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Bioactive *N*-isobutylamides from the flower buds of *Spilanthes acmella*

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Abstract

The hexane extract of dried flower buds of *Spilanthes acmella* afforded three *N*-isobutyl amides: spilanthol, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide. Their structures were determined by ^{1}H and ^{13}C NMR, MS and GC–MS spectroscopic methods. All were active against *Aedes aegyptii* larvae and *Helicoverpa zea* neonates at 12.5 and 250 μg/mL concentrations, respectively.

Keywords: *Spilanthes acmella*; Compositae; Heliantheae; *N*-isobutyl amides; Acetylenic compounds; Spilanthol

1. Introduction

Spilanthes acmella L. var. *oleracea* Clarke, the toothache plant, is an annual herb belonging to the family Compositae, the tribe Heliantheae, and the subtribe Ecliptinae. This variety has yellow flower heads which distinguishes it from *S. acmella* L. which has violet flowers (Nakatani & Nagashima, 1992). The flowers and leaves of these species have a pungent taste accompanied by tingling and numbness, and have been used as a spice for appetizers and as folk medicine for stammering, toothache, stomatitis and throat complaints (Nakatani & Nagashima, 1992). The genus is widely distributed throughout the tropics and subtropics and can be found in damp pastures, at swamp margins, on rocks near the sea and as a weed of roadsides and cultivations. Spilanthol (**1**), considered to be one of the most active constituents, was obtained from *S. oleracea* Jacq. in 1903 (Gerber, 1903) and later isolated from *S. acmella* (Gokhale & Bhide, 1945).

Since the isolation of spilanthol (**1**), a number of other *N*-isobutylamides such as 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, 2*E*,7*Z*-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide and 7*Z*-*N*-isobutyl-7-tridecene-10,12-diynamide from *S. acmella* have been reported (Nakatani & Nagashima, 1992). *N*-isobutyl amides have also been reported from other species such as *Spilanthes alba* (Bohlman, Ziesche, Robinson, & King, 1980), *Spilanthes oleracea* (Yasuda, Takeya, & Itokawa, 1980; Greger, Hofer, & Werner, 1985) and an *N*-2-phenylethylcinnamamide from *Spilanthes ocymifolia* (Borges-Del-Castillo, Vazquez-Bueno, Secundino-Lucas, Martinez-Martir, & Joseph-Nathan, 1984). Compounds isolated from the flowers of *S. acmella* included three *N*-isobutylamides, compounds **1**–**3**.

2. Results and discussion

Freshly harvested flowers (1.13 kg) of *S. acmella* were lyophilized and extracted with hexane to yield the hexane extract (10 g), followed by ethyl acetate and finally with methanol to yield the ethyl acetate extract (2.1 g) and methanol extract (28 g), respectively. These extracts were bioassayed against *A. aegyptii* larvae and *H. zea* (corn earworm) neonates. The hexane extract showed the highest activity. Fractionation of the hexane extract was carried out by medium pressure liquid

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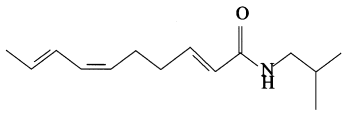

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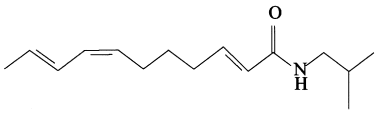

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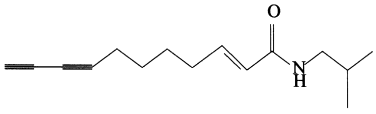

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chromatography (MPLC) on silica gel using hexane with increasing amounts of acetone and finally methanol as the eluting solvents. Six fractions, A–F, were collected and fraction E eluting with hexane–acetone (1:1) was the biologically active.

This bioactive fraction, E, was purified by prep. TLC to yield three major bands: I (365 mg, R_f 0.75), II (36 mg, R_f 0.45) and III (11 mg, R_f 0.25). Bands I and II were biologically active and were further purified by prep. TLC. Band I was subjected to prep. TLC to yield a 1:1 mixture of compounds **1** and **2** (270 mg). Band II was further purified by prep. TLC to yield compound **3** (5 mg).

Compounds **1** and **2** were isolated as a 1:1 mixture and further attempts to purify this mixture by prep. TLC and HPLC proved futile. The ^{1}H and ^{13}C NMR spectral analysis of this mixture showed the presence of two compounds since ^{1}H NMR gave two peaks at δ 4.30 and 4.45, respectively. Both of these peaks were exchanged with D_2O and were assigned to two amide protons. In the ^{13}C NMR spectrum most of the peaks were overlapped for compounds **1** and **2** except for a methylene carbon at δ 27.5. This peak was not as intense as the other peaks and suggested that compounds **1** and **2** differed from each other by one -CH_2- group. EIMS analysis of this mixture showed two peaks in the TIC and gave ions at m/z 221 and 235, respectively. These two peaks were 14 mass units apart due to a -CH_2- group difference. GC–MS analysis of this mixture was successful in separating the two compounds using a 30-m SPB20 column. Compound **1** gave an ion at m/z 221 which was calculated for $C_{14}H_{23}NO$. It gave fragment ions at m/z 141 and 81, respectively. Similarly, compound **2** gave an ion at m/z 235, calculated for $C_{15}H_{25}NO$, with fragment ions at m/z 141 and 95, respectively. This data allowed us to identify compound **1** as spilanthol (Nakatani & Nagashima, 1992) and compound **2** as undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide. This is the first report of these compounds with biological activity against *A. aegyptii* and corn earworm from the flower heads of *S. acmella*.

Compound **3** was identified as undeca-2*E*-en-8,10-diynoic acid isobutylamide, by comparing its ^{1}H NMR data with published spectral data (Bauer, Remiger, & Wagner, 1989). This is the first report of the ^{13}C NMR data and the biological activity of undeca-2*E*-en-8,10-diynoic acid isobutylamide, as well as its isolation from *S. acmella*.

Mosquitocidal assays on *A. aegyptii* using compounds **1**–**3** indicated that they were very active. Compounds **1**–**3** had a LD_{100} (24 h) at 12.5 μg/mL concentrations. However, compounds **1** and **2** showed 50% mortality at 6.25 μg/mL (S.D. 4.07), while compound **3** showed 30% mortality at 6.25 μg/mL (S.D. 3.04). Compounds **1**–**3** demonstrated significant antifeedant activity towards corn earworm (*H. zea*) neonates. The mixture of compounds **1** and **2** showed a 66% weight reduction of *H. zea* neonate larvae at 250 μg/mL concentration after 6 days (Fig. 1). However, compound **3** showed significantly higher weight reduction of *H. zea* at the same concentration. It reduced the weight of corn earworm at 250 μg/mL by 79% after 6 days (Fig. 1).

This investigation yielded two purely olefinic alkamides with the other being an acetylenic alkamide and all three compounds possessed the isobutyl side chain. They were all very active with the acetylenic compound **3** showing better activity against *H. zea* when compared to the olefinic alkamides **1**–**2**. The cooccurrence of these two types of alkamides in the same genus has been reported before and probably indicates

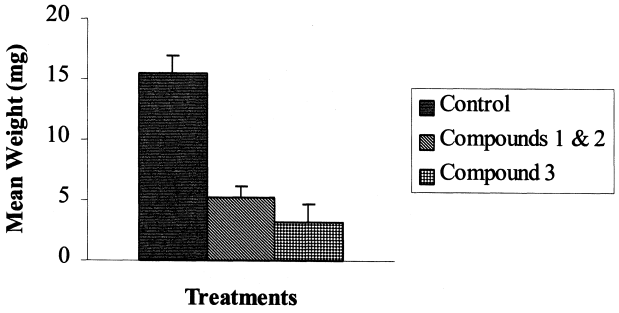

Fig. 1. Growth inhibitory assay of compounds **1**–**3** against *Helicoverpa zea* neonate larvae at 250 μg/mL concentration after 6 days. Statistical analysis was done using ANOVA ($p \leq 0.01$) and the means were compared by calculating least significant difference (LSD).

a close relationship of all these genera and hence the reason for placing them in the subtribe Ecliptinae.

3. Experimental

3.1. General

All spectra (^{1}H, ^{13}C, DEPT) were recorded at 300 MHz for ^{1}H and 75 or 125 MHz for ^{13}C. Chemical shifts were recorded in $CDCl_3$ and the values are in δ (ppm) based on δ residual of $CHCl_3$ 7.24 and $CDCl_3$ 77.0. Coupling constants, J, are in Hz. EIMS measurements were carried out at 70 eV. Electron impact GC–MS measurements were recorded using an SPB20 (80% dimethyl/20% diphenyl-siloxane) column (30 m $\times$ 0.32 mm) and operating at 70 eV. The column temperature was programmed to increase at 10°C min^{-1} from 50 to 350°C. Helium was used as the carrier gas. The silica gel used for MPLC was Merck Silica gel 60 (35–70 μm particle size). Preparative TLC was done on Analtech silica gel plates (20 $\times$ 20 cm, 500 μm).

3.2. Plant material

Matured flowers of *S. acmella* were harvested from plants grown in the Michigan State University, Department of Horticulture and National Food Safety and Toxicology Center greenhouses. The seeds were purchased from Seeds of Change, P.O. Box 15700, Santa Fe, NM, 87506-5700, catalog #724 (1996).

3.3. Extraction and isolation

The flowers (1.51 kg) were lyophilized and the dried flowers (454 g) were crushed and extracted with hexane (2 $\times$ 4 L, 48 h) to yield the hexane extract (10 g). The residue was then extracted with ethyl acetate (2 $\times$ 4 L, 48 h) followed by methanol (2 $\times$ 4 L, 48 h) to yield the ethyl acetate extract (2.1 g) and methanol extract (28 g), respectively. These extracts were bioassayed against *A. aegyptii* larvae and *H. zea* (corn earworm) neonates. The hexane extract was the most active among all these extracts and hence was selected for further study. Fractionation of the hexane extract (4.6 g) was carried out by medium pressure liquid chromatography (MPLC) on silica gel (250 g) using hexane with increasing amounts of acetone and finally with methanol as the eluting solvents. The fractions collected were: A (0.11 g, hexane–acetone, 15:1, 300 mL); B (0.04 g, hexane–acetone, 8:1, 200 mL); C (2.26 g, hexane–acetone, 8:1, 500 mL); D (0.11 g, hexane–acetone, 1:1, 500 mL); E (1.65 g, hexane–acetone, 1:1, 600 mL); and F (0.18 g, 100% methanol, 800 mL). Bioassays revealed that fraction E was active.

The bioactive fraction, E, was purified by prep. TLC (hexane–acetone 6:1, $\times$4) to yield three major bands: I (365 mg, R_f 0.75); II (36 mg, R_f 0.45); and III (11 mg, R_f 0.25). Bands I and II were biologically active and were further purified by prep. TLC. Band I was subjected to prep. TLC (hexane–$CHCl_3$ 1:3, $\times$4) to yield a 1:1 mixture of compounds **1** and **2** (270 mg). Band II was purified by prep. TLC (hexane–acetone 3:1, $\times$3) to yield compound **3** (5 mg).

3.4. Compound (*1*)

GC–EIMS 70eV, m/z (rel. int.): 221 $[M]^+$ (20), 206 (3), 141 (100), 126 (23), 98 (23), 81 (87), 69 (10), 53 (10). Compound **1** was identified as spilanthol by comparison of its ^{1}H and ^{13}C NMR spectral data with published values (Nakatani & Nagashima, 1992).

3.5. Compound (*2*)

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.84 (6H, d, J=6.9 Hz), 1.13–1.19 (2H, m), 1.69 (1H, m), 1.69 (3H, d, J=6.8 Hz), 2.14–2.25 (4H, m), 3.05 (2H, dd, J=6.9; 6 Hz), 4.45 (1H, bs, exchangeable with D_2O), 5.17 (1H, dt, J=10.5; 6.5 Hz), 5.65 (1H, dq, J=15; 6.5 Hz), 5.81 (1H, dt, J=15; 1.5 Hz), 5.95 (1H, m), 6.21 (1H, m), 6.73 (1H, dt, J=15; 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.1, 19.9, 20.1, 26.2, 27.5, 28.4, 31.9, 46.7, 124.1, 126.5, 127.4, 129.2, 129.7, 143.2, 166. GC–EIMS 70 eV, m/z (rel. int.): 235 $[M]^+$ (88), 220 (3), 192 (3), 163 (23), 141 (100), 126 (23), 108 (6), 98 (20), 95 (98), 68 (17), 57 (12). Compound **2** was identified as undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide from its EIMS, GC–MS and NMR data.

3.6. Compound (*3*)

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (6H, d, J=6.9 Hz), 1.55 (4H, m), 1.79 (1H, m), 1.95 (1H, t, J=1.2 Hz), 2.24 (4H, m), 3.13 (2H, t, J=6.5 Hz), 5.45 (1H, br s, exchangeable with D_2O), 5.76 (1H, dt, J=14.5; 1.5 Hz), 6.79 (1H, dt, J=14.5; 6.9 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 18.9, 20.1, 27.2, 27.4, 28.6, 31.3, 46.9, 64.7, 65.0, 68.4, 77.9, 124.0, 143.8, 165.9. Compound **3** was identified as undeca-2*E*-en-8,10-diynoic acid isobutylamide by comparing its ^{1}H NMR spectral data with published values (Bauer et al., 1989).

3.7. Mosquitocidal assay (Roth, Chandra, & Nair, 1998)

Fourth instar mosquito larvae, *A. aegyptii*, were reared from eggs in our laboratory. Ten to 15 larvae were placed in 980 μL of distilled water and 20 μL of DMSO solution containing test extracts or pure compounds were added. The test concentrations were 250 μg/mL for crude extracts and 100 μg/mL which were then serially diluted to 0.1 μg/mL for pure compounds.

A control was prepared with 980 μL of distilled water and 20 μL of DMSO solution to which larvae were added. The assay was conducted in triplicates and the mortality was recorded at 2, 4, 12 and 24 h intervals.

3.8. Corn earworm assay (Zhang, Nair, Murry, & Zhang, 1997)

Corn earworm eggs (*H. zea*) and dry insect diet containing the ingredients; 63.8% corn meal (gelatinized), 24.2% soy flour (defatted and toasted), 5% nonfat dry milk, 5% soy oil (refined and stabilized), 2% vitamin and a mineral premix was purchased from the North Carolina State Insectory, Department of Entomology, North Carolina State University, Raleigh, NC. The eggs were placed in an incubator at 27°C for 24–36 h. The insect diet was prepared as follows: agar (1.4%) in water was mixed and autoclaved for 5 min and the resulting solution was cooled to 55°C in a water bath. To this solution, the dry diet for corn earworm (940 mg) was added until the total diet weight reached 5 g. DMSO (25 μL) or DMSO solutions containing test extracts or purified compounds were then mixed with the diet separately. Three to four drops of this diet was then dispensed into 3.5 ml polystyrene vials and stored at 4°C for 24 h. To each vial one neonate larvae was placed using a fine point, sterilized brush. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature of 28°C and night temperature of 24°C. Each treatment had fifteen replicates. The larvae were weighed on an analytical balance after six days.

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References

Bauer, R., Remiger, P., & Wagner, H. (1989). *Phytochemistry*, *28*, 505.

Bohlman, F., Ziesche, J., Robinson, H., & King, R. M. (1980). *Phytochemistry*, *19*, 1535.

Borges-Del-Castillo, J., Vazquez-Bueno, P., Secundino-Lucas, M., Martinez-Martir, A. I., & Joseph-Nathan, P. (1984). *Phytochemistry*, *23*, 2671.

Gerber, E. (1903). *Archives of Pharmacy*, *241*, 270.

Gokhale, V. G., & Bhide, B. V. (1945). *Journal of the Indian Chemical Society*, *22*, 250.

Greger, H., Hofer, O., & Werner, A. (1985). *Monatshefte fuer Chemie*, *116*, 273.

Nakatani, N., & Nagashima, M. (1992). *Bioscience, Biotechnology and Biochemistry*, *56*, 759.

Roth, G. N., Chandra, A., & Nair, M. G. (1998). *Journal of Natural Products*, *61*, 542.

Yasuda, I., Takeya, K., & Itokawa, H. (1980). *Chemical and Pharmaceutical Bulletin*, *28*, 2251.

Zhang, D., Nair, M. G., Murry, M., & Zhang, Z. (1997). *The Journal of Antibiotics*, *50*, 617.