Pergamon

PII: S0031-9422(97)00534-7

*Phytochemistry*, Vol. 47, No. 4, pp. 631–634, 1998
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Printed in Great Britain
0031-9422/98 $19.00+0.00

# ANTIBACTERIAL CONSTITUENTS OF THE NEPALESE MEDICINAL HERB, *CENTIPEDA MINIMA*

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(*Received* 25 *February* 1997; *In revised form* 2 *June* 1997)

**Key Word Index**—*Centipeda minima*; Asteraceae; Nepal; Ethnomedicine; antibacterial; sesquiterpene lactones; 6-*O*-methylacrylylplenolin; 6-*O*-isobutyroylplenolin; 6-*O*-angeloylplenolin.

**Abstract**—*Centipeda minima*, a herb used medicinally to treat sinus infections in Nepal, was found to contain three antibacterial sesquiterpene lactones, identified as 6-O-methylacrylylplenolin, 6-*O*-isobutyroylplenolin, and 6-*O*-angeloylplenolin. 6-*O*-Methylacrylylplenolin had not been previously isolated from *C. minima*. All three had activity against *Bacillus subtilis* and *Staphylococcus aureus*, with 6-*O*-isobutyroylplenolin being the most active. © 1997 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

In the antimicrobial screening of medicinal plants of Nepal [1, 2], the crude methanolic extract of the whole plant of *Centipeda minima* (L.) A. Br. and Aschers (Asteraceae) was determined to be active against the bacteria *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium phlei* and *Microsporum gypseum*. The activity against *B. subtilis* was enhanced upon exposure to UV-A radiation. The plant has been documented as being used medicinally in Nepal to treat coughs, sinus infections and colds [1], headache [3] and blocked nose resulting from coughs and colds [4]. *C. minima* is also used throughout South East Asia to treat colds, nasal allergies and asthma [5]. There have been several studies of *C. minima* based on the fact that it is used as an anti-allergy treatment. Wu and coworkers [5, 6] have shown that extracts of *C. minima* inhibit histamine release, with flavonoids, sesquiterpene lactones and an amide being the active components. The same research group also determined that sesquiterpenes were responsible for the activity of *C. minima* as a platelet activating factor (PAF) antagonist [7].

There have been no reports of *C. minima* containing known antimicrobial compounds. The antibacterial constituents, determined through bio-activity guided fractionation, are described.

## RESULTS AND DISCUSSION

The three antibacterial compounds isolated were determined to be sesquiterpene lactones of the plenolin (13b-11,13-dihydrohelenalin) ester form (Fig. 1). 6-*O*-Methylacrylylplenolin (**1**) (α-methylacrylylplenolin, or arnicolide D), 6-*O*-isobutyroylplenolin (**2**) (arnicolide C), and 6-*O*-angeloylplenolin (**3**) (brevifolin, brevilin-A), were isolated in yields of 28.7 μg $g^{-1}$, 29.3 μg $g^{-1}$ and 27.7 μg $g^{-1}$ from air dried plant material. The $[M]^+$ of compound **1**, 332.16226, gave a molecular formula of $C_{19}H_{24}O_5$. The proton NMR spectrum compared well to that reported by Poplawski *et al.* [8]. Compound **2** was determined to be 6-*O*-

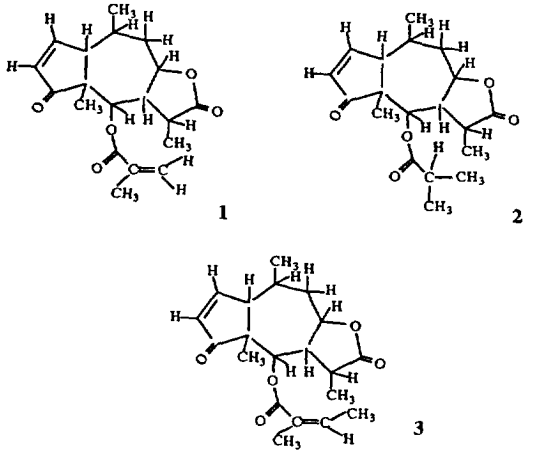

Fig. 1. Three antibacterial sesquiterpene lactones isolated from *Centipeda minima*; 6-*O*-methylacrylylplenolin (**1**), 6-*O*-isobutyroylplenolin (**2**), and 6-*O*-angeloylplenolin (**3**).

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isobutyroylplenolin based on mass spectral and NMR data. The $[M]^+$ peak of 334.17760 gave a molecular formula of $C_{19}H_{26}O_5$. The proton NMR spectrum compares well to that reported by Wu *et al.* [6]. The structure of compound **3** was assigned based on high resolution mass spectral and NMR data. The $[M]^+$ peak of 346.17775 gave a molecular formula of $C_{20}H_{26}O_5$. The proton NMR spectrum has been previously reported by Itoigawa *et al.* [9].

Compound **1** has not previously been isolated from a species of *Centipeda*. Poplawski *et al.* [8] isolated it from *Arnica montana* L. (Asteraceae), along with **2**. Herz and Sosa [10] isolated **1** from *Arnica acaulis* (Walt.) B.S.P., together with compounds **2** and **3**. Compound **2** had been previously isolated from *C. minima* by Bohlmann and Zhongliang [11]. It was previously known from *Arnica montana* [8]. Compound **1** has not previously been reported to have antibacterial activity. Compound **2** was reisolated from *C. minima* by Wu *et al.* [5] in a bioactivity guided fractionation looking for compounds showing significant antiallergy activity. While plenolin type sesquiterpene lactones are known to possess antibacterial activity, compound **2** has not been tested previous to this investigation. Compound **3** had been previously isolated from *C. minima* by Bohlmann and Zhongliang [11]. It was previously known from *Helenium brevifolium* [2], *H. alterniflolium* [13], and *H. autumnale* L. [9]. 6-*O*-Angeloylplenolin (**3**) was reisolated from *C. minima* by Iwakami *et al.* [7] in bioactivity guided fractionation research looking for compounds showing inhibitory activity on the binding of platelet activating factor (PAF) to rabbit platelets, a bioassay used to determine antiallergy compounds.

Sesquiterpene lactones possessing cytotoxic or antimicrobial activity number approximately 150 [14]. The compounds isolated from *C. minima* belong to the largest class of sesquiterpene lactones, the pseudoguaianolides [15]. Several pseudoguaianolides, such as helenalin and plenolin, have significant antibacterial activity. Both these compounds have an MIC of 100 $\mu g\ ml^{-1}$ against both *B. subtilis* and *S. aureus* [16]. Compounds **1** and **2** show similar activities, with MIC values against *B. subtilis* of 150 $\mu g\ ml^{-1}$. Compound **3** was less active than the parent plenolin structure, with an MIC of 300 $\mu g\ ml^{-1}$. All three compounds showed activity against both methicillin resistant and methicillin sensitive strains of *S. aureus* (Table 1).

The antimicrobial activity of sesquiterpene lactones seems to depend on the presence of a *beta* unsubstituted cyclopentenone ring moiety. Lee *et al.* [16] determined that the saturated compound corresponding to helenalin gave at least a 10-fold decrease in antibacterial activity. This antibacterial activity appears to be independent of the presence or absence of an α-methylene-γ-lactone or α-methyl-γ-lactone moiety. This is demonstrated by the fact that both helenalin and plenolin have the same activity against both *B. subtilis* and *S. aureus* [16]. However, this α-methylene-γ-lactone moiety is needed for significant cytotoxic activity [14], and antitumour activity [17].

The isolated sesquiterpene lactones did not account for the total antibacterial activity of the crude methanol extract. The activity of the sesquiterpene lactones was not increased in the presence of UV radiation, while the activity of the crude extract was. This light-enhanced activity was found in the petrol fraction after solvent partition [18]. This activity could possibly be attributed to photoactive polyynes. These compounds are known to be light active against bacteria and fungi, are soluble in petrol, and are common in the Asteraceae [19, 20]. The MICs of the isolated compounds were, however, lower than that of the original crude extract.

All three of the isolated compounds were determined to be bactericidal, not merely bacteriostatic. This was determined by trying to culture the media from the 96 well trays after 48 hr incubation. If the compounds were merely bacteriostatic, the bacteria would resume growth after being removed from the test compound. This was not the case. It would also be interesting to test these compounds for antileukaemia activity, as the crude extract of *C. minima* was quite active (unpublished results).

## EXPERIMENTAL

*General.* $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker AMX-500 (500 MHz) $CDCl_3$. The chemical shifts were recorded on a δ scale calibrated to $CDCl_3$ (7.24) as an int. standard. MS at 70 eV. UV spectra from HPLC fractions obtained using a Waters 994 PDA detector. Silica gel (70–230 mesh) was the stationary phase for CC. TLC was performed using silica gel G60 $F_{254}Al_2O_3$ backed plates. UV at 254 and 366 nm, with TLC plates stained with vanillin–$H_2SO_4$ spray reagent. VLC was performed using TLC grade silica (Merck Kiesel gel 60) as the stationary phase.

Table 1. Minimum inhibitory concentrations of the isolated sesquiterpene lactones in $\mu g\ ml^{-1}$

| Compound | *Staphylococcus aureus* (methicillin resistant) | *Staphylococcus aureus* (methicillin sensitive) |
|---|---|---|
| **1** | 300 | 75 |
| **2** | 300 | 38 |
| **3** | 600 | 75 |
| Gentamicin | Not active | 9 |

HPLC was performed using a Waters 660E controller, and the Waters 994 programmable photodiode array (PDA) detector, with either a Waters RCm 8 × 10, or 25 × 10, NV $C_{18}$ column. The UV detector at 220 nm.

*Antibacterial assays.* All fractions obtained from sepn procedures were assayed for antibacterial activity. TLC bioautography agar overlay was used to follow the bioactivity as fractionation occurred. The method used was from Saxena *et al.* [21]. Müller–Hinton medium (BBL) was prepd with 0.6% Bacto agar (6 g $l^{-1}$) and 0.002% phenol red (0.002 g $l^{-1}$). The medium was autoclaved at 121° for 15 min and then maintained as a liquid at 45° in a $H_2O$ bath prior to use. Bacterial cultures were grown overnight in Müller–Hinton medium and diluted to $10^6$ cells $ml^{-1}$ by serial dilution just before use. The final concn of bacteria in agar containing medium was $10^5$ cells $ml^{-1}$. Two identical chromatograms were run in appropriate solvent for each overlay. UV active compounds were detected at 254 and 366 nm on the reference chromatogram, which was then stained with vanillin–$H_2SO_4$ spray reagent. The other set was used for overlay. The bacteria used for the TLC overlay were *Bacillus subtilis*, standard laboratory strain from The University of British Columbia microbiological collection, Department of Microbiology, and methicillin resistant *Staphylococcus aureus*, a gift from Dr R. E. W. Hancock, Department of Microbiology, The University of British Columbia.

Minimum inhibitory concentrations of the purified compounds were determined by a modification of the method of Towers *et al.* [22]. A series of nine two-fold dilutions of the test compounds (dissolved in DMSO at a concn of 25 mg $ml^{-1}$ and diluted with Müller–Hinton broth (BBL) to a concentration of 2.5 mg $ml^{-1}$) and the control (gentamicin, 1 mg $ml^{-1}$ in sterile $H_2O$) were made with medium in a 96 well plate (Falcon 3072). Four replicates were performed, each one having 100 μl of serial dilution. One row was set up with medium only (control). A 18 hr culture of the bacteria, grown in Müller–Hinton broth at 37°, was diluted to $\sim 2 \times 10^4$ cells $ml^{-1}$ in fresh medium. Aliquots (100 μl) of this were added to the wells of the 96 well plate. The optical density (O.D.) at 429 nm of each solution in a test well was read before incubation, and both 24 and 48 hr later.

*Plant material and isolation procedures.* Whole plant material of *C. minima* (300 g dry wt) was collected from Devighat, central Nepal, in June of 1993. Vouchers have been filed in the National Herbarium and Plant Laboratories Godawari, Nepal, and in The University of British Columbia Herbarium (Vancouver, Canada).

*Extraction and isolation.* Plant material was air dried and ground in a Wiley grinder with a 2 mm wire mesh. The powder (300 g) was exhaustively extracted with 1000 ml aliquots of MeOH over a period of several days. Each extraction took a minimum of 24 hr. The sample was then suction filtered through Whatman No. 1 filter paper, and washed with another 1000 ml MeOH. The filtrate was evapd to dryness under red. pres. The crude methanolic extract was resuspended in MeOH–$H_2O$ (3:2). This was then partitioned successively in order of increasing polarity with petrol and $CH_2Cl_2$. The active $CH_2Cl_2$ fr. was then put through further bioactivity guided fractionation. A silica column (150 g) was run with $CH_2Cl_2$, with increasing percentage of MeOH, starting at 1% and doubling. The resulting frs (15 frs of 500 ml each) were collected and tested by the TLC overlay method. The active fr. was eluted with 4% MeOH in $CH_2Cl_2$ (fr. 6). This fr. (782 mg) was run through another silica column (120 g) and 45 frs of 20 ml each were collected. The activity was once again eluted at 4% MeOH in $CH_2Cl_2$ (frs 4–10). These frs were combined (41 mg) and run on a VLC (20 g), using $C_6H_6$ and EtOAc as the solvent, in increasing increments similar to the silica columns. Twenty frs of 20 ml were collected. The activity was located in frs 9 and 10, which were combined and run on the prep. HPLC. The resulting run on the HPLC (Waters RCm 8 × 10 NV $C_{18}$ column) with an isocratic solvent ratio of 7:13 (MeCN–$H_2O$) and an elution rate of 3 ml $min^{-1}$ gave three bioactive peaks, **1** ($R_t$ of 12.04 min, 8.6 mg), **2** ($R_t$ of 13.43 min, 8.8 mg) and **3** ($R_t$ of 17.64 min, 8.3 mg).

*Acknowledgements*—We thank the Natural Sciences and Engineering Research Council for the award of an NSERC Fellowship to one of us (R. T.) and for financial support of this research. Thanks are due to Prof. N. P. Manandhar, National Herbarium and Plant Laboratories, Godawari (Nepal), for his guidance with the ethnomedicinal field work. We also thank the people of the Langtang region of Nepal for their ethnobotanical information.

## REFERENCES

1. Taylor, R. S., Manandhar, N. P. and Towers, G. H. N., *Journal of Ethnopharmacology*, 1995, **46**, 153.
2. Taylor, R. S. L., Edel, F., Manandhar, N. P. and Towers, G. H. N., *Journal of Ethnopharmacology*, 1996, **50**, 97.
3. Manandhar, N. P., *Fitoterapia*, 1993, **64**, 266.
4. Manandhar, N. P., *Fitoterapia*, 1994, **65**, 7.
5. Wu, J.-B., Chun, T.-T., Ebizuka, T. and Sankawa, U., *Chemical and Pharmaceutical Bulletin*, 1985, **33**, 4091.
6. Wu, J.-B., Chun, T.-T., Ebizuka, Y. and Sankawa, U., *Chemical and Pharmaceutical Bulletin*, 1991, **39**, 3272.
7. Iwakami, S., Wu, J.-B., Ebizuka, Y. and Sankawa, U., *Chemical and Pharmaceutical Bulletin*, 1992, **40**, 1196.
8. Poplawski, J., Holub, M., Samek, Z. and Herout, V., *Collection of Czechoslovak Chemical Communications*, 1971, **36**, 2189.
9. Itoigawa, M., Kumagai, N., Sekiya, H., Ito, K.

and Furukawa, H., *Yakugaku Zasshi*, 1981, **101**, 605.
10. Herz, W. and Sosa, V. E., *Phytochemistry*, 1988, **27**, 155.
11. Bohlmann, F. and Zhongliang, C., *Kexue Tongbao*, 1984, **29**, 900.
12. Herz, W., Mitra, R. B., Rabindran, K. and Rohde, W. A., *Journal of the American Chemical Society*, 1959, **81**, 1481.
13. Herz, W., Gast, C. M. and Subramaniam, P. S., *Journal of Organic Chemistry*, 1968, **33**, 2780.
14. Bérdy, J., Aszalos, A., Bostian, M. and McNitt, K., *Antibiotics from Higher Forms of Life*: *Higher Plants*, Vol. VIII, Part 2. CRC Press, Boca Raton, Florida, 1982, p. 429.
15. Yoshioka, H., Mabry, T. J. and Timmermann, B. N., *Sesquiterpene Lactones*: *Chemistry*, *NMR and Plant Distribution*. University of Tokyo Press, Tokyo, Japan, 1973, p. 544.
16. Lee, K.-h., Ibuka, T., Wu, R.-y. and Geissman, T. A., *Phytochemistry*, 1977, **16**, 1177.
17. Rodriguez, E., Towers, G. H. N. and Mitchell, J. C., *Phytochemistry*, 1976, **15**, 1573.
18. Taylor, R. S. T., Medicinal Plants of Nepal: Ethnomedicine, Pharmacology and Phytochemistry. Ph.D. thesis, The University of British Columbia, Vancouver, Canada, 1996.
19. Bohlman, F. T., Burkhard, T. and Zdero, C., *Naturally occurring acetylenes*. Academic Press, New York, 1973.
20. Towers, G. H. N., *Progress in Phytochemistry*, 1980, **6**, 183.
21. Sazena, G., Farmer, S., Towers, G. H. N. and Hancock, R. E. W., *Phytochemnical Analysis*, 1995, **6**, 125.
22. Towers, G. H. N., Abramowski, Z., Finlayson, A. J. and Zucconi, A., *Planta Medica*, 1985, **3**, 225.