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กิจกรรมในการต่อต้านเชื้อราของสารเคมีในกลุ่ม flavaglines จากพืชสกุล *Aglaia*

Antifungal activity of flavaglines from *Aglaia* species

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Abstract

Lipophilic crude extracts of *Aglaia argentea*, *A. oligophylla, A. elaeagnoidea, A. spectabilis,* and *A. cucullata* (Meliaceae) were tested on their effectiveness on growth inhibition of posthavest pathogens. Bioassay-guided fractionation led to the isolation of three active flavaglines. These were elucidated and identified by using spectroscopic methods (NMR, UV and IR) as aglafoline and didesmethylrocaglamide from *A. argentea*, and rocaglaol from *A. oligophylla*. A 96-well microbioassay revealed that rocaglaol produced high growth inhibition of *Botrytis cinerea, Colletotrichum gloeosporioides,* and *Pestalotiopsis* sp. With EC_{50} values at 0.05 µg/mL against *Pestalotiopsis* sp., 1.2 µg/mL against *Botrytis cinerea*, and 52 µg/mL against *Colletrotrichum gloeosporioides*. The activity of rocaglaol is comparable with or sometimes even higher as commercial fungicides.

Keywords: antifungal, *Aglaia* sp., flavaglines, *Botrytis cinerea, Colletotrichum gloeosporioides, Pestalotiopsis* sp.

บทคัดย่อ

ประสิทธิภาพของสารสกัดหยาบในส่วนที่เป็น lipophilic ของพืชสกุล *Aglaia* วงศ์สะเดา (Meliaceae) ได้แก่ *Aglaia argentea*, *A. oligophylla, A. elae agnoidea, A. spectabilis,* และ *A. cucullata* ได้นำมาทดสอบการยับยั้งการเจริญของเชื้อราสาเหตุโรคหลังการเก็บเกี่ยว และเมื่อแยกและจำแนกสารบริสุทธิ์ด้วยวิธีการ spectroscopic (NMR, UV และ IR) ได้สาร flavaglines 3 ชนิด คือ aglafoline didesmethylrocaglamide จาก *A. argentea* และ rocaglaol จาก *A. oligophylla* ซึ่งสามารถยับยั้งการเจริญของเชื้อราได้ จากการวิเคราะห์ทางชีววิธีพบว่า rocaglaol มีประสิทธิภาพสูงในการยับยั้งการเจริญของเชื้อรา *Botrytis cinerea, Colletotrichum gloeosporioides* และ *Pestalotiopsis* sp. โดยพบว่า *B. cinerea* มีค่า EC_{50} เท่ากับ 1.2 µg/mL และ *C. gloeosporioides* มีค่า EC_{50} เท่ากับ 52 µg/mL และ *Pestalotiopsis* sp. มีค่า EC_{50}เท่ากับ 0.05 µg/mL ซึ่งมีประสิทธิภาพดีกว่าเมื่อเปรียบเทียบกับสารเคมีที่ใช้ป้องกันและกำจัดเชื้อราบางชนิด

คำสำคัญ antifungal, *Aglaia* sp., flavaglines, *Botrytis cinerea, Colletotrichum gloeosporioides, Pestalotiopsis* sp.

Introduction

The present studies showed a great diversity of unknown molecular structures with antifungal capacities in various tropical plant families. With respect to the much already known resistances of important phytopathogenic fungi against commercial fungicides, natural products provide a valuable source of new lead structures offering countless possibilities to overcome the increasing resistance. Compared to commercial fungicides secondary plant metabolites are also environmentally beneficial because of their rapid detoxification by microorganisms.

Agricultural research into plant-derived natural products has declined during recent decades, but this trend is now being reversed as it becomes evident that natural plant products still have enormous potential to inspire and influence modern pesticide research. Many plant-derived natural products are fungistatic rather than fungicidal. They are also considered bio-degradable, friendly and safe to the environment and delicate ecosystems, which suggests that the natural products from plants can be alternatives to synthetic pesticides.

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Such compounds, if properly formulated and applied, could be used directly or serve as templates for synthetic analogues (Lee et al., 2005).

Aglaia is a genus of more than 100 species belonging to the Mahogany family, Meliaceae. In this family, aromatic compounds with a cyclopenta[*b*]benzofuran skeleton represent a typical chemical character of the genus *Aglaia*. This class of compounds was named flavaglines. The genus has received increasing scientific attention due to its bioactive potential. The biologically very active cyclopenta[*b*]benzofurans are mainly accumulated in the roots and stem bark (Brader et al., 1998). Bioassays with lipophilic crude extracts of *Aglaia edulis* against *Spodoptera littoralis* displayed strong insecticidal activity. The insect toxicity of *A. basiphylla* was caused by the well known benzofuran flavaglines rocaglamide, desmethylrocaglamide, and aglafoline. Comparative feeding assays within the active pyrimidinone flavaglines showed that the free hydroxyl group in the aromatic ring A of marikarin diminishes the insecticidal activity (Bacher et al., 1999; Greger et al., 2000; Greger et al., 2001). In a study for antifungal activity, cyclopenta[*b*]benzofurans from *Aglaia odorata*, *A. elaeagnoidea*, and *A. edulis* were isolated and tested against the three plant pathogens *Pyricularia grisea*, *Fusarium avenaceum*, and *Alternaria citri*. Using the microdilution technique, the compounds displayed a strong spore germination inhibitory inhibition. *P. grisea*, responsible for rice blast disease, was the most susceptible fungus against all benzofurans, with rocaglaol as the most active derivative. Based on EC_{50}, EC_{90}, and MIC values, the antifungal activity of rocaglaol was clearly higher than those of the commercial fungicides blasticidin S and Benlate (Engelmeier et al., 2000).

Material and method

Plant material	Collecting date	Organ	Origin
Aglaia argentea Blume.	21.2.1999	**HG714** leaves / stem bark	Khao Chong
A. elaeagnoidea (A. Juss.) Benth.	19.4.2004	leaves	Thung Kai Botanic Garden
A.oligophylla Miq.	1999 and 2002	stem bark / root	Phytochemistry Dept. University of Vienna
A. spectabilis (Miq.) Jain & Bennet	22.4.2004	leaves	KB Kasetsart University Kasetsart University
A. cucullata (Roxb.) Pellegrin	15.2.2005	**HG967** leaves / stem bark	Kanom, Nakornsrithammarat

Extraction and isolation: Dried parts of *Aglaia* species were ground and extracted with MeOH at room temperature for 3 days, filtered, and concentrated. The aqueous residue was extracted with CHCl3. The obtained fraction was roughly chromatographed on silica (Merck Si 60, 0.2-0.5 mm, 600 _ 20 mm column), using hexane/EtOAc mixtures with EtOAc increasing from 15% to 100% and finally 100% MeOH, and further by preparative MPLC (400 _ 40 mm column, Merck Lichroprep Si 60, 25-40 μm, UV detection, 254 nm) using a step-gradient elution of 30% and 50% EtOAc in hexane and 100% EtOAc. Preparative TLC (Merck, Si gel 60, 0.5 mm) was used to finally purify the compounds (compare Brader et al., 1998; Bacher et al., 1999).

High performance liquid chromatography (HPLC): This method is based on reversed phase chromatography, which means that a polar mobile phase and a lipophilic stationary phase were used. The stationary phase consisted of silica gel with long carbon chains attached as substituents (Column Hypersil BDS, C18). Due to the different polarity of the compounds they are distributed to a different extent between the phases. Known compounds were identified by their retention times and UV spectra, which were compared with the existing data base of Department of Comparative and Ecological Phytochemistry, University of Vienna

Instrument: Hewlett Packard HP 1090 Series II LC, UV diode array detection at 230 nm. (UV detection at 252-360 nm)

Infrared (IR) spectroscopy: infrared transmission spectra were run in CCl_4 or in $CHCl_3$ using a Perkin Elmer 16 PC FT Infrared spectrophotometer.

NMR-Spectroscopy: ^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR) spectra were measured with a Bruker, AM 400 WB and AC 250 at the Institute of Organic Chemistry, University of Vienna.

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*. The same system was employed to determine the sensitivity of *Pestalotiopsis* sp. and *Colletotrichum gloeosporioides*. The various antifungal agents of the extracts were evaluated in comparison with known fungicidal standards: Benlate (50 % Benomyl), Carbendazim, Ronilan, and Kresoxim Methyl served as positive controls in the microtiter experiments. The chemical sensitivity of each fungus was evaluated. Each fungus was challenged in a dose-response format using test compounds with the final treatment concentrations of 0.1-200 µg/mL for pure compounds and 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 and 72 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 as outlined in the National Committee for Clinical Laboratory Standards (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).

Results

The isolation of three active flavaglines. These were elucidated and identified by using spectroscopic methods (NMR, UV and IR) as aglafoline and didesmethylrocaglamide from *A. argentea*, and rocaglaol from *A. oligophylla*.

Rocaglaol from *Aglaia oligophylla*

Root and stem bark organs of *A. oligophylla* were extracted separately and analyzed by HPLC linked with photodiode array detection. Since the cyclopenta[*b*]benzofuran rocaglaol was mainly accumulated in the root and stem bark, methanolic crude extracts from different individuals of *A. oligophylla* were prior to isolation.

Lipophilic fractions of root and stem bark from different collections (4888 mg) of *A. oligophylla* were combined and roughly separated by open column chromatography (Si gel). Fractions eluted with 50% and 75% EtOAc in hexane contained considerable amounts of rocaglaol and were combined (375.3 mg). Further separation was carried out by MPLC with 30% EtOAc in hexane to yield a fraction of 14.7 mg of still impure rocaglaol, which was purified by preparative TLC (CH_2Cl_2:EtOAc:MeOH 70:25:5) to give 4 mg of rocaglaol. The inconspicuous UV spectra showed small maxima at 275 nm (MeOH) and the compound produced the characteristic dark green coloration with anisadehyde reagent.

Aglafoline and, Didesmethylrocaglamide from *Aglaia argentea* (HG714, Khao Chong)

The lipophilic crude extract (740 mg) from 37.8 g dried stem bark was roughly separated by open column chromatography (Si gel). The combined fractions eluted with 50%, 75% and 100% EtOAc in hexane (481 mg) contained didesmethylrocaglamide and aglafoline. Further separation by MPLC using mixtures of EtOAc/hexane of increasing polarity starting with 50% EtOAc in hexane yielded a fraction of 1.4 mg pure aglafoline. Later

fractions eluted with 70% EtOAc in hexane afforded 1.8 mg of pure didesmethylrocaglamide. Fractions were purified by preparative TLC (CH_2Cl_2:EtOAc:MeOH = 70:23:7) as mentioned above.

The dried root bark from the same individual was not investigated in detail. However, HPLC comparison with reference compounds confirmed the occurrence of didesmethylrocaglamide, didesmethylrocaglamide formylester, aglafoline, and aglafoline formylester.

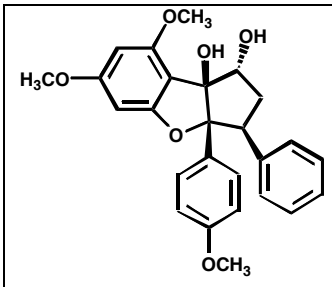
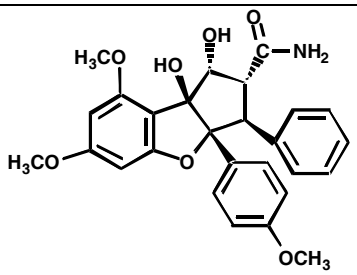
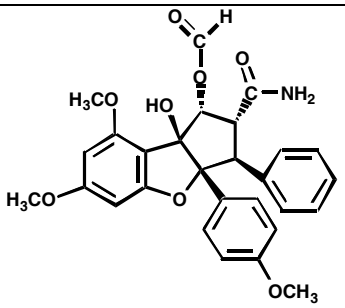
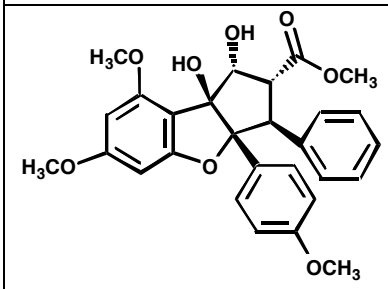
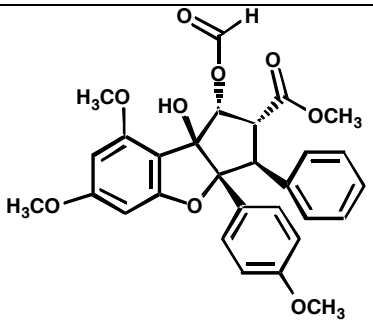
		
Rocaglaol $C_{26}H_{26}O_6$	Didesmethylrocaglamide $C_{27}H_{27}NO_7$	Didesmethylrocaglamideformylester $C_{28}H_{27}NO_8$
		
Aglafoline $C_{28}H_{28}O_8$	Aglafolineformylester $C_{29}H_{28}O_9$	

Figure 1 Molecular structures of flavaglines from *Aglaia oligophylla* (rocaglaol) and *Aglaia argentea* (HG714) which correspond with HPLC profiles and UV-spectra.

Table 1 $EC_{50,}$ EC_{90} and MIC values (µg/mL) of crude extract from *Aglaia* species as well as from three isolated pure flavaglines compare with various fungicides against *B. cinerea*, *Pestalotiopsis* sp., and *C. gloeosporioides* determined by microdilution bioassay.

		EC_{50} (95%FL)		EC_{90} (95%FL)		MIC
B. cinerea						
Aglaia argentea	leaves	>2500		>2500		>2500
	Stem bark	>2500		>2500		>2500
A. elaeagnoidea	leaves	>2500		>2500		>2500
A. spectabilis	leaves	>2500		>2500		>2500
A. cucullata	leaves	1635	(333->2500)	>2500		>2500
	Stem bark	631	(161->2500)	>2500		>2500
Didesmethylrocaglamide		**594**	**(137->200)**	**>200**		**>200**
Aglafoline		**128**	**(57-354)**	**>200**		**>200**
Rocaglaol		**1.2**	**(0.6-2)**	**2.2**	**(2-12)**	**1.6**
Benlate		0.01	(0-0.04)	62	(20-504)	>200
Carbendazim		0.1	a	>200		50
Ronilan		0.62	(0.3-1.3)	1.8	(0.9-14)	1.6
Kresoxim-Methyl		21	(9-70)	>200		>200
Pestalotiopsis sp.						
A. argentea	HG 714-leaves	2082	(898->2500)	>2500		>2500
	HG 714-bark	1448	(1315-1606)	>2500		>2500
Rocaglaol		**0.05**	**(0.002-0.2)**	**10**	**(4-69)**	**50**
Benlate		a	a	6	a	>200
Carbendazim		b		b		0.1
Ronilan		148	(27->200)	>200		>200
Kresoxim-Methyl		105	>200	>200		>200
C. gloeosporioides						
Rocaglaol		**52**	**(28-87)**	**86**	**(61-649)**	**>200**
Benlate		150	(135-176)	421	(326-609)	>200
Carbendazim		>200		>200		>200
Ronilan		>200		>200		>200
Kresoxim-Methyl		>200		>200		>200

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.

Pure compounds are presented in bold letters. a: no difference of inhibition in different concentrations of test compound (0.1-200 µg/mL).

b: missing endpoint impairs estimation of ECs. High activities are indicated by bold letters.

Summary

Lipophilic crude extracts of *Aglaia* species were analyzed for their antifungal activities. Based on bioautographic screening on TLC plates with the test fungus *Cladosporium herbarum* fungitoxic properties were observed. More detailed investigations were carried out in the two latter families from where active pure compounds were isolated and their structures elucidated in collaboration with the Institute of Organic Chemistry, University of Vienna. The molecular structures of the highly active the three flavaglines rocaglaol, aglafoline, and didesmethylrocaglamide isolated from *Aglaia oligophylla, A. argentea*.

The antifungal potency of corresponding crude extracts and isolated pure compounds were further tested in germ tube inhibition assays in 2-fold serial broth dilutions (microdilution) against the three phytopathogenic fungi *Botrytis cinerea*, *Pestalotiopsis* sp., and *Colletotrichum gloeosporioides* by ELISA optical density reading. Based on EC_{50}, EC_{90} and MIC values rocaglaol, isolated from stem and root bark of *Aglaia oligophylla* displayed the highest activity against all three fungi. With EC_{50} values at 0.05 µg/mL against *Pestalotiopsis* sp., 1.2 µg/mL against *Botrytis cinerea*, and 52 µg/mL against *Colletrotrichum gloeosporioides* the activity of rocaglaol is comparable with or sometimes even higher as commercial fungicides.

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