Antifungal Principle of *Ranunculus sceleratus*[1]

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So far antibiotic substances have largely been obtained from microorganisms, most of which act as antibacterials and do not show antifungal activity. Search for antibiotics from higher plants remained neglected for a long time but during recent years a large number of plants have been found to contain antibacterial substances. Antifungal substances from higher plants are comparatively little known, but the search for them continues on an increasing scale. Several plants have been screened for their fungitoxicity (Gilliver, 1947; Basu and Bose, 1956; Abdullaeva, 1959; Petrushova and Meshechaninova, 1961; Nene and Thapliyal, 1965; Thapliyal and Nene, 1967; Dhar et al., 1968; Gupta and Banerjee, 1970; Nicolls, 1970; Shekhawat and Prasad, 1971) but attempts to isolate and identify their active principle(s) are mostly wanting. The present paper records the isolation, identification, fungitoxicity, phytotoxicity and systemic activity of the antifungal active principle of *Ranunculus sceleratus* L. (Hindi: jaldhania; English: buttercup or water crowfoot), a widely occurring winter season herb with small, pale yellow flowers and nectaries at the base of the petals. It is also cultivated in gardens for its beautiful flowers and has been reported for its medicinal properties (Trivedi, 1965). Earlier, leaf extracts were found to possess strong fungicidal activity against several test fungi by the present authors (Misra et al., 1974; Misra, 1975; Misra and Dixit, 1976, 1977).

EXPERIMENTAL

Extraction of the active principle

As the volatile vapours arising from the crushed leaves of the plant also showed strong antifungal activity (Misra, 1975), it was thought that the active principle may be volatile in nature. Consequently, steam distillation of the leaves was carried out and the distillate was found to be equally effective, while leaves left after distillation possessed no activity. Therefore, isolation of the active principle was made from the steam distillate, as isolation from it is easier than from the crude extract of the plant.

Suitable solvent for extraction

Fifty ml of the steam distillate of the leaves were extracted separately with equal amounts of petroleum ether, ether and chloroform in separating funnels. After thorough shaking, the sets were left for an hour at room temperature. The solvent and the aqueous fractions were collected separately and tested for their fungitoxicity against *Alternaria tenuis* Nees, *Curvularia lunata* (Walker) Boedijn,

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Fusarium nivale (Fries) Cesati and *Helminthosporium gramineum* Rab. ex Schlecht by the method described by Nene (1971). Before the tests, the solvents from the solvent fractions were evaporated, and the viscous substances thus obtained were redissolved in 2 ml of the respective solvents. It was observed that only the chloroform fraction showed strong fungitoxicity, whereas the aqueous fraction was insignificantly active.

Isolation of the active principle

The isolation was performed following Baer et al. (1946). Five hundred grams of fresh leaves were washed and steam distilled to obtain 3 l distillate. The latter was extracted with 1 l of chloroform in several portions. The chloroform fraction was dried over anhydrous sodium sulphate and the solvent removed in vacuo below 40°C, and an oily substance weighing 5.0 g was obtained. The latter was strongly fungicidal, and was stored in the deep freeze for further investigation.

Thin layer chromatography of the substance

Thin layer chromatography of the oily substance on silica gel plates, run in benzene and developed in iodine vapour, indicated the presence of 2 spots—a larger with Rf. 0.65, and a smaller one with Rf. 0.1, indicating thereby the presence of one major, and one minor compound.

Properties of the oil

The oil was a pale yellow, volatile and irritating substance readily soluble in chloroform and slightly soluble in cold water. It gave a milky white emulsion with hot water. When treated with sodium hydroxide it showed reddish-brown colouration and reduced ammoniacal silver nitrate solution.

Since the physical and chemical characters (Baer et al., 1946) and antibacterial activity (Holden et al., 1947) were in conformity with the oily substance of *Anemone pulsatilla,* containing protoanemonin and anemonin, it was presumed that the oily substance of *R. sceleratus* might be a mixture of protoanemonin and anemonin.

Identification of the compound

The compound was identified with the help of ultra violet, infra red and nuclear magnetic resonance spectral studies.

Ultra violet spectrum

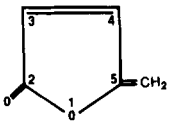
The compound showed an absorption peak at λ max^{EtOH}263 nm which indicated the presence of an extended α, β, unsaturated carbonyl-system in the molecule.

Infra red spectrum (neat)

Peaks at 1,775 and 1,650 cm^{-1} respectively were assigned to α, β unsaturated γ-lactone and a diene system.

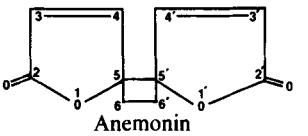
Nuclear magnetic resonance spectrum ($CDCl_3$)

The spectrum of the freshly prepared oily substance showed it to be a mixture of protoanemonin (93%) and anemonin (7%). The NMR signals for protoanemonin were observed as 2-proton multiplet at τ 4.85 for methylene protons numbered 6, i.e., C = CH_2 group. One proton multiplet for H-4 appeared at τ 3.67 while the H-3 proton appeared as a doublet at τ 2.40 (J = 6.0 Hz.):



Protoanemonin

In the NMR spectrum ($CDCl_3$) of anemonin the H-6 and 6′ appeared as a 4-proton multiplet at τ 7.47. The 2-proton doublets at τ 3.81 (J = 6.0 Hz) and τ 2.05 (J = 6.0 Hz) were respectively assigned to H-4 and 4′ and H-3 and 3′:



Anemonin

One major and one minor spot observed during thin layer chromatography supported the spectral studies.

Storage of the active principle

NMR of the only substance obtained from a 6-day-old steam distillate stored at room temperature indicated the presence of 88% protoanemonin and 12% anemonin. The oil on standing at room temperature crystallized, and its NMR spectrum showed the presence of 65% protoanemonin and 35% anemonin. These studies revealed that the monomer protoanemonin gradually dimerised into its dimer anemonin. Therefore, the compound must be stored in a deep freeze.

Lethal concentration of the active principle

Different dilutions (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000) of the active principle were prepared in chloroform by the serial dilution technique (Florey et al., 1949). A few intermediate dilutions, viz., 1:500, 1:5,000, 1:50,000 were also prepared from the aforesaid dilutions by adding requisite quantities of chloroform. These dilutions were tested for their fungitoxicity by the modified paper disc method (Nene, 1971) against the 4 test fungi mentioned.

It was noted that the freshly prepared active principle protoanemonin and its dimer anemonin completely inhibited the mycelial growth of all the test fungi up to 1:10,000 and 1:100 dilutions, respectively (Table 1).

Fungitoxic spectrum of the active principle

The active principle exhibited a broad fungitoxic spectrum, inhibiting as many as 26 additional test fungi at 1:10,000 dilution when tested by the method described earlier (Nene, 1971). The fungi inhibited were *Absidia spinosa* Lendner,

TABLE 1. LETHAL CONCENTRATION OF THE ACTIVE PRINCIPLE.

Dilutions[a] (V/V)	% inhibition of the test fungi							
	Freshly prepared oily substance (Protoanemonin)				Crystalline substance (Anemonin)			
	At	Cl	Fn	Hg	At	Cl	Fn	Hg
1:10	100	100	100	100	100	100	100	100
1:100	100	100	100	100	100	100	100	100
1:500	100	100	100	100	33.1	40.2	31.6	42.1
1:1,000	100	100	100	100	15.5	13.4	10.6	16.3
1:5,000	100	100	100	100	5.9	7.0	6.1	5.0
1:10,000	100	100	100	100	0	0	0	0
1:50,000	86.4	78.4	75.1	80.6	*	*	*	*
1:100,000	20.1	15.7	17.8	18.3	*	*	*	*

[a] Dilutions were prepared in chloroform by the serial dilution technique; 0 = no inhibition; * = growth accelerated.
At = *Alternaria tenuis*; Cl = *Curvularia lunata*; Fn = *Fusarium nivale*; Hg = *Helminthosporium gramineum*.

Alternaria solani (Ellis & Mart.) Jones and Grout, *Aspergillus aculeatus* Iizuka, *A. flavipes* Bainier and Sartory, *A. flavus* Link., *A. fumigatus* Fresenius, *A. japonicus* Saito, *A. niveus* Blochwitz, *A. nidulans* (Eidam) Winter, *A. niger* Van Tiegham, *A. sydowi* (Bainier and Sartory) Thom and Church, *A. terreus* Thom, *Cephalosporium sacchari* Butler, *Colletotrichum falcatum* Went, *Cunninghamella elegans* Lendner, *Monilia sitophila* (Montagne) Saccardo, *Mucor hiemalis* Wehmer, *Neocosmospora vasinfecta* E. F. Smith, *Periconia igniaria* Booth, *Pestalotia* sp., *Phomopsis* sp., *Rhizopus nigricans* Ehrenberg, *Thielavia terricola* (Gillman and Abbott) Emmons, *Trichoderma viridis* Pers. ex. Fries, *Verticillium terrestre* (Link) Lindan, Reinke and Berthold and *Zygorhinchus heterogamous* Vuillemin.

Phytotoxicity of the active principle

Tests were made following Grewal (1972) where seed germination, root application and foliar spray studies were carried out to determine the phytotoxicity of the active principle. Dilutions representing 1:5,000 and 1:10,000 concentrations of the active principle were tested for their phytotoxicity, and the tests were made on the tomato variety Marglove Supreme.

The findings on seed germination and root application are given in Table 2,

TABLE 2. PHYTOTOXIC STUDIES WITH THE ACTIVE PRINCIPLE.

Type of study	Observation on	Growth measurement (cm) at different dilutions and deviations from the control (± cm)				
		Control	1:5,000[a]		1:1,000[b]	
			T	D	T	D
Effect on seed germination	Root length	8.3	6.6	−1.7	6.8	−1.5
	Shoot length	7.6	5.0	−2.6	5.6	−2.0
	% seed germination	96	80	−16	88	−8
Effect on root application	Root length	7.0	0	−7.0	6.6	−0.4
	Shoot length	6.3	0	−6.3	6.3	0

[a] Hyperlethal dose; [b] lethal dose; T = treatment; D = deviation from the control.

which shows that the active principle possessed very little toxicity at its lethal concentration. Foliar spray with the active principle also induced no abnormality on the leaves or on the general behaviour of treated tomato plants.

Systemic activity of the active principles

This was determined following Grewal (1972) and Erwin et al. (1971). The application of the active principle was made both through roots and shoots of tomato variety Marglove Supreme, at concentrations 1:5,000 and 1:10,000.

In both studies no inhibition zone could be detected which indicated that the extracts were not systemically active at the dilutions tested.

DISCUSSION

Fungal attacks are very common in plants and animals and cause a great loss to agriculture and human health. The conventional synthetic fungicides are largely being discarded on account of their carcinogenicity, teratogenicity, phytotoxicity and residual effects. Search for antifungal antibiotics from different plant sources has recently been emphasized as they are devoid of such ill effects.

Ranunculus sceleratus has been found to exhibit strong fungitoxicity. The disease-free occurrence of the plant might be due to its highly toxic active principle protoanemonin. Bhakuni et al. (1969), however, have reported the plant to possess no activity while, on the contrary, Nene et al. (1968) have found it active. Bhakuni et al. (1969) missed the activity as they used dried materials and these have been found inactive (Misra, 1975). The active principle of the plant, having strong fungitoxicity and little phytotoxicity, can be exploited as an effective fungicide against a wide range of pathogens after it passes successful in vivo trials. It is further hoped that the active principle may also prove to be a valuable antibiotic for the cure of human fungal diseases in the future.

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