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Investigating the Antioxidant and Anti-Cancer Properties of Milk Thistle Extract Against the HepG2 Cells

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Abstract

Background: Hepatocellular carcinoma (HCC) is the third among all cancer-related causes of death worldwide. The primary cause of HCC development is oxidative stress. Milk thistle (MT) contains numerous phytochemicals that contain antioxidant and hepatoprotective properties. The goal of this study was to investigate MT's chemical composition and antioxidant activity, as well as its bioactive compound's in vitro anti-cancer efficacy against HepG2 human liver cancer cells.

Methods: We analyzed the bioactive components of MT using GC-MS and HPLC methods. The antioxidant activity was measured by the total phenolic, total flavonoid, DPPH, and radical ABTS scavenging. Furthermore, we evaluated the anti-hepatocellular carcinoma activity using the human cell line HepG2. The MTT test was used to calculate the IC50 values of MT. Cell cycle and apoptosis were measured by flow cytometry in the cell lines.

Result: Our findings demonstrated that 11 bioactive compounds with antioxidant potential were identified via GC/MS screening. Eighteen bioactive compounds with anti-cancer and antioxidant capabilities were found through HPLC screening. Furthermore, HPLC analysis demonstrated both high quality and number of amino acids. MT stops the growth of HepG2 cells at an IC50 level of 21.727±0.89 µg/ml. Moreover, MT arrested HepG2 cells in the G1 phase and induced apoptosis.

Conclusion: The findings of this study indicate that bioactive MT extract has potential therapeutic effects on cancer cells, highlighting the need for further research to explore its mechanisms of action and therapeutic applications in cancer treatment.

Introduction

Hepatocellular carcinoma (HCC) ranks as the third foremost cause of cancer-related mortality globally [1]. Increased reactive oxygen species (ROS) levels disrupt the body's defenses, making them harmful to cells. ROS can damage DNA, causing genomic instability and mutations [2]. Moreover, uncontrolled cell division and mutations from these species induce oxidative stress. According to Sharma et al., [3], oxidative stress is a major factor in the advancement of HCC. Moreover, oxidative stress accelerates liver disease to fibrosis, cirrhosis, and HCC [4]. Liver cancer treatment involves surgery, intervention therapy, chemotherapy, and liver transplantation, but results are unsatisfactory. Non-invasive chemotherapy is still a prominent treatment for advanced tumors. However, no systemic chemotherapy has been consistently successful, and liver cancer chemotherapy commonly causes drug-resistant phenotypes. Therefore, it is important to reduce chemotherapeutic drug resistance, which is a serious clinical issue [5].

Herbal antioxidants dramatically minimize oxidative stress. Its numerous phytochemicals make it useful for treating a variety of diseases. Among these natural species, milk thistle (MT) (Silybum marianum L.) is known as a significant and important medicinal plant [6]. The main bioactive ingredient in MT is silymarin. Most of its plant-derived constituents include flavonolignans, flavonoids, and polyphenolic compounds [7]. These compounds are antioxidants and have other biological qualities [8]. The bioactive MT extract has a positive effect on most liver diseases. It exhibits antioxidant and hepatoprotective abilities in preclinical tests [9] and has been used to treat liver disorders [10]. The therapeutic effects of bioactive MT extract are due to its antioxidant properties; it safeguards against lipid peroxidation by neutralizing free radicals and influencing enzyme systems that induce cellular damage, such as fibrosis and cirrhosis. It stabilizes membrane permeability by reducing lipid peroxidation, hence assisting the liver in maintaining glutathione levels [9]. The bioactive MT extract may be hepatoprotective by lowering oxidative stress and cytotoxicity in intact liver cells [10]. The aim of the present research was to examine MT's chemical composition and antioxidant activity, as well as its bioactive component's in vitro anti-tumor activity against HepG2 human liver cancer cells.

Methods

GC-Mass of fatty acids

Fatty acid methyl esters (FAME) are produced when fats react with methanol in the presence of 2M potassium hydroxide. The resultant mixture is subsequently introduced into hexane. The Agilent GC-MS system was outfitted with a GC (7890B) and an MS (5977A). A DB-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness) was fitted to the GC. Helium served as the carrier gas, flowing at a rate of 3.0 milliliters per minute. A 1 µl splitless injection volume was employed with a temperature program. The temperatures of the injector and detector were kept at 250°C and 320°C, respectively. The mass spectra of the components in the MT extract were obtained using electron ionization (EI) at 70 eV. The spectral range was set between m/z 50 and 600, allowing for the detection of a wide range of ionized fragments. The fragmentation patterns observed in the mass spectra were compared with the reference data from the NIST and Wiley Mass Spectral Libraries to identify the individual components of the extract.

HPLC analysis of amino acids

5 mL of HCl and 5 mL of H2O were mixed with 0.1 g of MT (total concentration: 6 M), heated for 24 hours at 120 °C, and then filtered. After drying, 1 mL of the filtrate was reconstituted in 0.1 M HCl and injected into an Agilent 1260 Series HPLC system for separation. The Eclipse plus C18 column (4.6 mm × 250 mm i.d., 5 µm) was employed for the separation of compounds. The mobile phases consisted of buffer [A] (sodium phosphate dibasic and sodium borate, pH 8.2) and [B] (a mixture of acetonitrile (ACN), methanol (MeOH), and water in a 45:45:10 ratio). The flow rate was set at 1.5 mL/min, and a serial linear gradient was applied for mobile phase programming. For detection, a fluorescence detector was used.

HPLC analysis of phenolic

HPLC is a preferred hyphenated analytical method for measuring specific bioactive secondary metabolites in herbal products [11]. An Agilent 1260 Series HPLC system was used to quantify and qualitatively analyze the phenolic components in the MT extract. Separation was carried out on a Zorbax Eclipse Plus C8 column (4.6 mm × 250 mm i.d., 5 µm particle size). The mobile phases consisted of [A] water and [B] 0.05% trifluoroacetic acid in acetonitrile, with a flow rate set at 0.9 mL/min. A linear gradient was applied to gradually program the mobile phase composition during the analysis. A 5 µL sample solution was injected, and the column was maintained at a constant temperature of 40°C to ensure stable separation. Detection was carried out using a multiwavelength detector set at 280 nm to monitor the phenolic compounds.

Antioxidant activity of MT extract

1,1-diphenyl-2-picryl-hydrazyl [DPPH•] was employed to quantify MT's capacity to scavenge free radicals. To

determine the percentage inhibition of the DPPH free radical, the following formula was utilized:

Inhibition [%] = 100 × [$A_{control} - A_{sample}$]/$A_{control}$

Using a Trolox acid calibration curve, antioxidant activity was measured in mg of Trolox equivalent (TE) per gram of sample. Radical ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity was evaluated according to Hwang and Do Thi [12]. Trolox was employed to establish the standard curve, and the findings were expressed as (mg TE/g) of material. The total phenolic (TP) content was evaluated utilizing the Folin-Ciocalteu methodology [13]. TP content was quantified in mg of gallic acid equivalent per gm of material (mg GAE/g). The total flavonoid (TF) amount was ascertained by employing the aluminum chloride ($AlCl_3$) colorimetric assay, in accordance with Zilic et al., [13]. TF content was measured in mg of catechin equivalent per gram of sample using a catechin calibration curve.

Cell lines and MTT assays to detect cell viability

HepG2 cells were cultured in minimum essential media enriched with 100 units/mL penicillin, 10 percent fetal bovine serum, and 2 mm glutamine at 37 °C in a humidified condition of 5% CO2 and 95% air. The MTT test for metabolic activity is used in almost all cell toxicity investigations [14]. A 96-well plate was used to seed HepG2 cells. Cells were exposed to samples at varying doses (0.4–100 µg/ml). Following a 48-hour treatment interval, MTT was introduced to each well and incubated at 37°C for 4 hours. Thereafter, DMSO was introduced to each well and incubated for 15 minutes to dissolve the formazan crystals. The absorbance was quantified at 450 nm, and the inhibitory concentration (IC50) was determined.

Cell cycle and apoptosis assay by flow cytometry

The distribution of the cell cycle was analyzed using flow cytometry [15]. Cells were inoculated on well plates and treated with test substances for 48 hours. Fixing the cells overnight in 70% ice-cold ethanol after two phosphate buffered saline (PBS) washes. The cells were subsequently treated for 30 min in a PBS solution containing propidium iodide and RNase. Apoptotic cell death is evaluated using annexin V-FITC labeling. Thereafter, these cells were stained with Annexin V-FITC/PI and incubated for 15 minutes at room temperature in darkness. The proportion of apoptotic cells was evaluated with the BD FACS Calibur flow cytometer.

Statistical analysis

The data were expressed as the mean ± SD. The analyses were performed utilizing the SPSS statistical software (version 16.0). The IC50 values were ascertained using the GraphPad Prism program (version 5.01).

Results

GC-Mass of fatty acids

GC-MS is a trusted analytical tool for profiling secondary metabolites such as fatty acids [16]. Using GC-MS, our study evaluated MT bioactive compounds. Figure 1 displays the eleven most prevalent volatile MT compounds. There were mainly 9,12-octadecadienoic acid, methyl ester (27.42%); n-hexadecanoic acid, methyl ester (26.7%); 9-octadecenoic acid, methyl ester (21.39%); octadecanoic acid, methyl ester (14.13%); eicosanoic acid, methyl ester (4.62%); docosanoic acid, methyl ester (1.83%); 17-octadecynoic acid, methyl ester (1.45%); nonanedioic acid, dimethyl ester (0.97%); tetradecanoic acid, methyl ester (0.88); and dodecanoic acid, methyl ester (0.39%) in the MT extract.

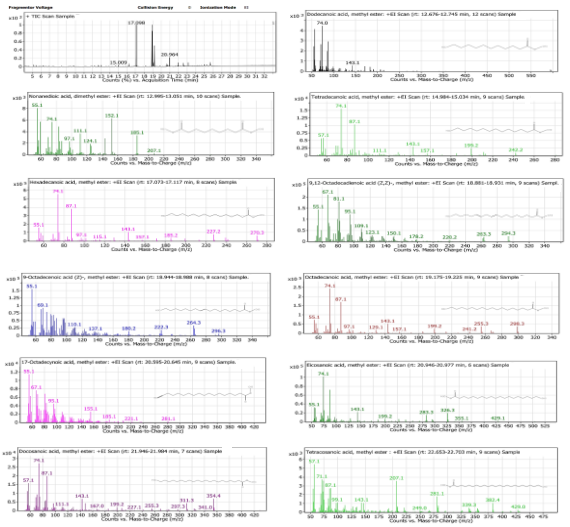

Figure 1: GC-MS chromatogram and major bioactive compounds were identified in the extract of MT. The peaks represent different bioactive compounds in the extract, determined by their retention times (RT) and mass spectra.

HPLC of amino acids

Figure 2 illustrates the amino acid content of the MT extract. MT contains a satisfactory quality and quantity of essential and non-essential amino acids. Among these essential amino acids are lysine (4.40 mg/g), leucine (2.48 mg/g), phenylalanine (2.17 mg/g), valine (1.94 mg/g), histidine (0.67 mg/g), threonine (1.54 mg/g), methionine (0.62 mg/g), and isoleucine (1.41 mg/g), as well as nonessential amino acids such as glutamic acid (4.58 mg/g), aspartic acid (4.42 mg/g), proline (3.77 mg/g), glycine (2.12 mg/g), alanine (1.99 mg/g), serine (1.83 mg/g), arginine (1.96 mg/g), and tyrosine (0.95 mg/g). In particular, glutamic acid, aspartic acid, lysine, proline, leucine, and

phenylalanine are more abundant than other amino acids.

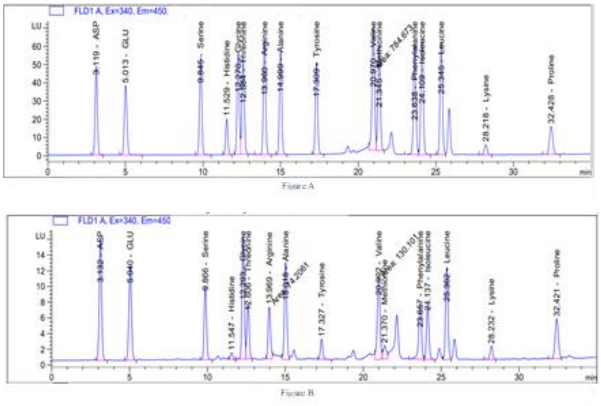

Figure 2: (A) displays the HPLC chromatogram of standard amino acids. (B) shows the HPLC chromatogram of the MT extract. The concentration of each amino acid in the extract is quantified based on the peak area.

HPLC of Phenolic

According to the HPLC analysis results, Figure 3 illustrates the 18 most predominant compounds in the MT extract. The main parts of the MT extract were rosmarinic acid (586.79 µg/g), gallic acid (145.05 µg/g), kaempferol (132.95 µg/g), chlorogenic acid (121.41 µg/g), coffeic acid (71.15 µg/g), syringic acid (33.27 µg/g), naringenin (30.78 µg/g), and ellagic acid (27.74 µg/g). The MT extract also contained coumaric acid (14.65 µg/g), vanillin (9.84 µg/g), methyl gallate (6.56 µg/g), catechin (4.58 µg/g), daidzein (3.74 µg/g), rutin (3.19 µg/g), quercetin (2.94 µg/g), hesperidin (2.62 µg/g), ferulic acid (1.18 µg/g), and cinnamic acid (0.91 µg/g). These compounds exhibit several biological roles, including anti-inflammatory and anti-tumor properties.

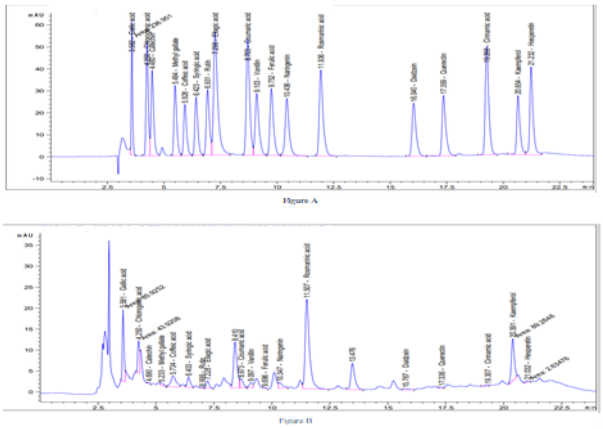

Figure 3: (A) displays the HPLC chromatogram of standard phenolic; (B) shows the HPLC chromatogram of the MT extract. The concentration of each phenolic in the extract is quantified based on the peak area. The figure exhibits the 18 main compounds in the MT extract.

Antioxidant analysis of MT extract

Table 1 showed that MT contained TP (3.77 mg GAE/g), TF (2.625 mg CE/g), DPPH (3.42 mg TE/g), and ABTS (4.47 mg TE/g). These findings suggest that MT exhibits potent antioxidant activity.

Parameter	Concentration
DPPH (mg TE/g)	3.42±0.08
TP (mg GAE/g)	3.77±0.09
TF (mg CE/g)	2.63±0.07
ABTS (mg TE/g)	4.47±0.44

Table 1: Data were expressed as mean ± SD. The DPPH• assay quantifies the free radical scavenging ability of the extract; the TABTS assay measures its capacity to neutralize the ABTS radical cation. The total phenolic (TP) and total flavonoid (TF) contents are important indicators of the extract's antioxidant potential.

Cytotoxic assay

Figure 4 presents the cytotoxicity effects of MT on HepG2 cells using the MTT assay. The IC50 value of MT was determined to analyze its impact on cell viability. Our results show that MT stops the growth of HepG2 cells at an IC50 level of 21.727±0.89 µg/ml, compared to 3.5969±0.15 µg/ml for the standard (staurosporine). These results show that MT can stop the growth of cancer cells, which is strong evidence of MT's powerful antiproliferative effect against HepG2 cells.

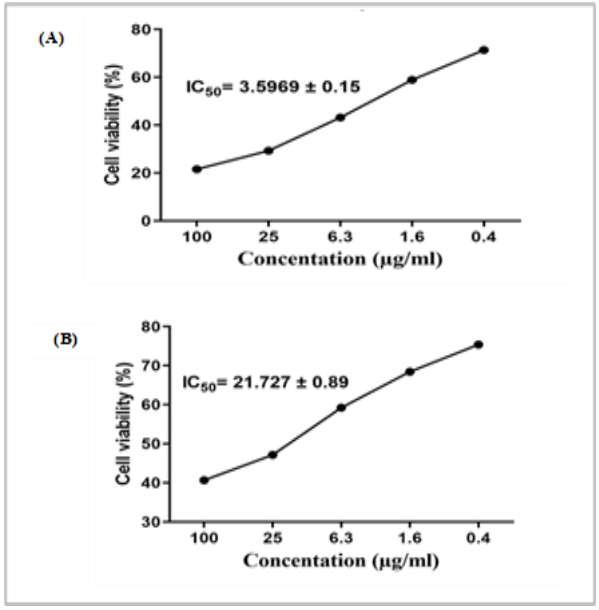

Figure 4: MTT assay: (A) showed the cytotoxic effect of the standard compound, staurosporine, on HepG2 cells. (B) showed the cytotoxic effect of the MT extract on HepG2 cells. The IC50 value for MT was 21.727±0.89 µg/ml, while the standard (staurosporine) was 3.5969±0.15 µg/ml.

Cell cycle and apoptosis analysis

Flow cytometry [17] assessed the cell's stage of arrest subsequent to MT treatments. MT was administered to HepG2 cells at an IC50 concentration. MT elevated the number of HepG2 cells in the G0/G1 phase relative to the control group, concurrently reducing the number of

cells in the S and G2/M phases (Figure 5A). Additionally, flow cytometry and Annexin V/FITC were employed to quantify the proportion of dead cells [18]. The proportion of living cells in the control group remained constant throughout the early and late phases of apoptosis, whereas the proportion of deceased cells in the MT-treated group increased significantly (Figure 5B).

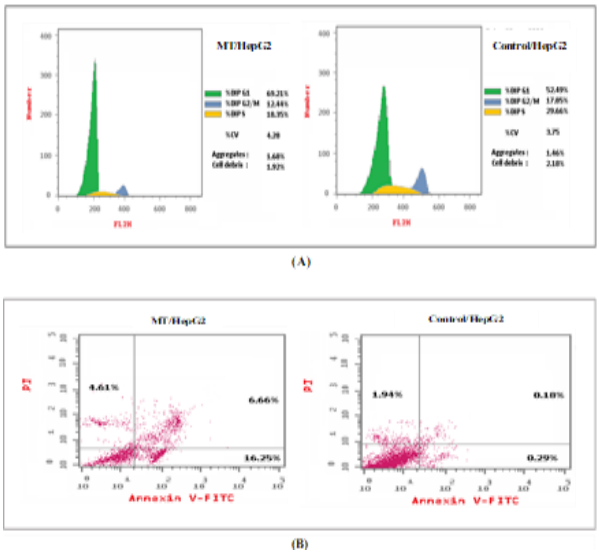

Figure 5: (A) Cell cycle analysis of control HepG2 cells and MT-treated HepG2 cells. The analysis was performed using flow cytometry to assess the distribution of cells in the G1, S, and G2/M phases. (B) Apoptosis analysis of HepG2 cells; the percentage of apoptotic cells in control HepG2 and MT-treated HepG2 cells was measured by flow cytometry.

Discussion

HCC is a severe threat and a main reason for cancer-related mortality worldwide [19]. Natural products represent a novel therapeutic approach in the search for more effective and newer hepatoprotective agents [20]. Medicinal plants include a large number of secondary metabolites that have a variety of biological actions, including antioxidant, antiviral, antibacterial, and anticancer effects [21]. As a result, the objective of this study was to examine MT's chemical composition and antioxidant activity, as well as its anticancer effect on HepG2 cells. The method of GC-MS is significant and has been modified to assess the numerous phytoconstituents and their structures found in different plant extracts. Also, GC-MS can provide quantitative data in addition to the linked mass spectral database, which is extremely valuable for establishing a link between bioactive chemicals and the pharmacological uses for which they are employed [22]. Our results using the GC-MS approach show that MT contained 11 different fatty acids. The majority of these fatty acids are well known for their anti-inflammatory, anti-cancer, and antioxidant properties [23]. Among them, n-hexadecanoic acid (palmitic acid) has anti-inflammatory [24] and anti-cancer effects [25].

HPLC is a widely used method for analyzing MT extracts, allowing for the identification and quantification of bioactive compounds. The HPLC analysis of the MT extract reveals the presence of both essential and non-essential amino acids in good quality and quantity. In particular, glutamic acid, aspartic acid, lysine, proline, leucine, and phenylalanine are more abundant than other amino acids. These amino acids are important for normal growth and function in the body. Antioxidant amino acids histidine, lysine, tryptophan, methionine, cysteine, arginine, and tyrosine have more antioxidant potential than the other 13 amino acids [26].

Polyphenols are antioxidant-rich plant components [27] and could play an important role in hepatoprotective activity [28]. Due to their capacity for scavenging, phenolic compounds are highly significant components of plants. MT is rich in polyphenolic compounds, including flavonoids and phenolic acids, which contribute to its therapeutic properties [29]. Most of these phenolic compounds in MT extract were reported to have antioxidant and anticancer activities against various types of cancer. The primary constituents in the MT extract were rosmarinic acid, which is a safe and effective tumor inhibitor and antioxidant [30]. Moreover, gallic acid has many health benefits; according to Badhani et al., [31], it is a potent antioxidant and apoptosis inducer. Moreover, kaempferol exhibits a number of pharmacological characteristics and is used in cancer treatment. These characteristics include antibacterial, anti-inflammatory, antioxidant, anticancer, cardioprotective, neuroprotective, and antidiabetic effects [32].

Our investigation demonstrated the antioxidant properties of MT extract, as evaluated by the most widely used assays: TP, TF, DPPH, and ABTS. The results of this investigation indicate that MT extract has an important quantity of flavonoids and phenols. Ali et al., [33] attribute the high TP content and antioxidant components of MT extract to the presence of polyphenols. These polyphenols also contribute to the antimicrobial activity of MT, making it potentially effective against multiresistant bacteria [34]. Its antioxidant activities safeguard cells from oxidative stress by neutralizing free radicals and diminishing lipid peroxidation [35]. Dhouibi et al., [36] have demonstrated the anti-cancer properties of MT extract against various types of cancer. Overall, the presence of polyphenols in MT enhances its antioxidant, anticancer, and antimicrobial properties, making it a valuable dietary supplement. Therefore, MT may have a

role in providing effective treatment for a range of illnesses.

Recent studies have found many natural chemo-preventive drugs that suppress cancer cells. Clinical research suggests that the cell cycle is the main target for treating cancer [37]. According to our findings, MT-induced inhibition of proliferation resulted in HepG2 cell cycle arrest. In line with our findings, Kang et al., [38] reported that cells prepare energy and materials for DNA replication in G1, while they relax and exit the cell cycle in G0. Therefore, G0/G1 arrest stops DNA synthesis and mitosis. Therefore, inducing cell cycle arrest is a useful technique to prevent unchecked cell growth [39]. Apoptosis is a crucial function of normal cells that is intended to preserve homeostasis by regulating the number of cells in specific locations and eliminating damaged or defective cells that cannot be repaired by physiological processes [40]. Our results showed that the fraction of dead cells in the MT-treated cells increased dramatically at both the early and late stages of apoptosis as compared to the control. This observation is consistent with previous research showing MT extract disrupts the expression of proteins linked to apoptosis and cell cycle regulators, which can modulate apoptosis in vitro and survival in vivo [41]. Also, Bektur Aykanatet al., [42] demonstrated that MT extract notably enhanced the induction of apoptosis in HepG2 cells. Therefore, the use of natural products like MT may be beneficial in altering metabolic pathways to cause cancer cell death.

This study concluded that MT extract contains many phytochemical compounds with antioxidant properties that can scavenge free radicals. Moreover, it can control the death of cancer cells by stopping the cell cycle and triggering apoptosis. So, our results show that MT is capable of acting as a useful adjuvant to cancer treatment and can act intelligibly in cancerous cells.

Author Contributions

Khateeb S. designed the research plan, methodology, data analysis, writing, review, and editing. Alrashidi A., Albalawi J., Alrwes M., Aloraini N. and Almudayni M.: writing (original draft) and methodology.

Conflict of Interest

The authors declare that there are no conflicts of interest related to the publishing of this paper.

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