partial synergistic effects were observed in Aucklandiae radix–Galla rhois and Gentianae macrophyllae radix–Galla rhois combinations, as supported by the lowest fractional inhibitory concentration index value of 0.66 for both combinations. Furthermore, Galla rhois showed the strongest synergistic effect on growth inhibition of *Trichophyton rubrum* with a fractional inhibitory concentration index value of 0.50 in combination with terbinafine. **Conclusions:** Our findings indicate that the combination of TOMs and TOM-terbinafine may be effective on treatment for chronic and recurrent dermatophytosis by improving fungistatic activity and led to decrease systemic toxicity in clinical practice.

1. Introduction

Fungal infections cause serious problems in immunocompromised populations, such as children and the elderly, patients infected with human immunodeficiency virus, those who have undergone transplantation, those undergoing chemotherapy, and those using long-term immunosuppressants. In recent years, the prevalence and mortality of opportunistic infections caused by *Candida albicans*, *Aspergillus* spp., and *Cryptococcus neoformans* in immunocompromised populations has been steadily increasing worldwide[1]. Certain fungal infections caused by the invasion of fungi into the skin are not considered severe; however, these can be chronic conditions. Dermatophytosis is the most common fungal infection caused by the superficial infection of dermatophytes, which subsist upon digested keratin in the skin, nails, and hair. A

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large portion of chronic dermatophyte infections are considered to be caused by *Trichophyton rubrum* (*T. rubrum*), which induces tinea pedis, tinea unguium, tinea cruris, and tinea corporis[2]. Since the 1950s, various types of fungicides have been developed for effective inhibition and treatment of fungal infections via chemical synthesis. Since the development of the polyene amphotericin B (Amp B), which binds to ergosterol in fungal cell membranes to disrupt their integrity, in the 1950s, various classes of antifungal agents have been developed. These include azoles and allylamines, which inhibit ergosterol synthesis, echinocandins that block β-1,3-glucan formation in fungal cell walls, and flucytosine or griseofulvin to inhibit DNA synthesis or mitosis. However, despite these efforts, few effective antifungal agents are currently available as many have shown limitations because of their resistance and toxicity to the human body. Although antifungal resistance is generally less of a global issue than antibacterial resistance, resistance to antifungal agents, such as fluconazole and flucytosine by Candida, first reported in patients infected with human immunodeficiency virus in the 1990s, continues to be a concern[3]. Although, azole antifungals, in particular, have a mechanism to inhibit the synthesis of ergosterol in the fungal cell membrane, the resistance of *Candida albicans* to azole antifungals by the efflux of antifungals and mutation of ERG11, causing a decrease in the affinity to the azoles antifungals was reported[4]. In addition, the administration of Amp B is limited to clinical treatment because of severe infusion-related toxicity resulting from the production of pro-inflammatory cytokines and nephrotoxicity[5], but several lipid-based formulations of Amp B have been developed to decrease this toxicity by limiting the exposure of human cells to Amp B[6,7]. Combination therapy of antifungal agents with different mechanisms has also been used to counteract issues of growing resistance and toxicity. Flucytosine is often used in combination with other antifungal agents primarily due to its narrow antifungal spectrum and emerging resistance.

Despite efforts to develop chemical antifungal agents, natural products, including various traditional oriental medicines (TOMs), and their constituents have attracted public attention as an alternative therapy to complement the treatment of diseases associated with fungal infections due to the emerging multidrug resistance of synthetic fungicides, which reduce the resistance and toxicity[8]. In this study, we evaluate *in vitro* fungistatic activities 36 TOMs against *T. rubrum*, which is a causative fungus for various types of tineas. In addition, because there have been few TOM compatibility studies on fungal infections, we evaluated the synergistic effects of TOM combinations as well as the interactions of TOM candidates with the conventional antifungal drugs terbinafine, which has been widely prescribed to treat various fungal diseases due to its relatively low toxicity. We hypothesized that dermatophytic growth may be more effectively suppressed using TOM combinations or TOMs in combinations with conventional antifungals and this may result in alleviation of toxicity by reducing the overall dose required.

2. Materials and methods

2.1. Chemicals and reagents

Sabouraud dextrose broth for fungal culture was purchased from BD DifcoTM (Sparks, MD, USA). Sodium chloride, terbinafine hydrochloride, gallic acid, methyl gallate, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant materials and preparation of plant extracts

A total of 36 TOMs (Table 1) were purchased from Yeongcheon Oriental Herbal Market (Yeongcheon, Republic of Korea) and identified by Professor Ki Hwan Bae, Chungnam National University, Republic of Korea. TOMs were deposited in the herb bank of the Korean Institute of Oriental Medicine. The extracts were prepared by extracting 50 g of TOMs in 1 000 mL of distilled water at 115 ℃ for 3 h (Gyeongseo Extractor Cosmos-600, Gyeongseo, Republic of Korea) or in 70% ethanol at 40 ℃ for 24 h. After filtering through testing sieves (150 μm; Retsch, Germany), the extracts were freeze-dried and placed in a desiccator at 4 ℃. The dried extract powders were stored at −20 ℃ until use. Samples for antifungal assays were prepared by dissolving the extract powders in 50% DMSO.

2.3. Fungal strain and inoculum preparation

T. rubrum ATCC 62345 was grown in Sabouraud dextrose medium for 7 d at 25 ℃, and cells were resuspended in 0.85% sterile saline. After filtering the cell resuspension through Whatman filter paper no. 40 (pore size: 8 μm) to collect microconidia with removal of hyphal fragments, the inoculum was diluted to (1×10^4)–(5×10^4) conidia/mL in Sabouraud dextrose broth for antifungal assay as suggested in previous reports[9,10].

2.4. Antifungal susceptibility testing

Fungistatic activity was evaluated by minimum inhibitory concentration (MIC) values, which were determined by a 2-fold dilution method using Sabouraud dextrose broth. Fungistatic activities of TOMs were measured in the range of 0–8 mg/mL, and terbinafine, tested as a positive control, ranged from 0–32 μg/mL. All tested samples contained a final concentration of 4% DMSO in the Sabouraud dextrose broth. MIC was determined as the lowest

Table 1
Traditional oriental medicines and parts used to determine fungistatic activity.

Herbal medicine	Plant species	Family	Part used
Glycyrrhizae radix praeparata	*Glycyrrhiza uralensis* Fisch	Leguminosa/Fabales	Root
Zingiberis rhizoma	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome
Cassiae semen	*Cassia tora* L.	Fabaceae	Seed
	Senna obtusifolia (L.) H.S.Irwin & Barneby		
Alpiniae officinari rhizoma	*Alpinia officinarum* Hance	Zingiberaceae	Rhizome
Pogostemon herba	*Pogostemon cablin* (Blanco) Benth	Lamiaceae	Aerial part
Cirsii herba	*Cirsium japonicum* var. *maackii* (Maxim.) Matsum.	Asteraceae	Whole plant
Rhei rhizoma	*Rheum palmatum* L.		
	Rheum tanguticum Maxim. ex Balf.		
	Rheum officinale Baill.	Polygonaceae	Rhizome
Aucklandiae radix	*Saussurea costus* (Falc.) Lipsch.	Asteraceae	Root
Adenophorae radix	*Adenophora triphylla* var. *japonica* (Regel.) Hara	Campanulaceae	Root
Sparganii rhizoma	*Sparganium stoloniferum* (Buch.-Ham. ex Graebn.) Buch.-Ham. ex Juz.	Typhaceae	Rhizome
Myristicae semen	*Myristica fragrans* Houtt.	Myristicaceae	Seed
Syzygii flos	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Myrtaceae	Flower bud
Gentianae macrophyllae radix	*Gentiana macrophylla* Pall.		
	Gentiana straminea Maxim.		
	Gentiana crassicaulis Duthie ex Burkill		
	Gentiana dahurica Fisch.	Gentianaceae	Root
Alpiniae katsumadaii semen	*Alpinia hainanensis* K.Schum..	Zingiberaceae	Seed
Ecliptae herba	*Eclipta prostrata* (L.) L.	Asteraceae	Whole plant
Scutellariae radix	*Scutellaria baicalensis* Georgi	Lamiaceae	Root
Phellodendri cortex	*Phellodendron amurense* Rupr.	Rutaceae	Bark
Mori cortex radicis	*Morus alba* L.	Moraceae	Root
Thujae orientalis folium	*Platycladus orientalis* (L.) Franco	Cupressaceae	Leaf, stem
Lacca rhois exsiccata	*Rhus verniciflua* Stokes	Anacardiaceae	Dried sap
Sophorae radix	*Sophora flavescens* Aiton	Fabaceae	Root
Echinopsis radix	*Echinops setifer* Iljin	Asteraceae	Root
Cantharides	*Mylabris cichorii* L.		
	Mylabris phalerata Pall.		
	Epicauta gorhami Marseul	Meloidae	Insect body
Psoraleae semen	*Cullen corylifolium* (L.) Medik.	Fabaceae	Seed
Belamcandae rhizoma	*Belamcanda chinensis* (L.) DC.	Iridaceae	Rhizome
Sophorae tonkinensis radix et rhizoma	*Sophora tonkinensis* Gapnep.	Fabaceae	Root, rhizome
Dendrobii herba	*Dendrobium nobile* Lindl.	Orchidaceae	Stem
Hirudo	*Hirudo niponica* Whitman	Hirudinidae	Insect body
	Whitmania pigra Whitman		
Curcumae rhizoma	*Curcuma phaeocaulis* Valeton		
	Curcuma kwangsiensis S.G.Lee & C.F.Liang		
	Curcuma aromatica Salisb.	Zingiberaceae	Rhizome
Scolopendra	*Scolopendra subspinipes* multilans Linne Koch	Scolopendridae	Insect body
Galla rhois	*Rhus javanica* L.	Anacardiaceae	Gall
Genkwa flos	*Daphne genkwa* Siebold & Zucc.	Thymelaeaceae	Flower bud
Helenii radix	*Inula helenium* L.	Asteraceae	Root
Piperis longi fructus	*Piper longum* L.	Piperaceae	Fruit
Cubebae fructus	*Piper cubeba* L.	Piperaceae	Fruit
	Litsea cubeba (Lour.) Pers.		
Stichopus	*Stichopus japonicus* Selenka	Stichopodidae	Body

concentration that showed no visible fungal growth after incubation at 25 ℃ for 7 d. The experiments were performed in triplicate.

2.5. Synergy study of TOM in combination with terbinafine

Based on the results of antifungal assays, four TOMs were selected to determine if their effects were synergistic. The fractional inhibitory concentration index (FICI) of all combinations at 7 mg/mL was determined through serial dilution. In addition, 0.1×MIC TOMs in combination with terbinafine were also investigated.

The FICI was calculated using the formula:

$$\sum FIC = (C_A/MIC_A) + (C_B/MIC_B).$$

Where, MIC_A and MIC_B are the MIC values of antifungal agents A and B alone, respectively, and C_A and C_B are the concentrations of antifungal agents in combination, respectively. The FICI was defined as $\sum$FIC $\leq$ 0.50, synergistic; 0.50 < $\sum$FIC $\leq$ 0.75, partially synergistic; 0.75 < $\sum$FIC $\leq$ 1.00, additive; 1.00 < $\sum$FIC $\leq$ 4.00, indifferent; and $\sum$FIC > 4.00, antagonistic[11,12].

3. Results

3.1. In vitro antifungal assays of TOMs and comparative analysis based on the extraction solvent

To investigate the ability of TOMs to suppress fungal propagation, fungistatic activities of 36 TOMs were assessed by measuring their MICs (Table 2). MIC of the extracts ranged from 1 to 8 mg/mL. When TOM showed no fungistatic activity at 8 mg/mL, it was indicated as > 8 mg/mL. Although all 36 TOMs exhibited fungistatic activities against *T. rubrum*, Table 2 shows the differences in fungistatic efficacy based on extraction solvents, which have different physicochemical properties. Ethanol extracts were more active in inhibiting *T. rubrum* growth than hot-water extracts in 25 of the 36 TOMs, and there were no TOMs showing better fungistatic activity in hot-water extracts. This property was particularly apparent in six ethanol extracts, namely, Aucklandiae radix (AR), Gentianae macrophyllae radix (GMR), Scutellariae radix (SR), Echinopsis radix, Belamcandae rhizoma, and Cassiae semen, in which the MIC values of the ethanol extracts were >2-fold lower than those of the corresponding hot-water extracts. Among them, the ethanol extracts of AR, GMR, and SR showed relatively low MIC values, indicating that they are effective in inhibiting *T. rubrum* growth. However, TOMs with MIC ratio of hot-water extract to ethanol extract (MIC_W/MIC_E) ≤ 2 other than Cantharides and Galla rhois (GR) showed weak fungistatic activities with MIC values more than 4 mg/mL in both hot-water and ethanol extracts.

The ethanol extracts of SR and Cantharides inhibited *T. rubrum* growth most effectively with an MIC value of 1 mg/mL, and the hot-water extract of Cantharides also exhibited inhibitory effect on *T. rubrum* growth with an MIC value of 2 mg/mL. In addition, the ethanol extracts of AR, GMR, and both extracts of GR also effectively suppressed the proliferation of *T. rubrum* with MIC values of 2 mg/mL.

3.2. Synergistic effects of selected TOMs and combinations with terbinafine

Four selected ethanol extracts of TOMs, including AR, GMR, SR, and GR exhibiting high fungistatic effects, were further investigated for a synergistic effect against *T. rubrum* (Table 3). FICI values for the various combinations ranged from 0.66 to 1.50, and partially synergistic (18.2%), additive (45.4%), and indifferent (36.4%) effects were observed. In particular, partial synergistic effects were observed in combinations of two TOMs: AR–GR and GMR–GR. Even though the combinations of three TOMs that included AR–GR or GMR–GR showed no synergistic effect, their FICI values were 0.79 and 0.83, close to the FICI value of 0.75 corresponding to partial synergy. Nevertheless, the combinations of AR–GMR showed the highest FICI value of 1.50, and FICI values of combinations between SR and other three TOMs (AR, GMR and GR) were 1.00, 1.10 and 1.20, respectively, indicating that the TOMs of each combinations interact indifferently for the inhibition of *T. rubrum* growth.

We also investigated the interaction of four TOMs with terbinafine, which inhibits ergosterol synthesis by suppressing squalene epoxidase activity for the synthesis of fungal cell membrane (Table 4). The results in Table 4 indicated that GR has a synergistic effect with terbinafine. The growth of *T. rubrum* was inhibited using only 40% MIC of terbinafine by the combination with 0.1×MIC of GR. However, terbinafine interacted with each AR, GMR and SR indifferently for fungistatic actions; the FICI values in these cases indicated more than 1.50.

Table 2

Fungistatic activities of traditional oriental medicines against *T. rubrum* determined by minimum inhibitory concentration (MIC).

MIC ratio	Herbal medicine	Antifungal activity on *T. rubrum* (MIC, mg/mL)	
		Hot-water extract	70% Ethanol extract
$MIC_W/MIC_E > 2$	Aucklandiae radix	>8	2
	Gentianae macrophyllae radix	8	2
	Scutellariae radix	8	1
	Echinopsis radix	>8	4
	Belamcandae rhizoma	>8	4
	Cassiae semen	>8	4
$MIC_W/MIC_E = 2$	Adenophorae radix	>8	8
	Helenii radix	8	4
	Sophorae tonkinensis radix et rhizoma	>8	8
	Glycyrrhizae radix praeparata	8	4
	Alpiniae officinari rhizoma	8	4
	Rhei rhizoma	8	4
	Sparganii rhizoma	>8	8
	Curcumae rhizoma	>8	8
	Dendrobii herba	>8	8
	Thujae orientalis folium	>8	8
	Cirsii herba	>8	8
	Ecliptae herba	>8	8
	Myristicae semen	8	4
	Phellodendri cortex	8	4
	Genkwa flos	>8	8
	Cubebae fructus	>8	8
	Cantharides	2	1
	Hirudo	>8	8
	Lacca rhois exsiccata	8	4
$MIC_W/MIC_E = 1$	Mori cortex radicis	8	8
	Sophorae radix	8	8
	Zingiberis rhizoma	8	8
	Alpiniae katsumadaii semen	4	4
	Psoraleae semen	8	8
	Syzygii flos	4	4
	Piperis longi fructus	4	4
	Scolopendra	8	8
	Galla rhois	2	2
	Pogostemon herba	8	8
	Stichopus	4	4

MIC_W/MIC_E: MIC ratio of hot-water extract to ethanol extract. Terbinafine was used as a positive control against *T. rubrum* and its MIC was 2 μg/mL.

Table 3
Synergistic effect of traditional oriental medicine (TOM) combinations for fungistatic activity.

No. of TOMs	Concentration in TOM blends (mg/mL)				MIC of combination (mg/mL)	FICI[a]	Interaction
	Aucklandiae radix (AR)	Gentianae macrophyllae radix (GMR)	Scutellariae radix (SR)	Galla rhois (GR)			
1	2.00	2.00	1.00	2.00	1.65	0.94	Additive
2	2.80	2.80	1.40	-	2.00	1.20	Indifferent
3	2.33	2.33	-	2.33	1.65	0.83	Additive
4	2.80	-	2.80	2.80	1.32	0.79	Additive
5	-	2.80	1.40	2.80	1.32	0.79	Additive
6	3.50	3.50	-	-	3.00	1.50	Indifferent
7	4.67	-	2.33	-	1.50	1.00	Additive
8	3.50	-	-	3.50	1.32	0.66	Partially synergistic
9	-	4.67	2.33	-	1.65	1.10	Indifferent
10	-	3.50	-	3.50	1.32	0.66	Partially synergistic
11	-	-	2.33	4.67	1.80	1.20	Indifferent

[a]FICI: fractional inhibitory concentration index.

Table 4
Synergistic effect of traditional oriental medicines (TOMs) in combination with terbinafine for fungistatic activity.

Combinations	FICI[a]	Interaction
0.2 mg/mL Aucklandiae radix (AR) + 3.8 μg/mL Terbinafine	2.00	Indifferent
0.2 mg/mL Gentianae macrophyllae radix (GMR) + 2.8 μg/mL Terbinafine	1.50	Indifferent
0.1 mg/mL Scutellariae radix (SR) + 2.8 μg/mL Terbinafine	1.50	Indifferent
0.2 mg/mL Galla rhois (GR) + 0.8 μg/mL Terbinafine	0.50	Synergistic

All four TOMs were at the 0.1×MIC (mg/mL). [a]FICI: fractional inhibitory concentration index.

4. Discussion

Previous studies have investigated fungistatic effects of various TOMs derived from herbs and animals to discover novel antifungal compounds for use against various fungi such as dermatophytes. The fungistatic activity of antifungal substances including TOMs and conventional drugs varies depending on the type of fungi, even in the same species. Therefore, in this study, we focused on *T. rubrum*, which is a major causative fungus for various types of tinea infection, to investigate the fungistatic activity of 36 TOMs. Based on the result of susceptibility test, we chose four TOMs with high fungistatic activities and examined their synergistic effects of TOM combinations as well as the interactions of TOM candidates with the conventional antifungal drug terbinafine.

In general, TOMs contain a variety of bioactive constituents, such as plant-derived secondary metabolites, which have several pharmacological effects on the human body, including antimicrobial effects. The result of fungistatic assay indicated that ethanol extracts inhibited *T. rubrum* growth more effectively than than hot-water extracts in 25 of the 36 TOMs; in particular, AR, GMR, SR, Echinopsis radix, Belamcandae rhizoma, and Cassiae semen. Many phenolic compounds in TOMs containing single or multiple ring structures are known to have fungistatic effects. Ring structures tend to make the phenolic compounds more hydrophobic and they dissolve better in organic solvents than hydrophilic solutions[13]. Based on the fungistatic activity assay, we selected top four TOMs, including AR, GMR, SR, and GR, with the lowest MIC values to analyze the synergistic effect of TOM blends and TOM-terbinafine combination. Despite the effective inhibition of *T. rubrum* growth in both hot-water and ethanol extracts of Cantharides, it was excluded from TOM candidate for the combination studies, because its use in various tineas including athlete's foot may be contraindicated due to induction of serious blisters and its inability to be applied to mucous membranes. For further analysis of fungistatic activities and synergistic effects of four TOMs, we scrutinized the previous reports on phytochemical findings of natural product based on the constituents in TOMs.

SR, an extract from the root of *Scutellaria baicalensis*, contains flavonoids, including baicalin, baicalein, wogonoside, and wogonin, that have demonstrated various pharmacological effects such as fungistatic activity[14-16]. The MIC value of ethanol extract, which was 8-fold lower than that of hot-water extract, indicates that SR contains some ethanol soluble constituents with high fungistatic acitivity on *T. rubrum*, which is presumably due to bacalein, being soluble in organic solvents but low in aqueous solution solubility and known to have antimicrobial property[17,18]. Baicalein and SR extract exhibited the suppression of fungal growth by inducing apoptosis and inhibiting (1,3)- β -D-glucan synthesis, leading to the destruction of fungal cell walls[19,20].

Major compounds of AR and GMR are terpenoids, which are believed to disrupt fungal cell membranes and cause mitochondrial dysfunction[21]. AR, a root of *Saussurea costus* (Falc.) Lipsch.,

contains sesquiterpene lactones, including costunolide, dehydrocostus lactone, and alantolactone. The polarity of sesquiterpene lactones appears to be an important factor in their antifungal activity; sesquiterpene lactones with low polarity are more effective in suppressing fungal growth than those with more polarity[22]. In this aspect, higher fungistatic activity of AR in ethanol extract rather than hot-water extract may result from higher solubility of sesquiterpene lactones in organic solvents, such as ethanol, due to the low polarity of sesquiterpene lactones for antifungal activity.

GMR, a dried root of *Gentiana macrophylla* Pall., *Gentiana straminea* Maxim., *Gentiana crassicaulis* Duthie ex Burkill, and *Gentiana dahurica* Fisch., contains various secoiridoid monoterpene derivatives as its active constituents, which inhibit ergosterol synthesis in fungi[21]. The main secoiridoid in GMR is gentiopicroside. Although limited reports exist on gentiopicroside, other secoiridoids in GMR have been reported to inhibit fungal growth[23]. Based on the results, we speculate that GMR contains the constituents with higher extraction efficiency in ethanol than in hot water, including secoiridoids, which demonstrated fungistatic effects.

GR is a gall formed by a leaf defense mechanism of *Rhus javanica* L. against wounds caused by the parasitic aphid *Schlechtendalia chinensis* and contains self-defensive compounds, primarily tannins, against external invasion. Although there are several previous reports that tannin-derived components in GR, including methyl gallate and gallic acid, promote growth inhibition of harmful intestinal bacteria[11,24] and phytopathogenic fungi by affecting a cAMP-related signaling pathway[25], both compounds did not exhibit any growth inhibitory effect of dermatophyte *T. rubrum* up to 2 mg/mL (data not shown). Our results that both hot-water and ethanol extracts of GR restricted fungal growth at 2 mg/mL, indicating that the constituents in both extracts, other than methyl gallate and gallic acid, are strongly involved in fungistatic action against *T. rubrum*.

Multi-herbal therapies include herbal blends of various ingredients that have different mechanism of actions. These blends can display synergistic or complementary pharmacological effects, thereby achieving a dose reduction in the overall combination or reducing the toxicity of a single herb. In this study, four selected TOMs with high fungistatic activity were further investigated for a synergistic effect against *T. rubrum*. The result showed that partial synergistic effects were observed with AR–GR and GMR–GR combinations, indicating that the constituents in AR and GMR, including terpenoids, and GR, containing tannin/tannin-derived components, may act on *T. rubrum* with different mechanisms leading to synergistic effects. On the other hand, the combinations of AR and GMR showed the highest FICI value of 1.50, suggesting that sesquiterpene lactones and secoiridoids act with different antifungal mechanisms but do not result in a synergistic effect. In addition, the combinations between SR and other three TOMs with FICI value more than 1.00 indicated that fungistatic mechanism of SR does not cause any synergistic effect with the action of other three TOMs.

We also investigated the interaction of four TOMs with conventional drug terbinafine which blocks the synthesis of fungal cell membrane by suppressing biosynthesis of ergosterol, and synergistic effect was observed in the combinatino of GR and terbinafine. This effect reduced the concentration of terbinafine needed in combinations with 0.1×MIC of GR compared with that required for treatment with terbinafine alone. Since terbinafine acts as an fungistatic mechanism to inhibit the synthetic pathway of ergosterol, a component of the fungal cell wall, the synergistic effect of GR and terbinafine seems to be due to the components in the GR with different mechanisms not related to ergosterol synthesis, excluding gallic acid and methyl gallate that exhibited no fungistatic activity against *T. rubrum*. Further study on fungistatic mechanism and synergistic interactions of TOMs, in particular GR, based on their constituents is warranted. Collectively, our findings indicate that multi-TOM therapy and TOMs in combination with conventional drugs may present an effective clinical treatment for superficial fungal infections such as tineas.

In summary, the present study focused on effective treatment of chronic and recurrent dermatophytosis using TOMs with high fungistatic activity. Our results demonstrated that majority of TOM ethanol extracts had higher fungistatic activities than the corresponding hot-water extracts, suggesting that fungistatic compounds in TOMs have hydrophobic properties. AR or GMR in combination with GR exhibited improved fungistatic activities indicative of a synergistic effect. Synergistic effects of GR was also observed in combination with terbinafine, suggesting that conventional drugs could be given at a reduced dose when administered concurrently with TOMs. Further investigation on changes in the fungal phenotype and metabolism and how these may impact clinical practice as well as the *in vivo* mechanisms underlying synergistic effects is warranted.

Conflicts of interest statement

The authors declare that they have no conflict of interests.

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