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Evaluation of The Effect of Flavonoids Isolated from Silybum Marianum on Lymphocytes of Adult Female Rats and Their Fetuses (MTT Assay)

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ABSTRACT

Objective: *This study aimed to evaluate the effects of flavonoids isolated from* Silybum marianum *(milk thistle) on the lymphocytes of adult female rats and their fetuses, focusing on cytotoxicity and compound characterization.* ***Method:*** *Fresh leaves collected from Sulaymaniyah, Iraq, in January 2025 were subjected to flavonoid extraction and analyzed using High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FT-IR) to identify phenolic compounds. Lymphocytes were isolated from rat blood samples via density gradient sedimentation, and MTT assays were performed to assess cell viability at different extract concentrations (25, 50, and 100 µg/ml).* ***Results:*** *HPLC analysis identified several flavonoids, with 4-hydroxyl benzoic acid (282.5 ppm) and catechol (233.1 ppm) being the most abundant. Cytotoxicity tests showed high lymphocyte viability for both adult females (92.34–81.35%) and fetuses (91.92–81.7%), with IC_{50} values of 324.9 µg/ml and 347.3 µg/ml, respectively.* ***Novelty:*** *The study demonstrates that* S. marianum *leaf extracts are rich in bioactive flavonoids with low cytotoxic effects on lymphocytes, suggesting potential for safe therapeutic use in antioxidant or protective applications.*

INTRODUCTION

Iraqi soil is abundant in medicinal plants, and the Sumerian and Babylonian clays demonstrate that Iraqis have long trusted the use of natural medicines [1]. The diverse climate and geography of Iraq result in a wide range of local health concerns and a variety of plant species [2]. Sillybum marianum is one of these significant wild medicinal herbs; in fact, it's one of the best-selling supplements in the US, generating almost $300,000 in revenue annually [3]. The annual plant known as milk thistle (Silybum marianum, family: Compositae) is indigenous to the Mediterranean region but has since expanded to other warm, dry, and warm regions [4]. It is also found in Iraq, particularly in the north [5]. There are two types of milk thistle: marianum, which is the most common and has a purple corolla, and albiflorum, which has a white corolla. The fruits of the two types are achenes, which are distinguished by their oblong shape and the long white pappus that is united at the base and then sheds [6], [7]. Silybum marianum extract is now utilized as a guide while looking for novel hepatoprotective drugs [8], [9]. Hepatocellular carcinoma [10], cirrhosis, alcoholic liver disease, chronic hepatitis C, growth promotant, chronic liver disease, and jaundice [11], [12] are among the liver conditions that silymarin's hepatoprotective action helps with. Additionally, Silybum marianum extract can help repair liver tissue following hepatoctomy by promoting the cell cycle and preventing apoptosis, which results in the proliferation of hepatocytes, regenerating the lost liver tissue, and restoring the normal mass of the liver [13], [14]. Natural medicines made from medicinal plants (as pure compounds or standardized extracts) provide various therapeutic applications because of the remarkable chemical variety they contain. Numerous bioactive component types can be found in botanical preparations used for therapeutic purposes [15], with natural polyphenols drawing particular interest as possible agents for the prevention and treatment of liver disorders [16]. The most prevalent type of substances found in milk thistle extract [17] are flavonolignans, which are naturally occurring hybrid molecules that biogenetically originate from flavonoids and lignans and have traditionally been employed for hepatoprotection. In addition, the extract's numerous additional health-promoting properties make it a promising substitute source for pharmaceutical and medical applications [17,18]. Therefore, the current study aimed to evaluate the effect of flavonoids isolated from Silybum marianum on lymphocytes of adult female rats and their fetuses (MTT assay).

RESEARCH METHOD

Plant material

The fresh leaves were collected at the full bloom stage for identification in January 2025 from Sulaymaniyah in Iraq. They were washed, air-dried at lab. Temperature then dried in an oven at 50°C till constant weight, and finally ground to fine powder.

S. marianum extraction

Using 95% methyl alcohol, 250 grams of dry powdered S. marianum leaves were extracted. To remove the remaining chlorophyll, the methanolic extract residue was cleaned with benzene before being cleaned with ethyl acetate, a subsequent selective organic solvent. The ethyl acetate fraction had the highest concentration of phenolic compounds, the majority of which were flavonoids, according to the results of the separation procedure [19].

HPLC analysis sample preparation

After dissolving 20 grams of milk thistle leaf powder in 60 milliliters of acetonitrile, the mixture was shaken for 40 minutes in an ultrasonic bath. To get eliminate of fibers and undissolved particles, the extracted samples were next filtered through Whatman filter paper (0.5 µm). After that, the extracts were pre-concentrated using a stream of nitrogen until they reached a level of roughly 0.5 mL. The mobile phase was then used to adjust the content to 1 mL. Then, under ideal circumstances for the effect of separation, 2 µL of the aqueous filtrate was injected onto the HPLC column [20].

Structural analysis of major components

FT-IR spectrum was analyzed to find the most important functional groups of flavonoid extract by KBr disk technique using FT-IR 8400S SIMADZU (Japan) in the central Lab./ Kufa university.

Animals model

20 experimental animals (albino rats) were utilized, five month-aged and weight ranged between 150-175 gm at period Junuriy 2025. After fertilization, a plug, blood, or bloody mucus was observed in the female's vagina 12–24 hours after mating, confirming fertilization. The gestation period was then calculated, and the females were dissected

after day 18 of gestation, and blood was drawn from the mothers and fetuses by cardiac puncture.

Lymphocytes isolation

Each female rat and embryos had their hearts punctured to extract 3 ml of blood using a disposable syringe that contained 10–20 units of heparin/ml (Becton Dickinson, China). According to Boyum, lymphocytes were separated using density gradient sedimentation. After being diluted 1:1 in RPMI 1640 medium (Sigma-Aldrich, USA), blood samples were put on top of 2 milliliters of Ficoll (HiSep™ LSM 1077, Himidea, India). Cooled centrifugation was performed for 20 minutes at 3000 rpm and 18 ^{o}C. Using a 10-ml sterile pipet, transfer the band containing the lymphocytes into a fresh centrifuge tube [21]. Since the blood drawn from the fetal heart is very small, blood samples from the fetuses from the same mother were combined in order to obtain the largest possible amount of blood.

Cell line

The separated lymphocytes (fetuses and mothers) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, USA), which was enhanced with 1% penicillin, 1% streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were moved to a fresh culture dish, treated with trypsin-EDTA (HiMedia, India), and then put in an incubator. At 37 °C and 5% CO_2 concentration, the cells were cultured in a CO_2 incubator (Biobase, China) [22].

Determining the cytotoxicity effects

Lymphocytes were added to a 96-well plate with a final volume of 200μL and a density of $1*10^4$ cells per well. After a confluent monolayer was created, cells were exposed to different quantities of *S. marianum* flavonoids extract (0, 25, 50, and 100) μg/mL. Finally, a plate reader operating at 570 nm was used to measure the absorbance.

Cell Viability

After 48 hours of treatment with different dosages of extract, the anti-proliferative effects were evaluated. To achieve this, the media was removed, 10 μL of MTT solution (5 mg/mL) was added, and the cells were incubated for two hours at 37 °C. Following the separation of the liquid fraction with suspended particles, 100 μL of Dimethyl Sulfoxide (DMSO) (HiMedia, India), was added to dissolve the blue crystals. The following formula was used to get the cell viability rate:

$$\text{Cell Viability} = (\text{Absorbance of treated cells} - \text{Absorbance of the blank}) / (\text{Absorbance of the control} - \text{Absorbance of the blank}) \times 100 \text{ [23].}$$

IC-50 value of the extract of *S. marianum* against lymphocytes cell line was then calculated.

Analyzing data

To perform statistical analysis, SPSS (version 20) was used. Experimental data were presented as mean ± standard deviation (mean ± SD). To ascertain the degree of significance between the treatment and control groups, one-way analysis of variance was utilized. When the difference was $p < 0.05$, it was deemed significant [24], [25].

RESULTS AND DISCUSSION

Results

Flavonoids of *S. marianum*

After conducting an HPLC examination of the extract containing flavonoids to detect their presence and concentrations, table 1 shows the presence of many flavonoid compounds and the concentration of each substance. It was found that 4-hydroxyl benzoic acid had the highest concentration, reaching 282.5 ppm, followed by Catechol 233.1 ppm, Cinnamaldehyde 103.7 ppm, Vanilic acid 58.8 ppm, Cinnamic acid 22.9 ppm, and Kaempferol 12.16 ppm. The least flavonoid compound present in the leaves was Callic acid, reaching 0.171 ppm, see figure 1.

Table 1. Concentrations of flavonoids in the leaf extract using HPLC.

Active ingredients	Quantity
Flavonoids	
4- hydroxyl benzoic	282.5 ppm
Catechol	233.1 ppm
Cinnamaldehyde	103.7 ppm
Vanilic	58.8 ppm
Cinnamic	22.9 ppm
Kaempferol	12.16 ppm
Chlorogenic	6.83 ppm
Eugenol	3.09 ppm
Caffeic	1.24 ppm
Pyrogallol	0.985 ppm
Rutin	0.379 ppm
Siliybin	0.313 ppm
Callic acid	0.171 ppm

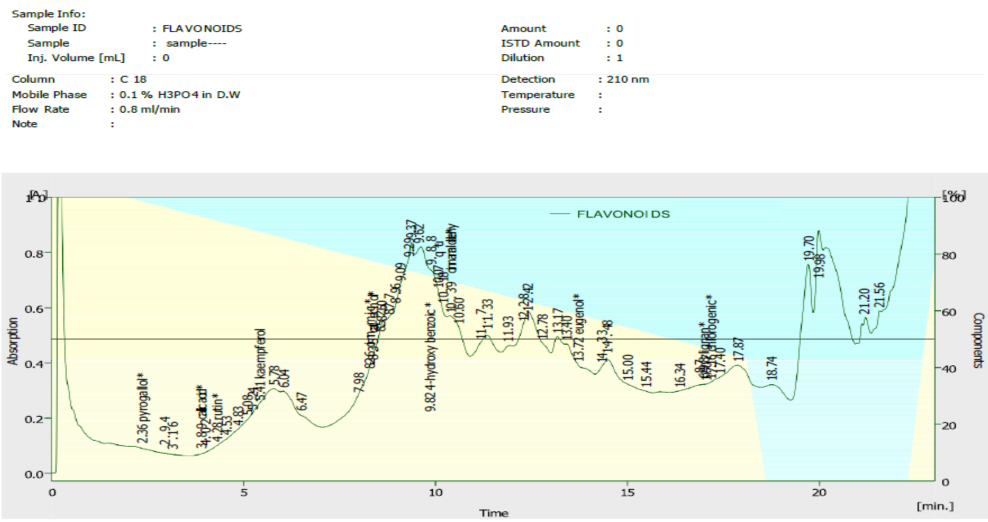

Figure 1. Concentrations of flavonoids in the leaf extract using HPLC.

The results indicate that the leave of S. marianum contain different concentrations of flavonoids, which include 4- hydroxyl benzoic, Catechol, Kaempfero, Caffeic, Pyrogallol, Rutin, Callic acid, Cinnamic, Siliybin and Vanilic with known pecks. Ahmed et al. [26] found seven different kinds of flavonoids in plant extract: kaempferol, apigenin 7-O-β-galactoside, apigenin 7-O-β-glucoside, kaempferol 3-O-α-rhamnoside-7-O-β-galacturonide, kaempferol 3-O-α-rhamnoside, and apigenin 7-O-β-glucuronide 6″-ethyl ester. According to Lv et al. [27], taxifolin biosynthesis takes place in the leaf and is subsequently transferred to the pericarp, where silymarin component synthesis takes place. However, Martin et al. [28] found other flavonoid types in the plant sections, such as isosilybin B, silychristin A, silychristin B, silybin A, silybin B, silydianin, and a trace amount of silymarin. The type of flavonoid that has been found, its bioavailability, and its possible method of action all influence its biological activity. In addition to the various pharmacological effects, such as anti-inflammatory, anti-cancer, antioxidant, neuroprotective activities, and anti-inflammatory [29], [30], [31], Wang et al. [32] demonstrated that taxifolin can modulate the NF-κB signaling pathway, improving oxidative damage.

Table 2 displays the most significant absorption peaks of the functional groups that are part of the isolated flavonoid compound's infrared spectrum. The isolated compound's aromatic structure, which includes phenolic hydroxyl groups, ether, and carbonyl groups, can be inferred from the IR spectra, see figure 2.

Table 2. the pure flavonoid compound's functional groups as determined by its IR spectra.

Wavenumber (cm-1)	Band	Functional group
3851.05-3466.89	O-H	Stretching of phenolic-OH
2953.85-2357.87	O-H	Bending of phenolic –OH
1745.49-1651.86	C=O	Stretching of ketone carbonyl
1462.43-1229.41	C=C	Stretching of olfenic C=C
1158.81-1111.03	C-O-C	Stretching of ether

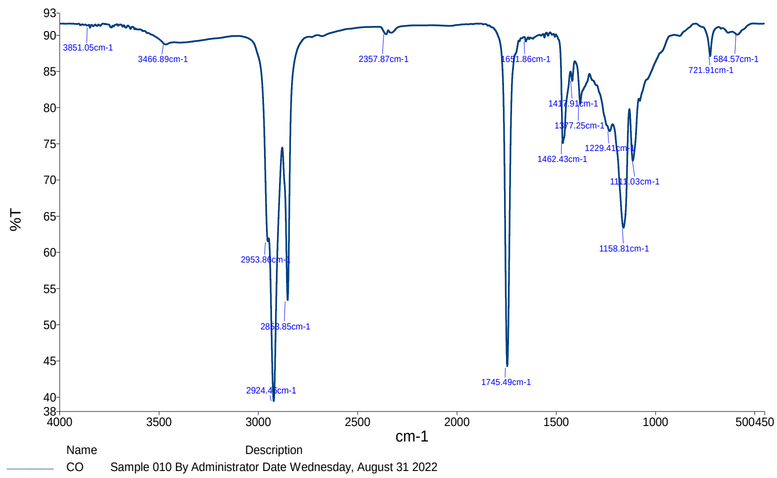

Figure 2. Infrared spectrum of the flavonoid molecule that has been purified.

Cytotoxicity effects

To determine the cytotoxicity effects of flavonoid extract of S. marianum on lymphocytes, three different concentrations of the extract of S. marianum were applied to the cells, including (25, 50 and 100) µg/ml. The results showed that the flavonoid extract had different effects on the cytotoxicity of the lymphocytes of adult females and fetuses, see Figure 3. The extract exhibited a dose-dependent inhibitory effect on lymphocytes cell line.

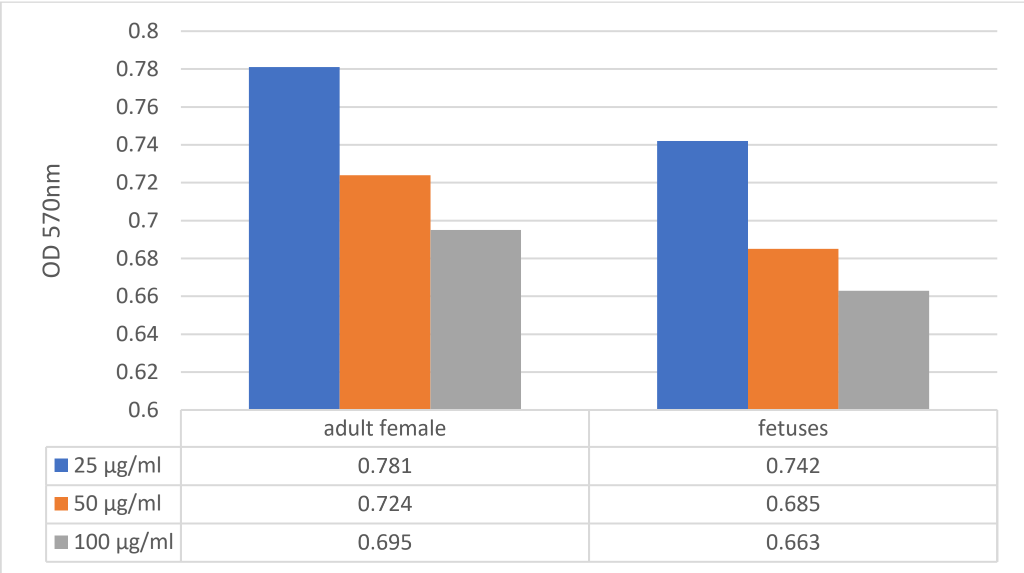

Figure 3. growth of cell lines of lymphocytes after treatment with various extract doses. * mean significant ($p \leq 0.05$) differences between groups.

Determining the cell viability

This test is significant because it clearly demonstrates how the extract suppresses lymphocyte proliferation and the percentage of inhibition at each concentration. The experiment's findings demonstrated that, at various doses, the extract of S. *marianum* may suppress the proliferation of adult female and fetal cells (Figure 4). It was demonstrated by the flavonoid extract that the highest concentration had the highest percentage of inhibition, whilst the lowest amount had the lowest percentage. The cell viability percentage for adult female lymphocytes was 92.34%, 85.06% and 81.35%, and for fetus's lymphocytes was 91.92%, 84.39% and 81.7% for the extract concentration of (25, 50 and 100) µg/ml respectively. MTT assay determined the IC-50 of flavonoids extract of S. *marianum* against adult female lymphocytes and fetus's lymphocytes. According to the calculations, the IC-50 for adult female lymphocytes was 324.9µg/ml and for fetus's lymphocytes was 347.3µg/ml.

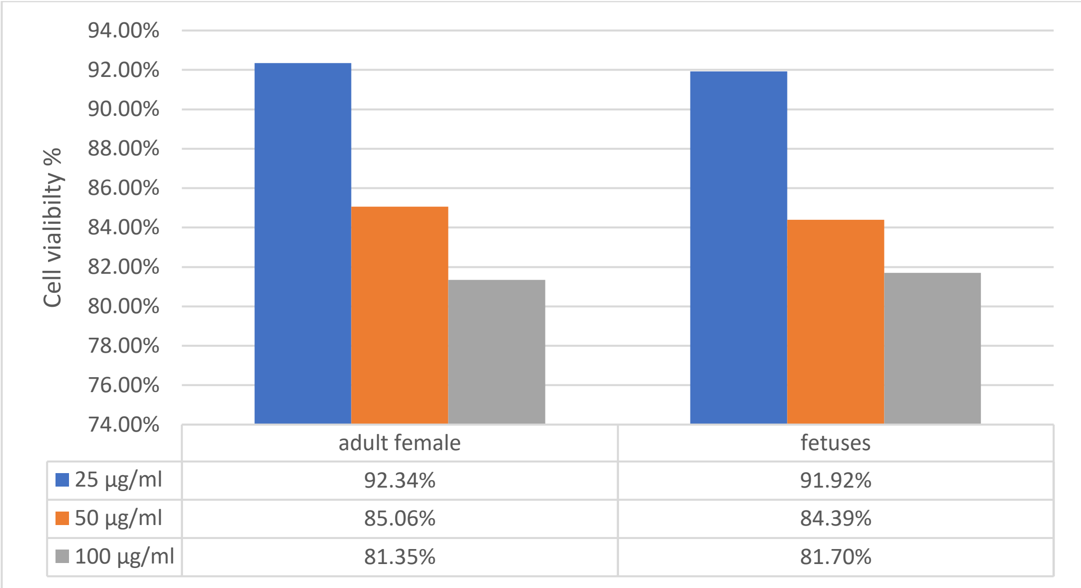

Figure 4. Inhibitory effects of various flavonoid extract concentrations on cell lines of lymphocytes.

* mean significant ($p \leq 0.05$) differences between groups.

In the current study, it was found that flavonoid compounds extracted from S. marianum had the greatest toxicity and inhibitory effect on lymphocytes of both adult females and fetuses. In a study conducted by Beltrami et al. [33] to detect the toxic effects of resins on Human Gingival Fibroblast Cell Lines. All materials tested showed cytotoxic effects on gingival fibroblasts. The Wilcoxon test for intragroup comparison showed that the percentage of viable cells decreased significantly for extracts, for all composite resins tested. The composite resins contained monomers that displayed cytotoxic properties. On the other hand, in a study conducted by Binder et al. [34] to reveal the effect of essential plant oils on the cell line NIH3T3 (mouse fibroblasts), it was found that essential oil led to damage to the nucleic acid of the cells and their destruction, which caused an increase in the rate of inhibition and a decrease in the percentage of cell viability as a result of the direct effect and the occurrence of programmed death in the cells. Evaluation of the biomaterial's extract's response to cell culture is done by cytotoxic tests [35]. By assessing the cell viability, the current work used the MTT method to assess the cytotoxic activity of *S. marianum* flavonoid extracts. The study used the CD50 value and cell viability percentage as indicators of cytotoxic action. When it comes to cytotoxic activity, the CD50 value is the sample concentration that prevents 50% of the cell from proliferating. A sample is considered less hazardous to cells if its CD50 value is higher [36], that noted in the current study where the IC-50 was very high. Additionally, another study used the MTT test to assess the cytotoxic activity of 23 crude methanol extracts from 19 medicinal plants in Bangladesh against four human cancer cell lines, healthy monkey kidney (VERO), and healthy mouse fibroblasts (NIH3T3). *Hymenodictyon excelsum* and *Aegiceras corniculatum* extracts exhibited high cytotoxicity across all evaluated cell lines. Whole plant extracts from *G. oppositifolius* and *E. viride* showed strong efficacy against MCF-7 cells [37]. High concentrations of certain flavonoids can function as pro-oxidants, causing oxidative stress, DNA damage, and possibly cell death by blocking enzymes linked to DNA and encouraging the production of free radicals [38], that may explain the results of high dose (50,100) ul/mg of flavonoids in current study.

CONCLUSION

Fundamental Finding : The current study concludes that the extract of *Silybum marianum* (S. marianum) leaves is rich in flavonoid compounds that possess both therapeutic and potentially toxic properties. The cytotoxicity analysis revealed that these flavonoids exhibited low toxicity at concentrations up to 100 µg/ml, with lymphocyte survival remaining above 80% at the highest tested concentration, indicating that the extract does not induce acute lymphocyte death under the study conditions. **Implication :** These results suggest that *S. marianum* leaf extract may be considered relatively safe for biological systems at moderate concentrations and could serve as a potential source of bioactive compounds for pharmacological applications, provided its dual therapeutic–toxic nature is carefully managed. **Limitation :** The study was limited to in vitro evaluation on lymphocytes and did not assess long-term exposure, in vivo responses, or the specific molecular mechanisms underlying the observed effects. **Future Research :** Further studies are recommended to explore the in vivo toxicity profile, dose-dependent responses, and pharmacodynamic pathways of *S. marianum* flavonoids, as well as their potential therapeutic applications and safety thresholds in clinical or preclinical models.

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