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Bioconversion of silybin to phase I and II microbial metabolites with retained antioxidant activity

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ABSTRACT

Microbial transformation of silybin (**1**), the major flavonolignan of milk thistle (*Silybum marianum*, Asteraceae), resulted in the isolation of four metabolites. The structures of the isolated metabolites were determined by spectroscopic methods. One phase I metabolite was produced by *Beauveria bassiana* and was characterized as 8-hydroxysilybin (**2**). Three phase II metabolites were produced by two *Cunninghamella* species and were identified as 2,3-dehydrosilybin-3-*O*-β-D-glucoside (**3**), obtained from *Cunninghamella* species; and silybin-7-sulfate (**4**) and 2,3-dehydrosilybin-7-sulfate (**5**), obtained from *Cunninghamella blakesleana*. Compared to **1** (IC_{50} 284 μg/mL), the generated metabolites displayed varying levels of antioxidant activities in the DPPH free-radical scavenging assay.

1. Introduction

The seed extract of milk thistle (*Silybum marianum*, family Asteraceae) is used in pharmaceutical products and in herbal preparations for the treatment of acute and chronic liver diseases and as an antihepatotoxic.[1,2] The dry extract is commonly known as silymarin which comprises a group of flavonolignan compounds with silybin (**1**, Fig. 1) as the major component, in addition to its analogs isosilybin, silychristin, silydianin and the flavonol taxifolin.[3] Beyond their reported antioxidant/hepatoprotective effects, recent studies have demonstrated that silymarin and silybin inhibit the growth of human carcinoma cells of different types, for example, prostate, breast, cervix, lung and liver; and that they inhibit DNA synthesis in these cells.[4–7] A silybin-phosphatidylcholine complex was found to possess antitumor activity against human ovarian cancer in an in vivo mouse model.[8] The same complex dose-dependently potentiated the activity of cisplatin against A2780 cells, both in vitro and in vivo.[9] Silybin has also been found to inhibit lung tumor angiogenesis in mice and may, thus, be a potential chemopreventive candidate.[10] Silybin is currently being evaluated in a number of clinical trials in patients with different types of cancer, alone and as an adjunct therapeutic agent.[11,12] In addition to its potential in cancer treatment and chemoprevention, silybin also possesses a neuroprotective effect as well as a possible role in the treatment of nephropathies, cardiopulmonary and gastrointestinal problems.[11]

Chemical derivatization of silybin has been adopted in an attempt to generate resolvable analogs of enhanced solubility/pharmacokinetic properties. Kren et al. was able to resolve the two silybin diastereomers in the form of acetylated monoglycosides with enhanced water solubility. The semisynthetic glycosides also exhibited increased antioxidant properties.[13] Another attempt to improve the bioavailability of silybin was performed by the same group whereby plant cell cultures of *Papaver somniferum* were used to biotransform silybin to silybin-7-*O*-β-D-glucoside.[14] One microbial transformation attempt of silybin A and B resulted in the formation of the 3-*O*-β-D- and the 7-*O*-β-D-glucosides of both diastereomers.[15] Carboxylic acid derivatives with increased water solubility and antioxidant activity were also semisynthesized from silybin.[16] The chemistry and pharmacology of silymarin/silybin are thoroughly covered in a comprehensive review by Gazak et al.[11]

As part of our drug discovery program, we have successfully utilized three fungal cultures, *Beauveria bassiana*, *Cunninghamella* sp.,

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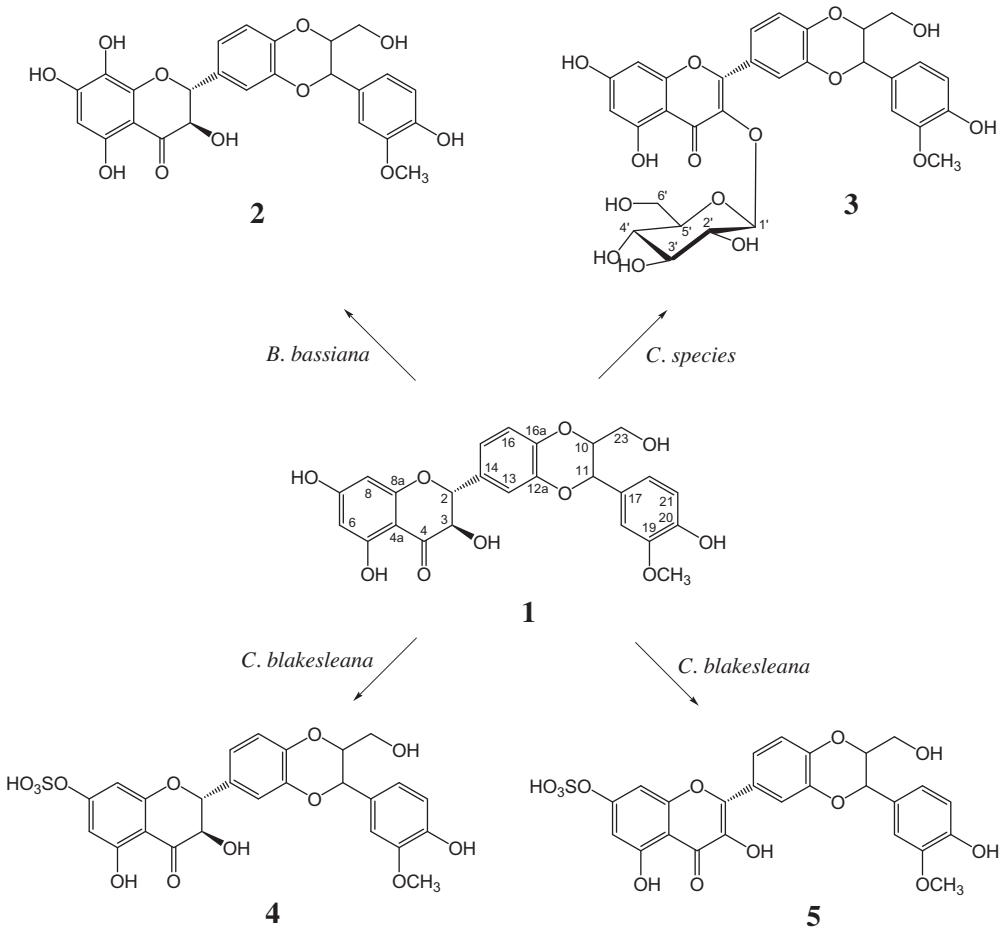

Figure 1. Fungal transformation of **1** to metabolites **2**–**5** by *B. bassiana*, *C. blakesleana* and *Cunninghamella* species.

and *C. blakesleana*, to generate four new phase I and phase II microbial metabolites of silybin. Two of the obtained metabolites were better antioxidants than silybin in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay. In this paper we describe the preparation, structure elucidation and antioxidant evaluation of the obtained silybin metabolites.

2. Results

Four microbial metabolites were generated by the selected fungal strains. Each metabolite existed as a mixture of diastereomers as evident by HPLC and specific double peaks in the ^{13}C NMR spectra (C-10 and C-11) of each metabolite. The chemical structures of the metabolites (Fig. 1) are reported for the first time as shown below and their NMR data are listed in Table 1, in comparison to the substrate, **1**.

8-Hydroxysilybin (**2**)—yellow solid; R_f 0.31 (chloroform–methanol, 9:1, v/v); ^1H and ^{13}C data, see Table 1.

2,3-Dehydrosilybin-3-*O*-β-D-glucoside (**3**)—brown solid; R_f 0.17 (chloroform–methanol, 85:15, v/v); ^1H and ^{13}C data, see Table 1.

Silybin-7-sulfate (**4**)—brownish yellow solid; R_f 0.20 (chloroform–methanol, 75:25, v/v); ^1H and ^{13}C data, see Table 1.

2,3-Dehydrosilybin-7-sulfate (**5**)—yellow solid; R_f 0.25 (chloroform–methanol, 75:25, v/v); ^1H and ^{13}C data, see Table 1.

In the DPPH scavenging assay, **2** had the highest (25% of positive control) and **4** had the lowest antioxidant activity of the tested compounds. Both the substrate, **1**, and metabolite **5** had intermediate antioxidant activities between **2** and **4**. Results are shown in Table 2.

3. Discussion

Analysis of the NMR data of the generated metabolites revealed that the γ-benzopyrone nucleus of silybin was the main target for biotransformation. A review of the current literature indicated that all the identified metabolites are reported here for the first time.

Metabolite **2** displayed a molecular ion at $m/z = 499.1222$ $[M+H]^+$ (calcd For $C_{25}H_{22}O_{11}$ + H: 499.1240) in positive-ion HRMS with an additional oxygen atom compared to **1**. The NMR data of **2** was almost identical with that of **1** except for the disappearance of the aromatic proton doublet at C-8 and the collapse of the aromatic doublet of H-6 to a singlet. The ^{13}C doublet at δ 95.2 was also replaced with a downfield singlet at δ 125.7 confirming the insertion of an oxygen atom at C-8. This assumption was further confirmed by HMQC and HMBC experiments. Thus, metabolite **2** was assigned as 8-hydroxysilybin.

Metabolite **3** displayed a molecular ion at $m/z = 643.1628$ $[M+H]^+$ (calcd For $C_{31}H_{30}O_{15}$ + H: 643.1663) in positive-ion HRMS. The mass difference between **1** and **3** indicated the introduction of a hexose moiety and the loss of two hydrogen atoms from **1**. The NMR data of the hexose was in agreement with published data for a silybin-3-β-D-glucoside obtained via microbial transformation.[15] The hexose moiety was thus assigned as glucose. The position of the glucosidic bond was determined to be at C-3 by observing the change in chemical shift of C-2, C-3, C-4 from δ 146.9, 137.6 and 176.9 to δ 156.1, 134.7 and 178.4, respectively. The ^1H NMR showed the disappearance of the two methine proton signals at δ 5.07 and 4.64, corresponding to H-2 and H-3, respectively in **1**. The corresponding carbons similarly disappeared with

Table 1
^{1}H and ^{13}C NMR chemical shift assignments of **1** and its metabolites **2–5**

Atom	**1**[a]		**2**[b]		**3**[c]		**4**[b]		**5**[d]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	5.07 (d, 11.5)	83.3 d	5.00 (d, 11.4)	83.8 d	–	134.7 s	5.04 (d, 11.6)	83.3 d	–	147.0 s
3	4.64 (d, 11.5)	72.3 d	4.51 (d, 11.4)	72.7 d	–	123.9 s	4.58 (d, 11.6)	72.7 d	–	135.0 s
4	–	197.3 s	–	197.2 s	–	178.4 s	–	198.3 s	–	177.0 s
4a	–	100.7 s	–	100.5 s	–	105.0 s	–	103.2 s	–	105.6 s
5	–	164.0 s	–	156.7 s	–	162.1 s	–	162.2 s	–	160.2 s
6	5.97 (d, 2.2)	96.2 d	5.98 (s)	96.0 d	6.22 (d, 2.0)	99.6 d	6.44 (d, 2.0)	100.6 d	6.60 (d, 2.0)	101.8 d
7	–	166.9 s	–	156.8 s	–	165.1 s	–	ND	–	159.7 s
8	5.94 (d, 2.2)	95.2 d	–	125.7 s	6.47 (d, 2.0)	94.6 d	6.50 (d, 2.0)	99.4 d	6.97 (d, 2.0)	98.1 d
8a	–	163.3	–	148.8 s	–	157.3 s	–	161.1 s	–	155.6 s
10	4.13 (m)	78.8 d	4.05 (m)	78.8 d	4.28 (m)	79.3 d	4.06 (m)	78.6 d	4.27 (m)	78.9 d
11	4.98 (d, 7.8)	76.4 d	4.89 (d, 7.8)	76.5 d	4.96 (d, 4.8)	76.5 d	4.90 (d, 8.0)	76.3 d	4.97 (d, 7.6)	76.3 d
12a	–	143.9 s	–	143.9 s	–	146.6 s	–	143.7 s	–	143.8 s
13	7.12 (d, 1.5)	116.6 d	7.15 (d, 1.8)*	116.7 d	7.08 (d, 9.2)	117.4 d	7.04 (d, 1.6)	116.2 d	7.83 (d, 2.0)	116.9 d
14	–	130.3 s	–	130.3 s	–	134.7 s	–	128.0 s	–	124.1 s
15	7.07 (br d, 7.8)	121.1 d	7.07 (dd, 8.0, 1.8)	121.2 d	7.78	123.7 d	7.05 (dd, 8.4, 1.6)	120.3 d	7.77 (dd, 8.4, 2.0)	121.7 d
16	6.94 (d, 7.8)	116.5 d	6.98 (d, 8.0)	116.6 d	7.75	118.4 d	6.99 (d, 8.4)	116.5 d	7.13 (d, 8.4)	117.4 d
16a	–	144.3 s	–	144.3 s	–	144.0 s	–	144.1 s	–	145.5 s
17	–	128.4 s	–	128.3 s	–	128.2 s	–	129.8 s	–	127.7 s
18	7.12 (d, 1.5)	111.0 d	6.99 (d, 1.50)	110.9 d	7.05 (d, 1.2)	112.6 d	7.10 (d, 1.6)	110.7 d	7.04 (d, 1.5)	112.1 d
19	–	147.7 s	–	148.0 s	–	148.5 s	–	147.8 s	–	148.1 s
20	–	147.1 s	–	147.2 s	–	147.9 s	–	146.8 s	–	147.4 s
21	6.85 (d, 8.3)	116.6 d	6.83 (d, 7.8)	115.1 d	6.82 (d, 8.1)	116.2 d	6.84 (d, 8.0)	116.2 d	6.82 (d, 8.0)	115.7 d
22	6.96 (dd, 8.3, 1.5)	120.8 d	6.88 (dd, 7.8, 1.2)	120.5 d	6.89 (dd, 8.1, 1.2)	121.4 d	6.88 (dd, 8.0, 1.6)	120.8 d	6.90 (d, 8.0, 1.5)	121.0 d
23	3.72 (d, 12.0) 3.49 (dd, 12.0, 2.5)	61.0 t	3.70 (d, 12.0) 3.48 (dd, 12.0, 4.8)	60.9 t	3.59 (d, 11.0) 3.30 (m)	61.8 t	3.70 (br d, 12.0) 3.49 (dd, 12.0, 4.0)	60.7 t	3.73 (br d, 12.4) 3.50 (dd, 12.4, 4.0)	60.5 t
OMe	3.85 (s)	55.5 q	3.85 (s)	55.3 q	3.79 (s)	56.6 q	3.86 (s)	55.1 q	3.84 (s)	56.1 q
1′	–	–	–	–	5.49 (d, 8.7)	101.8 d	–	–	–	–
2′	–	–	–	–	3.21 (m)	75.1 d	–	–	–	–
3′	–	–	–	–	3.23 (m)	77.3 d	–	–	–	–
4′	–	–	–	–	3.05 (m)	70.8 d	–	–	–	–
5′	–	–	–	–	3.10 (m)	78.4 d	–	–	–	–
6′	–	–	–	–	3.40 (d)	60.9 t	–	–	–	–

ND, not detected.
[a] In $(CD_3)_2CO$.
[b] In CD_3OD.
[c] In C_5D_5N.
[d] In $(CH_3)_2SO$.

Table 2
DPPH radical scavenging activities of **1** and its microbial metabolites **2–5**

Compound	IC_{50} (μM±S.D.)
1	284 ± 36.0
2	30.3 ± 4.5
3	Not tested
4	830.9 ± 124.5
5	99.7 ± 20.4
Ascorbic acid[a]	7.7 ± 0.5

[a] Positive control.

the appearance of two olifinic singlets at δ 134.7 and 123.9. The C-4 carbonyl signal also shifted upfield to δ 178.4 (δ 197.3 in **1**) further supporting the introduction of an α,β-unsaturated ketone system. Metabolite **3** was thus assigned as 2,3-dehydrosilybin-3-β-D-glucoside.

Metabolite **4** displayed a molecular ion at m/z = 561.0715 $[M-H]^-$ (calcd For $C_{25}H_{22}O_{13}S$—H: 561.0703) in negative-ion HRMS. The NMR spectra of **4** were almost identical with those of **1** except for the chemical shifts at C-7 and in its vicinity along with a loss of 80 mass units in the MS spectrum. This indicated that a sulfate group has been introduced at C-7. Therefore, metabolite **4** was assigned as silybin-7-sulfate.

Metabolite **5** displayed a molecular ion at m/z = 559.0547 $[M-H]^-$ (calcd For $C_{25}H_{20}O_{13}S$—H: 559.0546) in negative-ion HRMS. Compared to **4**, the NMR spectra of **5** showed an α,β-unsaturated ketone system similar to that of **3** (disappearance of H-2 and H-3 signals and appearance of ^{13}C downfield signals at δ 147.0, 135.0 and 177.0). This indicated that **5** is a 2,3-unsaturated analog of **4**. Therefore, metabolite **5** was assigned as 2,3-dehydrosilybin-7-sulfate.

Earlier microbial transformation of flavonoid compounds resulted in similar phase I and II biotransformation patterns, such as hydroxylation *ortho* to an existing hydroxyl group (as in metabolite **2**), and glycosylation/sulfation of an OH group (as in metabolites **3**, **4** and **5**). Other previously observed biotransformations, such as reduction of flavanone carbonyl, and *O*-methylation, were not achieved by the utilized fungal strains.[19–21]

The presence of free phenolic groups, isolated and in the form of *o*- and *p*-hydroquinones, and the ability to form aroxyl radicals are among the major factors contributing to the antioxidant activity of flavonoid compounds.[22] Results obtained from our DPPH radical scavenging assay are in line with this observation. Using **1** as a reference (IC_{50} 284 μM), introduction of an additional phenolic group resulted in an *o*-hydroquinone and an enhancement in activity (**2**, IC_{50} 30.3 μM) while blocking the C-7 phenolic group resulted in a drastic reduction in activity (**4**, IC_{50} 830.9 μM). On the other hand, the introduction of the α,β-unsaturated ketone in **5** seems to have overcompensated for the blocked C-7 hydroxy by imparting a phenolic character to the C-3 hydroxy group and a semiquinone moiety to the molecule. Thus, the antioxidant activity of metabolite **5** more than doubled in comparison with **1**. The antioxidant activity of **3** could not be evaluated due to its low yield.

4. Conclusions

This work further demonstrates the ability of microorganisms to perform phase I and II metabolic reactions on complex natural products. The three fungal species utilized in this study have been reported to perform similar transformations on various chemical substrates.[23,24] Bioconversions were not stereospecific for either of silybin A or B resulting in metabolites with a similar diastereomeric pairing. Two of the generated metabolites possessed enhanced antioxidant activity compared to the parent substrate. Further optimization of fermentation conditions may provide an efficient route for massive production of active analogs of silybin.

5. Experimental section

5.1. General experimental procedures

^1H and ^{13}C NMR were acquired on a Bruker DRX-400, 500 or a Varian Unity Inova 600 spectrometer. HR-ESI-MS data were obtained using a Bruker GioApex 3.0 or an Agilent 1100 SL. All column chromatographic separations were performed on silica gel 60 (230–400 mesh), while thin layer chromatography (TLC) was performed on silica gel-coated aluminum plates (Merck kieselgel 60 F254).

5.2. Substrate and chemicals

Bulk silymarin was purchased from USA NutraSource, Inc. (San Carlos, CA). Silybin was prepared from silymarin by precipitation with methanol, filtration and washing the residue with methanol. Purity was confirmed by HPLC and NMR analysis of the dry residue which showed an almost equimolar mixture of silybin A and B (silybin A and B were designated as a single compound, **1**. The same designation applies to isomeric pairs resulting from biotransformation of **1**). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich (St. Louis, MO). Fungal strains were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from the National Center for Agricultural Utilization Research (NCAUR) (formerly Northern Regional Research Laboratories (NRRL)), Peoria, Illinois. Stock cultures were maintained on agar slants of media recommended by ATCC and were stored at 4 °C. All screening and scale-up fermentations were run in a complex culture medium (medium-α) of the following composition: 5 g yeast extract (Difco Labs, Detroit, MI), 5 g bactopeptone (Difco Labs), 5 g NaCl, 5 g K_2HPO_4, 20 g dextrose, and distilled H_2O to 1 L. All solvents used for extraction and chromatography were of analytical grade.

5.3. Microbial transformation

A total of 42 fungal strains were screened for their ability to biotransform **1**. Of these, *Beauveria bassiana* (ATCC 13144), *Cunninghamella* species (NRRL 5695), and *C. blakesleana* (ATCC 8688A) were selected based on the yield and diversity of produced metabolites as judged by TLC. The fungus *Mucor ramannianus* produced a metabolite pattern similar to that of *C. blakesleana* but the latter was chosen for scale-up due to its relatively higher bioconversion yields.

A two-stage procedure was utilized for the screening and preparative fermentations.[17] For screening, cultures were grown in 25 mL of medium-α held in 125-ml Erlenmeyer flasks equipped with stainless steel caps. Compound **1** was added to 1-day-old stage II culture media as a 5% acetone solution (0.2 mg of substrate/ml of culture medium). Cultures were incubated at room temperature on a rotary shaker (New Brunswick Model G 10-21) at 150 rpm for a maximum period of 14 days with sampling and TLC monitoring at three-day intervals. Preparative scale fermentations followed the same general procedure with the difference that, for each organism, 10 mL of 5% solutions of **1** in acetone was equally divided on 10 1-L Erlenmeyer flasks, each containing 250 mL of stage II culture of the respective organism (50 mg/flask). Incubation periods for the preparative scale fermentations were 14 days. Work-up followed a routine procedure that comprised exhaustive extraction of both culture filtrates and residues. The combined biomass and filtrate extracts were concentrated under

vacuum at 40 °C to yield residues that were subsequently subjected to column chromatography for metabolite isolation and purification. Biotransformation progress was monitored by TLC. The necessary substrate and culture controls were simultaneously run alongside each screening and preparative scale fermentation.

5.3.1. Biotransformation of 1 with *B. bassiana*

At the end of the fermentation period, the media were filtered. Both the filtrate and biomass were exhaustively extracted with ethyl acetate to yield an orange residue (928 mg). The residue was ultrasonicated in chloroform and the chloroform-soluble fraction (330 mg) was flash chromatographed on silica using a gradient of 5–15% acetone in chloroform. Pure **2** (38.9 mg, 7.8% yield) eluted at 7% acetone in chloroform.

5.3.2. Biotransformation of 1 with *Cunninghamella* species

The ethyl acetate extract of the fermentation broth (737 mg) was flash chromatographed on silica using a gradient of 5–50% methanol in chloroform. Pure **3** (18.8 mg, 3.7% yield) eluted at 5% methanol in chloroform.

5.3.3. Biotransformation of 1 with *C. blakesleana*

The organic extract (ethyl acetate-isopropanol, 4:1, v/v) of the fermentation mixture (4.16 g) was chromatographed on a sephadex column eluted with methanol to yield 38.3 mg (7.7% yield) and 67.3 mg (13.5% yield) of **4** and **5**, respectively.

5.4. Antioxidant assay

The antioxidant activities of compounds **1**, **2**, **4** and **5** were evaluated as their ability to scavenge the stable free radical DPPH according to the modified method of Abourashed.[18] In brief, serial dilutions of test sample and solution of the reference antioxidant (grape seed extract) were prepared in methanol and mixed with a fixed concentration of DPPH (250 μM) in methanol. After one hour, 1 μL of each sample was applied to a TLC plate followed by densitometric evaluation of the dry spots at 530 nm. The reduction of purple color of the spots reflected the extent of radical scavenging. According to the equation DPPH Inhibition (%) = $([DPPH_{bl} - DPPH_{test}]/[DPPH_{bl}]) \times 100$, where $DPPH_{bl}$ is the peak area of the spot corresponding to blank DPPH solution and $DPPH_{test}$ is the peak area of the spot corresponding to DPPH + sample. A curve for sample concentration versus DPPH % inhibition was plotted and the 50% inhibition (IC_{50}) of each sample was calculated using the 3rd degree regression equation generated for the data points.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.046.

References and notes

1. Fetrow, C. W.; Avila, J. R. *Complementary & Alternative Medicines*; Springhouse: Springhouse, Pennsylvania, 1999.
2. Polyak, S. J.; Morishima, C.; Lohmann, V.; Pal, S.; Lee, D. Y. W.; Liu, Y.; Graf, T. N.; Oberlies, N. H. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 5995.
3. Hansel, R.; Schulz, J.; Pelter, A.; Rimpler, H.; Rizk, A. F. *Tetrahedron Lett.* **1969**, *10*, 4417.
4. Bhatia, N.; Zhao, J.; Wolf, D. M.; Agarwal, R. *Cancer Lett.* **1999**, *147*, 77.
5. Deep, G.; Oberlies, N. H.; Kroll, D. J.; Agarwal, R. *Int. J. Cancer* **2008**, *123*, 41.
6. Varghese, L.; Agarwal, C.; Tyagi, A.; Singh, R. P.; Agarwal, R. *Clin. Cancer Res.* **2005**, *11*, 8441.
7. Mateen, S.; Tyagi, A.; Agarwal, C.; Singh, R. P.; Agarwal, R. *Mol. Carcinog.* **2010**, *49*, 247.
8. Gallo, D.; Giacomelli, S.; Ferlini, C.; Raspaglio, G.; Apollonio, P.; Prislei, S.; Riva, A.; Morazzoni, P.; Bombardelli, E.; Scambia, G. *Eur. J. Cancer* **2003**, *39*, 2403.
9. Giacomelli, S.; Gallo, D.; Apollonio, P.; Ferlini, C.; Distefano, M.; Morazzoni, P.; Riva, A.; Bombardelli, E.; Mancuso, S.; Scambia, G. *Life Sci.* **2002**, *70*, 1447.
10. Singh, R. P.; Deep, G.; Chittezhath, M.; Kaur, M.; Dwyer-Nield, L. D.; Malkinson, A. M.; Agarwal, R. J. *Natl. Cancer Inst.* **2006**, *98*, 846.
11. Gazak, R.; Walterova, D.; Kren, V. *Curr. Med. Chem.* **2007**, *14*, 315.
12. Singh, R. P.; Agarwal, R. *Mol. Carcinog.* **2006**, *45*, 436.
13. Kren, V.; Minghetti, A.; Sedmera, P.; Havlicek, V.; Prikrylova, V.; Crespi-Perellino, N. *Phytochemistry* **1998**, *47*, 217.
14. Kren, V.; Kubisch, J.; Sedmera, P.; Halada, P.; Prikrylova, V.; Jegorov, A.; Cvak, L.; Gebhardt, R.; Ulrichova, J.; Simanek, V. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2467.
15. Kim, H. J.; Park, H.-S.; Lee, I.-S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 790.
16. Gazak, R.; Svobodova, A.; Psotova, J.; Sedmera, P.; Prikrylova, V.; Walterova, D.; Kren, V. *Bioorg. Med. Chem.* **2004**, *12*, 5677.
17. Abourashed, E. A.; Khan, I. A. *Chem. Pharm. Bull.* **1996**, *2000*, 48.
18. Abourashed, E. A. *Z. Naturfortsch.* **2005**, *60b*, 1212.
19. Herath, W.; Mikell, J. R.; Hale, A. L.; Ferreira, D.; Khan, I. A. *Chem. Pharm. Bull.* **2006**, *54*, 320.
20. Mikell, J. R.; Herath, W.; Khan, I. A. *Chem. Pharm. Bull.* **2011**, *59*, 692.
21. Herath, W.; Mikell, J. R.; Hale, A. L.; Ferreira, D.; Khan, I. A. *Chem. Pharm. Bull.* **2008**, *56*, 418.
22. Pietta, P.-G. *J. Nat. Prod.* **2000**, *63*, 1035.
23. Abourashed, E. A.; Clark, A. M.; Hufford, C. D. *Curr. Med. Chem.* **1999**, *6*, 359.
24. Azerad, R. *Adv. Biochem. Eng. Biotechnol.* **1999**, *63*, 169.