JOURNAL OF NATURAL PRODUCTS

Subscriber access provided by ISTANBUL TEKNIK UNIV

Antimicrobial Agents from Licaria puchuri-major and Their Synergistic Effect with Polygodial

Masaki Himejima, and Isao Kubo

J. Nat. Prod., **1992**, 55 (5), 620-625• DOI:
10.1021/np50083a010 • Publication Date (Web): 01 July 2004

Downloaded from http://pubs.acs.org on April 4, 2009

More About This Article

The permalink http://dx.doi.org/10.1021/np50083a010 provides access to:

- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

ACS Publications
High quality. High impact.

Journal of Natural Products is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Journal of Natural Products
Vol. 55, No. 5, pp. 620-625, May 1992

ANTIMICROBIAL AGENTS FROM *LICARIA PUCHURI-MAJOR* AND THEIR SYNERGISTIC EFFECT WITH POLYGODIAL

MASAKI HIMEJIMA and ISAO KUBO*

Division of Entomology and Parasitology, College of Natural Resources, University of California, Berkeley, California 94720

ABSTRACT.—The resistance of the seeds of *Licaria puchuri-major* (Lauraceae) to decomposition in nature seems to be due largely to chemical defense, since its *n*-hexane extract contains antimicrobial principles in quantity, with a broad antimicrobial spectrum. In order to identify the active principles, the *n*-hexane extract was steam-distilled to yield a distillate and a residue. Subsequent bioassay indicated that the distillate retained the original broad antimicrobial activity, while the residue exhibited almost no activity. Gc-ms analysis showed that the distillate contained four phenolic compounds, seven monoterpenes, and one sesquiterpene. In contrast, the residue contained, almost exclusively, lauric acid. In the detailed antimicrobial assay with the pure compounds identified, most of them showed broad, but moderate, antimicrobial activity.

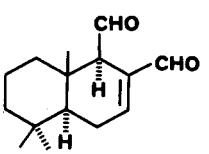
Some of the components identified in the distillate were combined with polygodial [**1**] in order to enhance their antifungal activity. Unexpectedly, while polygodial did not synergize the antifungal activity of any of the compounds tested, the antifungal activity of polygodial was significantly increased when combined with aromatic substances such as anethole, safrole, or methyleugenol.

The seeds of *Licaria puchuri-major* Mez (Lauraceae) are called "puchuri" and are used as a folk medicine in Brazil (1). Puchuri is usually gathered in tropical rain forests of the Amazon where most fallen plant materials rapidly decompose. However, the puchuri strongly resists decomposition. This resistance seems to be due to a combination of physical and chemical defense mechanisms, inasmuch as the seed is coated by a hard husk and contains large amounts of antimicrobial constituents. The fact that chemical constituents of puchuri have only rarely been investigated prompted us to identify its antimicrobial principles.

We now can control many human and animal pathogenic microorganisms with the antibiotics that are presently available. Nevertheless, the need for new antibiotics still exists. For example, systemic infections caused by fungi have become increasingly serious, especially when the host's defense mechanism is impaired. Various antifungal agents have been introduced, but control of many of the fungal diseases has not yet been achieved.

Large numbers of phytochemicals have already been isolated and shown to be antifungal principles (2,3). Although each of them may play an important role in the defense of living plants against microbial invasion, their antimicrobial activity is usually not potent enough to be considered for practical use. Hence, research to enhance their activity is needed. Combining two or more phytochemicals in order to enhance the total biological activity seems to be one of the most promising strategic approaches to this problem.

Recently, a bicyclic sesquiterpene dialdehyde, polygodial [**1**], was isolated from various plant sources (4–7) and reported to enhance the antifungal activity of several an-



1

tibiotics such as actinomycin D and rifampicin against *Saccharomyces cerevisiae* and *Candida utilis* (8–11). Specifically, the activity of actinomycin D against *Sac. cerevisiae* was increased sixteenfold when combined with polygodial. This was caused by an increase in the permeability of the plasma membrane of *Sac. cerevisiae* to these antibiotics (8). Based on this finding, some of the antifungal components identified in puchuri were combined with polygodial in order to enhance and broaden their activity. We focused our study on *Sac. cerevisiae* and *C. utilis*, as well as the dermatomycotic fungus *Pityrosporum ovale*.

This paper describes the identification of the antimicrobial principles of the seeds of *L. puchuri-major* and their antimicrobial activity. In addition, the antifungal activity of the puchuri compounds against *Sac. cerevisiae, C. utilis*, and *Pi. ovale*, when tested alone and in combination with polygodial, is discussed.

RESULTS AND DISCUSSION

In our preliminary assay, the *n*-hexane crude extract of the seeds of *L. puchuri-major* exhibited a broad antimicrobial activity when tested at a concentration of 1600 μg/ml. The growth of five Gram-positive bacteria, *Bacillus subtilis, Brevibacterium ammoniagenes, Propionibacterium acnes, Staphylococcus aureus*, and *Streptococcus mutans*, and three fungi, *C. utilis, Pi. ovale*, and *Penicillium chrysogenum*, was inhibited. However, the growth of another common fungus, *Sac. cerevisiae*, was not inhibited. The crude extract did not show any activity against three Gram-negative bacteria, *Escherichia coli, Pseudomonas aeruginosa*, and *Enterobacter aerogenes*. The *n*-hexane extract was divided into distillate and residue fractions by steam distillation, and the antimicrobial activity of each was tested at 1600 μg/ml. The distillate was found to have retained the original broad antimicrobial activity, with added activity against *Esch. coli* and *Sac. cerevisiae*. In contrast, the residue showed almost no activity against any of the microorganisms with the exception of *Pr. acnes*. This result indicated that the antimicrobial activity of the *n*-hexane extract was due to the distillate fraction.

In order to identify the active principles in the distillate, further analysis was performed by gc-ms. Four phenolic compounds, seven monoterpenes, one sesquiterpene, and one fatty acid were identified in the distillate. These components were identified as the following: phenolic compounds safrole, methyleugenol, eugenol, and anethole; monoterpenes α-terpineol, 1,8-cineole, 4-terpineol, geraniol, limonene, γ-terpinene, and linalool; the sesquiterpene caryophyllene; and lastly, the fatty acid lauric acid. The main components of the distillate were safrole and methyleugenol. This result is in general agreement with those reported earlier (12). In contrast, the residue contained lauric acid almost exclusively.

The antimicrobial activity of the individual components identified in the distillate was tested. As shown in Table 1, none of the components exhibited activity against *Ps. aeruginosa* or *Ent. aerogenes*. Among the Gram-positive bacteria tested, *Sta. aureus* was the least sensitive bacterium; methyleugenol, eugenol, and geraniol showed weak activity with an MIC of 800 μg/ml for each. In contrast, *Pr. acnes* was the most sensitive bacterium among the Gram-positive bacteria tested, with caryophyllene exhibiting the strongest activity with an MIC of 6.25 μg/ml.

A large amount of lauric acid was identified in the residue by spectroscopic techniques (ir, ms, and nmr). Lauric acid inhibited the growth of only *Pr. acnes* among the microorganisms tested, although it is known to be one of the most active antimicrobial fatty acids against several food-borne microorganisms (13). The antimicrobial activity of fatty acids, especially in foods, is well documented (14).

The antimicrobial spectrum of most of the compounds tested was broad but moderate to weak. However, these compounds may play an important role in the chemical de-

TABLE 1. Antimicrobial Activity, MIC (μg/ml) of the Components in the Distillate of *Licaria puchu;ri-major*.

Component	Microorganism[a]											
	Bs	Ba	Pac	Sa	Sm	Ec	Pae	Ea	Sc	Cu	Po	Pc
Safrole	400	>800	50	>800	100	400	>800	>800	200	200	100	>800
Methyleugenol	800	800	200	800	400	800	>800	>800	800	800	200	800
Eugenol	400	800	50	800	400	400	>800	>800	800	800	100	200
Anethole	400	200	100	>800	200	200	>800	>800	200	200	100	>800
α-Terpineol	800	>800	100	>800	400	800	>800	>800	800	800	400	400
1,8-Cineole	>800	>800	800	>800	>800	>800	>800	>800	>800	>800	>800	>800
4-Terpineol	>800	>800	800	>800	>800	>800	>800	>800	800	800	100	800
Geraniol	400	400	200	800	200	800	>800	>800	400	400	100	200
Limonene	800	>800	50	>800	100	>800	>800	>800	50	200	200	>800
γ-Terpinene	>800	>800	50	>800	100	>800	>800	>800	50	100	800	>800
Linalool	800	800	200	>800	400	>800	>800	>800	800	400	400	800
Caryophyllene	50	100	6.25	>800	>800	>800	>800	>800	>800	>800	>800	>800
Lauric acid	>800	>800	50	>800	>800	>800	>800	>800	>800	>800	>800	>800

[a]Bs, *Bacillus subtilis*; Ba, *Brevibacterium ammoniagenes*; Pac, *Propionibacterium acnes*; Sa, *Staphylococcus aureus*; Sm, *Streptococcus mutans*; Ec, *Escherichia coli*; Pae, *Pseudomonas aeruginosa*; Ea, *Enterobacter aerogenes*; Sc, *Saccharomyces cerevisiae*; Cu, *Candida utilis*; Po, *Pityrosporum ovale*; Pc, *Penicillium chrysogenum*.

fense of the living plant against microbial attacks, since the total quantity of antimicrobial principles in puchuri is more than 5% of the fresh weight; this is a relatively large quantity.

Most of the components identified in the distillate, with the exception of 1,8-cineole and caryophyllene, exhibited antifungal activity against *Sac. cerevisiae, C. utilis* and *Pi. ovale*. However, the MIC of each compound was moderate to weak. Hence, we attempted to increase their antifungal activity through combination with other substances. The components of the distillate were combined with the known antifungal synergizer, polygodial [**1**] (8), to see if it could enhance their activity.

Table 2 shows the MICs of the components in the distillate against the fungi, when

TABLE 2. Antifungal Activity of Components in the Distillate of *Licaria puchuri-major* in Combination with Polygodial [**1**].[a]

Component	MIC against fungus tested (μg/ml)		
	Saccharomyces cerevisiae	*Candida utilis*	*Pityrosporum ovale*
Anethole	50 (200)[b]	50 (200)	25 (100)
Safrole	100 (200)	50 (200)	25 (100)
Methyleugenol	200 (800)	200 (800)	50 (200)
Eugenol	400 (800)	400 (800)	50 (100)
Limonene	50 (50)	50 (200)	50 (200)
Geraniol	200 (400)	200 (400)	50 (100)

[a]The concentration of polygodial is ½MIC.
[b]The number in the parentheses is the MIC of the compound alone.

combined with polygodial at the concentration of ½MIC against: *Sac. cerevisiae*, 1.56 μg/ml; *C. utilis*, 3.13 μg/ml; and *Pi. ovale*, 25 μg/ml. The antifungal activity of limonene against *Sac. cerevisiae* was not increased by polygodial, and that of other components was increased by only 2–4-fold. The activity of these compounds against *C. utilis* and *Pi. ovale* was also increased only 2–4-fold. Thus, the dramatic synergistic effects observed when actinomycin D was combined with polygodial (8) were not observed. In contast, as shown in Table 3, the antifungal activity of polygodial was significantly increased when combined with sub-inhibitory concentrations (equivalent to ½MIC against each fungDus) of the puchuri components. Phenolic compounds that do not possess a free phenolic group, such as anethole, safrole, and methyleugenol, seem to exhibit more enhancing effects with polygodial than phenolic compounds that have a free phenolic group, such as eugenol. In particular, the MICs (μg/ml) of polygodial against *Sac. cerevisiae* and *C. utilis* were lowered from 3.13 to 0.049 and from 6.25 to 0.20, respectively. This was a 64- and 32-fold increase, respectively, through combination with anethole. Anethole also enhanced the activity of polygodial against *Pi. ovale*, but to a lesser extent, with an increase in the activity of polygodial of only 8-fold. Methyleugenol showed a 128-fold enhancement of the activity of polygodial against *Pi. ovale*; its MIC (μg/ml) was lowered from 50 to 0.39, as shown in Table 3. Anethole was very effective in enhancing the antifungal activity of polygodial against *Sac. cerevisiae* and *C. utilis*, and methyleugenol was the most effective against *Pi. ovale*. Eugenol and the monoterpenes did not seem to have any meaningful enhancing activity. An explanation for these combination effects is currently under investigation.

Although polygodial itself is one of the most potent antifungal compounds, it became much more potent when it was combined with aromatic compounds such as anethole, safrole, and methyleugenol. Therefore, the combination of polygodial with these compounds may be a very effective means of controlling human pathogenic fungi.

TABLE 3. Antifungal Activity of Polygodial in Combination with Components of the Distillate of *Licaria puchuri-major*.

Component[a]	MIC against fungus tested (μg/ml)		
	Saccharomyces cerevisiae	*Candida utilis*	*Pityrosporum ovale*
None	3.13	6.25	50
Anethole	0.049	0.20	6.25
Safrole	0.39	0.78	6.25
Methyleugenol	0.78	1.56	0.39
Eugenol	1.56	3.13	25
Limonene	3.13	1.56	25
Geraniol	1.56	3.13	12.5

[a]The concentration of component was ½MIC.

EXPERIMENTAL

PLANT MATERIAL.—The seeds of *L. puchuri-major* were purchased in Belem, and also collected near Belem, Brazil. The plant was identified by Dr. J.M. Pines, Museu Paraense Emilio Goeldi, Belem where a voucher specimen was deposited.

CHEMICALS.—Polygodial [**1**] was previously isolated from various plant sources (4–7). The authentic safrole, methyleugenol, eugenol, anethole, limonene, linalool, 1,8-cineole, 4-terpineol, α-terpineol, and geraniol were purchased from Sigma Chemical Co. (St. Louis, MO). γ-Terpinene and caryophyllene were provided by Takasago International Corporation (Tokyo, Japan). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

MICROORGANISMS AND MEDIA.—All the microorganisms used for the antimicrobial assay were purchased from American Type Culture Collection (Rockville, MD). They are: *Ba. subtilis* ATCC 9372, *Br. ammoniagenes* ATCC 6872, *Sta. aureus* ATCC 12598, *Str. mutans* ATCC 25175, *Pr. acnes* ATCC 11827, *Esch. coli* ATCC 9637, *Ps. aeruginosa* ATCC 10145, *Ent. aerogenes* ATCC 13048, *Sac. cerevisiae* ATCC 7754, *C. utilis* ATCC 9226, *Pi. ovale* ATCC 14521, and *Pe. chrysogenum* ATCC 10106.

The freeze-dried microorganisms were reactivated in the following manner. *Ba. subtilis, Sac. cerevisiae, C. utilis, Pi. ovale* and *Pe. chrysogenum* were cultured with shaking at 30°. *Br. ammoniagenes* and *Ent. aerogenes* were grown in stationary culture at 30°. The remaining microorganisms were grown in stationary culture at 37°.

Bacteria, with the exception of *Str. mutans*, were grown on media consisting of 0.8% nutrient broth (BBL), 0.5% yeast extract (DIFCO) and 0.1% glucose. For the culture of *Str. mutans*, 3.7% brain heart infusion broth (DIFCO) was utilized. Fungi, with the exception of *Pi. ovale*, were grown on media consisting of 2.5% malt extract broth (BBL). For the culture of *Pi. ovale*, 1% bactopeptone (DIFCO), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were utilized.

ANTIMICROBIAL ASSAY.—The minimal inhibitory concentration (MIC) was measured by twofold serial broth dilution (15). The test compound was dissolved in DMF, and 1% of the sample solution was added to the appropriate medium. The highest concentration used for the assay was 800 μg/ml unless otherwise specified because of solubility limitation of the samples in DMF and/or the H_2O-based media. All microorganisms were grown in stationary culture, except *Pe. chrysogenum* which was cultured with shaking. After 2 days (5 days for *Pe. chrysogenum*), the growth of the microorganisms, except *Pi. ovale* and *Pe. chrysogenum*, was examined for turbidity (OD at 660 nm). That of these two fungi was determined with the naked eye. The lowest concentration of the test compound in which no growth occurred was defined as the MIC. It should be noted that the concentration of DMF in each test medium was always 1%; this did not affect the growth of any of the microorganisms tested.

The combination data were obtained by the checkerboard method (16). The twofold dilutions of polygodial were tested in combination with concentrations of twofold dilutions of the other compounds. Each fungus was tested at least twice.

GC-MS ANALYSIS.—Analytical gc of the distillate was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and fitted with a glass capillary column (0.25 mm i.d. × 30 m) which was coated with carbowax bonded 20 m. The carrier gas was He at 1.5 ml/min, and the oven was programmed to increase temperature from 50° to 230° at 4°/min. Gc-ms analysis

was carried out on a Hitachi M-808 double focusing instrument equipped with a Hewlett-Packard 5890 gas chromatograph. Gc conditions were identical to the above analytical gc runs. Mass spectral data were acquired and processed by a built-in computer system (M-0101) developed by Takasago International Corporation (Tokyo, Japan). The components of the distillate were identified on the basis of a comparison of the gc retention time and ms fragmentations with those of the authentic samples.

ACKNOWLEDGMENTS

We thank Dr. H. Tsuruta, Takasago International Corporation, for gc-ms measurements and for providing the authentic γ-terpinene and caryophyllene, and Dr. J.M. Pines, Museu Paraense Emilio Goeldi, for identification of the plant.

LITERATURE CITED

1. M.L. van den Berg, "Plantas Medicinais na Amazonia," Conselho National de Desenvolvimento Cientifico e Tecnologico, Programa Tropico Umido, 1982, p. 44.
2. L.A. Mitscher, R.P. Leu, M.S. Bathala, W.N. Wu, J.L. Beal, and R. White, *Lloydia*, **35**, 157 (1972).
3. L.A. Mitscher, S. Drake, S.R. Gollapudi, and S.K. Okwute, *J. Nat. Prod.*, **50**, 1025 (1987), and references cited therein.
4. I. Kubo, Y.W. Lee, M.J. Pettei, F.P. Pilkiewicz, and K. Nakanishi, *J. Chem. Soc., Chem. Commun.*, 1013 (1976).
5. I. Kubo, I. Miura, M.J. Pettei, Y.W. Lee, F.P. Pilkiewicz, and K. Nakanishi, *Tetrahedron Lett.*, 4553 (1977).
6. K. Nakanishi and I. Kubo, *Isr. J. Chem.*, **16**, 28 (1977).
7. C.S. Barnes and J.W. Loder, *Aust. J. Chem.*, **15**, 322 (1962).
8. I. Kubo and M. Taniguchi, *J. Nat. Prod.*, **51**, 22 (1988).
9. M. Taniguchi, Y. Yano, E. Tada, K. Ikenishi, S. Oi, H. Haraguchi, K. Hashimoto, and I. Kubo, *Agric. Biol. Chem.*, **52**, 1409 (1988).
10. M. Taniguchi, Y. Yano, K. Motoba, T. Tanaka, S. Oi, H. Haraguchi, K. Hashimoto, and I. Kubo, *Agric. Biol. Chem.*, **52**, 1881 (1988).
11. Y. Yano, M. Taniguchi, E. Tada, T. Tanaka, S. Oi, H. Haraguchi, K. Hashimoto, and I. Kubo, *Agric. Biol. Chem.*, **53**, 1525 (1989).
12. M.L. da Silva, J.G.S. Maria, C.M. de Malta Rezende, and O.R. Gottlieb, *Phytochemistry*, **12**, 471 (1973).
13. J.J. Kabara, D.M. Swieczkowski, A.J. Conley, and J.P. Truant, *Antimicrob. Agents Chemother.*, **2**, 23 (1972).
14. J.J. Kabara, in: "Antimicrobials in Foods." Ed. by A.L. Branen and P.M. Davidson, Marcel Dekker, New York, 1983, pp. 108–140.
15. M. Taniguchi and Y. Satomura, *Agric. Biol. Chem.*, **36**, 2169 (1972).
16. C.W. Norden, H. Wentzel, and E. Keleti, *J. Infect. Dis.*, **140**, 629 (1979).

Received 26 August 1991