The weights and feed intake were the same for the control group as well as for the groups receiving the drug. No visual differences could be observed in the rats receiving the drug over those in the control group. Furthermore, the rats showed no sensitivity to the drug.

Four rats out of each series (*i.e.*, two males and two females) were checked at the end of 8 weeks and no abnormalities were found with respect to blood glucose, cholesterol, phospholipids, and urea nitrogen suggesting that no chemical changes resulted from the use of the drug.

SUMMARY AND CONCLUSIONS

In carefully controlled studies carried out over a period of 8 weeks, involving 120 rats, no statistically significant changes occurred in the blood picture with respect to red cell, white cell, and differential counts when varying amounts of dipyrone were administered orally.

No visual differences could be observed in the rats receiving the drug over those in the control group.

No rats died as the result of taking the drug.

No abnormalities were found with respect to blood glucose, cholesterol, phospholipids, and urea nitrogen on examining animals from each group suggesting that no chemical changes resulted from the use of the drug.

In this study using massive doses of dipyrone no indications of agranulocytosis were produced.

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Phytochemical Investigation of *Abies concolor*

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Extracts of the bark of *Abies concolor* have shown antitumor activity against the adenocarcinoma of the duodenum test system of the Cancer Chemotherapy National Service Center. One of the active materials appears to be a complex tannin. The isolation of the active fractions has been reported utilizing solvent extraction, column, paper, and thin-layer chromatography.

In a routine screen of Arizona and Mexico plants for antitumor activity, it was found that extracts of the bark of *Abies concolor* (Gordon and Glendinning) Hoopes were shown to have activity against the adenocarcinoma of the duodenum (7D1) test system of the Cancer Chemotherapy National Service Center, Bethesda, Md. The plant, also known as white fir, is a soft-wooded, resinous evergreen monoccious tree, 100–200 ft. tall, 3–6 ft. in trunk diameter. It is distributed from Wyoming west to Oregon and south into Baja California, Arizona, New Mexico, and Sonora, at elevations of 3000–10,000 ft.

The collection used in this study was obtained from upper Sabino Creek, 8000 ft. elevation, Santa-Catalina Mountains, Pima County, Ariz.[1]

EXPERIMENTAL

Preliminary Extraction.—The bark (3.5 Kg.) of *A. concolor* was extracted with chloroform–ethanol (1:1) in a Lloyd extractor for 2 days. After evaporation, this crude extract was submitted to the adenocarcinoma of the duodenum test system (7D1). The crude material showed a decrease in tumor size of approximately 86 and 84% at a dose of 400 and 200 mg./Kg., respectively (Table I).

Table I.—*In Vivo* Tumor Inhibition

	Dose, mg./Kg.	% T/C[a]
n-Hexane extract	175	32
	200	32
Brown powder	100	26
	200	11

[a] The criteria for activity is defined as being a % T/C (test/control) value of less than 42 in a satisfactory dose response test (1).

One kilogram of the crude material was extracted with 2000 ml. of *n*-hexane in a Soxhlet for 72 hr. The green *n*-hexane extract upon evaporation yielded 200 Gm. of gummy material. A brown powder was obtained in the Soxhlet. Both of the materials were active against the test system.

n-Hexane Extraction

Twenty-five grams of the green material obtained from the *n*-hexane extraction was extracted with 10 × 100 ml. of petroleum ether (36–60°). The petroleum ether was evaporated to dryness in a Rinco evaporator under reduced pressure. A light green residue (10 Gm.) was obtained.

Part A.—Five grams of this residue was chromatographed on a neutral alumina column (30 × 3 cm.) (Fisher certified reagent catalog No. A-950 Brockman, activity I, 80–200 mesh). The column

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[1] Identification confirmed by Robert Barr, Research Associate, College of Pharmacy, and Dr. Charles Mason, Curator of the Herbarium, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited.

was successively washed with the following solvents until extinction: (*a*) benzene, (*b*) chloroform, (*c*) ether.

(*a*) The benzene fraction was evaporated to a small volume. A white substance was separated (45 mg.). This substance was purified by preparative thin-layer chromatography using 20 × 20 cm. plates covered with a slurry of Silica Gel G (35 Gm. in 50 ml. of water), and activated in an oven at 110° for about 2 hr. Benzene–petroleum ether (3:7) was used as the developing solvent. The compound obtained from the plates was crystallized from ethanol and had a melting point of 82–83°. The I.R. curve showed that it was a simple long chain ketone. The semicarbazone derivative was oily. The oxime was prepared. It had a melting point of 59°. The compound was identified as palmiton by comparing the melting point of the compounds and oxime derivative (2) and I.R. curves. The remaining part of the benzene fraction was evaporated to dryness. An oily compound (2.0378 Gm.) was obtained. Preparative thin-layer chromatography indicated a series of materials. These appear to be small molecular weight terpenes. Since a thorough work has been performed by Carlberg (3), and since this part had no activity, no further work has been done.

(*b*) The chloroform fraction was evaporated to dryness and yielded 2.2 Gm. of material. Again by means of the preparative thin-layer chromatography, using chloroform–petroleum ether (7:3) as the developing solvent, two main spots and a series of minor compounds were obtained. Since the two main compounds were close to each other, they were extracted from the plates with chloroform and rechromatographed with petroleum ether–ether (1:1) as the developing solvent. A better separation was achieved. The yields of the compounds were 430 mg. for compound I with an R_f value 0.5, and 700 mg. of compound II with an R_f value of 0.7. Compound I was recrystallized from methanol and had a melting point of 136–137°. The I.R. spectrophotometry and mixed melting points indicated that the compound was *p*-menthandiol-(1,4). Compound II was recrystallized from acetone and had a melting point of 70–71°. The I.R. spectrophotometry and mixed melting points indicated the compound to be docosonol-1.

(*c*) The ether fraction, when evaporated, yielded 0.5318 Gm. of material. Preparative thin-layer chromatography with chloroform–ether (99:1) as developing solvent showed four spots (R_f values were 0.2, 0.27, 0.5, 0.95). The yields were 54.2 mg., 66.8 mg., 53.4 mg., and 203.8 mg., respectively. The first spot had a melting point of 88–89°. The I.R. curve has bands at 3509, 2941, 2874, 1718, 1626, 1595, 1508, 1464, 1422, 1266, 1156, 1022, 975, 845, 808, and 720 cm.$^{-1}$. The U.V. curve has a maximum at 321, 243, and a minimum at 263 mμ. The other three compounds were simple ketones and alcohols. No further investigation of the above materials was carried on since they were inactive.

Part B.—The remaining material after the petroleum extraction (13 Gm.) was diluted to 10 times its weight with 50% aqueous potassium hydroxide (4). The mixture was left to cool and 4 times the volume of ethanol was added. The mixture was then refluxed under nitrogen atmosphere for 3 hr. At 30-min. intervals a small amount of the mixture was subjected to thin-layer chromatography using petroleum ether–ether–acetic acid (70:30:2) solvent system. This was done in order to see if the hydrolysis was completed. After the 3-hr. refluxing period the volume was increased 3 times with water. The solution was extracted with ether (10 × 100 ml.), the ether was washed with water, and dried over anhydrous sodium sulfate. The yield of the nonsaponifiable portion was 9 Gm. One gram of this material was applied to a preparative thin layer of silica gel, and chloroform–benzene–ether (6:2:3) was used as the developing solvent system. Three spots were obtained with R_f values of 0.97, 0.6, and 0.4. The material at 0.97 R_f was oily and in minimal concentration. The middle spot yielded 395 mg., which was recrystallized from alcohol and then from acetone. It had a melting point of 76–77°. Comparison of the I.R. curves and mixed melting points showed that it was lignoceryl alcohol. The third compound (117.6 mg.) was recrystallized from alcohol. It had a melting point of 138–139°. The I.R. curve comparison and mixed melting point showed that it was β-sitosterol.

After the extraction of the nonsaponifiable part with ether, the aqueous solution was acidified and extracted with ether (10 × 100 ml.). Upon evaporation 6.5 Gm. of brown material was obtained. A portion of this material (2.5 Gm.) was separated using preparative thin layers with petroleum ether–ether–acetic acid (90:10:1) as the developing system. Four fractions were obtained. R_f values were 0, 0.01, 0.04, and 0.2, and the amounts 550 mg., 1.200 Gm., 200 mg., and 280 mg., respectively. All of these fractions were subjected to further separation using reverse phase paper and thin-layer chromatography. Papers were covered with 7 and 12% of paraffin in benzene and the developing solvent was 85% aqueous methanol. Fraction 4 gave a yellow spot on a blue background when sprayed with bromophenol blue. Methyl derivatives were prepared and only one spot was obtained in the case of fraction 4 when sprayed with Sudan IV solution (5). A dimethyl polysiloxane,[2] 5% in ether, was used to impregnate the silica gel plates for the reverse phase thinlayer chromatography. Acetic acid–formic acid–water (40:40:20) was used as the mobile phase. Again fraction 4 revealed one single spot. This fraction (280 mg.) was recrystallized from alcohol. It had a melting point of 79–80°. The I.R. curve comparison and the mixed melting points revealed that it was lignoceric acid. The compounds thus far isolated from the *n*-hexane extracts do not exhibit antitumor activity in the 7D1 test system.

Alcohol Soluble Part

The material remaining after the *n*-hexane extraction was an amorphous red-brown powder which charred above 250°. Elementary analysis showed 16.9% inorganic residue and the absence of nitrogen, sulfur, and halogens. Magnesium and hydrochloric acid (6), ferric chloride (6), gelatin test (7), and Wilson's boric acid test (8) suggested that this material was a mixture of complex tannin and flavonoid substances. Attempts to purify this crude material were made using the following chromatographic substrates: silica gel, aluminum oxide (neutral),

[2] Marketed as Dow Corning 200 fluid by the Dow Corning Corp., Midland, Mich.

TABLE II.—R_f VALUES OF DELPHINIDIN AND CYANIDIN IN DIFFERENT SOLVENT SYSTEMS

No.	Solvent System	Known		Unknown	
		Cyanidin	Delphinidin		
1	H_2O–HCl–Acetic acid (10:3:30)	0.52	0.31	0.52	0.30
2	*n*-Butanol–acetic acid–H_2O (4:1:5) (upper phase)	0.91	...	0.93	...
3	Formic acid (88%)–3 *N* HCl (1:1)	0.23	0.12	0.23	0.12
4	*n*-Butanol–2 *N* HCl (1:1) (upper phase)	0.62	...	0.62	...

magnesium trisilicate, and polycaprolactam pulver "S".[3]

The first three columns adsorbed the material. When polycaprolactam pulver "S" is mixed with the same amount of diatomaceous earth[4] and packed into a 35 × 3 cm. column, a yellow substance was obtained from ethanol elutions. This was a flavonoid substance. Column chromatography with polycaprolactam powder utilizing preparative thin-layer chromatography yielded the same material, but no antitumor activity was demonstrated by this material. Solvent purification was then employed. Fifty grams of the crude material was dissolved in alcohol and upon the addition of water a precipitate appeared (6.5 Gm.). This precipitate was washed with ether. One gram of a yellow flavonoid substance was obtained. The U.V. spectrum of the flavonoid showed maxima at 285 and 206 mμ and a minimum at 260 mμ. The ether and water-insoluble portion was active against adenocarcinoma of the duodenum test system (7D1), 11% T/C at 200 mg./Kg., 26% T/C at 100-mg. doses. This part gave long streaking blue spots when applied on paper with solvent systems such as (*a*) *n*-butanol–acetic acid–water (4:1:5), (*b*) 50% acetic acid, and (*c*) 2% acetic acid and when sprayed with ferric–ferricyanide, a long pink spot with *p*-toluene sulfonic acid, two violet-pink spots with vanillin-HCl, and no sugar spots with aniline phthalate. The I.R. curve revealed a large hydroxyl band at 3400, and phenyl group at 1595, 1505, and 855 $cm.^{-1}$. The U.V. spectra showed adsorption maxima at 280 and 206 mμ and a minimum at 260 mμ. This compound, when boiled with hydrochloric acid, caused the solution to turn a light pink color and had only 2.5% inorganic material. The compound was water and ether insoluble, nonhydrolyzable, and the classification tests indicated the presence of a phlobaphene. Further purification was accomplished by dissolving the material in alcohol and precipitation by the addition of ether. Column chromatographic separation or hydrolysis with HCl resulted in a loss of activity. The water-soluble portion was extracted with isoamyl alcohol. A slightly brown-red solution was obtained. This also gave streaking spots with the same solvent systems that were used on the phlobaphenes, and the same results were obtained when sprayed with the reagents as indicated above. This compound had less activity, 49% T/C at a dose of 100 mg./Kg. No residue was obtained upon burning. The I.R. curve was very similar to that of the phlobaphene. The U.V. curve gave maxima at 281 and 206 mμ and a minimum at 260 mμ. Being water soluble and ether insoluble, the differential classification reactions indicated above showed that this was a complex tannin. In the three compounds separated the U.V. curve did not show any shift when 1.2 ml. of a 1% $AlCl_3$ in alcohol solution was added, which shows the absence of 3-hydroxy flavones or flavonones with *o*-hydroxy carbonyl groups. When the alcoholic solution was made 0.006 *N* alkaline with KOH, the flavonoid spectra had a bathochromic shift of 20 mμ, phlobaphenes 22 mμ, and the complex tannin 14 mμ. The maximum 280–281 mμ indicated a flavan nucleus, and this is corroborated by the absence of a shift in the spectra with $AlCl_3$ and a definite shift in the spectra of the alkaline solution.

Boiling the complex tannin with hydrochloric acid for approximately 2 hr. changed the color to a bright red. Paper chromatography of this hydrolyzed compound using several solvent systems indicated the presence of cyanidin and delphinidin (Table II).

After hydrolysis, the compound did not yield any sugar spots on paper. Drastic hydrolysis of anthocyanidins isolated using the same method as reported previously (9) yielded protocatechuic acid, phloroglucinol, and gallic acid.

SUMMARY

A. concolor has been investigated to determine the chemical agents responsible for its activity against adenocarcinoma of the duodenum. A complex tannin and phlobaphene fractions appear to be the active compounds in the plant. In addition to the above, palmiton, *p*-menthandiol-(1,4), docosonol-1, lignoceryl alcohol, β-sitosterol, and lignoceric acid were separated and identified by using column and paper chromatographic techniques.

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