MAS Journal of Applied Sciences Uygulamalı Bilimler Dergisi ISSN: 2757-5675 masjaps.com

OPEN ACCESS

DOI: http://dx.doi.org/10.5281/zenodo.13939065

Araştırma Makalesi / Research Article

Developing Rp-Hplc Method and Determination in Vitro Cytotoxicity of Silymarin Obtained From *Silybum marinum* Plant

Erten AKBEL [1*], İbrahim BULDUK [2], Funda KARABAĞ [3]

[1] Uşak University, Faculty of Health Sciences, Department of Physiotherapy and Rehabilitation Uşak

[2] Afyon Kocatepe University, Faculty of Engineering, Department of Chemical Engineering, Afyonkarahisar

[3] Uşak University, Faculty of Engineering and Natural Sciences, Department of Molecular Biology and Genetics Uşak

*Corresponding author: erten.akbel@usak.edu.tr

Received: 08.07.2024 **Accepted**: 25.08.2024

Abstract

The Asteraceae family's *Silybum marinum* plant, commonly known as thistle, yields silymarin in its seeds. A frequently employed hepato-protective treatment for conditions like cirrhosis, fatty liver due to alcohol and hazardous chemicals, and hepatitis is silymarin. Conventional milk thistle extract is derived from seeds containing a silymarin content of 4-6%. The extract has 20–35% fatty acids, including linoleic acid, and 65–80% silymarin (a flavonolignan complex). Silymarin constitutes a complex blend of polyphenolic compounds that also contains a flavonoid (taxifolin) and seven closely related flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silychristin, and silydianin). The two main diastereoisomers of silymarin, silybin A and silybin B, are approximately equally mixed in silibinin, a semi-purified fraction of silymarin. The impact of silymarin on liver, pancreatic, prostate, and skin cancers has been the subject of numerous patents. Owing to silymarin's antioxidant and anti-inflammatory properties, and its ability to modulate various proteins and genes, silymarin exhibits antichemopreventive effects. Moreover, silymarin mitigates the damaging effects on healthy cells or organs. Consequently, silymarin holds potential as an adjuvant therapy for cancer. In this study, the cytotoxicity of silymarin extracts obtained via the HPLC technique—known for its sensitivity, utility, and established efficacy in determining silymarin quantity from the *Silybum marianum* plant—was assessed using a colorimetric test on A-549 cells. It was found that 100 µM was the LD50 when silymarin was administered to A-549 cells in dosages of 25, 50, 75, and 100 µM.

Keywords: HPLC, *Silybum marianum*, silymarin, cytotoxicity

1. Introduction

In the past decade, there has been a marked uptick in scientific interest in exploring the usage of naturally occurring compounds or micronutrients and their potential impacts on human health (Delmas, 2020). These plant-derived compounds have cellular targets that closely resemble those of modern pharmaceuticals (Vaidya et al., 2018). Indeed, it has been recently reported that over 1600 patents have been issued for flavonoids and more than 3000 for polyphenols (Delmas, 2020). A well-studied blend of polyphenolic antioxidants, known as silymarin, is sourced from the seeds of the thistle plant (*Silybum marianum*) (Surai, 2015). This plant, a member of the Asteraceae family, is under scrutiny as a potential natural remedy for various ailments, including cancer, heart disease, and neurological disorders (Bartolome et al., 2013). Despite its limited bioavailability, silymarin holds biological significance for human health, particularly in illnesses characterized by inflammation and oxidative stress (Delmas, 2020). Silymarin boasts a variety of noteworthy attributes, including antiviral, anti-inflammatory, and anticancer properties (Karimi et al., 2011). Numerous investigations have demonstrated silymarin's ability to halt the growth of several types of tumor cells, including those affecting the prostate (Davis-Searles et al., 2005), breast (Kim et al., 2021), colon (Bayram, 2017), ovary (Koltai and Fliegel, 2022), lung (Vargas et al., 2021), and bladder (Eser et al., 2012). In this study, a sensitive, efficient, and thoroughly validated method for quantifying silymarin in plant materials was developed. The devised approach ensured the best basic solubility of the seven recognized silymarin components within less than 15 minutes. The cytotoxicity of the obtained extracts was also assessed using a colorimetric assay, specifically the MTT test, on A-549 cells.

2. Materials and Methods

2.1 Reagents

Sigma Chemical Co. supplied the silibinin reference standard (≥98.0%), trifluoroacetic acid (≥99.0%), acetonitrile (≥99.9%), methanol (≥99.9%), and silymarine. A Milli-Q System was used to produce the ultrapure water employed in the experimental procedures, which had a conductivity of less than 0.05 S cm^{-1}. All other substances were of sufficient purity for analysis. In this research, *Silybum marianum* was cultivated on the grounds of the Faculty of Agriculture and Natural Sciences at Uşak University in Turkey. Various organs of the plant were partitioned and air-dried for 15 days in a darkened setting. The dried material was pulverized to a fine powder in a mortar.

2.2 Standart solutions

A precise 25 mg quantity of the reference standard was transferred into a 50 mL volumetric flask, followed by the addition of 15 mL of methanol. Methanol was then added until the volume was reached after sonication of the flask contents for five minutes. This resulted in the creation of a stock standard solution with a concentration of 500 g mL^{-1}. The stock solution was subsequently diluted with methanol to create a series of standard solutions (5-30 gmL^{-1}, n=6). Each solution was then filtered through a membrane filter with a porosity of 0.45 μm.

2.3 Extraction of plant material

Powdered *Silybum marianum* seeds, weighing 500 mg, was weighed with precision. Subsequently, 50 mL of methanol was added, and the mixture was subjected to extraction in an ultrasonic bath for 15 minutes. Upon completion of the extraction process, the solution was filtered through Whatman filter paper (with white bands).

2.4 Analytical instrument and conditions

The HPLC analyses were carried out using an Agilent 1260 system, which includes a quad-gradient pump, an autosampler, a UV detector, and ChemStation software. A novel

chromatographic method has been devised and verified for the precise and accurate quantification of silymarin in milk thistle plant extracts. Chromatographic separation was executed using an Agilent Extend C18 (250x4.6 mm, 5 µm) column. Ultrapure water containing 0.1% Trifluoroacetic acid and acetonitrile (67/33, v/v) served as the mobile phase at a flow rate of 1.0 mL min^{-1}. Detection of the eluents was conducted at a wavelength of 288 nm using a UV detector. An injection volume of 20 µl was employed.

2.5 Cell culture

Before starting cell culture, it was ensured that all plasticware and premade sterile media were obtained from trusted commercial sources. Lysates and media samples, prepared post-incubation, underwent cell viability assays based on a standard method. A549 cells were cultivated in RPMI 1640-based medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS), maintaining sterile conditions. The cells were then cultured in T25 and T75 flasks within a CO_2 incubator with CO_2 levels set at 5% and a temperature of 37 °C. Subculture was initiated once cell density covered approximately 85% of the flask surface. When cell numbers were deemed sufficient, they were utilized for subsequent experiments.

2.6 Cytotoxicity assay

In this study, commercial silymarin with a content of 45% w/w of apigenin 7-glucoside, silydianin, isosilybin, silychristin, and silybin (A and B) was employed. The assessment of silymarin's effects on breast and lung cancer cell viability was conducted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To achieve this, a stock solution of silymarin (in DMSO with a final concentration of 1%) was prepared (Polyak et al., 2013).

3. Results and Discussions

On the HPLC device, chromatograms were captured for standard solutions of silymarin and silibinin, with distinct peak regions for each component being noted. These peak area-concentration plots were subsequently converted into calibration graphs. The silymarin complex, comprising several physiologically active, closely related constituents, includes silychristin, silydianin, silybin A, silybin B, dehydrosilybin, isosilybin A, and isosilybin B. The chromatogram's concentration was determined using the silibinin calibration graph, by summing the areas of all seven peaks. The quantity of silibinin in the extracts was calculated utilizing the proposed analytical method. Figure 1 depicts the chromatogram of the silymarin standard solution (50 g mL^{-1}). The HPLC chromatogram of the silibinin standard solution (30 mg μL^{-1}) is shown in figure 2. Lastly, figure 3 presents the chromatogram of the methanol extract from thistle seeds. Extracts of milk thistle seeds were analyzed with the developed analytical method and the silymarin content was determined. It was determined that milk thistle seeds extracts contained 28 µg μL^{-1} silymarin.

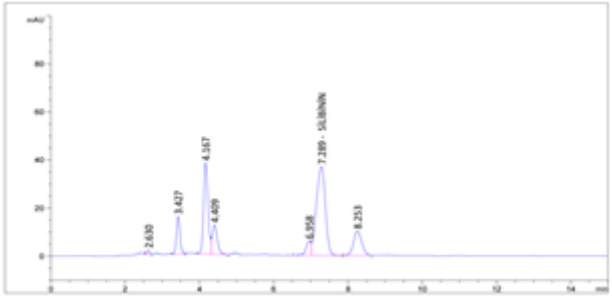

Figure 1. The HPLC Chromatogram of Silymarin Solution (50µg mL^{-1})

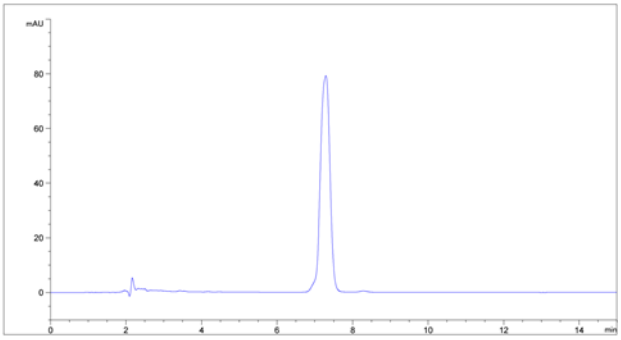

Figure 2. The HPLC Chromatogram of Silibinin Standart Solution (30µg mL^{-1})

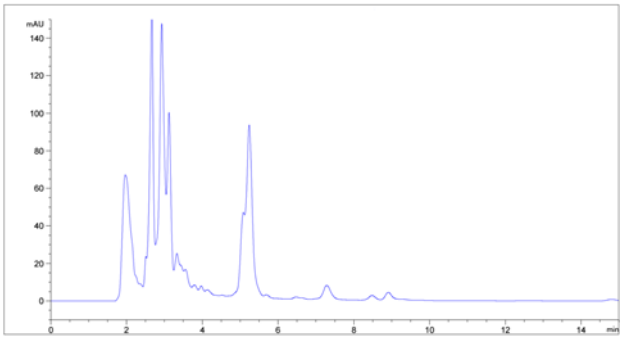

Figure 3. The HPLC chromatogram of the milk thistle extract

3.1. Cytotoxic effects of silymarin on a549 cells

25 M, 50 M, 75 M, and 100 M silymarin were applied to cells cultivated in 96 microplates until they reached logarithmic phase for 48 hours in order to assess the percentage survival rates of A549 cells after application of various concentrations of substance. After that, MTT cytotoxicity testing was carried out (Figure 4). Cell viability after the application of silymarin was found to be 98%, 89%, 74%, and 52%, respectively. Adipocytes were shown to be less proliferative after application of 100 M BGM. A549 cells did not experience any anti-proliferative effects after being exposed to 25 M silymarin (Figure 4).

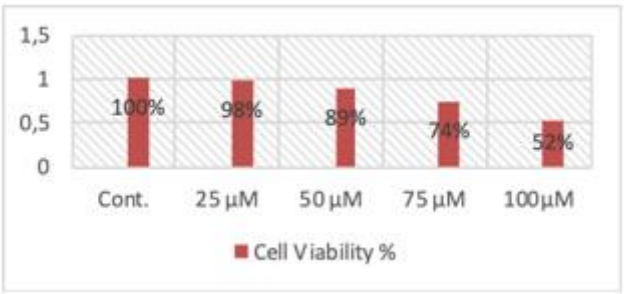

Figure 4. A549 cells are cytotoxic to silymarin at various doses. A MTT test was conducted. For 48 hours, A549 cells were exposed to 25 mM, 50 mM, 75 mM, and 100 mM BGM.

Silymarin, commonly known as milk thistle or *Silybum marianum* L., is a blend of isomers and flavonoids found in the seeds and fruits of this plant. Apart from its primary constituent, Silibin (or silibinin), Silymarin also comprises flavonolignans like Silyadianin, Silycristin, Isosilbin, and Taxifolin (Antika and Dewi, 2021). The polyphenolic flavonoid, Silymarin, is made up of Silybin, Silychristin, Isosilybin, and Silydianin, which constitute 33.4%, 12.9%, 8.35%, and 3.5% of its structure,

respectively (Wang et al., 2023). Both the flavonoid Silymarin and its constituent silibinin are compounds known for their hepatoprotective effects. However, literature reveals that they act in four distinct ways: (i) as antioxidants, scavenging and regulating intracellular glutathione content; (ii) as stabilizers of cell membranes and regulators of permeability, preventing hepatotoxic substances from entering hepatocytes; (iii) as promoters of ribosomal RNA synthesis, stimulating liver regeneration; and (iv) as inhibitors of the transformation of stellate hepatocytes into myofibroblasts, a process leading to collagen fiber deposition and ultimately, cirrhosis (Fraschini et al., 2002). A considerable number of articles have been published in the past 30 years focusing on Silymarin, a complex blend of flavonolignans, and its individual constituents, highlighting their activities and limitations. In other classes of compounds isolated from this plant, primarily terpenoids and steroids, a general framework for their biological activities is lacking (Giordano et al., 2021). Specifically, the effects of silymarin have been explored in various diseases, such as anti-cancer, anti-inflammation, hepatoprotection, and neuroprotection. Multiple studies suggest that consuming milk thistle may decrease the risk of developing certain types of cancer, including breast, skin, prostate, and lung cancer. The most widely accepted theory of carcinogenesis posits that changes in DNA lead to various types of cancer. It has been reported that silymarin can protect against DNA damage caused by different carcinogens, suggesting a cancer preventive property. Additionally, silymarin and silibinin have been shown to significantly reduce methyl methanesulfonate-induced DNA damage in human blood cells, as proven by the alkaline comet assay. It was also found that silymarin blocks the binding of DNA topoisomerase proteins and the expression of cell division cycle-related genes, acting as an antitumor agent by inhibiting the growth of human hepatocellular carcinoma (Antika and Dewi, 2021). The power and frequency of ultrasonic waves have a greater effect than other methods to extract flavonoid content (Karabegović et al., 2018). In addition to chromatography, which was a critical factor in developing an unconventional extraction process (Azmir et al., 2013), the extraction method from seeds or various plant parts also plays a crucial role in isolating flavonolignans. The traditional method involves first degreasing the plant material using hexane or petroleum ether, or partially degreasing it by pressing, and then extracting the silymarin with ethyl acetate, methanol, or acetone. An accelerated solvent extraction technique was used to shorten the time and solvent requirements for silymarin extraction. Moreover, ultrasonic-assisted extraction increased the amount of silymarin obtained (Chambers et al., 2017). Therefore, ultrasonic-assisted methanol extraction was used in our study. Silymarin and silibinin protect mitochondria by reducing oxidative stress. In addition, silymarin has antifungal and antibacterial properties. As viral infections pose a significant public health threat, it has been observed that silymarin and its derivatives exhibit strong antiviral activity against several viruses, including hepatitis C, hepatitis B, dengue virus, the enterovirus family, mayaro virus, and chikungunya virus (Polyak et al., 2013; El Menshawe et al., 2014; Teng et al., 2016; Lalani et al., 2020). Several patents have been published on the effect of silymarin on liver, pancreatic, prostate, and skin cancers, highlighting the antioxidant and anti-inflammatory effects of silymarin and its ability to regulate different proteins and genes, leading to its antichemopreventive effect. Silymarin also reduces the toxic effects on vital organs or healthy cells. Therefore, it is plausible to consider that silymarin may play a role in adjuvant cancer treatment (Emadi et al., 2022). An assay for in vitro cytotoxic activities in a standardized cell culture system was conducted to

determine which cancer cells were affected. This research was conducted on the human lung cancer cell line A549. Based on the studies, no effect was observed in A549 cells treated for 24 hours within the range of 10 to 100 µM of individual silymarin compounds (Bijak et al., 2017). In our study, a concentration of 100 µM of silymarin extracted from the seed within 48 hours reduced cell viability to approximately 50%. Therefore, since the compounds did not have an effect when applied individually, they may have a synergistic effect when combined. In another study with silymarin-loaded solid lipid nanoparticles, the concentration of silymarin applied for 24 hours as IC50 was found to be 25µM. When compared with our study, it is concluded that silymarin loaded on solid lipid particles is more effective (Sezer, 2021). As a similar example, in another study, silymarin loaded on iron oxide nanoparticles was examined in the A549 cell line and showed higher in vitro anticancer activity against A549 cancer cells (Manikandan and Raffıea Baseri, 2022). In summary, the specific targeting of nanocarriers such as iron oxide to cancer cells increases the power of silymarin. To examine the effects of silymarin on metastatic lung cancer, Anip973 cells, the IC50 value was determined as 18.6 mg/ml, as the inhibitory effect of silymarin on anip 973 cells applied at concentrations between 5-40 mg/ml for 24, 48 and 72 hours was significant at the 48 hour time point (Liv et al., 2011). From this point of view, when we compare the A549 human lung cancer line with the metastatic anip973 cell line; It has been observed that much higher concentrations of silymarin are required to reduce the viability of metastatic cells. Since metastic tumor cells are much more aggressive, this result showed that higher silymarin concentration is required than the A549 cell line we used. On the other hand, in a different study applied to MCF-7 breast cancer cell line of silymarin, the dose found as IC50 was 77.36nM/ml. It has succeeded in showing the same effect in breast cancer and lung cancer cell lines at approximately the same dose (Gheybi et al., 2019).

5. Conclusions

In line with all the results found, it is thought that silymarin may be effective on cancer. Based on the literature information, new significant information can be added to the field of cancer in the light of the trials made by creating a synergetic effect with other agents used in combination with silymarin, especially in combination with silymarin.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

References

Antika, L.D., Dewi, R.M., 2021. The pharmacological properties of silymarin and its constituents. *Natural Product Sciences*, 27(2): 68-77.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Omar, A.K.M., 2013. Techniques for extraction of bioactive compounds from plant materials: *A review*. *Journal of food engineering*, 117(4): 426-436.

Bartolome, A.P., Villaseñor I.M., Yang, W.C., 2013. Bidens pilosa L. (Asteraceae): Botanical properties, traditional uses, phytochemistry and pharmacology. Evidence-based complementary and alternative medicine, 51.

Bayram, D., 2017. Silibinin in vitro ortamda CCL-228-SW 480 kolon kanseri hücre hattı üzerine apoptotik etkisinin araştırılması. *Süleyman Demirel Üniversitesi Sağlık Bilimleri Dergisi*, 8(2): 1-7.

Bijak, M., Synowiec, E., Sitarek, P., Sliwiński, T., Saluk-Bijak, J., 2017. Evaluation of the cytotoxicity and genotoxicity of flavonolignans in different cellular models. *Nutrients*, 9(12): 1356.

Chambers, C.S., Holečková, V., Petrásková, L., Biedermann, D., Valentová, K., Buchta, M., Křen, V., 2017. The silymarin composition and why does it matter???. *Food Research International*, 100(3): 339-353.

Davis-Searles, P.R., Nakanishi, Y., Kim, N.C., Graf, T.N., Oberlies, N.H., Wani, M.C., Wall, M.E., Agarwal, R., Kroll, D.J., 2005. Milk thistle and prostate cancer: differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. *Cancer Research*, 65(10): 4448-57.

Delmas, D., 2020. Silymarin and derivatives: from biosynthesis to health benefits. *Molecules*, 25(10): 2415.

El Menshawe, S.F., Ahmed, A.S., Abdelaty, L.N., Aboseif, M.A., 2014. Study of hepatoprotective effect of silymarin and ursodeoxycholic acid in chronic Hepatitis C Patients. *Medicine Science*, 3(4): 1655-1674.

Emadi, S. A., Rahbardar, M. G., Mehri, S., and Hosseinzadeh, H., (2022). A review of therapeutic potentials of milk thistle (*Silybum marianum* L.) and its main constituent, silymarin, on cancer, and their related patents. *Iranian Journal of Basic Medical Sciences*, 25(10):1166.

Eser, N., Göçmen, C., Erdoğan, Ş., Büyüknacar, H.S.G., Kumcu, E.K., Açıkalın, A., Önder, S., 2012. Effect of silymarin on bladder overactivity in cyclophosphamide-induced cystitis rat model. *Phytomedicine*,19(8-9): 840-845.

Fraschini, F., Demartini, G., Esposti, D., 2002. Pharmacology of silymarin. *Clinical Drug ınvestigation*, 22: 51-65.

Gheybi, F., Alavizadeh, S.H., Rezayat, S. M., Zendedel, E., Jaafari, M., 2019. Chemotherapeutic activity of Silymarin combined with doxorubicin liposomes in 4T1 breast cancer cells. *Nanomedicine Research Journal*, 4(1): 29-34.

Giordano, M., Luongo, G., Davinelli, S., Ladhari, A., Nappo, G.R., Giordano, M., 2021. *Silybum marianum*: not Just Silymarin and Flavonolignans. *Records of Natural Products*,15(4),

Karabegović, I.T., Stojičević, S.S., Veličković, D.T., Nikolić, N.Č., Lazić, M. L., 2018. Direct ultrasound-assisted extraction and characterization of phenolic compounds from fresh houseleek (Sempervivum marmoreum L.) leaves. *Hemijska industrija*, 72(1): 13-21.

Karimi, G., Vahabzadeh, M., Lari, P., Rashedinia, M., Moshiri, M., 2011. "Silymarin", a promising pharmacological agent for treatment of diseases. *Iranian Journal of Basic Medical Sciences*, 14(4): 308.

Kim, S.H., Choo, G.S., Yoo, E.S., Woo, J.S., Lee, J.H., Han, S.H., Jung, J.Y., 2021. Silymarin inhibits proliferation of human breast cancer cells via regulation of the MAPK signaling pathway and induction of apoptosis. *Oncology Letters*, 21(6): 1-10.

Koltai, T., Fliegel, L., 2022. Role of silymarin in cancer treatment: facts, hypotheses, and questions. *Journal of Evidence-Based Integrative Medicine*, 27: 2515690X211068826.

Lalani, S.S., Anasir, M.I., Poh, C.L., 2020. Antiviral activity of silymarin in comparison with baicalein against EV-A71. *BMC Complementary Medicine and Therapies*, 20(97): 1-12.

Li, W., Mu, D., Song, L., Zhang, J., Liang, J., Wang, C., Wang, X., 2011. Molecular mechanism of silymarin-induced apoptosis in a highly metastatic lung cancer cell line anip973. *Cancer Biotherapy and Radiopharmaceuticals*, 26(3): 317-

Manikandan, J., Raffiea Baseri, J., 2022. Green Synthesized Fe_3O_4 Nanoparticles as Silymarin Drug Carrier and their Anticancer Activity against Liver-HepG2 and Lung-A549 Cancer Cells. *Asian Journal of Chemistry*, 34(9): 2363-2372.

Polyak, S.J., Ferenci, P., Pawlotsky, J.M., 2013. Hepatoprotective and antiviral functions of silymarin components in hepatitis C virus infection. *Hepatology*, 57(3): 1262-1271.

Sezer, C.V., 2021. An in vitro assessment of the cytotoxic and apoptotic potency of silymarin and silymarin loaded solid lipid nanoparticles on lung and breast cancer cells. *Pakistan Journal of Zoology*, 53(4):1407-1415.

Surai, P.F., 2015. Silymarin as a natural antioxidant: An overview of the current evidence and perspectives. *Antioxidants*, 4(1): 204–247.

Teng, Y.C., Shen, Z.Q., Kao, C.H., Tsai, T.F., 2016. Hepatocellular carcinoma mouse models: Hepatitis B virus-associated hepatocarcinogenesis and haploinsufficient tumor suppressor genes. *World Journal of Gastroenterology*, 22(1): 300.

Vaidya, A., Roy A., Chaguturu, R., 2018. How to rekindle drug discovery process through integrative therapeutic targeting? *Expert Opinion on Drug Discover*, 13(10): 893-898.

Vargas-Mendoza, N., Angeles-Valencia, M., Morales-González, Á., Morales-Martínez, M., Madrigal-Bujaidar, E., Álvarez-González, I., Morales-González, J.A., 2021. Effect of silymarin supplementation in lung and liver histological modifications during exercise training in a rodent model. *Journal of Functional Morphology and Kinesiology*, 6(3): 72.

Wang, Y., Yuan, A.J., Wu, Y.J., Wu, L.M., Zhang, L., 2023. Silymarin in cancer therapy: Mechanisms of action, protective roles in chemotherapy-induced toxicity, and nanoformulations. *Journal of Functional Foods*, 100: 105384.

To Cite: Akbel, E., Bulduk, İ., Karabğ, F., 2024. Developing Rp-Hplc Method and Determination in Vitro Cytotoxicity of Silymarin Obtained From *Silybum marinum* Plant. *MAS Journal of Applied Sciences*, 9(Special Isssue): 899–906.
DOI: http://dx.doi.org/10.5281/zenodo.13939065.