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ANTIMICROBIAL AGENTS FROM *TANACETUM BALSAMITA*

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ABSTRACT.—A series of C_7–C_{11} α,β-unsaturated aldehydes was characterized together with several fatty alcohols and acids as antimicrobial agents from the dried flowers of a Brazilian medicinal plant, *Tanacetum balsamita*. Among them, the α,β-unsaturated aldehydes were found to exhibit a broad antimicrobial spectrum.

Many pathogenic microorganisms can be controlled with the antibiotics presently available. However, the need for new antibiotics has increased due to current problems of resistance associated with antibiotic use (1,2). Receiving less attention is the need for new antimicrobial agents in non-medical products, such as cosmetics and disinfectants. Concerns of non-medically related microbial resistance are significant because of widespread and unregulated use of antimicrobial agents by the general public. Thus, new and effective antimicrobial agents from non-microbial sources are needed (3). Compounds isolated from plants have the potential to fill this need as their structures are different from those from microbial sources, and hence their modes of action may very likely differ (4).

In our continuing search for antimicrobial agents from plants (5), the *n*-hexane extract of the bitter-tasting dried flowers of *Tanacetum balsamita* L. (Compositae), locally known as "catinga-de-mulata" in Brazil, showed a broad spectrum of activity when tested against fourteen selected microorganisms (6) at 2000 μg/ml. It should be noted that in addition to *T. balsamita*, at least two other dried flowers are also called "catinga-de-mulata" in Brazil. *T. vulgare* is botanically related to *T. balsamita* and is more widely distributed, while the other "catinga-de-mulata" is not related taxonomically to *Tanacetum* and belongs to the Labiatae. Interestingly, they all possess very distinct aromas by which they are named "catinga" (translated as "distinct odor" in English) and are all used for similar medicinal purposes (8). Despite their long use, there has been little previous study to verify their pharmacological effects.

The *n*-hexane extract of the dried flowers of *T. balsamita* was separated by Si gel cc and the active fractions were combined and divided into distillate and residue fractions by steam distillation, and subsequently evaluated for antimicrobial activity. At 2,000 μg/ml, the distillate was found to have retained the original broad antimicrobial activity, whereas the residue was shown to be devoid of activity. Thus, 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 chemical analysis was performed by gc-ms. As a result, eight α,β-unsaturated aldehydes, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, (*E*)-2-undecenal, (*E*),(*E*)-2,4-decadienal, (*E*),(*Z*)-2,4-decadienal, and 3-methyl-2-butenal; three fatty acids, hexanoic acid, octanoic acid, and palmitic acid; and one saturated aldehyde, hexanal, were identified, with the major components being hexanal and (*E*),(*E*)-2,4-decadienal. In contrast, the residue contained a known furanoditerpenoid, centipedaoic acid (9), as its main constituent.

The antimicrobial activities of the individual components identified in the distillate, except for (*E*),(*Z*)-2,4-decadienal which was available in only minute amounts, were tested against four-

teen selected microorganisms. The highest concentration tested was at 800 μg/ml because of limited test compound solubility in the water-based media. The minumum inhibitory concentration (MIC) results are listed in Table 1. However, since the MIC values are determined by measuring the turbidity for bacteria and by naked eye observation for fungi, such activities are not fully characterized. Therefore, the minimum lethal concentration (MBC for bacteria and MFC for fungi) values were also obtained. Bactericidal kinetic assays were also conducted for selected microorganisms.

The antimicrobial activity of the eleven compounds tested was variable. Notably, (*E*)-2-heptenal showed activity against all the microorganisms tested, while palmitic acid did not exhibit any activity up to 800 μg/ml. All the other compounds tested showed some activity against one or more microorganisms. All the non-isoprene α,β-unsaturated aldehydes exhibited broad activity.

Among the fourteen microorganisms employed, fungi were found to be the most susceptible, especially *Trichophyton mentagrophytes* and *Penicillium chrysogenum*. Nine of the compounds tested showed activity against these two filamentous fungi with MIC values ranging from 1.56 to 400 μg/ml. Among them, (*E*)-2-undecenal and (*E*),(*E*)-2,4-decadienal were the most potent with MIC values of 1.56 μg/ml each against *T. mentagrophytes*, and 6.25 μg/ml and 12.5 μg/ml, respectively, against *P. chrysogenum*. The α,β-unsaturated aldehydes tested also exhibited activity against *Candida utilis*, *Saccharomyces cerevisiae*, and *Pityrosporum ovale*, with MIC values ranging from 12.5 to 800 μg/ml. These MICs were also found to be the MFCs. This is a rather unexpected result since most plant secondary metabolites show, in general, more potent activity against Gram-positive bacteria than fungi (10,11). Besides the α,β-unsaturated aldehydes, hexanoic acid and octanoic acid exhibited some antifungal activity (6), while hexanal and palmitic acid did not show any noticeable activity.

The α,β-unsaturated aldehydes also showed activity against the Gram-positive bacteria tested with MICs ranging between 6.25 and 800 μg/ml. Among them, *Propionibacterium acnes* was the most sensitive and *Streptococcus mutans* was the least. The MBC and MIC ratio was no greater than two except against the spore-forming *Bacillus subtilis*. We have recently reported that long-chain alcohols

TABLE 1. Antimicrobial Activity of *T. balsamita* Constituents.

Microorganism[b]	Test Compound [MIC (μg/ml)][a]									
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**	**12**
Bs	200	100	50	25	25	50	800	200	200	>800
Ba	400	200	100	50	25	100	>800	400	400	>800
Sa	400	200	50	25	12.5	50	>800	>800	400	>800
Sm	400	400	100	100	50	100	>800	>800	1600	>800
Pac	100	50	25	12.5	6.25	12.5	800	400	200	800
Pae	800	>800	>800	>800	>800	>800	>800	>800	>800	>800
Ea	400	200	200	>800	>800	>800	>800	>800	>800	>800
Ec	400	200	200	>800	>800	>800	>800	>800	>800	>800
Pv	100	50	25	25	12.5	25	800	400	200	>800
Sc	100	50	25	25	25	25	800	400	200	>800
Cu	100	50	25	25	12.5	25	800	400	200	>800
Po	50	25	25	25	25	12.5	200	400	200	800
Pc	50	50	50	12.5	6.25	12.5	50	400	100	>800
Tm	25	25	12.5	3.13	1.56	1.56	200	100	50	>800

[a]Key to test compounds: **1** (*E*)-2-Heptenal; **2** (*E*)-2-octenal; **3** (*E*)-2-nonenal; **4** (*E*)-2-decenal; **5** (*E*)-2-undecenal; **6** (*E*),(*E*)-2,4-decadienal; **8** 3-methyl-2-butenal; **9** hexanoic acid; **10** octanoic acid; **12** hexanal.

Bs, Bacillus subtilis; Ba, Brevibacterium ammoniagenes; Sa, Staphylococcus aureus; Sm, Streptococcus mutans; Pac, Propionibacterium acnes; Pae, Pseudomonas aeruginosa; Ea, Enterobacter aerogenes; Ec, Escherichia coli; Pv, Proteus vulgaris; Sc, Saccharomyces cerevisiae; Cu, Candida utilis; Po, Pityrosporum ovale; Pc, Penicillium chrysogenum; Tm, Trichophyton mentagrophytes.

do not show bactericidal activity against this organism up to 800 μg/ml (12). This is not surprising as alcohols are known to have little effect on spores (13). The current study indicates that the aldehydes tested also lacked bactericidal activity against *B. subtilis*. In addition to the α,β-unsaturated aldehydes, hexanoic acid and octanoic acid showed some activity against the Gram-positive bacteria while hexanal and palmitic acid did not exhibit any significant activity.

Although a number of antimicrobial agents have been characterized from plants, only a few have shown activity against Gram-negative bacteria (7). *Proteus vulgaris* was the most susceptible, with the MIC values of the α,β-unsaturated aldehydes ranging from 12.5 to 800 μg/ml. In contrast, *Pseudomonas aeruginosa* was the least sensitive and (*E*)-2-heptenal was found to be the only compound active against this bacterium (MIC of 800 μg/ml). In addition to *P. vulgaris*, the growth of *Escherichia coli* and *Enterobacter aerogenes* was inhibited by (*E*)-2-heptenal, (*E*)-2-octenal, and (*E*)-2-nonenal. Most importantly, the activity of these three α,β-unsaturated aldehydes was found to be bactericidal. Thus, their MICs and MBCs were the same. Since few phytochemicals have been reported to exhibit antibacterial activity against Gram-negative bacteria, their activity was further studied. For example, the bactericidal effect of (*E*)-2-nonenal was confirmed by the time-kill curve method. Cultures of *E. coli*, with a cell density of 6×10^5 colony forming units (CFU) per ml, were exposed to two different concentrations of (*E*)-2-nonenal. The number of viable cells was determined following different periods of incubation with (*E*)-2-nonenal. Figure 1 shows that the MBC and MIC values are the same, wherein the 1/2 MIC slowed growth, but the final cell count was not significantly different from the control. These results also demonstrate that lethality occurs quickly, within the first 8 hours, indicating a membrane

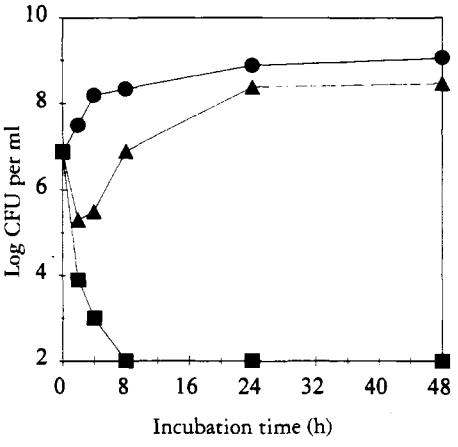

FIGURE 1. Effect of (*E*)-2-nonenal on the growth of *E. coli*. A 48-h culture was inoculated into NYG broth containing 200 μg/ml (■), 100 μg/ml (▲), and 0 μg/ml (*E*)-2-nonenal (●) (control).

disruptive effect. Compared to the α,β-unsaturated aldehydes, hexanal, the only saturated aldehyde characterized, did not show any activity against Gram-negative bacteria up to 800 μg/ml. Hexanoic acid exhibited activity against *E. coli* and *P. vulgaris* (MIC values of 800 and 400 μg/ml, respectively). Another fatty acid, octanoic acid, inhibited only the growth of *P. vulgaris* (MIC value of 200 μg/ml). From the above data, it appears that the original broad antimicrobial activity of the *T. balsamita* distillate may be ascribed predominately to the presence of α,β-unsaturated aldehydes.

These α,β-unsaturated aldehyde constituents of *T. balsamita* may have the potential to fill the need for new antimicrobial agents. However, their individual activities are not potent enough to be considered for practical use alone; studies to enhance their activity are required. Combining aldehydes with other substances in order to enhance the total biological activity seems to be a most promising strategic approach to this problem. The selection of other substances for such antibiotic combinations may be based on our previous studies (14). The antifungal activity against *C. albicans* and

S. cerevisiae of a cyclic sesquiterpene dialdehyde, polygodial, was dramatically enhanced through combination with a phenylpropanoid, anethole (15–17). The MIC of polygodial against *C. albicans* was reduced from 3.13 to 0.098 μg/ml when it was combined with the 1/2 MIC of anethole (15). Based on this previous observation, (*E*)-2-undecenal was tested against *C. utilis* and *S. cerevisiae* in combination with the 1/2 MIC of anethole. In contrast to polygodial, anethole did not significantly enhance the activity of (*E*)-2-undecenal against these fungi. In this combination, the MIC of (*E*)-2-undecenal was only reduced from 25 to 6.25 μg/ml against *C. utilis*, and from 25 to 1.56 μg/ml against *S. cerevisiae*. It appears that the synergistic activity of anethole against these fungi with the acyclic α,β-unsaturated aldehydes differs somehow from the cyclic sesquiterpene dialdehyde, polygodial. In addition, since anethole inhibited the growth of *E. coli*, (*E*)-2-octenal, and (*E*)-2-nonenal were also assayed in combination with the 1/2 MIC of anethole. However, both combinations were only additive against this Gram-negative bacterium. Even though these combinations did not significantly enhance antimicrobial activity, others may be quite efffective. This approach to developing therapeutic agents is promising because combinations inherently deter resistance, and because moderately potent compounds in combination may be equally effective but exhibit fewer side-effects than individually potent compounds. These α,β-unsaturated aldehyde constituents of *T. balsamita* should be studied further to determine their potential as antimicrobial agents.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The general procedures employed herein have been described previously (18–20). (*E*)-2-Heptenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, (*E*),(*Z*)-2,4-decadienal, hexanoic acid, octanoic acid, palmitic acid, and hexanal were purchased from Aldrich Chemical Co. (Milwaukee, WI). (*E*)-2-Undecenal, (*E*),(*E*)-2,4-decadienal, and 3-methyl-2-butenal were obtained from Wako Pure Chemical Industries (Osaka, Japan). For the antimicrobial experiments, all compounds were first dissolved in *N,N*-dimethylformamide (DMF) which was purchased from EM Science (Gibbstown, NJ).

PLANT MATERIAL.—The dried flowers of *T. balsamita* were purchased at marketplaces in Juazeiro do Norte, Brazil. The plant was identified by Dr. J.M. Pines, Museu Goeldi, Belem, Brazil, where the voucher specimen was deposited.

EXTRACTION AND ISOLATION.—The dried and pulverized flowers of *T. balsamita* (500 g) were extracted with *n*-hexane (4×) at room temperature. The solvent was removed under reduced pressure to give a brown crude extract (32.8 g), which was chromatographed over a Si gel (660 g, 230–400 mesh) column using *n*-hexane containing increasing quantities of EtOAc as eluent to give 8 fractions. Subsequent antimicrobial bioassays indicated fractions 1 and 2 to be active. The active fractions (4.8 g) were combined and steam-distilled to yield a distillate (0.5 g) and residue (3.9 g). Bioassay (2,000 μg/ml) showed the distillate to have retained the original spectrum of antimicrobial activity. In contrast, the residue containing primarily centipedaoic acid (9) exhibited no activity. Subsequent analysis to identify the constituents in the distillate was performed by gc-ms as described previously (19).

MICROORGANISMS AND MEDIA.—All microorganisms used for the assay were purchased from American Type Culture Collection (Rockville, MD): *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Proteus vulgaris* ATCC 13315, *Pityrosporum ovale* ATCC 14521, *Penicillium chrysogenum* ATCC 10106, and *Trichophyton mentagrophytes* ATCC 18748.

The culture medium for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose, with the exception of *S. mutans*. For the culture of *S. mutans*, 3.7% brain heart infusion broth (Difco) was used. The culture medium for the fungi was 2.5% malt extract broth (BBL), with the exception of *P. ovale* and *T. mentagrophytes*. For the culture of *P. ovale*, 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were used, and for *T. mentagrophytes*, 1% bactopeptone and 4% glucose were utilized.

ANTIMICROBIAL ASSAYS.—Throughout this experiment the broth dilution method was employed and the highest concentration of the indi-

vidual compounds tested was 800 μg/ml. The test compound was first dissolved in DMF and serial two-fold dilutions were performed in DMF. Thirty microliters of the sample solution were then added to sterile media, resulting in a 1% DMF concentration which did not affect the growth of any of the microorganisms employed. The growth test tube was inoculated with 1% of a 2-day-old culture of the test organisms (5-day-old for *P. chrysogenum* and *T. mentagrophytes*) and then incubated at 30 or 37°, depending on optimal growth conditions. All microorganisms were cultured stationary except *P. chrysogenum* and *T. mentagrophytes*, which were cultured with shaking. After 2 days of cultivation (3 days for *P. ovale* and 5 days for *P. chrysogenum* and *T. mentagrophytes*), the growth of the microorganisms, except *P. chrysogenum* and *T. mentagrophytes*, was examined by turbidity (OD at 660 nm). That of *P. chrysogenum* and *T. mentagrophytes* was examined with the naked eye. The MIC value was the lowest concentration of the test compound that completely prevented growth.

The bactericidal or fungicidal effects of the test samples were examined as follows. After determining the MIC, a 30-μl aliquot was taken from each clear tube and added into 3 ml of fresh, sample-free medium. After 2 days (3 days for *P. ovale* and 5 days for *P. chrysogenum* and *T. mentagrophytes*) of incubation, the MBC or MFC was determined as the lowest concentration of the test sample in which no recovery of microorganisms was observed.

A bactericidal kinetic assay for *E. coli* was performed in NYG broth containing appropriate concentrations of test compound. The initial inoculum was approximately 6×10^5 CFU/ml. Sample was removed at 0, 2, 4, 8, 24, and 48 hours of incubation The number of viable cells was determined by serial ten-fold dilutions and plating onto NYG agar. The plates were incubated at 37° for 24 h before counting.

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