Extraction and Determination of Chemical Ingredients from Stems of Silybum Marianum

Yasin Ghafor, Nian N. Mohammad and Dler. M. Salh
Chemistry Department, Faculty of Science and Educational Science, Sulaimaniyah University, Kurdistan Region, Iraq
E-mail:dlerchem@yahoo.com

Abstract
High performance liquid chromatographic (HPLC) and Inductively coupled plasma (ICP-OES) have been used to analyze the extracts of Silybum marianum (milk thistle) stems for carbohydrates, flavonoid, phenolic acids, Fatty acids, amino acids, flavonolignans and metal ions content. Proximate compositions of milk thistle showed high carbohydrate content especially fructose, high ratio of unsaturated to saturated fatty acid content, a high ratio of essential amino acids especially histidine, and highest ratio of the biologically active flavolignin silybin A. The active antioxidant ingredient coumaric acid showed a highest concentration compared to other phenolic acids and analysis of metal ions showed that Si and Al was the major elements present in the stems of the herb.
Keywords: Silybum Marianum, HPLC, ICP-OES, Carbohydrate, Flavonoid, Phenolic Acid

1. Introduction

Milk thistle or St. Mary's thistle [Silybum marianum] is an annual or biennial herb. The plant is native to the Mediterranean and North Africa but has spread to other warm and dry climates in the America, Australia, and Europe.

The plant reaches to heights 10 feet. It has a stem of 20-150 cm high, erect, ridged and branched in the upper part. A distinguishing characteristic of milk thistle is the white patches found along the veins of the dark green leaves (Figure 1). The principal components of silymarin are silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B and silydianin (Figure 2). The first six compounds exist as equimolar mixtures as trans diastereoisomers (Dr Venketeshwer Rao, 2012).

Figure 1.Milk thistle with white patches along the veins of dark green leaves.

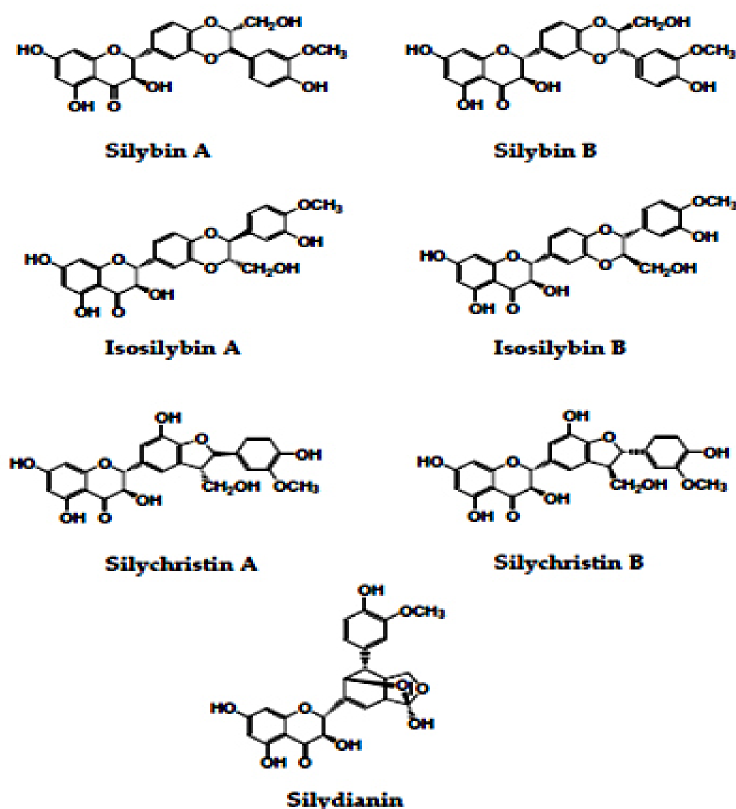


Figure 2.Chemical structures of silymarin components.

Milk Thistle, also known as Silymarin has enjoyed a long history of use in European folk medicine. Centuries ago, Romans recognized the value of this herb for liver impairments. They routinely used the seeds and roots of the plant to restore and rejuvenate a diseased liver (Woodland Publishing Inc 1995).
Many research done on this plant for determination and investigate the physicochemical properties, compsitions and constituents of milk thistle (Silybum marianum) (Ikhtiar Khan, et al. 2007, Szabolcs Nyiredy, et al. 2008. T. RADJABIAN1, et al. 2008 and M. Malekzadeh1, et al. 2011)
The aim of this study is to determine the constituents of the Silybum marianum stems and investigate the physicochemical properties of milk thistle grown in Kurdistan regional of Iraq.

2. Materials and Experiments

Milk thisle [Silybum marianum] stems, were harvested by hand in its optimum state in [Sulaimaniyah city, Chwar chra area] in Kurdistan region–north Iraq in 2013. After a morphological and chemical characterization, the samples were prepared for determination of their active constituents.

2.1. Preparation of the Sample for the Determination of metal ion Contents in milk thistle stems

A sample of milk thistle stem was finely ground to powder in a mortar. 10 g from this powder weighed and put in a muffle furnace at 550-600 ˚ C for at least 3 hours. The ash was then dissolved by adding 50 ml of concentrated HNO_3 and then diluted to 100 ml using deionized water. Measurements of metal ions concentration have been performed using ICP.

2.2. Preparation of the Sample for the Determination of Flavonoid and Phenolic Contents in milk thistle stems

Extraction of flavonoids and phenolic acid compounds were performed by crushing 1.0 g of wet sample into small pieces in a paste- mortar, followed by suspending fine crushed samples into 5ml of ethanol-water (80:20, v/v) in glass tubes. The suspension was then subjected to ultra-sonication (Branson sonifier, USA) at 60% duty cycles for 25 minutes at 25 ˚C followed by centrifugation at 7500 rpm for 15 minutes. The clear supernatant of each sample was subjected to charcoal treatment to remove pigments prior to evaporation under vacuum (Buchi Rotavapor). Dried samples were then resuspended in 1.0 ml HPLC GRADE methanol by vortexing and the mixture were passed through 2.5µm disposable filter and stored at 4˚C for further analysis. Finally, 20 µL of the sample was injected into HPLC system according to optimum condition using phenomenex C-18, 3 µm particle size (50x2.0 mm I.D.) column and a mobile phase which consists of acetonitrile: methanol:0.1% formic acid

(6:3:1, v/v) at a flow rate of 1.2 ml/min. The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu. The eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

2.3. Preparation of the Sample for the Determination of Fatty acids in milk thistle stems

5 g of sample were minced in blender with 20 ml ionized water. Fat separation was carried out according to ISO-IDF (2001) using mixture of 20 ml (50:50, v/v), n-pentane and diethyl ether after first adding of 20 ml of 0.1M ammonium hydroxide solution to the extract. Fat separation was also carried out using the rapid method proposed by Feng et al. (2004). 30 ml of raw extract was centrifuged at 14,000 rpm for 30 minutes using a Beckman (Fullerton, CA) J2-MC centrifuge. The fat layer was transferred to a microtube and left at room temperature for approximately 30 minutes before being microcentrifuged at 14,800 rpm for 20 minutes at room temperature. After the second centrifugation, the top layer was removed for analysis. In this method, separated fatty acid were stored in amber vials, exposed to a stream of N2 gas and frozen at -20 ˚C until analysis. The direct measurement of fatty acid samples were made on medium reversed phase liquid chromatography. The mixture of fatty acids was separated on FLC column, 3 µm particle size (50x 4.6 mm I.D.) C-18DB column using mobile phase that consists of acetonitrile: tetrahydrofuran (THF): 0.1% phosphoric acid in THF (50.4: 21.6:28, v/v) at flow rates 1.5 ml/min and by using a UV set at 215 nm. The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu. The eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

2.4. Carbohydrate measurements in milk thistle stems

5g of the sample were crushed and extracted at room temperature using deionized water. To facilitate the best contact between plant tissues and extraction solvent, the sample was shaken for 15 minutes and finally the suspension was centrifuged at 3000 rpm for 10 minutes. Prior to injection, the aqueous extracting phase was filtered through a single use 0.22 µm nylon syringe filter (Aldrich) and passed on cartridge on Guard A (Dionex) to remove anion contaminants. Such a solution turned out to be colorless. The same solution was injected and the sample separation carried out. The sample was separated on FLC anion exchange shimpack A1 column, 3 µm particle size (50x 4.6 mm I.D.) using mobile phase 21 mM NaOH spiked with 1 mM barium acetate at a flow rate 1.3 ml/min and using refractive index detector.

2.5. Analysis of flavonolignans from silbyium marianium

5 g of sample powder were dissolved in 30 ml acetonitrile, then agitated in ultrasonic bath for 30 minutes, then the extract were filtered on whatman filter paper 0.5 µm to remove the fibers and undissolved textures, then 20 µL of the aqueous filtrate were injected to HPLC column, analyzed accurate on HPLC column as in above separated condition for quantitatively identify the active constituents. The mixture of flavonolignans was separated on FLC column, 3 µm particle size (50x 4.6 mm I.D.) Zorbax clips XDB_C-18 column using mobile phase that consists of deionized water with 1% formic acid (A): methanol (B), by gradient program from 0% B to 100% B in 10 minutes at flow rates 1.0 ml/min and by using a UV set at 254 nm. The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu. The eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

2.6. Analysis of amino acids by PITC

For the extraction of the amino acids, 10 g of the sample weighed and 15 ml of 6N HCl was added to it. The sample was then put in a sealed vessel under reduced pressure for the hydrolysis of the protein. The content of the vessel was then dried under reduced pressure and allowed to stand overnight in a desiccators. HCl was removed by adding 15ml of 6N NaOH solution. The sample was filtered and derivatized then injected on to HPLC. The general protocol for derivatization was by mixing 10 µL aliquots of standard or sample with 10 µL of PITC (phenyl isothiocyanate) reagent. After 1 minute, 50 µL of 0.1 M sodium acetate pH (7.0) were added. The solution mixed for 5 minute and 20 µL sample was subjected to analysis. The HPLC system consist of two Shimadzu model LC-6A pumps (Koyoto, Japan), SIL-6A automated system controller for generation of elution gradients and a Shimadzu SPD-6AV UV-Vis detector equipped with 8 µ flow cell. The sample introduces into the column through Rheodye 7125 sample injector with 20 µ injection loop. The data were processed and analyzed by RC-6A data processors. The column used was Shimpack XR-ODS (50x4.6 mm I.D.), 3 µm particle size. Gradient were formed between two degassed solvents. Solvent A consists of 5% methanol in 0.1N sodium acetate buffer pH 7.0. Solvent B consists of methanol. The separation was performed using flow rates 1 ml/min and detection using UV set at 254 nm.

3. Results and Discussion

In this work Stems of Silybum marianum (milk thistle) plant were collected from Sulaimaniyah governorate and evaluated for the active constituents. Concentration in ppm of different ingredients in the stems of silbyium marianium were determined. These ingradients found in its edible stems have antioxidant, hepatoprotective, anticancer and hypolipidemic folk. The stems of the plant have been evaluated for its oil, aminoacids, carbohydrates, flavolignins, phenolic acids and mineral contents. The proximate composition of milk thistle

stems presented in table 1:

Table 1.The proximate composition of milk thistle stems

No.	Ingredients	Total concentration in ppm
1	Carbohydrates	40.207
2	Amino Acids	221.634
3	Fatty acids	61.113
4	Flavonolignans	251.704
5	Phenolic acids	87.175

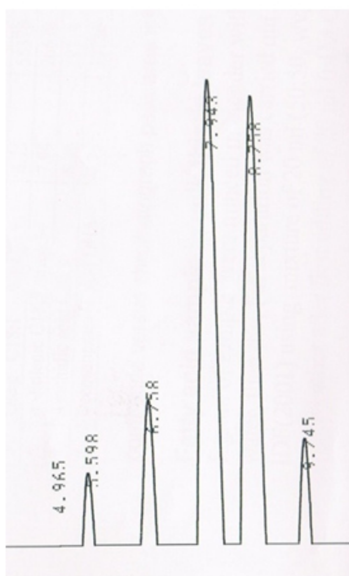
Minerals content: ICP-OES was used for the determination of minerals in the stems of silbyium marianium (table 2). The results indicates that the stems of the plant contains highest concentration of Si and Al, 398.4 and 166.1 ppm, respectively, and lowest concentration of As (ca. 0.028 ppm). It is well known that seeds of milk thistle if consumed, is a good source for minerals. It can be proposed that the stems of milk thistle also could be a fair source of minerals.

Table 1. ICP-OES analysis for minerals

No.	Elements	Concentration in ppm
1	V	0.82
2	Cr	1.37
3	Mn	9.75
4	Fe	3.25
5	Co	0.04
6	Ni	0.96
7	Zn	7.12
8	Cd	0.11
9	Pb	2.34
10	Si	398.4
11	Al	166.1
12	Sn	0.6
13	As	0.02779
14	Ag	1.136
15	Bi	9.005

3.1 Analysis of Fatty acids:

The stems of Milk Thistle contain ca.61ppm fixed oil. Fatty acid composition of milk thistle oil is presented in figure 3. The composition of the oil revealed that linoleic acid (11ppm) and Myristic acid (11ppm) are the predominant fatty acids. Milk thistle oil is fairly high in polyunsaturated fatty acids particularly an essential fatty acid i.e., α-linoleic acid (Omega-3) which is about 8.7 ppm, this fatty acid is believed to be helpful in lowering cholesterol, when induced in the diet.



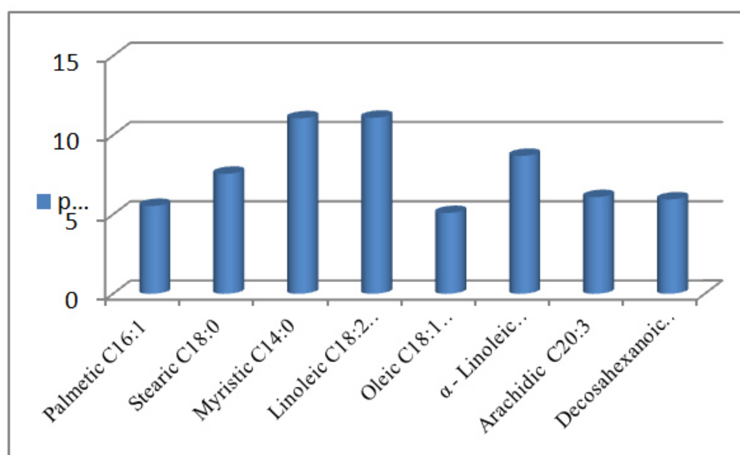


Figure 3.HPLC analysis of Fatty Acid content

3.2 Analysis of Amino acids:

Milk thistle stems were analyzed for amino acid composition and the results are presented in figure 4. The stems contain an excellent quality and quantity of essential amino acids, ranging from 6.7-31.4 ppm. The HPLC analysis revealed eighteen amino acids. Among these Histidine, Arginine, Glycine, and Threonine were the major amino acids.

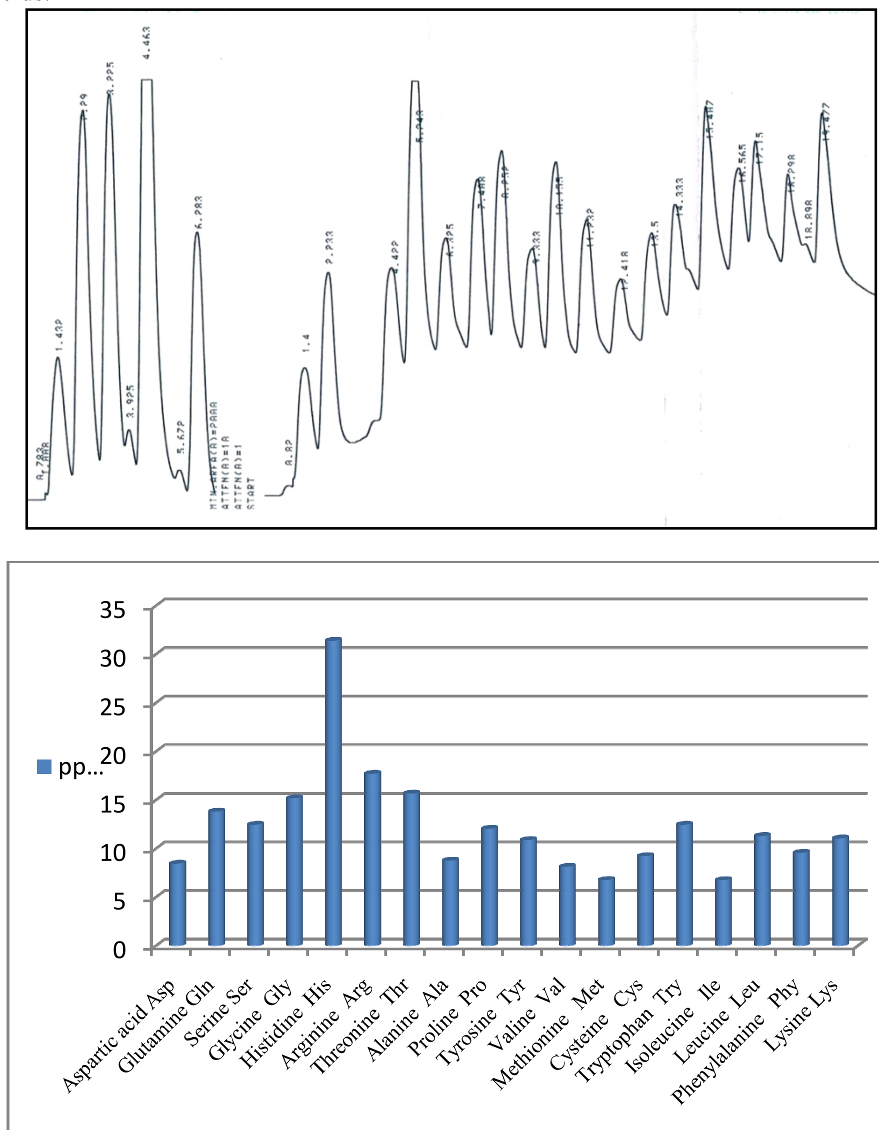


Figure 4.HPLC analysis of amino Acid content

3.3 HPLC Analysis of Carbohydrates

The harvested *stems* showed the highest fructose (ppm) and lowest Mannitol (ppm) content. (figure 5)

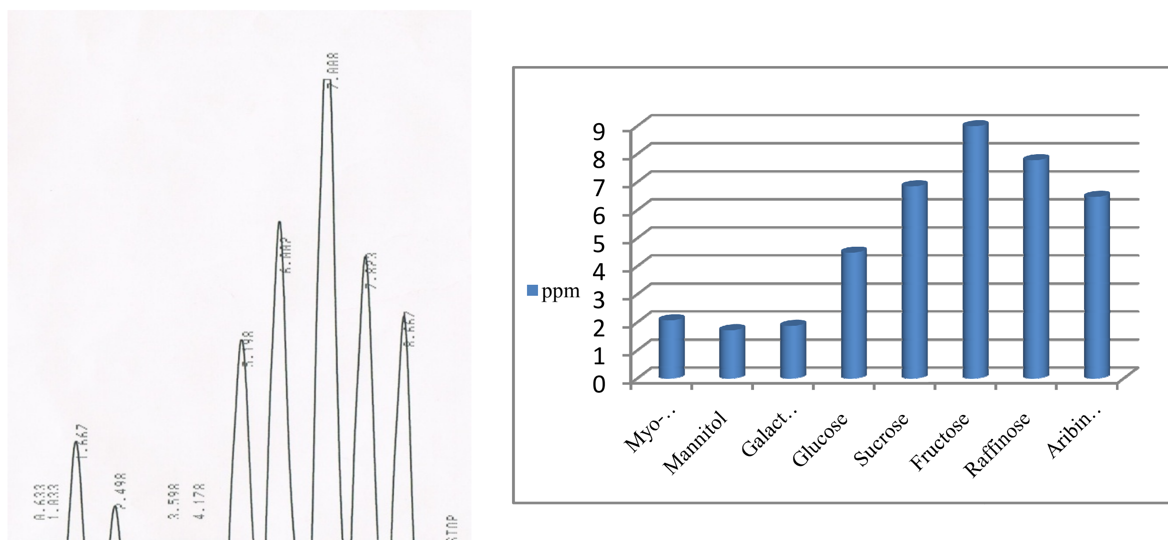


Figure 5.HPLC analysis of Carbohydrate contents

3.4 HPLC analysis of Phenolic acid and flavonoids:

The total content of phenolics and flavonoids in the plant stems extracts were analyzed using HPLC. The results are shown in figure 6.

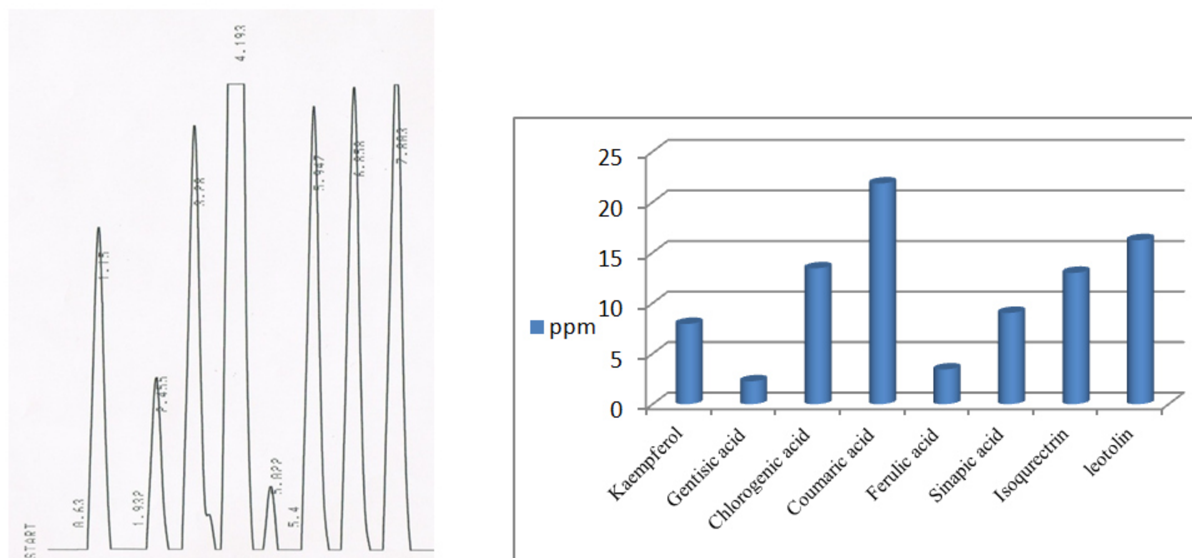


Figure 6: HPLC analysis of Flavonid and phenolic acids

3.5 HPLC analysis of flavolignin:

The principal components of silymarin (silybin, isosilybin, silychristin and silydianin) were determined using HPLC method. The results of analysis of the content of individual flavonolignans content in the fruits stem is shown in figure 7. The results showed that silybins are the major Silymarin in the stems and silydianin is the minor constituents.

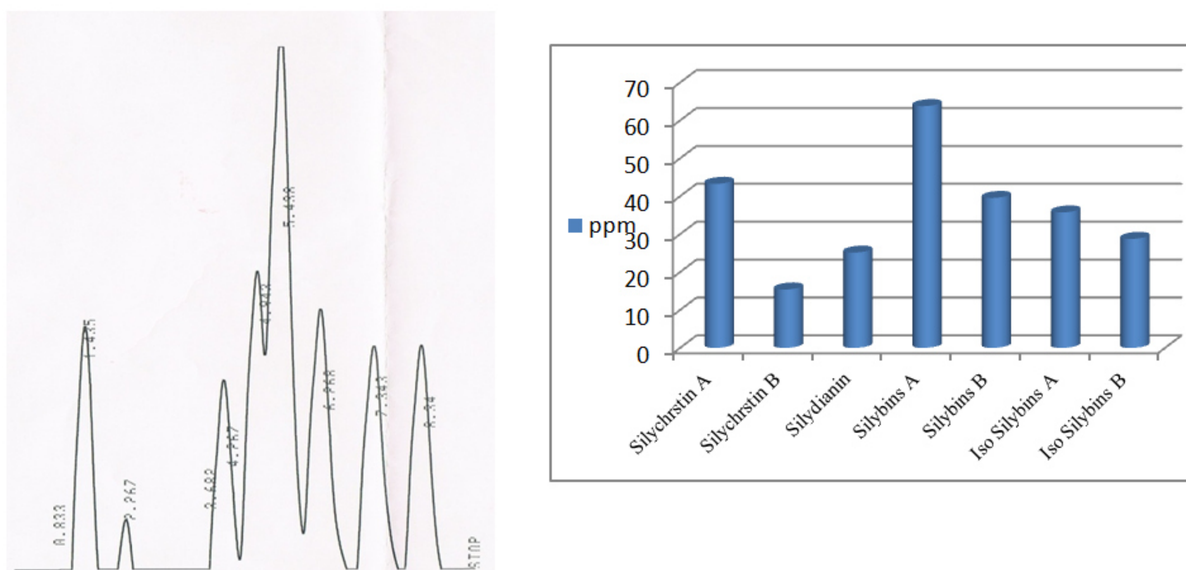


Figure 7: HPLC analysis of Flavonolignans conten

4. Conclusion

The analysis of silybum marianum stems showed lower concentration of all ingredients except Si which is found in higher quantity compared to other parts of the herb.

References

Dr Venketeshwer Rao, (2012), "Phytochemicals - A Global Perspective of Their Role in Nutrition and Health", Ch. 12

Woodland Publishing Inc (1995), "MILK THISTLE, A REMARKABLE FLAVONOID, ANTIOXIDANT AND LIVER PROTECTANT", P.O. Box 160 Pleasant Grove, UT 84062, P. 7

Ikhtiar Khan, Hidayat Ullah Khattak, Ihsan Ullah and Fazlullah Khan Bangash, (2007), Jour. Chem. Soc. Pak. Vol. 29, No. 6

Szabolcs Nyiredy, Zoltan, Sandor Antus, Zsuzsanno Samu, (October 2008). Chromatographia, Volume 68, Issue 1 Supplement, pp 5-11

T. RADJABIAN1**, SH. REZAZADEH2 AND H. FALLAH HUSEINI2, (2008), Iranian Journal of Science & Technology, Transaction A, Vol. 32, No. A2,

M. Malekzadeh1, S. I. Mirmazloum2*, H. Rabbi Anguorani3, S. N. Mortazavi3 and M. Panahi4, (2011), Journal of Medicinal Plants Research Vol. 5(8), pp. 1485-1488