Antifungal activity of mongolian medicinal plant extracts

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Abstract

The *in vitro* antifungal activity of extracts obtained from 14 medicinal plants of the mongolian flora were investigated by measuring their minimal inhibitory concentration (MIC) against fungi cause of cutaneous diseases such as *Candida* species, dermatophytes and *Malassezia furfur*. Among the species examined, *Stellaria dichotoma* L.*, Scutellaria scordifolia* L. *Aquilegia sibirica* Fisch. Et Schrenk. and *Hyoscyamus niger* L. extracts demonstrated antifungal activity against all studied fungi. In particular, *S. scordifolia* L. methanol extract, obtained at room temperature, showed the best activity against *Candida* spp., *Malassezia furfur* and dermatophytes with $GMMIC_{50}$ values of 22 μg/mL, 64 μg/mL and 32 μg/mL, respectively. The flavones, luteolin and apigenin, identified in *S. scordifolia* extracts, and rutin identified in *S. dichotoma* and *Hyoscyamus niger* L. extracts, could be responsible of the observed antifungal activity.

Keywords

Experimental section

Plant materials and preparation of plant extracts

The medicinal herbs (Table 1 in the main manuscript) were harvested in July in the Hentii botanical region of Mongolia and immediately put under vacuum in plastic bags. Systematic identification of all plants was authenticated by Professor Choijamts Gotov. The collected plant samples were chopped into small pieces and extracted with solvent at the Department of Chemistry and Technology of Drug (Sapienza, University of Rome). The extracts were obtained from the aerial part of the plant. Only for *Stellera chamajeasme* L. were used the roots. For the extraction process, the fresh plant materials were extracted using two different conditions.

Room temperature methanol extraction: a portion of each plant sample (100g) was transferred into a flask and extracted with methanol (3 × 400 mL) at room temperature (27 ± 2 °C) overnight. Hot methanol extraction: a portion of each plant sample (100g) was transferred into a flask and extracted with methanol (500 mL) at reflux temperature (64.7 °C) for 2h. In all cases, the collected methanol extracts were filtered through a buchner under vacuum through a Whatmann n. 1 filter paper and concentrated using a rotary evaporator (R-210, BÜCHI Labortechnik AG, Flawil, Switzerland) with the water bath set at 40°C. Acetone was added to each extract and evaporated *in vacuo* in order to remove water, until the obtainment of a solid residue. The powdered residues were transferred into appropriate containers, dried under high vacuum (RC-6, Vacuubrand, Wertheim, Germany) and stored at 4 °C in argon atmosphere before the analysis.

Antifungal susceptibility tests

To evaluate the minimal inhibitory concentration (MIC), the susceptibility *in vitro* assay was performed according to CLSI standardized methods (CLSI. M38-A2, 2008; CLSI M27-A3, 2008; CLSI M27–S4, 2012). Antifungal susceptibility of *Candida* spp and *M. furfur* was determined according to standardized methods for yeast (CLSI M27-A3, 2008; CLSI, 2012; Simonetti et al. 2016) and antifungal susceptibility of dermatophytes was determined according to standardized methods for filamentous fungi (CLSI M27-A3, 2008; CLSI, 2012). In particular, *Candida* spp strains were grown on SAB at 35°C for 24h. *M. furfur* strains were grown on modified Dixon agar at 32 °C for 4 days. The final concentration of the inoculum was 15×10^6 CFU/mL. Dermatophytes were grown on potato dextrose agar (Sigma Aldrich, St. Louis, Missouri, USA) at 28–30 °C until good conidial growth was present. The conidia suspension was prepared at final concentration of $10^3 - 3 \times 10^3$ CFU/mL (CLSI. M38-A2, 2008). The concentrations of extracts ranged from 256 µg/mL to 0.5 µg/mL. The MIC_{50} was the lowest concentration of extracts that caused ≥50% growth inhibition; the MIC_{80} was the lowest concentration that caused 80% growth inhibition, and the MIC_{100} was the lowest extract concentration that inhibited 100% of growth.

Chemical analyses

Samples and reagents

On the base of antifungal activity results, crude extracts from *Stellaria dichotoma* L., *Scutellaria scordifolia* L., *Aquilegia sibirica* and *Hyoscyamus niger* L. were selected to be analyzed by HPTLC.

Each plant extract was obtained using methanol (32213, Sigma Aldrich).

Dichloromethane, ethyl acetate, formic acid, acetic acid and methanol of HPLC grade were purchased from VWR International (Milan, Italy), and used as mobile phase. Chromatographic analysis was performed on HPTLC glass-backed plates (20 cm × 10 cm, 2 μm in thickness), silica gel 60 without fluorescence marker from Merck (Darmstadt, Germany). As derivatization solutions (for immersion), natural products reagent (1 g 2-Aminoethyl diphenylborinate was dissolved in 200 mL ethyl acetate) and anisaldehyde-sulfuric acid (10 mL of sulfuric acid are carefully added to an ice-cooled mixture of 170 mL methanol and 20 mL acetic acid, to this solution, 1 mL anisaldehyde is added) were prepared and used.

A CAMAG HPTLC system (Muttenz, Switzerland) was used, comprising an applicator, Linomat 5 automatic sample spotter, Automatic Developing Chamber ADC2, TLC Visualizer, TLC Plate Heater III, TLC Scanner 3 and TLC Immersion Device. All instruments were controlled via the software platform 'WinCATS' 1.4.4 Planar Chromatography Manager (CAMAG).

Chromatographic conditions

The HPTLC plates were washed (pre-chromatography) with methanol and dried for 15 min at 120 °C prior to use. Samples were applied as bands of 7 mm in length, at a constant application rate of 150 nL/s, keeping the distance between adjacent bands as 9.4 mm. The extracts (30 mg/mL in ethanol) were analyzed in comparison with known polyphenols (e.g., chlorogenic acid, caffeic acid, gallic acid, apigenin, luteolin, catechin, kaempferol, quercetin and rutin).

Chromatography was performed in the Automated Developing Chamber ADC2 up to a migration distance of 70 mm (from the lower plate edge) using a mixture of ethyl acetate, dichloromethane, formic acid, acetic acid and water 100:25:10:10:11 (v/v) as mobile phase. The plate was dried for 3 min at 120 °C on TLC Plate Heater III. A post-chromatographic derivatization was performed with natural product reagent and anisaldehyde-sulfuric acid solution using a TLC Immersion Device (CAMAG) with an immersion speed of 3.5 cm/s and an immersion time of 1 s and heated on a TLC plate heater for 2 min at 120 °C. After every post-chromatographic derivatization plates were detected at TLC Visualizer under UV light (254 nm and 366 nm) and white light.

References

1. CLSI. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard—Second Edition M38–A2.

2. CLSI. Clinical and Laboratory Standards Institute. 2008. Reference method or broth dilution antifungal susceptibility testing of yeasts; Approved Standard—Third Edition M27-A3.

3. CLSI. Clinical and Laboratory Standards Institute. 2012.Reference method for broth dilution antifungal susceptibility testing of yeasts; Fourth Informational Supplement M27–S4.

Table S1. Antifungal activity of plant extracts against *C. albicans, C.krusei, C. parapsilosis and C. tropicalis*

	C. albicans ATCC10231			*C. albicans* ATCC 90028			*C. krusei* PMC0631			*C. parapsilosis* DSM5784			*C. tropicalis* DSM11953		
	MIC(µg/mL)														
Extracts	**50**	**90**	**100**	**50**	**90**	**100**	**50**	**90**	**100**	**50**	**90**	**100**	**50**	**90**	**100**
3A	256	>	>	256	>	>	128	256	256	256	>	>	256	>	>
3B	256	>	>	256	>	>	64	128	256	256	>	>	128	256	>
4A	32	64	128	64	128	256	16	32	32	32	64	128	64	64	256
4B	64	128	128	64	128	256	32	64	64	64	128	256	64	64	256
5A	>	>	>	256	>	>	128	256	256	>	>	>	256	>	>
5B	>	>	>	256	>	>	128	256	256	>	>	>	256	>	>
10A	>	>	>	>	>	>	256	>	>	>	>	>	256	>	>
10B	256	>	>	256	>	>	256	>	>	>	>	>	256	256	>

Antifungal activity was determined according to Clinical and Laboratory Standards Institute guidelines. The values shown are the median from three independent determinations. MIC=

minimal inhibitory concentration. MIC_{50}, MIC_{80} and MIC_{100}= lowest drug concentration that prevented 50%, 80% and 100% of growth with respect to the untreated control.

Table S2. Antifungal activity of plant extracts against *C. glabrata*

	C. glabrata PMC0840			*C. glabrata* PMC0897			*C. glabrata* PMC0850R			*C. glabrata* PMC08202			*C. glabrata* PMC0807R		
	MIC(µg/mL)														
Extracts	50	90	100	50	90	100	50	90	100	50	90	100	50	90	100
3A	8	16	32	128	256	>	32	64	128	128	256	>	32	128	256
3B	8	16	32	128	256	>	32	64	128	128	256	>	32	128	256
4A	4	8	16	64	128	128	16	16	32	16	32	64	8	32	64
4B	16	32	64	128	256	256	32	64	128	128	256	256	32	128	256
5A	32	64	64	256	>	>	128	128	256	256	>	>	128	256	>
5B	16	16	32	256	>	>	128	128	256	128	256	>	256	>	>
10A	8	16	32	256	256	>	>	>	>	128	256	>	256	>	>
10B	8	16	64	256	>	>	128	128	256	256	>	>	128	256	>

Antifungal activity was determined according to Clinical and Laboratory Standards Institute guidelines. The values shown are the median from three independent determinations. MIC= minimal inhibitory concentration. MIC_{50}, MIC_{80} and MIC_{100}= lowest drug concentration that prevented 50%, 80% and 100% of growth with respect to the untreated control

Table S3. Antifungal activity of plant extracts against *M. gypseum, T. mentagrophytes* and *Malassezia furfur.*

	M.gypseum DSM 3824			*T. mentagrophytes* DSM4870			*Malassezia furfur* DSM529		
				MIC(µg/mL)					
Extracts	50	80	100	50	80	100	50	80	100
3A	>	>	>	256	>	>	128	128	
3B	>	>	>	256	>	>	128	128	256
4A	32	64	64	32	32	64	64	64	128
4B	64	128	128	64	64	128	128	128	256
5A	256	256	256	256	256	256	128	128	256
5B	256	>	>	256	>	>	128	128	256
10A	>	>	>	256	>	>	256	256	256
10B	>	>	>	256	>	>	128	128	256

Antifungal activity was determined according to Clinical and Laboratory Standards Institute guidelines. The values shown are the median from three independent determinations. MIC= minimal inhibitory concentration. MIC_{50}, MIC_{80} and MIC_{100}= lowest drug concentration that prevented 50%, 80% and 100% of growth with respect to the untreated control.
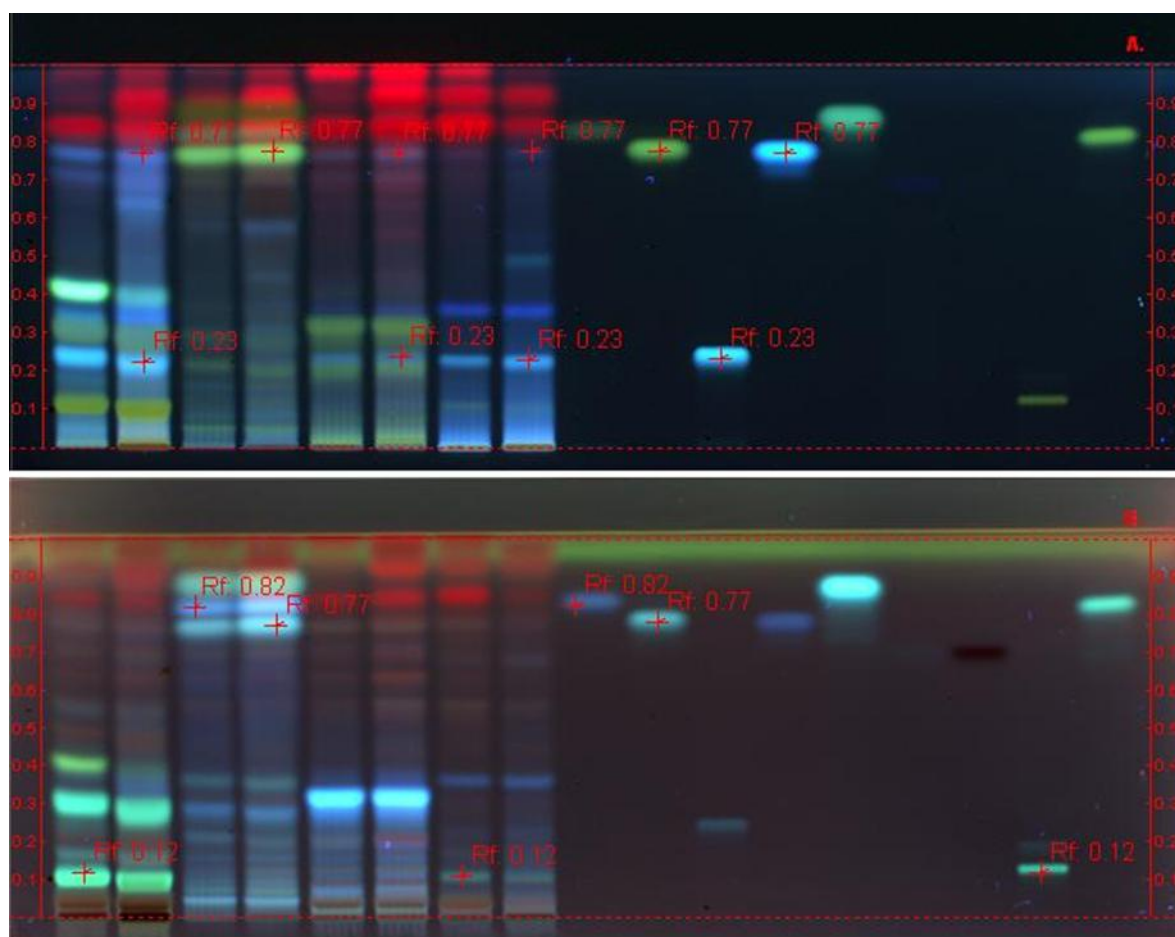
Figure S1 a and S1 b. HPTLC plates Tracks: 1. 3B; 2. 3A; 3. 4B; 4. 4A; 5. 5B; 6. 5A; 7. 10B; 8. 10 A; 9. Apigenin; 10. Luteolin; 11. Chlorogenic acid; 12. Caffeic acid; 13. Kaempferol; 14. Gallic acid; 15. Catechin; 16. Rutin; 17. Quercetin. a) Visualization, UV 366 nm, derivatization with natural product reagent. b) Visualization, UV 366 nm, derivatization with Natural Product reagent and anisaldehyde.
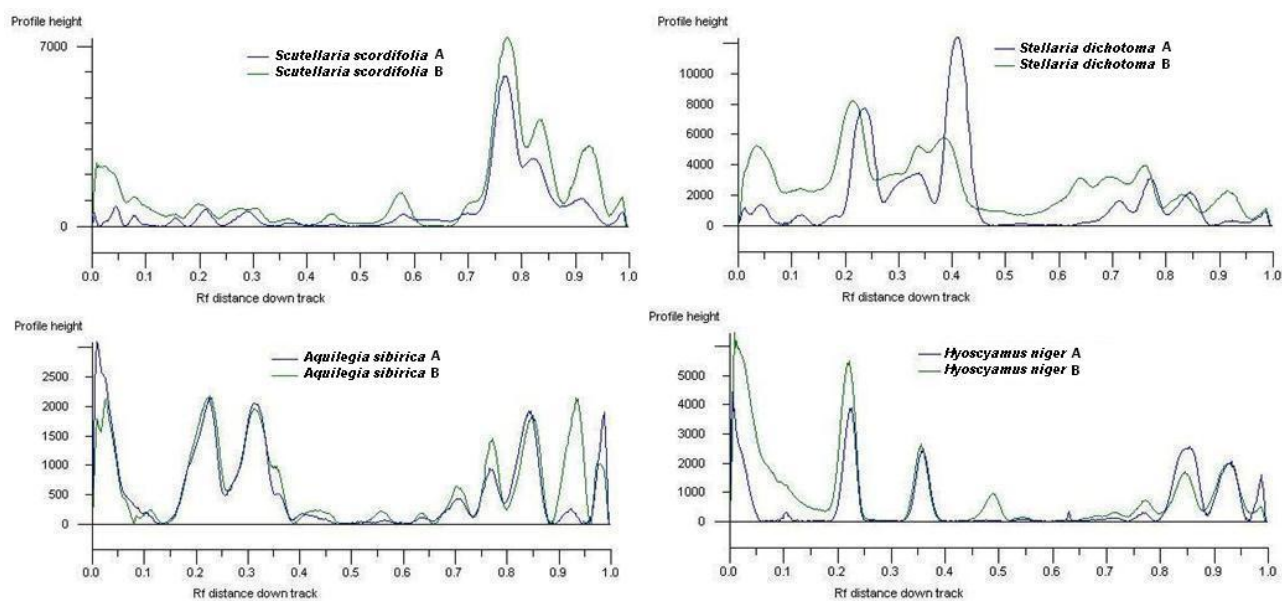
Figure S2. HPTLC chromatograms of *Stellaria dichotoma* L., *Scutellaria scordifolia* L., *Aquilegia sibirica* Fisch. Et Schrenk and *Hyoscyamus niger* L.