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Research Article

ANTIBIOTIC STUDIES ON AN EXTRACT FROM *LEPTOTAENIA MULTIFEDA* [1, 2, 3]

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Numerous surveys (1–8) have demonstrated the wide occurrence of antibiotically active materials in higher plants. In a recent investigation Matson and Flowers (9) examined 440 plants found in Utah and neighboring states. Certain of these plants have long been used for medicinal purposes by both American Indians and white men. Among the families which appeared to contain a higher percentage of species and genera containing antibiotic substances was the *Umbelliferae* family. Matson and Flowers found in preliminary experiments that an aqueous extract of the root and leaves of *Leptotaenia multifeda,* commonly known as "Indian carrot," inhibited growth of *Micrococcus aureus* and *Escherichia coli.* The root of *L. multifeda* has been used by the Gosiute Indians in the form of a paste for application upon wounds, cuts or bruises where the skin was broken (10). This dressing was particularly applied in case of infection.

Some of the preliminary work of the present paper was reported by Ravve (11). At about the same time a publication by Carlson and Douglas (12) described the antibiotic activity of oil fractions obtained from the root of a related plant, *Leptotaenia dissecta.* Further work on the extraction and purification of an oil from the root of *L. multifeda* and a study of its antibiotic properties is herein described.

METHODS

Extraction and purification. L. multifeda was collected on the foothills of the Wasatch Range northeast of Salt Lake City, Utah. The plants were stored in a refrigerator until used. Among the several methods studied for the extraction of oil from the root, the following represents a preferred procedure. The water-washed and peeled root was macerated in a meat grinder, placed in a flask and covered with ethyl acetate. After 24 to 48 hours at room temperature the mixture was filtered, and the solvent was removed under reduced pressure from the yellow filtrate. The recovered ethyl acetate was suitable for reextraction purposes. The extraction was conducted at room temperature in order to minimize any tendency toward loss of activity through polymerization. The extraction of a given batch of root was repeated three times. This procedure yielded approximately 9% of crude extract by weight of the cleaned, peeled, wet root of *L. multifeda.*

The crude extract was washed 10 or more times with water. This treatment gave material which will be designated as extract purified by water washing.

A more complete purification procedure involved treatment with sodium bisulfite. The crude oil was first washed thoroughly with a dilute, aqueous solution of sodium bicarbonate and then thoroughly washed with water. To 150 ml. of a saturated solution of sodium bisulfite in 95% ethanol was added a solution of 25 ml. of oil in 150 ml. of absolute alcohol. The resulting precipitate was filtered and washed with several portions of ethanol. There was no visible evidence of unreacted oil. The bisulfite addition complex was added to a two phase mixture of 30% aqueous acetic acid and petroleum ether (b.p. 30–60° C.). The petroleum ether, containing the liberated oil, was washed 10 or more times with water and dried over anhydrous sodium sulfate. Solvent removal under reduced pressure left the regenerated oil in 50 to 60% yield based on the crude oil. The resulting product was dissolved in alcohol-free benzene and was passed through a column packed with Magnesol [4]-Celite [5] (five to one, by weight) to remove polymerized material. Solvent removal left a light yellow oil in approximately 50% yield based on the bisulfite purified extract. Elution of the column with 200 to 1 benzene-ethanol (by volume) followed by solvent removal gave a brown viscous oil.

The use of ethylene glycol as a carrier in distillations under reduced pressures appeared to be useful if small quantities only of the oil were desired. At 60° C. and several microns pressure ethylene glycol and the oil, which were immiscible, distilled in the volume ratio of about five to one, respectively.

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[4] A product of Westvaco Chlorine Products Co., South Charleston, West Virginia.

[5] No. 535. A product of Johns-Manville Co., New York, New York.

Agar plate assay. To 250 ml. of cooled melted agar medium was added 5 ml. of an 18 to 24 hour broth culture of the test organism. An amount of 20 ml. of the seeded agar was poured into each petri plate and allowed to harden at room temperature. A sterile filter paper disc 10 mm. in diameter was immersed in the material to be assayed. Excess liquid was allowed to drain, and the disc was placed in the center of the poured plate. Plates were observed after 18 to 24 hours incubation at 37° C. The diameter of the zone of inhibition was measured to the nearest millimeter and noted as complete or partial. The figures given are averages of three or more plates. Dilutions of the materials tested were made in sterile mineral oil, Saybolt viscosity 335–350 at 38° C. Mineral oil controls were made with each organism. Penicillin G sodium at a concentration of 10^{-2} Gram per ml. of aqueous solution was used for comparative purposes. In addition Duracillin diluted with peanut oil to contain the same number of penicillin units per ml. as the aqueous solution was employed.

Broth tube assay. To 4.5 ml. of serial dilutions of the oil in sterile mineral oil was added 0.05 ml. of a one to 100 dilution of an 18- to 24-hour broth culture of the test organism. The tubes were shaken to distribute the organisms thoroughly through the mineral oil. After one-fourth, one-half, one, eight, and 24 hours, loopfuls (diameter of loop 4 mm.) of the suspensions were withdrawn from each tube and added to sterile broth. All tubes were observed for growth 48 hours after inoculation.

Toxicity assay. The animals used were 20-Gram, male, Swiss, white mice eight to ten weeks old. All mice received one subcutaneous injection. In certain instances the oils were diluted with sterile peanut oil to facilitate injection. The crude extract was free of ethyl acetate. Sterile peanut oil was used as the control.

RESULTS

Ethyl acetate extraction of the washed and peeled macerated root of *L. multifeda* provided a rapid and convenient means of obtaining a yellow, water insoluble oil in high yield.

The crude extract was tested *in vitro* for antibacterial properties against 14 representative Gram-positive and Gram-negative organisms (Table I). Complete or partial inhibition of growth was exhibited with ten organisms including all of those belonging to the Gram-positive group. In most cases in which the oil was active, it was effective at a concentration of 10^{-3} in mineral oil. At comparable concentrations (10^{-2}) penicillin and the crude oil were roughly equally effective against the organisms susceptible to both agents.

The extract purified by water washing was also evaluated in agar plate tests (Table II). On the whole and particularly at the lower concentrations, thorough washing with water appeared to have resulted in greater activity. The aqueous extracts from the washing treatments did not show any appreciable activity against Gram-positive organisms.

The effectiveness of the water-washed extract against three organisms in nutrient broth tests was also determined (Table III). These results were comparable with those obtained in agar

TABLE I

Activity of a crude extract

Organism	Diameter of zone of inhibition in mm.								
	Straight	Conc. in mineral oil						Penicillin G, 10^{-2}, in	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Peanut oil	Water
Micrococcus aureus	80	55	47	25	14	12	n	20	24
Corynebacterium diphtheria	42	34	28	18	14	n	n	40	43
Diplococcus pneumoniae III	37	40	28	22	n	n	n	17	22
Streptococcus pyogenes	33	44	30	25	15	n	n	27	32
Bacillus subtilis	24	32	28	20	n	n	n	27	33
Vibrio comma	45	33	35	26	n	n	n	41	50
Neisseria catarrhalis	37	39	29	22	12	n	n	26	31
Escherichia coli	40p	37p	15p	n	n	n	n	19	25
Pseudomonas aeruginosa	33p	18p	n	n	n	n	n	n	n
Proteus vulgaris	17p	13p	n	n	n	n	n	33p	37p
Salmonella schottmuelleri	n	n	n	n	n	n	n	27	33
Aerobacter aerogenes	n	n	n	n	n	n	n	13	18
Klebsiella pneumoniae	n	n	n	n	n	n	n	14	17
Serratia marcescens	n	n	n	n	n	n	n	n	n

n—no inhibition
p—partial inhibition

TABLE II

Activity of extract purified by water washing

Organism	Diameter of zone of inhibition in mm.								
	Straight	Conc. in mineral oil						Penicillin G, 10^{-2}, in	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Peanut oil	Water
Micrococcus aureus	78	58	29	24	14	11	n	20	24
Corynebacterium diphtheria	56p	48p	39p	30p	18p	n	n	40	43
Diplococcus pneumoniae III	51	44	36	28	18	14	n	17	22
Streptococcus pyogenes	39	24	29	27	21	11	n	27	32
Bacillus subtilis	51	39	34	22	13	n	n	27	33
Vibrio comma	61	43	32	31	14	n	n	41	50
Neisseria catarrhalis	74	67	39	35	15	13	n	26	31
Escherichia coli	45p	17p	14p	11p	n	n	n	19	25
Pseudomonas aeruginosa	24p	14p	12p	n	n	n	n	n	n
Proteus vulgaris	15	14	13	n	n	n	n	33p	37p
Klebsiella pneumoniae	n	n	n	n	n	n	n	14	17
Serratia marcescens	12	15	13	n	n	n	n	n	n

n—no inhibition
p—partial inhibition

TABLE III

Activity of extract purified by water washing as determined in nutrient broth

Time in hours	Conc. in mineral oil																	
	Micrococcus aureus						*Escherichia coli*						*Streptococcus pyogenes*					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
¼	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
½	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
1	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
8	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
24	−	−	−	−	+	+	−	+	+	+	+	+	−	−	−	−	+	+

− indicates no growth
+ indicates growth

TABLE IV

Activity of bisulfite treated extract after and prior to passage through Magnesol-Celite

Organism	Diameter of zone of inhibition in mm.					
	Straight	Conc. in mineral oil				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Micrococcus aureus	−	78(45)	61(20)	32(15)	17(14)	11(11)
Streptococcus pyogenes	90(37p)	85(25p)	30(27p)	30(19p)	20(15p)	6p(11p)
Bacillus subtilis	−	82(28)	68(21)	38(18)	17(12)	13(n)
Vibrio comma	85(85p)	83(77p)	58(42p)	30(26p)	19(16p)	6p(14p)
Escherichia coli	34p(n)	16(n)	14(n)	13p(n)	n(n)	−
Serratia marcescens	23p(n)	23p(n)	15p(n)	14p(n)	n(n)	n(n)

Figures in parentheses are activities prior to passage through Magnesol-Celite.
− not done
n—no inhibition
p—partial inhibition

TABLE V

Effect of the oil on acid-fast organisms

Organism	Diameter, zone of inhibition in mm.				
	Crude extract	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Mycobacterium lacticola	55	49	41	22	12
M. phlei	57	42	36	17	12
M. tuberculosis, var. *hominis*	40	40	22	15	11

plate tests in that the oil was much more active against the Gram-positive organisms.

Regeneration of the oil from the crystalline sodium bisulfite addition complex was accomplished by treatment with aqueous acetic acid. Although the reaction mixture was cooled, some polymerization appeared to have occurred as evidenced by the increased viscosity of the resulting oil. When this material in benzene solution was passed through a column packed with Magnesol-Celite (five to one, by weight), an oil with greater fluidity was obtained upon removal of solvent. The activity of this material was determined (Table IV) and found to be significantly greater than either the crude or the water-washed extract. The dark viscous oil adsorbed on the column exhibited but little activity against *M. aureus*.

The crude oil was found to be effective against both pathogenic and non-pathogenic acid-fast organisms (Table V). In all cases zones of complete inhibition were obtained at a concentration as low as 10^{-4} in mineral oil.

TABLE VI

Toxicity of extract toward mice

	Dose in *ml.*	No. of mice	Fatalities
Crude	0.025*	3	0
	0.05†	3	3
Water washed	0.30	2	0
	0.50	2	0
Bisulfite purified	0.05†	3	0
	0.18‡	3	0
Passed through column	0.18	2	0
Adsorbed on column	0.15	2	0
Peanut oil control	0.50	3	0

* Diluted to 0.05 ml. with peanut oil
† Diluted to 0.10 ml. with peanut oil
‡ Diluted to 0.27 ml. with peanut oil

Crude, water-washed, and bisulfite-purified specimens of the oil were tested for toxicity toward mice. Bisulfite-purified extract passed through Magnesol-Celite and material adsorbed on the column were also evaluated. The low toxicity of the water-washed and bisulfite-purified oils was indicated by the fact that subcutaneous injections of from 0.9 to 2.5 % of body weight of the mice did not noticeably affect their behavior (Table VI). Tolerance of the crude extract before washing with water was considerably lower.

The heat sensitivity of the crude oil was indicated by the reduction of activity against *M. aureus* and also by the increase in viscosity when the oil was maintained at 100° C. or higher for one hour (Table VII). Oil obtained by steam distillation of the crude extract at atmospheric pressure (640 mm.) likewise showed reduced activity against this organism. No appreciable loss of activity against *M. aureus*, however, was noted when the crude oil was heated in ethyl acetate at 69° C. for two hours.

TABLE VII

Effect of heat on a crude extract

Conc. in mineral oil	Activity against *Micrococcus aureus* Diameter of zone of inhibition in mm.			
	Control unheated	1 Hour at °C.		
		100	150	200
10^{-1}	47	40	15	27
10^{-2}	36	19	18	11
10^{-3}	25	15	11	n
10^{-4}	21	11	n	n
10^{-5}	14	n	n	n

DISCUSSION

The fact that an antibiotically active oil can be extracted from *L. multifeda* in high yield has greatly facilitated the investigation of this material since ample quantities for the desired laboratory studies were readily obtained.

While the oil was quite effective in agar plate tests against certain Gram-negative organisms such as *Vibrio comma* and *Neisseria catarrhalis*, it appeared to be much more generally active against members of the Gram-positive group. This was indicated by the complete inhibition obtained even at concentrations of 10^{-3} with all Gram-positive organisms used. These included

M. aureus, Corynebacterium diphtheria, Diplococcus pneumoniae III, Streptococcus pyogenes, and *Bacillus subtilis.* These tests suggest that the oil diffused through an essentially aqueous medium. A water-insoluble antibiotic of this nature might afford effective action for a comparatively long period of time *in vivo.* The oil was also effective *in vitro* against *Mycobacterium tuberculosis,* var. *hominis* and the two other acid-fast organisms tested. The results indicate the desirability of *in vivo* tests, which are in progress.

Washing the crude extract with water provided an effective means of removing material which was somewhat toxic to mice. The fact that subcutaneous injections of water-washed oil equivalent to 2.5% of the weight of the mice did not produce any deaths, or even any noticeable difference in behavior, suggested that the active material was only slightly if at all toxic to mice when administered in this way.

Heat stability tests showed that the oil underwent polymerization readily at 100 to 200° C. with a gradual loss in activity against *M. aureus.* This tendency toward polymerization upon heating complicated the purification of the oil since conventional distillation procedures even at low pressures and in the presence of antioxidants were not feasible. Distillation in the presence of immiscible liquids such as water or ethylene glycol offered a means of obtaining small quantities of distilled oil. A purification procedure involving regeneration of the oil from a sodium bisulfite addition complex, however, was found to be a convenient means of obtaining an oil of greatly enhanced antibacterial activity.

The oil, obtained by ethyl acetate extraction of the root of *L. multifeda,* appeared to be quite different in certain respects from the oil fractions Carlson and Douglas (12) obtained by the steam distillation of the root of *L. dissecta.* Both of these plants belong to the *Umbelliferae* family. The yellow oil obtained from *L. dissecta* was reported to decolorize upon standing in air and to be bactericidal against *E. coli* in a short time in broth tests. This behavior was clearly in contrast with that shown by the oil from *L. multifeda,* which showed no tendency toward decolorization even after several weeks in air, and which was only slightly effective against *E. coli* in broth tests and then only after long contact. Further, the active oil obtained by Carlson and Douglas was described as being heat stable while the extract of *L. multifeda* undergoes polymerization at temperatures of 100° C. or higher with a concomitant reduction in activity.

Major emphasis thus far has been placed on the extraction and purification of the oil with a view to devising a satisfactory procedure for obtaining the active material in reasonably pure form. In preliminary chemical studies the oil was found to decolorize readily aqueous potassium permanganate and bromine in carbon tetrachloride. This behavior along with other results suggests that the antibiotic may be an unsaturated carbonyl compound containing only carbon, hydrogen, and oxygen. Further chemical studies are in progress.

SUMMARY

1. A light yellow, water-insoluble oil possessing antibiotic properties has been obtained in high yield by the ethyl acetate extraction of the macerated root of *L. multifeda.*

2. The oil showed considerable activity *in vitro* against the five Gram-positive organisms and also against certain of the nine Gram-negative bacteria tested.

3. Penicillin G and the crude or the water washed extract at concentrations of 10^{-2} were roughly comparable in agar plate tests in antibacterial activity against the Gram-positive and Gram-negative organisms sensitive to both.

4. Complete inhibition of acid-fast organisms, including *M. tuberculosis,* var. *hominis,* by the extract was observed at a concentration as low as 10^{-4} in mineral oil.

5. A procedure involving regeneration of the sodium bisulfite addition complex of the oil resulted in a purified material having substantially enhanced bactericidal activity.

6. Water washed or bisulfite purified extract showed no visibly noticeable toxic effect in mice when relatively large doses were given subcutaneously.

7. Preliminary chemical studies suggest that the oil may be an unsaturated carbonyl compound containing only carbon, hydrogen and oxygen.

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