Suppressors of Cancer Cell Proliferation from Fig (*Ficus carica*) Resin: Isolation and Structure Elucidation

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A mixture of 6-*O*-acyl-*β*-D-glucosyl-*β*-sitosterols, the acyl moeity being primarily palmitoyl and linoleyl with minor amounts of stearyl and oleyl, has been isolated as a potent cytotoxic agent from fig (*Ficus carica*) latex and soybeans. Identity was established by spectroscopic methods (NMR, MS) and confirmed by chemical synthesis. Both the natural and the synthetic compounds showed in vitro inhibitory effects on proliferation of various cancer cell lines.

Fig (*Ficus carica*, *Moraceae*) products are widely used both as a food and as medicine in the Middle East. The latex released on picking the fruits is used to treat skin tumors and warts.[1]

The first scientific investigation of the activity of fig latex was done by Ullman et al. in the 1940s.[2−4] High doses of fig latex injected into rats were found to be lethal. Smaller doses injected into mice bearing a benzpyrene-induced sarcoma caused inhibition of the growth of the tumor and even the disappearance of small tumors.[2] The dialysate of the latex contained the active ingredient. Although isolation of the active components was not pursued further, some pharmacological work was reported by the same group.[3,4] Fig latex has also been tested for its antihelmintic activity, but was found to cause acute toxicity with hemorrhagic enteritis.[5] The leaf decoction affected lipid catabolism in hypertriglyceridemic rats[6] and had hypoglycemic action in type-I diabetic patients.[7]

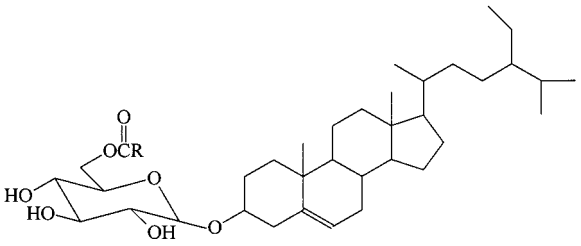
Several phytochemical investigations of *Ficus carica* leaves have been published, but with no biological data. Athnasios et al.[8] have isolated psoralen, *β*-sitosterol, bergapten, and taraxasterol from the petroleum ether extract of the leaves. Others have isolated triterpenoids.[9−10]

In this paper we describe the isolation and identification of a potent cytotoxic agent from fig latex, which is also present in soy products.

Fig latex is present in the plant in extremely small quantities and must be collected drop by drop. Many procedures were examined in order to obtain a maximum yield of the active cytotxic compounds from the fig resin. The optimal procedure (see Experimental Section) involved removal of the inactive polymeric material by filtration, followed by extraction of the aqueous filtrate with various solvents. An extract that was found to inhibit Raji cell proliferation was further chromatographed by several methods, and the activity was monitored with an assay based on the proliferation of Raji cells. Ultimately a small amount of solid active material was isolated (0.002% overall yield from 500 mL of resin, 0.008% of the nonaqueous material in the resin).

The compound migrated as a single band (R_f 0.7) on thin-layer chromatography (TLC) silica plates developed with chloroform−methanol (9:1). Purity was determined as >99% by normal-phase HPLC analysis.

The cytotoxic agent was identified by spectroscopic methods and confirmed by chemical synthesis as a mixture of 6-*O*-acyl-*β*-D-glucosyl-*β*-sitosterols (6-AGS). Acylated sitosteryl glucosides have been isolated from soybeans,[11] millet seeds,[12] wheat flour,[13] and other plants[14] and have also been synthesized.[15]



R= $(CH_2)_{14}CH_3$
$(CH_2)_{16}CH_3$
$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
$(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

The major problem with the structure elucidation of the active material was the variability of its composition and the small amounts available. Nevertheless, from the NMR data (Supporting Information), it was clear that 6-AGS always contains a C_{29}-sterol (*m*/*z* 397, see below, and δ_H 0.629 s, Me-18, and 1.003 s, Me-19), a hexose unit (δ_C 99.6 d, an anomeric C-atom, and 70.3 d, 73.6 d, 75.7 d, 76.4 d, and 62.1 t, indicative of C adjacent to oxygen), and a long aliphatic chain, which differed in its NMR profile from batch to batch. In certain isolation batches this was a saturated chain, while in others there were double bonds (δ_C 126.0−130.2 ppm) and a $-CH{=}CH-CH_2-CH{=}CH-$ group (δ_H 2.76 t and δ_C 25.6 t, a double allylic methylene).

FABMS gave a major molecular ion at *m*/*z* 837 (MH^+), accompanied by weak higher peaks, as well as two strong fragments at *m*/*z* 696 (M − 141, M − $C_{10}H_{21}$) and *m*/*z* 397 (100%). The molecular ion was confirmed by obtaining 22

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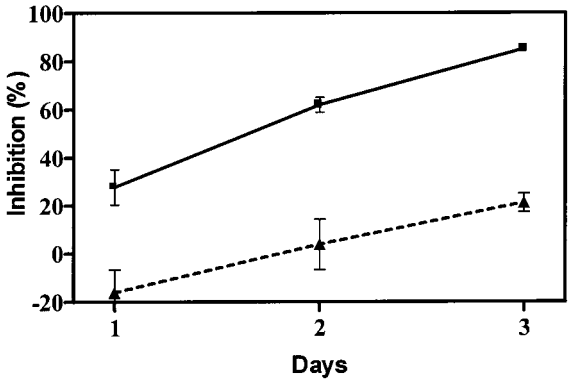

Figure 1. Kinetics of the antiproliferative activity of 6-AGS (50 μg/mL) on Raji cells: ■, purified fig latex with 6-AGS; ▲, purified fig latex without 6-AGS.

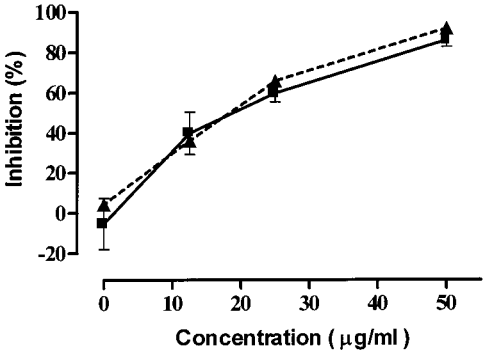

Figure 2. Effect of various concentrations of 6-AGS from figs (■) and of synthetic 6-PGS (▲) on proliferation of Raji cells. Values represent average with SD of 10 repeated assays.

Table 1. Cytotoxicity of 6-AGS[a]

		inhibition of growth (%)	
cell line	origin of cell line	25 μg/mL	50 μg/mL
Raji	Burkitt B cell lymphoma	46	69
DG-75	Burkitt B cell lymphoma	41	87
Jurkat	T-cell leukemia	62	81
HD-MAR	T-cell leukemia	57	66
DU-145	prostate cancer	61	75
MCF-7	mammary cancer	57	66

[a] Various cell lines were incubated for 3 days in the presence of two concentrations of 6-AGS. The inhibition of the growth of various cells by 6-AGS was calculated as a percentage of cells grown without addition of inhibitory compounds. Values represent average of three repeated experiments.

and 38 mu shifts by the observation of Na^+ or K^+ adducts, respectively, in the analyzed sample.

Comparison of various samples of 6-AGS revealed the presence of β-sitosterol and glucose in all samples, with differences in the acylating fatty acid moieties. The molecular ion of the triacetate, *m/z* 964, suggested a $C_{17}H_{31}$-CO_2H unsaturated acid as the glucose acylating molecule (964 = 414 (β-sitosterol) +306 (glucose triacetate) +280 ($C_{17}H_{31}CO_2H$) − 36 ($2H_2O$)). The major unsaturated acid posseses a −CH=CH−CH_2−CH=CH− unit.

The final structural determination of the fatty acid moiety was established by means of basic hydrolysis of 6-AGS followed by GC−MS analysis. The acyl moiety was found to consist primarily of palmitoyl and linoleyl (possessing the double allylic methylene unit) and minor amounts of stearyl and oleyl.

For comparison and bioactivity purposes the synthesis of several 6-acyl derivatives was undertaken following a previously published method,[15] with minor modifications (Supporting Information). The spectroscopic properties of the individual synthetic 6-acyl derivatives of β-sitosteryl-β-D-glucoside were identical to those of the isolated product. The two compounds showed the same R_f and retention time in chromatography (TLC and HPLC, respectively) and had similar optical rotation values ($[\alpha]_D$ for 6-stearyl derivative −58.8° (*c* 1.19 mg/mL, $CHCl_3$) and −57.1° for the natural product (*c* 1.16 mg/mL, $CHCl_3$)).

The cytotoxic activity of various fractions from the fig latex clearly paralleled the concentration of 6-AGS. It was, however, difficult to demonstrate directly the activity of the purified 6-AGS because of the low solubility of the compounds in various solvents, such as acetone, DMSO, ethanol, THF, or mixtures of these solvents with solubilizing agents (Tween-80 and cremophor). Cytotoxic activity of the purified compounds was obtained only by solubilization in a THF−Tween-80 mixture; however the results were variable due to partial solubility. The activity of 6-AGS was substantiated by experiments in which 6-AGS, purified from fig latex, was added to partially purified fig latex rendered nontoxic by removal of 6-AGS (Figure 1). As shown in Figure 2 the growth of Raji cells was inhibited by over 50% following exposure to 6-AGS at concentrations of 25−50 μg/well. Similar results were obtained by addition of synthetically prepared derivatives. Of the compounds tested so far, the palmitoyl derivative (6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol) (6-PGS) was the most effective (Figure 2). The stearyl and linoleoyl derivatives were somewhat less active. 6-AGS was cytotoxic not only to Raji cells but also to other cell lines (Table 1).

The finding that the cytotoxic agents isolated from fig latex are 6-*O*-acyl-β-D-glucosyl-β-sitosterols is of considerable interest. Acylglucosylsterols are widely distributed in higher plants,[11−17] some of which, such as soybeans and chickpeas, comprise an important food source, especially in East Asia. In Japan, where soya products are widely consumed, certain cancer diseases are less prevalent than in North America and Europe,[18] and it may be of importance to determine if the compounds described herein contribute to this phenomenon. Similar compounds have been found to possess antimutagenic activity.[19] When orally administered to mice, 6-*O*-palmitoyl-β-D-glucosyl-β-stigmasta-5,25(27)-diene was found to be antimutagenic at a dosage range of 12.5−50 μg/g.[19] Other reports have indicated that 6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol is orally bioavailable,[16] and it has been shown that β-sitosteryl-β-D-glucoside is absorbed from the intestinal tract by lipoproteins in vivo,[20] via active transport across the intestinal membrane.

Acylglucosylsterols have also been isolated from snake and chicken epidermis. It was speculated that since the molecule contains two lipophilic arms (the fatty acid and the sterol moieties) stretching outward from a central hydrophilic glucosyl unit, it could function as a molecular rivet that can hold together adjacent lamellar structures and thus might form the water barrier mechanism of birds and snakes.[21,22] In a similar manner, such a molecular interaction may affect adverse changes in membrane permeability of malignant cells and thus may lead to their death. It will be important to determine the in vivo activity of such compounds and their mode of action.

Finally, compounds comprising aglycone, sugar, and acid esters are distributed in higher plants. Such compounds include carotenoid glucoside fatty acid esters[23,24] and acylated saponins.[25] Of particular interest is the steroidal saponin fatty acid ester OSW-1, which has been found to

be an exceptionally potent antitumor agent.[26] It may therefore be of interest to study the influence of acylation of various saponins on the biological activity of the glucoside and to determine the possible antiproliferative action of such compounds.

Experimental Section

General Experimental Procedures. FABMS was recorded on a Fison Autospec Q instrument. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-500 spectrometer. All chemical shifts are reported with respect to TMS ($\delta_H = 0$) and $CDCl_3$ ($\delta_C = 77.0$). The HPLC system consisted of a Waters 660 pump and a Waters 996 PDA detector. A HP model G1800B GCD having a 28 m × 0.25 mm i.d. × 0.25 μm film thickness cross-linked 5% HP ME siloxane column, programmed from 90 to 280 °C at a rate of 5 °C/min, interfaced with a HP model 5971 mass selective detector was used. Si gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.2 mm) were used for analytical TLC.

Extraction and Separation. The fig latex was collected over the summer months in various parts of Israel. The latex was kept refrigerated until processed. The latex (500 mL) was filtered through Whatman No. 1 filter paper by suction filtration, separating polymeric material from the aqueous filtrate. Further purification of the filtrate was then attained by filtration using a 5 μm disposable filter membrane. The clear aqueous filtrate was extracted three times with petroleum ether and then further extracted three times with 1000 mL portions of ethyl acetate for 1 h at 62 °C with stirring. The ethyl acetate was evaporated to dryness under vacuum. The residue was dissolved in methanol and filtered once more to eliminate remaining polymeric gum. A residue (2.2 g) was obtained after evaporation of the methanol. This residue, contrary to the pure 6-AGS mixture, was soluble in ethanol, thus allowing facile testing. The extract was tested for cytotoxic activity against several human cancerous cell lines including the Raji lymphoma cells. This cell line was selected as a bioactivity-guided fractionation assay for the isolation procedure.

The methanolic extract containing 2 g of solid material was chromatographed on silica gel columns with a binary solvent system of increasing polarity, using chloroform−methanol as eluent. The fractions were evaporated, and the residues were assayed for cytotoxic activity (see below). The activity was recovered principally in the fraction eluted in 3% methanol in chloroform. The active fraction (45 mg) was then subjected to gel filtration chromatography using Sephadex LH-20 and a mixture of methanol−chloroform−*n*-hexane (1:1:2, v/v/v) as elution solvent. The fractions thus separated were tested for bioactivity and the activity was localized to the relatively pure first eluting fraction. The isolated compound was further purified by precipitation from a chloroform−acetonitrile mixture to give 8 mg of a white solid.

HPLC Analysis. A normal-phase HPLC analysis was carried out using an Absorbshpere HS silica 5U column (250 × 4.6 mm, Altech) as a stationary phase. The analysis was run under isocratic conditions (propane-2-ol−*n*-hexane, 20:80 v/v) at a flow rate of 1.0 mL/min. The compound was dissolved in the mobile phase at a concentration of 0.5 mg/mL and had a retention time of 6.57 min.

Assessment of Cytotoxic Activity. Various compounds isolated from fig latex were examined for their capacity to inhibit the growth of cancer cells in vitro. Numerous solvents were used for the solubilization of the isolated fractions, such as acetone, DMSO, ethanol, Tween-80, and cremophor. The Raji B-cell lymphoma line, derived from an African Burkitt's lymphoma,[27] was used for routine assays of the cytotoxic activity of latex fractions. Raji cells were suspended in RPMI 1640 medium, supplemented with 20% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/mL penicillin, and 0.01 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Aliquots of suspensions of lymphoma cells were dispensed at 200 μL volumes into wells of 96-well tissue culture plates at densities of 0.02×10^6 cells/well.

Various concentrations of latex-derived compounds were introduced into four wells, and their efficacy was tested 3 days after initiation of the cultures, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The principle of this assay is that cells which survived following exposure to various compounds can reduce MTT to a dark-colored formazan, while dead cells are incapable of doing so. The assay was performed as described previously.[28−30] In each MTT assay every concentration of the cytotoxic substance was tested in five replicates in microplate wells. Assays with every cell line were carried out in two to three repeated experiments. Assays with Raji cells were preformed in over 10 repeated assays. The inhibitory effect of various compounds was calculated as percentage inhibition in comparison with the values obtained in untreated wells to which no compounds were added.

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Supporting Information Available: NMR data (^{13}C, 125 MHz and ^{1}H, 500 MHz) of 6-AGS and synthesis of 6-O-acyl-β-D-glucosyl-β-sitosterols. This material is available free of charge via the Internet at http://pubs.acs.org

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