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Chemical Composition and Biological Activity of Milk Thistle Seeds (*Silybum marianum* (L.) Gaertn.)

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Abstract

Milk Thistle Seeds (MTS) purifies the liver from all toxic and harmful substances, supports the regeneration of liver cells. In this study, the chemical composition of milk thistle seeds (*Silybum marianum* (L.) Gaertner = *Carduus marianus* L.) and also its biological activity were determined. Essential oil was analyzed using by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques. After that, the essential oil was tested against bacteria and fungi by agar well diffusion and micro dilution methods. The essential oil yield was 1.1% (v/w). Eight constituents were comprised the 97.3% of the total oil extract of the Milk thistle seeds. The major compounds were determined as oleic acid (45.6%), linoleic acid (29.0%), ethylbenzene (7.0%) and stearic acid (5.7%). The seed essential oils of MTS significantly inhibited the growth of Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Streptococcus sp.)* bacteria ($p < 0.05$). The oils also showed fungicidal activity against *Candida tropicalis* and *C. globrata.*

Key words: Biological activity, Essential oil, Milk Thistle Seeds, GC-GC/MS

1. Introduction

Silybum marianum (L.) Gaertn., synonym *Carduus marianus* L. common name is Milk Thistle, a member of the Compositae family, is an annual or biennial herbaceous plant, native to the Mediterranean basin, but now naturalized and widespread throughout the world (Kaur et al., 2011; Sidhu and Saini, 2012). Its fruits (i.e. achenes), often referred to as seeds, have been valued for their medicinal properties (Gazak et al., 2007; Kroll et al., 2007), have been utilized as medicine for over 2000 years and were known for liver protecting properties since ancient Greek civilization (Alemardan et al., 2013).

Milk Thistle is grown commercially as a medicinal plant in Europe, Egypt, China and Argentina (Hammer et al., 1992; Veres et al., 2012). However, milk thistle is considered a weed in sowed annual legume pastures (Sulas et al., 2008), waste areas, cereal crops, decreasing wheat yields (Khan et al., 2009), and along roadsides (Karkanis et al., 2011). On the other hand, milk thistle is currently being regarded as an interesting crop for bioenergy production in Mediterranean environment (Sulas et al., 2008; Ledda et al., 2013), and as a source for biodiesel production (Ahmad et al., 2014). It is grown also as an ornamental plant (Bhattacharya, 2011) and as a tolerant species for soils polluted by heavy metals (Rio-Celestino et al., 2006; Perrino et al., 2014).

The special milk thistle extract, silymarin, stimulates the liver regeneration, and its constituents act as antioxidant, anti-inflammatory and hepatoprotective agents, and therefore are effective in the treatment of mushroom (*Amanita* sp.) poisoning, hepatitis, cirrhosis and fibrosis of the liver. In addition, milk thistle fruit extracts have antiviral (Polyak et al., 2010) and antitumor (Scambia et al., 1996) activities and their constituents are underintense research in the clinical therapy of cancer for chemoprevention, treatment, and amelioration of chemotherapy-associated side effects. The standardized extract of *S. marianum* fruits is known as silymarin and has long been used for the treatment of chronic inflammatory liver diseases (Flora et al., 1998) and more recently for prostate cancer chemoprevention (Deep and Agarwal, 2010).

These leaves, stems, flowers and seeds of milk thistle were used for various purposes in different countries for centuries. In recent years, considerable attention has been laid on medicinal plants with antioxidant and antimicrobial activity. Main components of these seeds are rich in crude oil, starches, mucilage, minerals tannins, and flavonolignans (Andrzejewska et al., 2011). Mineral elements are assumed to have immense value as each of these elements show a distinctive individual role in the structural and functional integrity of the organization of living systems (Ghafor et al., 2014). There is a significant demand on the production of this plant from European countries in recent years. It is noteworthy that milk thistle is an important pharmaceutical plant for pharmaceutical industries and it has gained interest in Turkey as well. In this context, some agricultural studies were conducted in order to determine the potential production area of this plant. The oil extracted from these seeds can be used as a cure for many diseases including viral hepatitis and cirrhosis (Gurbuz et al., 2000; Elwekeel et al., 2013).

In this study, we aimed to identify the chemical composition of *Silybum marianum* seed. The analysis of essential oil composition of *Silybum marianum* seed by Gas Chromatography-Mass Spectrometry (GC-MS), also to show their antimicrobial activities on some bacteria and fungi.

2. Material and Methods

2.1. Plant material

The seed of *S. marianum* were collected from natural habitats in Adana province of Turkey, in May 2015. The seed were dried in the shade at room temperature. The voucher specimen for *S. marianum* (Dogan 2401) has been deposited in the Firat University Herbarium (FUH) in Elazig province of Turkey.

2.2. Extraction of essential oil

The essential oil was extracted by hydrodistillation using a modified Clevenger apparatus coupled to a 2 L roundbottom flask. A total of 100 g of seed material and 1 L of water were used for the extraction. The chemical analyses were performed in the Plant Products and Biotechnology Research Laboratory at Firat University. The extraction was performed over a 3-h period. The oil was transferred to black-colored vials, wrapped in parafilm and aluminum foil and stored at 4 °C until analysis. The yields of the oils were calculated on the basis of the dry mass.

2.3. Gas chromatography (GC) analysis

The essential oil was analyzed using a HP 6890 GC equipped with a flame ionization detector (FID) and a HP-5ms (30 m × 0.25 mm i.d., film thickness 0.25 µm) capillary column was used. The column and analysis conditions were the same as in GC-MS, as expressed below. The percentage composition of the essential oils was computed from GC-FID peak areas without correction factors.

2.4. Gas chromatography/mass spectrometry (GC-MS) analysis

GC-MS analyses of the oils were performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with a Hewlett Packard 5973 mass spectrometer system equipped with a HP-5ms capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) (J&X Scientific). The oven temperature was programmed from 70 to 240 °C at the rate of 5 °C/min. The ion source was set at 240 °C and the electron ionization at 70 eV. Helium was used as the carrier gas at a flow rate of 1 mL/min. The scanning range was 35–425 amu. Diluted oil in *n*-hexane (1.0 µL) (Merck) was injected into the GC-MS. The identification of constituents was performed on the basis of the retention index (RI) determined by co-injection with reference to a homologous series of *n*-alkanes (C8–C25) under identical experimental conditions (Adams, 2001). Further identification was performed by comparison of their mass spectra with those from NIST 98 Libraries (on ChemStation HP) and Wiley 7th Version. The relative amounts of individual components were calculated based on the GC (HP-5ms column) peak area (FID response) without using correction factors.

2.5. Antimicrobial activity

2.5.1. Microbial strains and culture media

All media were supplied by LAB Ltd (UK). All microbial strains were obtained from the Microbiology Laboratory, Department of Biology, Science Faculty of Firat University. Stock cultures of Gram-positive *S. aureus* (ATCC 6538P) and *Bacillus subtilis* (ATCC 6633), Gram-negative *E. coli* (ATTC 25922), *Streptococcus sp.* (ATCC 8059) and *Candida tropicalis* (ATCC 13803) and *C. globrata* (ATCC 66032) were subcultured and maintained in nutrient broth at 37 °C for 24 h. Briefly, 100 µL of test bacteria/fungi were grown in 10 mL of fresh media until they reached a count of approximately 10^8 cells/mL for bacteria or 10^5 cells/mL for fungi. Then, 100 µL of microbial suspension was spreaded onto agar plates corresponding to the broth in which they were maintained.

2.5.2. Antimicrobial screening

The agar well diffusion and micro dilution methods were employed for the determination of the antimicrobial activities of the essential oils and standard antibiotics (NCCLS, 1999). The results of the essential oils were discussed with standard antibiotics as the positive controls [amikacin (30 µg) AK30, gentamicin (10 µg) CN10, tetracycline (30 µg) TE30 and netilmycin (30 µg) NET30]. All tests were performed in triplicate.

2.5.3. Agar well diffusion method

A suspension of the test microorganism, 0.1 mL of 108 cells/mL, was spreaded on the Muller Hinton Agar (MHA). The wells (6 mm diameter) were cutted from the agar and different concentrations (0.5, 1, 2, 3 and 4 µg/mL) of essential oils were delivered into them. After incubation for 24 h at 37 °C, all plates were examined for each zones of growth inhibition, and the diameters of these zones were measured in millimeters.

2.5.4. Determination of Minimum Inhibitory Concentration (MIC)

Bacterial cultures were obtained in Mueller-Hinton broth (Difco) for all the bacterial strains after 24 h of incubation at 37 ± 0.1 ºC. Yeasts were propagated in Sabouraud dextrose broth (Difco) after incubation for 24 h at 25 ± 0.1 ºC. Testing was carried out in Mueller-Hinton broth and Sabouraud dextrose broth at pH 7.4 for bacteria and yeast, respectively. The final inoculum size for bacteria and fungi was 10^5 CFU/mL. Essential oil were dissolved in DMSO at an initial concentration of 1000 µg/mL and then 4 serially diluted in culture medium to 125 µg/mL. A set of tubes containing only inoculated broth was kept as control. Antimicrobial activity was determined after incubation for 24 h at 37 ^{o}C for bacteria and after incubation for 48 h at 25 ^{o}C for the yeasts. MIC was defined as the lowest concentration of the compounds that inhibited the visible growth of a microorganism. Each experiment in the antibacterial and antifungal assays was replicated three times to define the MIC values.

3. Results and Discussion

3.1. Chemical composition of essential oil

The essential oils yield was 1.1% (v/w). Eight constituents were comprised the 97.3% of the total oil extracted from the milk thistle seeds. The major compounds were determined as oleic acid (45.6%), linoleic acid (29.0%), ethylbenzene (7.0%) and stearic acid (5.7%). Our results showed that *S. marianum* seed has rich in terms of faty acids. The results of the GC-MS analysis of the oils are presented in Table 1.

The biochemical evaluation of essential oil composition of *Silybum marianum* seed by Gas Chromatography-Mass Spectrometry (GC-MS) was analyzed by Mhamdi et al. (2016). This study showed the presence of 14 volatile components with the predominance of γ-cadinene (49.8%) and α-pinene (24.5%). Whereas, the analysis of fatty acids composition, showed the predominance of linoleic (50.5%) and oleic (30.2%) acids (Mhamdi et al., 2016). Another study was reported that *S. marianum* seed oil has high unsaturated fatty acid content which constitutes (73.0%) of the total fatty acids. Linoleic acid content was 53.30% of the total

composition followed by oleic acid (20.80%) (El-Mallah et al., 2003). It can be said that our results have shown compatibility with the other *S. marianum* seed essential oil studies especially on major compounds.

Table 1. Chemical Composition of MTS analyzed by GC-MS.

Name of Compounds	RI	%
Ethylbenzene	968	7.0
m-Xylene	974	2.4
p-Xylene	990	1.0
2,4-Decadienal	1289	1.5
n-Hexadecanoic acid	1690	5.1
Linoleic acid	1807	29.0
Oleic acid	1811	45.6
Stearic acid	1824	5.7
Total		97.3

MTS: Milk Thistle Seeds, RI: Retention index relative to C8–C25 n-alkanes on HP-5 column.

3.2. Antibacterial and antifungal activities of essential oils

The results of antibacterial and antifungal activities are summarized in Tables 2, 3 and Figure 1. The results indicated that the inhibition zones (IZs) resulting from the antibacterial activities ranged between 27 and 36 mm at 0.5, 1, 2, 3 and 4 µg/mL oil concentrations ($p < 0.05$). Essential oils showed antibacterial activities against the studied bacterial strains (Table 2). The inhibition zones resulting from the antifungal activities ranged between 27 and 35 mm at 0.5, 1, 2, 3 and 4 µg/mL oil concentrations ($p < 0.05$) (Table 3).

Essential oil significantly inhibited the growth of Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli* and *Streptococcus* sp.) bacteria strains. Essential oil showed strong antibacterial activity against the *E. coli* (36 ± 1.2), *Streptococcus sp.* (35 ± 0.8), *B. subtilis* (34 ± 1.2) and *S. aureus* (33 ± 0.5), respectively ($p < 0.05$) (Table 2). It also showed highly effective fungicidal activity against *C. tropicalis* (35 ± 1.2) and *C. globrata* (34 ± 0.7) ($p < 0.05$) (Table 3). Results obtained by measurements of minimal inhibition concentration (MIC) indicated that, *B. subtilis* and *E. coli* were the most sensitive microorganisms tested with the lowest MIC values 125 µg/ml in the presence of the oil isolated from MTS. *S. aureus, Streptococcus* sp., *C. tropicalis* and *C. globrata* were other sensitive microorganisms against the oil with an MIC value at 500 µg/ml (Fig 1).

The essential oil of MTS exhibited strong antimicrobial activity against the microorganisms. As can be seen from Table 2 and Fig 1, the oil achieved the highest inhibition zone (36 ± 1.2) and the lowest MIC value (125 µg/ml) for *E. coli*, which shown that this microorganism was the most sensitive to MTS essential oil. Other sensitive microorganism against the essential oil of MTS was *B. subtilis* with an inhibition zone (34 ± 1.2) and the lowest MIC value (125 µg/ml).

Yue et al. (2017) reported the antibacterial activity of protease hydrolysates from *Silybum marianum* protein isolates (SMPIs). The researchers were tested the neutral protease, papain, pepsin and alkaline protease enzymes on *E. coli*, *S. aureus*, *Sarcina lutea* and *B. subtilis*. The results showed that neutral protease, papain and pepsin hydrolysates exerted inhibitory effects on all the tested bacteria. However, alkaline protease

hydrolysates of SMPI showed stimulatory effects on replication of used bacteria. The antibacterial mechanism of SMPI hydrolysates was studied using scanning electron microscopy, and the results showed effective inhibition of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). It is speculated that the underlying mechanism of SMPI hydrolysates may involve injury to *E. coli* and *S. aureu*s cell membranes (Yue et al., 2017).

In another study, the antimicrobial activities of seed ethanol extracts and seed oil were tested in vitro against *Pseudomonas aeruginosa*, *E. coli*, *S. aureus*, *Aspergillus niger* and *C. albicans* using the disc diffusion method. Seed and seed oils obtained from obvious doses of potassium sulfate (0, 30, 60, 90 and 120 kg ha -1) fertilizer applications displayed antimicrobial activities against *E. coli*, *A. niger* and *P. aeruginosa*. The application of 90 kg ha-1 of potassium sulfate on seed oil resulted in the highest antimicrobial activities (Yaldiz, 2017).

In addition, there are many literature revealing the powerful antioxidant effect of this plant. Ahmad *et al.* (2013) evaluated the antioxidant activity by DPPH method in different parts of *S. marianum* and found that the tested plant materials had significant free radical scavenging activity, suggesting that such plant materials can be used as a source of antioxidants for different diseases. Besides they evaluated the antioxidant activity in different parts of the plant (leaves, stems, seeds, roots) and found highest antioxidant capacity in young leaves of a white seed variety (Ahmad et al., 2013).

Table 2. Antibacterial activity of essential oils of MTS against Gram-positive and Gram negative bacterial strains.

Bacterial strains	Essential Oils (µg/ml)					Antibiotic			
	0.5	1	2	3	4	NET	AK	CN	TE
S. aureus IZ (mm)	29±1.2	30±0.8	31±1.6	32±0.4	33±0.5	18±0.0	22±0.0	20±0.0	15±0.0
B. subtilis IZ (mm)	27±1.7	28±2.0	31±1.2	32±0.8	34±1.2	20±0.0	19±0.0	19±0.0	16±0.0
E. coli IZ (mm)	29±1.2	31±0.8	33±1.2	35±1.2	36±1.2	17±0.0	15±0.0	16±0.0	18±0.0
Streptococcus sp. IZ (mm)	31±1.6	32±2.0	33±0.6	34±0.2	35±0.8	16±0.0	17±0.0	15±0.0	19±0.0

IZ, Diameter of inhibition zone (mean±SD) ($p < 0.05$); NET, netilmycin; AK, amikasin; CN, gentamycin; TE, tetracyclin.

Table 3. Antifungal activity of essential oils of MTS against fungal strains.

Fungal strains	Essential Oils (µg/ml)					Antibiotic			
	0.5	1	2	3	4	NET	AK	CN	TE
C. tropicalis IZ (mm)	27±0.4	31±0.3	32±0.4	34±0.5	35±1.2	NA	NA	NA	NA
C. globrata IZ (mm)	28±0.8	31±0.8	32±0.4	33±0.8	34±0.7	NA	NA	NA	NA

IZ, Diameter of inhibition zone (mean ± SD) ($p < 0.05$); NA, not analyzed.

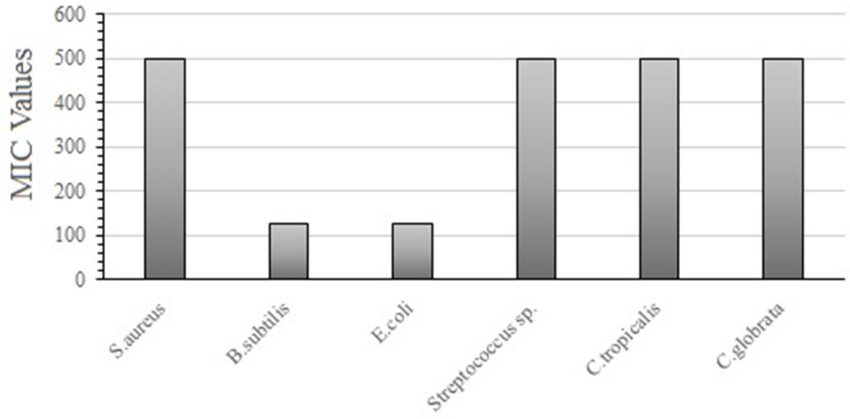

Figure 1. The MIC values (µg/ml) of essential oil of MTS against different bacteria and yeasts.

4. Conclusion

Based on the results of the current study it can be concluded that *S. marianum* seeds can be a good source of unsaturated fatty acids which has quite antimicrobial activities. However, it is notable that further in vivo studies on the potential bioactivities of *S. marianum* seeds would be necessary to increase understanding of their function and metabolism.

Conflict of interest

The authors declares that there is no conflict of interests.

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