Passicol, an Antibacterial and Antifungal Agent Produced by *Passiflora* Plant Species: Qualitative and Quantitative Range of Activity

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Fungal molds and yeasts, an actinomycete, and gram-positive and gram-negative bacteria were tested qualitatively with an antimicrobial substance, here called "Passicol," obtained from *Passiflora* species. A wide range of organisms were susceptible to Passicol. Some bacteria and a yeast were tested quantitatively by a broth dilution method. Passicol was nontoxic to mice and rabbits.

Nicolls (10) described antifungal activity present in plants of the *Passifloraceae*, particularly in the *Passiflora* species *P. caerulea* (passion flower), *P. edulis* (purple passion fruit), and *P. mollissima* (banana passion fruit). Activity was obtained from living tissues.

Birner and Nicolls in an accompanying paper (3) showed that the antimicrobial active principle, now named "Passicol," in *P. mollissima* fruit rinds had ultraviolet absorption characteristics of the polyacetylenic group of compounds. Formation of the active compound by gentle bathing of living plant pieces in water in the presence of air, extraction by ethyl acetate, concentration, and a spectrophotometric assay method are described in the companion paper. Whereas Nicolls (10) described inhibition only of fungi, and the fungi chosen affected mainly plant species, in this study we examined fungi of a more medical interest, as well as gram-positive and gram-negative bacteria and an actinomycete.

MATERIALS AND METHODS

Cut stem pieces of *P. caerulea* were used to test the effect of Passicol, as it occurred in the plant, on a number of microorganisms seeded and grown on agar-based media in petri dishes (Table 1, Fig. 1 and 2).

Nearly all extracts of the active compound were obtained from immature fruit rind of *P. mollissima* by methods previously described (3). The spectrophotometric assay method used here was also described (3). Optical density at 375 nm was a mea-

sure of activity of Passicol-containing extracts. All optical density measurements were made with 10-mm cells.

Disc diffusion susceptibility test on agar (qualitative method). Zones of inhibition around pieces of plant tissue and plant diffusates and extracts, on agar media seeded with fungus spores or bacteria, were measured after 1 or more days of growth at 22 or 37 C. With most organisms, only 1 day of growth was necessary, but some slow-growing fungi such as *Microsporum* and *Trichophyton* required several days of growth.

The results were not always quantitatively comparable, as the initial density of inoculum was variable. Where possible, a comparable spore density was used.

Qualitative plating methods. Melted agar medium (7 to 8 ml) was first poured into a petri dish and allowed to solidify. Then 5 to 6 ml of cooled melted agar medium seeded with spores or vegetative growth of the organisms to be tested was poured evenly over the first layer in the petri dish. In a few tests with blood-agar medium, a light smear of bacteria was spread on the surface of the agar in the petri dish.

Active extracts were placed on sterile filter-paper discs 6.5 mm in diameter or in stainless-steel Heatley cups, also 6.5 mm in diameter, set in the agar. The small amounts of liquid diffusates and extracts used on paper discs were measured with a 100- μ liter pipette.

After ethyl acetate extracts were placed on the filter-paper discs, the solvent was quickly dried off over a Bunsen burner flame before the discs were placed in position on the agar. Control discs were treated in the same way with the organic solvent only. Care was necessary, as ethyl acetate itself can in-

hibit growth of microorganisms.

As a comparison, an aqueous solution of Passicol was used. This was prepared by evaporating ethyl acetate active extract under vacuum and dissolving the residue in phosphate buffer solution (pH 7).

Quantitative broth dilution tests. The material used was an aqueous suspension of the active compound in 1% phosphate buffer which, after removal of ethyl acetate, was brought to pH 7 by the addition of sodium bicarbonate. The strength of this suspension was measured by its optical density at 375 nm by dilution of a sample. The suspension used for quantitative work had a calculated optical density of 257.

For quantitative work on gram-positive and gram-negative bacteria and a yeast, 1 ml of half-strength aqueous Passicol suspension was mixed with 1 ml of nutrient broth, making a 1:4 dilution of the Passicol, and then doubling dilutions in broth were made in successive tubes. A control of phosphate buffer was used in each experiment. The inoculum used was a 0.02-ml drop of a 1:1,000 dilution of an overnight broth culture of the test microorganism. Immediately after inoculation, a viable count was performed by the Miles and Misra (9) method by taking 0.1 ml, diluting it suitably, and counting the colonies growing on nutrient agar plates from a spread 0.02-ml drop. After 18 hr of incubation at 37 C, another viability count was performed on a sample of 0.1 ml taken from each tube, similarly diluted, and plated in quadruplicate. Viable counts were also carried out on the phosphate buffer control. In this way, it was possible to establish the concentrations at which the extract was bacteriostatic or bactericidal. Glucose broth and 10% blood-agar plates were used for streptococci, and Sabouraud broth agar was used for the yeast.

Optical density of Passicol extracts. All measurements were made by ultraviolet spectroscopy, at the required wavelength after dilution suitable for the instrument range, and were expressed as equivalent undiluted values.

RESULTS

Qualitative results. Table 1 compares the effects of Passicol on the growth in vitro of a range of microorganisms, including fungal molds and yeasts, an actinomycete, and gram-positive and gram-negative bacteria. Pieces of stem tissue of *P. caerulea*, 1.5 mm thick and 1 to 2 mm in diameter, and ethyl acetate extracts with an optical density of 7.95 at 375 nm were used to test inhibitory effects on organisms growing on various agar-based media. The fungi *Microsporum* and *Trichophyton* required additional amounts of extract on each of 2 or 3 days because of their slow growth.

It can be seen from Table 1 that a wide range of microorganisms was inhibited by Passicol. Increasing concentrations of Passicol in phosphate buffer solutions (pH 7) applied in stain-

less-steel Heatley cups set in the agar medium gave increasingly wide zones of inhibition (Table 2). Increasing concentrations of Passicol applied in ethyl acetate extracts dried on paper discs did not always give wider zones of inhibition on seeded agar, and the results of the latter method cannot be considered quantitative. Its advantages, however, are speed and convenience in testing the susceptibility of a number of different organisms.

Figures 1-3 show the inhibitory effects of Passicol against a number of microorganisms. Wide zones of inhibition were produced by transversely and longitudinally cut pieces of *P. caerulea* stem with the fungi *Microsporum gypseum*, *M. cookei*, and *Aspergillus fumigatus* (Fig. 1a-c); smaller zones were produced with the yeast *Rhodotorula* sp. (Fig. 1d). One stem piece on the *Rhodotorula* plate (Fig. 1d) was cut at the ends only and shows that Passicol diffusing from cut tissues produces the inhibition zones. All microorganisms shown in the figures, except *A. fumigatus*, were grown at 22 C; *A. fumigatus* was grown at 37 C. Several longitudinally cut pieces of *P. caerulea* were placed together to produce the central zone in Fig. 1c. Other microorganisms inhibited by cut stem pieces are shown in Fig. 2a-d. The effect of Passicol in ethyl acetate extracts (optical density, 62.0 at 375 nm) is also shown for the four organisms in Fig. 2a-d. The central part of the zones around the filter-paper discs is shown to be stronger than the outer part with the two *Candida* species (Fig. 2c and d).

As a control measure, filter-paper discs immersed in ethyl acetate solvent only were treated in the same way as the Passicol-containing ethyl acetate extracts (Fig. 3a and b). Filter-paper discs containing Passicol ethyl acetate extracts show inhibition zones in Fig. 3a-c. As a comparative test, filter-paper discs immersed in phosphate buffer solutions containing Passicol were placed on agar seeded with *A. fumigatus* (Fig. 3d).

Table 2 compares the diameters of inhibition zones produced when increasing concentrations of crude Passicol extracts in phosphate buffer (pH 7) solutions were tested against *Trichophyton mentagrophytes* (ringworm) and *Candida albicans* growing at 28 C. Optical density readings at 375 nm are given for each concentration. The results confirm under quantitative conditions the more qualitative observations recorded for these organisms in Table 1. Buffer solutions used as controls gave no inhibition zones.

TABLE 1. Effects of Passicol on a range of fungi and bacteria and on one actinomycete

Organism	Inhibition zones ^a with		Amt of ethyl- acetate extract (μliters)	Medium ^b
	Stem tissue	Ethyl- acetate extract		
Molds				
<i>Arthroderma flavescens</i>	29	35	10	S.A.
<i>Aspergillus fumigatus</i>	25	42	10	S.A.
<i>Cladosporium herbarum</i>	18	35	10	P.D.A.
<i>Epidermophyton floccosum</i>	15	17	10	S.A.
<i>Microsporium canis</i>	20	22	3 × 10 ^c	S.A.
<i>M. cookei</i>	25	23	2 × 10 ^c	S.A.
<i>M. gypseum</i>	17	26	2 × 10 ^c	S.A.
<i>Penicillium notatum</i>	18	27	10	P.D.A.
<i>Trichophyton mentagrophytes</i>	—			
<i>T. quinckeanum</i>	—	39	2 × 10 ^c	S.A.
		30	2 × 10 ^c	S.A.
Yeasts				
<i>Candida albicans</i>	8	25	10	S.A.
<i>C. krusei</i>	6	17	10	S.A.
<i>Cryptococcus neoformans</i>	15	35	10	S.A.
<i>Rhodotorula</i> sp.	7	24	10	S.A.
<i>Torulopsis glabrata</i>	7	24	10	S.A.
Actinomycete				
<i>Nocardia asteroides</i>	17	33	10	S.A.
Gram-positive bacteria (aerobic)				
<i>Corynebacterium xerosis</i>	—	34	25	N.A.
<i>Staphylococcus aureus</i>	8	32	10	N.A., S.A.
Gram-positive bacteria (anaerobic)				
<i>Clostridium sporogenes</i>	—	8	25	B.A.
Gram-negative bacteria				
<i>Escherichia coli</i>	0	15	10	S.A.
<i>Salmonella</i> sp. C2	0	14	10	S.A.
<i>S. stanley</i>	0	12	10	N.A.
<i>Shigella sonnei</i>	0	19	10	S.A.

^a Inhibition zones produced by cut pieces of *Passiflora caerulea* stem tissue (1.5 mm thick and 1 to 2 mm in diameter) and by Passicol-containing ethyl acetate extracts of *P. mollissima* (optical density of extracts = 7.95 at 375 nm). Zones are measured in millimeters by diameter of the cleared area including the piece of stem or filter-paper disc. Organisms were grown at 22 C except for *Clostridium*, which was grown at 37 C.

^b S.A. = Sabouraud agar medium; P.D.A. = potato dextrose agar; N.A. = nutrient agar; and B.A. = blood-agar.

^c Additional amounts required on 2 or 3 days because of slow growth.

Some microorganisms were grown at both 22 and 37 C to compare the effects of different temperatures on susceptibility to Passicol. Filter-paper discs containing dried Passicol from 10 μliters of ethyl acetate extract (optical density of 7.95 at 375 nm) were used for each test. Microorganisms tested were *A. fumigatus*, *Torulopsis glabrata*, *Nocardia asteroides*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* group C2, *S. stanley*, and *Shigella sonnei*. In each case, zones of inhibition were smaller at the higher temperature of 37 C. This may be due to faster growth of organisms as well as to the greater lability of Passicol at high temperatures.

Quantitative results. Quantitative tests were performed on six microorganisms, and

TABLE 2. Inhibition of *Trichophyton mentagrophytes* (ringworm) and *Candida albicans* by increasing crude concentrations of Passicol in phosphate buffer (pH 7) solutions set in cups in agar^a

Organism	Concn of solution ^b	Diam of inhibition zone (mm) ^c
<i>T. mentagrophytes</i>	2.47	22
	4.94	23
	7.41	26
<i>C. albicans</i>	2.47	13
	4.94	17
	7.41	18

^a The organisms were grown at 28 C.

^b Optical density at 375 nm.

^c Controls (buffer solutions) showed no inhibition.

Table 3 shows the corresponding minimal concentration necessary for bactericidal and bacteriostatic effects. The calculated optical density of the undiluted solution at 375 nm was 257.0. A solution was considered to be bactericidal if after overnight incubation all organisms were killed, and bacteriostatic if the viable count was the same as it was at the time of inoculation.

From Table 3, it can be seen that the *Streptococcus* strain was very much more susceptible to the extract than the *Staphylococcus* strain was and that the effect on *Candida* was intermediate. The extract was relatively ineffective in its action on the gram-negative

bacteria, and it was ineffective against *Pseudomonas*.

An interesting fact about the rate of action of the extract was noted. For this experiment, each broth dilution tube was inoculated separately with a standard inoculum of a well-shaken diluted suspension of bacteria, and an immediate count was performed about 1 min after inoculation. The number of colonies counted from the staphylococci at 1:4 and 1:8 dilutions of the suspension was only half the number counted in the greater dilutions and in the control. A similar effect was observed with the streptococci, as shown in Table 4. This could not be due to carry-over of the extract,

FIG. 1. Passicol in living stem tissue of *Passiflora caerulea* causing zones of inhibition on agar seeded with spores of (a) *Microsporium gypseum*, (b) *M. cookei*, (c) *Aspergillus fumigatus*, and (d) *Rhodotorula* sp. A, transverse section of stem; B, longitudinally sliced piece of stem; C, piece of stem with ends only cut. The large zones in Fig. 1a could be explained by a weaker spore inoculum.

FIG. 2. Antibiotic activity due to *Passiflora* in *Passiflora caerulea* stem tissues and in extracts of *P. mollissima* fruit rind. Zones of inhibition on agar seeded with spores: (a) *Nocardia asteroides*, (b) *Cryptococcus neoformans*, (c) *Candida albicans*, and (d) *C. krusei*. A, transverse section of stem; B, longitudinally sliced piece of stem; C, ethyl acetate extracts (optical density of 62 at 375 nm) on filter-paper discs 6.5 mm wide.

as the sample was diluted at least a further 500 times for individual colony counts, and it is known that the extract is not active at these concentrations.

To test the speed of action, bacterial counts were performed on staphylococci immediately after inoculation and 2, 4, and 18 hr later (Table 5).

At concentrations at which the solution was bacteriostatic, it was noted that staphylococcal colonies tended to be much smaller than those in the controls, and the size of individual colonies showed great variation. If the plate was replaced in the incubator for a further 24 hr,

most colonies grew to normal size. The organisms when stained with Gram stain showed normal morphological characteristics.

Toxicity test. Three mice and one rabbit were used for the toxicity test. For each mouse, 1 ml of the crude ethyl acetate extract, having an optical density of 192.0 at 375 nm, was dried under vacuum and then dissolved in 0.85% sodium chloride solution. The pH was adjusted to 7.0 by the addition of sodium bicarbonate, the final volume being 1 ml. The insoluble material was separated by centrifugation, and the supernatant fluid was injected intravenously into each mouse. All three mice survived.

FIG. 3. *Passicol* extracts causing zones of inhibition on agar seeded with (a) *Nocardia asteroides*, (b) *Candida albicans*, (c) *Shigella sonnei*, and (d) *Aspergillus fumigatus*. Ethyl acetate extracts were used for the first three organisms (a-c); C represents a control (solvent only) disc producing no inhibition zones, and A and B represent *passicol*-containing ethyl acetate extracts with optical densities at 375 nm of 12 and 28, respectively. Phosphate buffer (pH 7) solutions were used with *A. fumigatus* (d): D, weaker; E, stronger.

The rabbit, which weighed 835 g, received an intravenous injection of 5 ml of an aqueous solution prepared by dissolving crude dry material in phosphate buffer, neutralizing with sodium bicarbonate, and then adding 40 mg of sodium deoxycholate. The solution had an optical density of 188.7 at 375 nm. The rabbit has survived for many months.

DISCUSSION

The accompanying paper (3) indicated that the antifungal and antibacterial agent *Passicol*,

which occurs in all living tissues of *Passiflora* plants, belongs to the polyacetylenic group of compounds.

Crude extracts of *P. mollissima* (banana passion fruit) tissues have the ultraviolet absorption spectra with multiple maxima characteristic of polyacetylene compounds (11). Vegetative tissue extracts of *P. caerulea* (passion flower) and *P. exoniensis* show similar ultraviolet absorption spectra, but detailed studies have been made only on extracts from *P. mollissima* fruit rind. It has been known for some time that acetylenic and polyacetylenic

TABLE 3. *Minimal concentrations of Passicol needed to give a bactericidal or a bacteriostatic effect against gram-positive and gram-negative bacteria and the yeast Candida albicans*^a

Organism	Bactericidal concn	Bacteriostatic concn
<i>Staphylococcus aureus</i> (Oxford)	1:8	1:32
Group A hemolytic <i>Streptococcus</i>	1:40	1:160
<i>Escherichia coli</i>	—	1:4
<i>Salmonella enteritidis</i>	—	1:4
<i>Pseudomonas aeruginosa</i>	—	—
<i>C. albicans</i>	1:16	1:32

^a Broth dilution tests were used. The calculated optical density of undiluted Passicol solution was 257.0 at 375 nm.

TABLE 4. *Broth dilution tests with Passicol suspensions showing numbers of staphylococcal and streptococcal colonies counted immediately after inoculation*

Dilution of Passicol suspension	No. of colonies	
	Staphylococci	Streptococci
1:4	11.5×10^4	5.5×10^3
1:8	14.5×10^4	4.5×10^3
1:16	29.5×10^4	7.5×10^3
1:32	26.0×10^4	9.0×10^3
Control	25.5×10^4	8.0×10^3

TABLE 5. *Colonies of Staphylococcus aureus present at various times after the addition of a 1:4 broth dilution of Passicol*

Time of incubation (hr)	No. of colonies ^a
0	71
2	42
4	30
18	Nil

^a Colonies were counted on plates onto which 0.02-ml amounts of a 1:1,000 dilution of an *S. aureus* culture had been spread.

antibiotic activity occurs in some higher fungi and higher plants, but most of the active factors are too toxic or too unstable to be of practical value (4, 6, 11).

Acetylenic compounds which inhibit germination or mycelial growth of some fungi have been described (1, 8, 12). Wyerone acid, a monocetylenic compound, was obtained from fungus-infected broad bean (8). It inhibited germination of the fungi *Botrytis fabae*, *B. cinerea*, and *B. allii*. Maximal ultraviolet absorption of the compound was at 350 nm. An

antifungal polyacetylenic compound from *Phytophthora*-infected safflower inhibited mycelial growth of the fungus *P. dreschleri* (12). The polyacetylenic compound Safynol gave 10 ultraviolet absorption maxima ranging from 354 to 215 nm (1).

Capillin is an antibiotic from *Artemisia capillaris*, a plant of the *Compositae* family, which Sorensen (11) stated has long been known to be fungicidal. Mycomycin was the first fungal polyacetylene antibiotic described (7) and characterized (5). Some other fungal polyacetylene antibiotics were described by Anchel (2).

Passicol inhibits the growth of many microorganisms in vitro. The active compound seems different from other acetylenic compounds (1, 8), as maximum ultraviolet absorption occurs at 340, 360, and 375 to 380 nm (3).

The relative ease of production of Passicol from the fruit rind of banana passion fruit could make it a useful product. The plant vines grow well in sheltered, frost-free positions in Tasmania and Victoria, in southern Australia, and produce fruit for 8 months of the year for many years. The nontoxic properties of Passicol, the wide range of microorganisms inhibited, and particularly its antifungal properties are of special interest.

Passicol can also be produced from fruit rinds of the purple passion fruit, which are waste products of passion fruit juice manufacture.

Although the activity of Passicol remained high indefinitely in ethyl acetate extracts, it was gradually lost in aqueous solutions and quickly lost (usually within 1 or 2 days) in the dried residues. It was found, however, that the addition of some substances, for example, dextran, milk, and milk products, gave protection and considerable stability to dry Passicol-containing residues when they were added to aqueous diffusates and phosphate buffer solutions before freeze-drying. Most of the additive substances so far tested have been of high molecular weight.

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