Agricultural Sci. J. 39 : 3 (Suppl.) : 119-123 (2008) ว. วิทย. กษ. 39 : 3 (พิเศษ) : 119-123 (2551)

การยับยั้งเชื้อราสาเหตุโรคพืชด้วยสารสกัดจากพืชในสกุล Rutaceae 16 ชนิด

Antimicrobial activity of 16 plant extracts of the Rutaceae family against phytopathogenic fungi

เนตรนภิส เขียวขำ[1], Harald Greger[2] **และ สมศิริ แสงโชติ**[1]
Netnapis Khewkhom[1], Harald Greger[2] and Somsiri Shangchote[1]

Abstract

The lipophilic extract of 16 plant extracts of the Rutaceae family was selected for investigation of antifungal properties. Antifungal active compounds bioautography bioassays against *Cladosporium herbarum* have been detected. Comparative studies of *Toddalia sp.* (leaves), *Limonia acidissima* (leaves), *Vepris bilocularis* (leaves), *Coleonema pulchellum* (roots), *Triphasia trifoliata* (leaves), *Pleiospermum alatum* (leaves and stem), *Acronychia pedunculata* (leaves), and *Atalantia sp.* (leaves) extract showed clear inhibition zones on TLC plates against *C. herbarum*. In microdilution bioassay, the leaf extracts of *Fortunella hindsii* exhibited the strongest fungicidal activity with a MIC value at 312.5 µg/mL and showed an inhibition of spore germination at EC_{50} of 114 µg/mL for *Botrytis cinerea*. *L. acidissima* leaf extracts displayed a MIC of 1250 µg/mL and showed an inhibition of spore germination with $EC_{50\ 0}$ values at 589 µg/mL for *B. cinerea*. The leaf extracts of two different collections of *Glycosmis mauritiana* (RUT 213/7) and (RUT 400) revealed clear differences for the antifungal activity: the EC_{50} value of the former was at 70 µg/mL, but 1249 µg/mL for the latter. *G. mauritiana* (RUT 213/7) revealed clear differences for the antifungal activity: the EC_{50} value at 70 µg/mL. Stem extracts of *Pleiospermum alatum* showed activities with values at EC_{50} 262 µg/mL against *B. cinerea*. Spore germination of *Pestalotiopsis* sp. was inhibited by leaf extracts of *L. acidissima* with EC_{50} 199 µg/mL, and *Orixa sp.* with EC_{50} 492 µg/mL. All of them showed MIC values over 2500 µg/mL. Leaf extracts of *L. acidissima* showed EC_{50} 366 µg/mL against *Colletotrichum gloeosporioides* whereas *G. mauritiana* (RUT213/7) showed a value of 784 µg/mL.

Keywords: bioactive, fungistatic, Rutaceae

บทคัดย่อ

การศึกษาคุณสมบัติการยับยั้งการงอกของสปอร์ของสารสกัดหยาบในส่วนที่เป็น lipophilic ของพืชสกุล Rutaceae โดยการทดสอบสารสกัด *Toddalia sp.* (ใบ), *Limonia acidissima* (ใบ), *Vepris bilocularis* (ใบ), *Coleonema pulchellum* (ราก), *Triphasia trifoliata* (ใบ), *Pleiospermum alatum* (ใบและเปลือกลำต้น), *Acronychia pedunculata* (ใบ), and *Atalantia sp.* (ใบ) ยับยั้งการเจริญของเชื้อรา *Cladosporium herbarum* บนแผ่น TLC วิธี bioautography เมื่อทดสอบด้วยวิธี microdilution พบว่าสารสกัดจากใบของ *Fortunella hindsii* ยับยั้งการงอกของสปอร์เชื้อรา *Botrytis cinerea* ดีที่สุด โดยมีค่า MIC เท่ากับ 312.5 µg/mL และค่า EC_{50} of 114 µg/mL ส่วนสารสกัดจากใบของ *L. acidissima* มีค่า MIC เท่ากับ 1250 µg/mL และมีค่า EC_{50} เท่ากับ 589 µg/mL พบความแตกต่างระหว่าง ใบ *Glycosmis mauritiana* RUT 213/7 และ RUT400 โดยมีค่า EC_{50} เท่ากับ 70 µg/mL ในตัวอย่างแรก แต่ในตัวอย่างหลังมีค่าเท่ากับ 1249 µg/mL สารสกัดจากเปลือกลำต้นของ *Pleiospermum alatum* มีค่า EC_{50} เท่ากับ 262 µg/mL สารสกัดส่วนใบ *L. acidissima* ยับยั้งการงอกของสปอร์ *Pestalotiopsis* sp โดยมีค่า EC_{50} เท่ากับ 199 µg/mL และสารสกัดจากใบของ*Orixa sp.* มีค่า EC_{50} เท่ากับ 492 µg/mL โดยมีค่า MIC มากกว่า 2500 µg/mL. สารสกัดจากส่วนใบของ *L. acidissima* ยับยั้งการงอกของสปอร์ *Colletotrichum gloeosporioides* โดยมีค่า EC_{50} เท่ากับ 366 µg/mL ขณะที่ *G. mauritiana* (RUT213/7) มีค่า EC_{50} เท่ากับ 784 µg/mL.

คำสำคัญ: bioactive, Fungistatic, Rutaceae

[1] ภาควิชาโรคพืช / ศูนย์นวัตกรรมเทคโนโลยีหลังการเก็บเกี่ยว มหาวิทยาลัยเกษตรศาสตร์ กรุงเทพฯ 10900 ประเทศไทย
[1] Department of Plant Pathology/ Postharvest Technology Innovation Center, Kasetsart University, Bangkok 10900, Thailand
[2] Section of Comparative and Ecological Phytochemistry, University of Vienna, Rennweg 14,A-1030 Vienna, Austria

Introduction

Rutaceae contain many members of economic importance. Most notable are the species of *Citrus* that produce both the citrus fruits of commerce (lemons, oranges, mandarins, tangerines, limes, kumquats, etc.) and the essential oils used in perfumery, the species of *Pilocarpus* that are the source of pilocarpine, a drug used to treat glaucoma. They are a large family comprising 155 genera with 1600 species, mostly tropical and subtropical in distribution. The family is usually placed in Rutales or in Sapindales. They are usually woody plants with typically compound, estipulate leaves, seldom more than two ovules per carpel, a nectary disk (sometimes modified into a gynophore), usually no more than twice as many stamens as sepals or petals, and a superior ovary. (Chase *et al*., 1999) A broad spectrum of plants produces antifungal secondary metabolites. These may be preformed compounds, which are found in healthy plants and which may represent chemical barriers to prevent infection by potential pathogens. There is increasing evidence from studies of phytopathogenic fungi that at least some plant compounds that inhibit fungal growth *in vitro* are likely to act as antimicrobial phytoprotectants in plants and that fungal mechanism of tolerance or detoxification can be important for pathogenicity (Osbourn, 1999). The search for simple bioactive compounds of plant origin against plant pathogenic fungi is of interest for ecologically safe products. For example, the essential oils are known to contain a natural mixture of monoterpenes, sesquiterpenes, diterpenes, phenylpropanes, and hydrocarbons, with a variety of functional groups that lead to antifungal and antimicrobial activities.

Material and Method

Plant Material: Plant material was obtained from wild collection as well as from plants collection in Botanical Garden Vienna (HBV) and Bangkok.

Extraction: Plant samples were ground and extracted with MeOH at room temperature for 3 days, filtered, and concentrated. The aqueous residue was extracted with CHCl3.

Bioautography: This technique was used to detect active compounds of the crude extracts. They were dissolved and spotted on two TLC plates (Merck 60 F 254, 0.25 mm) using a disposable glass micropipette for each sample. The developed plates were dried and observed under a CAMAG UV-Lamp at wavelengths of 254 and 366 nm. One plate was sprayed with a MeOH–HOAc–H_2SO_4–anisaldehyde reagent (85:10:8:0.5) and activated at 100-110 °C for 10 min. to visualize UV-invisible compounds in the extracts. The other plate was used for the bioautography assay system in order to detect antifungal activity directly on the developed TLC plate. In this case the silica gel plates were sprayed with *Cladosporium herbarum* spore suspensions adjusted to a final concentration of 10^5 conidia/mL in 1.7% malt extract broth (Merck). Inoculated plates were then placed in a humid chamber to monitor the activity of single compounds after 3 days at 25 °C in darkness. Clear zones of fungal growth inhibition indicated the presence of antifungal constituents.

Microdilution assay: A standardized 96-well microtiter plate assay developed for the discovery of natural products of fungicidal activity (Hadacek and Greger, 2000) was used to evaluate naturally occurring antifungal agents against *B. cinerea, Pestalotiopsis* sp. and *Colletotrichum gloeosporioides*. Each fungus was challenged in a dose-response format using test compounds with the final treatment concentrations of 1- 2500 µg/mL for crude extracts. Each dose dilution was repeated 4 times for evaluation. Microtiter plates (Greiner) were incubated at room temperature. The fungal growth was then evaluated by measuring the absorbance of each well at 620 nm using an ELISA optical density reading (SLT Labinstrument: SLT 400 ATC). The mean of the absorbance values and standard errors were used to evaluate the fungal growth at 48 h. Means for percent inhibition of each fungus at each dose relative to the untreated positive growth controls were used to evaluate fungal growth inhibition.

Data analysis: Minimum inhibitory concentrations (MIC) were determined as the lowest compound concentration completely inhibiting spore germination (NCCLS method M27-A, 1997). EC_{50} and EC_{90} values were calculated by

probit-log analysis (SPSS statistical analysis software) as described for quantitative assays (Hadacek and Greger, 2000; Engelmeier et al., 2000).

Result and Discussion

As shown in Figure1, chromatographic fractionation combined with bioautography of plant extracts from Rutaceae can be used to isolate antifungal constituents. Comparative studies of *Toddalia sp.* (leaves), *Limonia acidissima* (leaves), *Vepris bilocularis* (leaves), *Coleonema pulchellum* (roots), *Triphasia trifoliata* (leaves), *Pleiospermum alatum* (leaves and stem), *Acronychia pedunculata* (leaves), and *Atalantia sp.* (leaves) showed clear inhibition zones on TLC plates against *C. herbarum.* On the other hand, extracts from *Pamburus missionis* (roots), *Garcinia mangostana* (fruit), *Fortunella hindsii* (leaves), *Citrus reticulata* (leaves), *Coleonema puldrum* (root), *Feroniella sp.* (leaves), *Orixa sp.* (leaves), *Pleiospermum alatum* (twigs), and *Acronychia pedunculata* stem bark, and *Atalantia roxburghiana* leaves had no antifungal activity in bioautographic tests.

In microdilution bioassays of plant extracts of the Rutaceae family against *B. cinerea*, *Pestalotiopsis* sp., and *C. gloeosporioides* EC_{50}, EC_{90} and MIC values were determined. The leaf extracts of *Fortunella hindsii* exhibited strongest fungicidal activity with a MIC value at 312.5 µg/mL and showed an inhibition of spore germination at EC_{50} and EC_{90} of 114 µg/mL and 335 µg/mL for *B. cinerea*. In comparison *Fortunella hindsii* had no antifungal activity in the bioautography test. However, in some cases the results of the bioautographic tests are not in agreement with the antifungal activities already determined by microdilution assay. Results of fungal inhibition studies are often expressed as the dose required to give 50% inhibition or the minimum inhibitory concentration (MIC) in ppm (Gorham, 1995). *Limonia acidissima* leaf extracts displayed a MIC of 1250 µg/mL and showed an inhibition of spore germination with EC_{50} and EC_{90} values at 589 µg/mL and 1158 µg/mL for *B. cinerea*. It should be pointed out that the leaf extracts of two different collections of *Glycosmis mauritiana* (RUT 213/7) and (RUT 400) revealed clear differences for the antifungal activity: the EC_{50} value of the former was at 70 µg/mL, but 1249 µg/mL for the latter. Correspondingly, the EC_{90} value of RUT 213/7 was at 241 µg/mL, but 2120 µg/mL for RUT 400 against *B. cinerea*. Leaf extracts of *Pleiospermum alatum* showed activities with values at EC_{50} 262 µg/mL and EC_{90} 347 µg/mL against *B. cinerea* and MIC over 2500 µg/mL. Spore germination of *Pestalotiopsis* sp. was inhibited by leaf extracts of *Limonia acidissima* with EC_{50} 199 µg/mL and EC_{90} 831 µg/mL, and *Orixa sp.* with EC_{50} 492 µg/mL and EC_{90} 855 µg/mL. All of them showed MIC values over 2500 µg/mL. Leaf extracts of *Limonia acidissima* showed EC_{50} 366 µg/mL against *C. gloeosporioides* while *Glycosmis mauritiana* (RUT213/7) showed a value of 784 µg/mL, both of them showed MIC over 2500 µg/mL (Table 1). Of special interest is the formation of different amides in the leaf extracts of *Glycosmis* species (Greger et al., 1996) The lipophilic leaf extracts contained a series of antifungal phenethyl- and styrylamine-derived amides and imides, most of them derived from the sulfur containing cysteine. These compounds were active against the rice blast disease (*Pyricularia oryzae*). The most active growth inhibitors were found in *Glycosmis* cf. *mauritiana* such as penimide A, dehydroniranin B, and methylillukumbin A. The imide ritigalin and six phenethyl/styrylamine-derived amides isolated from the lipophilic leaf extracts of *Glycosmis* cf. *mauritiana, Glycosmis* cf. *cyanocarpa,* and *Glycosmis crassifolia* also displayed pronounced antifungal and/or insecticidal activity against *Cladosporium herbarum* and *Spodoptera littoralis*, respectively (Greger, 1996).

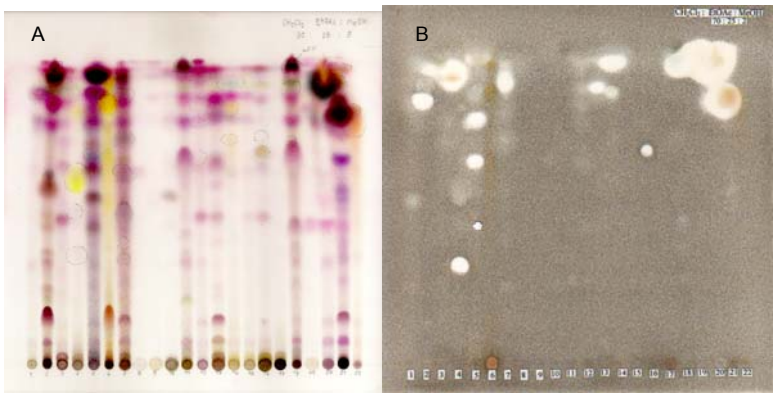

Figure 1 A: TLC of crude extracts of different Rutaceae family plants sprayed with anisaldehyde reagent. B: Corresponding antifungal activity observed by direct spraying of a conidia suspension of *Cladosporium herbarum*. Solvent system: $CHCl_3$:EtOAc:MeOH (70:25:5).

(1) *Pamburus missionis* root
(2) *Toddalia sp.* leaves
(3) *Limonia acidissima* leaves
(4) *Vepris bilocularis* leaves
(5) *Coleonema pulchellum* root
(6) *Garcinia mangostana* fruit
(7) *Fortunella hindsii* leaves
(8) *Triphasia trifolia* leaves
(9) *Citrus reticulata* leaves
(10) *Coleonema puldrum* root
(11) *Feroniella sp.* leaves
(12) *Orixa sp.* leaves
(13) *Pleiospermum alatum* leaves
(14) ___,___ stem
(15) ___,___ twigs
(16) *Acronychia pedunculata* leaves
(17) ___,___ stem bark
(18) *Atalantia sp.* leaves
(19) *Atalantia roxburghiana* leaves

Conclusion

The lipophilic extract of 16 plant extracts of the Rutaceae family were selected for investigation of their antifungal properties. For the detection of antifungal active compounds bioautography bioassays on thin layer chromatography plates against *C. herbarum* have been carried out. Antifungal active extracts were further proved in a quantitative bioassay, the germ tube inhibition test in serial dilution against the 3 pathogenic fungi. The leaf extracts of *F. hindsii* exhibited the strongest fungicidal activity with a MIC value at 312.5 µg/mL against *B. cinerea* in microdilution bioassay.

Acknowledgement

The Ministry of University Affairs and Asian development Bank (ADB) and Postgraduate Education and Research Development Project in Postharvest Technology, Kasetsart University of Thailand

Reference

Chase, Mark W.; Cynthia M. Morton & Jacquelyn A. Kallunki. 1999. Phylogenetic relationships of Rutaceae: a cladistic analysis of the subfamilies using evidence from RBC and ATP sequence variation. *American Journal of Botany* 86 (8): 1191–1199.

Engelmeier, D., Hadacek, F., Pacher, T., Vajrodaya, S., Greger, H., 2000. Cyclopenta[b]benzofurans from *Aglaia* species with pronounced antifungal activity against rice blast fungus (*Pyricularia grisea*). Journal of Agricultural and Food Chemistry 48, 1400-1404.

Hadacek, F., Greger, H., 2000. Testing of antifungal natural products, methodologies, comparability of results and assay choice. Phytochemical Analysis 11, 137-147.

Greger, H., Zechner, G., Hofer, O., Vajrodaya, S., 1996. Bioactive amides from *Glycosmis* species. Journal of Natural Product 59, 1163-1168.

Osbourn, A.E., 1999. Antimicrobial phytoprotectants and fungal pathogens, a commentary. Fungal genetics and Biology 26, 163-168.

Table 1 EC_{50}, EC_{90} and MIC values (µg/mL) of plants extract of the Rutaceae family against *B. cinerea*, *Pestalotiopsis* sp. and *C. gloeosporioides* determined by microdilution bioassay.

		EC_{50} (95%FL)		EC_{90} (95%FL)		MIC
B. cinerea						
Acronychia pedunculata	leaves	>2500		>2500		>2500
	stem	>2500		>2500		>2500
Atalantia roxburghiana	leaves	2045	(1893-2225)	>2500		>2500
	fresh root	>2500		>2500		>2500
Coleonema pulchrum	root	>2500		>2500		>2500
Feroniella sp.	leaves	2128	(1226->2500)	>2500		>2500
Fortunella hindsii	**leaves**	**114**	**(78-169)**	**335**	**(214-804)**	**312.5**
Glycosmis pentaphylla	leaves	>2500		>2500		>2500
Glycosmis mauritiana	RUT215i-l	>2500		>2500		>2500
	RUT213/7-l	70	(62-79)	241	(203-299)	>2500
	RUT400	1249	(967-1579)	2120	(1658->2500)	>2500
Limonia acidissima	leaves	589	(501-693)	1158	(946-1585)	1250
Citrus reticulata	leaves	>2500		>2500		>2500
Orixa schozank	leaves	>2500		>2500		>2500
Pamburus missionis	leaves	>2500		>2500		>2500
Pleiospermium alatum	leaves	>2500		>2500		>2500
	stem	262	(243-278)	347	(325-380)	>2500
	twig	1708	(877-9475)	>2500		>2500
Toddalia sp.		>2500		>2500		>2500
Vepris bilocularis	leaves	>2500		>2500		>2500
***Pestalotiopsis* sp.**						
Fortunella hindsii	leaves	>2500		>2500		>2500
Glycosmis mauritiana	RUT213/7-l	2001	(1543->2500)	1763	(1267-2614)	>2500
Limonia acidissima	leaves	199	(115-332)	831	(466-2601)	>2500
Orixa sp.	leaves	492	(342-689)	855	(627-1979)	>2500
Pamburus missionis	leaves	>2500		>2500		>2500
Pleiospermum alatum	stem	630	(32-2300)	>2500		>2500
C. gloeosporioides						
Fortunella hindsii	leaves	1374	(442->2500)	>2500		>2500
Glycosmis mauritiana	RUT213/7	784	>2500	>2500		>2500
Limonia acidessima	leaves	366	(123->2500)	>2500		>2500
Pamburus missionis	leaves	1159	(606-2407)	>2500		>2500
Pleiospermum alatum	stem	842	(122->2500)	>2500		>2500

EC_{50} and EC_{90} were determined by probit-log analysis, FL: fiducial limits. **MIC** (Minimum inhibitory concentration) defined the lowest concentration of the dilution series, which completely inhibited spore germination. High activities are indicated by bold letters.