Hypolipidemic, Anti-Inflammatory, and Antineoplastic Activity and Cytotoxicity of Flavonolignans Isolated from Hydnocarpus wightiana Seeds

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HYPOLIPIDEMIC, ANTI-INFLAMMATORY, AND ANTINEOPLASTIC ACTIVITY AND CYTOTOXICITY OF FLAVONOLIGNANS ISOLATED FROM *HYDNOCARPUS WIGHTIANA* SEEDS

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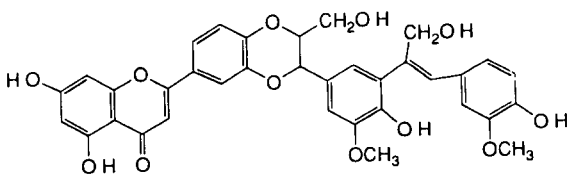
ABSTRACT.—Flavonolignans isolated from *Hydnocarpus wightiana* seeds, namely hydnowightin [**1**], hydnocarpin [**2**], and neohydnocarpin [**3**], demonstrated potent hypolipidemic activity in mice, lowering both serum cholesterol and triglyceride levels at 8 mg/kg/day ip. Hydnowightin demonstrated the best lipid-lowering effect of the three compounds. Good anti-inflammatory and antineoplastic activity was demonstrated by hydnocarpin in mice in vivo. The other two derivatives were not as active in these screens. Cytotoxicity against the growth of murine and human tissue cultured cells was shown. All three compounds were moderately active against murine L-1210 leukemia growth. All three compounds demonstrated good activity against the growth of human KB nasopharynx, colon adenocarcinoma, osteosarcoma, and HeLa-S^3 uterine growth. Hydnocarpin was the only compound of the three which was active against glioma growth. Hydnocarpin and neohydnocarpin demonstrated significant activity against $Tmolt_3$ leukemia cell growth.

Hydnocarpus (Flacourtiaceae), a genus of trees and sometimes shrubs, is distributed in southeast Asia, mainly in the Indo-Malayan regions. Four species occur in India. *Hydnocarpus wightiana* is a deciduous evergreen tree, which grows up to 50 feet or more in height. The fruit of the tree is globose and contains 15–20 seeds (1). The seeds of several species of *Hydnocarpus* yield fatty oils, generally known as the chaulmoogra oils. *H. wightiana* seeds constitute the major source of hydnocarpus oil, which is by far the predominant oil of the chaulmoogra group. The oil is used mainly in the treatment of leprosy and is effective in early stages of the disease in decreasing the size of the nodules. However, the oil and its derivatives are being replaced by sulfone drugs like promin, diazole, and promizole. The oil has a potential for use in the soap industry, and it may have a potential use as an energy source. Pseudotsuganol, the first true flavonolignan, has recently been isolated from the bark of Douglas fir (2). A preliminary isolation of flavonolignans from *H. wightiana* (3,4) and the characterization of the products by ^{13}C-nmr (5) have been reported.

Ruen *et al.* (6) and Wagner and co-workers (7,8) studied the protection by silymarin against lipid peroxidation in rats afforded by phalloidin intoxication. Silymarin-*N*-methyl glucamin salt 51148-13-1, when administered to rats treated with CCl_4, prevented lipid peroxidation in liver lipid metabolism. Silymarin is marketed as an antioxidant in West Germany for use against hepatic lipid-peroxidation-induced toxicity. A number of these derivatives are thought possibly to be effective in liver cirrhosis.



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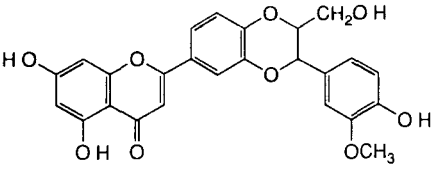
The current study was undertaken to expand the possible medicinal effects of three flavonolignans; hydnowightin [**1**], hydnocarpin [**2**], and neohydnocarpin [**3**]. Because lipid peroxidation and generation of free radicals play a role in a number of disease states, e.g., carcinogenic activity, inflammation, and atherosclerotic plaques, we have decided to test these compounds for anticancer, hypolipidemic, and anti-inflammatory activity (9).



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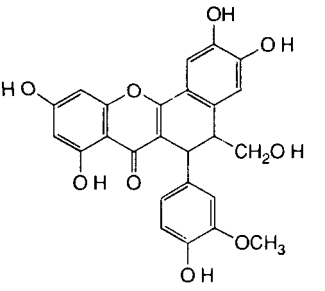

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RESULTS AND DISCUSSION

Among the flavonolignans from *H. wightiana*, hydnocarpin [**2**] is obtained in the highest yield. Hydnocarpin can also be easily obtained by direct crystallization from the crude extract of the seed hulls on allowing them to stand at room temperature. Hydnocarpin was found to show good and maximum activity in most of the pharmacological tests conducted in the present studies. The three flavonolignans tested for biological activity proved to be safe at 8 mg/kg/day, demonstrating no observable side effects: i.e., there was no significant loss of body weight, food consumption was normal, and behavioral, CNS, and motor activities were normal at multiple doses over 16 days. All of the agents were effective in lowering serum triglyceride levels 32–41% by day 16 at 8 mg/kg/day in mice (Table 1). Hydnowightin [**1**] and hydnocarpin [**2**] were more effective than neohydnocarpin [**3**]; they lowered serum cholesterol levels 38–41%. All of the agents were more effective than clofibrate at 150 mg/kg/day. Hydnocarpin at 8 mg/kg demonstrated anti-inflammatory activity, lowering the induced edema by 42%, which is comparable to phenylbutazone at 50 mg/kg but not as effective as indomethacin.

TABLE 1. The Effects of Flavonolignans on Serum Lipid Levels of CF_1 Male Mice at 8 mg/kg/day ip ($n = 6$; percent of control, mean ± SD).

Flavonolignan	Serum Cholesterol		Serum Triglycerides
	Day 9	Day 16	Day 16
Hydnowightin [**1**]	80 ± 7	59 ± 5[a]	59 ± 6[a]
Hydnocarpin [**2**]	83 ± 5	62 ± 6[a]	68 ± 7[a]
Neohydnocarpin [**3**]	85 ± 6	79 ± 7[a]	60 ± 5[a]
Clofibrate 150 mg/kg	88 ± 4	87 ± 5	75 ± 5[a]
1% Carboxymethyl cellulose	100 ± 6[b]	100 ± 5[c]	100 ± 6[d]

[a] $P \leq 0.001$, Student's t-test.
[b] 125 mg%.
[c] 128 mg%.
[d] 137 mg%.

Hydnocarpin also produced the best activity against the reduction of Ehrlich ascites carcinoma growth in CF_1 mice, demonstrating 84.75% inhibition of tumor growth. This was not as effective as 6-mercaptopurine, the standard used (Table 2). In the cytotoxicity screens (Table 3), all three compounds were active, i.e., $ED_{50} \leq 4$ μg/ml in the murine L-1210 lymphoid leukemia, KB nasopharynx, colon adenocarcinoma, HeLa-S^3 uterine carcinoma, and bone osteosarcoma. None of these compounds were active against lung bronchogenic growth. Only hydnocarpin was active against brain glioma growth. Hydnocarpin and neohydnocarpin were active against $Tmolt_3$ leukemia growth. These three agents demonstrated pharmacological activities that show promise for new drug development within this group of natural products. Other natural products, such as sesquiterpene lactones, have been shown to afford hypolipidemic, antineoplastic, and anti-inflammatory activity. A positive correlation exists between the ability of the agent to inhibit cell growth and its ability to suppress cholesterol synthesis (10–12).

TABLE 2. The Effects of Flavonolignans on Inflammation and Ehrlich Carcinoma Growth in CF_1 male mice ($n = 6$; percent of inhibition, mean ± SD).

Flavonolignan	Anti-inflammatory	Anti Ehrlich carcinoma
Hydnowightin [1]	24 ± 3[a]	12.1
Hydnocarpin [2]	42 ± 4[a]	84.75[a]
Neohydnocarpin [3]	6 ± 2	43.07[a]
Phenylbutazone 50 mg/kg	47 ± 5[a]	ND[b]
Indomethacin 10 mg/kg	78 ± 6[a]	ND
6-mercaptopurine 0.5 μg/kg/day	—	99.9%[a]
0.05% Tween 80/H_2O	0 ± 8	0

[a]$P \leq 0.001$, Student's *t*-test.
[b]ND = not determined.

The relationship between cellular division and cholesterolgenesis is well established in rapidly dividing cells (13–15). Brown and Goldstein (16) demonstrated that hydroxysterols that inhibit HMG CoA reductase activity inhibit cell growth of human fibroblasts. Compactin, another HMG CoA reductase inhibitor, blocks the growth of ML-236 B tissue culture cells as well as DNA synthesis (17). Apparently the presence of mevalonate in the cell is critical to DNA replication and activation of DNA polymerase α (18–20).

A wide variety of flavonoids and related derivatives are known to be cytotoxic, anti-

TABLE 3. The Cytotoxicity of Flavonolignans Against the Growth of Murine and Human Tissue Culture Lines ($n = 5$; ED_{50} μg/ml).

	Murine	Human						
	L-1210	$Tmolt_3$	KB Nasopharynx	Adenocarcinoma Colon	HeLa-S^3	Lung	Osteosarcoma	Glioma
Hydnowightin [1]	3.07[a]	4.33	1.96[a]	2.38[a]	3.01[a]	7.49	2.50[a]	5.02
Hydnocarpin [2]	3.65[a]	2.94[a]	1.15[a]	2.00[a]	2.02[a]	8.18	2.14[a]	2.59[a]
Neohydnocarpin [3]	3.29[a]	3.05[a]	1.06[a]	2.03[a]	2.12[a]	4.53	2.49[a]	4.07
Standards								
5-Fluorouracil	1.41[a]	2.14[a]	1.25[a]	3.09[a]	2.47[a]	5.64	—	1.28[a]
Cytosine arabinoside	2.76[a]	2.67[a]	2.84[a]	3.42[a]	2.13[a]	4.60	—	1.88[a]
Hydroxyurea	2.67[a]	3.18[a]	5.29	4.79	1.96[a]	7.37	7.54	2.27[a]

[a]Values of ≤4 μg/ml are considered significantly active according to the NCI protocol (15).

fungal, and antibacterial. Isoflavonoids have been reported to possess anti-inflammatory activity by blocking prostaglandin synthetase activity (21), and isoflavonoglycosides have been reported to be useful in lowering serum cholesterol and triglycerides of mice (22).

EXPERIMENTAL

CHEMICAL COMPONENTS OF THE SEED HULLS OF *H. WIGHTIANA*.—*Chemical components in the petroleum ether extract.*—*H. wightiana* seeds (5 kg) (National Education Society, Mysore, India) were air dried. The hulls and kernels were separated. The hulls (1 kg) were extracted with petroleum ether (4 liters) for 3 h. The extract on concentration yielded a light yellow oil. The oil was dissolved in MeOH (50 ml) and was saponified using 10% aqueous NaOH solution (20 ml). The MeOH was removed under reduced pressure, and the residue was neutralized with HCl and extracted with Et_2O. The Et_2O extract was washed with H_2O, dried, concentrated, and adsorbed on Si gel (100 g) and subjected to liquid cc. Compounds such as β-sitosterol, lupeol, β-amyrin, and betulinic acid were found to be present in the extract.

Chemical components in the EtOH extract.—The defatted seed hulls, after removal of the petroleum-ether-soluble portion, were extracted with EtOH. The EtOH extract was concentrated to a brown pasty mass that was macerated with petroleum ether to remove any oil. After this, the brown pasty mass (15 g) was dissolved in MeOH and kept in the refrigerator. On standing for 48 h, pale yellow prisms (crystals) were precipitated; the crystals were identified as hydnocarpin [**2**] (3.1 g), mp 262–264°. This compound was insoluble in petroleum ether, C_6H_6, $CHCl_3$, and EtOAc and sparingly soluble in dioxane.

The mother liquor, after separation of **2**, was subjected to liquid cc using Si gel. Gradual elution with C_6H_6-Me_2CO (6:5) yielded mixtures of compounds **1–3** and apigenin, chrysoeriol, methoxyhydnocarpin, luteolin, isohydnocarpin, and β-sitosterol-β-D-glucoside.

ISOLATION OF HYDNOWIGHTIN, NEOHYDNOCARPIN, AND β-SITOSTEROL-β-D-GLUCOSIDE.—The mixture containing **1–3**, methoxyhydnocarpin, luteolin, isohydnocarpin, and β-sitosterol-β-D-glucoside was rechromatographed over Si gel eluting with increasing proportions of Me_2CO in C_6H_6. The later fractions contained a mixture of **1**, **3**, and β-sitosterol-β-D-glucoside. Repeated chromatography of the mixture gave two fractions containing **1** and **3** (mixture A) and **3** and β-sitosterol-β-D-glucoside (mixture B).

Mixture A was further fractionated by subjecting it to lipid cc using Si gel with $CHCl_3$/MeOH as eluting solvents. Pure **1** was obtained by $CHCl_3$/MeOH (100:2) elution, and it was crystallized from C_6H_6/Me_2CO as a pale yellow powder: mp 239–241° yield 0.5 g. C_6H_6/Me_2CO elution failed to give any resolution of mixture B. Hence the mixture was subjected to rechromatography over Si gel by elution with $CHCl_3$ and increasing the polarity with MeOH. The elution with $CHCl_3$-MeOH (100:5) afforded pure **3** and a separate mixture of **3** and β-sitosterol-β-D-glucoside. Compound **3** was crystallized from C_6H_6/Me_2CO as a pale yellow powder, mp 235–237°, yield 0.45 g and was identified as neohydnocarpin.

The mixture of **3** and β-sitosterol-β-D-glucoside was subjected to cc on Si gel by elution with C_6H_6/EtOAc, and pure β-sitosterol-β-D-glucoside was eluted using C_6H_6-EtOAc (1:1.5).

BIOLOGICAL SCREENING.—All compounds were suspended in the vehicle by homogenization (10×). Drug solutions were sterilized by passing through an Acrodisc 45 μm (23).

Ehrlich ascites carcinoma tumor screen.—CF_1 male mice (ca. 25 g) were inoculated with 2×10^6 Ehrlich ascites carcinoma cells in isotonic sterile saline ip on day zero. On days 1–9, drugs suspended in 0.05 Tween 80 H_2O were administered at 8 mg/kg/day. On day 10, the mice were sacrificed, and the ascites fluid from the peritoneal cavity was collected. The volume and ascrit (packed-cell volume) were determined for each animal, and the present inhibition of tumor growth was calculated (24).

Cytotoxicity assays for murine and human tissue culture cell lines.—The following cell lines were maintained by literature techniques (25,26): murine L-1210 lymphoid leukemia, P-388 lymphocytic leukemia, human Tmolt$_3$ acute lymphoblastic T cell tumor, colorectal adenocarcinoma SW-480, lung bronchogenic MB-9812, osteosarcoma TE-418, KB nasopharynx, HeLa-S^3 suspended cervical carcinoma, and brain glioma EH118MG. On day zero, 10^4 tissue culture cells were mixed with sterile drugs 1–100 μg/ml (1 mM stock solution in 0.05% Tween 80 H_2O) and growth medium to a final volume of 1 ml. Cells were counted on day 3 since growth was still logarithmic at this time. The cells were counted with a Hemocytometer using the trypan blue exclusion technique. The ED_{50} values were calculated from a semi-log plot as representing the concentration of drugs required to kill 50% of the tumor cells on day 3.

Anti-inflammatory activity.—CF_1 male mice (ca. 25 g) were administered test drugs at 8 mg/kg in 0.05% Tween 80 H_2O ip 3 h and again 30 min prior to the injection of 0.2 ml of 1% carrageenan in 0.9% saline into the plantar surface of the right hind foot. Saline was injected into the left hind foot to obtain a

base line value. After 3 h, both feet were excised at the tibiotarsal (ankle joint) according to the modified method of Winter *et al.* (27) and Hendershot and Forsaith (28). The control mice afforded a 78±3 mg increase in paw weight.

Hypolipidemic activity.—CF_1 male mice (28 g) were administered drug suspended in 1% carboxymethylcellulose at 8 mg/kg/day ip for 16 days. On days 9 and 16, the mice were bled by tail vein bleeding into capillary tubes. The serum was obtained by centrifugation at 3000 g for 3 min. The total serum cholesterol was determined by the Liebermann-Burchard reaction (29), and the serum triglyceride was determined by a commercial kit (Bio-Dynamics/bmc triglyceride kit).

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