

Antifungal Activity of Red-stemmed *Ipomoea aquatica* Forsk. Extracts against Dermatophytes and *Malassezia* sp.

นิพนธ์ต้นฉบับ

Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อประเมินฤทธิ์ต้านเชื้อราในหลอดทดลอง (*in vitro*) ของสารสกัดจากยอดและดอกทั้งแบบสดและแห้งของผักนึ่งไทยพันธุ์ก้านแดงในการยับยั้งเชื้อราโรคราก 3 สายพันธุ์ (*Trichophyton rubrum*, *Epidermophyton floccosum* และ *Microsporum gypseum*) และโรคลิ้น (*Malassezia*) 2 สายพันธุ์ (*M. furfur* และ *M. globosa*) **วิธีการศึกษา:** ใช้วิธี disc diffusion ประเมินฤทธิ์ของสารสกัดดังกล่าวในการยับยั้งเชื้อราโรครากและลิ้น และหาค่าความเข้มข้นต่ำสุดของสารสกัดที่สามารถยับยั้งเชื้อทั้ง 5 สายพันธุ์ (MIC) ด้วยวิธี broth micro-dilution **ผลการศึกษา:** สารสกัดจากยอดแห้งและดอกแห้งสามารถยับยั้งเชื้อ *T. rubrum*, *E. floccosum*, *M. gypseum* และ *M. globosa* ได้ สารสกัดจากยอดสดสามารถยับยั้งเชื้อ *E. floccosum* และ *M. globosa* ได้ ส่วนสารสกัดจากดอกสดสามารถยับยั้งได้เพียงเชื้อ *E. floccosum* เท่านั้น อย่างไรก็ตามสารสกัดทั้ง 4 ชนิดไม่สามารถยับยั้งเชื้อ *M. furfur* และเมื่อทดสอบด้วยวิธี broth micro-dilution พบว่าสารสกัดจากยอดผักนึ่งไทยพันธุ์ก้านแดงทั้ง 4 ชนิดยับยั้งเชื้อ *E. floccosum* ได้ดีที่สุดที่ความเข้มข้นต่ำสุดเท่ากับ 25 มก./มล. **สรุป:** สารสกัดจากยอดแห้งและดอกแห้งสามารถยับยั้งเชื้อราโรครากและลิ้นแบบ *in vitro* ได้ซึ่งมีผลสอดคล้องกับการใช้พืชนี้ตามภูมิปัญญาพื้นบ้านของไทยในการรักษาโรครากและลิ้น

คำสำคัญ: ฤทธิ์ต้านเชื้อรา, ผักนึ่งไทยพันธุ์ก้านแดง, เชื้อรา, ราก, ลิ้น

Abstract

Objective: To evaluate the *in vitro* antifungal activity of the extracts of fresh and dry apical buds and flowers of red-stemmed *Ipomoea aquatica* Forsk. on the inhibition of three dermatophytes (*Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum*) and two *Malassezia* species (*M. furfur* and *M. globosa*). **Method:** The disc diffusion was performed to determine the antifungal activity of the extracts, and the minimal inhibitory concentration (MIC) was measured using a broth micro-dilution method. **Results:** The extracts of dry apical buds and flowers of red-stemmed type of *Ipomoea aquatica* Forsk. inhibited *T. rubrum*, *E. floccosum*, *M. gypseum* and *M. globosa*. Fresh apical bud extracts inhibited *E. floccosum* and *M. globosa*, whereas those of fresh flowers were for only *E. floccosum*. However, none of four types of extracts exhibited antifungal activity against *M. furfur*. When tested with the broth micro-dilution technique, all four extract inhibited *E. floccosum* the most at the MIC of 25 mg/ml. **Conclusion:** The extracts of dry apical buds and flowers of red-stemmed *Ipomoea aquatica* Forsk. could inhibit dermatophytes and *Malassezia* sp. *in vitro*. The result was consistent with the use of Thai folkore remedy against skin infections of dermatophytes and *Malassezia* sp.

Keywords: Antifungal activity, red-stemmed *Ipomoea aquatica* Forsk., fungus, dermatophytes, *Malassezia* sp.

Introduction

Skin fungal infections caused by dermatophytes, such as ringworm and tinea versicolor, have been found about 20 – 25% of the population worldwide. Skin dermatophyte infections are prevalent in tropical regions of the world because of the predominantly high moisture and temperature. The diseases are mainly prevalent among people who are poor and living in large and dense communities that allow for contacting a skin fungal infection. Pathologically, ringworm and tinea versicolor are the infestation of dermatophytes in the keratinized tissues including the skin, nail and hair follicles; hence the alternative name “cutaneous mycoses.” Based on the World Health organization (WHO) epidemiologic study, the most common dermatophyte of ringworm is the genus of *Trichophyton*, followed by *Epidermophyton* and *Microsporum*,

respectively. The most common fungi causing tinea versicolor is the genus of *Malassezia*, a tropical normal flora which becomes pathogenic with immunocompromised host, extreme humidity, or the host’s malnutrition. Once pathogenic, *Malassezia* spp. morphologically changes. As the result, skin pigmentation change is seen as the lesion. The most common pathogenic species of *Malassezia* is *M. globosa*, followed by *M. sympodialis* and *M. furfur*.^{1,2}

In Thai traditional medicine, various local wisdom-based herbal remedies have been used to treat fungal skin infections. These included galanga rhizome with rice whisky, fresh holy basil leaves, garlic cloves, etc. These individual herbs have been found to have an acceptable antifungal activity. However, with an abundant number of herbs in

Thailand, herbal antifungal remedies are worth further investigated. Among various herbs unexplored for this activity, *Ipomoea aquatica* Forsk. (water morning glory, water spinach, or river spinach) could reasonably be subject to an exploration of *in vitro* antifungal activity screening.

Water morning glory (*Ipomoea aquatica* Forsk.), a semiaquatic annual climbing vine plant abundantly found across Thailand and tropical and subtropical regions worldwide, is in the family of Convolvulaceae. Two strains of *Ipomoea aquatica* Forsk. namely green/white-stemmed with white flowers and red-stemmed with pink/purple flowers are found in Thailand.³⁻⁶ Red-stemmed water morning glory has been known as a folk-lore remedy for fungal skin infections in Thailand. Six to ten flower buds of red-stemmed water morning glory are cleaned with water, and then crushed by mortar and pestle. The crushed herb is used to mask the infected area for 2 to 3 hours before washing off. To cure fungal skin infection effectively, masking the infected area with the ground herb 3 to 4 daily is recommended.⁷⁻⁹ In addition, a study by Ghani (1989) revealed that the apical buds of *Ipomoea aquatica* Forsk. has an antifungal activity against *tinea versicolor*.¹⁰ There has been a lack of studies on *in vitro* antifungal activity of *Ipomoea aquatica* Forsk. especially the red-stemmed strain. Most previous studies did not specify the strain(s) of *Ipomoea aquatica* Forsk. tested. With its abundance all across Thailand, *in vitro* study on antifungal activity of the red-stemmed water morning glory is needed. This study aimed to examine *in vitro* antifungal activity of red-stemmed strain of *Ipomoea aquatica* Forsk. against dermatophytes and *Malassezia*. The information obtained could help verify the remedial benefit of Thai folk-lore medicine. Such traditional medicine usage could then be confidently encouraged.

Methods

Chemicals and devices

Absolute ethanol was from Fisher BioReagent™, and Sabouraud dextrose medium with agar (SDA) and without agar (i.e., broth) (SDB) was from BD Difco™. A cork borer with a diameter of 5 mm was used.

Strains of fungi and culture

Three strains of dermatophytes causing ringworm including *T. rubrum*, *M. gypseum*, and *E. floccosum* and two species of *Malassezia* yeasts causing *tinea versicolor* namely

M. furfur and *M. globosa* were tested for *in vitro* antifungal activity. *Candida albicans* ATCC90028 was used as a control. All micro-organisms were supported by the Mycology Laboratory, Department of Microbiology, Faculty of Medicine, Chiangmai University, Chiangmai, Thailand. All fungi specimens were kept at 4 °C. Before the experiment, these fungi strains were subcultured on SDA and incubated at 25 °C for 48 – 72 hours.

Plant collection

In this study, red-stemmed strain of *Ipomoea aquatica* Forsk. was collected from Sainoi district of Nonthaburi province, Thailand. The collected plant was identified. Based on plant taxonomy and morphology, red-stemmed strain of *Ipomoea aquatica* Forsk. had a reddish green or reddish purple stems with dark-green spear-shaped obtuse-based simple leaves. The leaves were 3 – 5 cm wide with leaves close to the apical buds were shorter (2 – 4 cm long) while those close to the root were longer (5 – 10 cm long). Flowers of red-stemmed *Ipomoea aquatica* Forsk. are inflorescent with white sepals and purple-white petals.^{4,11} Apical buds and flowers of red-stemmed *Ipomoea aquatica* Forsk. were tested for *in vitro* antifungal activity.

Plant extraction

The extract of dry plants

Apical buds and flowers of *Ipomoea aquatica* Forsk. were separately washed with water. Apical buds were then incubated at 50 °C while flowers at 40 °C till dry. Dry samples were ground by blender and macerated with 95% ethanol based on the methods modified from the work of Khatun et al. (2012)¹² with a ratio of dry sample to solvent of 1:5 for 3 days at room temperature. The extract was filtered and the solvent was evaporated by rotary evaporator at 40 °C.¹² Crude extract of dry flower was viscous with greenish red color and a yield of crude extract of 10.35% w/w. For the dry apical bud, its crude extract was also viscous with a dark-green color and a yield of 4.55% w/w. Crude extracts of both parts of the plant were kept in amber colored glass container at 40 °C before the antifungal assay.

The extract of fresh plants

Apical buds and flowers of *Ipomoea aquatica* Forsk. were separately washed with water. Samples were crushed using mortar and pestle and macerated with 95% ethanol based on the method used for dry plant samples.¹² Crude extract of

fres flower was viscous with greenish brownish red color and a yield of crude extract of 1.20% w/w. For the fresh apical bud, its crude extract was also viscous with a dark-green color and a yield of 6.68% w/w. Crude extracts of both parts of the plant were kept in amber colored glass container at 40 °C before the antifungal assay.

Disc diffusion assay for antifungal activity

The test for antifungal activity of the plant samples was modified from that of Huang, Xie and Gong (2000).¹³ Petriplates containing SDA with a colony of *T. rubrum*, *M. gypseum*, or *E. floccosum* of a size of about 2 cm were bored with a cork borer of a 0.5 cm distance from the colony edge. 40 microliters of the extract with a concentration of 100 mg per mL was pipetted and transferred into the bored SDA medium. Each plate was kept at 25 °C for 7 days. The inhibition zone of was observed and measured in millimeter of the zone diameter.¹³

For *M. furfur* and *M. globosa*, suspension of each of the two *Malassezia* species was prepared in the SDB broth and adjusted to a McFarland turbidity standard number of 0.5. The suspension of the fungus was gently placed on the surface of SDA plate with three planes using sterile cotton buds. The SDA medium was bored with a cork borer. 40 microliters of the extract with a concentration of 100 mg per mL was pipetted and transferred into the bored SDA medium. Each plate was incubated at 37 °C for 48 – 72 hours. The inhibition zone of was observed and measured in millimeter of the zone diameter.¹³

In all experiments, miconazole with a concentration of 10 mcg per mL was used as a positive control. All tests were carried out in triplicate.

Determination of minimal inhibitory concentration (MIC)

Extracts with apparent inhibition zone from the disc assay were further tested for MIC by broth microdilution modified from the method of Baron and Finegold (1990).¹⁴ With a two-fold serial dilution by SDB broth in the 96-well microplate, the extract concentrations were ranged from 100 to 0.781 mg per mL. The final volume of in each well was 100 microliters. The inocula of *T. rubrum*, *M. gypseum*, and *E. floccosum* was adjusted to the concentration of 1×10^5 CFU/mL. A volume of 50 mL of inoculum suspensions was transferred to each well.

The microplates were then incubated at room temperature and the MIC for dermatophytes was recorded at days 5, 7 and 10.

For *Malassezia* fungi, procedures previously described were repeated. The microplates were then incubated at 37 °C for 48 to 72 hours and the MIC for each of these *Malassezia* species was recorded.¹⁴

Positive control was done with miconazole with a concentration of 30 mcg per mL or higher against *C. albicans* ATCC90028. All tests were carried out in triplicate. The MIC for the test fungi was recorded as the lowest concentration of extracts that inhibited the growth of the fungi as compared to that of control.

Results

Antifungal activity based on disc diffusion method

It was found that the extracts of dry apical buds and dry flowers of red-stemmed *Ipomoea aquatica* Forsk. had the highest antifungal activity by disc diffusion test (Table 1). The fresh flower extract could inhibit only one strain of dermatophytes (*E. floccosum*). In addition, inhibition zone in *M. furfur* samples was not found with any plant extracts.

Table 1 Antifungal activity of extracts of red-stemmed *Ipomoea aquatica* Forsk. by disc diffusion method presented as inhibition zone.

Extract (100 mg/ml)	Diameter of inhibition zone (mm) by fungi				
	<i>T. rubrum</i>	<i>E. floccosum</i>	<i>M. gypseum</i>	<i>M. furfur</i>	<i>M. globosa</i>
Fresh apical bud	N	6.0 ± 0.0	N	N	10.0 ± 0.0
Dry apical bud	9.3 ± 0.3	6.0 ± 0.0	8.7 ± 0.1	N	9.3 ± 0.3
Fresh flower	N	6.0 ± 0.0	N	N	N
Dry flower	10.7 ± 0.1	6.0 ± 0.0	8.0 ± 0.8	N	10.0 ± 0.0
Miconazole (10 µg/mL)	20.2 ± 2.3	33.2 ± 1.5	23.5 ± 2.6	16.8 ± 1.3	14.3 ± 1.8
95% ethanol	N	N	N	N	N

Note: N = no inhibition zone.

Minimal inhibitory concentration (MIC)

All species of test fungi, except *M. furfur*, were tested for MIC by micro-dilution method. It was found that dry apical bud extracts had the highest activity against all 4 fungi (Table 2). The highest inhibitory activity of dry apical bud extract's was found in *T. rubrum* and *E. floccosum* (MIC = 25 mg/ml for both strains).

Table 2 Minimal inhibitory concentration (MIC) of extracts of red-stemmed *Ipomoea aquatica* Forsk. against fungi by broth micro-dilution method.

Extract	MIC (mg/mL) by fungi			
	<i>T. rubrum</i>	<i>E. floccosum</i>	<i>M. gypseum</i>	<i>M. globosa</i>
Fresh apical bud	-	25	-	> 50
Dry apical bud	25	25	50	> 50
Fresh flower	-	50	-	-
Dry flower	50	25	50	> 50

Note: (-) denotes no MIC test because no inhibition zones were observed in disc diffusion assay.

Discussions and Conclusion

The minimal inhibitory concentrations of the extracts of red-stemmed *Ipomoea aquatica* Forsk. against four fungi were close to each other, i.e., 25 – 50 mg/mL, except for *M. globosa* (MIC > 50 mg/ml). Our finding was consistent with the study of Ghani (1989) revealing that *Ipomoea aquatica* Forsk. had inhibitory action against fungi.¹⁰ The inhibitory effect of *Ipomoea aquatica* Forsk. on fungi is offered by 3,5-di-O-caffeoyl-quinic acid (or isochlorogenic acid), a phenolic compound.³ Isochlorogenic acid was also reported for inhibitory activity against certain fungi species.¹⁵

The presence of antifungal activity of the red-stemmed extract of *Ipomoea aquatica* Forsk. in our study could be attributable to a relatively low temperature used in the experimental process. As it has been known that stability of phenolic compounds is temperature-dependent¹⁶, it has been reported that phenolic compounds are decomposed at temperature of 60 °C or higher.¹⁷⁻¹⁹ Since incubation temperature of 40 - 50 °C was used in this study, the decomposition of the active compound(s) in the extracts of *Ipomoea aquatica* Forsk. could be limited, and antifungal effects could be observed.

We found that the extract of the red-stemmed *Ipomoea aquatica* Forsk. exhibited the inhibitory effect only on some species of fungi causing ringworm and tinea versicolor. This could be due to the fact that the effect phenolic compounds of on the test fungi could be diminished by the remaining enzymatic activity in the plant. In this study, we did not halt enzymatic activity using the boiling alcohol. With less phenolic compounds, it therefore was possible that inhibitory effects on the test fungi that needed higher MIC could not be observed.²⁰

Antifungal activity of the extract of the red-stemmed *Ipomoea aquatica* Forsk. was lower than that of miconazole, a standard drug to treat these fungal infections. Our finding was however inconclusive since only one solvent was used.

Different types and amounts of active compounds could have been extracted with different solvents; hence the possibility of different levels of antifungal effects.

Since most previous works tested the antibacterial effects of the *Ipomoea aquatica* Forsk.^{14,21-23}, our research was different in three aspects. First, our study was the first to explore antifungal effect of the plant. Second, specific strain of the plant, i.e., the red-stemmed one, was tested. Third, in addition to the test of the plant's leaves, flower was also tested in our study. This study provided additional information regarding the antifungal activity of red-stemmed *Ipomoea aquatica* Forsk. which could be useful for further research on this plant as the traditional herb remedy.

In conclusion, in investigating the antidermatophytic activity of red-stemmed *Ipomoea aquatica* Forsk. on *T. rubrum*, *E. floccosum*, *M. gypseum*, *M. furfur* and *M. globosa*, using disc dilution and broth micro-dilution methods, the extracts of dry apical bud and dry flower of the plant exhibited the highest inhibitory effects. Most species, except *M. furfur*, were susceptible to the plant extracts. The antifungal activity of the red-stemmed *Ipomoea aquatica* Forsk. in our study was found to be relatively low and was incomparable to those well-known modern medicines and some Thai traditional medicines including garlic clove (*Allium sativum*), galanga rhizome (*Languas galanga*), and white crane flower (*Rhinacanthus nasutus* (L.) Kurz). The use of other solvents could possibly allow for more potent extracts against fungi causing ringworm and tinea versicolor.

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Editorial note

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