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Effect of Milk Thistle (*Silybum marianum* (L.) Gaertn) Seed Extract on Bacterial Activities and Growth of Human Liver Cancer Cells

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Abstract

Silymarin is a polyphenolic flavonoid mixture isolated from milk thistle which is believed to be responsible for the plant's hepatoprotective action. Silymarin is hepatoprotective flavonoid drug available as bio marker in *Silybun marianum* (common name milk thistle). Silymarin is being used in treatment of various liver disease of different etiology because of its hepatoprotective action. Phytochemicals are playing a vital role for the treatment of different types of diseases and still used in both traditional and modern medication system. The phytochemical analysis of milk thistle seeds extract indicated that the plant is rich in secondary compounds. The results revealed that milk thistle seeds contain high amount of total phenolic, flavonoid and antioxidant compounds. The antibacterial activities of the ethanol seeds extract of milk thistle was tested against Gram-positive bacteria (*Staphylococcus aures*) and Gram-negative bacteria (*Salmonella entirica*). Antibacterial effects of crude extract were performed using modified Kirby-Bauer disc diffusion technique to determine the zone of inhibition. The results demonstrated that ethanol seeds extract of milk thistle is shown strong inhibition zone against *Staphylococcus aures* and *Salmonella entirica* compared to the control. Also, milk thistle seeds extract showed highly anticancer activity. This medicinal plant could be developed into affordable and safe standardized herbal products and may serve as a source of new molecules for broad-spectrum anticancer, antimicrobial agents.

Keywords: Antioxidant; Anticancer; Antibacterial; Flavonoid; Phenolic components.

1. Introduction

Herbal plants have long been used as medicines in folk and traditional medicinal practice based on the use of plants and plant extracts [1]. Herbal medicines derived from plant extracts are being increasingly utilized in traditional and folk medicine treatments for many human diseases for thousands of years throughout the world [2, 3]. The medicinal value of many plants species reported having pharmacological properties due to the presence of various kinds of phytochemicals (biologically active components) including alkaloids, terpenoids, flavonoids, glycosides, saponins, steroids, and other phenolic compounds which are therefore, should be utilized against the disease-causing pathogens [4-6]. Milk thistle (*Silybum marianum* L.) plant belongs to family *Asteraceae* which native to the Mediterranean area [7]. Milk thistle known for its medicinal properties having essential biochemical constituents including isomeric mixtures of flavonolignans, including taxofillin, silychristin, silydianin, silybin and isosilybin collectively known as silymarin (SM) [8]. Silymarin has antioxidant and antibacterial activity and utilized as part of hepatic disorders, including hepatotoxicity secondary to acute and chronic viral hepatitis and mushroom poisoning [9, 10]. Milk thistle has been reported to have protective effects on the liver and to greatly improve its function. It is typically used to treat liver cirrhosis, chronic hepatitis (liver inflammation), toxin-induced liver damage (including the prevention of severe liver damage from Amanita phalloides (death cap mushroom poisoning), and gallbladder disorders [11]. Barceloux [12] reported that Silymarin were used for the treatment of several liver diseases characterized by degenerative necrosis and functional impairment including chronic liver disorders. The objectives of this study were to determine the antioxidant activity, the total flavonoid content, the total phenolic content, the anticancer activity, and antibacterial activity of the ethanolic extract of milk thistle seeds.

2. Materials and Methods

2.1. Plant Material

Milk thistle seeds were collected from farm in Sharkia Governorate (30.7327° N, 31.7195°E), Egypt in season 2018/2019. The seed were cleaned with double distilled water and air dried, then stored at 4°C until used.

3. Methods

3.1. Preparation of Ethanolic Extracts

Milk thistle seeds were extracted according to the method of Panovska, *et al.* [13], briefly, 100 g of dry powder were extracted with 500 mL of 70% ethanol in a screw-capped flask and shaken at room temperature for 24 h and filtered through filter paper (Watman No. 1, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), the extract was dried using rotary evaporator (LabTech, Sorisole, Italy) and stored at -20°C until using for further study.

3.2. Phytochemical Analysis

Screening of the milk thistle seeds extract for various phytochemical constituents were carried out according to the standard methods of Waweru, *et al.* [14].

3.3. Determination of Total Phenolic Content

Total phenolic compounds of milk thistle seeds extract were determined according to Ghasemzadeh, *et al.* [15] using Folin-Ciocalteu procedure. Analytical grade of gallic acid was used to generate standard curve. Seeds extract of milk thistle (1 mg mL^{-1}), 200 µL was mixed 5 mL of deionized water and 0.5 mL of Folin-Ciocalteu phenol reagent. After 5 minutes, 2 mL of 7.5% (w/v) sodium bicarbonate solution was added to the mixture. The mixture was intermittently shacked and kept in total darkness for 1 h at room temperature. The absorbance was measured at 760 nm using spectrophotometer (JENWAY 6405 UV/visible, Essex, UK). The content of total phenolic compounds was calculated using gallic acid calibration curve and expressed as grams of gallic acid equivalents (CAE) in 100 g of the seeds extract [16]. The data presented was for three technical replicates.

3.4. Determination of Total Flavonoids

Total flavonoid compounds of milk thistle seeds extract were determined as described by Panovska, *et al.* [13], using aluminum chloride colorimetric method. Briefly, 500 µL of ethanol extract was mixed with 500 µL of 2% (w/v) aluminum chloride solution. The mixture was shacked for 10 min at room temperature. The absorbance of the mixture was measured at 430 nm with spectrophotometer (JENWAY 6405 UV/visible) against a blank sample without aluminum chloride. Different concentrations of quercetin were dissolved in 80% ethanol and used to generate a calibration curve. The content of total flavonoids was calculated and expressed as grams of Quercetin equivalents (QE) in 100 g of the extract by comparison with the quercetin calibration curve that prepared under the same condition. The data presented was for three technical replicates.

3.5. GC-MS Analysis

GC-MS analysis was carried out on Thermo Trace Ultra-GC coupled with THERMO ISQ (Gas Chromatograph–Mass Spectrometer instrument, Thermo Scientific, Massachusetts, USA). The GC-MS was associated with TG-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness) and operated in electron impact mode at 70_eV. Helium gas (99.999%) was used as a carrier gas at a constant flow of 1 mL min^{-1}. An injection volume of 0.5 µL was employed (split ratio of 10:1), injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C min^{-1} to 200°C, then 5°C min^{-1} to 280°C, with hold for 9 min. Mass spectra of the four components recorded at 70_eV; scan interval time was 0.2 second and fragments from 40 to 450 Da. Total GC running time was 36 min. min.

3.6. Determination of Antioxidant Activity

The antioxidant activity of seeds extract of milk thistle was evaluated according to the method described by Mensor, *et al.* [17] using DPPH (1,1-Diphenyl-2-picryl-hydrazyl) photometric assay. Different concentrations of milk thistle extract (50, 100, 150, and 200 µg mL^{-1}) were prepared in ethanol. One mL of 1 mM DPPH ethanol solution were added to 500 µL of concentration of the sample solutions. The mixture allowed to react at room temperature for 30 min. The absorbance was determined against control at 518 nm and the radical scavenging capacity of the samples was measured as a decrease in the absorbance of DPPH radical and was calculated using the following equation.

$$\text{Radical scavenging activity (\%)} = 100 - \{[(Abs_{sample} - Abs_{blank})\, x\, 100]/Abs_{control}\}$$

Milk thistle seeds extract solution (500 µL) and ethanol (1 mL) was used as a blank. One mL of 1 mM DPPH solution and 1 mL ethanol was used as control. The positive controls were those using the sample solutions.

The SC_{50} (the concentration of the sample that scavenges 50% of the DPPH radicals) was calculated by linear regression where the x-axis represented the concentration of milk thistle seeds extract and the y-axis represented the average percent of the radical scavenging activity from three technical replicates.

3.7. Antimicrobial Activities

Antimicrobial activities of the milk thistle seeds extract were determined using a modified Kirby-Bauer disk diffusion method [18, 19]. Plates were inoculated with Gram-positive bacteria (G+); *Staphylococcus aures* and *Listeria monocytogenes* as well as gram-negative bacteria (G-); *Salmonella entirica* and *Escherichia coli.* The plates were incubated at 35 – 37°C for 24 – 48 hours., and then the inhibition zone diameters were measured in millimetres [18].

3.8. Anticancer Activity

Human tumor carcinoma cell lines (liver) that used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, USA). The tumor cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Samples were prepared by dissolving 1:1 Stock solution and stored at -20°C in dimethylsulfoxide (DMSO) at 100 mM. Different concentrations of the milk thistle seeds extract (0.1, 0.5, and 1.0 µg mL^{-1}) were used. The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara [20].

4. Results and Discussion

4.1. Milk Thistle Seeds Extract Chemical Composition and Active Components Content

Milk thistle seeds extract exhibited highly content of phenolics, followed by flavonoids, saponins, steroids, and coumarins (Table 1). Phytochemicals are playing a vital role for the treatment of different types of diseases and still used in both traditional and modern system of medication [1, 7, 21]. Plants produce many secondary metabolites such as flavonoids, carotenoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols, beta-carotene, ascorbic acid and alpha tocopherols, which have used antioxidants activity [22].Milk thistle seeds extract has high value of total phenolic compound, and total flavonoid compound (Table 2). The natural antioxidants such as phenolics and flavonoids compounds have wide spectrum pharmacological effects like antibacterial, anti-allergic, neuroprotective activities, anti-inflammatory and anticancer, also protect plants from the attack of pathogenic microbial [21, 23] Milk thistle can be a major source of natural or phytochemical antioxidants.

Table 3 and Figure 1 present the Chemical composition of the studied components of milk thistle seeds extract and Total Ion Chromatogram (TIC) measured for extracted milk thistle seeds, recpectivily.

4.2. Radical Scavenging Activity of Milk Thistle Seeds Extract

The milk thistle seeds extract showed an increase in antioxidant activity by increasing concentration of extract residue (Table 4). The highest scavenging activity was reported with 200 µg mL^{-1} milk thistle seeds extract, therefore exhibiting a concentration dependent pattern of free radical scavenging ability. As showed by Shahwar, *et al.* [24] there was a great association between antioxidant activity and phenolic compound concentration. Silymarin has been reported to act as an excellent antioxidant, scavenging free radicals and inhibiting lipid peroxidation thereby protecting cells against oxidative stress [25]. Silibinin exhibits membrane protective properties and it may protect blood constituents from oxidative damage. Also, Kvasnička, *et al.* [26] reported that silymarin is a well-established milk thistly seeds containing standardized dry extract, primarily flavonolignans (about 70% –80% w/w) and polymeric and oxidized polyphenolic compounds composed of a blend of flavonoids. In any biological system, generation of free radicals during metabolism are beyond the antioxidant capacity for that system, which lead to oxidative stress [27]. Oxidative stress play a key role in several diseases such as heart diseases, cancer, neurodegenerative diseases as well as aging process [28] and dietary antioxidant could have a big impact to lower the risk of such diseases even when they present in a very low concentration[29]. Natural antioxidants or phytochemical antioxidants have no or very low side effect compared with the synthetic antioxidants when taken *in vivo* [30].

4.3. Antimicrobial Activity

The intensive use of antibiotics is often followed by the presence of resistant strains of microorganisms. In view of the resistance of compounds having antibacterial activity is an urgent one in order to cope with the harmful effects of these microorganisms. For these reasons, ethanol extract of milk thistle seeds was tested against different Gram-positive bacteria (G+); *Staphylococcus aures* and *Listeria monocytogenes* as well as gram-negative bacteria (G-); *Salmonella entirica* and *Escherichia coli*. Inhibition zones are recorded as shown in Table 4. Control (DMSO) was in the same conditions did not produce any inhibition zones. It is showed that the extract gave rise to concentration dependent inhibition zones in the four types of bacteria (gram positive and negative). The results of the present study showed that the ethanol extract of milk thistle seeds inhibited the growth of the tested bacteria strongly (Figure 2) especially at concentration 0.5 µg mL^{-1} or higher. Milk thistle seeds extract at 1.0 µg mL^{-1} showed the highest inhibition (67%) against *Listeria monocytogenes* compared with Chloromphenicol, while the lowest inhibition (40%) was at 0.5 µg mL-1 against *Salmonella entirica.* This may be due to presence of antioxidant activities of phenolic and flavonoid contents of milk thistle seeds. According to these results, *Salmonell entirica* (G-) and *Staphylococcus aures* (G+) were found to be more sensitive to the milk thistle seeds extract than other bacteria. These results are in agreement with Abed, *et al.* [9]who reported that milk thistle seeds extract has antibacterial activity against *Staphylococcus saprophyticus*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae.*

4.4. Anticancer Activity

The results in Figure 3 showed slightly active substances in milk thistle seeds extract that may have anti-cancer effects using Sulphorhodamine-B (SRB) assay [20]. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content. One active substance known as silymarin has strong antioxidant properties and has been shown to inhibit the growth of human prostate, breast, and cervical cancer cells in test tubes [1]. Further studies are needed to determine whether milk thistle is safe or effective for people with these forms of cancer.

5. Conclusion

In the present study, the milk thistle seeds extracts exhibited different inhibitory activities against the tested bacterial. This study showed that milk thistle seeds extracts can be used as a natural antioxidant, antimicrobial as well as anticancer. In general, the results of this study may open the possibility of future using of natural product extracts as a safe medical treatment. Further investigations are needed to evaluate the plant extracts activities against a wide range of bacterial diseases and cancer cell lines.

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Figure-1. Total Ion Chromatogram (TIC) measured for extracted milk thistle seeds

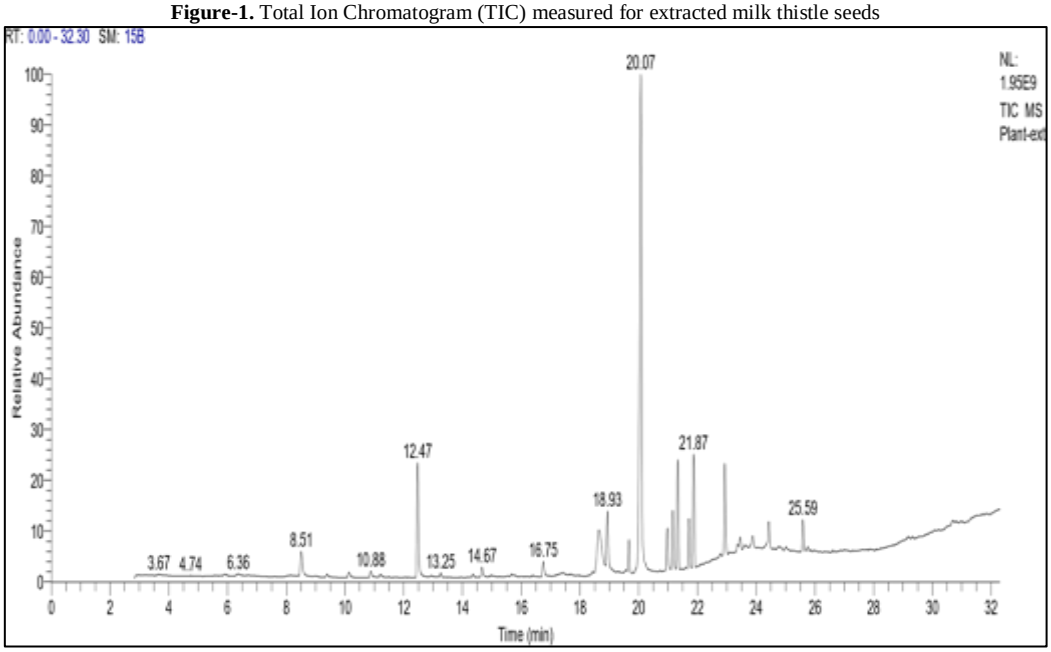

Figure-2. Effect of ethanolic extract of milk thistle seeds on microorganisms' activity

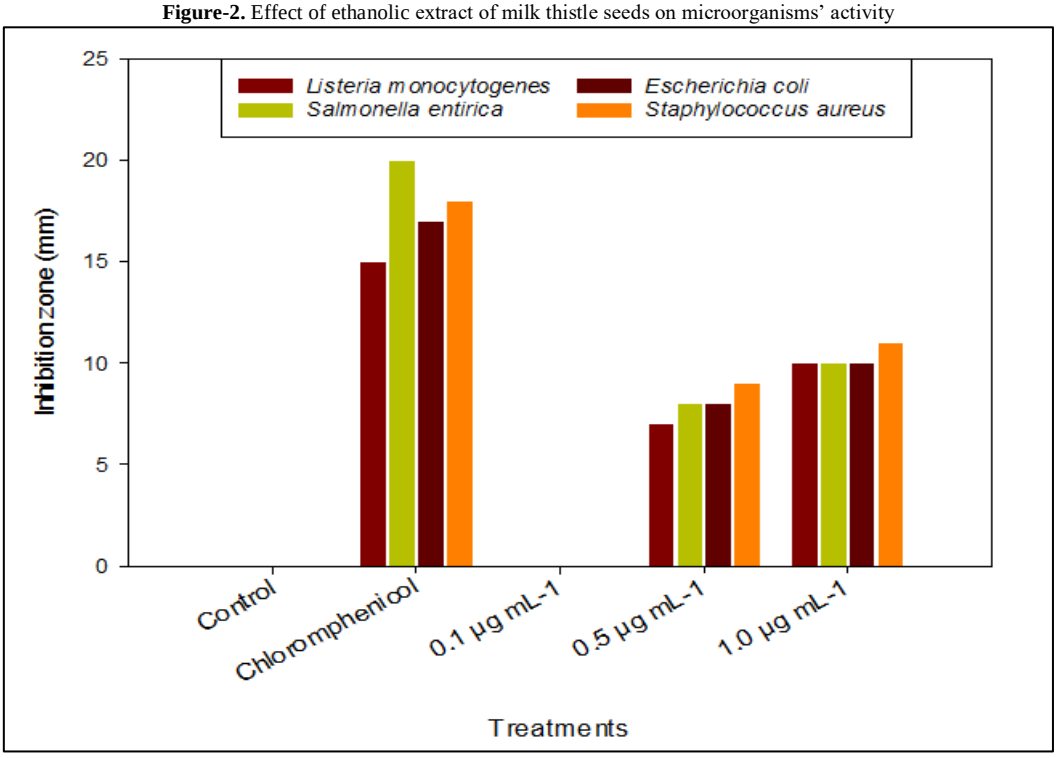

Figure-3. Effect of milk thistle seeds extract on HEPG2-1 cell line of liver cancer cells

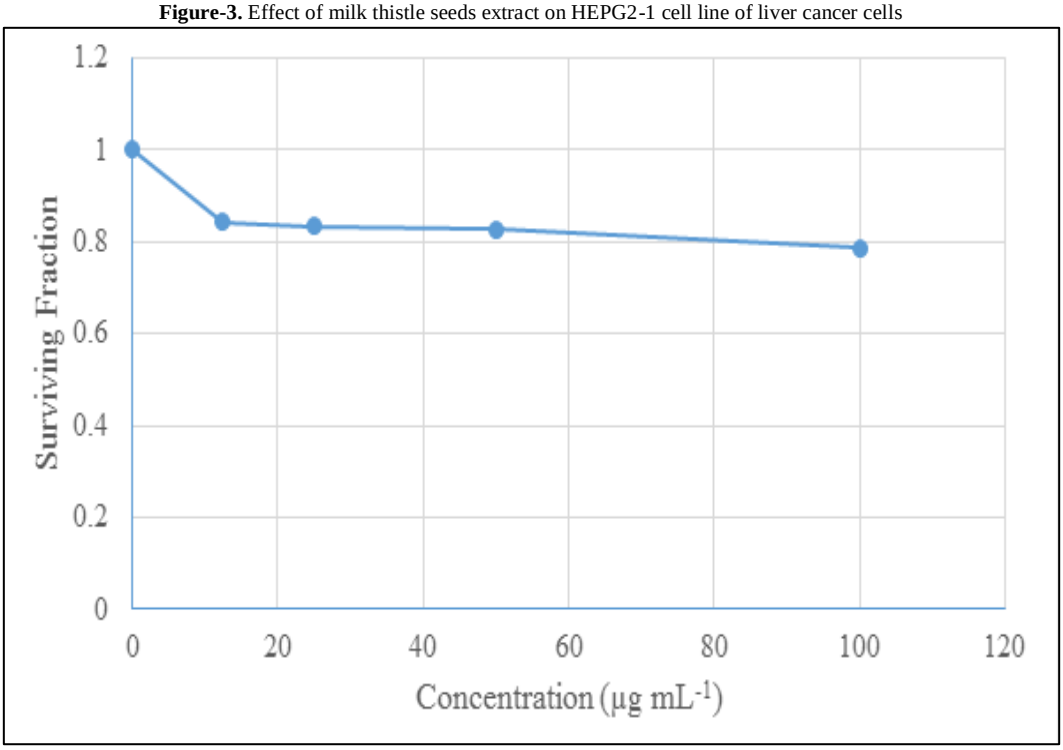

Table-1. Phytochemicals analysis of milk thistle seeds

Phytochemical contents	Results
Phenolic	+++
Flavonoids	++
Saponins	+
Steroids	+
Coumarins	+
Carbohydrates	+
Protein	+
Tannins	-
Phlobatannins	-
Glycosides	-
Emodins	-
Anthocyanins	-

Table-2. Total phenolic and total flavonoid contents of milk thistle seeds extract

	Total phenolic (mg GAE g^{-1} DW)	**Total flavonoid (mg querceting^{-1} DW)**
Milk thistle seeds extract	245.183	88.151

Table-3. The Chemical composition of the studied components of milk thistle seeds extract

No	**RT**	**Compound Name**	**Area %**	**Molecular Formula**	**Molecular Weigh**	**SI**
1	8.51	Phenol, 2-methoxy	2.11	$C_7H_8O_2$	124	842
2	12.47	2-Methoxy-4-vinylphenol	7.75	$C_9H_{10}O_2$	150	928
3	14.67	Phenol, 2-methoxy-4-(1-propenyl)	0.73	$C_{10}H_{12}O_2$	164	809
4	16.75	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)	1.11	$C_{10}H_{12}O_3$	180	824
5	18.63	Ethyl à-d-glucopyranoside	5.64	$C_8H_{16}O_6$	208	822
6	18.93	Hexadecanoic acid, methyl ester	3.82	$C_{17}H_{34}O_2$	270	715
7	19.66	Hexadecanoic acid, ethyl ester	2.15	$C_{18}H_{36}O_2$	284	866
8	20.07	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy	40.43	$C_{10}H_{12}O_3$	180	812
9	20.97	2-Propenoic acid,3-[4-(acetyloxy)-3-methoxyphenyl]-, methyl ester	2.91	$C_{13}H_{14}O_5$	250	806
10	21.16	9-Octadecenoic acid (Z)-, methyl ester	3.87	$C_{19}H_{36}O_2$	296	858
11	21.33	Ethyl (9z,12z)-9,12-Octadecadienoate	6.79	$C_{19}H_{34}O_2$	294	868
12	21.71	Ethyl 9-Octadecenoate	3.20	$C_{20}H_{38}O_2$	310	849
13	21.87	9,12-Octadecadienoic acid, ethyl ester	7.01	$C_{20}H_{36}O_2$	308	891
14	22.93	11-Eicosenoic acid, methyl ester	6.40	$C_{21}H_{40}O_2$	324	844
15	24.43	13-Docosenoic acid, methyl ester	2.00	$C_{23}H_{44}O_2$	352	762
16	25.59	1,2-Benzenedicarboxylic acid, diisooctyl ester	2.08	$C_{24}H_{38}O_4$	390	756

Table-4. DPPH scavenging activity of milk thistle seeds extract

Parameters	**Free radical scavenging activity "DPPH" (%)**			
	50	**100**	**150**	**200**
	µg mL^{-1}			
Milk thistle seeds	60.561%	75.510%	87.551%	91.684%