Significantly greater antioxidant anticancer activities of 2,3-dehydrosilybin than silybin

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Abstract

Silybin or silymarin extract has been used to treat liver diseases, and has now been entered into clinical trials for cancer treatment. Here, we compared antioxidant and anticancer activities between silybin and its oxidized form 2,3-dehydrosilybin (DHS). With IC50 at three-fold lower concentrations than silybin, DHS inhibited reactive oxygen species generation in glucose–glucose oxidase system and HepG2 cells. Compared with silybin, DHS elicited greater protection against H2O2-induced HepG2 cell death and galactosamine-induced liver injury in vivo. It is known that oxidants induce releases of metalloproteinases (MMP)-2,-9 which are responsible for invasive and metastasis potentials of transformed cells. DHS at 10 μM markedly inhibited MMP-2,-9 releases as well as invasiveness, while silybin at 90 μM had marginal effects. DHS but not silybin at 30 μM induced apoptosis and loss of mitochondrial membrane potentials. LD50 of DHS was five-fold lower than that of silybin. Our data suggest that DHS may be more useful therapeutically than silybin.

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1. Introduction

Epidemiological data show that naturally occurring phytochemicals including flavonoids taken up in the diet play a major role in prevention from malignancies [1]. Naturally occurring flavonoids and isoflavones have received increased attention in the past few years for their chemopreventive properties. Similar to curcumin [2] and flavopiridol [3], silybin is one of the flavonoid antioxidants now being tested in clinical trials to treat different types of cancer in the USA [4] and Europe [5]. Silybin is a major biologically active compound of milk thistle plant (Silybum marianum L. Gaertn.) extract, silymarin [6]. Silymarin extract is widely used as traditional herbal/dietary supplement around the world including the USA for its strong antihepatotoxic activity against almost every kind of human liver disease. In the past three decades, silybin is used clinically at least in Europe and Asia as a drug, such as, Legalon® and Silipide IdB1016 [7]. Antioxidant activity of silybin is considered as one of its therapeutic properties. However, the one-electron reduction potential at neutral pH for silybin is relatively high at 0.76 V [Silybin-O2−H+/Silybin-OH] [8]. Silybin is thus not considered to be a superb antioxidant in comparison to other antioxidants such as vitamins C and E [9]. Recent studies have attempted to identify other silymarin active compounds which exhibit improved antioxidant and anticancer activities [10].

An oxidized form of silybin so-called 2,3-dehydrosilybin (DHS) (Scheme I) was found to exist in fruit of spotted milk
thistle plants cultivated in Russia and its common independent states [11]. While silybin is white, DHS is yellow and thought to be responsible for yellowish color in all silymarin extract preparations. A recent study has reported that DHS inhibited microsomal lipid peroxidation better than silybin [12]. Compared with silybin, DHS was more cytotoxic in killing cancer cell lines and was a better inhibitor of P-glycoprotein [13]. Because of significant therapeutic uses of silybin, it is rationalized that detailed comparison between silybin and DHS with pertinent endpoints should be performed. In this study, we systematically compared silybin and DHS in terms of protection of liver injury in in vitro and in vivo models as well as anticancer activities in cell systems. The latter include studies on effects of silybin and DHS on metalloproteinases (MMP)-2,-9 releases, apoptosis and cell viability. We provide evidence that compared with silybin, DHS exhibited significant greater antioxidant and anticancer activity by three- and five-fold, respectively. In addition, DHS was more selective than silybin in killing cancer cells but not less-transformed cells. Improved biochemical characteristics of DHS reported here support the notion that DHS may be of potential therapeutic uses.

2. Materials and methods

2.1. Materials and cell culture

DMEM, RPMI 1640, penicillin/streptomycin, nonessential amino acids, silybin, pyridine, Coomassie brilliant blue, luminol, superoxide dismutase (SOD), LPS (E.Coli 055:B5) and glucose oxidase were from Sigma (Deisenhofen, Germany). Alamar Blue™ reagent, and tetramethylrhodamine methylester (TMRM) were obtained from Invitrogen (Freiburg, Germany). Deferoxamine and gelatin were from Merck (Darmstadt, Germany). Galactosamine was from AppliChem (Darmstadt, Germany). Keratinocyte growth medium kit was from Cambrex (Verviers, Belgium). Dichlorofluorescein diacetate (DCF-DA) and dihydroethidine (DHE) were from Molecular Probes, Leiden, Netherlands.

HepG2 cells were cultured in RPMI 1640 containing 10% FCS and 1% nonessential amino acids in a 5% CO2 incubator at 37 °C. EPI and FIB cells cultured in DMEM containing 10% FCS were cell lines we previously developed from human gingival keratinocytes [14]. Fibroblast-like FIB cells, which exhibited anchorage-independent growth, were regarded as more transformed cells, while EPI cells were designated as less-transformed cells. EPI and FIB cells were used to compare with cancer HepG2 cell line to test cytotoxic effects of silybin and DHS.

2.2. Synthesis of DHS

DHS was synthesized according to published method [12]. Briefly, 6 g silybin was dissolved in 400 ml pyridine and heated to 90 °C under reflux for 77 h with stirring. The majority of pyridine was removed using a rotary evaporator under 45 mbar at 60 °C. To remove residual pyridine, 50 ml toluene was added and dried under a rotary evaporator at 80 °C. Ethyl acetate was added and products were filtered through silica gel, and then washed with acetone. Yellow filtrates were dried under a rotary evaporator to obtain fine powder of DHS. DHS was again washed with hot ethanol. After filtration and air-dried, 3.2 g DHS was obtained with ~50% yield. Mass spectrum of DHS showed correct mass: m/z 481.1 (M+H)⁺, and 503.2 (M+Na)⁺.

2.3. Measurement of ROS generation

For antioxidant activity in glucose and glucose oxidase (G/GO) system, we employed a luminol-hypochlorite chemiluminescence detection system [15]. Reactive oxygen species (ROS) generation was initiated by adding 10 μM H2O2 to 490 μL PBS containing 5 mM glucose and 50 μM luminol. To assess specific ROS species, luminescence was measured in the presence of SOD (6.7 or 20 μg/ml), catalase (2 or 10 μg/ml) or desferoxamine (1–20 μM). Luminescence intensity was measured using a luminescence photomultiplier (AutoLumat LB953, Berthold Eg&G, Wildbad, Germany).

Fluorescence microscopy was utilized to monitor ROS generation in cells. HepG2 cells were seeded at 5 × 10⁴ cells per well of 96-well plates. After 5-day culture, cells were treated with 20 μM DHE or 10 μM DCF-DA [which is hydrolysed intracellularly to dichlorofluorescein (DCFH)] in serum-free medium for 20 min. After washing twice with PBS, cells were treated with 5 or 15 μM DHS, 5 or 15 μM silybin or DMSO in serum-free medium. An aliquot of H2O2 was added to obtain a final concentration of 500 μM. After 20-min incubation, cells were washed twice with PBS and added with serum-free medium. Fluorescence was recorded with a fluorescence microscope (Olympus IX50, Olympus, Hamburg, Germany).

2.4. Cell viability assay

Cell viability was assessed by using Alamar Blue™ assay kit. HepG2 cells were seeded at 5 × 10⁴ cells per well of 96-well plates. After 6-day culture, cells were washed with PBS and treated with serum-free medium containing 2.5 or 5 μM silybin, 2.5 or 5 μM DHS or DMSO. After 1-h incubation, an aliquot of H2O2 was added to obtain a final concentration of 100, 250 or 500 μM. Treated cells were incubated further for 19 h, and cell viability was determined with a
Multiscan ex microplate reader (Thermo Electron Corp., Vantaa, Finland). For comparison of cell lines with different malignancies, FIB, EPI or HepG2 cells were seeded at 10 × 10³ cells per well in 96-well plates and cultured overnight. After treatment with 10–90 μM silybin, 5–30 μM DHS or DMSO in serum-free medium for 3 days, cell viability was then determined. Lower DHS concentrations were used because of its limited solubility at 30 μM in medium.

2.5. Effects of silybin and DHS on liver injury in vivo

Silybin or DHS solubilized in 2% carboxy methylcellulose. Male C57BL/6 mice were injected intraperitoneally with 60 μg/kg silybin, DHS or carboxy methylcellulose control. After 1 h, mice were intraperitoneally injected with 700 μg/kg galactosamine (GalN) and 10 μg/kg lipopolysaccharide (LPS) for 6 h. Experimental protocols for use of animals were approved by the University of Heidelberg and State Authority Animal care and Use Committee. Serum AST, ALT and LDH activities were determined using diagnostic kits (Randox Laboratories, Krefeld, Germany).

2.6. Zymogram analysis of MMP-2 and -9 and invasiveness

FIB cells and EPI cells were seeded at 2 × 10⁵ cells in each well of 12-well plates. Next day, cells were treated 20–90 μM silybin, 10–20 μM DHS or DMSO in 1 ml keratinocyte growth medium. After 3 days, collected medium was concentrated five times by lyophilization. Samples dissolved in lysis buffer were subjected to gel electrophoresis in a 10%-polyacrylamide gel containing 2.5 mg/ml gelatin. Gels were then washed twice with 2.5% Triton X-100 each and incubated overnight at 37 °C in buffer containing 50 mM Tris, 200 mM NaCl and 10 mM CaCl₂, pH 7.4. Gels were stained with Coomassie brilliant blue followed by washing with water containing 10% acetic acid and 30% methanol. Gelatinase activities of conditioned medium were determined by photographing gels with a gel documentation system (Peqlab, Erlangen, Germany). Experiments were reproducible at least twice.

The effects of silybin or DHS on invasiveness of FIB cells were studied using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Heidelberg) in a 24-well plate with inserts containing 8 μm-pore membranes. FIB cells (50 × 10³ cells in 0.5 ml serum-free medium per well) were added to the upper compartment in the presence of 0, 5, 10 μM silybin or DHS, FCS at 5% was used as chemoattractants at the lower compartment. After 3 days, cells that invaded through Matrigel membrane were fixed with methanol and stained with 1% toluidine blue. Total number of invading cells was counted under a light microscope. Experiments in each group were conducted in triplicate.

2.7. Flow cytometric determination of DNA fragmentation and ΔΨm

Cell death in the form of apoptosis was studied by DNA fragmentation analysis (to measure % sub-G1 peak) and loss of mitochondrial membrane potentials (ΔΨm) by using flow cytometry with a BD FACS Calibur™ (10,000 events were measured in each sample). After treatment, % sub-G1 was measured in ethanol-fixed cells that had been treated with RNase A (100 μg/ml) at 37 °C for 30 min, and stained with propidium iodide (50 μg/ml). ΔΨm was analyzed by accumulation of tetramethylrhodamine methylster (TMRM), a membrane-permeable cationic fluorescence dye [16]. After treatment and trypsinization, cells were stained with 1 μM TMRM in serum-free DMEM for 30 min at 37 °C. The intensity of TMRM was determined by FACS analysis.

HepG2 at 50 × 10³ cells were seeded in each well of 24-well plates and cultured for 4 days to reach ~70% confluence. To induce apoptosis by H₂O₂, HepG2 cells had been treated with 0.3 mM l-buthionine sulfoximine in serum-free medium overnight according to published procedure [17]. Cells were then treated with DHS or silybin I h followed by 100 μM H₂O₂. After 24-h incubation, % sub-G1 was determined. % sub-G1 and ΔΨm loss were measured in HepG2, FIB and EPI cells treated with 30 μM DHS or silybin for 24 h.

2.8. Gel electrophoresis analysis of DNA fragmentation

DNA fragmentation in HepG2, FIB, EPI cells (0.24 × 10⁶ cells seeded in each well of six-well plates) was treated with 30 μM DHS or silybin for 24 h in serum-free medium. Treated cells were lysed with 1 ml lysis buffer (50 mM Tris–HCl, pH 7.4, 10 mM EDTA, 0.5% SDS containing 500 μg/ml proteinase K), and incubated for 2 h at 65 °C. DNA was extracted with 1 ml phenol, pH 8.0, then with 1 ml 1:1 phenol:chloroform, and finally with 1 ml chloroform. DNA was precipitated with 2.5 volumes cold ethanol and 0.1 volume 3 M sodium acetate, pH 5.2, and kept at ~20 °C overnight. DNA was collected by centrifugation at 16,000 × g at 4 °C for 15 min. DNA pellets were air-dried and dissolved in Tri-EDTA containing 100 μg/ml RNAse A, and incubated at 37 °C for 30 min. DNA was loaded onto a 1.2%-agarose gel. After exposure to ethidium bromide, gels were photographed.

2.9. Statistical analysis

Data are represented as mean ± standard deviation. Statistical analysis was performed by ANOVA tests. A p < 0.05 was considered significant.

3. Results

3.1. DHS scavenges ROS better than silybin in G/GO system and in HepG2 cells

Chemiluminescence measurements of G/GO system revealed that generated ROS were highly reactive species because measured luminescence signals were significantly inhibitable by catalase or deferoxamine (Fig. 1A, left panel). G/GO system produces mainly H₂O₂ [18] generating metal-catalyzed reactions involving H₂O₂, hydroxyl and superoxide radicals, and iron (II) and (III) (present in buffer). This G/GO system was then used to compare antioxidant activity of DHS and silybin. In Fig. 1B right panel, dose response curves demonstrated that DHS inhibited ROS generation more effectively than silybin showing IC₅₀ of 0.74 and 2.57 μM for DHS and silybin respectively.

To investigate whether DHS could scavenge ROS better than silybin in cells, we measured antioxidant activities in HepG2 as model liver epithelial cells. The potent scavenging of DHS may indicate a potential use of DHS to treat liver diseases. In Fig. 1B, HepG2 cells treated with H₂O₂ produced green and red fluorescence indicative for an oxidized form of DCFH and DHE, respectively. Similar to G/GO system, DHS scavenged intracellular ROS generated in HepG2 cells at three-fold lower concentrations than silybin.

3.2. DHS exhibits better protection than silybin in vitro and in vivo models

To correlate inhibition of ROS generation by silybin or DHS with concomitant cytoprotection, we determined cell viability and apoptosis of H₂O₂-treated HepG2 cells in the presence of silybin or DHS. Significant loss of cell viability was obtained after overnight treatment with 100–500 μM H₂O₂ (Fig. 2A). At 250 and 500 μM H₂O₂, moderate ~20% protection was obtained with 5 μM DHS while silybin had marginal ~10% protection (Fig. 2A). To correlate with cell viability data, DNA fragmentation as DNA damage due to cell death was measured by FACS analysis (% sub-G1) in H₂O₂-treated HepG2 cells [17]. H₂O₂ treatment significantly increased % sub-G1 by two-fold (Fig. 2B). Silybin at 5 μM did not change these levels (left
panel), while DHS at 5 μM markedly inhibited these increases to control levels (right panel). Similar to viability data in Fig. 2A, silybin or DHS at 2.5 μM did not affect % sub-G1 (not shown). Compared with silybin, greater cytoprotection from H2O2-induced cell death by DHS was consistent with improved antioxidant activity of DHS.

Fig. 1. Compared antioxidant activities between silybin and DHS in G/O system and in HepG2 cells. (A) ROS-dependent chemiluminescence in G/O system was produced by adding glucose oxidase to PBS containing glucose and luminol. Left panel shows the effects of SOD, catalase (cat) or desferoxamine (def), where significant inhibition was indicated (*, p<0.01 vs. con, n=3). Right panel shows that DHS has greater chemiluminescence inhibition than silybin (Δ, p<0.01 vs. silybin, n=5). (B) Inhibition of H2O2-induced oxidation of DCFH or DHE in HepG2 cells was better by DHS than silybin. DCF-DA or DHE-preloaded HepG2 cells were treated with DHS or silybin and then with 500 μM H2O2. After 20-min incubation and washing, cellular fluorescence was recorded with a fluorescence microscope. Pictures shown are representatives of three experiments.
In vivo liver injury was induced by GaIN/LPS as measured by increases of liver enzyme AST/ALT/LDH activities (Fig. 2C). At 60 mg/kg, DHS suppressed increases of AST, ALT and LDH activities while silybin significantly inhibited only ALT activity.

3.3. DHS inhibits MMP-2,-9 releases and invasiveness better than silybin

ROS regulate expression of proteins that are important for degradation of extracellular matrices, such as gelatinase matrix
metalloproteinases-2,-9 [19], which are key enzymes for degrading type IV collagen and thought to play a critical role in tumor invasion and metastasis [20]. In this study, we used our model EPI and FIB keratinocytes representing less and more transformed cells, respectively [14]. As shown in Fig. 3A top panel, EPI cells secreted latent MMP-9 (92 kDa) and MMP-2 (72 kDa). FIB cells released a protein at 86 kDa which was not active MMP-9 (82 kDa) [20]. It has been described that MMP-9 at 86 kDa is an intermediate after first cleavage of latent MMP-9 by MMP-3 [21]. In both cell lines (Fig. 3A, bottom panel), silybin at 90 μM showed some inhibition of MMP-2,-9 releases. For MMP-9 releases, DHS at 15 μM showed complete inhibition in FIB cells but marginal inhibition in EPI cells. This indicates that DHS has inhibition selectivity towards more transformed cells. DHS at 20 μM caused complete inhibition of MMP-2,-9 releases in both EPI and FIB cells. Effective inhibitory concentrations of MMP-2,-9 releases by DHS were ~5 folds lower than those by silybin.

ROS have been shown to increase invasive potentials of transformed cells [22], we therefore studied effects of DHS or silybin on invasiveness and migration of model EPI and FIB cells [14]. Using Boyden invasion chamber, FIB cells had significant migration through the filters observed as blue-stained cells indicated with an arrow in Fig. 3B left panel. EPI cells did not migrate (data not shown). DHS at 5 μM significantly inhibited number of blue-stained FIB cells. The inhibition of invasion was not due to cytotoxicity of DHS under 3-day treatment of FIB cells as later shown in Fig. 5B. Quantitatively, DHS inhibited invasion of FIB cells in a dose-dependent manner while silybin at 10 μM elicited no inhibition (Fig. 3B, right panel). The observed inhibition of invasiveness by 5–10 μM DHS was consistent with effects of

![Fig. 3](image_url)

Fig. 3. Anti-invasive effects of DHS or silybin in less-transformed EPI and more transformed FIB cells. (A) EPI cells released latent MMP-9 and MMP-2, while FIB cells released partially active MMP-9 (upper panel). Inhibition of MMP-2,-9 releases by EPI and FIB cells was obtained at 15 μM DHS and 90 μM silybin. (B) Using Boyden invasion chamber, invading FIB cells (which had migrated through filters) were observed as blue-stained cells indicated with an arrow in left panel. From number of invading cells counted (right panel), DHS at 10 μM completely inhibited invasion of FIB cells (*, p<0.05 vs. con, n=3).
DHS on oxidant inhibition and cytoprotection against H₂O₂-induced injury.

3.4. DHS induces apoptosis more effectively than silybin

To study anticancer activity, we compared silybin and DHS ability to perturb cell cycle profile and induce cell death. We measured propidium iodide-stained DNA of FIB cells treated with silybin or DHS for 24 h (Fig. 4A). Silybin at 100–200 μM are typical concentrations to induce apoptosis in cancer cells [23], in our study we used lower maximum solubility of DHS at 30 μM. As shown in histograms in Fig. 4A, DHS caused a decrease of G₀/G₁ intensity with concomitant increase of sub-G₁ peak (as marked with M₁), indicating cell cycle arrest. DHS caused a significant increase in % sub-G₁ by ~two-fold (Fig. 4B, left panel). To elucidate the molecular events involved in apoptosis, we compared loss of mitochondrial membrane potential (ΔΨₘ) under the same conditions. Consistently, DHS induced loss of ΔΨₘ (Fig. 4B, right panel). Silybin did not have any effects on G₀/G₁ intensity, % sub-G₁ and loss of ΔΨₘ.

We then further investigated whether DHS was selective in inducing % sub-G₁ increases in cell lines with increased stages of malignancies: HepG2, FIB, and EPI cells (Fig. 5). DHS at 30–50 μM markedly caused increases of % sub-G₁ in HepG2 and FIB cells but only marginally in EPI cells (Fig. 5A). DNA fragments as % sub-G₁ not only can represent condensed nucleosomes from apoptosis but also autolytic DNA from necrosis [24]. We then measured 200-bp DNA fragments detectable on agarose gels to identify apoptosis in treated HepG2, FIB, and EPI cells. DNA smears and 200-bp DNA ladders were observed in HepG2 and FIB cells treated with 30 μM DHS; while no DNA ladders were detected with 30 μM silybin (Fig. 5B). DNA smears but no ladders were detected in DHS- or silybin-treated EPI cells (Fig. 5B) and untreated EPI cells (not shown). Thus, % sub-G₁ increased by DHS in HepG2 and FIB cells was consistent with apoptosis as mode of cell death. In EPI cells, DHS caused marginal % sub-G₁ increases that were apparently associated with necrotic DNA breakdown.

To confirm selectivity property of DHS, we determined cell viability of HepG2, FIB and EPI cells treated with up to 100 μM silybin and 30 μM DHS. In Fig. 5C left panel, silybin exhibited the same cytotoxicity profiles in all three cell lines with LD₅₀’s ~40–50 μM. DHS exhibited differential cytotoxic effects with lower LD₅₀ of ~8 μM for HepG2 cells, ~20 μM for FIB cells, and ~27 μM for EPI cells (Fig. 5C, right panel). DHS was more cytotoxic towards HepG2 cells than FIB and
EPI cells and that silybin did not exhibit such selectivity. DHS was more potent than silybin by five-fold in the killing of HepG2 cells.

4. Discussion

Silybin is a widely used drug and supplement for various forms for liver diseases and acute liver injury partly due to antioxidant activity of silybin. Similar to curcumin and flavopiridol, silybin exhibits anti-tumor activity in vitro and in vivo. Since DHS can be prepared in large quantities in a pure form from silybin, DHS may be used to substitute silybin when data have clearly demonstrated improved properties of DHS. Our data strongly support this notion since DHS exhibited significant greater antioxidant and anticancer activities than silybin. With extensive literature supporting protective role of silybin in

Fig. 5. DNA fragmentation as % sub-G1, 200-bp ladders and cell viability following DHS and silybin treatment of cell lines with different degree of malignancies. (A) % sub-G1 as a function of silybin or DHS dose was measured in HepG2, FIB and EPI cells after 24-h treatment. Data are mean±std (n=6). (B) DNA fragmentation was detected as 200-bp DNA ladders on agarose gels in HepG2, FIB and EPI cells treated with 30 μM silybin or DHS for 24 h. (C) Cell viability was determined after treatment of HepG2, FIB and EPI cells with silybin or DHS for 3 days. Data are mean±std (n=6).
Silybin has a relatively high one-electron reduction potential of 0.76 V [8] and this is consistent with previous finding that silybin does not react with superoxide radical very well [25]. The improved antioxidant activity of DHS being 3 times better than silybin is likely due to an unsaturated bond in conjugation with 4-oxo group in ring C of DHS (Scheme I) contributing to hydrogen-donating capacity to react with oxidants [26]. It has been recently shown that antioxidant activity of a flavone (as a model of DHS) is much improved compared with a flavonone (as a model of silybin) [27]. It is known that antioxidant activity is directly proportional to the compound’s lipophilicity [28], the increased antioxidant activity of DHS is consistent with lipophilicity of DHS being higher than that of silybin [12]. DHS may react with membrane lipids and proteins, and consequently the scavenging of ROS by DHS is more effective than by silybin as we observed in HepG2 cells. Silybin has a weak iron-chelation activity [29]. DHS contains functional groups for chelation, i.e., 3-OH and 4-carbonyl groups of ring C [26]. This improved iron-chelation may also contribute to improved antioxidant activity of DHS compared with silybin. Significant improvement in antioxidant activity of DHS correlates well with superior cytoprotection by DHS against H2O2-induced cell injury in vitro and GalN/LPS-induced liver injury in vivo. At 60 mg/kg, silybin was partially protective, while DHS exhibited greater protection in all three liver enzymes studied. As silybin is an active ingredient for treatment of liver diseases with pathogenesis involving oxidative stress from neutrophils, macrophages and Kupffer cells, it is perceived that DHS may function therapeutically for treatment of liver diseases better than silybin.

ROS at low concentrations not only mediate transformation of normal to tumor cells but also activate expression and releases of MMP-2,-9 [19,21] which are proteinases of Type IV collagen which is a major structural protein of basement membrane. It is believed that MMPs play central role in invasion and metastasis of malignant tumors of different histogenetic origin [30]. Here, a novel model for MMP-2,-9 releases from EPI and FIB cells was utilized for testing efficacies of DHS and silybin. FIB cells expressed superoxide radical-generating NADPH oxidase isoform 1 (Nox1) at higher levels than EPI cells [31]. Since it has been shown that Nox 4 induces MMP-9 [32], it is surmised that ROS from Nox1 in EPI and FIB cells may mediate MMP-2,-9 releases. Similar to normal human keratinocytes and HaCaT cells [33], EPI cells released latent MMP-9. FIB cells secreted partially active MMP-9 [21] suggesting that FIB cells likely contain MMP-3 whereby its induction is known to be redox dependent [34]. It is known that expression of all three MMPs-2,-3, and -9 is increased in malignant cells [35] thus MMP-2,-9 releases by EPI and FIB cells were appropriate targets for comparing DHS and silybin. We found that significant greater inhibition of MMP-2,-9 releases by DHS than by silybin, and this corroborates with greater antioxidant activity of DHS compared with silybin. Superior ROS scavenging by DHS resulting in an inhibition of MMP-2,-9 releases also reflects much improved anticancer activity of DHS. While inhibition of MMP-2 releases was achieved by ~100 μM silybin [36] or ~100 μM epigallocatechin-3-gallate [37], DHS caused complete inhibition of MMP-9 releases at much lower ~15 μM which is similar to that reported for curcumin [38].

It is known that increased MMP-9 activity is correlated with cell migration and invasion [39]. More specifically, H2O2 treatment of epithelial cells induces MMP-2,-9 releases as well as enhanced cell invasion, and that an MMP inhibitor markedly prevented invasion [22]. FIB cells released partially active MMP-9 and also produced significant migration through Boyden invasion chambers. Non-toxic dose of DHS but not silybin inhibited both MMP-9 releases and invasion of FIB cells. DHS inhibiting ROS affected primary ROS targets, i.e., MMP-2,-9, thus this process may potentially slow-down progression towards enhanced malignancies. In this context, DHS may be more efficient chemopreventive agent.

At concentrations higher than those used in antioxidant activity studies, we also found that DHS was more cytotoxic than silybin. While silybin did not induce apoptosis, DHS was capable of inducing apoptosis in HepG2 and FIB cells. DHS was slightly cytotoxic in EPI cells likely via necrosis. DHS (LD50 ~8 μM) was five-fold more effective than silybin (LD50 ~50 μM) in the killing of HepG2 cells. LD50 of silybin at 100 μM has been reported [23], and that lower LD50 of DHS was similar to that of curcumin [40]. In a similar manner as silybin, DHS may inhibit cell survival and induce apoptosis via cell cycle arrest and down regulation of survivin, Akt, and NFκB [2,4,36]. Consistently, we observed cell cycle arrest in FIB cells treated with DHS but not silybin. The observed loss of ΔΨm elicited by DHS may also be associated with cytochrome c release from mitochondria leading to apoptosis. While DHS scavenges ROS better than silybin by three-fold, cytotoxicity of DHS in killing of HepG2 cells is greater at five-fold suggesting that metabolism and cytotoxicity of DHS may rely on its flavone structure, i.e., unsaturation on ring C and enhancement of electrophilicity. For an example, a potent anticancer flavopiridol (which is also a flavone) inhibits cyclin-dependent kinases by binding with the proteins’ ATP-binding pockets [41]. The inhibition of MMP-2,-9 and invasion of FIB cells by DHS five-fold better than silybin cannot be explained only by superior antioxidant activity of DHS but may also be explained by inhibitory effects by DHS on other key proteins related to cell migration and invasion. Such DHS effects unrelated to its antioxidant activity may be elicited by its flavone structure.

As a result of ROS scavenging by DHS, DHS phenoxy radical is produced and can be recycled upon further reaction with other antioxidants, such as, vitamins E and C present in cells and tissues. Such futile redox cycle results in steady state DHS which leads to cytotoxicity. DHS being more electrophilic than silybin is capable of interacting with proteins, such as, multidrug resistant protein 1 whereby the binding sites for DHS on cytosolic and transmembrane domains have been described [42]. Such binding results in inhibition of efflux function of multidrug resistant P-glycoproteins allowing DHS to be accumulated intracellularly hence eliciting cytotoxicity more efficiently. Consistent with our data, potent cytotoxicity of DHS is correlated with a stronger inhibition of P-glycoproteins by DHS than by silybin [42]. DHS may be able to sensitize other anticancer drugs.
such as cisplatin, adriamycin and TNF-α for the killing of cancer cells.

The switch from protection to cytotoxicity with increasing DHS concentrations relies on the ROS levels generated in the system whether they were acutely elevated (i.e., during liver injury) or moderately elevated (i.e., in cancer). DHS is more reactive than silybin; on one hand, DHS is easier oxidizable than silybin and, on the other hand, it is more electrophilic capable of reacting with key cancer-related proteins. The latter reactions would require high DHS concentrations while ROS scavenging is operative at low DHS concentrations.

Anticancer drugs now tested in clinical trials would be expected to have high selectivity towards cancer cells but not normal cells. We observed selectivity of DHS in inhibiting MMP-9 releases and invasion as well as cytotoxicity and apoptosis induction. DHS not only had lower LD₅₀’s than silybin by five-fold, DHS exhibited anticancer activities selective towards killing of transformed cells. Consistently, dietary flavonoid quercetin (which contains rings A and C similar to DHS) has been shown to selectively induce inhibition and apoptosis in hepatic tumor cells but not in normal hepatocytes [43]. Molecular mechanisms for such selectivity are not known and may be related to differences in locations of key cancer-related proteins on plasma membrane of transformed and non-transformed cells.

In conclusion, we have demonstrated that DHS exhibited antioxidant and anticancer properties than silybin by a factor of three- and five-fold, respectively. With extensive uses of silybin, our work here raises a possibility that DHS may function better than silybin. DHS may thus represent an improved flavonoid for treatment of treat liver diseases and cancer.

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References


[37] E. Pezzato, L. Sartor, I. Dell’Aica, R. Dittadi, M. Gion, C. Belluco, M. Lise, S. Garbisa, Prostate carcinoma and green tea: PSA-triggered base-
ment membrane degradation and MMP-2 activation are inhibited by (−) epigallocatechin-3-gallate, Int. J. Cancer 112 (2004) 787–792.


