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Anthelmintic constituents of ferns

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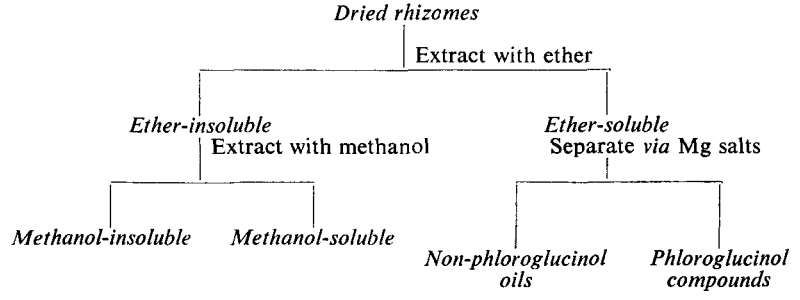
Extracts, oils and some individual phloroglucinol compounds prepared from the fern *Dryopteris dilatata* and *Dryopteris filix-mas* were tested for taeniacidal activity against the dwarf tapeworm *Hymenolepis nana*. In *D. dilatata* activity is concentrated in the phloroglucinol compounds (mainly aspidin, phloropyrone and aspidinol) of which aspidin is the most potent. In *D. filix-mas* the mixed phloroglucinol compounds (mainly flavaspidic acid and aspidinol) are active, but no more so than the total ether extract. Flavaspidic acid seems to be the most active phloroglucinol constituent of the fern. Desaspidin which occurs in a third fern, *Dryopteris austriaca*, is more potent than either aspidin or flavaspidic acid. A method of detecting phloroglucinol compounds by thin-layer chromatography is described.

POWDERED rhizomes of *Dryopteris filix-mas* and extracts of the plant, have been used for centuries as anthelmintics. However, the main disadvantages of such preparations are their variable composition, and toxicity. Several phloroglucinol compounds have been isolated from *D. filix-mas* and have been considered to be the active principles (Boehm, 1897; Widén, 1944) although activity has been claimed for other constituents, such as tannins and resins (Borkowski & Kowalewski, 1958). Again, aspidin, the most active phloroglucinol compound when tested *in vitro* against *Enchytraeus* is ineffective *in vivo* against *Hymenolepis fraterna* infections in mice (Büchi, 1957).

A related fern, *Dryopteris dilatata*, is reputed to be more effective than *D. filix-mas* as a taeniafuge (Rosendahl, 1911). The object of the work here reported was to compare the activities of the constituents of *D. dilatata* and *D. filix-mas* using the dwarf tapeworm *Hymenolepis nana* for both *in vitro* and *in vivo* assessments of activity.

The dried, powdered rhizomes of *D. dilatata*, for which we are indebted to Dr. G. A. Nelson of the Department of Pharmacology, University of Leeds, were fractionated according to the following scheme.



The rhizomes were collected near Golden Acre, Leeds, England, in November, 1959, dried at room temperature, and ground into a fine powder which was stored in the dark at 2° in stoppered bottles.

Ether-soluble material. Powdered rhizomes (500 g) were percolated to exhaustion at room temperature with ether and the solution evaporated down to give a dark green oil (23·2 g).

From Smith Kline and French Research Institute, Welwyn Garden City, Herts.

Treatment of ether-soluble material. The ether-insoluble material was dried in a vacuum and extracted continuously with hot methanol in a Soxhlet apparatus for 24 hr. The methanol extract was evaporated to dryness, finally in a vacuum, to give a pale brown solid (100 g). The methanol-insoluble marc (*ca* 370 g) was dried in a vacuum.

Separation of ether-soluble material into phloroglucinol and non-phloroglucinol components. The approved assay of the active principles of *D. filix-mas*, based on the amount of material extracted by aqueous barium hydroxide is not an accurate guide to the activity of the extract (Pabst & Bliss, 1932), and the isolation of the phloroglucinol constituents by means of their magnesium salts according to Ackermann & Mühlemann (1946) appears to be preferable.

The oil obtained by ether extraction of the rhizomes of *D. dilatata* was treated by the method of Büchi (1957) to give mixed phloroglucinol compounds (1·7 g). The material insoluble in magnesium hydroxide solution was dried in a vacuum and extracted to exhaustion in a Soxhlet apparatus with ether. Removal of the solvent from the extract gave non-phloroglucinol materials (7·5 g).

As a source of phloroglucinol compounds and non-phloroglucinol oils from *D. filix-mas*, a commercial sample of male fern extract, before dilution with arachis oil was used. This contained 37·8% filicin.

Isolation of non-phloroglucinol oils from male fern. Male fern extract (100 g) in ether (1,000 ml) was stirred with light magnesium oxide (300 g) suspended in water (2,500 ml) containing sodium sulphite (2·5 g). A further 500 ml of ether was added and the mixture stirred mechanically for 1½ hr. The mixture was filtered at the pump and sucked as dry as possible. The solid was dried on trays in air, then ground to a fine powder. The filtrates were extracted with ether, and the ether extract, together with 1 litre of ether, were stirred with the powder at room temperature for 3 hr. The mixture was filtered and the solid washed with ether. The filtrates were distilled down and dried in water-pump vacuum in a warm water-bath to give a greenish oil (21·3 g).

Isolation of aspidin from the phloroglucinols from D. dilatata. The crude phloroglucinol mixture (2·9 g) isolated as above was dissolved in ether, and exhaustively extracted with 2% aqueous sodium carbonate and the extract acidified with hydrochloric acid. The precipitate was centrifuged down, washed with water and dried in a vacuum to give 2 g of material. Recrystallisation of this from petroleum (b.p. 40–60°) and then from 95% ethanol gave crude aspidin (0·55 g), m.p. 110–112°. This material (0·5 g) in benzene (10 ml) was purified through a column of silica gel (Lights Silica Gel for Chromatography 100–200 mesh), 38 cm long by 2 cm diameter made up in light petroleum (b.p. 60–80°) and fitted at the top with a two-necked separating funnel (500 ml) nearly full with light petroleum (b.p. 60–80°) and equipped with a mechanical stirrer. A second separating funnel, stoppered and containing benzene, was arranged with its stem just dipping below the surface of the light petroleum in the first funnel so that, as the petroleum passed down the column it was replaced by a progressively enriched mixture of petroleum

and benzene. Fractions of 5 ml were taken and bulked according to the spots on the chromatograms, to give crude aspidin (0·325 g) which was crystallised from methanol as pale yellow needles (0·27 g), m.p. 124–125°.

Isolation of aspidinol from oils of D. dilatata. Oils (22 g) obtained by the ether extraction of dried rhizomes of *D. dilatata* were distilled in high vacuum in a short-path distillation apparatus and the fraction (1·3 g) which distilled at 110–125° (bath temp.) at 10^{-4} mm was collected. The pale-yellow solid was repeatedly recrystallised from benzene to give aspidinol (0·2 g), m.p. 140–145°.

Isolation of flavaspidic acid. Male fern extract (200 g) was treated by the method of Widén (1944) to give flavaspidic acid (5 g), m.p. 155–157° crystallised from benzene.

Thin-layer chromatography of phloroglucinol compounds from ferns. The mixtures of phloroglucinol compounds obtained by the above methods were subjected to paper chromatography on unbuffered and buffered paper impregnated with formamide and developed with benzene: chloroform using the methods of Klevstrand (1957) and Penttilä & Sundman (1961a). Although these procedures gave distinct separations of components, the Rf values could not be used as unequivocal guides to their identities.

We have found that a more convenient method of detection was by thin-layer chromatography on Kieselgel G. By this means the components could be quickly detected and the method could be applied not only to the phloroglucinol components after partial purification *via* their magnesium salts but also to the crude ether extracts of the rhizomes.

TABLE 1. CHROMATOGRAPHIC DATA

Substance	Rf value	Colour with Fast Blue Salt B Reagent
Flavaspidic acid	0	Reddish-mauve
Phloropyrone	0·17	Yellow
Desaspidin	0·25	Purple-red
Aspidin	0·35	Yellow
Aspidinol	0·6	Purple

The thin layers were made by shaking Kieselgel G (Merck) (34 g) with distilled water (60 ml) for 90 sec and then applying to the plates (10 × 20 cm) in 250 μ thickness. The plates were dried as recommended. The Shandon "Unoplan" apparatus was used. Mixtures of crude phloroglucinols (20–50 μg) or pure phloroglucinol compounds (10 μg) were applied to the plates in chloroform and developed in the tank supplied with the apparatus lined with filter paper soaked in the developing solvent. The plates were developed in chloroform:acetone (3:1 by volume) at 21·5° for approximately 50 min, by which time the solvent front had travelled 10 cm from the starting line. If greater separation of the spots was required, the plates were developed for 1½ hr. After drying, the plates were sprayed with 0·1% aqueous Fast Blue Salt B (Merck). The phloroglucinol compounds appeared as coloured spots (Table 1).

The major spots on the chromatograms were identified by comparison with pure substances isolated by the rather laborious methods from the ferns.

Biological testing

Various extracts, oils, and individual phloroglucinol compounds from the ferns were tested for activity against the dwarf tapeworm *H. nana* by an *in vitro* method based on that of Sen & Hawking (1960) and an *in vivo* method based on that of Steward (1955).

Worms for testing *in vitro* were obtained from mice killed 12–15 days after infection. The worms were freed from the small intestine by squeezing the intestine between a moistened sheet of glass and the non-cutting edge of a fine pair of angled scissors. The length of the intestine was slowly and carefully pulled between the two. The faeces were placed in a dish containing boiled and cooled tap water. The mixture was gently stirred to free the worms. Worms approximately $\frac{1}{2}$–$\frac{3}{4}$ in. in length were removed and placed in a second dish of boiled and cooled water where they were gently washed. Then they were placed in the nutrient broth medium containing penicillin and streptomycin as described by Sen & Hawking (1960). The nutrient broth containing the tapeworms was kept in an incubator at 37° until the worms were required (1–3 hr later). Special care was taken in adjusting the pH of the nutrient broth to exactly 8·5 using a direct reading pH meter. 5 mg of the substance to be tested was warmed to 37–40° in 0·5 ml of absolute ethanol. When the substance was dissolved, the volume was made up to 5 ml with nutrient broth. Various dilutions were prepared, and 3 ml of each dilution was placed in a small Kjeldahl flask. One worm was placed in each flask. The flasks were sealed with parafilm and incubated at 37°. The worms were examined at 2, 18 and 24-hr intervals. Only the 24-hr results are shown. In each experiment, controls consisting of worms in nutrient broth alone, and in nutrient broth containing ether extract of *D. filix-mas*, were set up.

The method of *in vivo* testing was based on that of Steward (1955). Mice from stock not infected with *H. nana* weighing from 14–16 g were given by stomach tube approximately 500 *H. nana* ova per mouse. Only mature ova from proglottids of adult worms were counted using a haemacytometer chamber. An appropriate dilution was made so that there were 1000 ova/ml. The diluted ova were allowed to stand at room temperature for 24–48 hr before infecting the mice.

The substances to be tested were given by mouth in a 1% sodium glycocholate solution on the 12th day of the infection. A group of infected mice received 1% sodium glycocholate only. Food was not withheld before treatment nor were the mice purged. The mice were killed on the 15th day and the tapeworms, removed from the intestine as described in the *in vitro* method, were counted. This method of removal eliminated the necessity to withhold food for 24 hr before killing. In assessing the results a form of computation similar to that of Steward (1955) was used.

Results

By the chromatographic procedure described, the major phloroglucinol compounds in *D. filix-mas* appeared as flavaspidic acid and aspidinol whereas those in *D. dilatata* were aspidin, aspidinol and an unknown substance having an Rf value of 0·17. This last substance was removed from a series of plates on which 150 mg of mixed phloroglucinol compounds had been separated, using the small scale "vacuum-cleaner" technique of Ritter & Meyer (1962). By this procedure 4·5 mg of the substance was obtained. Its infra-red spectrum showed a strong absorption peak at 1630 cm^{-1} and in the ultra-violet had maximum at 215, *ca* 311 and 355 mμ, indicating it to be phloropyrone, the structure of which has been elucidated by Penttilä & Sundman (1961b).

TABLE 2. MINIMUM CONCENTRATIONS REQUIRED TO KILL *Hymenolepis nana* AT pH 8·5 AND 37° IN 24 HR

Substance	Minimum lethal concentration
D. dilatata—	
Ether-soluble material	1:500,000
Mixed phloroglucinol compounds	1:1 million
Oils	1:10,000
Methanol soluble fraction of ether-insoluble material	>1:10,000
D. filix-mas—	
Ether extract (37·8% filicin)	1:500,000
Mixed phloroglucinol compounds	1:500,000
Oils	1:10,000
Individual phloroglucinol compounds—	
Aspidin	1:5 million
Aspidinol	1:100,000
Desaspidin	1:10,000 to 1:500,000
Flavaspidic acid	1:500,000
Phloropyrone	1:100,000

Various extracts of the two ferns, oils, and individual phloroglucinol compounds, were tested for anthelmintic activity. The results of *in vitro* tests are shown in Table 2. The ether extracts of the ferns are both active (1:500,000), as are the mixed phloroglucinol compounds (from *D. dilatata* 1 in 1 million, from *D. filix-mas* 1:500,000). The oils are far less active (1:10,000) and the methanol-soluble fraction of the ether-insoluble residue of *D. dilatata* was inactive. Of the individual phloroglucinol compounds, aspidin was most active (1:5 million). Changes in pH altered the minimum lethal concentration of aspidin. At pH 6 the minimum lethal concentration was 1:50 million, at pH 7, 1 in 20 million and at pH 9, 1 in 5 million. Next in potency were flavaspidic acid and desaspidin (1:500,000). However, the results with desaspidin were irregular, ranging from 1:10,000 to 1:500,000. Desaspidin did not dissolve either in 0·5 ml absolute ethanol or in the nutrient broth. Aspidinol and phloropyrone were weaker at 1:100,000.

All of the above extracts, oils, and individual compounds (except phloropyrone, of which there was an insufficient quantity) were tested in mice infected with *H. nana*. The results are summarised in Table 3. In general the results of these tests show greater variability than the

TABLE 3. THE ACTION OF SOME FERN CONSTITUENTS ON *Hymenolepis nana in vivo*

Treatment	Oral dose per kg	Mice surviving / Mice treated	Treated/Untreated ratios: Mice free from infection	Treated/Untreated ratios: Average computations	% Activity
D. dilatata					
Ether-soluble material ..	100 mg	8/8	0/8 to 0/8	141/191	26
	100 mg	7/7	0/7 to 0/7	177/147	0
Mixed phloroglucinols ..	100 mg	7/7	2/7 to 0/7	37/141	74
Oils	100 mg	9/10	0/9 to 1/10	190/165	0
	400 mg	19/20	2/19 to 2/19	77/53	0
	25 g	6/7	0/6 to 0/9	60/124	52
Methanol-soluble fraction of ether-insoluble material ..	100 mg	9/10	0/9 to 1/10	259/165	0
D. filix-mas					
Extract of *D. filix-mas* (containing 37·8% filicin) ..	50 mg	20/20	4/20 to 3/19	130/145	10
	100 mg	20/20	4/20 to 0/20	83/188	56
	250 mg	20/20	8/20 to 3/20	36/165	78
	400 mg	20/20	7/20 to 1/20	20/217	91
Mixed phloroglucinol compounds	100 mg	7/7	0/7 to 0/6	222/413	46
Oils	100 mg	7/7	1/7 to 0/7	60/67	10
	400 mg	7/7	1/7 to 0/7	51/67	24
	1000 mg	7/7	0/7 to 0/7	71/90	21
	25 g	1/8	1/1 to 0/8	2/8	100
Phloroglucinol compounds					
Aspidinol	100 mg	7/7	0/7 to 1/7	155/100	0
Flavaspidic acid	100 mg	5/6	2/5 to 1/10	66/140	53
	100 mg	6/6	1/6 to 1/10	37/140	74
	100 mg	12/12	2/12 to 4/12	85/111	23
	200 mg	3/10	0/3 to 1/10	2/140	99
	200 mg	3/10	3/3 to 1/10	0/140	100
	200 mg	6/12	3/6 to 4/12	25/111	77
Aspidin	10 mg	10/10	2/10 to 2/10	86/65	0
	50 mg	10/10	3/10 to 2/10	36/65	45
	100 mg	10/10	5/10 to 1/10	7/89	92
	100 mg	20/20	2/20 to 0/20	192/173	0
	100 mg	7/7	2/7 to 0/7	14/279	95
	100 mg	10/10	1/10 to 0/10	116/144	19
	200 mg	10/10	6/10 to 0/10	3/111	97
	200 mg	10/10	2/10 to 0/10	28/111	75
Desaspidin	10 mg	8/8	0/8 to 0/8	78/85	9
	50 mg	8/8	0/8 to 0/8	27/86	69
	100 mg	8/8	8/8 to 0/8	0/227	100
	250 mg	8/8	8/8 to 0/8	0/227	100

in vitro tests. However, of the plant extracts studied, the ether extract of *D. filix-mas*, and the mixed phloroglucinols from both *D. filix-mas* and *D. dilatata*, were all active at 100 mg/kg. The oils from the two ferns showed some activity in very high doses (25 g/kg = 0·5 ml/mouse approx.). The methanol soluble fraction of the ether insoluble material from *D. dilatata* showed no activity at 100 mg/kg, nor, surprisingly, did the ether extract of *D. dilatata*. Of the individual phloroglucinol compounds desaspidin, aspidin and flavaspidic acid were all active at 100 mg/kg, but aspidinol was inactive.

Discussion

In these experiments two methods of testing for taeniacidal activity were used. The *in vitro* method has the advantage that it requires only small quantities of test materials, but results from these tests alone could

be misleading since it is well known that drugs active *in vitro* may be inactive against the same worm *in vivo*. Moreover, these tests would indicate drugs that are toxic to all living organisms. Steward's method of *in vivo* testing compares the number and size of worms found at autopsy in the intestines of a treated group of mice, with the number and size of worms in a "control" untreated group of mice. Steward believed that large worms were more readily removed by treatment than smaller worms, and this was the basis of his computation. But another possibility is that sublethal doses of taeniacides may so damage the tapeworms that they are broken into fragments. The scolices and rostral segments of the worm remain attached to the intestinal wall and at autopsy these would be counted as small worms. Fragmentation of worms occurs in the clinical use of inadequate doses of taeniacidal drugs, hence the importance of finding the scolex in the faeces to be sure that treatment has been effective. However, larger doses of the drug cause elimination of the complete worm. Our experiments using Steward's method show that low doses of extract of *filix-mas* or of mepacrine reduce the average size (length) of the worms without reducing the number of the worms. Higher doses reduce both the number and size of the worms. Whatever the precise mechanism of action of taeniacides in this test, drugs that are effective in the clinic are also active in this test (Steward, 1957; Sen & Hawking, 1960) and these findings have been confirmed by the present authors.

In general the minimal lethal concentrations recorded in the *in vitro* experiments show less variation than the results obtained in the *in vivo* tests. An exception to this is desaspidin, which did not dissolve in the 0·5 ml of absolute ethanol from which dilutions were prepared and where the minimal lethal concentration varied in different experiments between 1:10,000 to 1:500,000. Sen & Hawking (1960) experienced similar difficulty in testing the insoluble drugs tetrachloroethylene and carbon tetrachloride. The variability of the *in vivo* tests may be illustrated by the results obtained with aspidin 100 mg/kg. In different experiments the percentage activity was 0, 19, 92 and 95 though the activity repeatedly determined *in vitro* remained constant at 1:5 million. In assessing the anthelmintic activity of the various fern extracts and constituents, data from both *in vitro* and *in vivo* experiments will be considered.

D. dilatata. The ether-soluble material was active *in vitro* and possibly active *in vivo* (100 mg/kg). No activity was found in the methanol extract of ether-insoluble material. Of the constituents of the ether-soluble material, the mixed phloroglucinol compounds were highly active in both tests. The oil fraction was inactive *in vitro* and inactive *in vivo* unless massive quantities were administered. Thus the main anthelmintic activity of the fern residues in the phloroglucinol compounds. The major phloroglucinol compounds were aspidin, aspidinol and phloropyrone. Of these aspidin is active in both tests, aspidinol was weakly active *in vitro* and inactive *in vivo* at 100 mg/kg, phloropyrone was weakly active *in vitro* and was not tested *in vivo*. It is concluded that the phloroglucinol

compounds are the main active constituents of *D. dilatata*, the most active single constituent probably being aspidin. Büchi (1957) found that aspidin was inactive against *H. fraterna* infections in mice but he used a low dose (10 mg/kg).

D. filix-mas. The starting material was ether extract said to contain 37·8% filicin. This was active in both tests. The oils were weakly active *in vitro* (1 : 10,000) and had possibly slight activity *in vivo*. The mixed phloroglucinol compounds were active in both tests but not appreciably more active than the original ether extract from which they were prepared. It may be that (*a*) there is an active substance present in the ether extract which is neither phloroglucinol compound nor oil, (*b*) that the various constituents enhance each others activity, (*c*) that active phloroglucinol compounds are in part destroyed by the extraction process, or (*d*) the apparent discrepancy may be due to the insensitive nature of the test procedures. Of the major phloroglucinol compounds in *D. filix-mas*, flavaspidic acid seems to be the most potent, being active in both tests, though at 200 mg/kg some of the treated mice died. However, desaspidin obtained from *D. austriaca* (Aebi, Büchi & Kapoor, 1957) proved to be the most potent phloroglucinol compound *in vivo*. In practice the potency of an anthelmintic is of less interest than the therapeutic index. Östling (1962) gives the oral LD50 values (mg/kg) in mice as desaspidin 260, phloropyrone 530, flavaspidic acid 700, and aspidin 995. So desaspidin appears to be the most potent and the most toxic of the phloroglucinol compounds. Nevertheless, both desaspidin and flavaspidic acid have been successfully used in the treatment of human tapeworm infestations (Östling, 1962; Anttonen, 1954).

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References

Aebi, A., Büchi, J. & Kapoor, A. (1957). *Helv. chim. acta*, **60**, 266–274.
Ackermann, M. & Mühlemann, H. (1946). *Pharm. acta Helvet.*, **21**, 157–177.
Anttonen, V. M. (1954). *Ann. Med. exp. Fenn.*, **32**, 15–25.
Boehm, R. (1897). *Arch. exp. Path. Pharmak.*, **38**, 35–58 and later papers.
Borkowski, J. & Kowalewski, Z. (1958). *Acta polon. pharm.*, **15**, 15–21.
Büchi, J. (1957). *J. Pharm. Belg.*, **12**, 391–417.
Klevstrand, R. (1957). *Dansk Tidsskr. Farm.*, **31**, 217–221.
Östling, G. (1962). *Nord. med.*, **67**, 15–24.
Pabst, M. L. & Bliss, A. R. (1932). *J. Amer. pharm. Ass.*, **21**, 431–441.
Penttilä, A. & Sundman, J. (1961a). *J. Pharm. Pharmacol.*, **13**, 531–535.
Penttilä, A. & Sundman, J. (1961b). *Acta chem. scand.*, **15**, 839–848.
Ritter, F. J. & Meyer, G. M. (1962). *Nature, Lond.*, **193**, 941–942.
Rosendahl, H. (1911). *Pharm. J.*, **87**, 35.
Sen, A. B. & Hawking, F. (1960). *Brit. J. Pharmacol.*, **15**, 436–439.
Steward, J. S. (1955). *Parasitology*, **45**, 255–265.
Widén, B. (1944). *Acta bot. fenn.*, **37**, 4–79.