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Antibacterial and antifungal activity of sulfur-containing compounds from *Petiveria alliacea* L.

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Abstract

A total of 18 organosulfur compounds originating from *Petiveria alliacea* L. roots have been tested for their antibacterial and antifungal activities. These represent compounds occurring in fresh homogenates as well as those present in various macerates, extracts and other preparations made from *Petiveria alliacea*. Of the compounds assayed, the thiosulfinates, trisulfides and benzylsulfinic acid were observed to be the most active, with the benzyl-containing thiosulfinates exhibiting the broadest spectrum of antimicrobial activity. The effect of plant sample preparation conditions on the antimicrobial activity of the extract is discussed.
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1. Introduction

Petiveria alliacea L. (Phytolaccaceae) is a perennial shrub that grows primarily in South and Central America, some areas of Africa, and the southeastern United States. It is popularly used in folk medicine for treating a wide variety of disorders. Various preparations from this plant reportedly exhibit anti-inflammatory, anticancer and stimulant effects, among many others. Recently, we reported the isolation and characterization of several novel sulfur-containing amino acids from *Petiveria alliacea* roots, including $(R_S R_C)/(S_S R_C)$-*S*-benzylcysteine sulfoxides (petiveriins A and B) (**1**) and $(R_S R_C)/(S_S R_C)$-*S*-(2-hydroxyethyl)cysteine sulfoxides (6-hydroxyethiins A and B) (**2**). We also showed that these cysteine derivatives are enzymatically cleaved by a C–S lyase enzyme upon disruption of the tissue, yielding four thiosulfinates (**4**–**7**) (Kubec and Musah, 2001; Kubec et al., 2002). Additionally, we identified the lachrymatory principle of the plant as the unique sulfine, (*Z*)-thiobenzaldehyde *S*-oxide (**3**), which is yet another likely derivative of the petiveriins (Kubec et al., 2003). Compounds (**3**)–(**7**) are the main sulfur-containing components present in freshly prepared homogenates of *Petiveria alliacea*, with (**3**) and (**6**) predominating. However, both thiosulfinates and sulfines are known to be labile compounds that undergo many subsequent reactions, particularly when subjected to heat and/or storage in non-polar solvents. Thus, compounds (**3**)–(**7**) can readily decompose, giving rise to a variety of secondary products including sulfides, benzylsulfinic and sulfonyl acids, (*E/Z*)-stilbenes, and benzaldehyde (Smythe, 1922; Kice et al., 1960; Furukawa et al., 1973; Chatgilialoglu et al., 1980; White and Dellinger, 1985).

There are considerable discrepancies in the bioactivity assay results reported for *Petiveria alliacea*-derived compounds and extracts. Whereas some authors have reported the plant to be devoid of antimicrobial activity (Torre Melis et al., 1994; Pérez and Anesini, 1994a, 1994b; Cáceres et al., 1998), others have observed significant antimicrobial properties in various *Petiveria alliacea* extracts (Misas et al., 1979). Moreover, several polysulfides, some of which were demonstrated to exhibit relatively strong antifungal activity, have been isolated from *Petiveria alliacea* roots (Szczepanski et al., 1972; Benevides et al., 2001).

From our previous studies, we are aware that the compounds present in *Petiveria alliacea* extracts vary as a function of differences in sample preparation. We propose that the aforementioned discrepancies in *Petiveria alliacea* antimicrobial assay results are a consequence of differences in extraction and sample preparation procedures that ultimately result in significant

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changes in the relative concentrations of bioactive compounds present, with freshly prepared plant macerates possessing a different organosulfur compound profile than those from dried plant and/or heat-treated extracts. Thus, we embarked on the present study in order to determine the possible antimicrobial activity of the compounds present in freshly prepared macerates, and to also determine the antimicrobial activity of their major decomposition products.

2. Materials and methods

2.1. Chemicals

$(R_C R_S)/(R_C S_S)$-*S*-Benzylcysteine sulfoxide (**1**), $(R_S R_C)/(S_S R_C)$-*S*-(2-hydroxyethyl)cysteine sulfoxide (**2**) and (*Z*)-thiobenzaldehyde *S*-oxide (**3**) were prepared as previously described (Kubec and Musah, 2001; Kubec et al., 2002, 2003). All four thiosulfinates [*S*-(2-hydroxyethyl) phenylmethanethiosulfinate (**4**), *S*-benzyl (2-hydroxyethane)thiosulfinate (**5**), *S*-benzyl phenylmethanethiosulfinate (**6**), and *S*-(2-hydroxyethyl) 2-(hydroxyethane)thiosulfinate (**7**)] were synthesized as described previously (Kubec et al., 2002). Benzyl sulfide (**8**), bis(2-hydroxyethyl) sulfide (**10**), benzyl disulfide (**11**) and benzyl trisulfide (**14**) were purchased from Aldrich. Benzyl 2-hydroxyethyl sulfide (**9**), benzyl 2-hydroxyethyl disulfide (**12**), bis(2-hydroxyethyl) disulfide (**13**), benzyl 2-hydroxyethyl trisulfide (**15**) and bis(2-hydroxyethyl) trisulfide (**16**) were obtained as described in the literature (Ayodele et al., 2000). Sodium benzylsulfonate (**17**) was synthesized according to a published method (Johnson and Ambler, 1914), and benzylsulfinic acid (**18**) was prepared by hydrolysis of benzylsulfinyl chloride. The identities of the compounds were confirmed by ^{1}H, ^{13}C NMR and IR spectroscopy. Their purity was >99%.

2.2. Microbial strains

The microorganisms tested were obtained from the American Type Culture Collection (Manassas, VA, USA). They were *Aspergillus flavus* (ATCC 9643s), *Mucor racemosus* (ATCC 7924), *Pseudallescheria boydii* (ATCC 760), *Issatchenkia orientalis* (ATCC 6258), *Candida tropicalis* (ATCC 750), *Candida albicans* (ATCC 10231), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698), *Mycobacterium smegmatis* (ATCC 19420), *Streptococcus agalactiae* (ATCC 13813), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Stenotrophomonas maltophila* (ATCC 13637), and *Klebsiella pneumoniae* (ATCC 13883).

2.3. Disk diffusion tests

Assays were performed according to the standard guidelines (M2-A7) of the National Committee for Clinical Laboratory Standards (NCCLS), using a modified Kirby–Bauer disk diffusion method. All the organisms were stored at −80 °C until use. BBL blank paper discs (6 mm diameter) were purchased from Becton Dickinson and Company (Sparks, MD, USA). Cells were grown at 30 °C in Mueller–Hinton broth to an $OD_{420} = 1.9$ (approximately 10^5 CFU/mL), and were passaged at least twice on proper agar (brain heart infusion agar for bacteria, Sabouraud dextrose agar for yeasts, and potato dextrose agar for fungi). Broth cultures were swabbed onto agar to achieve a lawn of confluent bacterial growth. Paper disks impregnated with a test compound (50 and 200 μg) or antibiotic (10 μg for gentamycin and ampicillin, 30 μg for chloramphenicol and tetracycline) were placed on each plate. The plates were incubated at 30 °C for 24 h. Active compounds were those for which a zone of inhibition of >6 mm was observed.

2.4. Determination of minimum inhibitory concentration (MIC) values

The microdilution broth methods (M7-A5 for bacteria, M27-A for yeasts, and M38-P for fungi) of the NCCLS were employed. Briefly, 100-μL aliquots of inoculum dilution that were twice the test concentration were dispensed into sterile 96-well plates (Corning Incorporated, Corning, NY, USA) to final inoculum concentrations of approximately 5×10^5 CFU/mL for bacteria, 2.5×10^3 CFU/mL for yeasts, and 5×10^4 CFU/mL for fungi. Determination of MIC was performed with a dual-wavelength microplate reader (model EL311; Bio-Tek Instruments, Winooski, VT, USA), measuring the optical density at 570 nm, with reference reading at 690 nm. MIC is defined as the lowest concentration that completely inhibits organism growth. Thus, the growth in each well was compared with that of the control (drug-free) well. Tetracycline and amphotericin B (Sigma) were used as controls for the antibacterial and antifungal tests, respectively.

3. Results

In the present study, we investigated the antibacterial and antifungal properties of 18 sulfur-containing compounds originating from *Petiveria alliacea* L. roots. The compounds include those present in intact tissue (**1** and **2**), as well as their primary decomposition products, namely (*Z*)-thiobenzaldehyde *S*-oxide (**3**) and thiosulfinates (**4**–**7**), which occur in freshly disrupted plant tissue. Additionally, the degradation products of (**3**–**7**), which are likely to be present in various *Petiveria alliacea* preparations (**8**–**18**), were also tested. The structures of the tested compounds are shown in Fig. 1.

The antibacterial activity of these compounds was initially evaluated by the disk diffusion method, using five strains of Gram-positive bacteria (*Bacillus cereus*, *Mycobacterium smegmatis*, *Micrococcus luteus*, *Streptococcus agalactiae*, *Staphylococcus aureus*) and four strains of Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophila*, *Klebsiella pneumoniae*). (*Z*)-Thiobenzaldehyde *S*-oxide (**3**), the three benzyl-containing thiosulfinates (**4**–**6**), two trisulfides (**15**, **16**), and benzylsulfinic acid (**18**) exhibited relatively strong antibacterial activity. Unlike its benzyl containing counterparts, the fourth thiosulfinate tested, *S*-(2-hydroxyethyl) 2-(hydroxyethane)thiosulfinate (**7**), showed only weak activity.

Nonvolatile precursors:

R = —CH₂—⟨phenyl⟩ (**1**)

—CH₂CH₂OH (**2**)

Primary products:

(**3**)

R¹ = —CH₂—⟨phenyl⟩, R² = —CH₂CH₂OH (**4**)

R¹ = —CH₂CH₂OH, R² = —CH₂—⟨phenyl⟩ (**5**)

R¹ = R² = —CH₂—⟨phenyl⟩ (**6**)   R¹ = R² = —CH₂CH₂OH (**7**)

Secondary products:

R^1-(S)$_n$—R^2 R¹ = —CH₂—⟨phenyl⟩, R² = —CH₂CH₂OH, n = 1 (**9**), n = 2 (**12**), n = 3 (**15**)

R¹ = R² = —CH₂—⟨phenyl⟩, n = 1 (**8**), n = 2 (**11**), n = 3 (**14**)

R¹ = R² = —CH₂CH₂OH, n = 1 (**10**), n = 2 (**13**), n = 3 (**16**)

⟨phenyl⟩—CH₂SO₃Na (**17**)   ⟨phenyl⟩—CH₂SO₂H (**18**)
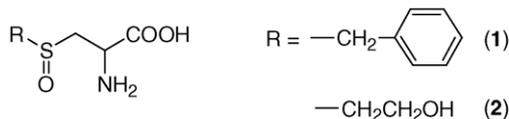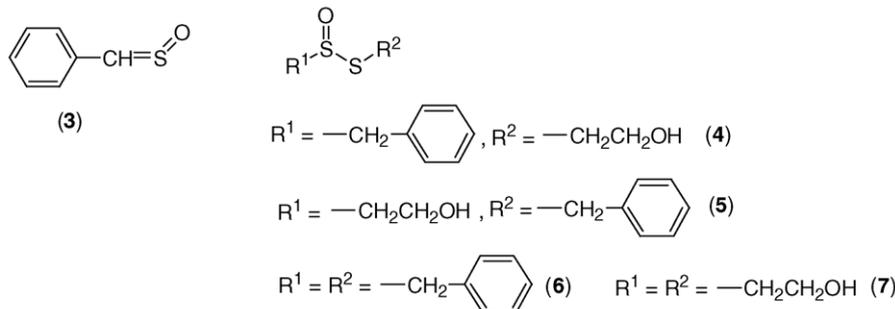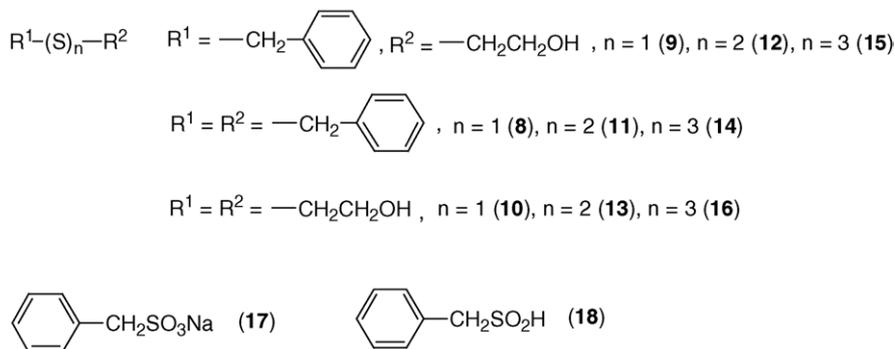
Fig. 1. Structures of the *Petiveria alliacea*-derived organosulfur compounds tested.

The other compounds were not found to be active at the levels tested. With the exception of *Pseudomonas aeruginosa*, the growth of Gram-negative bacteria was significantly inhibited by unsymmetrical thiosulfinates (**4** and **5**).

Based on the results of the disk diffusion assay, the antibacterial activity of 8 compounds (**3**–**7**, **15**, **16**, and **18**) out of the total of 18 were further evaluated to determine the minimum inhibitory concentration (MIC) of each. The results of this assay are outlined in Table 1. MIC is defined as the minimum concentration of a compound required for complete inhibition of microorganism growth. Since none of the compounds were observed to be active against *Pseudomonas aeruginosa*, this bacterium was not included in the MIC assay. Compounds (**4**)–(**6**) were found to be the most active. No significant difference

Table 1
Minimum inhibitory concentration (in µg/mL) of *Petiveria alliacea*-derived compounds against Gram-negative and Gram-positive bacteria

#	*Bacillus cereus*	*Mycobacterium smegmatis*	*Micrococcus luteus*	*Streptococcus agalactiae*	*Staphylococcus aureus*	*Escherichia coli*	*Stenotrophomonas maltophila*	*Klebsiella pneumoniae*
3	512	512	512	512	512	—[b]	—[b]	—[b]
4	16	128	16	16	64	32	32	128
5	16	128	16	32	64	32	32	128
6	16	256	16	16	32	—[b]	—[b]	—[b]
7	128	128	128	—[b]	—[b]	128	128	256
15	128	128	64	256	256	—[b]	—[b]	—[b]
16	32	64	128	—[b]	128	256	128	256
18	128	128	32	64	64	—[b]	—[b]	—[b]
Te[a]	0.25	0.5	2	1	2	2	4	1

[a] Tetracycline.
[b] No inhibition observed.

Table 2
Minimum inhibitory concentration (in µg/mL) of *Petiveria alliacea*-derived compounds against fungi

#	*Aspergillus flavus*	*Mucor racemosus*	*Pseudallescheria boydii*	*Candida albicans*	*Candida tropicalis*	*Issatchenkia orientalis*
1	256	128	256	256	256	256
3	128	128	64	128	64	64
4	32	32	32	16	32	32
5	64	32	32	16	32	16
6	32	16	16	16	16	16
7	>512	512	>512	>512	>512	>512
8	256	256	64	128	128	128
9	>512	512	512	>512	>512	>512
11	128	256	512	256	256	256
12	128	256	256	128	128	128
13	>512	>512	32	>512	>512	>512
14	256	64	64	128	256	128
15	128	64	32	16	32	32
16	>512	>512	16	8	8	16
18	>512	64	16	2	4	2
Ap[a]	4	8	4	<1	<1	<1

[a] Amphotericin B.

between the activities of the two regiomeric thiosulfinates (**4** and **5**) was observed. Sulfine (**3**) showed activity only against some of the Gram-positive bacteria. In general, the compounds tested exhibited only modest antibacterial activity, compared with that of the reference compound tetracycline.

Antifungal activities of the compounds tested are summarized in Table 2. Those compounds showing no antifungal activity by the disk diffusion assay method (**2**, **10**, **17**) are omitted from the table. As can be seen, the sulfine (**3**), the benzyl-containing thiosulfinates (**4–6**), all three trisulfides (**14–16**) and benzylsulfinic acid (**18**) possess varying degrees of activity, with (**18**) being the most active. Interestingly, one of the cysteine sulfoxide precursors, *S*-benzylcysteine sulfoxide (petiveriin, **1**), also exhibited modest antifungal properties.

4. Discussion

The purpose of this study was to assess the antimicrobial potential of various sulfur-containing compounds derived from *Petiveria alliacea* L. We believe the compounds tested in the present study to be the most important of these. They may serve, in part, as a component of the defense system of the plant, protecting it from fungi and predators.

The primary products of enzymatically mediated decomposition of petiveriins A and B [i.e. the sulfine (**3**) and thiosulfinates (**4–6**)], were found to be the most active, whereas the products of further thiosulfinate decomposition (i.e. sulfides and disulfides) exhibited much lower or no activity in the antimicrobial assays. Our study has confirmed the antifungal activity of benzyl 2-hydroxyethyl trisulfide (**15**) that was first reported by Szczepanski et al. (1972) and subsequently corroborated by Ayodele et al. (2000). All three trisulfides tested (**14–16**) were significantly more active than the corresponding mono- and disulfides (**8–13**).

There are two important distinctions between our studies, and those in which *Petiveria alliacea* extracts were reported to be devoid of antimicrobial activity (Torre Melis et al., 1994; Pérez and Anesini, 1994a, 1994b; Cáceres et al., 1998). Firstly, our study focused on compounds that have been observed in *Petiveria alliacea* fresh root macerates, whereas the previous studies focused on leaf extracts. Because the cysteine sulfoxides (**1** and **2**) that serve as precursors of the organosulfur compounds are present in the leaves in only very low concentrations, the primary products of their C–S lyase-mediated decomposition, such as thiosulfinates (**4–7**), are present at much lower levels compared to the root. Indeed, we have found that the concentration of *S*-benzylcysteine sulfoxides in the root is ∼3 mg/g fresh weight whereas in the leaves it is on the order of 0.08 mg/g fresh weight (Kubec and Musah, 2001). Given our observation that the thiosulfinates possess the highest antibacterial activity, the reported absence of antibacterial activity in *Petiveria alliacea* leaf extracts may be a consequence of significantly lower levels of cysteine sulfoxide precursors in the leaves of the analyzed plants. The second important difference between our study, and those in which *Petiveria alliacea* extracts were reported not to have antibacterial activity, is related to sample preparation. Whereas the compounds used in our study are those found both in extracts of fresh macerates and also in heat-treated samples, other studies were based on an analysis of dried samples, or fresh samples that were extracted after heating in water or alcohol (Torre Melis et al., 1994; Pérez and Anesini, 1994a, 1994b; Cáceres et al., 1998). The cascade of reactions that ultimately results in the formation of the organosulfur compounds that we have observed in fresh macerates is mediated by C–S lyase enzymes. Inactivation of these enzymes through sample drying and/or application of heat would effectively curtail the formation of the sulfine (**3**), thiosulfinates (**4–7**), and consequently, other antimicrobially active compounds (e.g. **15** and **16**). Thus, the reported absence of antimicrobial activity in previous studies may have been the result of the absence of antimicrobial compounds, since these compounds would not be present in the extracts if the enzymes that mediate their formation were destroyed through treatment with heat.

Based on our findings, we suggest that the discrepancies in the data that have so far been published on the antimicrobial activity of *Petiveria alliacea* could be the result of differing procedures used during preparation of the tested samples. Whereas freshly prepared samples are likely to contain mostly the sulfine (**3**) and thiosulfinates (**4–7**), other preparations (obtained following heat treatment, distillation, and/or drying of plant material) will mainly consist of various sulfides, stilbenes, benzaldehyde and other secondary decomposition products. This implies that fresh samples prepared by mild procedures are likely to show a significantly higher degree of antimicrobial activity than those prepared employing harsher conditions. The latter ones may, however, still possess significant antifungal properties. Furthermore, root-derived preparations are likely to exhibit much higher antimicrobial activity compared to leaf extracts.

Acknowledgements

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