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Identification and Biological Characteristics of an Antifungal Compound Extracted from Cocklebur (*Xanthium strumarium*) against *Phytophthora drechsleri*

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Crude extracts of *Xanthium strumarium* inhibited mycelial growth and zoospore germination of *Phytophthora drechsleri*, the causal agent of Atractylis rot, *in vitro*. Fresh sap from *X. strumarium* at 50-fold dilution was highly effective in controlling the disease incidence in pot and field trials. Purified extracts from cocklebur inhibited mycelial growth and zoospore germination *in vitro* at a concentration of 12.5 μg/ml and 15.6 μg/ml, respectively. Hyphal tips affected by the compound showed malformation. The antifungal compound purified from *X. strumarium* was identified as 4-oxo-1 (5), 2,11, (13)-xanthatriene-12,8-olide, known as "deacetyl xanthumin".

Keywords : antifungal compound, Atractylis, deacetyl xanthumin, *Phytophthora dechsleri*, *Xanthium strumarium*.

Traditional medicinal plants are now being cultivated massively in the fields, where disease epidemics are becoming serious. Fungicide spraying, although inevitable for high productivity, is usually not desirable. Hence, alternative methods to control plant diseases are urgently needed. A *Phytophthora* disease of "sabju" called Atractylis rot, seriously occurred in Korea recently. The causal agent of the disease was identified as *P. drechsleri* (Kim et al., 1997).

Using extracts from plants containing natural antifungal compounds for plant disease control is considered to be one of the desirable methods for plant protection in agriculture. Although it is known that allicin from *Allium sativum* is a natural antifungal compound that inhibits fungi including *Aspergillus* (Yoshida et al., 1987), the use of allicin as a fungicide has not been widely practiced yet. Many attempts have also been made to detect antifungal activities against storage mold (Ohmoto et al., 1981; Ohmoto and Sung, 1982; Reuter and Sendl, 1994) and seed-borne fungal pathogen (Paik et al., 1998). Antibacterial compounds extracted from plants have also been previously reported (Cavallito et al., 1944; Coffey and Bower, 1984; Jang, 1998; Stoll and Seebeck, 1948; Yoshida et al., 1987), but these have not been developed commercially.

This study attempted to: identify plant sources with antifungal activity against *Phytophthora*, the aggressive fungal pathogen causing Atractylis rot; extract the antifungal compounds from the plants; screen the antifungal compounds efficacy; and identify the structure of these antifungal compounds.

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Materials and Methods

Isolates and inoculum preparation. The target fungus, *Phytophthora derchsleri*, has been previously described (Kim et al., 1997). *P. drechsleri* 9601 was grown on V-8 juice agar at 25% for 3 days in the dark. Mycelial discs taken by cork borer, 5 mm in diameter, from the actively growing colony margins were transferred to a petri-dish with 20 ml of sterile distilled water to induce massive sporangia production under fluorescent light illumination for 12 h.

Control of Atractylis rot using cocklebur sap in pot and field trials. Fresh leaf and stem of cocklebur plant were squeezed by using a flesh juice maker. The suppressive effects of the sap on the outbreak of *Phytophthora* rot of Atractylis were tested in pots and in the fields. The fresh sap was diluted to 10×, 20×, 50×, and 100 times with water, while the boiled sap had the same dilutions. The amount of sap drenched was 20 ml in 1/5000-a pot, where ten seedlings of 'sabju' were grown. The experiment was carried out in a complete randomized design with five replications. After drenching 20 ml of the zoospore suspension of *P. drechsleri* at 50 sporangia per ml, disease severity was observed after 7 days.

For the field trials, the fresh sap of cocklebur was diluted 50× and 100×, while the boiled sap was also diluted at the same concentrations. The amount of sap drenched per plant was 20 ml of

each dilution in the endemic field plots. Atractylis plants tested were 1-year-old *Atractylis macrocephala* grown in experimental fields and carried out in a randomized complete block design with three replications. The effects of the sap on disease suppression were rated after 1 month of treatment.

Purification of active compound from cocklebur. Air-dried *Xanthium strumarium* (2 kg) was ground and mixed with chloroform (4 L) for 48 h by vigorous shaking to extract the plant's natural compound. The chloroform extract was added to 5% aq. solution of lead acetate to precipitate fatty acid, phenolics and chlorophylls, and then filtered. The aqueous layer was re-extracted with chloroform (300 mL×5 times). The combined organic layers were washed with brine and dried over Na_2SO_4 and concentrated to a brown mass (62 g), which was loaded on silica gel (0.5 kg) column filled with hexane. Elution was with chloroform, then with chloroform enriched with methanol. The fractions (2.1 g) of $CHCl_3$-MeOH (40:1) were purified by repeated chromatography on silica gel [hexane-EtOAc (40:1), then hexane- EtOAc (2:1)] to give antifungal compound (32 mg) (Table 5).

Bioassay of purified antifungal compound. The purified substance was bioassayed for determination of inhibitory concentration against mycelial growth and zoospore germination of *P. drechsleri*. The purified compounds (50, 25, 12.5, 6.25, and 3.125 μg/ml) were treated in 5 mm wells on PDA media with *P. drechsleri* growing in the center. The inhibition of zoospore germination by purified substance was tested at concentrations of 7.8-125 ppm zoospore suspension on a petri dish. After 24 h, the inhibition of mycelia and germ tube of zoosporangium were observed under the microscope. The sap of fresh cocklebur plants was also tested *in vitro* for inhibiting zoospore germination by serial 2-fold dilutions to 256×.

Structural identification of the antifungal compound. The purity of the compound was clarified by HPLC (Waters Water-201 USA), and the structural information was interpreted by NMR spectrum (Bruker DRK-500, Germany). Then, the molecular weight was analyzed by using the Mass spectrometer (GC14A Shimadzu, Japan). IR spectrum was obtained by IFS66 (Bruker DRK-500, Germany).

Results

Effect of cocklebur sap against Atractylis *Phytophthora* rot. The dilution end concentration of the cocklebur sap inhibiting zoospore germination was 128× as shown in Table 1. In the sap from cocklebur fresh plants, the amount of antifungal compound was analyzed by HPLC. Based on the HPLC chart, the amount of antifungal compound in the sap was approximately 1 mg/1 ml (data not shown).

The disease control efficacy test was carried out in pot and field trials. As shown in Table 2, there was a positive effect of the sap in the pot trials, which resulted in control values on *Atractylis* rot at 67-100% at 50-fold diluting concentration. With 100-fold diluting concentration, the effect was not as high.

Table 1. Effect of crude extracts of cocklebur plants on the germination of zoospores of *Phytophthora drechsleri*

Germination of zoospores	Reciprocal dilution				
	16	32	64	128	256
	–*	–	–	–	+

*Extracts from juice maker without adding water were serially diluted. – = no germination; + = with germination.

Table 2. Effects of crude extracts of cocklebur on the incidence of *Phytophthora* rot of Atractylis in pot trials

Dilutions	Disease severity[a] (%)	Control value (%)
Crude extract 20 X	0	100
50 X	13.7	67.0
100 X	29.5	28.9
Boiled extract 20 X	0	100
50 X	0	100
100 X	13.3	67.9
Control (Untreatment)	41.5	–

[a] Ten plants/pot with five replications were rated for disease severity in 7 days.

Table 3. Controlling effects of the sap of cocklebur on *Phytophthora* rot of Atractylis in field trials

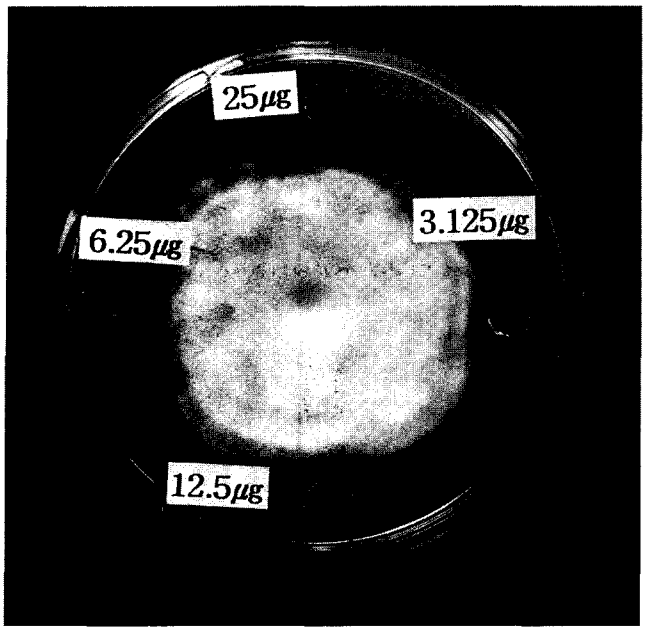
Dilution	Infection rate[a] (%)	Control value (%)
Crude sap 50X	2.2	84.2
100X	3.7	73.5
Boiled sap 50X	6.0	57.1
100X	4.4	68.5
Control (Untreated)	14.0	–

[a] One hundred and twenty plants per plot with 3 replications was inspected for disease infection.

As shown in Table 3, the effect of the cocklebur sap was also observed in the field trials. At 50-fold diluting concentration, the disease control value was over 80%.

Bioassay of purified compound. The purified compound inhibited the mycelial growth of *P. drechsleri* as shown in Fig. 1. The minimum inhibitory concentration for mycelial growth was 12.5 μg/ml. The compound induced malformation of the mycelial tip. The coral type malformation of the mycelial tip caused by the compound was observed under the microscope. The degree of malformation of hyphae was dependent on the concentration of the antifungal compound applied. Malformation was severe at high concentrations above 12.5 μg/ml (Table 4). However, at concentrations as low as 3.125 μg/ml, the walls of the hyphae were only roughened and no further inhibiting effect was observed.

The compound also inhibited the motility and germination of zoospore of *P. drechsleri* at a concentration of 15.6 μg/ml. Below 7.8 μg/ml, zoospores germinated but the



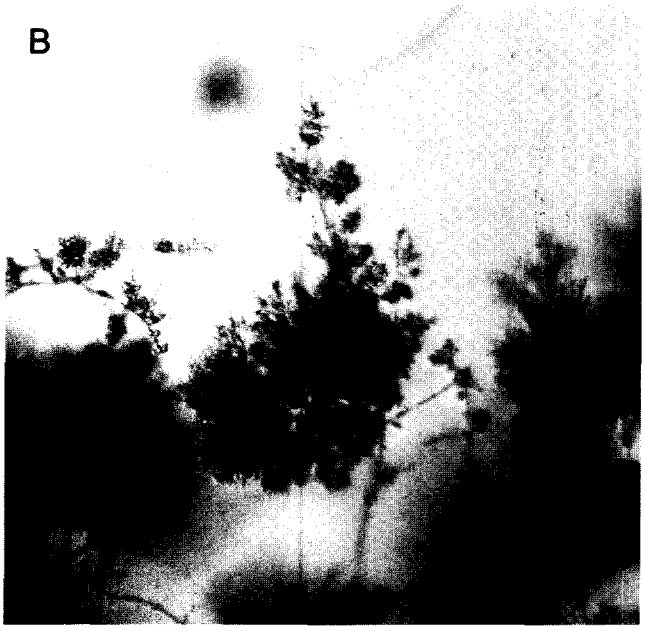


Fig. 1. Inhibition of mycelial growth by antifungal compound extracted from cocklebur. (**A**) Mycelial inhibition on media; (**B**) malformation of hyphal tip affected by antifungal substance.

germ tubes were malformed with rough surface (Table 5).

Structural identification of antifungal compound. As shown in Table 6, maximum absorption at *ca.* 235 nm in ultraviolet absorption spectra and strong bands at *ca.* 1750 and 1670 cm^{-1} in the infrared absorption spectra revealed the presence of an α,β-unsaturated lactone group. To begin with the analysis of the COSY spectrum of the compound, a starting point was selected. A convenient entry point of the COSY was the H-13a/b vinyl protons resonating at 5.55

Table 4. Inhibitory effect of purified antifungal compound on mycelial growth of *Phytophthora drechsleri*

	Concentration (μg/ml)			
Mycelial growth	25	12.5	6.25	3.12
	–[a]	–	+	+

[a] – = no growth, + = growth.

Table 5. Concentration of the purified antifungal compound from extracts of cocklebur for inhibiting zoospore germination of *Phytophthora drechsleri*

	Concentration (μg/ml)				
Germination of zoospore	125	62.5	31.25	15.62	7.81
	–[a]	–	–	–	+

[a] – = no germination, + = with germination.

Table 5. Concentration of the purified antifungal compound from extracts of cocklebur for inhibiting zoospore germination of *Phytophthora drechsleri*

	Concentration (μg/ml)				
Germination of zoospore	125	62.5	31.25	15.62	7.81
	–[a]	–	–	–	+

[a] – = no germination, + = with germination.

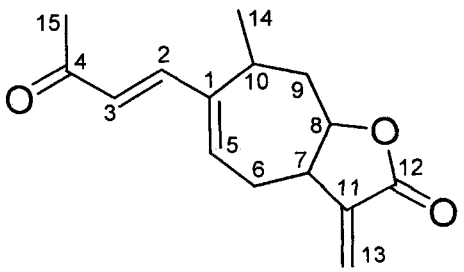


Fig. 2. Structure of antifungal compound, deacetyl xanthumin, extracted from *Xanthium strumarium*.

and 6.26 ppm, because nonequivalent methylene protons were linked to the same carbon (122.5 ppm) from the HMQC experiment. The H-13a/b vinyl protons were linked to their allylic H-7 resonating at 3.38 ppm, which was connected to its neighboring H-6a/b and H-8 protons resonating at 2.49, 2.57, and 4.62 ppm, respectively. The H-8 proton was directly linked to the H-9 vicinal protons (1.86 ppm), which was connected to H-9 resonating at 2.12 ppm. The H-9 proton was linked to H-14 (1.14 ppm), and the connectivity network terminates at H-14. Similarly, H-6 was connected to H-5 resonating at 6.18 ppm. The large coupling constant value (16 Hz) between the H-2 and H-3 indicates that both protons exist *trans* conformation. The isolated H-15 was confirmed from the DEPT and Mass data. From these spectroscopic data, the compound was identified as deacetyl xanthumin (Fig. 2).

Discussion

Chemical control for plant disease is the most prominent measure nowadays. For staple crops, chemical control is the most effective and stable method. However, for medicinal crops, it is not desirable to use chemical fungicide for many reasons, such as, residue and efficacy in soils, although metalaxyl is the most effective to *Phytophthora* diseases. Moreover, it is very difficult to determine optimum timing for fungicide treatment to control soil-borne disease.

The systemic fungicide, metalaxyl, although effective to *Phytophthora* (Cohen et al., 1979; Hamm et al., 1984), should be timely applied, i.e., before inoculation or within 2 days after inoculation (Coffey and Bower, 1984). Unfortunately, tolerant strains to metalaxyl have been reported to develop due to the variation of *Phytophthora* (Cavallito et al, 1944; Chang and Ko, 1990; Joseph and Coffey, 1984).

This study demonstrated that extracts of cocklebur effectively inhibited the mycelial growth and germination of zoospores of *P. drechsleri*. Considering the high amount of antifungal compound in the cocklebur fresh sap and its antifungal effect, application of cocklebur sap is highly probable to protect Atractylis from *Phytophthora* rot, instead of chemical fungicides.

Although researches on plant extracts for antifungal activity were numerous, the efficacies were rarely proven against plant fungal diseases. Powell and Ko (1986) reported that root extracts of garlic in the genus *Allium* inhibited the germination of zoospores and chlamydospores of *P. palmivora*, and indicated the possibility of finding antagonistic plants sources in nature. Ohmoto and Sung (1982) reported that the antifungal compound, β-asarone from *Acorus calamus,* was effective against mycotoxin producing *Aspergillus* spp.

Reports on chemical structures of compounds from extracts of cocklebur were abundant. However, their effectiveness against fungal disease has not been reported yet. Saxena and Mondal (1994) extracted xanthanolide from cocklebur, while Marco et al. (1993) extracted xanthanol and isoxanthanol from *X. strumarium* subsp. *italicum*. Meanwhile, Agata et al. (1993) and Malik et al. (1993) extracted sesquiterpene lactones from *X. strumarium*. Reports on extracts of new compounds from *Xanthium* are also available (Cole et al., 1980; Cutler and Cole, 1983; McMillan et al., 1976; Minato and Horibe, 1965).

This is the first report of the antifungal effects of sesquiterpenoid from cocklebur. The structure of antifungal compound extracted from cocklebur in this study was that of deacetyl xanthumin identified by Minato and Horibe (1965). In summary, the antifungal compound extracted from *X. strumarium* from this experiment was identified as sesquiterpene lactone of xanthanolide type, 4-oxo-1(5),2, 11,(13) -xanthatriene-12,8-olide having the molecular formula $C_{15}H_{18}O_3$ and MW 246. As the function of the compound has not been reported previously, this is considered as the first report of antifungal effect especially on *Phytophthora*.

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References

Agata, I., Goto, S., Hatano, T., Nishibe, S. and Okuda, T. 1993. 1,3,5-Tri-O-caffeoylquinic acid from *Xanthium strumarium*. *Phytochemistry* 33:508-509.

Cavallito, C. J., Buck, J. S. and Suter, D. M. 1944. Allium, the antibacterial principles of *Allium sativum*. II. Determinationm of the chemical structure. *J. Am. Chem. Soc.* 66:1952-1954.

Chang, T. T. and Ko, W. H. 1990. Resistance to fungicides and antibiotics in *Phytophthora parasitica*: Genetic nature and use in hybrid determination. *Phytopathology* 80:1414-1421.

Chang, T. T. and Ko, W. H. 1992. Variation in growth rate and colony morhphology of *Phytophthora parasitica* induced by metalaxyl. *Ann. Phytopath. Soc. Japan* 58:72-77.

Chen, C. P., Lin, C. C. and Namba, T. 1987. Development of natural crude drug resources from Taiwan (VI). *In vitro* studies of the inhibitory effect on 12 microorganisms. *Shoyakugaku Zasshi* 41:215-225.

Coffey, M. D. and Bower, L. A. 1984. *In vitro* variability among isolates of six *Phytophthora* species in response to metalaxyl. *Phytopathology* 74:502-506.

Cohen, Y., Reuveni, M. and Eyal, H. 1979. The systemic antifungal activity of ridomil against *Phytophthora infestans* on tomato plants. *Phytopathology* 69:645-649.

Cole, R. J., Stuart, B. P., Lansden, J. A. and Cox, R. H. 1980. Isolation and redefinition of the toxic agent from cocklebur (*Xanthium strumarium*). *J. Agri. Food Chemistry* 28:1330-1332.

Cutler, H. G. and Cole, R. J. 1983. Carboxyatractyloside: a compound from *Xanthium strumarium* and *Atractylis gummifera* with plant growth inhibiting properties. The possible "inhibitor A". *J. Natural Products* 46:609-613.

Hamm, P. B., Cooley, S. J. and Hansen, M. 1984. Response of *Phytophthora spp.* to metalaxyl in forest tree nurseries in the Pacific Northwest. *Plant Dis.* 68:671-673.

Jang, D. S. 1998. Biological activities of sesquiterpene lactones isolated from *Hemisteptia lyrata* Bunge, *Chrysanthemum zawadskii* Hirbich var. *latilobum* Kitamura and *Chrysanthemum boreale* Makino. Gyeongsang National University Ph.D thesis. 191 p.

Joseph, M. C. and Coffey, M. D. 1984. Development of laboratory resistance to metalaxyl in *Phytophthora citricola*. *Phyto-*

pathology 74:1411-1414.

Kim, D. K., Jee, H. J. and Kim, H. K. 1997. Occurrence of rhizome rot of *Atractylodes* spp. caused by *Phytophthora drechsleri*. *Korean J. Plant Pathol.* 13:433-437.

Malik, M. S., Sangwan, N. K. and Dhindsa, K. S. 1993. Xanthanolides from *Xanthium strumarium*. *Phytochemistry* 32:206-207.

Marco, J. M., Sanz-Cervera, J. F., Corral, J., Carda, M. and Jakupovic, J. 1993. Xanthanolides from *Xanthium*: absolute configuration of xanthanol, isoxanthanol and their C-4 epimers. *Phytochemistry* 34:1569-1576.

McMillan, C., Mabry, T. J. and Chavez, P. I. 1976. Experimental hybridization of *Xanthium strumarium* (Compositae) from Asia and America, 2: Sesquiterpene lactones of F1 hybrids. *American J. Botany* 63:317-323.

Minato, H. and Horibe, I. 1965. Studies on sesquiterpenoids XI. Structure and stereochemistry of xanthumin, a stereoisomer of xanthinin. *J. Chem. Soc.* 1965:7009-7019.

Ohmoto, T., Kano, M., Koike, K., Hitokoto, H. and Morozumi, S. 1981. Antimycotic substance in the crude drugs. *Shoyakugaku Zasshi* 35:71-77.

Ohmoto, T. and Sung, Y. I. 1982. Antimycotic substances in the crude drugs II. *Shoyakugaku Zasshi* 36:307-314.

Paik, S. B., Chung, I. M. and Doh, E. S. 1998. Screening of medicinal plants with antifungal activity on major seedborne disease. *Korean J. Medicinal Crop Sci.* 64:277-285.

Powell, C. R. and Ko, W. H. 1986. Screening for antagonistic plants for control of *Phytophthora palmivora* in soil. *Ann. Phytopathol. Soc. Japan* 52:817-824.

Reuter, H. D. and Sendl, A. 1994. *Allium sativum* and *Allium ursinum*: Chemistry, pharmacology and medicinal applications. In: *Economic and Medicinal Plant Reaserch Vol.6*, edited by Wagner, and Farnsworth, N. R. Published by Academic Press. 55-113.

Saxena, V. K. and Mondal, S. K. 1994. A xanthanolide from *Xanthium strumarium*. *Phytochemistry* 35:1080-1082.

Stoll, A. and Seebeck, E. 1948. Uber Alliin, die genuine Muttersubstanz des Knoblauchols. 1. Mitteilung uber Allium-Substanzen. *Helv. Chim. Acta* 31:189-210.

Yoshida, S., Kasuga, S., Hayashi, N., Ushiroguchi, T., Matsuura, H. and Nakagawa, S. 1987. Antifungal activity of ajoene derived from garlic. *Appl. Environ. Microbiol.* 53:615-617.