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In vitro and QSAR studies of cucurbitacins on HepG2 and HSC-T6 liver cell lines

Judit Bartalis, Fathi T. Halaweish *

Department of Chemistry & Biochemistry, SDSU, Shepard Hall 121, Box 2202 Brookings, SD 57007, USA

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ABSTRACT

The aim of this study was to evaluate cucurbitacins (Cucs) liver protective activity in vitro and conduct QSAR studies against lipophilicity and ab initio descriptors. Nine Cucs were isolated from Cucurbitaceae plants and eight prepared by C2-alkylation or C16-acylation. Ten Cucs demonstrated protective activity on human hepatocyte-derived HepG2 cells exposed to CCl_4 (EC_{50} = 2.4–45.3 μM) with good margin to toxicity (T/A). All Cucs exhibited anti-proliferative effect on serum-activated rat stellate cells, HSC-T6 (EC_{50} = 0.02–4.12 μM) with high T/A. While silybin is nontoxic, its protection is lower compared to Cuc D (**3**), iso-D (**4**), I (**5**), B (**11**), E (**12**), I–Me (**6**), L-Me (**7**), and E-Me (**13**) on both cell lines. Strong correlations were found for lipophilicity with both protection and toxicity on HepG2. Lipophilicity correlated only with toxicity on HSC-T6. Consequently, we suggest that Cucs are potential hepatoprotective agents against fibrosis that deserve further examination.

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1. Introduction

The use of complementary and alternative medicine (CAM), especially herbal therapy, has a long tradition among patients with liver disease. The most commonly available and potent herbs used for liver disease are *Sylibum marianum*, *Phyllanthusamarus*, *Glycyrrhizaglabra* (liquorice), *Camellia sinensis* (tea), *Curcuma longa* (turmeric), *Picrorhizakurroa* and *Cynarae folium* (artichocke). Mixtures of herb extracts such as TJ-9 or cpd 861, CH-100, and LIV.52 are also commonly prescribed in Asia for this purpose. Silymarin, a standardized extract from Sylibum marianum earned a big market share in Germany for liver cures.[1,2] However, lack of well-designed studies (heterogeneous patient populations, no standardized preparations, poorly defined end points), and complexity of any herbal extract lead to conflicting reports in the literature. There are no convincing data to suggest a definite histological and/or virological improvement with most of these agents; therefore, the FDA is not recommending CAM therapy for patients with acute or chronic liver disease in the USA.[3,4] Consequently, it is imperative to conduct further studies for a better understanding of mechanism of liver protection at a molecular level for various herbs extracts or purified active compounds. In addition, more control is needed in the design of human studies.

While cucurbitacins (Cucs), highly oxygenated triterpenes, are not the most widely recognized active components of herbs used for the treatment of liver problems, plants with cucurbitacin content, for instance *Anagallis arvensis*, *Ecballium elaterium*, *Cucumismelo*, and *Melonispedicellus*, to name a few, have been used in folk medicine for their hepatoprotective and hepatocurative properties in the treatment of jaundice and cirrhosis.[5,6]Only a few in vivo studies have been conducted on mice or rats using pure Cucs or cucurbit plant extracts. Elaterium (dried juice of *Ecballium elaterium*), Cuc B and E prevented and protected animals against chemically induced hepatotoxicity by normalizing serum levels of liver enzymes and lipoproteins, and by decreasing steatosis and inflammation.[7–9] Cuc B also prevented and controlled hepatitis and cirrhosis in experiments dealing with chronic exposures.[9] In other studies, Cuc B and iso-B improved bile flow during experimentally induced cholestasis.[10] Administration of elaterium to animals with jaundice decreased serum bilirubin.[11] This effect was attributed to Cuc E (**12**), B (**11**) and D (**3**) from elaterium, which modified the albumin conformation allowing bilirubin to bind with a higher affinity to the protein.[12]

Other well known pharmaceutical properties of herbal medicines attributed in folk medicine to Cucs are the laxative, anti-inflammatory, and antimicrobial properties.[5] Additionally, research studies indicate that Cucs have strong cytotoxic activities in vivo as well as on different tumor panels and cancer cell lines; the mechanism of action of Cuc B (**12**), D (**3**), I (**5**), E (**12**), and E-Me-ether (**13**) have been documented to some extent.[5,7]

* Corresponding author. Tel.: +1 605 688 4269; fax: +1 605 688 6364.
E-mail address: Fathi.Halaweish@sdstate.edu (F.T. Halaweish).

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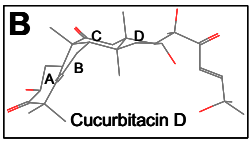
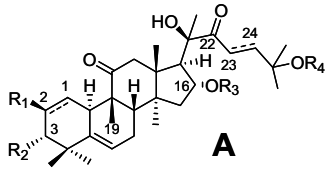
Recent published work sparked the interest of cucurbitacin and highlighted mechanism of activities by inhibition of Jak/STAT pathway (NILS), induction of G2 arrest and apoptosis via reactive oxygen species in SW480 cells.[13] Demonstration of potential antioxidant/free-radical scavenging and inhibition of lipid peroxidation have been also document as potential mechanism for the hepatoprotective activities of cucurbitacins.[14,15] The activities are, however, at doses very close to the toxic dose on animals. These data were compiled for a fairly large number of analogs by Cassady and Suffness.[16] Based on QSAR studies involving semi-empirical calculations, van Dang et al suggested the design of Cuc E derivatives bearing a strongly electron donating ester group on C25 atom that would increase the anticancer activity and lower animal toxicity.[17] We reported earlier that the increasing lipophilicity of Cucs enhanced their toxicity on human hepatocyte-derived HepG2 cells.[18] Since HepG2 is one of the standard cell lines used to predict human acute toxicity,[19] lipophilicity might be an important factor to consider during the design of novel cucurbitacin derivatives.

To the best of our knowledge, the hepatoprotective effect of Cucs has not previously been investigated on cultured cells. Therefore, we screened the activity of 17 natural and semi-synthetic Cucs (Scheme 1) on parenchymal and a non-parenchymal cell lines: human hepatocyte-derivedHepG2 cells, and rat stellate HSC-T6 cells. Cucs protective activity was monitored on HepG2 cells against CCl_4-induced toxicity, and their antiproliferative activity on serum-activated HSC-T6 cells. Cytotoxicity of Cucs was also monitored in order to define correct doses for the above assays. Furthermore, we studied the effect of molecular properties predicted by quantum mechanical calculations and lipophilicity on in vitro activities. Such correlations will help in quantifying the influence of various structural properties on hepatoprotective activity and help in designing novel derivatives with improved hepatoprotective activities.





No.	Cucurbitacin	R_1 [d]	R_2	R_3	R_4	Other
1	I Glu[a, b]	Glu	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
2	E Glu[a, b]	Glu	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
3	D[b]	OH	=O	H	H	$\Delta^{23,24}$
4	iso-D[b]	=O	αOH	H	H	$\Delta^{23,24}$
5	I [b]	OH	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
6	I-Me[c]	O-Me	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
7	L-Me[c]	O-Me	=O	H	H	$\Delta^{1,2}$
8	I-Et[c]	O-Et	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
9	I-iPr[c]	O-iPr	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
10	I-nPr[c]	O-nPr	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
11	B[b]	OH	=O	H	Ac	$\Delta^{23,24}$
12	E[b]	OH	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
13	E-Me[c]	O-Me	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
14	E-Et[c]	O-Et	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
15	E-iPr[c]	O-iPr	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
16	E-nPr[c]	O-nPr	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
17	E-Me-Ac[c]	O-Me	=O	Ac	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$

[a] β-D-glucopyranose; [b] isolated from plants; [c] generated by semi-synthesis;
[d] C2 hydroxyl is positioned in β for all analogs.

Scheme 1. Structures of natural and semi-synthetic cucurbitacins. (A) Generic chemical structure, indicating the numbering and stereochemistry, (B) 3D structure of Cuc D inferred from the crystallographic data of datiscoside.

2. Materials and methods

2.1. Materials for the cell culture assays

Continuous cell lines, HepG2 human hepatocellular carcinoma cells and HeLa human cervix cancer cells were purchased from ATCC (American Type Culture Collection), and HSC-T6 rat stellate cells were received as a gift from Dr. S. Friedman (Mnt. Sinai Hospital, NY, NY). Rational behind choosing HeLA cell line is to demonstrate the known differential cytotoxicity of cucurbitacins analogues toward other cell line and point out that cucurbitacins analogues will exhibit lower toxicity toward other cells while it is very effective as hepatoprotective agents. Cell culture media, Eagle's Minimum Essential Media, penicillin G, fungizone, GKN, and trypsin were purchased from Gibco (Grand Islands, NY), and fetal bovine serum (FBS) from HyClone (Logan, UT). GKN is a glucose-containing buffered saline containing 11 mM D-glucose, 5.5 mM KC1, 137 mM NaC1, 25 m MNa_2HPO_4, and 5.5 mM $NaH_2PO_4{\cdot}2H_2O$. Chemicals used for cell viability assessment are the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium-bromide (MTT) dye solution from Promega (Madison, WI) or MTT powder from Sigma (NJ), sodium dodecyl sulphate (SDS) (Sigma), concentrated HCl (Acros), and phosphate buffered saline (PBS) 1X solution (Gibco). The MTT powder is dissolved at 5 mg/ml concentration in PBS 1X and stored in the refrigerator, shielded from light. 10% SDS solution is prepared by dissolving it in 0.01 N HCl in PBS 1X. Bromobenzene, silybin, CCl_4 for cell culture assays were purchased from Acros. Rat platelet derived growth factor (PDGF) BB dimer was purchased from Sigma. All chemicals were dissolved in DMSO: ethanol (1:1). Stock concentrations: CCl_4 and bromobenzene 10^{-1} M, silybin (standard): 10^{-1} M (cytotoxicity measurement), and 10^{-2} M (hepatoprotection assay); and Cucs: 10^{-2} M. Further dilutions were conducted in the cell culture media.

2.2. Isolation and structural modification

Compounds **1** and **2** were mainly isolated from *Cucurbita texana* and *Citrullus lanatus* (Cucurbitaceae); compounds **3**–**5**, **11**, and **12** were isolated from *Cucurbita texana*. Additional quantities of **5** and **12** were generated from their corresponding glycosides by enzymatic hydrolysis. Compounds **6**–**10** were synthesized from cucurbitacin I and L by alkylation. Compounds **13**–**17** were synthesized from cucurbitacin E by alkylation or acetylation. The isolation procedure and synthesis are described in details by the authors.[18] In the process of studying antioxidant, anti-inflammatory activities, the mostly common cucurbitacin analogues were chosen to explore if any of these compounds will show these activities before we conduct a full study for all analogues in the project.

2.3. Antioxidant assays (experimental details are presented in the Supplementary data SM1)

We investigated the *DPPH free radical scavenging activity*[20] of Cuc B (**12**) and ascorbic acid (standard). The total antioxidant activity of Cuc B (**12**) and trolox (standard) was evaluated by the *ABTS*$^{\cdot+}$ *decolorization assay*.[21,22]

2.3.1. Inhibition of lipid peroxidation

Lipid peroxidation in rat liver microsomes was induced by NADPH-generating system G6P/NADP/G6PD or by ascorbate in presence of an ADP-iron complete.[23,24] Liver microsomes were

isolated freshly from rats.[23] We investigated the activity of Cuc B, E, E-Me ether and quercetin (standard).

2.4. Anti-hyaluronidase activity

The anti-inflammatory activities of Cucs and phenylbutazone (standard) were studied as a measure of preventing the hyaluronidase enzyme that breakdown hyaluronic acid found in the connective tissue. Degradation of hyaluronic acid was monitored colorimetrically by the modified Morgan-Elson method.[25] Find more detail in *SM2*.

2.5. Cell culture assays

Cells were batch cultured in EMEM with 10% FBS for 10 days (HepG2), 7 days (HeLa) or 4–5 days (HSC-T6). HepG2 cells, HeLa cell, and HSC-T6 cells were seeded for 24 h in EMEM cell media containing 2% FBS considering 30,000, 15,000, and 100,000 cells/ml concentration, respectively. Live cells were visualized by the MTT dye assay (Promega, Madison, WI) and the absorbance measured at 570 nm after 24 h incubation.[26] The cytotoxicity at 50% and hepatoprotection at 50%, $IC_{50}$and EC_{50}, were deduced from the log IC_{50} and log EC_{50} values. Three independent experiments with at least 3 replicates each were considered for all experiments.

2.5.1. HepG2 cells

2.5.1.1. Cytotoxicity assay. HepG2 cells seeded for 24 h in 96-well plates[27] were exposed to different concentrations of Cucs. Five-fold serial dilution of compounds was carried out in the plate for 5 consecutive wells (final volume 100 μl) and incubated for 24 h. Negative (no cells, NC) and positive (no test chemicals, PC) controls were also considered for each plate to avoid interference of spectrophotometer reading from test chemicals.[28]

$$\text{Viability}(\%) = \frac{A_{\text{Sample}} - A_{\text{NC}}}{A_{\text{PC}} - A_{\text{NC}}} \cdot 100$$

2.5.1.2. Hepatoprotection assay. Cells seeded for 24 h were subjected to Cucs or silybin (standard) in the presence of CCl_4 for another 24 h. The amount of toxin was optimized (4.5×10^{-3} M) to damage about 40–60% of the cells. Silybin was incubated with the toxin at the following final concentrations: 1, 14, 25, 50, 100, 200 μM, and the effective concentration was calculated at 50% protection (EC_{50}). Cucs hepatoprotection was studied firsthand at two dose levels, at 0.5 and 0.2 of their IC_{50} values. Additional experiments at doses 0.13 $IC_{50}$and 0.06 IC_{50} were considered for those indicating activity in the first step. Control samples consisted of cells subjected to CCl_4 (T) or media alone (NC). The equation below is considered for the calculation of protective activity. The choice of hepatoprotection dose was picked up outside the cytotoxic dose (Table 1) based on experimental data to develop this step (<0.2 IC_{50} which are 0.13 and 0.06 the IC_{50})

$$\text{Protection}(\%) = \frac{A_{\text{Sample}} - A_{\text{T}}}{A_{\text{T}} - A_{\text{NC}}} \cdot 100$$

2.5.2. HeLa cells

The cytotoxicity assay was similar to the one presented above for HepG2 cells.

2.5.3. HSC-T6 cells

For both *cytotoxicity* and *anti-proliferation* the following steps were followed. Cells were seeded for 24 h in 2% FBS in 96-well plates. After this period, the cell culture media was discarded and test compounds or silybin (standard) were added in fresh media supplemented with 10% FBS. Two-fold dilution of the compounds was executed externally and the prepared solutions added to seven consecutive wells. Controls were also established with 10% FBS (PC), 0% FBS (ZC), and media with 10% FBS with no cells (NC). Plates were incubated for 24 h. The only difference between the two assays was the concentration levels of test compounds. Anti-proliferation assays required non-toxic concentration levels. Therefore, cytotoxicity was measured initially and non-toxic doses chosen for the anti-proliferation assay. The inhibitory activity is calculated by the equation indicated below and viability by the equation presented above for the appropriate assay on HepG2 cells.

$$\text{Inhibition}(\%) = \frac{A_{\text{PC}} - A_{\text{Sample}}}{A_{\text{PC}} - A_{\text{ZC}}} \cdot 100$$

2.6. Structure building and ab initio calculations

The absolute configuration of datiscoside C, C16-glycoside of Cuc D, was established earlier by X-ray crystallographic analysis.[29]

Table 1
Demonstration of differential cytotoxicity of cucurbitacin analogs on HepG2, HeLa, and HSC-T6 cell lines (SD <10%)

#	Cucs.	HepG2 cells		HeLa cells			HSC-T6 cells		
		IC* (Model 1)	EC* (Model 2)	IC/EC	IC_{50}* (μM)	RIC	IC* (Model 3)	EC* (Model 4)	IC/EC
	Silybin	Non-toxic	45.0				Non-toxic	18.83	
1	I Glu	390.00	195.00	2	nd		256.00	4.15	62
2	E Glu	226.67	45.33	5	37.0	6	102.00	3.28	31
3	D	77.33	9.00	9	0.7	107	26.00	0.07	379
4	iso-D	80.33	13.80	6	nd		8.70	0.06	137
5	I	15.80	3.16	5	nd		3.60	0.02	150
6	I-Me	15.00	5.25	3	nd		6.37	0.11	60
7	L-Me	19.00	5.00	4	nd		6.70	0.18	36
8	I-Et	5.50	<50%		nd		3.53	0.34	10
9	I-iPr	7.33	<50%		nd		2.53	0.25	10
10	I-nPr	5.00	<50%		nd		2.47	0.33	8
11	B	27.67	10.50	3	0.8	37	4.42	0.02	205
12	E	15.25	3.20	5	0.1	197	2.03	0.04	50
13	E-Me	12.00	2.40	5	nd		2.83	0.08	35
14	E-Et	5.10	<50%		1.1	5	3.50	0.27	13
15	E-iPr	4.27	T		nd		2.50	0.32	8
16	E-nPr	3.70	T		1.4	3	2.75	0.34	8
17	E-Me-Ac	26.00	5.20	5	3.2	8	11.50	0.91	13

IC_{50} (μM): IC, EC_{50} (μM): EC; RIC: relative cytotoxicity/IC_{50} of HePG2 to HeLa, nd: not measured; T-toxic at 12.5% IC_{50} and inactive an 6.25% IC_{50}.
St. Dev. was less that 5% is all samples.

This fundamental structure was considered for the calculation of various physicochemical properties, which was confirmed by Van Dang et al.[17] to provide the lowest molecular energy among structures with various configurations. The X-ray coordinates were converted to atomic coordinates by means of the Crystal Maker v.3.0 (Crystal Maker Software Ltd, Oxford shire, UK). Modifications to this structure were carried out in Chem3D, ChemOffice 2002 v 7.0.1(Cambridge Soft, Cambridge, MA) to generate the 17 Cuc analogs. A representative 3D structure for Cuc D (**3**) is presented in B and the corresponding coordinates in *SM4*. According to the X-ray studies, rings A and C are in chair conformation while rings B and D have half-chair conformation. The molecule shows folded conformation with cis fusion of rings B and C and trans-fusion of rings C and D.

The preliminary geometry optimization of each molecule was conducted in Gaussian 98 (Gaussian Inc., Wallingford, CT)[30] on Windows platform by using ChemOffice as interface. The closed-shell restricted PM3 semi-empirical calculation was selected for this purpose. Next, the ab initio geometry optimization was conducted in Gamess v 6 1999 (Iowa State University, Ames, IA),[31] using the IBM eServer Cluster 1350 high-end computer, an AMD Opteron™ processor-based Linux cluster. Molecules optimized by means of the density functional theory RHF and basis set 6-31G(d) were evaluated for their surface area (SA) and cavity volume (CV) in water. This calculation implicated the Polarizable Continuum Model. The geometry of molecules was further optimized by using MP2/6-31G(d), and additional parameters inferred included total energy, atomic net charges, dipole moment and bond orders. As a remark, the MP2 theory does not allow the calculation of SA and CV. The Geodesic sphere algorithm[32] was considered for the calculation of electrostatic potential-derived atomic net charges. The sum of fitted atomic charges was constrained to reproduce the total molecular charge. The net charges were examined for following atoms: C1, C2, C2-O, C3, C11, C16, C22, C24, C25, and C25O. Bond orders were evaluated for bonds between C1–C2 and C2–C3 atoms and labeled BO12 and BO23.

2.7. Lipophilicity

The 1-octanol/water partition coefficient log *P* for Cucs was estimated on-line by means of ALOGPS software.[33] This estimation is based on the neural network ensemble analysis fora large pool of organic compounds available from the database of Syracuse Research Corporation.[34]

2.8. Multiple linear regression analysis

Multiple linear equations were established between independent descriptors termed *predictors* and in vitro data measured in our lab termed *response variables*. Independent descriptors were defined as sets of data with multiple squared correlation coefficient r^2 <0.60. Equations were built for all four biological activities: in vitro cytotoxicity on HepG2 cells (Model 1, $n = 17$), cytotoxicity on HSC-T6 cells (Model 3, $n = 17$), hepatoprotection on HepG2 cells (Model 2, **1**–**7**, **11**–**13**, and **17**), and inhibition of HSC-T6 cells proliferation (Model 4, $n = 17$). The fitting quality of these equations was evaluated by the correlation coefficient *r*, *F*-test, and *t*-test, and only those predictors were chosen that indicated a *t*-test confidence level higher than 95% (p <0.05).The established equations were subjected to cross validation by the 'leave-one-out' method and the 'cross-validated' r^2 determined by the equation below.[35] The numerator represents the predictive residual sum of squares (PRESS) which includes $y_{\mathrm{pred,i}}$, the value for y_i predicted from the model while excluding the observed y_i value. The denominator includes the $\langle y \rangle$ that represents the mean value for the experimentally observed set of values. The S/O was introduced as a specific fitting error, which represents the ratio of standard error and range of observation. All calculations were conducted in Microsoft Excel.

$$r^2_{cv} = 1 - \frac{\sum_{i=1}^{N}(y_{pred,i} - y_i)^2}{\sum_{i=1}^{N}(y_i - \langle y \rangle)^2}$$

2.9. Design of novel cucurbitacin analogs

All predictors of the QSAR equations were taken in consideration to design novel cucurbitacin derivatives. Overall, the following modifications to the basic structure were considered: reduction of carbonyl groups to hydroxyl on C3, C11, or C22 for Cuc D, I, B, and E; esterification of hydroxyl on C2 or C3 with acetyl or propionyl group for Cuc D (**3**) and E (**12**), and reduction of $\Delta^{23,24}$ and connection of a hydroxyl to C24 for Cuc D (**3**). The PM3 potential energy was estimated for possible conformational isomers and the more stable conformer with the lowest energy selected for subsequent ab initio calculations. Novel derivatives are labeled with the name of the parent compound followed by the number representing the carbon center at which the modification was made and by the new group such as OH, *n*Pr, *n*Bu, or Ac.

3. Results

3.1. Hepatoprotection activity

Initial trials using the hepatotoxin bromobenzene[27] were abandoned due to inconsistent results. Since bromobenzene precipitated out of the cell media, the toxicity of both precipitate and supernatant were evaluated during method development. On the other hand, the well known hepatotoxic agent CCl_4 showed reproducible and dose-dependent cytotoxicity on HepG2 cells. At 4.5 mM, CCl_4 reduced cells' viability by 50–60%. Silybin (standard) exhibited no toxicity on cells and protected them against the damaging effect of $CCl_4$with EC_{50} 45.0 μM. At concentration above 100 μM silybin completely protected the liver cells (Fig. 1). Without silybin (0.00 concentration), cell viability was 0% (data is not shown). Cucurbitacin glycosides and aglycons exhibited medium to high toxicity on cells; see IC_{50} values listed in Table 1. Their hepatoprotective activity was investigated at 6.25%, 12.5%, 25%, and 50% IC_{50} dose levels. Majority of Cucs demonstrated activity without reaching 100% protection due to the close proximity of higher doses to the toxic level. The highest protection (74–83%) was achieved by Cucs **3**–**7** and E (**12**). Several analogs exhibited lower than 50% Hepatoprotection such as Cuc I-Et (**8**), I-*i*Pr (**9**), I-*n*Pr (**10**), and E-Et (**14**). Furthermore, Cuc E-*i*Pr (**15**) and E-*n*Pr (**16**) exhibited toxicity at dose levels equal or larger than 12.5% IC_{10} and no activity below this level. The EC_{50} was estimated for those compounds that reached at least 50% protection, with a toxic-to-active dose, T/A, spanning between 2 and 9, a relatively narrow and low margin (Table 1).

3.2. Cytotoxicity on HeLa cells

Cytotoxicity of selected Cucs was measured on HeLa cells under similar experimental conditions to those applied on HepG2 liver cells (Table 1). Cucurbitacin E (**12**), D (**3**), and B (**11**) in decreasing order exhibited the largest differences in activity between the two cell lines; the ratio of IC_{50} values were 197, 107, and 37, respectively. HeLa cell data was presented to show that cucurbitacins have differential cytotoxicity and that they are extremely toxic toward other cells while they are protecting liver cells.

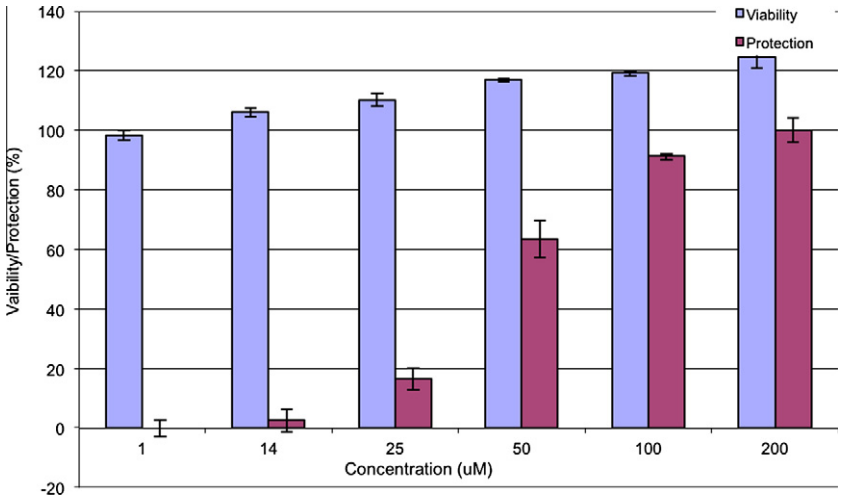


Figure 1. Hepatoprotective effect of silybin on HepG2 cells in presence or absence of CCl_4 (4.5×10^{-3} M).

3.3. Effect of Cucs on HSC-T6 cell growth

Among several growth factors shown to be involved in the process of stellate cell activation, platelet derived growth factor (PDGF) is the most suitable stellate cell mitogen identified.[36] However, proliferation of HSC-T6 cells in presence of PDGF under the experimental conditions presented by Zhang[37] or Yang[38] was not evident. Further modifications to these experiments (incubation period, starvation period, concentration of PDGF, type of PDGF) did not induce cell mitosis in presence of PDGF (see details in *SM3*). Instead, fetal bovine serum (FBS) containing higher concentration of growth factors[39] was selected to induce cell mitosis. The serum induced marked proliferation and the proliferation rate for three independently conducted experiments was 46.7±10.0%.

Proliferation of cells was inhibited by all Cuc derivatives and by silybin (standard) (Table 1). Under similar experimental conditions, silybin at doses up to 300 μM did not exhibit any toxicity on cells while Cuc glycosides and aglycons exhibited medium to high toxicity (Table 1).

3.4. Morphological changes

Healthy HSC-T6 stellate cells exhibited an activated phenotype as reflected in their fibroblast-like (spindle) shape and rapid proliferation in monolayer culture..[40] Normal HepG2 cells grow in three-dimensional clusters, are less angular than the stellate cells, and do not have clearly defined subcellular structures.[41] Although HepG2 cells were still attached to the bottom of the well, they rounded up, and contracted, and the intercellular adhesion diminished in presence of CCl_4. When Cucs were added to the cells in presence of CCl_4, cells' viability improved significantly, while improvements in morphology were not visible. Significant improvement in cell shape and size in addition to cell viability was observed in presence of silybin. Non-toxic amounts of cucurbitacin disturbed to some extent stellate cells, manifested in similar cell shape alterations to those noted for HepG2, while silybin did not cause any morphology changes. Cell morphology of treated cell was examined against untreated cells through the use of light microscope.

3.5. Antioxidant and anti-inflammatory activities

The antioxidant activity of Cuc B (**11**) was studied by the *DPPH* assay. Experimental conditions were optimized on ascorbic acid that demonstrated a 50% activity at 64 μM at 10 min incubation period. The antioxidant activity of Cuc B (**11**) was also studied by the $ABTS^{\cdot+}$ assay. Experimental conditions were optimized with trolox, which showed a 50% inhibition at 0.58 mM within 6 min monitoring period. The anti-lipid peroxidation activity of Cuc B (**11**), E (**12**), and E-Me (**13**) was studied by the *Microsomal Lipid Peroxidation* assay. Experimental conditions were optimized with quercetin that inhibited lipid peroxidation in a dose-dependent manner showing 100% inhibition at 0.1 mM in both chemical and enzymatic assays. The anti-inflammatory activity of Cuc B was also monitored by the *Anti-Hyaluronidase* assay. This assay was validated with phenylbutazone, which at 71.68 mM inhibited the enzyme activity by 50%.

3.5.1. QSAR studies

QSAR equations were built for all four distinctive bioactivities, cytotoxicity (Model 1) and anti-hepatotoxic activity (Model 2) on HepG2 cells, cytotoxicity (Model 3) and anti-proliferation activity (Model 4) on HSC-T6 cells. Seventeen descriptors were computed by quantum mechanics or estimated online (log *P*) for each cucurbitacin. These descriptors can be categorized as follow (a) *hydrophobic*: 1-octanol/water partition coefficient log *P*, (b) *steric*: surface area (SA), and cavity volume (CV), (c) *electrostatic*: atomic net charges (labeled by the atom type and number) and dipole moment, and (d) *theoretic*: energy calculated by the MP2 density functional theory (E).Our calculations established four groups of dependent descriptors: SA, CV and E; C2 and C3 net charges;C3 and BO12; and C11 and C16.Table 2 presents those descriptors that are part of the QSAR equations (rest are listed in *SM6*). The logarithmic values of bioactivities were chosen as response variables since they yielded to stronger correlations with physicochemical properties (find r^2 for individual descriptors in *SM5*). The regression equations are presented for all models in Table 1 along with the fitting (r, p, S/O) and 'leave-one-out' predictive values (PRESS, r_{cv}). Interestingly, the hydrophobic character of Cucs (log *P*) exhibited the strongest individual association with both toxicity and protection on HepG2 cells ($r^2 \geqslant 0.77$) (*SM5*). On the other hand, none of the descriptors correlated individually at a significant level with both cytotoxic and inhibitory activities on the stellate cells. All models are statistically significant by the *F*-test (p <0.001) with good correlation coefficients (r >0.92). Scatter plots between experimental and predicted bioactivities are illustrated in Figure 2 for all models, where values outside of SD <40% criteria are highlighted and labeled. Calculations predicted weaker protective activities for Cuc I and E-Me-Ac on hepatoma cells (Fig. 2B), lower toxicity

Table 2
Theoretical descriptors of cucurbitacins determined by ab initio calculations and lipophilicity (log P), surface area (SA) and Carbon C2, C16 and C25 used in the present work for the multiple linear equations (find rest of descriptors in *SM6*)

No.	Cucs.	log *P*	SA	Dipole	C2	C16	C25
1	I Glu	2.09	702.686	8.566	0.241	0.509	0.727
2	E Glu	2.28	746.792	6.155	0.274	0.488	0.797
3	**D**	3.12	551.214	7.242	0.385	0.407	0.656
4	iso-D	3.07	549.704	5.603	0.556	0.407	0.652
5	I	3.33	546.788	7.048	0.184	0.419	0.662
6	I-Me	3.81	570.337	7.699	0.109	0.438	0.656
7	L-Me	3.79	574.428	6.287	0.141	0.477	0.657
8	I-Et	4.15	593.845	7.563	0.093	0.412	0.653
9	I-*i*Pr	4.54	613.994	7.526	0.141	0.418	0.673
10	I-*n*Pr	4.52	617.017	7.491	0.226	0.443	0.685
11	B	3.69	597.806	5.139	0.355	0.470	0.776
12	E	3.72	591.214	4.332	0.172	0.413	0.774
13	E-Me	4.15	613.876	4.493	0.262	0.439	0.772
14	E-Et	4.68	634.972	4.431	0.237	0.426	0.761
15	E-*i*Pr	4.78	651.808	4.773	0.326	0.416	0.758
16	E-*n*Pr	4.93	659.574	5.291	0.189	0.504	0.783
17	E-Me-Ac	4.29	663.843	3.952	0.243	0.627	0.774

for Cuc E (Fig. 2C), and better inhibitory activities for Cuc E-Me-Ac and I Glu (1) on stellate cells (Fig. 2D) relative to experimentally observed values. The cytotoxicity of all test compounds on hepatoma cells are predicted within a standard error less than 40% (Fig. 2A). Cross validation of Models 1 and 4 yielded very good linear relationships between observed and predicted variables (r_{cv} >0.95, S/O <0.07), and less linear for the other two models (r_{cv} >0.84, S/O <0.12).

3.6. Theoretical design of novel derivatives

Considering the strong impact of log *P* lipophilicity on the cytotoxicity of Cucs expressed on both cell lines (Table 3), we initiated efforts to theoretically modify the existing structure to lower lipophilicity and hence increase polarity. Major changes to the structures of Cuc D (**3**), I (**5**), B (**11**), and E (**12**), such as reduction of carbonyl groups or esterification are specified in the Methods section. These changes influenced the rest of the descriptors in the QSAR equations leading to a more complex effect on activity. Table 4 presents the predicted activities for the newly designed derivatives on both HepG2 and HSC-T6 cell lines and the T/A (see related predictors in *SM7*). Additionally, we studied Cuc L and compared its bioactivity with that of the experimentally available Cuc L-Me. For comparison, the observed and predicted bioactivities for the parent compounds are also provided. The differential cytotoxicity of cucurbitacins on HeLa cells vs. HepG2 cells and non-correlation found on HSC-T6 suggest that there is no mechanistic interference at the cellular level between the two bioactivities.

4. Discussion

4.1. Hepatoprotection activity on hepatoma cells

The HepG2 hepatoma cells isolated from primary human liver carcinoma[42] express many of the functions attributed to normal hepatocytes and often lost by primary hepatocytes. In addition, they have the biosynthetic capabilities of normal liver parenchyma cells.[43] HepG2 cells are widely used for studying basal cytotoxicity[19] and for the detection of carcinogens and mutagens.[44] HepG2 cells represent a good in vitro model to predict the hepatotoxicity of new drugs and their metabolites in human[45,46] and may be useful in the study of the regulation of drug-metabolizing enzymes.[47]

CCl_4 is one of the most intensively studied hepatotoxins in vivo.[48] CCl_4 leads to changes in the regulation of numerous genes in HepG2 cells.[49,50] Several reports indicate good correlation between studies conducted on animal models and on HepG2 cells

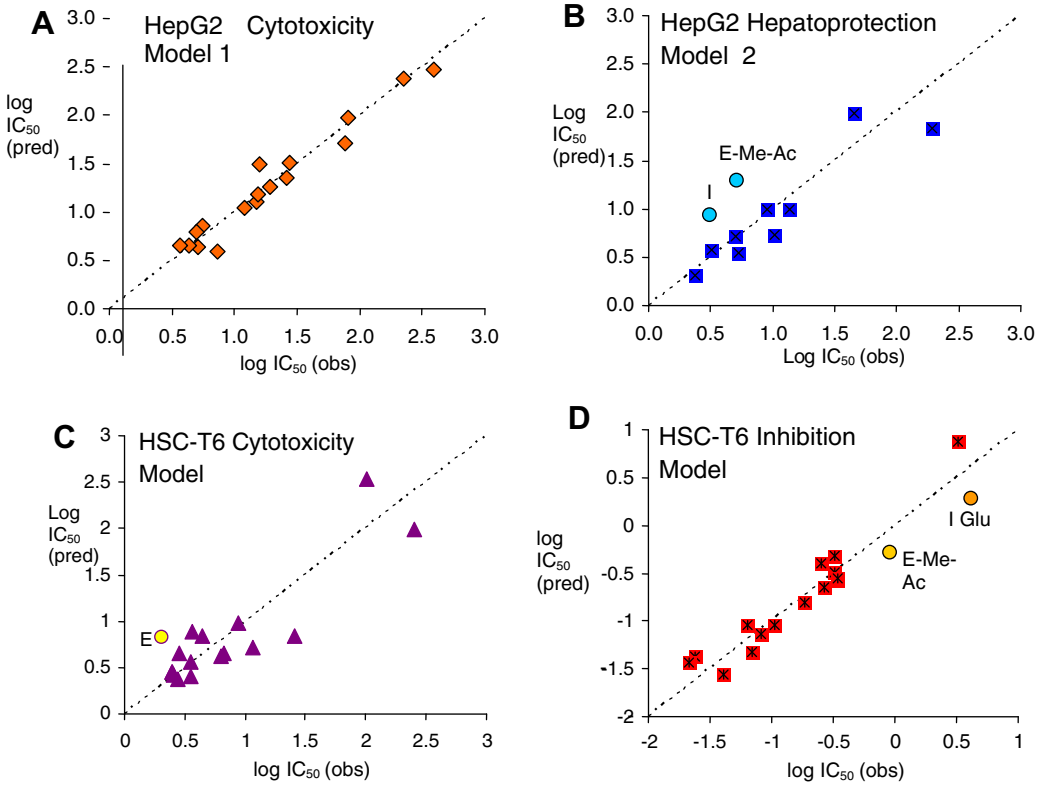


Figure 2. Correlation of log IC_{50} of observed (obs) and predicted (pred) values for Models 1–4 based on the 'leave-one-out' method (see Table 3 for the cross-validated *r* and PRESS values). Predicted values with a standard deviation >40% to observed values are labeled on the graphs.

Table 3
Regression analysis for Models 1–4 of the cytotoxicity and hepatoprotection of cucurbitacins towards HepG2 and HSC-T6 cell lines

Model cell line	Activity	Regression equation				r	r_{cv} (PRESS)	S/O
1 HepG2	Cytotoxicity F[20,3]	$\log IC_{50} = -0.640 \log P + 2.428C16 + 0.936C2 + 2.392$						
		F-test	p <0.001			0.985	0.977 (0.266)	0.058
		t-test	p_{logP} <0.001	p_{C16} <0.001	p_{C2} <0.01			
2 HepG2	Hepatoprotection F[11,2]	$\log EC_{50} = -0.738 \log P + 3.136C16 + 2.016$						
		F-test	p <0.001			0.945	0.835 (0.980)	0.109
		t-test	p_{logP} <0.001	p_{C16} <0.05				
3 HSC-T6	Cytotoxicity F[20,2]	$\log IC_{50} = -0.564 \log P + 0.004SA + 0.412$						
		F-test	p <0.001			0.923	0.863 (1.452)	0.117
		t-test	p_{logP} <0.001	p_{SA} <0.01				
4 HSC-T6	Inhibition F[20,3]	$\log EC_{50} = 0.017SA - 10.696C25 - 0.118Dipole - 3.051$						
		F-test	p <0.001			0.979	0.954 (0.609)	0.065
		t-test	p_{SA} <0.001	p_{C25} <0.001	p_{Dipole} <0.05			

Table 4
Descriptors and predicted bioactivity for novel cucurbitacins

No.	Cucs	HepG2		$\frac{IC_{50}}{EC_{50}}$	HSC-T6		$\frac{IC_{50}}{EC_{50}}$
		IC_{50}	EC_{50}		IC_{50}	EC_{50}	
1	Cuc D	77.33	9.00	9	26.0	0.07	379
2	D (Pred)	55.73	9.77	6	9.00	0.05	180
3	D3OH te	93.51	18.58	5	15.80	0.06	250
4	D11OH te	77.14	14.08	5	14.75	0.04	336
5	D22OH (t to C21)	153.77	36.35	4	15.82	0.02	728
6	D3te&11OH te	117.70	22.51	5	23.31	0.10	240
7	D23DH&24OH (c to C25OH)	233.49	60.93	4	25.49	0.12	216
8	D2OAc	24.78	3.58	7	7.07	0.34	21
9	D2OPr	9.05	1.23	7	3.38	0.75	4
10	Cuc I	15.80	3.16	5	3.60	0.02	150
11	I (Pred)	28.34	7.44	4	6.57	0.04	174
12	I3OH t	25.79	8.39	3	7.74	0.06	138
13	I11OH te	30.48	7.96	4	9.62	0.05	207
14	I22OH (t to C21)	54.28	17.00	3	7.73	0.01	553
15	Cuc L-Me	19.00	5.00	4	6.70	0.18	36
16	LMe (Pred)	18.09	5.17	3	4.72	0.16	29
17	L	38.25	11.37	3	7.24	0.04	169
18	Cuc B	27.67	10.50	3	4.42	0.02	205
19	B (Pred)	31.94	5.80	6	6.72	0.03	226
20	B3OH t	42.21	7.31	6	10.82	0.06	196
21	B11OH te	56.72	10.21	6	11.31	0.04	290
22	B22OH (t to C21)	53.41	9.95	5	11.62	0.005	2534
23	*iso*-B	49.90	5.73	9	6.71	0.03	214
24	Cuc E	15.25	3.20	5	2.03	0.04	50
25	E (Pred)	15.02	3.67	4	6.07	0.03	200
26	E3OH c	35.03	4.49	8	6.73	0.04	165
27	E11OH te	13.44	3.36	4	7.22	0.04	180
28	E22OH (t to C21)	22.89	6.42	4	6.66	0.01	565
29	E2OAc	8.94	1.58	6	4.88	0.39	13

t—trans, c—cis; e—ecuatoria.

for the hepatoprotection of various natural products against CCl_4;[51,52] therefore, it is suitable to use CCl_4 for our studies.

Eleven cucurbitacin analogs increased the viability of parenchymal liver cells by at least 50% in the presence of CCl_4. Among them, aglycons yielded several fold higher activity, defined as EC_{50}, than silybin. However, unlike silybin, none of the cucurbitacins protected cells by 100%. In addition, the margin between their activity and toxicity was marginal (T/A = 2–9). In this context, others also reported relatively small margins, ca. 5, between activities, such as antitumor and anticancer activities, and toxic animal dose for a large number of Cucs including all natural aglycons studied by our group[53–56] rendering them unfeasible as potential drugs without further studies. Therefore, additional hepatoprotection activity studies will require strict control of dosing and perhaps specific structural modifications to increase the margin to toxicity.

4.2. Differential cytotoxicity on HeLa cells

The potentially high cytotoxicity of cucurbitacins including Cuc D, I, B, and E on HeLa cervical cancer cells has been documented.[53] We examined the cytotoxicity of cucurbitacins on HeLa cells and on HepG2 cells under similar experimental conditions. The IC_{50} values do not correlate with each other suggesting different mechanism of action underlying the activities on the two types of cells.

4.3. Anti-proliferative activity on stellate cells

Hepatic stellate cells play a central role in liver fibrogenesis in experimental models of liver fibrosis as well as in human chronic liver disease. Activation of stellate cells is characterized by elevated proliferation rate, loss of vitamin A storage, expression of α-smooth muscle actin, and synthesis and excretion of some extracellular matrix components.[57,58] The HSC-T6 cell line developed by Friedman et al. is a stable phenotype, well characterized, and it serves as a useful tool for studying cell mechanism and biology, as well as for screening drug candidates.[59]

The proliferative effect of PDGF is well documented on primary stellate cells, but not on the immortalized cell line HSC-T6.[36,40] Several studies suggest that PDGF alone does not induce proliferation, because it requires the presence of other growth factors.[60–63]

This perhaps explains the reason why we did not achieve considerable cell proliferation by using PDGF.

Cucurbitacin analogs demonstrate very good inhibitory effect on HSC-T6 cells grown in serum-supplemented media (Table 1). Although they exhibit moderate to high toxicity, it is remarkable to mention that the toxic dose for Cucs is much larger than the active dose thus increasing their potential as hepatoprotective agents for controlling fibrosis. Due to its lower toxicity compared to the rest of the aglycons and the high T/A, Cuc D is one of the best candidates to be considered for further evaluation of its activity. Furthermore, data suggests high potential for CucI Glu (1) and E Glu (2) as a result of moderate toxicity and good margin to activity. Under stricter dosage, Cuc iso-D, I, I-Me, L-Me, B, E, and E-Me may also be very good candidates, since a T/A higher than 30 was realized. Alkylated diosphenols with the exception of methyl derivatives did not exhibit good T/A due to lower activity. On the other hand, acetylation of Cuc E-Me reduces toxicity besides lowering activity. Silybin is over 4-fold less active than the least active cucurbitacin, CucI Glu (1). However, it does not exhibit any cytotoxicity; therefore its overall pharmaceutical value is suggested to be better than the value of any of the test compounds. This is clearly highlight the potential of Cux E-Me as hepatoprotective agent for controlling fibrosis.

4.4. Antioxidant and anti-inflammatory activities

We postulated that cucurbitacins protect the liver, at least partially, by their inhibitory effect on lipid peroxidation and/or by the radical scavenging activity. However, Cucs did not express any antioxidant activity using the lipid peroxidation assay and the DPPH and $ABTS^{\cdot+}$ radical scavenging assays.

Our data suggest that cucurbitacins exhibit anti-inflammatory activity through different mechanisms other than the anti-hyaluronidase activity[64,65] and that cucurbitacins lack the free radical scavenging and anti-peroxidative activity.

4.5. Prediction and correlation of natural, semi-synthetic and theoretically designed cucurbitacins

4.5.1. Hepatoma cell line

We explored earlier the correlation between lipophilicity and toxicity on HepG2 cells for the array of Cucs presented here.[18] The current work is a continuation of efforts to understand the effect of various physicochemical parameters in addition to lipophilicity on both toxicity and protection of test compounds on the hepatoma liver cells. To our surprise, the hydrophobicity factor strongly influences both activities by increasing Hepatoprotection in parallel with cytotoxicity. According to our data collected on natural, semi-synthetic, and theoretically designed Cucs, lipophilicity increases by alkylation or esterification and decreases by glycosilation or reduction of carbonyl groups to hydroxyl. The net charge on C16 carbon has similarly a parallel effect on both activities, to some extent weaker on hepatoprotection (p <0.05) than on cytotoxicity (p <0.001) (Table 3). In this regard, esterification of C16–OH yielding Cuc E-Me-Ac correlates with decreased cytotoxicity and weakened Hepatoprotection. Our cytotoxicity data for Cuc E-Me-Ac parallels earlier observations of lower in vitro anticancer activities caused by C16 acetylated derivatives.[16,66] The third term in Model 1, the C2 net charge does not influence the hepatoprotective activity. In this regard, a single rather than double bond between C1 and C2 carbons (see Cuc D or B against Cuc I or E) or a carbonyl group rather than a hydroxyl on C2 carbon in the vicinity of a C3–OH (see Cuc iso-D against Cuc D) amplifies the positive charge on C2, hence contributing to the lower toxicity.

Since both log P and C16 net charge influence both activities on HepG2 cells in a similar manner, it becomes challenging to increase the T/A factor or suggest significant improvements to the structures. This is evident from Table 4 where the predicted bioactivities of novel cucurbitacins are presented. We were able to predict a larger T/A only for two novel cucurbitacins, Cuc iso-B and E3OH. The improved T/A attributes to a larger C2 net charge that increases the third term of the QSAR equation by 59% and 184%, respectively, without leading to significant changes in the other terms. Furthermore, the regression equations predict lower cytotoxicity for several compounds by keeping T/A $\geqslant$ 4 for Cuc D3OH, D11OH, D22OH (**5**), D3&11OH, D23DH&24OH (**7**), B11OH (**21**), B22OH (**22**), and E22OH (**28**). In these cases the cytotoxicity decreases mainly due to a higher polarity, but in some cases other factors also contribute positively to the change, such as C16 net charge in case of **5**, **7**, and **28**, and C2 net charge in case of **21** and **22**.

4.5.2. Stellate cell line

The two models for the stellate cells reveal significant independence from each other in compared to the hepatoma cell models, since the major descriptors, log P in the cytotoxicity model and surface area and C25 net charge in the inhibition model, are not related (Table 3). The surface area (SA) contributes significantly less to cytotoxicity (p <0.01) than to inhibition (p <0.001) as part of the QSAR equation, and individually correlates well only with inhibition (r^2 = 0.73 vs 0.25, *SM5*). A larger positive charge on C25 carbon achieved by acetylation and a larger dipole moment can improve the anti-proliferation activity. In this context, esterification of C25–OH can boost its positive charge, but it negatively alters the dipole moment. Reduction of C22 carbonyl significantly increases by the dipole moment and improves to some extent the C25 net charge as well. Thus, novel derivatives with the best T/A are Cuc D22OH, I22OH, B22OH, and E22OH. Reduction of C22 carbonyl does not alter log P or the SA significantly; therefore, the high toxic-to-active dose ratio is largely attributed to a significant increase in activity and less significant decrease in cytotoxicity. Quantitatively there is a clear negative effect of the acetyl group in case of Cuc E-Me-Ac on the inhibitory activity, expressed by the larger SA and the smaller dipole moment versus Cuc E-Me. The bigger SA also contributes to a decreased toxicity. However, the T/A is smaller than that of the parent compound, thus its overall value is inferior.

The effect of esterification at C2 hydroxyl with acetyl or propionyl groups was studied on Cuc D and E; see novel compounds **8**, **9**, and **29**. The regression equations suggest an elevated toxicity and reduced activity attributing it to a bigger SA and elevated log P.

Since lipophilicity demonstrates a large impact on cucurbitacins cytotoxicity on both cell lines and on the hepatoprotection activity on the hepatoma cells, it is important to consider it during drug design. Lipophilicity influences compounds transport through the lipid bilayer of the cell membrane and it plays a dominant role in drug-receptor interactions inside the cell.[67] As a general rule of thumb, compounds with log P >4 are not considered for drug design because of the adverse effects on protein binding, drug absorption, and the ability to accumulate in fatty tissue.[68] The principle of 'minimum hydrophobicity' is to keep log P as low as possible, preferably below 2.[67] Cucurbitacins do not enter into this optimal category. For the same reason, they are less soluble and theoretically cannot be taken orally. Survey of literature indicates that pure Cucs or cucurbitacin-containing plant extracts were injected intraperitoneally or subcutaneously in animals. They were also administered orally as aqueous suspension.[17,55,69–71] In folk medicine, Cuc B and elaterium were used externally for the treatment of sinus, or applied as a milk solution into nostrils to clear away icterus and to cure severe headache. This later application

caused severe congestion of the upper respiratory tract because of the high toxicity.[72]

5. Conclusions

Eleven cucurbitac inside monstrate cytoprotective activity on HepG2 cells against CCl_4-induced toxicity without reaching 100% protection and at a marginal toxic-to-active dose (T/A). Since Models 1 and 2 include two similar variables, improvements to the T/A factor on HepG2 cells can only be achieved by increasing the net charge on C2 without altering rest of the terms in the QSAR equation. The cytotoxicity of novel compounds can be reduced by altering one or more terms in Model 1.Cucurbitacins exhibit stronger cytotoxicity on HeLa cells than on HepG2 cells. All natural cucurbitacins demonstrate high anti-proliferation effect on serum-activated HSC-T6 cells with relatively high T/A, while the semi-synthetic derivatives exhibit lower T/A. Cucurbitacins trigger morphological alterations on both cell lines at EC_{50}, unlike silybin. Models 3 and 4 are more independent, therefore it is easier to improve T/A. Since lipophilicity has a large impact on cytotoxicity on both cell lines and on the hepatoprotection activity on the hepatoma cells, it is important to keep it as low as possible during drug design. Cucurbitacins do not provide antioxidant activity established by the DPPH, $ABTS^{\cdot+}$, and by the lipid peroxidation assays. Their anti-inflammatory activity may be involved in liver protection. However, this activity is not apparent from the protection of the connective tissue against the destructive effect of the hyaluronidase enzyme.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.037.

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