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Interaction of isolated silymarin flavonolignans with iron and copper

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ABSTRACT

Silymarin, the standardized extract from the milk thistle (*Silybum marianum*), is composed mostly of flavonolignans and is approved in the EU for the adjuvant therapy of alcoholic liver disease. It is also used for other purported effects in miscellaneous nutraceuticals. Due to polyhydroxylated structures and low systemic bioavailability, these flavonolignans are likely to interact with transition metals in the gastrointestinal tract. The aim of this study was to analyze the interactions of pure silymarin flavonolignans with copper and iron. Both competitive and non-competitive methods at various physiologically relevant pH levels ranging from 4.5 to 7.5 were tested. Only 2,3-dehydrosilybin was found to be a potent or moderately active iron and copper chelator. Silybin A, silybin B and silychristin A were less potent or inactive chelators. Both 2,3-dehydrosilybin enantiomers (A and B) were equally active iron and copper chelators, and the preferred stoichiometries were mainly 2:1 and 3:1 (2,3-dehydrosilybin:metal). Additional experiments showed that silychristin was the most potent iron and copper reductant. Comparison with their structural precursors taxifolin and quercetin is included as well. Based on these results, silymarin administration most probably affects the kinetics of copper and iron in the gastrointestinal tract, however, due to the different interactions of individual components of silymarin with these transition metals, the biological effects need to be evaluated in the future in a much more complex study.

1. Introduction

Silymarin is officially approved by the European Medicinal Agency for the supportive treatment of alcoholic liver disease. It is also used traditionally – in the form of nutraceuticals - for other liver disorders and various studies have shown its potentially positive effects both in humans and animals [1,2]. This extract from milk thistle (*Silybum marianum* (L.) Gaertn.) fruits (cypselae) is a complex mixture containing flavonolignans together with other compounds; its composition varies markedly depending on the cultivation conditions, method of extraction and other factors [3]. Major silymarin flavonolignans, formed by the coupling of a flavonoid (taxifolin) with a lignan (coniferyl alcohol) include silybin, isosilybin, silychristin and silydianin. With the exception of silydianin, all these compounds exist naturally as pairs of diastereomers in various proportions, referred to as *e.g.* silybin A and silybin B (Fig. 1). Silymarin also contains the flavonol taxifolin, a relatively undefined polymeric fraction constituting approximately 30%, and minor constituents, mostly the 2,3-dehydroflavonolignans (racemic) (Fig. 1) [3]. Most of the beneficial effects of silymarin have been attributed to its major component silybin (also called silibinin in pharmacopoeias, a diastereomeric mixture of silybin A and B ca 1:1, content roughly 30%), which is the most abundant and easiest to isolate [4].

Since the extract is frequently used both as an approved drug and a popular food supplement, the general population's exposure to it is becoming important. Due to the presence of the flavonoid core functionalized with hydroxy- and oxo-groups, interactions with transition metals and related anti/pro-oxidant activity are to be expected. This property might also lead to a positive clinical effect(s), since silybin decreased iron absorption in patients with hereditary hemochromatosis [5]. There exist additional data supporting the potential clinical use of silybin against iron toxicity [5–7]. However, limited information is available to date on the interaction of silymarin components with transition metals, in particular no data are available for optically pure

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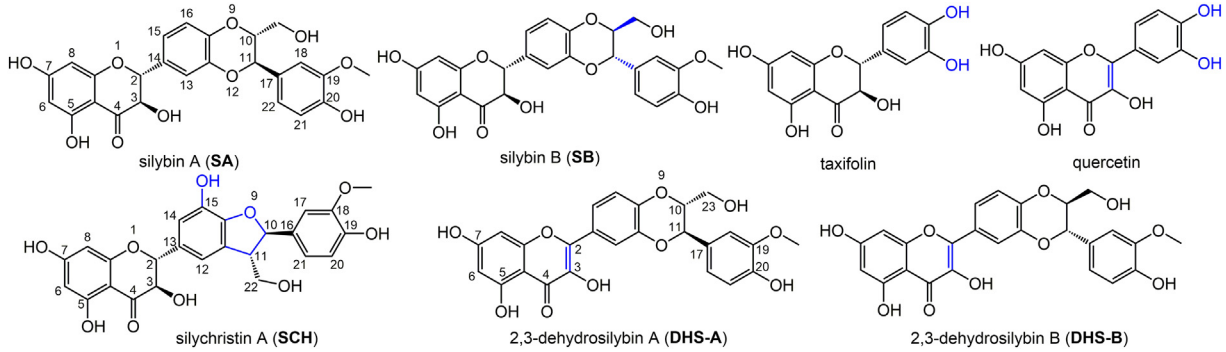

Fig. 1. Chemical structures of selected silymarin flavonols and flavonolignans. Major structural differences compared with silybin A are highlighted in blue color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

flavonolignans. There has only been a single focused study, which demonstrates the complexation of silybin (mixture of diastereomers A and B) with ferric ions under non-competitive conditions [8]. The *in vitro* complexation of silybin with Ni^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} was studied by *ex situ* voltammetry [9], but nothing is known about the possible metal chelation of other silymarin flavonolignans and especially in terms of their pure stereomers. In principle, the interaction of silymarin components with iron and copper can take place in every human cell. However, the systemic bioavailability of all flavonolignans from silymarin is below or around 1% [10,11]. Therefore, it is likely that the interaction with biological components mostly occurs in the gastrointestinal tract. Iron is absorbed mainly in the duodenum in the form of ferrous ions and reductants significantly influence it [12]. As for copper, the data are less conclusive, but again its reduction to cuprous ions before absorption is highly plausible [12]. Since both iron/copper reduction and chelation might influence the above-mentioned processes, the aim of the present study was to assess in detail the interaction of pure isolated components of silymarin with both transition metals, iron and copper at physiologically relevant pH levels *in vitro*. This study is a part of a more complex project devoted to the study of the potential influence of natural compounds on iron/copper absorption.

2. Material and methods

2.1. Chemicals and equipment

Silybin A (99.2%), silybin B (99.0%) and silychristin A (SCH, 96.3%) were isolated from silymarin (Liaoning Senrong Pharmaceutical, Panjin, China; batch No. 120501) as described previously [13–15]. 2,3-Dehydrosilybin (DHS, mixture of enantiomers, 99.9%), 2,3-dehydrosilybin A (DHS-A, 97.4%), and 2,3-dehydrosilybin B (DHS-B, 95.7%) were prepared by the oxidation of silybin, silybin A and silybin B, respectively [16].

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinedisulfonic acid sodium salt (ferrozine), $FeSO_4{\cdot}7\ H_2O$, $FeCl_3{\cdot}6\ H_2O$, ferric tartrate ($Fe_2(C_4H_4O_6)_3$), hydroxylamine hydrochloride (HA), $CuSO_4{\cdot}5H_2O$, CuCl, disodium bathocuproinedisulfonate (BCS), dimethylsulfoxide (DMSO), taxifolin, quercetin and chemicals for buffer preparations were purchased from Sigma-Aldrich Inc. (Germany) and methanol from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water (Milli-Q RG, Merck Millipore, Massachusetts, USA) was used throughout this study. Stock solutions of ferrozine, Cu/Fe salts and BCS (all 5 mM) and HA (100 mM) were prepared in ultrapure water with the exception of CuCl (5 mM), which was dissolved in an aqueous solution of 0.1 M HCl and 1 M NaCl. All experiments for the stoichiometry determination were performed in semi-micro polystyrene or ultraviolet-transparent cuvettes (BrandTech Scientific Inc., UK) and the absorbance was measured with the Helios Gamma spectrophotometer equipped with VisionLite 2.2 software (ThermoFisher Scientific Inc., USA), while competitive experiments were performed in 96-well microplates (BRAND GmbH + CO KG, Germany) with the spectrophotometer Synergy HT Multi-Detection Microplate Reader spectrophotometer (BioTec Instruments, Inc., USA).

2.2. pH conditions

Experiments were performed at four pH levels relevant to the gastrointestinal tract, where iron and copper absorption takes place (4.5. 5.5, 6.8 and 7.5). Acetate buffers (15 mM of sodium acetate and 27.3 or 2.7 mM of acetic acid, respectively) were used for the two lower pH values, whereas HEPES buffers (15 mM of sodium HEPES and 71.7 and 14.3 mM of HEPES, respectively) were used for pH 6.8 and 7.5. Based on our previous experiments, chloride was used for ferric ion solutions at pH 4.5 and 5.5, and tartrate at pH 6.8 and 7.5. As the oxidation of Fe^{2+} accelerates significantly at pH 7.5; HA at a final concentration of 5 mM was added to this buffer to avoid oxidation [17]. With Cu^{+} ions, HA was added at the same concentration to all buffers to keep the ion in its lower valence state [18].

2.3. Competitive methods

The general principle of these methods is based on the fact that indicators (BCS, hematoxylin or ferrozine) are also metal chelators and compete with the tested compounds for binding the metal. Such methods are therefore competitive and give an idea about the affinity of the tested compound for the metal and the stability of the complex formed. First, the flavonolignans were mixed with the respective metal ion to allow the complex formation and then an indicator was added to the mixture. In order to test their stability, the absorbance was measured immediately and after a certain period of time depending on the method. An unstable complex will lose its metal ion over time due to the formation of the complex indicator-metal and hence the absorbance will be significantly higher in the second measurement than immediately after mixing. The methods can also be used for the assessment of reduction, since both BSC and ferrozine react only with the ions in their lower valence state (Cu^{+}, Fe^{2+}). All experiments were performed in 96-well microplates at room temperature (25 °C) and at least five different concentrations were measured. A detailed methodology was described in our previous papers [17,18] and it is described briefly below.

2.3.1. Ferrozine method

Ferrozine forms a magenta-colored complex with Fe^{2+}; total iron (Fe^{2+} + Fe^{3+}) chelation at pH 4.5 can be measured after the reduction of ferric ions by HA. At other pH levels the reduction of ferric to ferrous ions by HA is not complete, but the degree of reduction relative to that caused by HA at pH 4.5 can be determined at all pH levels.

Various concentrations of flavonolignan solutions in DMSO (50 μL)