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Silybin is metabolized by cytochrome P450 2C8

in vitro

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Running title: Metabolism of silybin

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Abbreviations:

CYP, cytochrome P450; IC_{50}, concentration of compound required for 50% inhibition; K_m, Michaelis-Menten constant; V_{max}, maximum reaction velocity; HPLC, high performance liquid chromatography; µLC/MS, micro liquid chromatography coupled with mass spectrometry.

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ABSTRACT

Silybin (a flavonolignan, the main component of silymarin, extract from seeds of *Silybum marianum*) has been used to date mostly as hepatoprotectant showing, however, also other interesting activities, e.g. anticancer or hypocholesterolemic effects. It is also known that silybin can inhibit activities of the CYP enzymes. In this study, a weak interaction of silybin with human microsomal CYPs 2E1, 2A6, 2B6, 2C19 and 2D6 (IC_{50} ≥ 250 μM) was found; a moderate inhibition was observed for CYPs 1A2 and 2C8. The most prominent inhibition effect was found with CYP3A4 and CYP2C9 (IC_{50} ≤ 50 μM). Using the MS detection, production of O-demethylated (the main metabolite) as well as of the hydroxylated derivatives of silybin formed by CYP enzymes was detected. The effect of different CYP inhibitors on the formation of O-demethylated product was also studied. In particular, a relatively specific inhibitor of the CYP2C8 (quercetin) markedly inhibited the formation of this metabolite. With the help of recombinant enzymes (bactosomes) it was confirmed that it is the CYP2C8 enzyme which is responsible for the reaction leading to the O-demethylated silybin.

Introduction

Silybin (also silibinin, CAS 22888-70-6) is a flavonolignan and an active component of silymarin, extract from *Silybum marianum* (milk thistle) seeds (Gazak et al., 2007). Its hepatoprotective effects have been known for hundreds of years; novel studies indicate that the molecular basis of this effect is its antioxidative and radical scavenging property (Flora et al., 1998; Gazak et al., 2007). Recent discoveries of its other activities (chemopreventive, anticancer, neuroprotective effects) are responsible for an increasing number of papers in peer-reviewed journals devoted to this natural compound. Interference of silybin with cell cycle regulating pathways are expected to be the mechanism underlying the majority of the effects described (Singh and Agarwal, 2006).

Although silybin (Fig. 1) is generally considered to be safe with only few adverse effects (involving mostly gastrointestinal discomfort), a possibility of drug interactions based on metabolism mediated by cytochromes P450 (CYP) was recently investigated. Nifedipine oxidation, one of the major CYP3A4 activities, was found to be inhibited by silybine in the micromolar range (Beckman-Knopp et al., 2000; Zuber et al., 2002; Sridar et al., 2004). Also, CYP2D6 and CYP2C9 as well as glucuronidation activities were found to be inhibited. These facts have led to studies on the potential influence of silybin on pharmacokinetics of a typical CYP3A4 substrate indinavir (Di Cenzo et al., 2003; Mills et al., 2005). The differences in the AUC of indinavir due to concomitant application of silymarin (i.e. of the silybin-containing extract) were found not significant. The possibility of direct interaction of silybin with CYP enzymes and its conversion to metabolites by human liver was

further studied; presence of O-demethylated and hydroxylated derivatives of silybin was detected by HPLC-electrospray ionization-ion trap mass spectrometry (Gunaratna and Zhang, 2003). However, no systematic study on the metabolism of silybin by individual CYP enzymes with an identification of a particular CYP form responsible for the formation of metabolite(s) is available.

This work is aimed at a detailed investigation of the role of individual human liver microsomal CYP enzymes in the metabolism of silybin (i) using inhibition of prototypical microsomal CYP activities by silybin, (ii) by inhibition of silybin metabolism by known substrates or inhibitors of specific CYP forms and finally (iii) by confirmation of the involvement of CYP2C8 in the formation of O-demethylated product, the main metabolite of silybin.

MATERIALS AND METHODS

Chemicals

Silybin was a gift from Ivax-CR a.s. (Opava, Czech Republic). Chlorzoxazone, 6-hydroxychlorzoxazone, diclofenac, 4-hydroxydiclofenac, bufuralol, 6-hydroxybufuralol and 6β-hydroxytestosterone were supplied by Ultrafine Chemicals (Salford, UK). P450-Glo® substrate for determination of CYP2C8 activities by luminescence spectrometry was the product of Promega (Madison, WI, USA) obtained through East Port (Prague, Czech Republic). 7-Ethoxy-4-(trifluoromethyl)coumarin was supplied by Fluka (Buchs, Switzerland). All other chemicals were purchased from Sigma Aldrich CZ (Prague, Czech Republic).

Microsomes and recombinant enzymes

Pooled human liver microsomes were purchased from Advancell (Barcelona, Spain). Microsomes were obtained in accordance with ethical regulations of the country of origin (Spain). They were from ten donors (five males and five females) with a protein content of 38.4 $mg.ml^{-1}$; the CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1 and CYP3A4 enzymes activities were accessible at the Advancell website (http:/www.advancell.net, batch reference number 102091201). Bactosomes (bacterial membrane fractions from *Escherichia coli*), containing recombinant human cytochrome P450s enzymes (CYP1A2, 2C8, 2C9, 3A4, 2D6, 2B6 and 2E1) co-expressed with human NADPH-cytochrome P450 reductase, were purchased from Cypex (Dundee, UK).

Methods

CYP activities

Individual CYP activities were measured according to established protocols. The following microsomal CYP activities were tested: CYP3A4, testosterone 6β-hydroxylation (Guengerich et al.,1986); CYP2C9, diclofenac 4´-hydroxylation (Crespi et al., 1998); CYP2E1, chlorzoxazone 6-hydroxylation (Lucas et al., 1996); CYP1A2, 7-ethoxyresorufin *O*-deethylation (Chang and Waxman 1998); CYP2D6, bufuralol 1´-hydroxylation (Crespi et al., 1998a); CYP2A6, coumarin 7-hydroxylation (Waxman and Chang, 1998); CYP2B6, 7-ethoxy-4-(trifluoromethyl)coumarin *O* –deethylation (Donato et al., 2004); CYP2C19, S-mephenytoin 4´-hydroxylation (http://www.cypex.co.uk/intro.htm, Cypex 2C19 QC assays); CYP2C8, luciferin-ME demethylation (Promega Technical Bulletin No.325, http://www.promega.com).

For determination of metabolites formed from specific substrates, HPLC system Shimadzu Class VP (Kyoto, Japan) with UV (6β-hydroxytestosterone, 6-hydroxychlorzoxazone, 4´-hydroxydiclofenac, 4´-hydroxymephenytoin) or with fluorescence detection (1´-hydroxybufuralol) was used. A TECAN GENios absorbance/fluorescence/luminescence reader (Tecan, Vienna, Austria) was used for detection of other metabolites (7-ethoxyresorufin, 7-hydroxycoumarin, 7-ethoxy-4-(trifluoromethyl)coumarin, luciferin).

Inhibition of CYP enzymes by silybin in microsomal fraction

First, for each enzyme assay the K_m and V_{max} values were determined to get the substrate concentration suitable for inhibition experiments. The K_m values corresponded well to known literature data (e.g. Walsky and Obach, 2004). Substrate concentrations were used near the K_m (Table 1). Inhibition experiments were performed with six levels of silybin concentration 10, 50, 100, 150, 200, 400 μM; the stock solution was 25 mM in 60% (v/v) DMSO (dimethyl sulfoxide), except for measurement of activities of CYP2E1 and 2C19 (here the stock solution contained 17.3 mM silybin in acetonitrile). Experimental conditions were the same as for determination of individual CYP activities; preincubation of reaction mixtures with inhibitor (silybin) for 30 min was kept in all determinations. Inhibition of individual CYP activities was in all cases evaluated by plotting respective remaining activity against the inhibitor concentration. When an inhibition of a particular CYP activity was pronounced, the K_i values were determined as averages from Dixon plots with three substrate concentrations used (corresponding to 0.5 K_m, K_m and 2 K_m). Parameters of the enzyme kinetics (K_m, V_{max}) as well the IC_{50} and intercept values (for K_i

determination) were obtained using the Sigma Plot 8.0.2 scientific graphing software (SPSS, Chicago, IL, USA).

Inhibition of metabolite formation by carbon monoxide

The incubation was performed in 0.05 M K-phosphate buffer (pH 7.4) containing NADPH generating system (0.5 mM NADP, 3.7 mM citric acid, 0.5 unit/ml of isocitric acid dehydrogenase), 5 mM $MgCl_2$ and 250 pmol of microsomal CYP, in total volume of 1 ml. After 10 minutes of preincubation at 37°C, carbon monoxide (Linde Technoplyn, Prague, CZ) was applied by gentle bubbling through the reaction mixture for 1 min. Then silybin was added as 20 µl of 5 mM stock solution in 60% (v/v) DMSO giving the final concentration of 100 µM and the reaction mixture was incubated for 30 min in a shaking water bath. The reaction was stopped by the addition of 2 ml ethyl acetate. The next procedure was performed in accordance with Gunaratna and Zhang (2003). After vigorous stirring and centrifugation (5 000g 10 min) 1 ml of supernatant was evaporated under a stream of nitrogen and samples were dissolved in 100 µl of mobile phase (27/5/68 acetonitrile, methanol, 0.1% formic acid) and transferred into vials of autosampler. Formation of metabolites was followed by high performance liquid chromatography according to the original work (Gunaratna and Zhang, 2003).

Inhibition of microsomal silybin metabolism by specific inhibitors of CYP forms

To examine the effects of different CYP inhibitors on the metabolism of silybin in human microsomes, inhibition of individual CYP activities was studied. Furafylline (22.5 µM) was used as a specific inhibitor of CYP1A2; sulfaphenazole (3 µM) to

inhibit the CYP2C9; triacetyloleandomycin (3 µM) to influence the CYP3A4; inhibitor diethyldithiocarbamate (75 µM) was used for CYP2E1, quinidine (0.3 µM) for CYP2D6, 8-methoxypsoralen (1 µM) for CYP2A6, 7-pentoxyresorufin (2 µM) for CYP2B6; S-mephenytoin (160 µM) was used to inhibit the CYP2C19 activity and an inhibitor of CYP2C8 quercetin (100 µM) was also added to the reaction mixture (Baldwin et al., 1995; Newton et al., 1995; Dierks et al., 2001; Goldstein et al., 1994.). Every inhibitor used was preincubated with the reaction mixture (see the preceding paragraph) containing human liver microsomes for 30 min, and then the silybin (50 µM) was added.

Identification of CYP enzyme involved in metabolite formation in bactosomes

The metabolism of silybin by CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP3A4, CYP2D6, CYP2E1 was screened using bactosomes containing individual CYP enzymes.

The experiments were done according to the supplier's recommendation (Cypex, Dundee, UK). The incubation was carried out in 0.1 M TRIS-HCl buffer (pH 7.4) containing 5 pmol CYP-enzyme (together with CYP-reductase), 5 mM $MgCl_2$, NADPH generating system (0.5 mM NADP, 3.7 mM citric acid, 0.5 unit/ml of isocitric acid dehydrogenase), and 25 or 50 µM silybin. The reaction was terminated by the addition of 1 ml of ethyl acetate. Samples were centrifuged at 5000g for 10 min to take out the precipitated protein. The supernatant was evaporated under a stream of nitrogen.

Identification of silybin metabolites by µLC/MS

Residue obtained after incubation with microsomes or with bactosomes was dissolved in acetonitrile (50 µl) and further diluted by 200 µl of mobile phase A (see bellow). µLC/MS analyses were carried out using Waters CapLC XE System (Milford, USA), C18 column Gemini (150 mm x 300 µm I.D.; Phenomenex, USA) at a flow rate of mobile phase 6 µl/min. The elution was realized at isocratic condition with 65% of mobile phase A (5.7 mmol/l acetic acid + 5% acetonitrile) and 35% of mobile phase B (acetonitrile), 1 µl of sample solution was injected using an autosampler. Accurate mass measurement and MS/MS experiments were performed to confirm the identity of metabolites on Q-TOF mass spectrometer (Waters Micromass Q-Tof Premier Mass Spectrometer, Milford, USA). Optimized parameters of electrospray were: capillary voltage -2.8 kV (negative mode), sampling cone 45 V, source temperature 80°C, desolvation temperature 180°C, cone gas flow 50 l/hr and desolvation gas flow 400 l/hr. A collision energy ramp in range 5-30 eV was used for fragmentation experiments. Data were obtained in a single V mode.

RESULTS

Inhibition of specific activities in human liver microsomes by silybin

The results of *in vitro* inhibition of nine CYP enzymes by silybin in microsomal fraction are given in Figure 2. Silybin displayed a weak or no interaction with CYPs 2E1, 2A6, 2B6, 2C19 and 2D6 (IC_{50} ≥ 250 µM), a moderate inhibition was observed for CYPs 1A2 and 2C8. The most prominent inhibition effect was found with CYP3A4 and CYP2C9 (IC_{50} 49.8 µM and 34.1 µM, see Table 1).

Identification of metabolites by LC/MS analysis

Incubation of silybin with human liver microsomes confirmed the formation of metabolites of silybin (Gunaratna and Zhang, 2003). The major metabolite was identified by μLC/MS analysis as O-demethylated silybin; the minor ones were silybin mono- and di-hydroxy derivatives. Figure 3 shows chromatograms for selected m/z values (silybin m/z=481; O-demethylated metabolite m/z=467; mono-hydroxy m/z=497 and di-hydroxy m/z=513) and fragmentation spectra of quasimolecular ions of the mentioned metabolites. It is evident that modification of a molecule of silybin occurs in two different positions in the case of mono-hydroxy metabolite (two peaks for m/z=497 with retention time 4.49 min and 5.31 min, respectively). It has to be mentioned that chromatographic peaks in retention time of silybin (6.7-6.8 min) correspond to products of modification of silybin in the ion source. This fact was verified by an analysis of standard (unmodified) silybin and it underlines the necessity of chromatographic separation. Identity of metabolites was verified by interpretation of MS/MS spectra and by accurate mass measurements. Experimental masses of metabolites were in good agreement with the calculated ones. Their relative errors were 0.6 ppm or less.

Effect of carbon monoxide and of the specific CYP inhibitors on the formation of metabolites of sylibin

To prove the role of cytochromes P450 in the metabolism of silybin, inhibition of this reaction by carbon monoxide was examined. Results indicated a considerable reduction (by 80%) of the main metabolite level in the reaction mixture suggesting the role of CYP-mediated mechanism of metabolite formation (Fig. 4).

To find which CYP form is responsible for the formation of silybin metabolite(s), the effect of different inhibitors of particular CYP activities was studied. An overview of inhibitors used together with their effect on the formation of O-demethylated silybin is shown in Fig.5. Among the inhibitors used, only quercetin (relatively specific inhibitor of CYP2C8; Walsky et al., 2005), appeared to be the most potent inhibitor giving 80% inhibition of O-demethylated silybin formation.

Incubation of silybin with recombinant enzymes (bactosomes)

Although the inhibition experiments described in the preceding paragraph indicated a possible involvement of several CYP forms in an interaction with silybin and, hence, a possibility of their ability to form silybin metabolite(s), experiments using bacterial membrane fractions from *Escherichia coli* containing recombinant human cytochrome P450 enzymes (CYP1A2, 2C8, 2C9, 3A4, 2D6, 2B6 and 2E1) co-expressed with human NADPH-cytochrome P450 reductase (bactosomes) confirmed (Fig. 6) a significant role only of CYP2C8 (and a minor contribution of CYP3A4, results not shown) in the formation of the main metabolite of silybin, i.e. of its O-demethylated derivative by HPLC/MS.

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DISCUSSION

The results of the inhibition of prototypic activities of individual microsomal CYP enzymes by silybin revealed that there are at least four CYP activities

influenced by the presence of this compound, namely, the CYP1A2, 2C8, 2C9 and 3A4 ones (Fig. 2). In the earlier literature, data showing a significant inhibition of CYP3A4 and CYP2C9 were published (Beckman-Knopp et al., 2000; Zuber et al., 2002; Sridar et al., 2004). The character of inhibition was also studied using standard Dixon plots (results not shown) indicating the presence of a noncompetitive inhibition of the CYP2C9 activity and of a competitive mechanism of inhibition of the CYP3A4 one. In cases when the inhibition was more pronounced, IC_{50} and K_i were also determined (Table 1). The results obtained here with the CYP3A4 and CYP2C9 are in agreement with the previous ones; on the other hand, they do not confirm the results of a certain degree of inhibition obtained also with the CYP2E1 and CYP2D6 (Beckman-Knopp et al., 2000; Zuber et al., 2002). Taken together, the results are hence indicative of an interaction between silybin and at least two microsomal CYP enzymes.

Silybin was shown recently to yield several metabolites when incubated with human liver microsomes (Gunaratna and Zhang, 2003). The main metabolite was found to be the O-demethylated product; the mono- and di-hydroxy silybins were the minor ones. In this work, the formation of these metabolites in human liver microsomes has been confirmed (Fig. 3); as the next step, we were interested in the identification of the particular CYP form involved in the formation of the main metabolite.

Carbon monoxide is known to bind strongly to the heme iron of all cytochromes P450 yielding a complex which is unable to bind molecular oxygen and perform the catalytic reaction (Cooper et al., 1977). The results (Fig. 4) have shown a clear inhibition of the formation of the O-demethylated silybin; also, the levels of

hydroxylated metabolites were diminished. The inhibitors used in the literature to examine the function of a particular CYP enzyme have been subsequently employed to find which form of CYP is responsible for the formation of the main silybin metabolite.

Among the inhibitors used, quercetin, an inhibitor of CYP2C8 (Walsky et al., 2005), has been found to inhibit the O-demethylation of silybin to the greatest extent. A minor inhibiting effect of sulfaphenazole and triacetyloleandomycin (inhibitors of CYP2C9 and CYP3A4) was in line with findings on the inhibition of specific CYP activities by silybin in microsomes (Fig. 2) as well as with earlier results of Beckman-Knopp et al. (2000) and of Sridar et al. (2004). The use of bactosomes, expressing a single CYP form, has confirmed conclusively that it is the CYP2C8 form which is catalyzing the O-demethylation of silybin. Bactosomal preparation with CYP3A4 has also been able to produce the demethylated metabolite, but to a much lesser extent. No formation of metabolites has been found with bactosomes expressing the CYP2C9 or other CYP enzymes. The inhibition of silybin metabolism by sulfaphenazole as well as the inhibition of CYP2C9 activity by silybin (Figs. 3 and 2) can apparently be explained by a strong structural similarity of the CYP2C8 and CYP2C9 forms (77.8%, P450 database at http://cpd.ibmh.msk.su) which is reflected also in a broad overlap of substrates and inhibitors of both forms; however, only the CYP2C8 is able to metabolize silybin.

The results obtained here both with the metabolism of silybin as well as with the inhibition of CYP activities does not seem to constitute a rational base for the clinical importance of silybin-drug interactions. The percent inhibition of the metabolic pathway may be expressed (in an ideal case of competitive inhibition, Segel (1975),

Boxenbaum (1999)) as %Inhibition = 100 $C_I/(C_I + K_i)$ where C_I is the inhibitor concentration and the K_i has the known meaning of the inhibition constant. As the silybin concentration in the systemic circulation does exceed 1 μM (Weyhenmeyer et al., 1992; van Erp et al., 2005), the degree of inhibition as estimated from the *in vitro* experiment should be expected in the order of several percentage points. Silybin hence should be taken as a relatively safe drug whose metabolism does not interfere significantly with major CYP-catalyzed routes of drug biotransformation.

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Footnotes

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Legends to Figures

Figure 1

Structure of silybin.

Figure 2

Effect of silybin on specific activities of cytochromes P450. Concentrations of silybin in reaction mixture were 0, 10, 50, 100, 150, 200, 400 µM. Experiments were performed in duplicates with results expressed as means; as a rule, the data obtained did not differ more than 5%.

Figure 3

Metabolites of silybin identified by µLC/MS analysis. The major metabolite was identified O-demethylated silybin; the minor ones were silybin mono- and di-hydroxy derivatives.

Figure 4

HPLC analysis of silybin metabolites generated by human hepatic microsomes before and after treatment with carbon monoxide. M1 – M3 are metabolites of silybin.

Figure 5

Effect of selective inhibitors of CYP activities on the rate of formation of demethylated silybin by human hepatic microsomes. Furafylline, sulfaphenazole, triacetyloleandomycin (TAO), diethyldithiocarbamate (DEDC), quinidine, 8-methoxypsoralen, 7-pentoxyresorufin, S-mephenytoin and quercetin were used to inhibit the respective CYP activities. Experiments were performed in duplicates with results expressed as means; as a rule, the data obtained did not differ more than 5%.

Figure 6

HPLC analysis of silybin metabolized by CYP2C8-bactosomal extract. M1 is the O-demethylated silybin. Blank sample was obtained with the reaction stopped by an immediate addition of ethyl acetate. The results obtained with the other CYP – containing bactosomes were similar to the blank.

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Table 1

Enzyme activities of microsomal CYP enzymes.

P450 - enzyme	Substrate	K_m (µM)	Substrate conc. (µM)	Silybin IC_{50} (µM)	Silybin K_i (µM)
CYP1A2	Ethoxyresorufin	2.4	2.5	220±31	165±28
CYP2A6	Coumarin	8.2	10	NA[a]	NA[a]
CYP2B6	7-ethoxy-4-(trifluoromethyl)coumarin	14.1	15	NA[a]	NA[a]
CYP2C8	Luciferin-ME	ND[b]	150	≥250	NA
CYP2C9	Diclofenac	15.9	16	34.1±11	75±12
CYP2C19	S-mephenytoin	81.0	80	NA[a]	NA[a]
CYP2D6	Bufuralol	25.3	25	≥250	NA[a]
CYP2E1	Chlorzoxazone	52.0	50	NA[a]	NA[a]
CYP3A4	Testosterone	77.5	100	49.8±14	21±6

Inhibition by silybin expressed as IC_{50} or K_i ± S.E.M.

[a]NA, not accessible; [b]ND, not determined

Figure 1

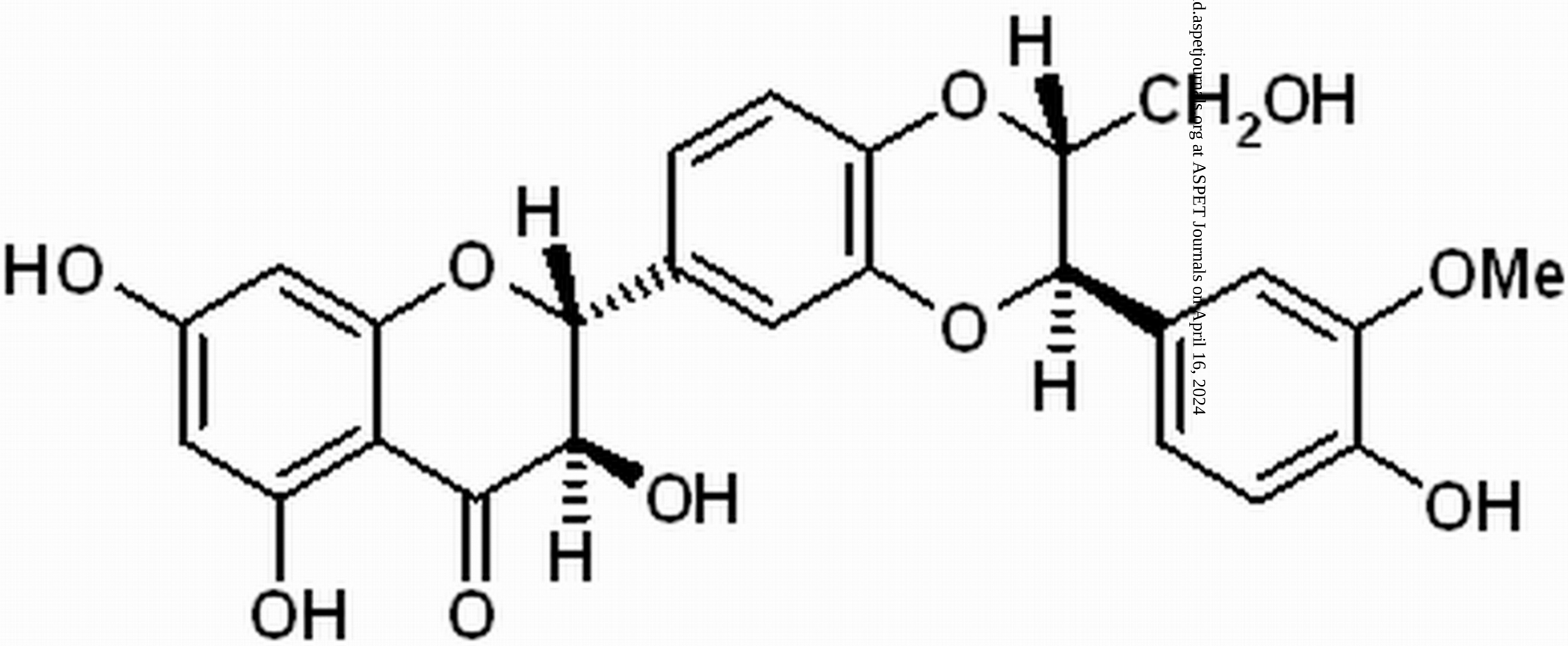


Figure 2

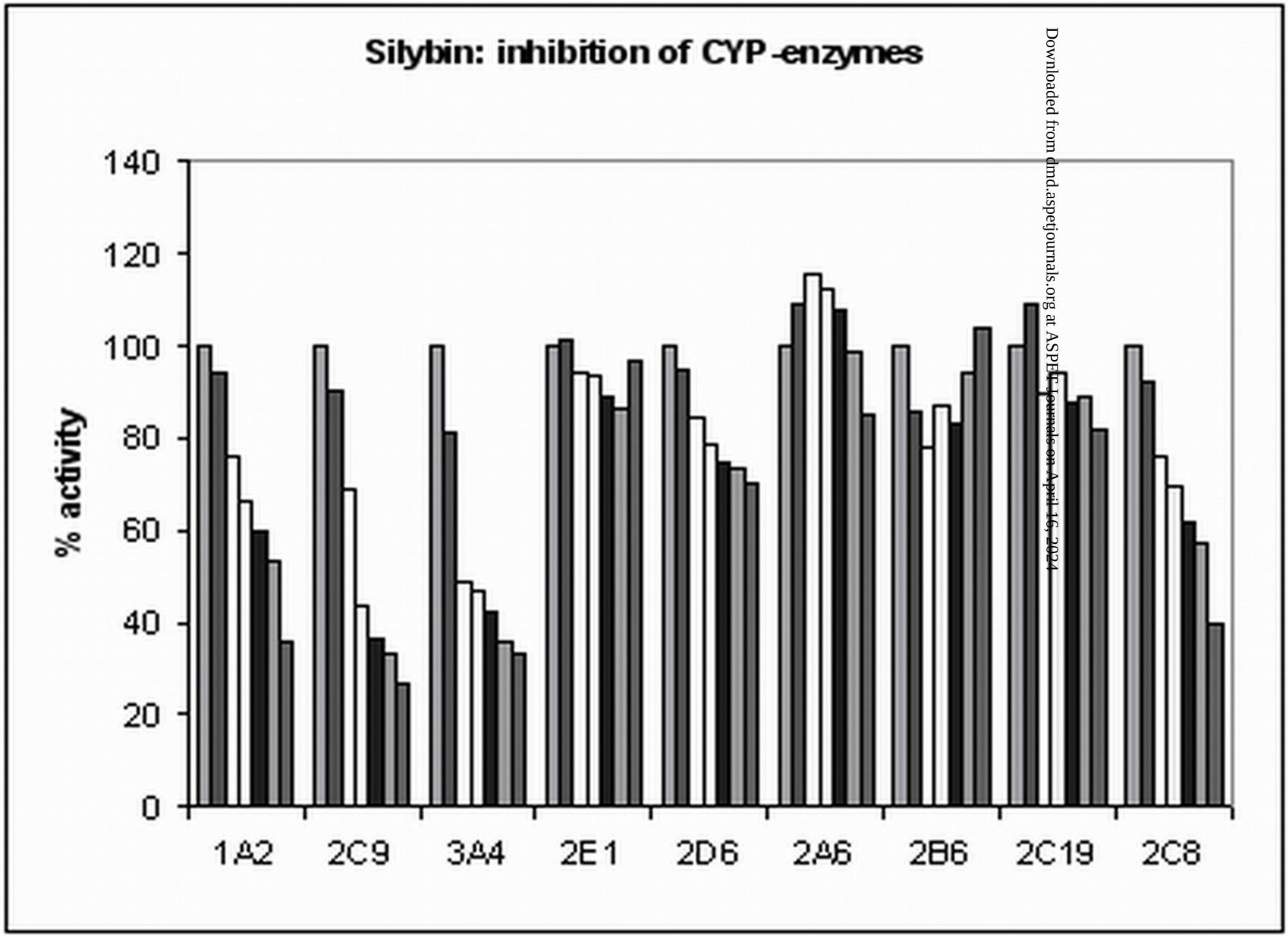
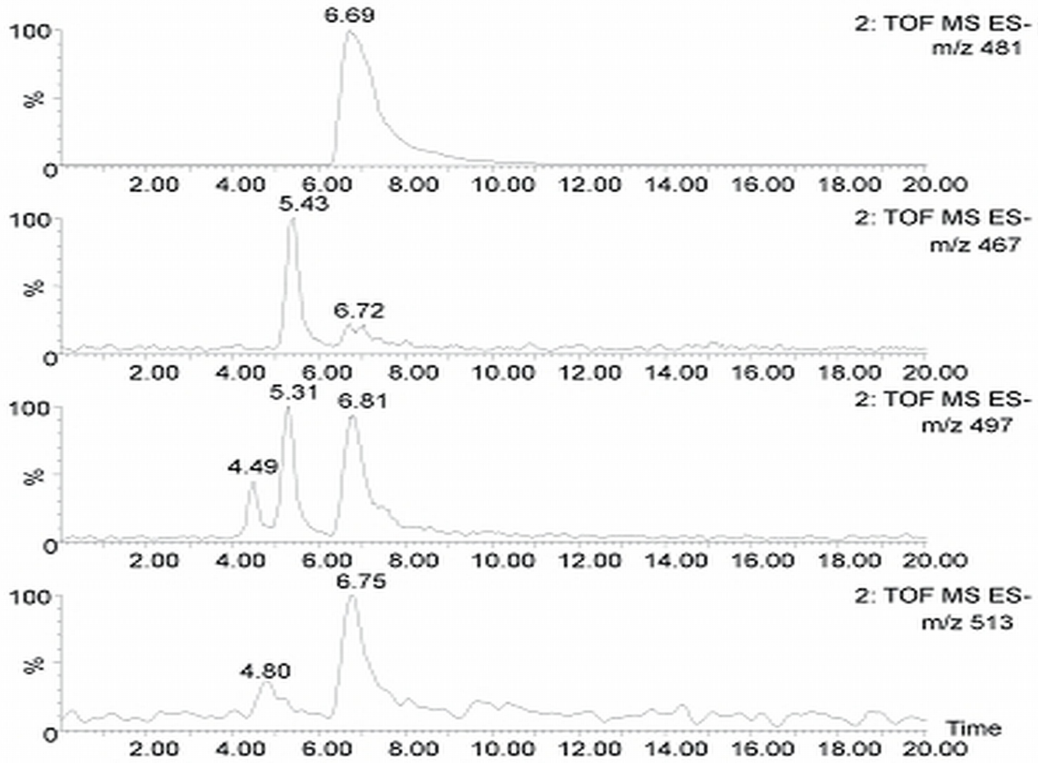
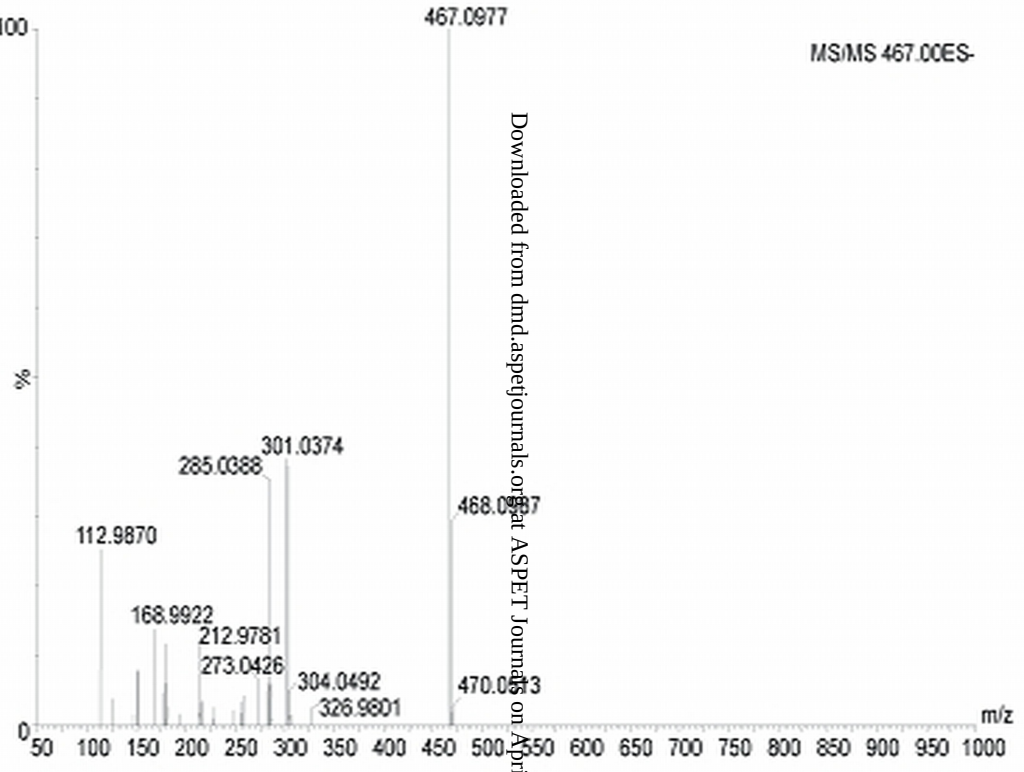
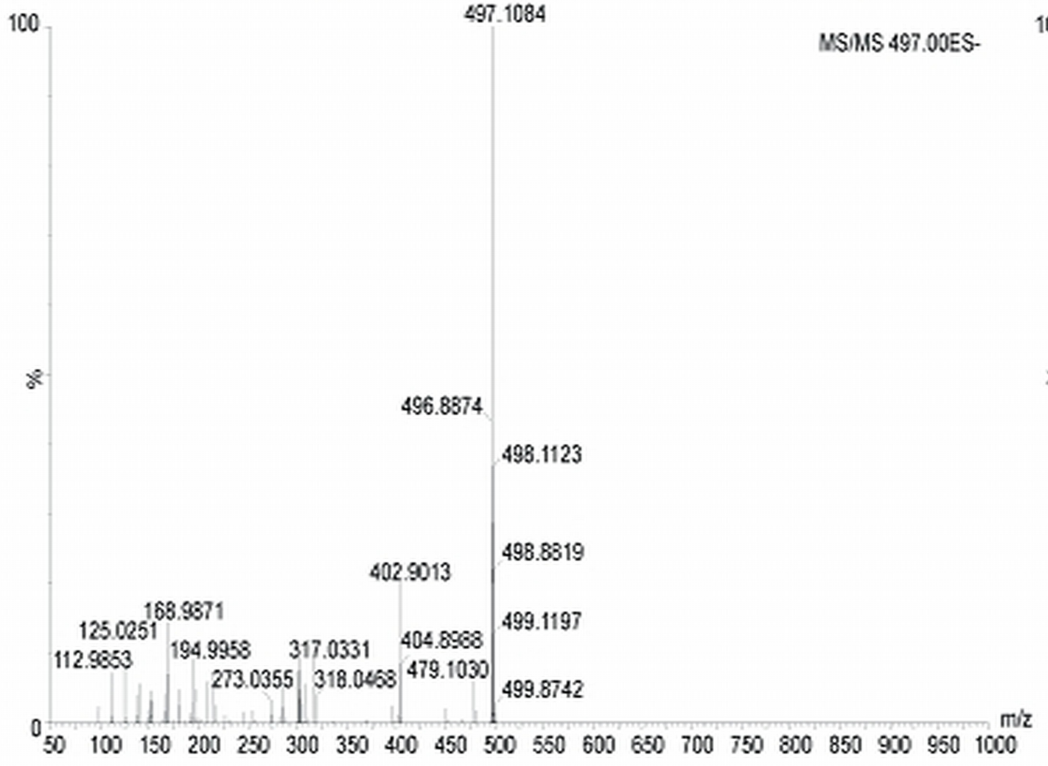


Figure 3







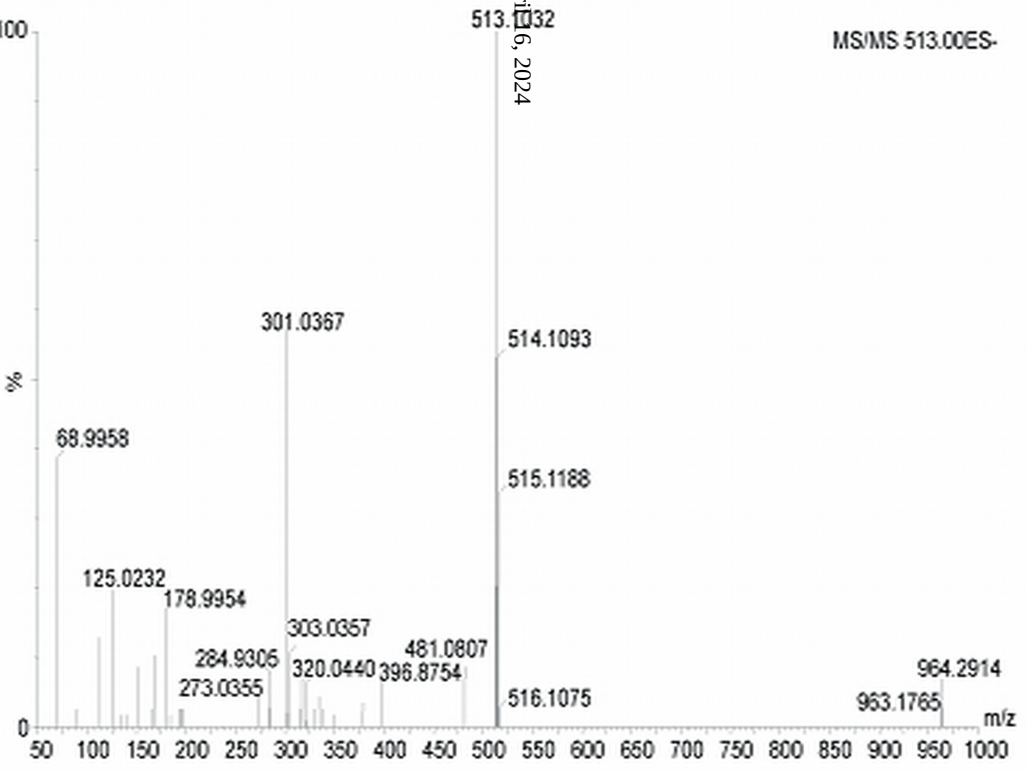


Figure 4

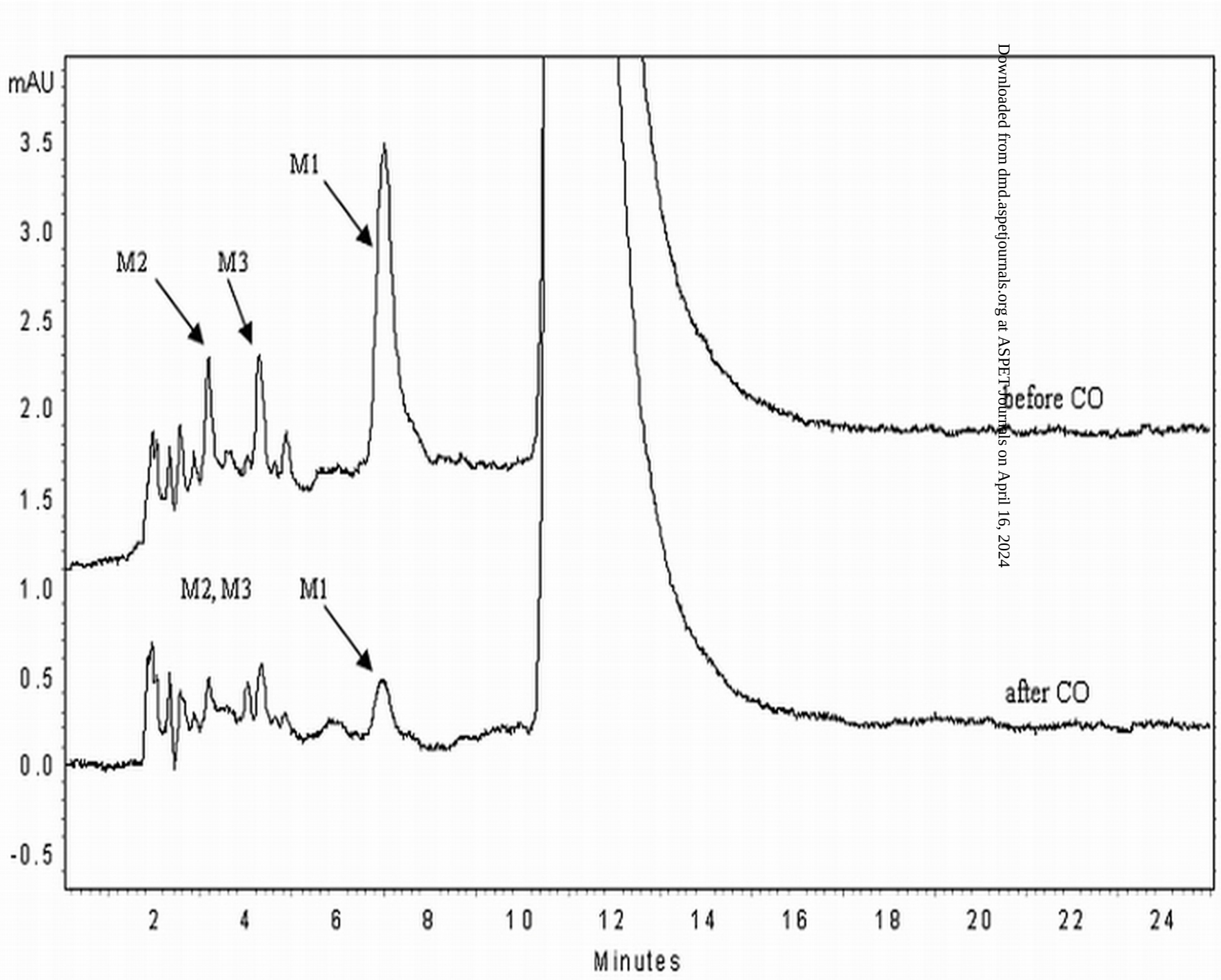


Figure 5

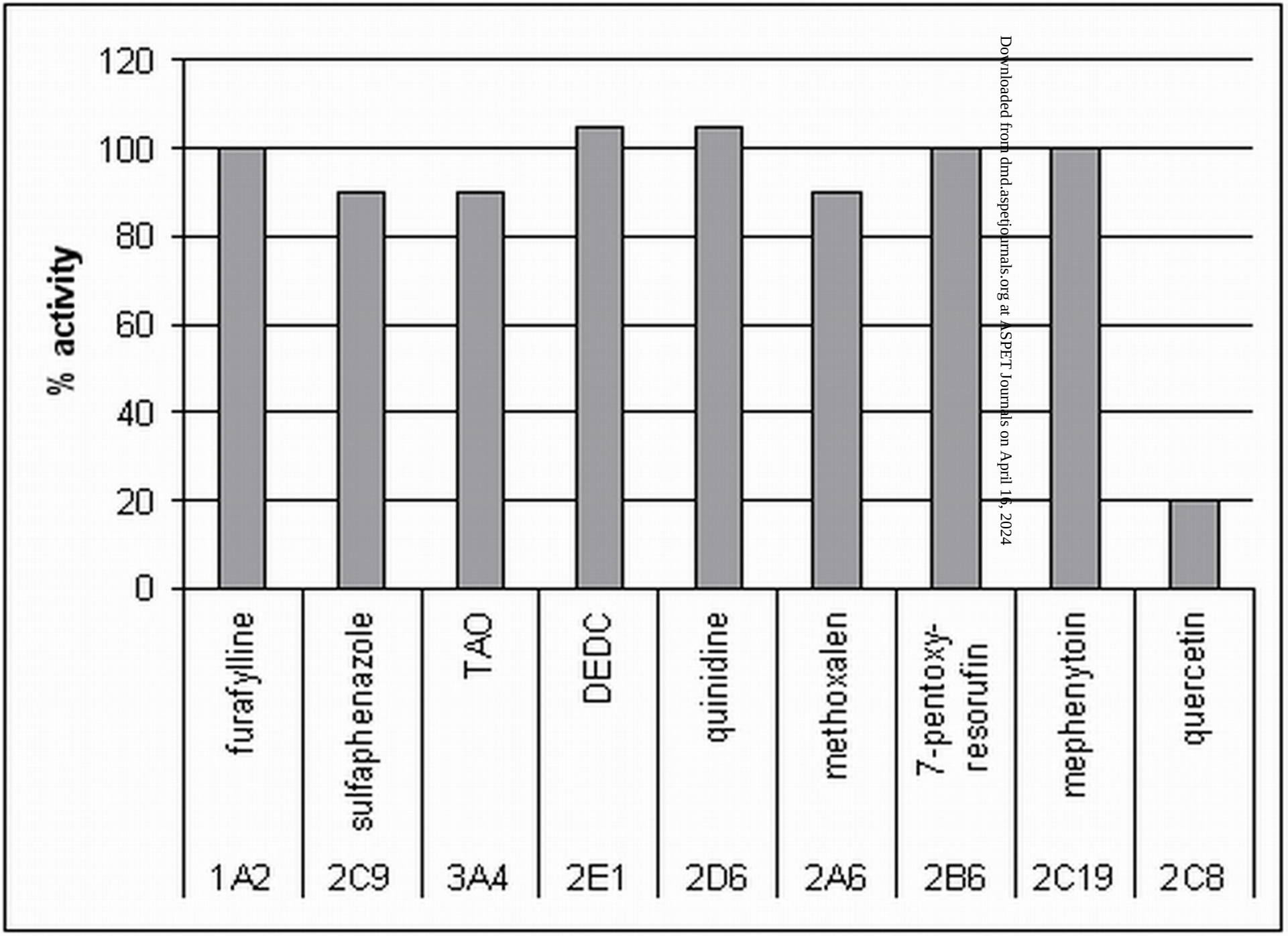


Figure 6