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Biological *in Vitro* and *in Vivo* Studies of the Milk Thistle Seed Oil

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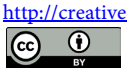

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Abstract

The milk thistle plant is one of the famous plants that have been gaining popularity for its therapeutic potential for centuries. Milk thistle seed oil (MTSO) has been subjected to extensive research. The fixed oil was extracted from the seeds of *Silybum marianum* (L.) using petroleum ether as a solvent by a soxhlet device. GC-MS was used to identify the chemical composition of the oil. The antioxidant activity of MTSO was tested by the ABTS method, which showed the ability to inhibit lipid peroxidation. In addition, antimicrobial and antifungal investigations were examined. It proved that MTSO has an inhibitory effect against Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*), and antifungal effects (*Candida albicans*). MTSO has a slightly higher effectiveness against fungi than bacteria. Moreover, the cytotoxic activities of the oil on hepatocellular and colorectal carcinoma were examined. MTSO has shown a moderate cytotoxic effect on the HCT-116 cell line and a weak effect on HePG-2. Whereas; *in vivo* study has been done on five diagnosed patients who have impaired liver function, and were recruited for the study. Their weight ranged from 100 ± 30 kg, their age range was between 39 - 50 years. Each patient was given ten drops of MTSO daily and added to a little water for a four-week study period. MTSO has effects to improve the function of the injured liver. The present work aims to study Milk thistle seed oil, for estimating its pharmacological properties for the liver. This study focused on showing the importance of milk thistle seed oil in our lives as a source of antioxidants, anti-bacterial and anti-fungal, and as an anti-cancer of the liver and colon. It also sheds light on its importance as a treatment for impaired liver, function and fatty liver, due to its improvement in all liver function markers, so it can be hired as an effective human therapy.

Keywords

Milk Thistle, Antioxidant, Antimicrobial, Anticancer,

Liver Function Markers

1. Introduction

Nowadays, medicinal plants function as a remarkable role in the pharmaceutical industry, as they are considered the prime source of pharmaceutical compounds. So, many researchers attempt to develop methods for extracting these compounds from medicinal plants. Also, they seek to attain effective compounds that have biological properties [1]. So, the great demand for natural products is nowadays due to their natural compounds that benefit humans in various fields and have no side effects. For example, thyme has received excessive attention from professionals due to its antioxidant, antimicrobial, and antimicrobial properties. Also, it has anti-cancer properties and is beneficial in treating stress and respiratory diseases. In addition, It is used in modern applications and studying its effect on Covid 19 [2]. Regarding the pharmacological influences of medicinal plants on the liver, another study analyzed 32 types of medicinal plants. They found that 20 plants of them showed protective and pharmacological properties for the liver. These properties are due to the compound's biological efficacy in these plants [3].

It has been reported that Milk thistle, *Silybum marianum* (L.) Gaertn is a wild thorny herbaceous plant that grows in many areas and is considered a weed up to two meters in height. This plant has a purple rosette with spiky edges and large glossy green leaves of various sizes. The fruits of this herbaceous plant are distinguished by the presence of one seed, and these seeds have a hard, shiny shell of brown color. Milk thistle belongs to the family *Asteraceae* [4] [5].

It has been reported that the seeds of milk thistle (MTS) grown in the northern delta are an acceptable source of protein (25.25%), fat (29.68%), fiber (29.95%), and carbohydrates (38.16%) [6]. The protein of milk thistle seeds (MTS) contained significant quantities of essential amino acids such as leucine, isoleucine, lysine, threonine, and valine compared to sunflower seed protein. But the MTS is insufficient in sulfur-containing amino acids. The amino acid score determined threonine was the most heightened amino acid score of 490, whereas phenylalanine and tryptophan were the first and second identified amino acids [6].

Opyd and Jurgoński (2021) studied the impact of MSTO supplementation on liver disorders in genetically obese rats [7].

The present work aims to study Milk thistle seed oil, for estimating its pharmacological properties for the liver.

2. Materials and Methods

2.1. Materials

2.1.1. Medicinal Plant Materials

In this study, Milk thistle *Silybum marianum* seed oil MTSO was purchased

from the local market in Mansoura, Egypt in November 2020.

2.1.2. Microbial Strains

Escherichia coli and *Staphylococcus aureus* were selected as examples of Gram-negative and Gram-positive bacteria, respectively. *Candida albicans* have also been used as an example of fungi. These microbes were used to estimate the antimicrobial activities of the oil under study.

2.1.3. Cell Lines

The HepG-2 and HCT-116 (hepatocellular and colorectal carcinoma, respectively) cell lines were frozen in liquid nitrogen (−180˚C) and brought from ATCC via a Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. These cancer cell lines were used for evaluating the anticancer activities of oil under study.

2.1.4. Patients

Five diagnosed patients in the clinic of Prof. Dr. Walid El-Sherbiny, Professor of Internal Medicine and Hepatology, Faculty of Medicine, Mansoura University, were recruited for the study. These patients have impaired liver function. These patients were a weight range of 100 ± 30 kg, their age range was between 39 - 50 years, and their BMI (body mass index) was between 24.9 - 40 kg/m^2. Before conducting the study, patients received a complete and detailed explanation about this study, and each patient subsequently signed informed consent. The Scientific Research Ethics Committees in both faculties of medicine and specific education, at Mansoura University, Mansoura, Egypt, approved the study protocol. The excluded patients from this study were those with a history of other liver diseases and those treated with lipid-lowering drugs during the previous four weeks or the four-week study period.

2.1.5. Chemicals

All the chemicals and solvents were of pro-analysis purity and were obtained from Sigma (Sigma–Aldrich GmbH, Germany).

2.2. Methods

2.2.1. Extraction of Milk Thistle Seed Oil

Milk thistle seed oil (MTSO) was extracted using a soxhlet device. Two hundred grams of ground thistle seeds were placed in 1000 ml of petroleum ether as a solvent for 24 h. The MTSO was obtained by evaporating petroleum ether in a rotary evaporator at 40˚C [8].

2.2.2. Preparation and GC/MS Study of Milk Thistle Seeds Oil

The preparation of milk thistle seed oil (MTSO) was performed according to the reported method by Hermenean *et al.* (2015) [9]. Briefly, the powder of the dried milk thistle seeds (MTS) was defatted in the Soxhlet extractor by hexane for 6 h. Then, the petroleum ether was evaporated under a vacuum from the collected hexane extract in a rotary evaporator. After hexane evaporation, a yellow oil

(MTSO) was the residue.

The MTSO was analyzed using a Shimadzu QP-2010 GC/MS instrument (Shimadzu, Kyoto, Japan). The GC/MS analysis for MTSO is according to the method stated by Hermenean *et al.* (2015) [9]. One mg of MTSO was dissolved in one ml of hexane to prepare the MTSO stock solution. For GC/MS measurements, one µl of the MTSO stock solution was injected into the gas chromatograph. Separation of the compounds in MTSO was carried out by a Zebron ZB-5MS column (30 m × 0.25 mm × 0.25 µm). The temperature of the column oven was initially at 60˚C, elevated to 300˚C at a rate of 10˚C/min. The temperature injector was 200˚C, and the temperatures of the electron ionization (EI) ion source and the interface were 300˚C. Helium was used as carrier gas. The split injection was conducted with a ratio of 10:1. The ionization energy was 70 eV for the mass spectroscopy detector, the scan range was 40 - 500 amu., and the scan rate was 0.20 s per scan. The detected components' identification in MTSO was carried out by comparing their mass spectra and retention times (RT) with those of the NIST 98 and data of the GC/MS system of Wiley libraries and literature data [10].

2.2.3. *In Vitro* Antioxidant Activity Evaluation

1) *ABTS Radical Scavenging Method*

The ABTS radical scavenging method was carried out to estimate the antioxidant activity of each sample of both oils and separated compounds under study. The antioxidant activity was tested according to the stated method by Re *et al.* (1999) [11]. 0.1 ml of the tested sample was added to 3.9 ml of ABTS radical solution. After 30 min from the initial mixing, the absorbance was measured at 734 nm. Ascorbic acid was used as a standard. All determinations were carried out in triplicate. The obtained results were expressed as ascorbic acid equivalent. The ABTS radical scavenging activity % of the studied MTSO was calculated using the following equation [12]:

$$\begin{aligned}&\mathrm{ABTS}^{\bullet+}\ \text{scavenging}\ \%\left(\text{Inhibition}\ \%\right)\\&=\frac{\text{Absorbance of control}-\text{Absorbance of sample}}{\text{Absorbance of control}}\times 100\%\end{aligned}$$

2) *Oxidative Hemolysis Inhibition Method*

The oxidative hemolysis inhibition method was used to study the antioxidant activity of the tested oils and separated compounds. This activity is estimated based on inhibiting membrane damage to red blood cells by free radicals. Erythrocytes were used as oxidizable targets and peroxyl radicals as pro-oxidants in this method. Briefly, after drawing blood samples from all participants, erythrocytes were isolated. Different concentrations of the tested oil were mixed with a 10% suspension of erythrocytes according to the stated method in Elgendy and Semeih (2019) [13]. The Absorbance can be determined at 540 nm. Ascorbic acid was used as a standard. The following equation calculated the hemolysis percentage.

$$\text{Hemolysis percentage} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

2.2.4. *In Vitro* Anti-Microbial Activity Evaluation

The evaluation of the antibacterial impact of each sample of both oils and separated compounds under study was, according to the reported method by Rizk *et al.* (2018) [14]. The bacterial strains used were *Escherichia coli* and *Staphylococcus aureus* (Gram-negative and Gram-positive, respectively). One fungus (*Candida albicans*) was used to study the antifungal activity of the tested oils and compounds. Each sample of oils and separated compounds under study was dissolved separately at a concentration of 1 mg/ml in DMSO. Paper discs of Whatman filter paper were sterilized in an autoclave, then soaked in the oil and compounds under study. Afterward, the soaked paper discs were put in the Petri dishes. These dishes contained nutrient agar media (beef extract 3 g + peptone 5 g + agar 20 g) and were inoculated separately with the microbes mentioned above. Finally, the inhibition zones were recorded after incubating these dishes for 24 h at 36˚C. Each treatment was repeated three times. Ampicillin was utilized as a standard for antibacterial activity, whereas clotrimazole was used for antifungal activity.

The below formula calculated the antimicrobial activity % of the oil under study:

$$\text{Antimicrobial Activity \%} = \frac{\text{Zone of inhibition by studied compound}\left(\text{diametre}\right)}{\text{Zone of inhibition by standard}\left(\text{diametre}\right)} \times 100$$

2.2.5. *In Vitro* Anticancer Activity Evaluation

The anticancer activity determination of each sample of both oils and separated compounds under investigation was carried out according to Mosmann (1983) [15]. Two cancer lines; HepG-2 and HCT-116 (hepatocellular and colorectal carcinoma, respectively), were used. The MTT method was used for the anticancer activity determination, and 5-Fluorouracil (an anticancer drug) was used for comparison. The below formula calculated the relative cell viability % after treatment of these cancer cells from the oil sample under study:

$$\text{Relative cell viability \%} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

The IC_{50} value for the oils and separated compounds under investigation is the concentration that causes the death of fifty percent of the cells.

2.2.6. Evaluation of the Therapeutic Effect of the Tested Oils and Compounds against Hepatic Injury

1) Study Design

The therapeutic influence of milk thistle seed oil was evaluated in nonalcoholic fatty liver disease (NAFLD) patients. Five patients in the group were close in

age, gender, and BMI. The milk thistle seed oil (MTSO) group (n = 5) was given ten drops of MTSO daily and added to a little water.

2) Laboratory Assessments

Blood samples were drawn twice from all patients at the study's beginning and after four weeks of the intervention. Blood from each patient was withdrawn on the morning after 12-hour overnight fasting, then left for coagulation, and centrifuged for 15 minutes at 3000 rpm to collect serum for further analyses. After serum isolation, serum was preserved at −20˚C until the required studies. The serum was then assayed for serum AST and ALT (hepatic marker enzymes). These enzymes were evaluated by enzymatic method using diagnostic kits according to Reitman and Frankel's (1957) method [16].

Moreover, serum lipid profile; serum TG, TC (total cholesterol), HDL-C, and LDL-C can be measured by enzymatic method using diagnostic kits according to the methods stated in, [17] [18] [19] [20] respectively. The following equation calculated VLDL-C:

The atherogenic indices were calculated using the formulas stated by Gol *et al.* (2021).

- Atherogenic Index of Plasma (AIP) = $\log\left(\frac{\text{TG}}{\text{HDL}-\text{C}}\right)$.
- A value of AIP lower than 0.11 points to a low risk of CVD (cardiovascular disease). However, AIP values in the intermediate and high CVD risks are between 0.11 to 0.21 and upper than 0.21, respectively [21].

2.2.7. Statistical Analysis

Statistical calculations were carried out by SPSS V17.0 (SPSS Inc, Chicago, USA). A comparison of the effect of oil was accomplished on hepatic marker enzymes and serum lipid profile between all groups. A T-test was used for comparison between the means of the two groups. A paired-samples T-test was conducted comparing the means for the same group before and after therapy. The difference is considered to be significant if the calculated $P \leq 0.05$.

3. Results and Discussion

There is a great demand for finding new, safe, and inexpensive effective drugs for the management of liver disorders. Principal candidates in this finding process are natural plant products because of their curative effects. Various chemical and biological investigations are nowadays carried out with natural plant products. From these products, plant extracts and fixed oils. Milk thistle seed oil was chosen to be studied here. Chemical composition, antioxidant, antimicrobial, and anticancer activities for the fixed oil of milk thistle from the plant was tested. As well as, its effect on disordered liver patients was studied.

3.1. Phytochemical Analysis for the Milk Thistle Seed Oil (MTSO) (Fixed Oil)

The maximum MTSO extracted by a Soxhlet was found to be 1%. **Table 1**

Table 1. The GC-MS of chemical constituents of MTSO.

Compounds	Molecular Formula	Retention time (min)	Area %
Ribitol	$C_5H_{12}O_5$	4.13 - 4.40	5.80
1-deoxy-d-ribitol	$C_5H_{12}O_4$	4.77	2.76
á-Curcumene	$C_{15}H_{24}$	6.45	2.61
R(+)-Limonene	$C_{10}H_{16}$	7.01	1.98
1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$	7.71	0.95
Retinol	$C_{20}H_{30}O$	16.67	1.85
2,6,10,10-Tetra methyl bicyclo[7.2.0] undeca-1,6-diene	$C_{15}H_{24}$	16.99	1.05
Retinal	$C_{20}H_{28}O$	20.07	4.19
Oxiraneundecanoic acid, 3-pentyl-, methyl ester,	$C_{19}H_{36}O_3$	24.28	1.29
Oleic acid	$C_{18}H_{34}O_2$	25.34 - 25.84	7.55
10-Octadecenoic acid, methyl ester (oleic acid methyl ester)	$C_{19}H_{36}O_2$	27.02	5.13
Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	27.94	7.66
Linoleic acid	$C_{18}H_{32}O_2$	28.30	34.69
Hi-oleic safflower oil	$C_{21}H_{22}O_{11}$	30.14	0.93
Ethyl iso-allocholate	$C_{26}H_{44}O_5$	30.74 - 30.85	3.38
2-Hydroxy-3-[(9e)-9-octadec enoyloxy]propyl(9e)-9 octadecenoate (1,3-dielaidin)	$C_{39}H_{72}O_5$	36.52	3.98
(Z,Z)-1,3-Dioctadecenoylglycerol	$C_{39}H_{72}O_5$	36.70 - 36.81	2.16
Unidentified compounds			12.04
Total identified			**100%**

showed a detailed description of all of the compounds detected in the MTSO by GC/MS and their RT value. The GC/MS analysis of MTSO revealed the existence of 17 components. Among these identified compounds, it was found that the major components in this oil were linoleic acid (34.69%) followed by linoleic acid ethyl ester (7.66%), then oleic acid (7.55%) and oleic acid methyl ester (5.13%). In addition, it is clear from this table that the phytochemical analysis of MTSO revealed that the major components in this oil are fatty acids. This result agrees with that obtained by Adetuyi *et al.* (2021); Kleymenova (2021) [22] [23].

From data represented in **Table 1** and **Figures 1-4**, it was found that the main peak was at a retention time (RT) of 28.30 min. This peak was for a compound that had a relative abundance of 34.69%, molecular formula $C_{18}H_{32}O_2$, and molecular weight of 280.452. It is clear from the above-mentioned results that the compound which is the major compound in MTSO was linoleic acid

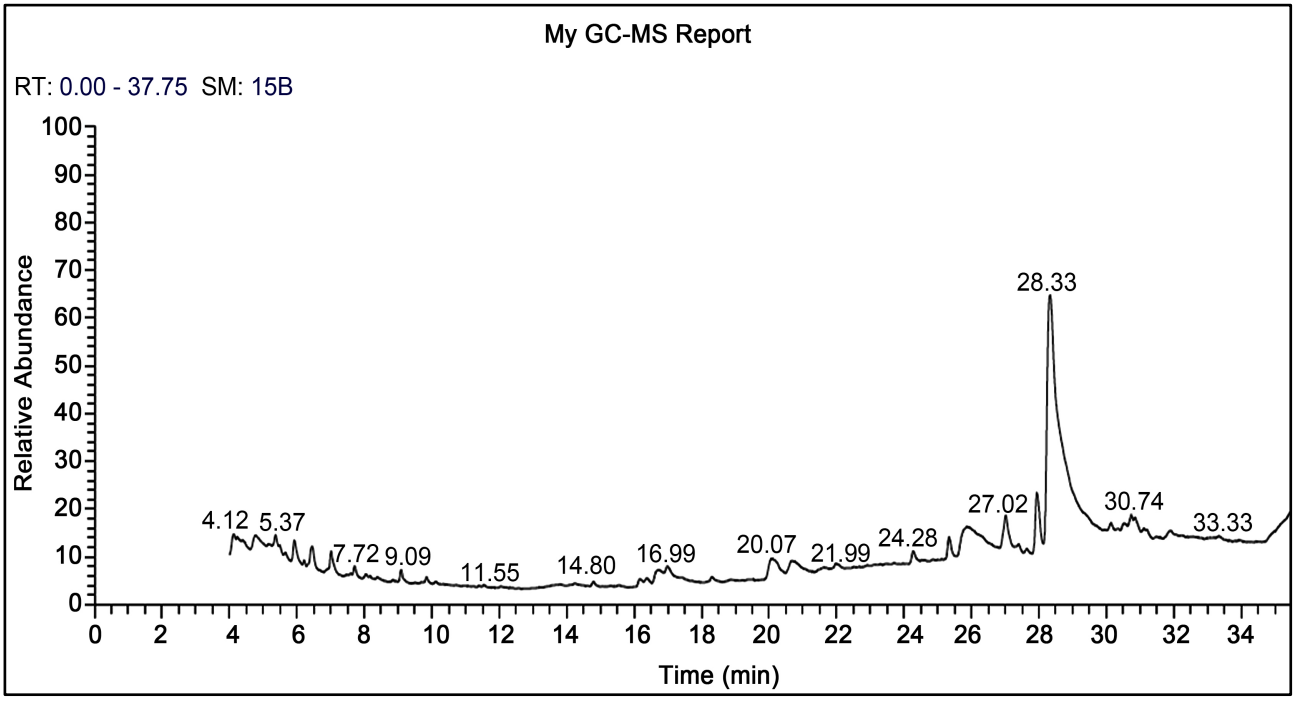

Figure 1. Chromatogram of the components of milk thistle seed oil (MTSO) by GC/MS.

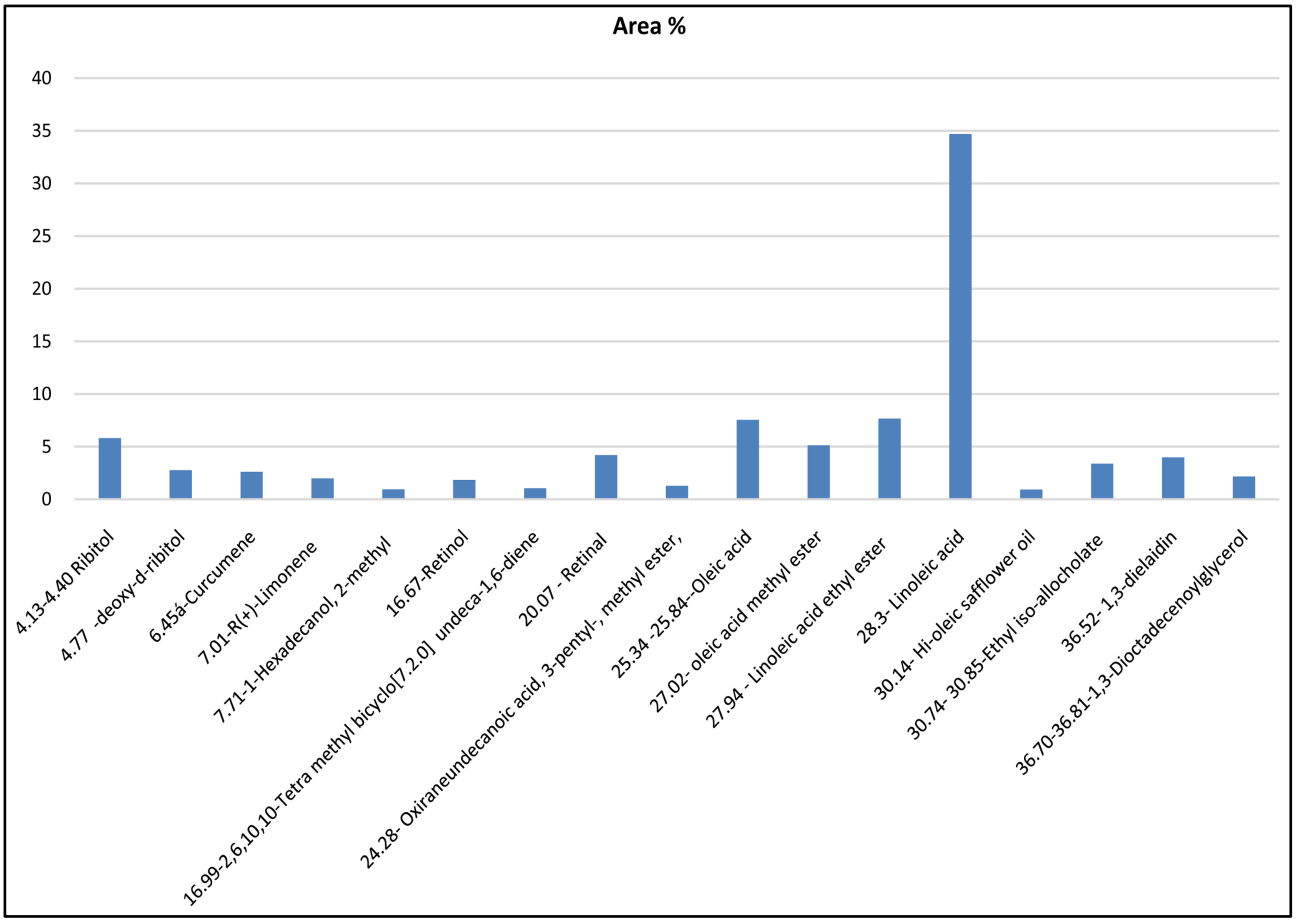

Figure 2. The retention time (RT) and area % for chemical constituents of milk thistle seed oil (MTSO).

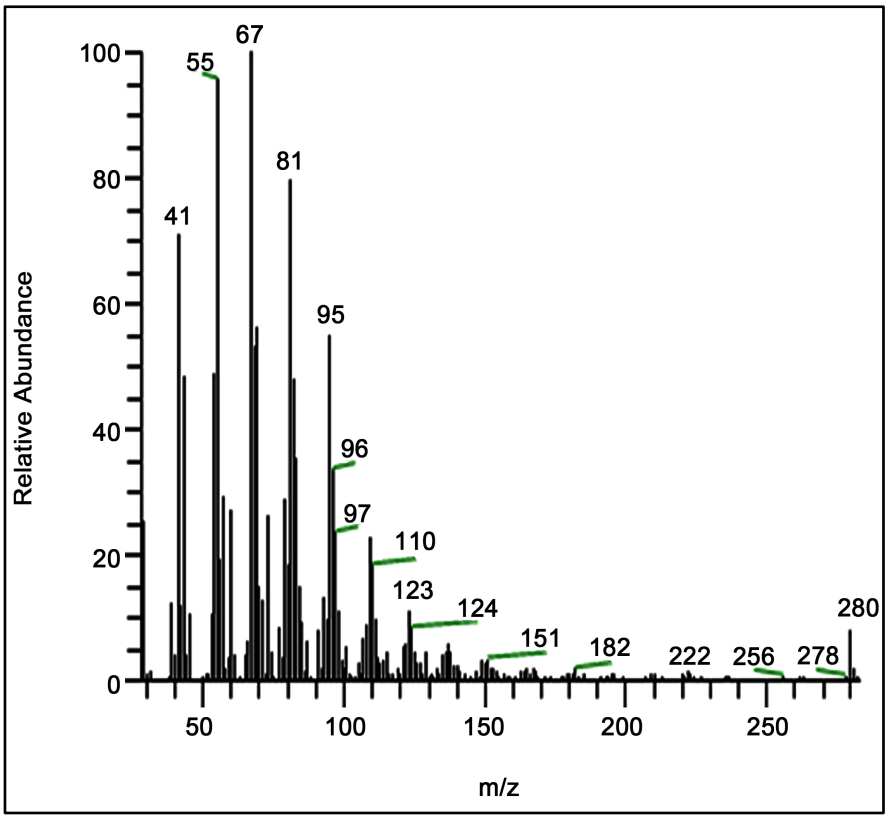

Figure 3. Fragmentation diagram of linoleic acid (the major component of MTSO).

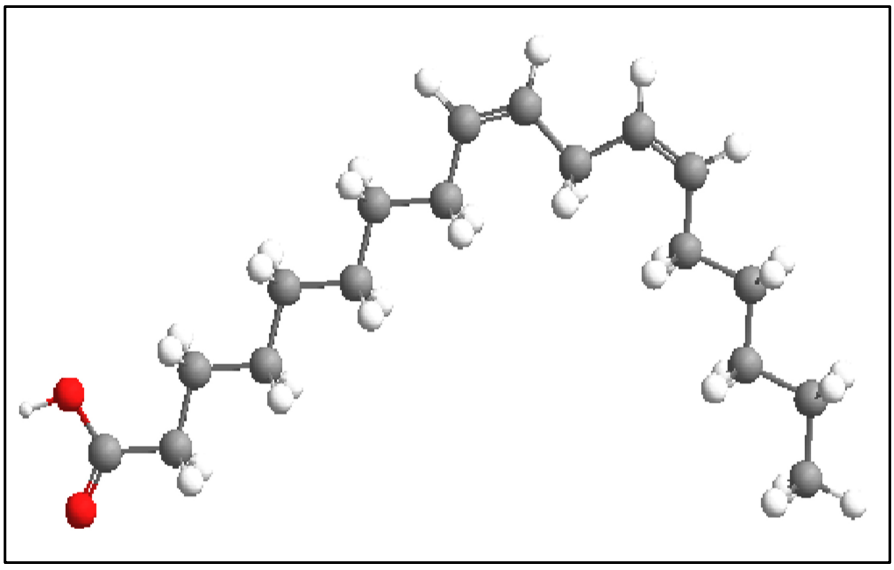

Figure 4. Chemical structure of linoleic acid.

[9,12-Octadecadienoic acid (Z, Z), III]. These results agree with that obtained by Kleymenova (2021) [23] who reported that the major component of the MTSO was Linoleic acid. The fragments of linoleic acid are 280 $(M)^+$, 222 $(M\text{-}CO_2CH_2)^+$, 182 $(M\text{-}C_7H_{14})^+$, and 67 (C_5H_7), with base peak 280 m/z are shown in **Figure 3**. The chemical structure of linoleic acid is shown in **Figure 4**.

3.2. Antioxidant Activity of the Milk Thistle Seed Oil (MTSO)

3.2.1. Antioxidant Activity of MTSO by ABTS Assay

It has been reported that free radicals are implicated in lipid peroxidation reactions and are also represented a major role in many chronic diseases. Therefore, the capability to scavenge free radicals is an important antioxidant feature to protect the human body from the harmful effects of free radicals [24]. In many *in vitro* studies, Elgendy *et al.* (2017); Lim *et al.* (2022) [25] [26] reported that there were many of the essential oils exhibited a noticeable antioxidant activity.

It has been stated that the ABTS test was first developed as a simple and convenient method used to measure total antioxidant capacity (TAC) [27]. The ABTS assay is cheap and operationally simple, so it was used in this work. From the data shown in Table 2 and Figure 5, it is clear that MTSO has weak antioxidant activities.

The result for MTSO disagrees with that obtained by Nowak *et al.* (2021) [28] who reported that MTSO did not show an ABTS radical scavenging activity.

Table 2. Antioxidant activity screening of the MTSO.

Compound	2,2'-azino-bis(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS)		
	Absorbance of sample	% inhibition ($ABTS^{\bullet+}$ scavenging %)	P-Value (Significance)
Ascorbic acid	0.061	88.0	-
MTSO	0.452	11.4	0.000***

Each value represents the Mean ± standard deviation for three determinations. $ABTS^{\bullet+}$ scavenging (% inhibition) = (A control − A test)/A control × 100%. Ascorbic acid (vitamin C) was used as a standard antioxidant (positive control). ***Very high significant difference ($P \leq 0.001$).

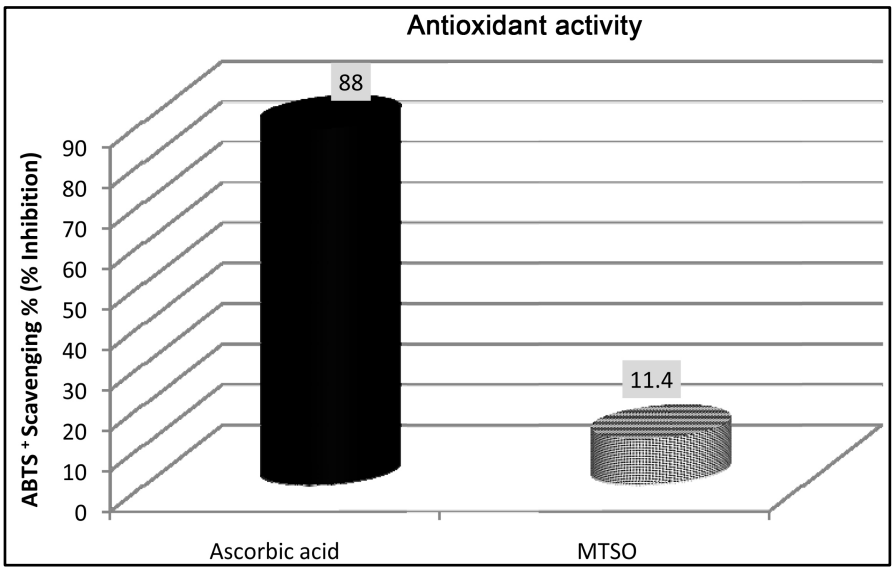

Figure 5. Antioxidant activity screening (% inhibition) of MTSO by ABTS method.

Therefore, it can be considered a good source of natural antioxidants and can be used for preventing or minimizing lipid oxidation in food products, maintaining nutritional quality, and prolonging the shelf life of food.

3.2.2. Anti-Oxidant Activity Screening Assay Erythrocyte Hemolysis

The MTSO was examined for anti-oxidant activity as reflected in the ability to inhibit lipid peroxidation rate erythrocyte hemolysis. It manifested potent anti-oxidative activity in the lipid peroxidation assay, which showed high inhibitory activity in the hemolysis assay [29].

T-test result indicated that Milk thistle seed oil (MTSO) showed a very high significant difference ($P \leq 0.001$) with ascorbic acid.

The results as shown in Table 3 and Figure 6 indicate that Milk thistle seed oil (MTSO), has good Anti-hemolytic activity.

Anti-hemolytic Assay

3.3. Anti-Microbial Activity and Anti-Fungal Activities of the Milk Thistle Seed Oil (MTSO)

Data concerning the *in vitro* anti-microbial activities (anti-bacterial and anti-fungal activities) are represented in Table 4 and Figure 7. On the bases of the diameter of the inhibition zone, the greater the diameter of the inhibition, the greater in % of ant-imicrobial activity. Concerning the anti-bacterial activity of the oil, it is clear that the MTSO had weak inhibitory activity against the growth

Table 3. Anti-Hemolytic assay (% of Hemolysis) of the MTSO compared to Vit – C.

Compound	% Hemolysis	P-Value (Significance)
Vit - C	3.8	
Milk thistle seed oil (MTSO)	13.5	0.000***

***Very high significant difference ($P \leq 0.001$); **High significant difference ($P \leq 0.01$).

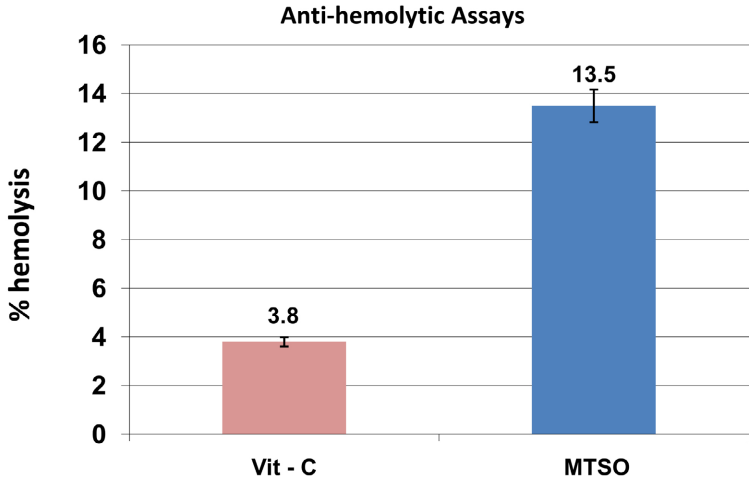

Figure 6. Anti-hemolytic assay (% of hemolysis) of MTSO.

Table 4. Anti-microbial activity of the oils and separated compounds under study.

Compound	*Escherichia coli*			*Staphylococcus aureus*			*Candida albicans*		
	Diameter of inhibition zone (mm)	Activity index (%)	P-Value (Significance) with Ampicillin	Diameter of inhibition zone (mm)	Activity index (%)	P-Value (Significance) with Ampicillin	Diameter of inhibition zone (mm)	Activity index (%)	P-Value (Significance) with Colitrimazole
Ampicillin	26	100		26	100		0	0	
Colitrimazole	0	0		0	0		27	100	
MTSO	6	23.1	0.000***	7	29.2	0.000***	9	33.3	0.000***

Milk thistle seed oil (MTSO). ***Very high significant difference ($P \leq 0.001$).

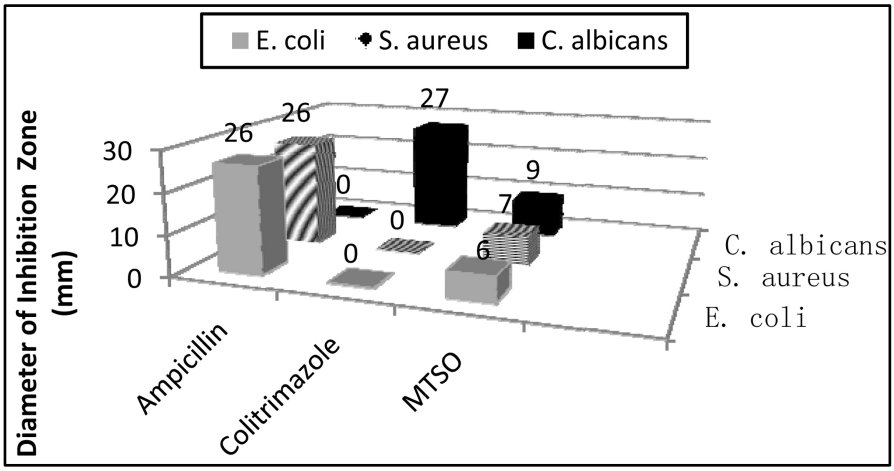

Figure 7. Anti-microbial activity of the MTSO on *E. coli* and *S. aureus and C. albicans.*

of *Escherichia coli* and *Staphylococcus aureus*. The results for MTSO were completely consistent with those who found that the n-hexane and chloroform extracts (at room temperature) exhibited a weak impact against the studied bacteria [30].

Among the suggested mechanisms for the anti-bacterial activity of the tested oil, is the disturbance of the cell membrane increasing in permeability, loss of cell contents, damage of enzymes, and dysfunction of the system-activated proton transfer [31].

Concerning the anti-fungal activity of the milk thistle seed oil (MTSO) against *Candida albicans,* it is clear that MTSO had weak inhibitory activity against *Candida albicans*. The results for MTSO were completely consistent with those who found that the n-hexane and chloroform extracts (at room temperature) exhibited a weak impact against *Candida albicans* [30].

3.4. *In Vitro* Anticancer Activity of the Milk Thistle Seed Oil (MTSO)

Data concerning the anticancer activity of the tested oil on HepG-2 and HCT-116 cell lines are tabulated in Table 5, Table 6 and Figure 8.

In Vitro Anticancer Activity Evaluation in HePG-2 and HCT-116 Cell Lines

The activity of the milk thistle seed oil against HepG-2 and HCT-116 cell lines appeared in Table 5, Table 6 and Figure 8. It showed significant anticancer activity against them.

The IC_{50} value for the oil is the concentration that causes the death of fifty

Table 5. Relative viability % of HePG-2 and HCT-116 cell lines after treatment by the MTSO.

Cancer cell line	Concentration of MTSO (µg/ml)						
	100	50	25	12.5	6.25	3.125	1.56
HCT-116	33.6	47.2	61.5	73.4	95	100	100
HePG-2	38.7	52.9	68.1	76.5	95.4	100	100

Table 6. Cytotoxic activity of the tested oil against hepatocellular carcinoma (HepG-2) and colorectal carcinoma (HCT-116) cell lines.

Oil/compounds	HepG-2	HCT-116
	IC50 (µg/ml)	IC50 (µg/ml)
5-FU	7.9 ± 0.28	5.3 ± 0.31
MTSO	57.17 ± 3.2	44.87 ± 2.9

IC_{50} (µg/ml): 1 - 10 (very strong), 1 - 20 (strong), 21 - 50 (moderate), 51 - 100 (weak), and above 100 (non-cytotoxic).

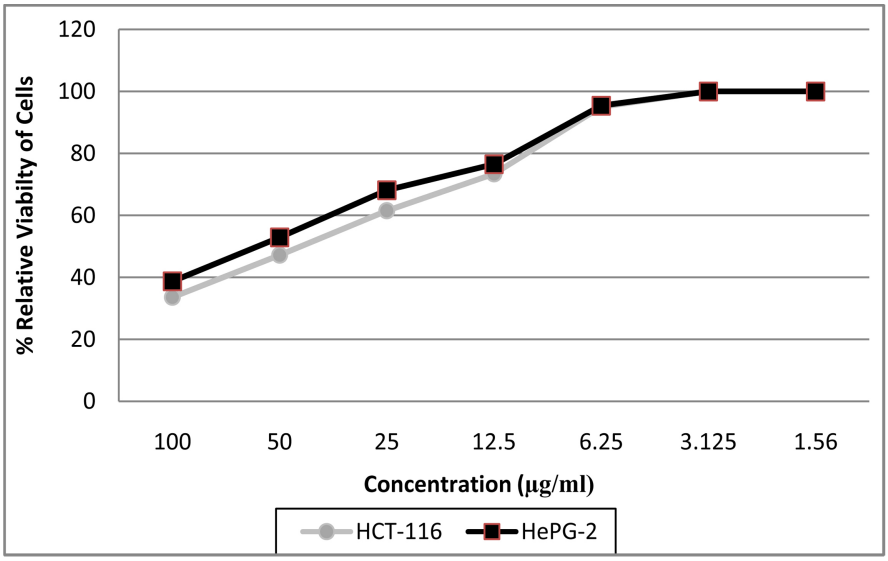

Figure 8. Relative viability % of HePG-2 and HCT-116 cell lines after treatment by the MTSO.

percent of the cells. Data concerning the anticancer activity of the Milk thistle seed in the HePG-2 and HCT-116 cell lines on the bases of IC_{50} value are tabulated. The MTSO showed a moderate cytotoxic effect on HCT-116 cell lines and a weak effect on HePG-2. Therefore, MTSO may be played an important role in colon cancer prevention or treatment more than liver cancer.

3.5. *In Vivo* Hepato-Therapeutic Effect of the Milk Thistle Seed Oil (MTSO) for Hepatic Disorder Patients

In *Vivo* hepato-therapeutic effect was carried out to investigate the effect of MTSO in thirty diagnosed patients, having elevated liver function tests. Biochemical studies were carried out to declare the effect of MTSO on liver functions, some serum antioxidant enzyme activities, and serum lipid profile.

Biochemical Analysis

Lipid profile and liver biomarker analysis:

Data concerning the liver functions and Serum lipid profile markers; are represented in Table 7, Table 8 and Figures 9-14. The studied liver functions are alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The studied Serum lipid profile, TG (triglycerides), TC (total cholesterol), LDL-C (low-density lipoprotein cholesterol), and HDL-C (high-density lipoprotein cholesterol) were determined by enzymatic method using diagnostic kits. These markers were studied for patients before and after treatment. They were evaluated after oral administration of MTSO. The atherogenic index in NAFLD patients appeared in Table 9. The atherogenic indices were calculated using the formulas stated by Gol *et al.* (2021).

$$\text{Atherogenic Index of Plasma}\left(\text{AIP}\right) = \log\left(\frac{\text{TG}}{\text{HDL}-\text{C}}\right)$$

Table 7. Comparison between serum AST and ALT levels in NAFLD patients before and after oral administration of MTSO.

Age	Weight	AST (U/L)			ALT (U/L)		
		before	after	% Change	before	after	% Change
49.4 ± 12.6	102.0 ± 10.6	50.75 ± 10.82	33.25 ± 5.04	−34.48	68.67 ± 19.68	50.67 ± 14.17	**−26.21**

Table 8. Comparison between Serum lipid profile in NAFLD patients before and after oral administration of MTSO.

Cholesterol TC (mg/dl)			HDL-C (mg/dl)			LDL-C (mg/dl)			Triglycerides TG (mg/dl)		
before	after	% Change	before	after	% Change	before	after	% Change	before	after	% Change
192.2 ± 18.6	141.5*** ± 5.7	−26.4	43.6 ± 3.6	49.0 ± 5.0	+12.4	125.7 ± 15.5	106.0 ± 8.3	−15.7	201.0 ± 13.00	162.0 ± 19.00***	−19.4

*Statistically significant difference between before and after treatment at $p \leq 0.05$. **Statistically highly significant difference between before and after treatment at $p \leq 0.01$. ***Statistically very highly significant difference between before and after treatment at $p \leq 0.001$.

Table 9. Atherogenic indices in NAFLD patients before and after oral administration of MTSO.

Group	API Atherogenic Index of Plasma (AIP) = $\log\left(\frac{TG}{HDL-C}\right)$		
	before	after	% Change
MTSO	0.66	0.52	−21.2

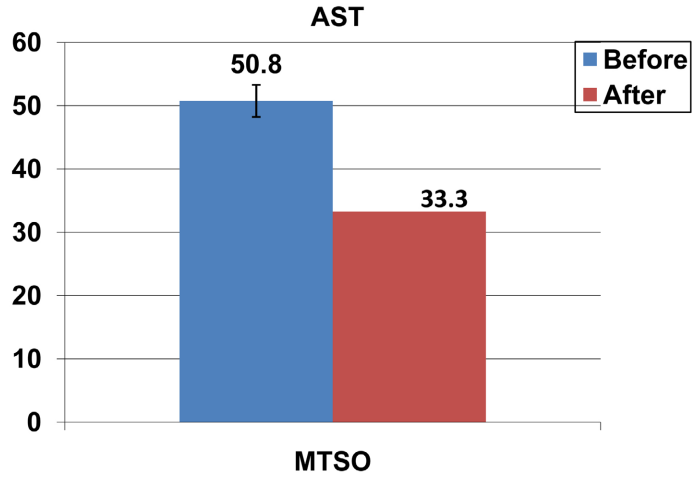

Figure 9. Mean AST of the MTSO group before and after treatment.

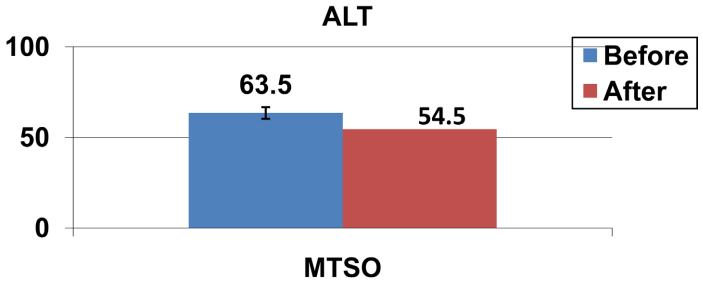

Figure 10. Mean ALT of the MTSO group before and after treatment.

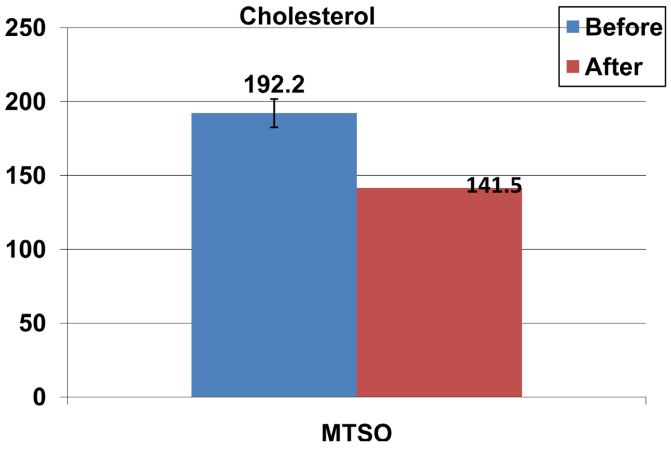

Figure 11. Mean cholesterol of the MTSO group before and after treatment.

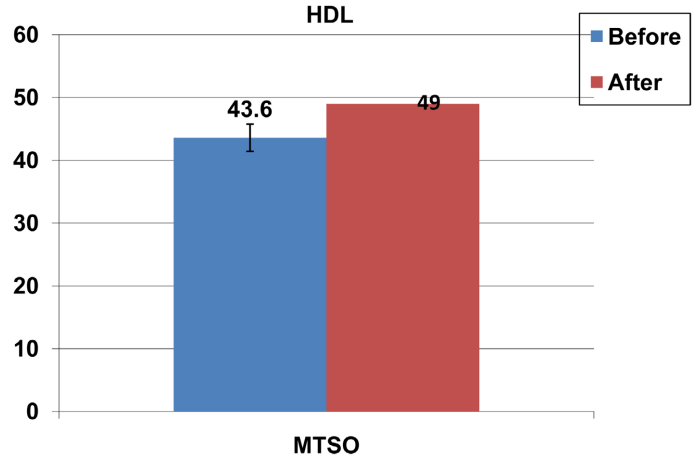

Figure 12. Mean HDL of the MTSO group before and after treatment.

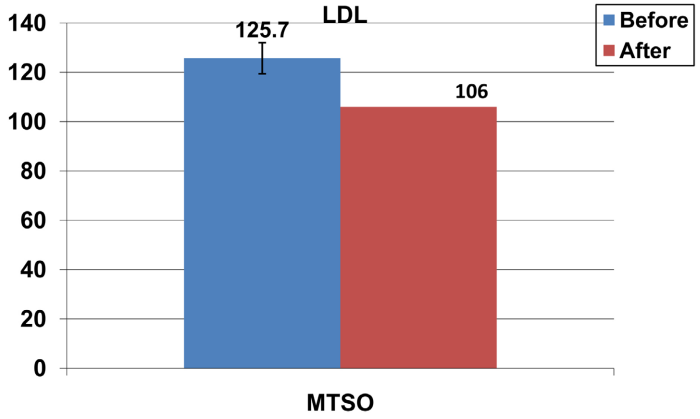

Figure 13. Mean LDL of the MTSO group before and after treatment.

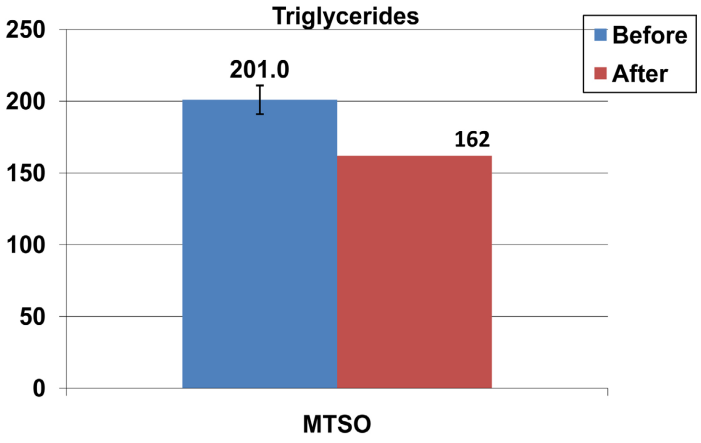

Figure 14. Mean TG of the MTSO group before and after treatment.

$$\text{Atherogenic Index of Plasma}\left(\text{AIP}\right)=\log\left(\frac{\text{TG}}{\text{HDL}-\text{C}}\right)$$

It is clear from Table 7 and Figure 9 and Figure 10 that MTSO decreased serum ALT and AST levels in NAFLD patients. It showed a moderate decrease

percentage.

The result for MTSO agrees with that obtained by Hermenean *et al*. [9], who found that MTSO decreased serum ALT and AST levels in CCl_4-intoxicated rats.

From the data in **Table 8** and **Figures 11-14**, it is clear that the serum TC, LDL-C, and TG levels were high in NAFLD patients' serum before treatment. Also, serum HDL-C level was low in NAFLD patients' serum before treatment. Similar results were obtained by Chatrath *et al*. [32]. The accumulation of lipids in hepatocytes causes liver damage and triggers inflammation, fibrosis, and cirrhosis [33].

MTSO showed lowered serum TC and TG. The decreasing triglyceride effect may be attributed to suppressing the hepatic fatty acid synthase. Regarding the value of cholesterol, the results showed that MTSO showed high efficiency in reducing the proportion of cholesterol in the blood. The TC-decreasing effect may be related to their inhibition mechanism on HMG-CoA reductase activity which is the rate-limiting enzyme in the cholesterol biosynthetic pathway. MTSO showed a higher potent in lowering serum TC levels.

MTSO showed high potential in lowering serum LDL-C levels. In addition, MTSO showed medium potential in increasing serum HDL-C levels.

It is clear from **Table 9**, MTSO decreased the Atherogenic indices. A value of AIP lower than 0.11 points to a low risk of CVD (Cardiovascular disease). However, AIP values in the intermediate and high CVD risks are between 0.11 to 0.21 and upper than 0.21, respectively.

The AIP value for the tested NAFLD patients decreased after oral administration of MTSO but did not approach the normal value of AIP. Therefore the NAFLD patients under investigation are still at risk of developing cardiovascular disease.

4. Conclusion

It has been concluded that MTSO has effects to improve the function of the injured liver. This study suggests that MTSO possesses *in vitro* antioxidant, antimicrobial and anticancer activities, and also has a hepato-therapeutic effect against liver function markers. MTSO showed remarkable activity in reducing the values of all these parameters in the blood. So, it can be used as an active therapy for humans.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Graphical Abstract to the Extraction of Milk Thistle Seed Oil