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Silybin, the main active component of *Silybum marianum*, affects blood coagulation: an in vitro pilot study †

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Abstract: The health-promoting properties of *Silybum marianum* have been acknowledged since antiquity. This plant is credited with substantial hepatoprotective properties and is also protective in cardiovascular diseases, diabetes mellitus, and neurodegeneration, mainly for its anti-inflammatory and antioxidant effects. Only a few experimental studies have described the impact of *Silybum marianum* extract on the blood coagulation process; furthermore, these data are unsatisfactorily fragmented and need to be supplemented to understand the plant's properties better. The predominant biologically active flavonolignan extracted from *Silybum marianum* is silybin, a mixture of two diastereomers, silybin A and silybin B, in approximately equimolar ratio. This study investigated the effect of silybin on the fundamental laboratory parameter for blood coagulation, namely prothrombin time (PT), an assay used to assess the extrinsic and common coagulation pathways. To evaluate the effect of silybin on PT, we prepared three solutions of silybin (Silybin (A + B mixture), PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) in 0.1% dimethylsulfoxide (DMSO, Sigma-Aldrich, Co., St. Louis, MO, USA): 10 μM, 50 μM, and 100 μM. PT was measured on a Coag 4D coagulometer (DIAGON Kft., Budapest, Hungary) using rabbit calcium thromboplastin (Dia-PT, DIAGON Kft., Budapest, Hungary) and control plasma which is pooled plasma obtained from healthy donors (Dia-CONT, DIAGON Kft., Budapest, Hungary). 10 μl of silybin solution was added to 40 μl of plasma, the sample was incubated for two minutes at 37 °C, and then 100 μl of thromboplastin, pre-warmed to 37 °C, was added to the mixture. The coagulometer automatically gives the PT result in seconds (sec). At the same time, PT was measured in the control plasma both without additional solutions and with the addition of tris-buffered saline (TBS) and 0.1% DMSO (10 μl of TBS or DMSO + 40 μl of plasma). Each measurement was made eight times. Student's t-test and the Friedman test with post-hoc analysis were used in the statistical analysis (Statistica 13, TIBCO Software Inc. Palo Alto, CA, USA). In the first step of our study, we tested how the dilution of the plasma sample affected PT. We did not observe statistically significant differences in PT between the control plasma and the control plasma supplemented with TBS (mean ± standard deviation 14.00±0.77 sec vs. 13.88±0.38 sec, p=0.606). We also found no statistically significant differences in PT between the control plasma and the control plasma with the addition of 0.1% DMSO (mean ± standard deviation 14.00±0.77 sec vs. 14.10±0.26 sec, p=0.728); therefore, we further analyzed the effect of silybin on PT using DMSO at this level (0.1%). The addition of silybin solutions to the control plasma resulted in a statistically significant PT shortener (p<0.001). Post-hoc analysis revealed a substantial shortening of PT under the influence of 50 μM (median 13.55 sec) and 100 μM solution (median 13.40 s) of silybin compared to plasma with the addition of 0.1% DMSO alone (median 14.10 sec) and plasma with the addition of the lowest, 10 μM, level of silybin (median 14.20 sec). At the same time, PT in the plasma with the addition of a 50 μM and 100 μM solution of silybin did not significantly differ statistically. Our in vitro analysis characterized the possible effect of *Silybum marianum* on the blood coagulation process. These results require further investigation to validate their validity and clinical utility.

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1. Introduction

The health-promoting properties of various plant species have been known for centuries. An example of such a plant is *Silybum marianum* (Figure 1), which has a prolonged therapeutic use history [1, 2]. The standardized extract of *Silybum marianum* seeds used in medicine is silymarin, whose main active constituent is silybin [3, 4]. Silybin, a mixture of two diastereomers, silybin A and silybin B, in approximately equimolar ratio, is an outstanding example of a natural remedy. It is predominantly used as a supportive element in liver disorders, cardiovascular diseases, diabetes mellitus, and neurodegeneration. This chemical compound exhibits several pharmacological properties, mainly hepatoprotective, anti-inflammatory, and antioxidant effects, modulating various cell-signaling pathways [3, 5].

Even though silybin has many biological properties, its effect on blood coagulation remains obscure. Only a few experimental studies have described that silybin inhibits platelet aggregation [6]. The study of the axis of silybin-blood coagulation is still in its infancy. For example, the effect of silybin on basic blood coagulation parameters is unknown and needs to be supplemented to understand the plant's properties better.

This study investigated the effect of silybin on the fundamental laboratory parameter for blood coagulation, namely prothrombin time (PT). PT is an elementary test to evaluate the extrinsic pathway and common pathway of coagulation. It's also used in day-to-day clinical routine, especially in monitoring anticoagulant therapy [7]. For these reasons, assessing the relationship between silybin and PT is reasonable.

Figure 1. *Silybum marianum* flowerhead (author: Agnieszka Mlicka).

2. Materials and Methods

2.1. Examination of the impact of silybin on prothrombin time (PT) of normal human plasma

Silybin (A+B mixture, product #: 89280, PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) was investigated *in vitro* for possible impact in prothrombin time (PT) assay. Three levels of silybin, 10 μM, 50 μM, and 100 μM were prepared using 0.1% dimethylsulfoxide (product #: D2650-5X5ML, DMSO, Sigma-Aldrich, Co., St. Louis, MO, USA). 10 μl of silybin was mixed with 40 μl of normal human plasma (product #: 91020, Dia-CONT I, DIAGON Kft., Budapest, Hungary) and incubated for 2 minutes at 37 °C, then 100 μl of pre-warmed to 37 °C rabbit calcium thromboplastin (product #: 81050, Dia-PT, DIAGON Kft., Budapest, Hungary) was added to the mixture and PT (in seconds, sec) was recorded. A Coag 4D semi-automated coagulation analyzer (DIAGON Kft., Budapest, Hungary) was used to perform PT. Each measurement was made eight times.

2.2. Examination of the impact of normal human plasma sample dilution on prothrombin time (PT)

To check the impact of dilution on PT, we measured it in normal human plasma with the addition of tris-buffered saline (TBS). 10 μl of TBS was mixed with 40 μl of plasma and incubated for 2 minutes at 37 °C. PT was recorded after adding to the mixture of 100 μl pre-warmed to 37 °C rabbit calcium thromboplastin. PT was also measured in normal human plasma samples without additional solutions. Each measurement was performed eight times by using a Coag 4D analyzer.

2.3. Examination of the impact of DMSO on prothrombin time (PT) of normal human plasma

Since the silybin solutions were prepared using 0.1% DMSO, we also tested the effect of this solvent on PT. 10 μl of 0.1% DMSO was mixed with 40 μl of plasma and incubated for 2 minutes at 37 °C. PT was recorded after adding to the mixture of 100 μl pre-warmed to 37 °C rabbit calcium thromboplastin. Each measurement was performed eight times by using a Coag 4D analyzer.

2.4. Statistical analysis

The difference in PT between normal human plasma and plasma samples supplemented with TBS and DMSO was assessed using the Student's t-test. These results are represented by mean and standard deviation. The Friedman test with post-hoc analysis demonstrated the difference in PT between the three silybin levels, and the results are presented as median (Me) and interquartile range (IQR). Two-sided $p < 0.05$ was considered statistically significant. All analyses were performed using Statistica 13 (TIBCO Software Inc. Palo Alto, CA, USA).

3. Results

3.1. Examination of the impact of normal human plasma sample dilution on prothrombin time (PT)

Firstly, the influence of dilution of the normal human plasma samples on PT was checked. The mean ± standard deviation of PT of control plasma was 14.00 ± 0.77 sec. In the case of the addition of TBS to the control plasma, the PT mean ± standard deviation was 13.88 ± 0.38 sec. There were non-statistically significant differences in PT of compared samples ($p = 0.606$).

3.2. Examination of the impact of DMSO on prothrombin time (PT) of normal human plasma

The next step was to examine the impact of DMSO on PT of control plasma. The mean ± standard deviation of the PT of control plasma was 14.00 ± 0.77 sec, while the mean ± standard deviation of the PT of control plasma with the addition of 0.1% DMSO was 14.10 ± 0.26 sec. The statistical analysis has shown non-statistically significant differences in that comparison. Hence, the effect of silybin on PT was examined with 0.1% DMSO.

3.3. Examination of the impact of silybin on prothrombin time (PT) of normal human plasma

The test was performed with three levels of silybin prepared in 0.1% DMSO (10 μM, 50 μM, and 100 μM). The results were compared to PT of normal human plasma with the addition of 0.1% DMSO alone. After adding the silybin solutions, we demonstrated a statistically significant shortened PT ($p = 0.0004$) in normal human plasma samples (Figure 2). When PT results for 50 μM (median 13.55 sec, IQR 13.40-13.65 sec) and 100 μM (median 13.40 sec, IQR 13.10-13.65 sec) silybin solutions were compared with PT obtained in normal plasma samples with 0.1% DMSO alone (median 14.10 sec, IQR 13.90-14.30 sec); the statistically significant differences in PT were observed with $p = 0.004$ and $p = 0.0005$, respectively. No difference was found when PT was compared between normal human plasma with 0.1% DMSO and 10 μM silybin solution (median 14.20 sec, IQR 14.00-14.35 sec, $p = 0.491$). Similarly, no difference was found between PT measured in plasma samples supplemented with 50 μM and 100 μM silybin solutions ($p = 0.235$).

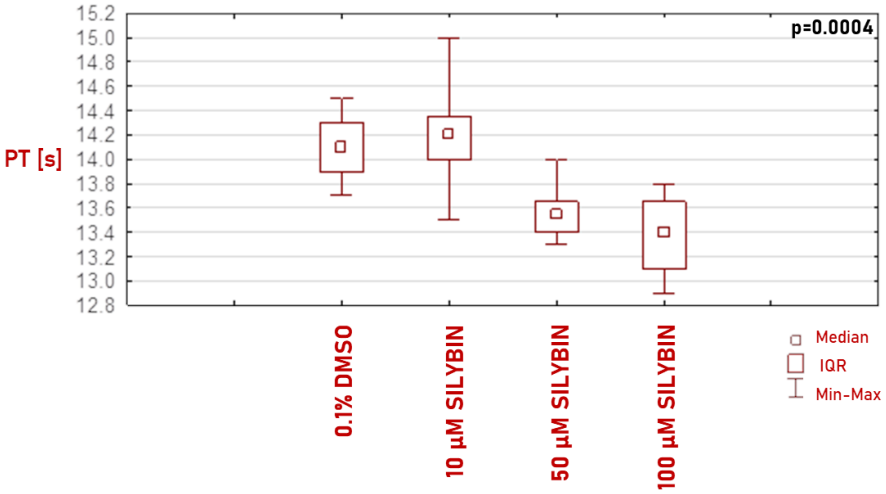

Figure 2. Effects of three different levels of silybin (10 μM, 50 μM, and 100 μM) on prothrombin time (PT) measured in normal human plasma.

4. Discussion

The aim of our study was to test the effect of silybin on prothrombin time (PT) in vitro. This assay is commonly used to assess the extrinsic and common coagulation pathways. To the best of our knowledge, this pilot study showed that silybin might modulate blood coagulation, which was presented as silybin level-dependent shortening of PT.

The beneficial properties of the extract from *Silybum marianum* have been known for years, although its impact on hemostasis is a new challenge for scientists [8]. Previous studies instead examined the effect of flavonolignans from *Silybum marianum* on platelet aggregation. However, the effect of silybin, the main biologically active compound of *Silybum marianum*, on the basic laboratory parameters of blood coagulation remains poorly understood. Bijak et al. have shown plant extract's influence on platelet aggregation inhibition [9]. However, Pourová et al. have proven only a slight effect of flavonolignans from *Silybum marianum* on platelets aggregation [10]. In our work, we decided to examine if silybin impacts in vitro blood coagulation measured by PT.

Our pilot study showed that higher levels of silybin (50 μM and 100 μM) could shorten PT measured in normal human plasma. It suggests that silybin may impact the extrinsic and common pathway of blood coagulation. Such an observation seems extremely valuable due to the wide application of silybin in medicine. However, our study failed to show which coagulation factor may be affected by silybin. In addition, we have only studied one active component from *Silybum marianum;* future research needs to determine how the plant extract modulates blood coagulation.

Our in vitro analysis characterized the possible effect of *Silybum marianum* on the blood coagulation process. These results require further investigation to confirm their validity and clinical utility.

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Conflicts of Interest: The authors declare no conflict of interest.

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