

ISOLATION OF UMBELLIFERONE AS A PRINCIPAL ALLELOCHEMICAL FROM THE PERUVIAN MEDICINAL PLANT *Diplostephium foliosissimum* (Asteraceae)

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ABSTRACT

Diplostephium foliosissimum (Asteraceae) is a peruvian medicinal plant which grows at high altitudes in the Andes mountains. In a previous screening of peruvian plant species we found high plant-inhibitory activity in the leaves of this species. Here, we isolated and quantified allelochemicals from *D. foliosissimum*. A crude methanol extract of leaves was fractionated, and the activity of each fraction was evaluated by bioassays using lettuce seedlings. Most of the inhibitory activity was present in the ethyl acetate fraction. Umbelliferone was isolated, identified, and quantified at 0.01 g g⁻¹ dry weight, and was confirmed to be the principal allelochemical involved in the inhibitory activity of *D. foliosissimum*.

Key words: allelochemical, *Diplostephium foliosissimum*, growth inhibition, *Lactuca sativa*, total activity, umbelliferone (7-hydroxycoumarin).

AISLAMIENTO DE LA UMBELIFERONA COMO PRINCIPAL ALELOQUÍMICO DE LA PLANTA MEDICINAL PERUANA *Diplostephium foliosissimum* (Asteraceae)

RESUMEN

Diplostephium foliosissimum (Asteraceae) es una planta medicinal peruana que crece a gran altura en las montañas de los Andes. En una selección anterior de especies vegetales del Perú se encontró alta actividad inhibidora de plantas en las hojas de esta especie. En este estudio hemos aislado y cuantificado aleloquímicos de *D. foliosissimum*. El extracto crudo de metanol de las hojas fue fraccionado, y la actividad de cada fracción se evaluó mediante bioensayos con plántulas de lechuga. La mayor parte de la actividad inhibitoria estuvo presente en la fracción de acetato de etilo. La umbeliferona fue aislada, identificada y cuantificada en 0,01 gg⁻¹ peso seco, y se confirmó como el aleloquímico principal involucrado en la actividad inhibitoria de *D. foliosissimum*.

Palabras clave: aleloquímico, *Diplostephium foliosissimum*, inhibición de crecimiento, *Lactuca sativa*, actividad total, umbeliferona (7-hidroxycumarina).

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INTRODUCTION

Allelopathy is the phenomenon by which plants produce compounds that influence the growth and development of other organisms. These compounds are called allelochemicals. One of their functions is considered to be to protect against other plants, herbivores, pathogens, and competitors. Allelopathic plants can be used as cover crops or green manure in sustainable agriculture, and allelochemicals are exploited in the development of new herbicides from natural sources¹.

We evaluated the allelopathic activity of 170 peruvian plant species and found *Diplostegium foliosissimum* (Asteraceae) to have one of the highest activities². *Diplostegium* comprises about 90 species distributed from Colombia and Venezuela to Bolivia and northern Chile, with one species in Costa Rica³. Around 23 species are endemic to Peru. Among them, *D. foliosissimum* grows on rocky slopes around 3200 to

3300 m above sea level in the regions of Amazonas, Ancash, Piura, and San Martín⁴. A study of the chemical composition of this plant reported linolenic and palmitic acids as the main fatty acids, a high content of saturated hydrocarbons, and triterpenoids⁵. The plant is used as a traditional medicine in the northern peruvian Andes: as an infusion for the treatment of gastric pain and as an alcohol tincture for the treatment of hypotension and systemic debilitation⁶. In this region the species is called "Poleo del Inca".

Here, we describe the isolation and identification of the principal allelochemical involved in the plant-inhibitory activity of *D. foliosissimum*.

EXPERIMENTAL PART

Plant materials

Fresh *D. foliosissimum* leaves were purchased in a local market in Huaraz City, Ancash region, Peru, in January 2009. The samples were identified in the Herbarium of the Department of Biology, Faculty of Science, at the National Agrarian University La Molina, Peru. The material was dried in a drying chamber at 60–70 °C for 24 h.

Bioassay of plant growth inhibitory activity

We used a lettuce seedling bioassay to evaluate the plant growth inhibitory activity. Toyo No. 1 filter paper (27 mm, Toyo Roshi Kaisha Ltd., Japan) was placed in a glass Petri dish (27 mm), soaked with test solutions at a series of concentrations, dried *in vacuo*, and rewet with 0.7 mL of distilled water. Five pre-germinated (20 h at 20 °C in the dark) lettuce seedlings (*Lactuca sativa* cv. Great Lakes 366, Takii Co. Ltd., Kyoto, Japan) were put in each Petri dish and incubated for 52 h at 20 °C in the dark. The control treatment used distilled water. After incubation, we measured the lengths of radicles and hypocotyls to calculate growth relative to the control. The bioactivity was evaluated as the degree of inhibition of growth of the highly sensitive radicle compared with growth in the control.

Extraction and isolation

Dried leaves (150 g) were soaked in methanol (1.2 L) for 1 week at room temperature. The methanol was then evaporated *in vacuo*, and the dried extract was diluted with 500 mL of distilled water and partitioned three times with *n*-hexane (ca. 300 mL each). The resulting aqueous layer was partitioned three times with ethyl acetate (ca. 300 mL each), and the residual aqueous layer was partitioned three times with 1-butanol (ca. 300 mL each).

The ethyl acetate fraction, which had the highest inhibitory activity, gave a white precipitate. The precipitate was washed three times with 30 mL of ethyl acetate, and each supernatant was filtered through a membrane (disposable syringe filter unit, DISMIC-13HP, 13HP020CN, 0.2 µm, PTFE, Toyo Roshi Kaisha, Ltd.) and used for bioassay.

Identification and quantification

Nuclear magnetic resonance (NMR) was used to identify the precipitate in the ethyl acetate fraction. ^1H and ^{13}C NMR spectra (measured in $\text{MeOH}-d_4$) were recorded with a JNM α -600 (Jeol, Tokyo, Japan) spectrometer.

The compound was analyzed by gas chromatography – mass spectrometry (GC-MS) on a GCMS-QP5050A apparatus (Shimadzu, Kyoto, Japan) equipped with an Equity-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness). The temperatures of the injector and the interface were 200 $^\circ\text{C}$. The oven temperature was held at 40 $^\circ\text{C}$ for 10 s, then raised to 220 $^\circ\text{C}$ at 8 $^\circ\text{C min}^{-1}$ and held for 5 min. The column flow rate was 1.5 mL min^{-1} .

The compound was quantified by high-performance liquid chromatography (HPLC, 626 pump with 996 photodiode array detector; Waters, Milford, MA, USA) with an ODS column (Inertsil ODS-3, 250 mm x 4.6 mm; GL-Sciences, Tokyo, Japan). It was eluted with a mixture of methanol and water (40:60 by volume) at 1.0 mL min^{-1} and detected at 326 nm. The concentration of the compound in the crude extract was calculated by comparing the peak areas of samples with those of standards (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan).

The total activity (TA) of an allelochemical is defined as biological activity per unit weight of the plant⁷: $\text{TA} = (\text{concentration or content of allelochemical in a plant}) / \text{specific activity} (\text{EC}_{50})$.

RESULTS

The plant growth inhibitory activity of the crude, *n*-hexane, ethyl acetate, 1-butanol, and water fractions was evaluated by bioassay at different concentrations. The crude extract inhibited radicle elongation by 50% at 0.49 mg FW mL^{-1} . The major activity of the crude extract was found in the ethyl acetate fraction (figure 1).

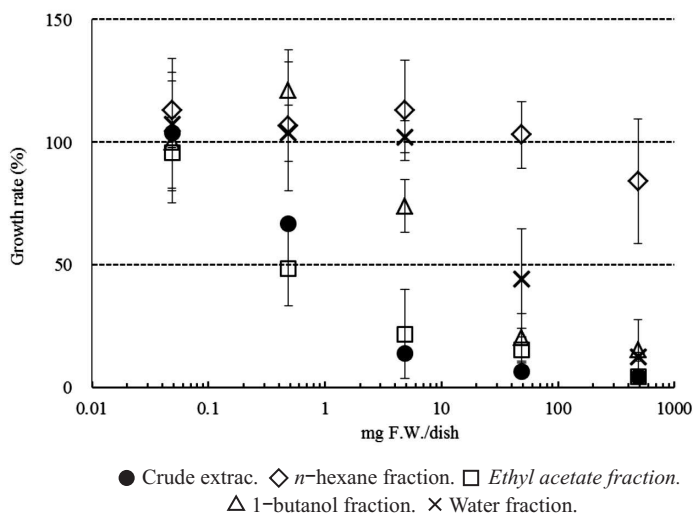


Figure 1. Growth rate of radicles of lettuce seedlings treated with crude extract of *D. foliosissimum* and subsequent *n*-hexane, ethyl acetate, *n*-butanol, and water fractions. Values are mean $\pm$ SD ($n = 5$).

The precipitate of the ethyl acetate fraction was washed three times with ethyl acetate, and the activity of each supernatant was assayed. All three supernatants gave nearly the same inhibitory activity (figure 2), indicating that the activity was derived from the precipitate, which was apparently slightly soluble in ethyl acetate. Thus, the precipitate was responsible for the strong inhibitory activity of this fraction.

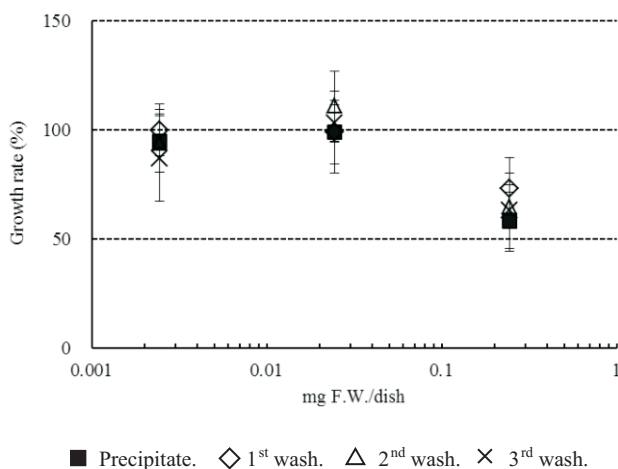


Figure 2. Growth rates of radicles of lettuce seedlings treated with ethyl acetate precipitate and first, second, and third washes of the precipitate. Values are mean $\pm$ SD ($n = 5$).

The ^1H NMR spectrum of the compound revealed a 1,2,4-substituted benzene together with a *cis*-1,2-substituted ethylene moiety, the stereochemistry of which was suggested by the coupling constant ($J = 9.6$ Hz). The ^{13}C NMR spectrum showed the presence of nine carbon atoms in the structure. These observations imply that the compound is a 6- or 7-substituted coumarin, and that the most probable substituent is a hydroxyl group, because there was no other signal in the ^1H and ^{13}C NMR spectra. GC-MS analysis gave the same retention time and mass spectrum as those of 7-hydroxycoumarin (umbelliferone). ^1H and ^{13}C NMR analyses of an authentic sample supported this identification. Therefore, the compound is umbelliferone. The content of umbelliferone in the dried leaves of *D. foliosissimum* was 0.01 g g^{-1} ($6.62 \times 10^{-3} \text{ mol L}^{-1}$). The EC_{50} was estimated as $1.85 \times 10^{-5} \text{ mol L}^{-1}$. These values gave a TA of umbelliferone in *D. foliosissimum* of 358 (no units).

In the seedling assay of crude extract and authentic umbelliferone at equivalent concentrations, the dose-response curves were almost identical (Fig. 3). Thus, umbelliferone is the principal allelochemical involved in the inhibitory activity of *D. foliosissimum*.

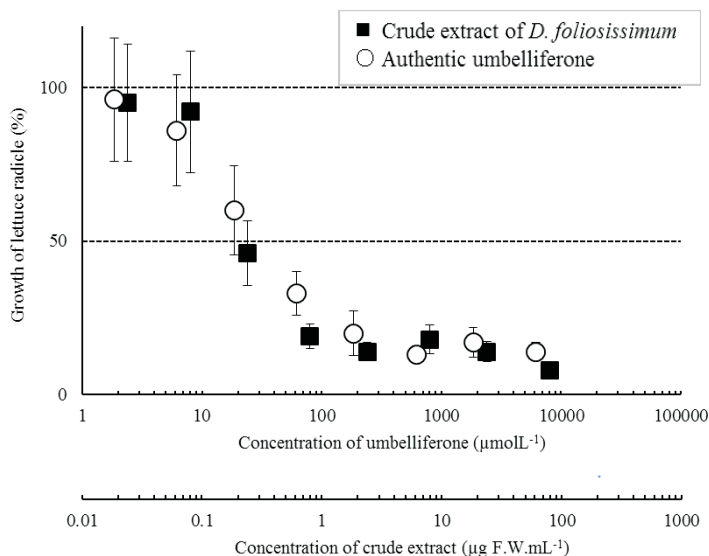


Figure 3. Growth rates of radicles of lettuce seedlings treated with crude extract of *D. foliosissimum* and authentic umbelliferone at equivalent concentrations. Values are mean $\pm$ SD ($n = 5$).

DISCUSSION

Coumarins are a large group of secondary metabolites distributed in the Apiaceae, Rutaceae, Asteraceae, and Fabaceae. They are involved in defense against pathogens and the response to some types of stress, among other things⁸. Umbelliferone is a hydroxycoumarin whose name derives from its isolation from plants of the Apiaceae (Umbelliferae). It is a well known compound reported from several medicinal plants, including *Angelica archangelica*, *Apium graveolens*, *Pimpinella anisum* (Apiaceae), *Artemisia abrotanum*, *Matricaria chamomilla* (Asteraceae), *Lavandula angustifolia* (Lamiaceae), *Aegle marmelos* (Rutaceae), and *Hydrangea paniculata* (Saxifragaceae)⁹. It is also considered to be an important defense against fungal infection in sweet potato roots (*Ipomoea batatas*) and grapefruit (*Citrus paradisi*)¹⁰.

The role of umbelliferone as an allelochemical has been studied previously. In 1950, the inhibitory effects of umbelliferone and other coumarin derivatives in *Avena sativa* roots were tested¹¹. Another study tested the inhibition of germination of the seeds of cucumber (*Cucumis sativus*), garden pea (*Pisum sativum*), and maize (*Zea mays*) by coumarin and analogues, but umbelliferone did not show significant inhibition at low concentrations¹². Umbelliferone was also identified as an allelochemical in the invasive weed *Hieracium pilosella* (syn. *Pilosella officinarum*). This species has a number of phenolic compounds with phytotoxic properties, of which umbelliferone was the most active, inhibiting roots in the leachate of dead leaves¹³. *Lantana camara*, an invasive allelopathic weed, also contains umbelliferone, which may be involved in the weed's interactions with other species and establishment of new colonies¹⁴.

Koo *et al.*¹⁵ compared the TAs of several allelopathic species. Juglone in *Juglans ailanthifolia* and coumarin in *Anthoxanthum odoratum* had TAs of 2000. Lower values came from 6-*O*-(4'-hydroxy-2'-methylenebutyryl)-1-*O*-*cis*-cinnamoyl- β -D-glucopyranose in *Spiraea thunbergii* (300), L-3,4-dihydroxyphenylalanine(L-DOPA) in *Mucuna pruriens* (250), 1-*O*-*cis*-cinnamoyl- β -D-glucopyranose in *S. thunbergii* (200), L-mimosine in *Leucaena leucocephala* (100), and cyanamide in *Vicia villosa* (40). Against these, the TA of umbelliferone in *D. foliosissimum* was almost 360.

CONCLUSIONS

Our results suggest the allelopathic potential of extracts of *D. foliosissimum*, which suppressed the growth of *L. sativa* seedlings in laboratory tests. The crude extract of the leaves inhibited the elongation of radicles by 50% at 0.49 mg FW mL⁻¹. Umbelliferone, present in dried leaves at 0.01 g g⁻¹ DW, was responsible for this activity. The total activity of umbelliferone in *D. foliosissimum* was almost 360. Although umbelliferone is a well known compound present in many species, this is the first report of its presence as a principal allelochemical in *D. foliosissimum*.

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REFERENCES

1. Weston LA. History and Current Trends in the Use of Allelopathy for Weed Management. *Horttechnology* 2005; **15**: 529-534.
2. Ono MCI, Miyaura R, Tapia y FML, Rengifo SEL, Fujii Y. Screening of 170 of Peruvian plant species for allelopathic activity using the sandwich method. *Weed Biol Manag* 2012; **1** (in press).
3. Bohm BA, Stuessy TF. *Flavonoids of the sunflower family (Asteraceae)*. New York: Springer Wien; 2001.
4. Beltrán H, Granda A, León B, Sagástegui A, Sánchez I, Zapata M. El libro rojo de las plantas endémicas del Perú. Asteráceas endémicas del Perú. *Rev Per Biol* 2006; **13**: 64-164.
5. Senatore F, De Feo V, Zhou ZL. Non polar constituents of two Peruvian Asteraceae. *Riv Ital Sostanze Gr* 1991; **Vol. LXVIII**. Settembre: 493-495.
6. De Feo V. Ethnomedical field study in northern Peruvian Andes with particular reference to divination practices. *J Ethnopharmacol* 2003; **85**: 243-256.
7. Hiradate, S. Isolation strategies for finding bioactive compounds: specific activity vs total activity. In: *Natural Products for Pest Management. ACS Symposium Series*. American Chemical Society, Washington, DC. USA: A.M. Rimando and S.O. Duke editors; 2006 **927**: 113-126.
8. Razavi SM. Plant coumarins as allelopathic agents. *Int J Biol Chem* 2011; **5**: 86-90.
9. Soedin P. Coumarins: Plants, structures, properties. Chapter II. Physical constants and spectral characteristics of coumarins (continued). *Chem Nat Compd* 1998; **34**: 517-548..

10. Afek U, Orenstein J, Carmeli S, Rodov V, Joseph MB. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. *Phytochemistry* 1999; **50**: 1129-1132.
11. Goodwin RH, Taves C. The effect of coumarin derivatives on the growth of Avena roots. *Am J Bot* 1950; **37**: 224-231.
12. Pollock BM, Goodwin RH, Greene S. Studies on roots. II. Effects of coumarin, scopoletin and other substances on growth. *Am J Bot* 1954; **41**: 521-529.
13. Makepeace W, Dobson AT. Interference phenomena due to mouse-ear and king devil hawkweed. *New Zeal J Bot* 1985; **23**: 79-90.
14. Sharma OP, Sharma S, Pattabhi V, Mahato SB, Sharma PD. A review of the hepatotoxic plant *Lantana camara*. *Crit Rev Toxicol* 2007; **37**: 313-352.
15. Koo J, Fujii Y, Yoshizaki S, Kobori H. Evaluation of total allelopathic activity of heartseed walnut (*Juglans ailanthifolia* Carr.) and its potential to control black locust (*Robinia pseudoacacia* L.). *Allelopathy J* 2010; **26**: 243-254.