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In Vitro Assessment of Milk Thistle Seeds as a Natural Anti-Aflatoxin B_1

Meryemana Dikeni Tohumlarının Doğal Anti-Aflatoksin B_1 Olarak *In Vitro* Değerlendirmesi

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Abstract

Milk thistle (MT) or *Silybum marianum* is a weed that grows in many parts of the world. Since centuries, its seeds are used as herbal remedies for many diseases. In this study, an in- vitro model was designed to mimic the temperature, pH, and the time of passage of seeds through the stomachs and the intestinal tracts of chickens to investigate the absorption capacity of MT seeds for aflatoxin B1 (AFB1). Forty-eight flasks, each containing 25 g of rice and 250 or 500 µg/kg AFB1 in the presence of 125, or 250 mg MT seeds were used in six treatments with two samples and four replicates, I) 250 µg/kg of rice contaminated with AFB1 as the control; II) 500 µg/kg AFB1 as a control; III) 125 mg MT seeds plus 250 µg/kg AFB1; IV) 125 mg MT seeds plus 500 µg/kg AFB1; V) 250 mg of MT seeds plus 250 µg/kg AFB1; VI) 250 mg MT seeds plus 500 µg/kg AFB1. A high proportion of milk thistle was bound by 250 µg/kg of AFB1 in treatment number V. The highest and lowest levels of AFB1, i.e., 331.64±9.57 µg/kg and 45.62±4.25 µg/kg, were determined in treatments II and V, respectively. The mechanism by which MT seeds diminish AFB1 is not fully understood. MT seeds are a rich source of fiber, and therefore, they may have a high capacity for binding to the aflatoxin. On the other hand, silymarin, a component of MT seeds, is a natural polyphenolic flavonoid that has antioxidant properties. Polyunsaturated fatty acids may also act as antioxidants, where by preventing the absorption or bioactivation of mycotoxins by enhancing their metabolism and excretion. It was concluded that the MT seeds were able to effectively diminish AFB1 *in vitro*.

Keywords: Aflatoxin B1 (AFB1), binder, *in vitro* trial, milk thistle

Öz

Meryemana dikeni (MD) veya Silybum marianum, dünyanın pek çok yerinde yetişen yabani bir ottur. Tohumları birçok hastalıkta bitkisel ilaç olarak yüzyıllardır kullanılmaktadır. Bu çalışmada, meryemana dikeni tohumlarının aflatoksin B1'e (AFB1) yönelik emilme kapasitesini araştırmak için; sıcaklığı, pH değerini ve tohumların tavukların mide ve bağırsak yollarından geçiş süresini taklit edecek bir *in vitro* model tasarlanmıştır. 48 adet şişe; her biri 25 g pirinç, 125 veya 250 mg meryemana dikeni tohumu, 250 veya 500 µg/kg AFB1 içerecek şekilde, 2 örnekli 6 uygulama grubu ile 4 tekrarlı olarak kullanılmıştır. I) kontrol olarak AFB1 ile kontamine 250 ug/kg pirinç; II) kontrol olarak 500 µg/kg AFB1; III) 125 mg MD tohumuna ilaveten 250 ug/kg AFB1; IV) 125 mg MD tohumuna ilaveten 500 µg/kg AFB1; V) 250 mg MD tohumuna ilaveten 250 µg/kg AFB1; VI) 250 mg MD tohumuna ilaveten 500 µg/kg AFB1. Meryemana dikeninin büyük bir kısmı, V. uygulama grubundaki 250 µg/kg AFB1 tarafından bağlanmıştır. En yüksek 331,64±9,57 µg/kg ve en düşük 45,62±4,25 µg/kg AFB1 seviyeleri, sırasıyla II. ve V. uygulamalarda belirlenmiştir. MD tohumlarının, AFB1 düzeyini azaltma mekanizması tam olarak anlaşılamamıştır. MD tohumları zengin bir lif kaynağıdır ve bu nedenle, yüksek aflatoksin bağlama kapasitesine sahip olabilir. Bunun yanı sıra, MD tohumlarının bir bileşeni olan silymarin, antioksidan özelliklere sahip doğal bir polifenolik flavonoiddir. Çoklu doymamış yağ asitleri, mikotoksinlerin metabolizma ve atılımlarını arttırarak, emilimlerini veya biyolojik aktivasyonlarını önlediğinden, antioksidan olarak rol oynayabilmektedirler. MD tohumlarının *in vitro* olarak AFB1 düzeyini etkili bir şekilde azaltabildiği sonucuna varılmıştır.

Anahtar kelimeler: Aflatoksin B1 (AFB1), bağlayıcı, *in vitro* deneme, meryemana dikeni

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Introduction

Milk thistle (MT)/ *Silybum marianum* (L.) is an annual or biannual plant found throughout the world. The medicinal parts are the ripe seeds. The seeds are rich in antioxidants (Davis-Searles et al., 2005), and have been used for centuries as medicine for the treatment of kidney, liver, and biliary tract diseases. The cytoprotective activity of MT is probably mediated by its antioxidant properties based on its interactions with specific receptors (Křen and Walterová, 2005). Aflatoxin B1 (AFB1) is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* which are associated with many human and animal foods such as wheat, rice, corn, barley, etc. (Yunus et al., 2011). AFB1 is the most potent, naturally-occurring carcinogen. Several approaches have been investigated with the aim of reducing the exposure of animals to aflatoxin. Physical, chemical, and biological techniques have been tested on contaminated animal feed. (Ramos and Hernandez, 1996) In the last few years, many studies have been conducted evaluating the fungicide effects of natural substances on growth and production of the aflatoxins (Kalemba and Kunicka, 2003; Santos et al., 2011). Many different types of compounds have been evaluated, including antioxidants, carotenoids, flavonoids, surfactants, and herbicides. Silymarin, obtained from MT seeds, is a mixture of flavonolignans that includes silybin, isosilybinin, silydianin, silychristin, and taxifolin (Kvasnicka et al., 2003; Post-White et al., 2007; Davis-Searles et al., 2005). The seeds also contain betaine, trimethyl glycine, and essential fatty acids, which may contribute to silymarins hepatoprotective and anti-inflammatory effects. Silymarin phytosomes have beneficial effects on poultry health during aflatoxicosis (Tedesco et al., 2004; Abascal and Yarnell, 2003). The oil extracted from MT seeds contain fatty acids, such as linoleic acid, oleic acid, linolenic acid, palmitic acid, and stearic acid (Fathi-Achachlouei and Azadmard-Damirchi, 2009). To the best of our knowledge, no research has been conducted to evaluate the capability of MT seeds in absorbing AFB1 under in-vitro conditions. Thus, that was the objective of this research.

Materials and Methods

In vitro trial

In this study, an *in vitro* model was designed to mimic the temperature, pH, and time for feed to pass through the stomachs and intestinal tracts of chickens.

Treatment schedule

Forty-eight flasks, each containing 25 g of rice and 250 or 500 µg/kg AFB1 in the presence of 125 or 250 mg MT seeds were used in six treatments with two samples and four replicates (Table 1).

The treatments consisted of:

I: 250 µg/kg AFB1 only as a control
II: 500µg/kg AFB1only as a control
III: 125 mg MT plus 250 µg/kg AFB1
IV: 125 mg MT plus 500 µg/kg AFB1
V: 250 mg MT plus 250 µg/kg AFB1
VI: 250 mg MT plus 500 µg/kg AFB1

AFB1 production

AFB1 was produced using a pure culture of *Aspergillus flavus* (PTCC NO: IR 111). *A. flavus* was obtained from the Center for Scientific and Industrial Research in Iran, and it was grown on potato dextrose agar (PDA) media. Each treatment was tested at pH values in the range of 4.5 to 6.5 and at temperature in the range of 24 to 28°C. This in vitro model was designed to simulate the absorption of MT toxins in the upper and middle portions of the gastrointestinal tracts of chickens. Production and quantification of AFB1 were done using the methods described by Shotwell et al. (1966). The AFB1 media content was determined by thin layer chromatography (TLC) according to the Association of Official Analytical Chemists (AOAC, 1995).

Experimental procedures

Compounded broiler feed, consisting of 25 g rice and the desired level of toxin and esterified MT, was placed in 250 ml Erlenmeyer flasks. The feed in the control flasks was left untreated. Citric acid-sodium phosphate buffer (100 ml, pH 6.5) was added to each flask. The contents were mixed in a horizontal shaker for thirty minutes. The flasks were incubated at 37°C for three hours, after which the contents were filtered, and the residue was dried at 37°C for two hours. The toxin was extracted from the residual material and quantified. The differences in the toxin content at the beginning and end of the trial in the MT-treated and control flasks were calculated. The percent of binding of each toxin in the different treatments was determined by subtracting the percent difference in the toxin content of the control flasks from that of the treated flasks.

% Toxin adsorption= $[B_t - E_t \times 100] / B_t$ - $[B_c - E_c \times 100] / BC$,

Table 1. Density and absorption ratio of AFB1 by MT seeds at pH values in the range of 4.5 to 6.5[1]

VITreatment	I	II	III	IV	V	VI
AFB1 density (µg/kg)	184.72+8.25	331.64±9.57	89.37±5.12	201.57±6.65	45.62±4.25	87.11±6.01
AFB1 absorption (%)	0	0	30.14±3.24	26.15±2.48	48.91±3.69	41.39±4.36

I) 250 µg/kg of rice contaminated with AFB1 only as a control; II) 500 µg/kg AFB1 only as a control; III) 125 mg of MT seeds plus 250 µg/kg AFB1; IV) 125 mg of MT seeds plus 500 µg/kg AFB1; V) 250 mg of MT seeds plus 250 µg/kg AFB1; VI) 250 mg of MT seeds plus 500 µg/kg AFB1.

[1]Pooled standard error of the mean.

Where B_t= AFB1 is content at the beginning in the treated flask, E_t= AFB1 is the content at the end in the treated flask, B_c= AFB1 is the content at the beginning in the control flask, and E_c= AFB1 is the content at the end in the control flask (Raju and Devegowda, 2002).

Extraction of flavonolignans

MT seeds (Figure 1) were obtained from the Center for Agricultural Research at Shahrekord University in Iran. The extraction of silymarin is a two-step process. First, the powdered seeds were de-fatted. Ten grams of finely powdered seeds were weighed (±0.1 mg) and extracted with n-hexane (4 h) and ethyl acetate (8 h) in a Soxhlet extractor. The ethyl acetate solution was evaporated under reduced pressure by a rotary evaporator (Knauer, Germany). The analysis of the silymarin samples was conducted using a liquid chromatograph (Knauer K2600, Germany) equipped with a Nucleosil C18 (150 × 4.6-mm ID, 5-µm) column. A mixture of methanol and water (50:50) served as the mobile phase. The elution was conducted in an isocratic mode at a flow rate of 1 mL/min, and the detection made at 288 nm. One analysis required twenty minutes (Table 2 and Figure 2), (Rajabian et al., 2008).

Analysis of fatty acids

The oil was converted into methyl esters via trans esterification with a five percent methanolic hydrogen chloride (Christie and Xianlin, 1982). The trans esterification reaction was monitored using thin layer chromatography (TLC) with silica gel G plates and n-hexane-diethyl ether-acetic acid (80:20:1) as the developing solvent. A Hewlett Packard HP 5890-A gas chromatograph-mass spectrometer (GC-MS) was used for the analysis of the mixed methyl esters at the following operating conditions: column, DB-23 (0.32 mm × 30 m); temperature programming, 150-230°C, 3^{arc} min^{-1}; injector, 230°C; detector, flame ionization detector (FID) at 240°C; carrier gas, helium at a flow rate of 1.3 mL/min and a split ratio of 100:1. The equipment was calibrated using standard fatty acid methyl esters. The results were recorded by an electronic integrator as a peak area percent (Table 3 and Figure 2), (Hassan El-Mallah et al., 2003).

Statistical analysis

The collected data was analyzed with statistical software, IBM Statistical Package for the Social Sciences (IBM SPSS Statistics; Armonk, NY, USA) version 21, using descriptive statistics.

Figure 1. Photograph of the MT seeds used in the experiment

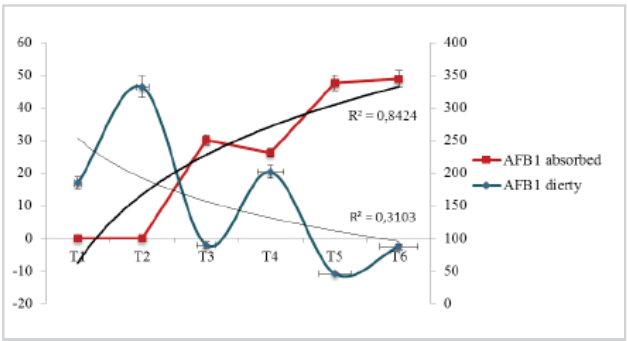

Figure 2. Effect of different quantities of MT seeds on the samples and absorption of AFB1 in *in vitro* conditions (%)

Table 2. Flavonolignan components (mean±SEM) in MT seeds as determined and quantified by HPLC analysis

Flavonolignan contents (mg/g DW)							
Milk thistle	TXF	SCN	SDN	SBN_A	SBN_B	$ISBN_A$	$ISBN_B$
	2.42±0.02	2.28±0.02	4.31±0.04	1.25±0.009	3.55±0.008	2.45±0.01	2.72±0.02

DW: dry weight; TXF: taxifolin; SCN: silychristin; SDN: silydianin; SBN: silybin; ISBN: isosilybin

Table 3. Fatty acid components (%) in MT seeds

	Palmitic. (C16:0)	**Stearic. (C18:0)**	**Oleic. (C18:1)**	**Linoleic (C18:2)**	**Linolenic (C18:3)**	**Arachidic (C20:0)**	**Behenic (C22:0)**
Inhibition index	1241	1024	1253	1299	1521	927	1012
Milk thistle seeds oil	7.44±0.007	4.54±0.008	26.85±0.52	48.55±0.72	2.57±0.004	0.34±0.0006	0.55±0.0008

Results

The results on AFB1 density alone or in combination with MT seeds and the percentage of absorption of AFB1 by different quantities of MT powder are presented in Table 1. Figures 2 and 3 show the content of flavonolignan in the MT seeds and the fatty acid pattern of oil from the MT seeds. The highest (331.64±9.57 µg/kg) and lowest (45.62±4.25 µg/kg) levels of AFB1 were determined in treatments II and V (R^2=0.3103), respectively (Figure 2). Also, the components of flavonolignan from the MT powder in the cell suspension cultures are reported in Table 2, and the fatty acid components of oil from the MT seeds are presented in Table 3. Also, the highest amounts of SBN_B and SDN were observed in 3.55 and 4.31, respectively (Table 2). Seven fatty acids were identified in MT seeds, including palmitic acid (7.44±0.007), stearic acid (4.54±0.008), oleic acid (26.85±0.52), linoleic acid (48.55±0.72), linolenic acid (2.57±0.004), arachidic acid (0.34±0.0006), and behenic acid (0.55±0.0008). The diastereo isomeric flavonolignans that consisted of these chemical components were separated successfully using the high-performance liquid chromatography (HPLC) method (Figure 3).

Discussion

The results demonstrated that MT seeds were able to diminish AFB1 effectively in vitro. It is evident that MT seeds have a wide-ranging efficacy against AFB1 density and absorbency. The mechanisms, by which MT seeds absorb aflatoxin in vivo, are not fully understood. An anti-aflatoxin compound should have a high capacity for binding to the aflatoxin (Ramos and Hernandez, 1996), or it may act by transforming the aflatoxin to less toxic metabolites (Abascal and Yarnell, 2003). As a binder, indigestible dietary fibers have the potential to adsorb mycotoxins (Smith, 1980; Williams et al., 1999; Aoudia et al., 2009). MT seeds are a rich source of fiber since 25% of their dry matter is crude fiber (Abu-Rajouh, 1996; Jadayil et al., 1999). Bio transforming agents, such as enzymes, bacteria, and fungi, can degrade mycotoxins into non-toxic metabolites (Haskard et al., 2001; Dorner et al., 1999; Buchanan and Lewis, 1984). It is likely that the most protective effect of MT, at least in part, is related to its flavonolignans, such as silybin and silymarin (Tedesco et al., 2004). In the present study, quantitative analyses showed that the amount of total silymarin varied from isosilybin A (SBN_A) and silydianin (SDN), (1.25 to 4.31 mg/g DW), respectively (R^2=0.1467). The highest amounts of SBN_B and SDN were obtained in 3.55 and 4.31, respectively (Table 2). Also, MT seeds are thought to be a strong source of antioxidants (Singh and Agarwal, 2002). Silymarin is a natural, polyphenolic, flavonoid antioxidant (Singh and Agarwal, 2002). Silymarin may act as an antitoxin in in vivo studies (Tedesco et al., 2004). Antioxidants could interact in vivo with mycotoxins by preventing their absorption, deactivation, or enhancing their metaboismor excretion (Dvorska et al., 2001, 2007).

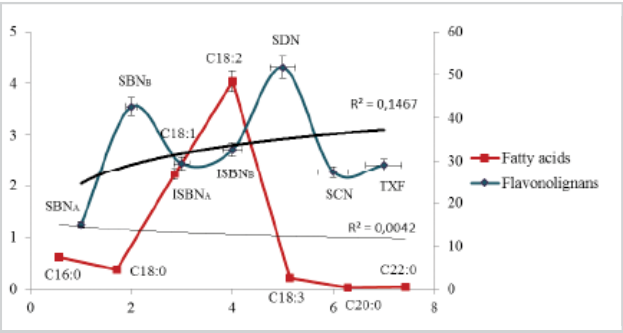

Figure 3. Flavonolignan content (mg/g dry weight (DW)) and fatty acids (%) pattern of MT seeds in *in vitro* conditions

In addition, polyunsaturated fatty acids (PUFA) may act as antioxidants (Richard et al., 2008). MT seeds are 25-30% oil and rich in unsaturated fatty acids (N=3). In this study, more than 70% of the fatty acids were unsaturated i.e., linoleic acid and linolenic acid (Sanchez-Machado et al., 2002; Kvasnicka et al., 2003). As a result, the higher the level of linoleic acidin the plant, the higher its ability to absorb toxins (Richard et al., 2008). Silymarin and silybin may increase the strength of the toxin binding dramatically.

In conclusion, MT seeds are a rich source of fiber; therefore, they have a high capacity for binding to the aflatoxin. Silybin and silymarin with linoleic acid may provide some special antitoxin capacity for MT seeds. The results demonstrated that MT seeds were able to effectively diminish AFB1 in vitro. MT seeds might prove beneficial in the management of aflatoxin-contaminated poultry feed when used in combination with other mycotoxin management practices.

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