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The In Vitro Anti-Inflammatory and Antioxidant Activitites of Extracts from the Silybum Marianum (L.) Gaertn

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Abstract: The *Silybum marianum* (L.) Gaertn is a medicinal herb containing many polyphenol and flavonoid compounds with important biological activities, especially antioxidant and anti-inflammatory properties. This study aimed to evaluate the antioxidant and anti-inflammatory activities of extracts from milk thistle. The raw materials were extracted using ethanol–water solvent and analyzed parameters including total polyphenol content (TPC), total flavonoid content (TFC), DPPH and ABTS free radical scavenging ability, ability to form complexes with Cu^{2+} metal ion and anti-inflammatory activity through the albumin denaturation inhibition model (BSA). The results showed that milk thistle extract has significant antioxidant activity with DPPH value reaching 23.59 mg TE/g and ABTS reaching 27.96 mg TE/g. The extract also demonstrated anti-correlation analysis showed that TPC and TFC had a strong positive correlation with ABTS activity and anti-inflammatory activity, showing the important role of phenolic compounds and flavonoids in the biological activity of the extract. The research results provide a scientific basis for the exploitation and application of the *Silybum marianum* in the field of medicinal herbs and functional foods.

Keywords: *Silybum marianum*, inflammation, antioxidant, polyphenol, flavonoid.

INTRODUCTION

Oxidative stress and inflammatory response are considered two important biological mechanisms related to the formation and progression of many chronic diseases such as liver disease, cardiovascular disease, cancer and metabolic disorders. Accumulation of free radicals and reactive oxygen species can cause damage to cell membranes, proteins and DNA, thereby impairing cell physiological function [Akaike, T. *et al*., 1990]. Therefore, finding natural compounds with antioxidant and anti-inflammatory properties is becoming an important research direction in the fields of biology and pharmacology.

The *Silybum marianum* (L.) is a plant in the asteraceae family, widely known for containing a flavonolignan complex called silymarin. Studies show that silymarin has many important biological activities such as antioxidant, anti-inflammatory and cell protection, especially liver cells [Al-Rasheed, N. *et al*., 2016]. In addition, extracts from milk thistle have also been reported to have anti-inflammatory effects in experimental models, showing potential applications in the field of medicinal herbs and functional foods [Balian, S. *et al*., 2006].

Although numerous studies have reported on extraction methods, chemical composition [Altemimi, A. *et al*., 2016; Ivancheva, S., & Stantcheva, B. 2000; Akbel, E., & Bulduk, I. 2022; Toklu, H. Z. *et al*., 2008; Aziz, M. *et al*., 2021], and biological activities of the *Silybum marianum* from different regions [Lekmine, S. *et al*., 2025; Hora, J. *et al*., 2025; Niedzwiecki, A. *et al*., 2016], information regarding the chemical profile and bioactivities of essential oil derived from *Silybum marianum* cultivated in Dak Lak Province remains scarce. Therefore, the study to evaluate the antioxidant and anti-inflammatory activities of *Silybum marianum* extract, while also considering appropriate extraction conditions, has scientific and practical significance in exploiting this medicinal source.

MATERIALS AND METHODS

General Experimental Procedures

The *Silybum marianum* (L.) Gaertn were collected in Dak Lak, Vietnam. The fresh leaves and stems were collected and cut, then washed and drained. Stems and leaves continue to be processed at 80- 5°C, for 3 - 5 minutes to kill enzymes and chopped, drained and dried at 55°C until moisture reaches 11 - 13%. The dried medicinal herbs are crushed and ground into fine powder

Chemicals

Sodium carbonate (Na_2CO_3), Folin-Ciocalteu, Methanol, Ethanol, 2,2-Diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Gallic acid, Quercetin, Trolox, Dimethyl sulfate (DMSO), Natri nitrite ($NaNO_2$), Aluminum chloride ($AlCl_3$), Sodium hydroxide (NaOH), Bovine albumin serum (BSA), Copper (II) sulfate ($CuSO_4$), Pyrocatechol violet (PV), Acetic acid

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(CH_3COOH), Sodium acetate (CH_3COONa), Potassium persulfate ($K_2S_2O_7$).

Extraction and Isolation

The *Silybum marianum* of powder will be extracted using the ultrasound-assisted soaking method using an ethanol/water solvent mixture. The conditions for investigating the extraction process were conducted through the parameters of solvent concentration, extraction time, extraction temperature and reference solvent/material ratio according to the publication of Drouet *et al*., 2019 and the layout model according to table 1. Then, extraction was carried out in an ultrasonic tank at a specified temperature and time. After extraction, the mixture was filtered through filter paper to collect the extract. The extract continues to be concentrated using a low-temperature vacuum rotary evaporator to remove the solvent and collect a concentrated extract for use in subsequent experiments.

Table 1. Control factors and levels of control

Sample	**factors**	**levels**		
		Low level	**Middle level**	**High level**
A	Solvent concentration (%)	25	50	75
B	Ultrasound time (min)	5	10	15
C	Extraction temperature (^oC)	20	40	60
D	Solvent/sample ratio (mL/g)	10:1	15:1	20:1

Note*: The study levels were determined from the previous screening experiment*

Antioxidant Assay

The ability to neutralize ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free radicals was determined according to the method of Nguyen and colleagues (2015)[Nguyen, V. T. *et al*., 2015]. ABTS solution was prepared by mixing 7.4 mM ABTS solution with 2.6 mM potassium persulfate and incubating in the dark for 12 hours to generate $ABTS^+$ free radicals. The solution was adjusted to an absorbance of approximately 1.1 ± 0.02 at 734 nm. The trolox standard curve was constructed using 20 µL of trolox solution dissolved in methanol to obtain a series of concentrations (0.01; 0.025, 0.05, 0.075 and 0.1 mg/mL) mixed with 180 µL of ABTS solution. The mixture was incubated in the dark for 2 hours. The absorbance of the solution was determined at 734 nm using a Hidex Sense fluorescence microplate reader system. Results are expressed as mg trolox equivalent per gram of sample.

The antioxidant activity of the extract was also evaluated through its ability to inactivate DPPH free radicals. The DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by diluting the stock DPPH solution with methanol (0.24 mg/mL) to obtain a solution with an absorbance of 1.1 ± 0.02 at 515 nm. The trolox standard curve was constructed using 50 µL of trolox solution dissolved in methanol to obtain a series of concentrations (0.01; 0.025, 0.05, 0.075 and 0.1 mg/mL) mixed with 150 µL of DPPH solution. The mixture was incubated in the dark for 30 minutes. The absorbance of the solution was determined at 515 nm using a Hidex Sense fluorescent microplate reader system.

Method for determining the ability to reduce Cu^{2+}

Cu^{2+} reduction ability was performed according of Thu Hang Nguyen *et al*. (Nguyen *et al*., 2020) [Nguyen, T. H. *et al*., 2020]. The quercetin standard curve was constructed using 30 µL of quercetin dissolved in acetate buffer (pH 6.0) to obtain a concentration range (0.005; 0.01; 0.025; 0.05, 0.1, 0.25 and 0.5 mg/mL) mixed with 200 µL of acetate buffer and 30 µL of solution. CuSO4 200 mg/L. After 2 minutes, add 8.5 µL of 2 mmol/L pyrocatechol violet solution to start the reaction. The mixture was shaken and incubated for 10 minutes at 25^oC. The absorbance of the solution was determined at 632 nm using a hidex Sense fluorescence microplate reader system. The absorbance of the extract sample was determined similarly to quercetin, replaced by 30 µL of extract sample. Cu^{2+} reduction ability was calculated as quercetin equivalent (mgQE/g sample) based on the quercetin standard curve.

Anti-inflammatory Assay

The anti-inflammatory activity of the extract was evaluated through the bovine serum albumin (BSA) denaturation inhibition model (Dharmadeva S *et al*., 2018) The ibuprofen standard curve was constructed using 0.5 mL of ibuprofen solution dissolved in acetate buffer (pH 5.5) to obtain a concentration range (0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07; 0.08; 0.09 and 0.1 mg/mL) mixed with 2.5 mL of solution. BSA solution (0.2%) and 1 mL acetate buffer solution, stir well. After incubation at 37 ± 2 oC for 30 minutes, the mixture was denatured on a water bath at 70 oC for 3 minutes and cooled quickly under running water. The absorbance of the solution was determined at 660 nm using a UV-Vis spectrophotometer (Jasco, Japan). The absorbance of the extract sample was determined similarly to ibuprofen, replacing it with 0.5 mL of extract solution. The anti-inflammatory ability is calculated according to the formula:

$$\%\mathrm{PI} = \frac{\mathrm{ABS_{Ctrl}} - (\mathrm{ABS_{sample}} - \mathrm{ABS_{color}})}{\mathrm{ABS_{Ctrl}}}$$

In there:

%PI is the protein denaturation inhibition rate (%)

$\mathrm{ABS_{Ctrl}}$ is the absorbance of a sample containing only BSA and buffer

$\mathrm{ABS_{sample}}$ is the absorbance of the sample to be measured

$\mathrm{ABS_{color}}$ is the absorbance of the sample containing only buffer solution and the sample to be measured

Statistical Analysis

Experiments were repeated at least 3 times. The results are expressed as the average of three replicates ± standard deviation. Statistically significant differences of the results were compared at the significance level $p \leq 0.05$ using SPSS 23.0 software. Graphs are presented using Excel 2016 software. Optimization data are processed using ANOVA in Design Expert 12 software

RESULTS AND DISCUSSION

Determination of the chemical composition of the *Silybum marianum extract*

The results of active ingredients of the ***Silybum marianum*** extract are shown in Table 2.

Table 2. The ingredients of Silybum marianum extract

Experience	TPC (mgGAE/g)	TFC (mgQE/g)
1	8.992 ± 0.0066^{h}	32.533 ± 0.0405^{i}
2	10.545 ± 0.0155^{de}	36.757 ± 0.0351^{e}
3	11.991 ± 0.0063^{a}	41.123 ± 0.0405^{a}
4	10.909 ± 0.0063^{c}	38.887 ± 0.0536^{c}
5	9.732 ± 0.0063^{g}	36.500 ± 0.0731^{f}
6	11.369 ± 0.0113^{b}	35.845 ± 0.0608^{g}
7	10.561 ± 0.0094^{d}	33.317 ± 0.0351^{h}
8	11.633 ± 0.0041^{ab}	38.197 ± 0.0351^{d}
9	10.303 ± 0.0063^{ef}	40.900 ± 0.0608^{b}

Note*: Mean ± SD (standard deviation) (n=3). Different letters (a-n) depict statistically significant differences between different extraction conditions according to Fisher's Least Significant Difference (LSD) test (α=0.05).*

The results of analysis of total polyphenol content in milk thistle (TPC) leaves (Table 3.1) show a statistically significant variation ($p<0.05$) in polyphenol extraction efficiency among the 9 treatments investigated, with values ranging from 8,992 to 11,991 mgGAE/g dry matter (CK), showing that the polyphenol collection efficiency of the combined ultrasonic water–ethanol extraction system is generally low and of high amplitude. narrow variation, this result is similar to that of some previous studies such as Abbassia Benchaachoua *et al*., 2018 (11.95 mg GAE/g dry matter) [Benchaachoua, A. *et al*., 2018].

This reflects the chemical properties of the main active ingredient system in milk thistle which is flavonolignan - a group of semi-polar to less polar compounds, not typical polar polyphenols. Therefore, the aqueous ethanol solvent mainly pulled out the auxiliary phenolic acids and flavonoid glycosides, while most of the flavonolignans were not effectively dissolved. The results are similar to Abbassia Benchaachoua *et al*., 2018, showing that the water-methanol and water-acetone solvent systems have better extraction efficiency than the water-ethanol solvent system [Benchaachoua, A. *et al*., 2018]. However, the current trend of using the water-ethanol solvent system is preferred in industrial production because it is friendly to the environment and operators. Meanwhile, research by Nowak *et al*., 2021 shows that the TPC extraction efficiency is highest when extracted with 70% ethanol solvent (4.14 GAE/g dry matter), higher than 96% ethanol as well as methanol, acetone and petrolium, especially using the Soxhlet extraction method, but lower than the results in this study. This shows that TPC depends heavily on the solvent, extraction method and where milk thistle samples are collected the was firts [Guemari, F. *et al*., 2020].

The treatment 3 (NT3) was determined to be the most suitable condition for the TPC extraction process, significantly different from most of the remaining treatments, showing that temperature and ultrasound time are the two factors that most strongly influence the release of polyphenols, because they help break down cell walls and accelerate diffusion.

The storage capacity of flavonoid content (TFC) in the treatments showed a large fluctuation from 32,533 to 41,123 mgQE/g, showing that the water–ethanol system has higher selectivity for flavonoids than for total polyphenols. This is appropriate because many flavonoids in milk thistle exist in the form of glycosides, have moderate polarity and are well soluble in aqueous ethanol. This trend is consistent with the results published by Abbassia Benchaachoua *et al*. (2018) when studying flavonoid extraction from milk thistle, in which the author also noted higher flavonoid extraction efficiency in water-ethanol solvent systems compared to single solvents. The total flavonoid content in milk thistle leaves has been published by many different authors such as Sun *et al*. 2016 (9 mg RE/g dry matter; Abbassia Benchaachoua *et al*. (2018) (TFC ranges from 2.63-16.12 mg C (catechin) E/g dry matter, depending on the solvent and extraction method). At the same time, the total Flavonoid content (TFC) (Table 2) also shows that treatment 3 (NT3) is superior, reaching the highest value of 41.123 ± 0.0405 mg QE/g. The strong positive correlation between TPC and TFC at NT3 is a chemically reasonable result, since flavonoids are a major subclass of polyphenols [Benchaachoua, A. *et al*., 2018].

Total flavonoid content (TFC) reached the highest value when combining high extraction temperature and prolonged ultrasound time (NT3), due to the cavitation phenomenon breaking down the lignocellulose structure of plant cell walls, thereby enhancing the release of bound flavonoids and promoting mass transfer [Chemat, F. *et al*., 2017]. On the contrary, when the ultrasound intensity or ethanol concentration is too high, TFC tends to decrease because flavonoids are a group of compounds that are sensitive to oxidation and structural decomposition under the strong impact of cavitation, reflecting the existence of an optimal threshold in ultrasound-assisted extraction [Chemat, F. *et al*., 2017].

Evaluation of biological activity of the Silybum marianum

The results of biological activity of the *Silybum marianum* extract are shown in Table 3.

Table 3. Biological activity of the Silybum marianum extract..

Experience	**DPPH (mgTE/g)**	**ABTS (mgTE/g)**	**Cu-chelation (IC$_{50}$, mg/ml)**	**BSA (IC$_{50}$, mg/ml)**
1	23.327±0.0635^{gh}	22.869±0.4312^{hi}	89.691±0.2275^{b}	40.446±0.0370^{i}
2	23.764±0.2046^{f}	26.755±0.2895^{de}	88.838±0.2862^{c}	46.114±0.0258^{de}
3	23.591±0.1500^{fg}	27.958±0.7308^{a}	91.202±0.2367^{a}	49.117±0.0406^{a}
4	23.924±0.2403^{cd}	27.925±0.4642^{ab}	76.822±0.3009^{d}	48.355±0.0203^{b}
5	24.392±0.3097^{a}	25.653±0.2046^{f}	66.120±0.1737^{f}	43.855±0.0165^{g}
6	23.992±0.2308^{c}	26.789±0.2411^{cd}	75.181±0.1137^{e}	47.198±0.0291^{c}
7	23.820±0.2191^{de}	23.068±0.6697^{g}	41.431±0.1737^{i}	44.204±0.0360^{f}
8	24.076±0.1639^{ab}	26.948±0.651^{c}	61.983±0.2862^{g}	46.221±0.0306^{d}
9	23.836±0.0302^{d}	22.909±0,279^{h}	57.584±0.2111^{h}	42.320±0.0239^{h}

Note: *Mean ± SD (standard deviation) (n=3). Different letters (a-n) depict statistically significant differences between different extraction conditions according to Fisher's Least Significant Difference (LSD) test (α=0.05).*

The antioxidant capacity according to DPPH (Table 3) is expressed in mg TE/g (equivalent to Trolox), therefore, the higher the value, the stronger the antioxidant capacity. Among the 9 treatments, treatment number 5 (NT5) gave the highest activity of 24,392 ± 0.0041 mgTE/g, followed by treatment number 8 (NT8) of (24,339 mgTE/g). It is worth noting that treatment number 3 (NT3), which is the best for TPC/TFC, only gave an average DPPH free radical scavenging activity of 23,958. mgTE/g. The relatively narrow variation shows that the DPPH free radical scavenging ability of the extract changes according to the extraction conditions but is not too strong, implying that the DPPH free radical scavenging ability may be dominated by a subgroup of fastreacting compounds instead of the total amount of polyphenols/flavonoids. Mechanistically, DPPH is often sensitive to the ability to donate H/e^- in the reaction medium (usually favoring the organic phase), so it may not be completely in phase with ABTS. and/or TPC or TFC. This observation is consistent with the fact that many studies on silymarin/flavonolignan extraction show that optimizing conditions (especially UAE) changes the active ingredient spectrum, thereby leading to the antioxidant response between different tests not being completely consistent [Vinatoru, M. *et al.*, 2017].

Similar to DPPH free radical scavenging ability, ABTS free radical scavenging activity (Table 3.2) is also measured in mgTE/g (the higher the better). Here, treatment number 3 (NT3) showed the highest activity, reaching 27.958 ± 0.0094 mgTE/g, showing that phenolic and flavonoid compounds extracted in NT3 have the ability to capture ABTS free radicals better than DPPH radicals. This is because ABTS is sensitive to both polar and semipolar compounds, including phenolic acids and flavonoid glycosides, which are effectively extracted in water–ethanol systems. Treatments with high TPC/TFC such as NT3 and NT4 also showed high ABTS free radical scavenging ability, proving that ABTS is an indicator that accurately reflects the biological quality of the extract in this study. Thus, ABTS is more suitable than DPPH when evaluating the effectiveness of water–ethanol solvent systems, and is consistent with studies on UAE showing that extraction conditions can significantly enhance flavonolignan content and total antioxidant capacity [Trabalzini, A. *et al.*, 2025; Drouet, S. *et al.*, 2019].

The ability to form complexes with Cu^{2+} showed the strongest difference between the parameters, with IC_{50} ranging from 41.431–91.202 mg/mL (Table 3.2). Treatment 7 showed the strongest chelating effect (IC_{50} = 41.431 ± 0.1737 mg/mL), while treatment 3 had the weakest (91.202 ± 0.2367 mg/mL). The difference in antioxidant capacity through the ability to form Cu-chelation and ABTS/DPPH shows that metal complexation activity is a separate mechanism, depending more on the coordination functional group (eg catechol/ortho-dihydroxy, carbonyl, carboxylate) and molecular configuration than on the ability to accept electrons or protons. This trend is consistent with studies on flavonolignan/silymarin showing that some components have the ability to chelate metal ions (e.g. Zn^{2+}) and this property has biological significance independent of free radical scavenging tests [Tvrdý, J. *et al.*, 2021]. The IC_{50} values of BSA ranged from 40.446–49.117 mg/mL (Table 3), in which treatment 1 was the strongest (40.446 ± 0.0370) and treatment 3 was the weakest (49.117 ± 0.0406). This shows that the optimal condition for ABTS (treatment 3) is not at the same time optimal for protein stability. Mechanistically, the BSA denaturation test reflects the ability to stabilize protein structure against denaturing agents through non-covalent interactions (hydrogen bonding, hydrophobicity, electrostatics), and is widely used as an in vitro anti-inflammatory screening model in plant extract studies [Lekouaghet, A. *et al.*, 2020]. NT1 gave the lowest IC_{50}, meaning the most active, despite having a low TPC/TFC. This demonstrates that the compounds responsible for the inhibition of protein denaturation are highly polar compounds (organic acids, polysaccharides, small phenolics) that are more soluble in water than ethanol. Therefore, when increasing the ethanol ratio to optimize flavonoids, BSA activity decreased, showing that these two biological targets cannot be optimized simultaneously in the same solvent system.

Influence of Agronomic Practices on the Antioxidant Activity of
Three Mediterranean Officinal Wild Plants: Silybum marianum,
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From the above results, it shows that biological activity indicators are divided into different groups of mechanisms: ABTS represents the total antioxidant capacity of the total polyphenol/flavonoid content [Trabalzini, A. *et al.*, 2025; Drouet, S. *et al.*, 2019], while Cu-chelation and BSA reflect separate activity axes (metal chelating and protein stabilization) that may depend on different compounds in the extract [Tvrdý, J. *et al*., 2021]. Therefore, applying multi-objective optimization (Taguchi–GRA) is necessary to avoid selecting conditions based on a single criterion.

CONCLUSION

The article evaluates the antioxidant and anti-inflammatory activities of extracts from the *Silybum marianum* and combines optimal procedures in the extraction. The results showed that *Silybum marianum* extract has significant antioxidant activity with DPPH value at 23.59 mg TE/g and ABTS to 27.96 mg TE/g. The extract also demonstrated anti-inflammatory activity with an IC_{50} value in the BSA model of approximately 49.12 mg/mL. Notably, NT3 extract achieved TPC/TFC equivalent to international research, proving that local medicinal sources have the potential to produce polyphenol for the liver protection and antioxidant functional foods.

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