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Synthesis and antioxidant evaluation of novel silybin analogues

LEIXIANG YANG[1], JINGXU GONG[1], FENG WANG[1], YONGMIN ZHANG[2], YANGUANG WANG[3], XIAOJIANG HAO[4], XIUMEI WU[5], HUA BAI[5], JOACHIM STÖCKIGT[6], & YU ZHAO[1,2]

[1]*Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan an Road, Hangzhou 310031, P.R. China,* [2]*ZJU-ENS Joint Laboratory of Medicinal Chemistry, Zhejiang University, Hangzhou 310031, P.R. China,* [3]*Department of Chemistry, College of Sciences, Zhejiang University, Hangzhou 310027, P.R. China,* [4]*The Key Laboratory of Chemistry of Natural Products of Guizhou Province and Chinese Academy of Sciences,Guiyang 550022, P.R. China,* [5]*Zhejiang Hisun Naturelite Pharmaceutical R & D Co., Ltd., 19-G, Huazhe Plaza, Hangzhou 310006, P.R. China, and* [6]*Lehrstuhl für Pharmazeutische Biologie, Institut für Pharmazie, Johannes-Gutenberg Universität Mainz, Staudinger Weg 5, Mainz D-55099, Germany*

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Abstract

In this work, we evaluated the antioxidant properties of the eight novel silybin analogues for their capacity to scavenge free radicals including superoxide anion radicals and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals *in vitro*. Compound **7d** demonstrated an excellent antioxidant effect in scavenging superoxide anion free radical with an IC_{50} value of 26.5 μM, while the IC_{50} of quercetin (the reference compound) was 38.1 μM. Compounds **7b**, **7e**, **7h** showed certain scavenging activities for both types of free radicals.

Keywords: *Antioxidant, superoxide anion, DPPH, silybin analogues, free radical scavenging*

Introduction

Oxidative stress (OS), which refers to the imbalance between cellular production of free radicals and the ability of cells to defend against them, links to a variety of diseases in nervous system, such as stroke (a disease in acute central nervous system injury) [1], Parkinson's disease [2] and Alzheimer's disease (AD) [3]. It also results in an enormous variety of pathological processes, including cardiac disease, autoimmune rheumatic disease, cancer, viral disease (such as AIDS) and aging [4–7]. Therefore, the discovery of new antioxidants has become an efficient way to treat various diseases related to free radicals.

Superoxide anion radical ($\cdot O_2^-$) is one kind of reactive oxygen species, which may be generated by a biological process *in vivo* such as hypoxanthine acted on by xanthine oxidase and is extremely active due to its unpaired electron. It can quickly capture electron from other molecules and lead to the generation of hydroxide radical, the most harmful free radical so far known. Furthermore, 1,1-diphenyl-2-picrylhydrazyl (DPPH), another kind of stable free radical existing *in vitro* may be utilized to screen the aryl free radical scavenging activity. Therefore, it is necessary to find superoxide anion scavengers and DPPH inhibitors as new antioxidants.

A well-known flavonolignan silybin (see Figure 1) was reported to have a variety of biological activities such as hepato-protection [8], antioxidant [9–11] and antibacterial [12]. However, its bioavailability and therapeutic efficiency are limited by its low water solubility. To explore new concepts of structure-activity relationship (SAR) of this type of flavonolignan

Correspondence: Y. Zhao, Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan an Road, Hangzhou 310031, P.R. China. Fax: 86 571 85270026. E-mail: dryuzhao@zju.edu.cn

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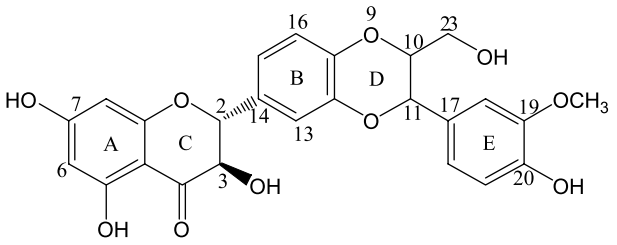

Figure 1. Silybin A (10 *R*, 11 *R*) and B (10 *S*, 11 *S*).

derivatives, eight silybin analogues with a modified B ring and/or E ring of the molecule were designed and synthesized and their free radical scavenging activities on superoxide anion and DPPH free radicals determined. The present study might be beneficial for an understanding of the mechanisms of the antioxidant activity of silybin analogues and lead to a further optimizing of their application.

Materials and methods

IR spectra were recorded on a Bruker Vector-22 spectrometer as kBr discs. ^{1}H NMR spectra were recorded on a Bruker AC2000 at 400 MHz and chemical shifts were expressed as δ (ppm). ESI were determined on a HP-5898B mass spectrometer. Column chromatography was performed on Qingdao silica gel (200–300 mesh), and thin layer chromatography (TLC) with Qingdao GF_{254} silica gel. Phenazine methosulfate (PMS, Sigma), nitroblue tetrazolium (NBT, Sigma), NADH (disodium, reduced, Amresco), Tris base (Gibco), quercetin (prepared in our laboratory), multi-well plates (Greiner) and multi-well plates reader (Bio-Tek Instruments, USA) were used in the experiments.

Chemistry

Synthesis of the target compounds is outlined in Figure 2. Condensation of 2,4,6-tri(methoxymethoxy)acetophenone **1** with substituted benzaldehydes **2** in a methanolic solution of KOH (15.0 eq) gave the chalcones **3** in 75–80% yields. Epoxidation of **3** with H_2O_2 in the presence of 2 N NaOH in methanol at room temperature afforded the corresponding chalcone epoxides **4** in yields of 80 − 85%. Treatment of **4** with hydrochloric acid in methanol and THF afforded the (±)-dihydroflavonols **5** as white solids in 55 − 65% yields [13]. Coupling of **5** with substituted cinnamyl alcohols in the presence of Ag_2CO_3 in dry benzene and acetone at about 60°C gave the target diastereoisomeric compounds (±)-**7** as main products in yields of 20 − 45% [14]. The relative configurations of C-2, C-3 and C-10, C-11 in **7** were both *trans*, based on the diagnostic ^{1}H NMR doublets of H-2 (ca. J = 12.0 Hz) and H-11 (J = 8.0 Hz) (see Table I). Some physico-chemical properties and spectral data of the compounds are given in Table I.

General procedure for the preparation of (±)-2,3-dihydro-2-(3,4-dihydroxy- 5-substituted)-3,5,7-trihydroxy-4H-1-benzopyran-4-one (5). A mixture of MeOH (5 ml) and conc. HCl (1 ml) was added dropwise to a solution of **4** (1 mmol), in MeOH (4 ml) and THF (1 ml) and the reaction mixture was heated at 55°C for 15 min. After cooling, the mixture was poured into ice-water and extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated. The residual oil was purified by column chromatography on silica gel using $CHCl_3$–MeOH (10:1) as eluent to afford (±)-5 as a solid.

General procedure for the preparation of (±)-2-[2,3-dihydro-3-(4-hydroxy-3,5-substituted-phenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-4H-1-benzopyran-4-one (7). A mixture of 1.48 mmol of **5**, 1.48 mmol of alcohol **6**, 60 ml of benzene and 20 ml of acetone was placed in oil bath at 60°C and the solution was stirred for 10 min. Ag_2CO_3 0.408 g (1.48 mmol) was then added and the reaction mixture was stirred vigorously for 20 h. The reaction was then worked up and the solvent removed by rotary evaporation to leave a yellow powder which was subjected to column chromatography over silica gel using $CHCl_3$–AcOEt–HCO_2H (25:2:0.25) as eluent to give (±)-7 as a pale yellow powder.

Antioxidant activity studies

Superoxide anion scavenging activity. The superoxide anion scavenging activity of the compounds was assayed spectrophotometrically as reported with a slight modification [14]. Superoxide anion radicals were generated in a non-enzymic phenazine methosulfate-NADH system by following the reduction of nitroblue tetrazolium. In this assay, the superoxide anion radicals were measured in plates which contained 78 μM of NADH, 50 μM of nitroblue tetrazolium, 5 μM of phenazine methosulfate and the test samples at different concentrations in 16 mM Tris–HCl buffer at pH 8.0. The color was monitored at 560 nm after 5 min incubation at room temperature. The blank samples did not contain phenazine methosulphate. Quercetin was used as a positive reference compound.

DPPH free radical scavenging activity. The quenching of free radicals by the compounds was assayed spectrophotometrically at 517 nm against the absorbance of the stable free DPPH radical [15]. The free radical scavenging efficiency of the compounds was

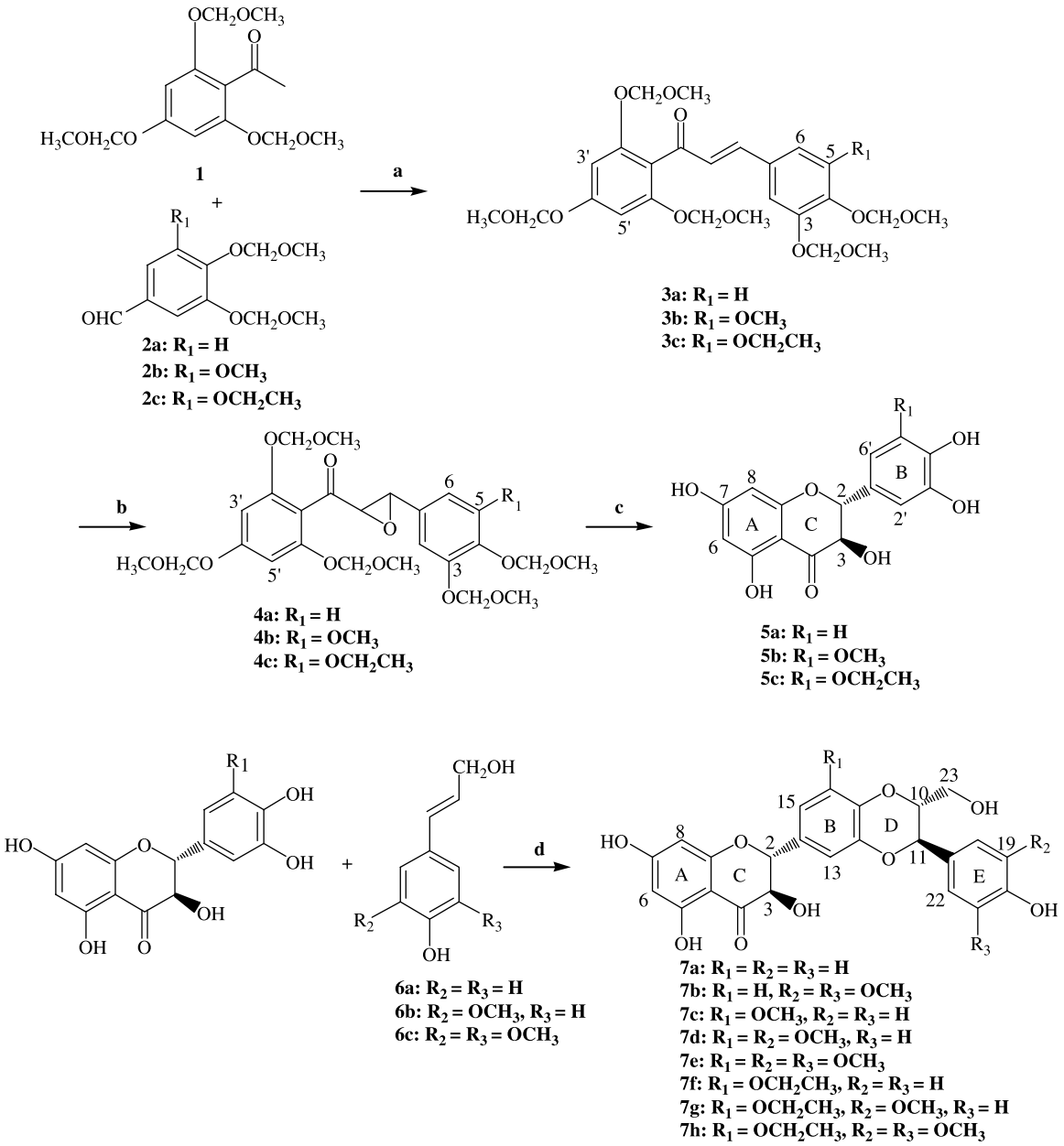

Figure 2. Synthetic route for the preparation of compounds **5** and **7**. Conditions and reagents **a**: KOH, MeOH, r.t. **b**: 2 N NaOH, H_2O_2, MeOH, r.t. **c**: HCl, MeOH, 55°C **d**: Ag_2CO_3, dry benzene–acetone, 60°C.

determined by decoloration of the DPPH radical. In brief, reaction mixtures contained various concentrations of the test compounds which were dissolved in DMSO and DPPH (0.4 mg/ml) dissolved in methanol. The methanolic solution of DPPH served as a control and quercetin was used as a reference free radical scavenger. The absorbance was measured at 517 nm after the mixture was incubated at 37°C for 30 min.

Results and discussion

Chemistry

All spectral data were in accordance with the assigned structures. Yields were not optimized in the reactions. The Ag_2CO_3-initiated reactions of **6a** and **6b** with **5a** yielded **7a** and **7b** both as a mixture of silybin and isosilybin type products, although the normal silybin type product was the major product. Interestingly, the synthesis of **7c**–**7h**, unlike those of **7a** and **7b**, did not provide the isosilybin type by-products during preparation. This regiospecific selectivity might be attributable to the presence of alkoxy groups on the B ring in the case of these compounds.

Antioxidant bioactivity

The superoxide anion radical scavenging activity was measured by the inhibition of NBT reduction and the

Table I. Physical and spectral data of compounds **5** and **7**.

No	Formula	Yield (%)	IR (cm^{-1})	^1H NMR data (400 MHz, Acetone-d_6) δ	ESI
5a	$C_{15}H_{12}O_7$	65	3385, 1642, 1467, 1285, 1164, 1086	4.59 (dd, 1H, J = 11.6, 4.4 Hz, H-3), 4.70 (d, 1H, J = 4.4 Hz, 3-OH), 5.01 (d, 1H, J = 11.6 Hz, H-2), 5.95 (d, 1H, J = 2.4 Hz, H-6), 5.99 (d, 1H, J = 2.4 Hz, H-8), 6.86 (d, 1H, J = 8.0 Hz, H-5′), 6.91 (dd, 1H, J = 8.0, 2.0 Hz, H-6′), 7.06 (d, 1H, J = 2.0 Hz, H-2′), 11.72 (s, 1H, 5-OH).	304
5b	$C_{16}H_{14}O_8$	43	3423, 1643, 1513, 1462, 1350, 1239, 1154, 1092	3.77 (s, 3H, OCH_3), 4.60 (dd, 1H, J = 11.6, 4.0 Hz, H-3), 4.69 (d, 1H, J = 4.0 Hz, 3-OH), 4.96 (d, 1H, J = 11.6 Hz, H-2), 5.91 (d, 1H, J = 2.0 Hz, H-6), 5.93 (d, 1H, J = 2.0 Hz, H-8), 6.71 (d, 1H, J = 2.0 Hz, H-2′), 6.72 (d, 1H, J = 2.0 Hz, H-6′), 11.69 (s, 1H, 5-OH).	334
5c	$C_{17}H_{16}O_8$	35	3421, 2980, 1637, 1459, 1247, 1163, 1083	1.34 (t, 3H, J = 6.8 Hz, CH_3), 4.08 (q, 2H, J = 6.8 Hz, CH_2), 4.62 (dd, 1H, J = 12.0, 4.0 Hz, H-3), 4.68 (d, 1H, J = 4.0 Hz, 3-OH), 4.97 (d, 1H, J = 12.0 Hz, H-2), 5.93 (d, 1H, J = 2.0 Hz, H-6), 5.97 (d, 1H, J = 2.0 Hz, H-8), 6.71 (s, 1H, H-2′), 6.73 (s, 1H, H-6′), 11.71 (s, 1H, 5-OH).	348
7a	$C_{24}H_{20}O_9$	35	3382, 2925, 1639, 1511, 1464, 1270, 1162, 1086, 833	3.59 (m, 1H, H-23a), 3.72 (m, 1H, H-23b), 4.08 (m, 1H, H-10), 4.62 (d, J = 11.6 Hz, 1H, H-3), 4.97 (d, J = 8.0 Hz, 1H, H-11), 5.06 (d, J = 11.6 Hz, 1H, H-2), 5.92 (s, 1H, H-6), 5.95 (s, 1H, H-8), 6.86–7.32 (m, 7H, Ar-H), 11.66 (s, 1H, 5-OH).	452
7b	$C_{26}H_{24}O_{11}$	31	3426, 2927, 1639, 1511, 1464, 1279, 1162, 1089, 833	3.55 (m, 1H, H-23a), 3.77 (m, 1H, H-23b), 3.86 (s, 6H, OCH_3), 4.16 (m, 1H, H-10), 4.66 (d, 1H, J = 11.6 Hz, H-3), 4.99 (d, 1H, J = 8.0 Hz, H-11), 5.11 (d, 1H, J = 11.6 Hz, H-2), 5.97 (d, 1H, J = 2.0 Hz, H-6), 6.00 (d, 1H, J = 2.0 Hz, H-8), 6.85–7.34 (m, 5H, Ar-H), 11.69 (s, 1H, 5-OH).	512
7c	$C_{25}H_{22}O_{10}$	54	3417, 2926, 1640, 1514, 1454, 1231, 1163, 1119, 834	3.48 (m, 1H, H-23a), 3.79 (m, 1H, H-23b), 3.86 (s, 3H, OCH_3), 4.04 (m, 1H, H-10), 4.67 (d, 1H, J = 11.6 Hz, 1H, H-3), 4.99 (d, 1H, J = 8.0 Hz, 1H, H-11), 5.04 (d, 1H, J = 11.6 Hz, H-2), 5.95 (s, 1H, H-6), 5.97 (s, 1H, H-8), 6.73–7.33 (m, 6H, Ar-H), 11.64 (s, 1H, 5-OH).	482
7d	$C_{26}H_{24}O_{11}$	35	3436, 2926, 1635, 1513, 1456, 1275, 1158, 1120, 823	3.54 (m, 1H, H-23a), 3.77 (m, 1H, H-23b), 3.87 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.15 (m, 1H, H-10), 4.76 (d, J = 11.6 Hz, 1H, H-3), 4.99 (d, 1H, J = 8.0 Hz, H-11), 5.13 (d, 1H, J = 11.6 Hz, H-2), 6.18 (s, 1H, H-6), 6.19 (s, 1H, H-8), 6.78–7.24 (m, 5H, Ar-H), 11.69 (s, 1H, 5-OH).	512
7e	$C_{27}H_{26}O_{12}$	45	3426, 2942, 1641, 1514, 1465, 1222, 1162, 1119, 837	3.52 (m, 1H, H-23a), 3.77 (m, 1H, H-23b), 3.81 (s, 3H, OCH_3), 3.85 (s, 6H, OCH_3), 4.07 (m, 1H, H-10), 4.65 (d, 1H, J = 11.6 Hz, H-3), 4.95 (d, 1H, J = 8.0 Hz, H-11), 5.04 (d, 1H, J = 11.6 Hz, H-2), 5.93 (s, 1H, H-6), 5.96 (s, 1H, H-8), 6.76–6.83 (m, 4H, Ar-H), 11.67 (s, 1H, 5-OH).	542
7f	$C_{26}H_{24}O_{10}$	38	3413, 2928, 1639, 1514, 1485, 1246, 1166, 1092, 837	1.32 (t, J = 7.2 Hz, 3H, CH_3), 3.47 (m, 1H, H-23a), 3.80 (m, 1H, H-23b), 4.12 (q, J = 7.2 Hz, 2H, CH_2), 4.17 (m, 1H, H-10), 4.64 (d, J = 11.6 Hz, 1H, H-3), 5.00 (d, 1H, J = 8.0 Hz, H-11), 5.03 (d, J = 11.6 Hz, 1H, H-2), 5.94 (s, 1H, H-6), 5.96 (s, 1H, H-8), 6.71–7.33 (m, 6H, Ar-H), 11.69 (s, 1H, 5-OH).	496
7g	$C_{27}H_{26}O_{11}$	32	3423, 2927, 1640, 1513, 1453, 1271, 1160, 1120, 825	1.38 (t, J = 7.2 Hz, 3H, CH_3), 3.52 (m, 1H, H-23a), 3.77 (m, 1H, H-23b), 3.85 (s, 3H, OCH_3), 4.11 (q, J = 7.2 Hz, 2H, CH_2), 4.12 (m, 1H, H-10), 4.65 (d, 1H, J = 11.6 Hz, H-3), 4.97 (d, 1H, J = 8.0 Hz, H-11), 5.03 (d, 1H, J = 11.6 Hz, H-2), 5.93 (s, 1H, H-6), 5.96 (s, 1H, H-8), 6.77–7.10 (m, 5H, Ar-H), 11.68 (s, 1H, 5-OH).	526
7h	$C_{28}H_{28}O_{12}$	32	3382, 2940, 1603, 1499, 1463, 1300, 1153, 1048, 856	1.39 (t, 3H, J = 7.2 Hz, CH_3), 3.55 (m, 1H, H-23a), 3.78 (m, 1H, H-23b), 3.80 (s, 6H, OCH_3), 4.12 (q, 2H, J = 7.2 Hz, CH_2), 4.12 (m, 1H, H-10), 4.68 (d, 1H, J = 11.6 Hz, H-3), 5.00 (d, 1H, J = 8.0 Hz, H-11), 5.06 (d, 1H, J = 11.6 Hz, H-2), 5.96 (s, 1H, H-6), 5.99 (s, 1H, H-8), 6.74–6.87 (m, 4H, Ar-H), 11.68 (s, 1H, 5-OH).	556

Table II. The scavenging effects of synthetic compounds **7a**–**7h** on superoxide anion and DPPH radicals.

Compound	Concentration (μg/mL)	Superoxide anion inhibition (%)	DPPH inhibition (%)
7a	40	15.5	11.3
7b	40	31.9	45
7c	40	25.1	14.5
7d	40	69.7[a]	13.9
7e	40	44.4	37.5
7f	40	27.6	17.8
7g	40	28.8	18.7
7h	40	44.3	41.2
Quercetin	40	64.4[b]	99.5[c]

[a] $IC_{50} = 2.65 \times 10^{-5}$ M. [b] $IC_{50} = 3.81 \times 10^{-5}$ M. [c] $IC_{50} = 3.20 \times 10^{-6}$ M.

results are summarized in Table II. As shown in Table II, the order of the antioxidant effect is **7d** > **7e** ≈ **7h** > **7b** > **7g** ≈ **7f** > **7c** > **7a**. It is observed that compound **7d** possessed the best superoxide anion radical scavenging activity in this synthetic series with an IC_{50} value of 26.5 μM. The reference compound, quercetin exhibited a weaker effect than that of **7d** with IC_{50} value of 38.1 μM.

The effects of alkoxy groups substituted at the C-16 position of silybin were studied. It can be seen that besides **7d**, compound **7e** and **7h** also exhibited moderate scavenging activities on superoxide anion radicals with IC_{50} values of 44.4 and 44.3 μM, respectively. On the contrary, compounds **7a** and **7b** with $R_1 = H$ showed relatively weak scavenging activities. This evidence suggests that introducing an alkoxy group at the C-16 position of silybin benefits the superoxide anion radical scavenging activity, probably due to the electron-donating properties of these groups and the subsequent modulation of the electron density. The methoxy-substituted compounds exhibited better scavenging activity than the ethoxy-substituted analogues, indicating that chain length might also play an important role.

Comparing the radical scavenging activities of **7b**, **7d** and **7h** with a methoxy substituent on the E ring with those of **7a**, **7c** and **7f**, it can be deduced that one or two methoxy groups attached on the E ring improves the antioxidant activity. This result is in agreement with previous findings that the alkoxy moiety substituted on the phenol ring can increase the efficacy of phenolic antioxidants [16]. The enhanced activities could be attributed to the electron-donating capacity of the methoxy groups.

In addition, the DPPH radical scavenging activity of the synthesized compounds was also evaluated and the results are incorporated into Table II. It was found that compounds **7b**, **7h** and **7e** have a similar inhibitory activity which is higher than for other synthesized silybin analogues. It was indicated that the possession of two methoxy groups on the E ring was superior in activity to those only having one or none methoxy functionalities. Contrary to the activity of superoxide anion radical scavenging, the analogue with a H-atom on the B ring exhibited higher scavenging activity on DPPH among the three compounds **7b**, **7h** and **7e**. To some extent, the activity for scavenging DPPH free radicals was related to the concentrations of test compounds so that in general, absorbance values decreased when the concentrations of test sample decreased. The inhibitory effects on DPPH radicals were less than 10% at concentrations of 4 and 0.4 μg/ml for most of the tested samples.

Moreover, it should be noticed that although compound **7d** showed an excellent inhibitory effect on superoxide anion radical, it exhibited rather weak DPPH radical scavenging activity which suggested that there existed selectivity among the synthesized compounds towards different free radicals due to the different mechanisms involved.

In conclusion, the preliminary bio-evaluation results showed that introducing methoxy groups on the B and E rings of silybin may elevate the ability of the compounds to scavenge superoxide anion radicals, while introducing methoxy groups on the E ring improves the capacity of capturing DPPH radicals. This study afforded some useful information for further development of silybin analogues as novel antioxidants.

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