# Characterization of the flavonolignans in *Silybum marianum* L. Grown Naturally in Iraqi-Kurdistan Region

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## Abstract:

**(Abstracted from MSc. Thesis entitles "Pharmacognostic and Pharmacologic study of Silybum marianum grown naturally in Iraqi-Kurdistan Region")**

**The antihepatotoxic principles found in the indigenous plant Silybum marianum L. (Milk thistle) was obtained using a multi-extraction/ separation and isolation procedures. Starting with identification of the plant and collection of the seeds. A pretreatment (defatting) step with light petroleum ether preceded the extraction with organic solvent (ethanol) in a soxhlet extractor. The alcoholic extract after drying was partitioned with n-hexane, Chloroform, Ethyl acetate, and n-butanol successively. Each fraction was separated by flash column chromatography. The sub-fractions after column chromatography were tested with thin layer chromatography (TLC) for the presence of the flavonolignans. Fractions showed spots with the same $R_f$-values were combined and furthermore isolated with high performance liquid chromatography (HPLC). Components were then purified and identified using UV, IR-spectroscopy. The results were compared with a standard sample of silybin (obtained from Sigma-Aldrich Co.). Results confirmed that the plant Silybum marianum L. which grows indigenous in Kurdistan Region/ Iraq, reserves the same active components (flavonolignans) present in the plant that grows elsewhere.**

**Key words:** Flavonolignans, Silybins, Chromatography.

## Introduction

Silybum marianum L. Gaertn (Astraceae) (Milk thistle) which contains flavonolignans is a cosmopolitan plant has been found to be one of the most notable folkloric medicinal plants that is under extensive studies for the extended chemical, pharmacological, pharmacokinetic properties have been pursued [6, 9, 13, 17, 20, 26], while in this region of the world, especially in Kurdistan, this plant has no record of being used as a medicinal plant. Silymarin
(mixture of flavonolignans) is found in the entire plant but concentrated more in the seeds [16, 20].

Many methods for the evaluation of the flavonolignan contents were proposed, we adopted the method applied by Kim *et. al*. [15] and the seeds were defatted before extraction to increase the yields [21, 23].

## Aims of the study:

The aim of this study is to evaluate for the first time the chemical composition of the naturally grown Milk thistle in Iraqi Kurdistan qualitatively as the source of flavonolignans.

## Materials and Method:

The plant was identified before collection by two taxonomists (College of Agriculture and College of Science/ Department of Biology), the time of seed collection and plant part was designated. During late May, the flower tops were collected in the Old Slaughter House Area/ South East of Sulaimaniyah City, Kurdistan Region-Iraq. Dried under shade and purified from adulterants. Herbarium specimen for proper identification and authentication was kept in the Department of Pharmacognosy, College of Pharmacy-University of Sulaimani.

The seeds were ground to coarse particles and subjected to defatting by light petroleum ether (40-60$^{o}$C) in a soxhlet apparatus for 24 hours [21, 23]. The solvent was evaporated under vacuum using rotary evaporator and the defatted seed were dried in a desiccator

under vacuum using a refrigeration compressor for 48 hours to ensure complete dryness.

Absolute ethanol denaturized (with 5% Methanol) was used for the extraction of the active constituents as the best solvent for extraction of the silybins [1, 2, 3, 14, 22, 23]. The ground defatted dried seed sample was placed in a soxhlet apparatus, and a continuous extraction was run for 10 hours at a temperature not exceeding (78$^{o}$C) [23].

The marc was air dried under vacuum, weighed and the alcoholic extract evaporated using rotary evaporator at 45$^{o}$C. The gummy residue which contains the active ingredients was weighed. To reconstitute the concentrated alcoholic extract, minimal amount of water was added, and then vacuumed in a desiccator containing ammonium sulfate as dehydrating agent and the sample was left to reach 20ml volume. A sample of the alcoholic extract spotted on TLC plate to monitor the presence of flavonolignans using $FeCl_3$ as a general reagent.

For further separation of the polar and non-polar components of the extract, Liquid–liquid partitioning was applied using *n*-hexane, chloroform, ethyl acetate and *n*-butanol successively and each fraction was labeled for further investigation [1, 2, 15]. All the five fractions obtained from partitioning were chromatographed on TLC plates, with precoated silica gel plates and paper strip chromatography [8]. The solvent system used for development was (*n*-butanol : Acetic acid : Water) (6 : 3 : 1) [11] . All the five fractions were tested with ferric chloride solution 1% in test tubes as well as spotted on filter papers for color development (figure3). The chromatograms were detected under UV short wave 256nm. Then they were developed by spraying with ferric chloride 1% solution and the spot appeared clearly. Each fraction gave spots with different $R_f$-values than other fractions and the $R_f$-values were calculated [8].

**Column chromatography:**

Each of the five fractions was column chromatographed in glass column (30cm). The column was prepared by plugging the lower end with glass wool and then was filled to the height of (30cm) with silica gel powder that was solvated with sufficient quantity of chloroform as a stationary phase [15, 25]. The fractions were eluted with (Chloroform : 0 100 : Methanol) as gradient elution solvent system at 20ml interval for each proportion. The ethyl acetate fraction was collected as 100 fractions each of 1ml. All the fractions mentioned above were concentrated to dryness in vacuum using rotary evaporator. Then each dried solid fraction was recovered from the round flask with ethanol 98% [15].

A TLC and Paper strip chromatography were applied for each fraction and the $R_f$-values of each spot were calculated. Those fractions which showed similar $R_f$-values were combined and collected as one sample and stored in deep freeze for further HPLC chromatography.

**HPLC fractionation:**

A preparative HPLC (for combined fractions showed similar $R_f$ –values after column chromatography) using gradient mixtures of methanol-$H_2O$ (50 : 50 ⟶ 65 : 35 for 60 minutes), then (65 : 35 ⟶ 100 : 0 for 20 minutes), as eluents [15, 24].

**Purification:**

Fractions A and B (from ethyl acetate fraction), showed the highest content of flavonolignans were applied for preparative TLC analysis [15]. Silica gel GF 254 has been used for preparative TLC. Slurry made according to the official method [8]. Volume of 0.35ml was applied for each fraction as stationary band at the base line and developed. Plates were dried and checked under UV at 254/ 361nm. Each sample was also applied at the right edge of each plate for comparison; the separated bands were

marked and scraped off, separated and dissolved in a mixture of chloroform: methanol (9 : 1), boiled, shaken for half an hour and filtered.

The upper layer was evaporated to dryness; each layer was checked to confirm purity by TLC using the same system as mentioned above and examined under UV-light 256.

**Weighing of the purified fractions:**

Fractions (A and B) from ethyl acetate were crystallized and weighed to calculate the percentage of yield of the active component in the silymarin mixture seed sample.

**Results and discussion:**

The dried defatted seeds weighed 74.32gm. The lost 25.68gm corresponded to the weight of the fatty materials content in the seed sample, 25% of the total weight of the seeds was close to the results obtained by Sunny *et. al.* [23].

(100.00gm – 74.32gm = 25.68gm)
(025.68gm – 23.44gm = 03.24gm)

This difference is possibly due to one or several of the following factors such as error of extraction including weighing error, personal error, instrumental error, variation of the taxon under study due to ecological and genotypic variations, and the error of recovery.

The error of recovery could be seen by the fact that *n*-hexane and the chloroformic extract which removed further lipophilic compounds would add to the total percentage of the lipophilic compounds which was extracted further, 25.68gm (weight loss during defatting) 1.25gm (weight loss with *n*-hexane extraction) 0.22gm (weight loss with chloroform extraction). The total weight loss during all the lipophilic solvents extraction equals to (27.15gm), this corresponds to 27.15% of the total lipid content in the seeds sample. However when defatting involved heavy petroleum ether (80-100$^{o}$C), the lipid content was (14.7gm). These results indicate that light petroleum ether is far better than heavy petroleum ether for the extraction of lipophilic compounds and hence defatting [4].

Similar results were previously obtained by Benthin *et. al.* [4], and Sunny *et. al.* [23], who recovered 25% fat from the whole seeds using petroleum ether alone. The extra defatting with *n*-hexane and chloroform has no records in the literatures [15].

**TLC results:**

TLC analysis of *n*-hexane fraction showed no spots when detected with ferric chloride 1% spray reagent or under UV, while the paper chromatogram showed a faint spot with ($R_f$-value = 0.4). Chemical test with ferric chloride solution 1% in the test tube showed no color change.

TLC of the chloroformic layer gave no indications for the presence of phenolic compounds when the spots were sprayed with ferric chloride 1%. The paper chromatography technique was applied to the total fraction and showed no spots. Chemical test with ferric chloride solution 1% in a test tube gave faint color change as indication for the presence of phenolic compounds.

Ethyl acetate fraction analyzed with TLC, giving positive color change of the spots with ferric chloride 1% solution indicating the presence of phenolic compounds. The paper chromatogram showed a clear spot of ($R_f$-value = 0.88) was detected, chemical test of ferric chloride solution 1% made in a test tube, a dark greenish- black color appeared.

**IR-Spectrum results**

The IR-spectrum showed the following absorption bands as listed in the (Table 1) below:-

**Table 1:** IR-stretching absorption bands for functional groups

| Functional group | Stretching | (γ) Absorption band 1/cm |
|---|---|---|
| Hydroxyl of alcohols and phenols | O-H | 3207, 3257, 3338, 3431, 3545, 3607, 3688 |
| Aromatic ring | C-H | 3080, 3113,3151 |
| Cyclic compounds | C-H | 3066 |
| $CH_3$ | C-H | 2845, 2893, 2926.3, 2999.41 |
| Carbonyl | C=O | 1747 |
| Aromatic ring | C=C | 1580,1610 |
| Ether | C-O | 1147 |

**HPLC results:**

Results of preparative HPLC analysis for all fractions using gradient mixtures of methanol-H2O (50 : 50 ⟶ 65 : 35 for 60 minutes, then 65 : 35 ⟶ 100 : 0 for 20 minutes) as eluents, the details are listed below:-

Table (2), HPLC results for all fractions

| # | Fraction | Retention time (minutes)* | Compound | % Area |
|---|---|---|---|---|
| 1 | *n*-hexane fractions (80:20/70:30) | 35.183 | Silybin A | 4.13 |
| 2 | *n*-hexane fraction (10:90) | 37.067<br>42.800 | Silybin B<br>Isosilybin B | 2.20<br>0.65 |
| 3 | *n*-hexane fraction (90:10) | 35.450 | Silybin A | 1.82 |
| 4 | Chloroform fractions ($CHCl_3$ 100/90:1/80:20/70:30/60:40/50:50) | 13.233<br>35.733 | Taxifolin<br>Silybin A | 0.70<br>1.80 |
| 5 | Ethyl acetate fractions (5 to 50) | 23.867<br>35.983 | Silychristin<br>Silybin A | 1.20<br>13.55 |
| 6 | Ethyl acetate fractions (54 to 64) | 35.517 | Silybin A | 0.90 |
| 7 | Ethyl acetate fractions (51 to 53) | 36.217<br>41.250 | Silybin A<br>Isosilybin A | 0.65<br>2.51 |
| 8 | *n*-butanol fraction ($CHCl_3$ 100) | 35.583 | Silybin A | 3.73 |
| 9 | *n*-butanol fraction (90:10/80:20/70:30/60:40/50:50/40:60/30:70/20:80) | 20.750<br>37.633<br>43.583 | Isosilychristin<br>Silybin B<br>Isosilybin B | 2.30<br>0.33<br>1.42 |

**Results for preparative TLC of ethyl acetate fractions A and B:**

Sample A (ethyl acetate fraction), weighed 0.50 gm:
Layer No.1($R_f$-value = 3.5/11 = 0.31).
Layer No.2 ($R_f$-value = 5.2/11 = 0.47).
Layer No.3 ($R_f$-value = 6/11 = 0.54).
Layer No.4 ($R_f$-value = 8.5/11 = 0.77).
Layer No.5 ($R_f$-value = 10/11 = 0.90).
Sample B (ethyl acetate fraction), weighed 0.30 gm:
Layer No.1 ($R_f$-value = 7/12 = 0.58).
Layer No.2 ($R_f$-value = 8.8/12 = 0.73).
Layer No.3 ($R_f$-value = 0.90).

**Extraction with absolute alcohol:**

Results gave a total fraction of 9.14gm/100gm using 98% ethyl alcohol. However in the literature there is no reference as to show the yield of the absolute 98% ethanolic extract. Only results were calculated in the literatures after running all the chromatographic methods for the isolation and separation of the active constituents (silymarin) and calculate the yield of isomers in the total silymarin fraction [15, 23].

**Column chromatography:**

<u>*n*-hexane (Fr.2)</u>

TLC, paper chromatography, and chemical reaction with ferric chloride solution 1% for all the sub-fractions obtained from column chromatography, showed presence of different polyphenolic compounds as they gave four different $R_f$-values with TLC [11]. These compounds are stereoisomers having similar properties since they gave same $R_f$-values with paper chromatography analysis and confirmed by

HPLC analysis (figure 4) mostly less polar silybins (silybin A, silybin B, and isosilybin B) [15].

Presence of these compounds in the beginning sub-fractions explained by the fact; that washing the sample with large volume of *n*-hexane eluted some of the flavonolignans [10].

**Chloroform (Fr.3):**

TLC analysis for the sub-fractions obtained from column chromatography, showed presence of spots in the first five sub-fractions with the same $R_f$-values while paper chromatography analysis also showed spots with the same $R_f$-values in the same sub-fractions, except for fraction (100:0) which showed two spots with different $R_f$-values indicating the presence of two flavonolignans [15, 24]. Ferric chloride reaction on filter paper also gave same positive results for the same sub-fractions [14]. These results were confirmed by HPLC analysis with the presence of [taxifolin (polar) and silybin A (less polar)] (figure 4) [15, 24].

**Ethyl acetate (Fr.4):**

This fraction should contain the highest concentration of the flavonolignans [23, 27]. TLC analysis for the sub-fractions obtained from column chromatography showed separation of three components in the sub-fractions (5 to 50/ 54 to 64/ 51 to 53) with three $R_f$-values (0.57/ 0.65/ 0.7 respectively) (Figure 2). This has been confirmed by HPLC analysis giving three retention times for (silychristin, silybin A, and isosilybin A) and were in agreement with Krol and Kim *et. al.* (Figure 4) [7, 15].

Ferric chloride reaction on filter paper also confirmed the presence of the flavonolignans in these sub-fractions which confirms the presence of phenolic compounds (Figure 3) [11].

***n*-butanol (Fr.5):**

TLC analysis of the sub-fractions obtained from column chromatography showed eight spots with only two $R_f$-values (0.92 and 0.96) while paper chromatography analysis showed four spots with the same $R_f$-values indicating the presence of two flavonolignans in the fraction as obtained by Wagner and Kim *et. al.* [11, 15].

HPLC analysis separated four components (silybin A, silybin B, isosilychristin, and isosilybin B) (Figure 4). Presence of four separated components by HPLC while only two appeared on TLC plates may be due to very small concentration of the other two components (0.33 for silybin B and 1.42 for isosilybin). Also these stereoisomers (silybin A, silybin B, and isosilybin B) have similar physical characteristics rendering them more difficult for separation by TLC [11].

Ferric chloride reaction on filter paper also showed positive reaction for the same sub-fractions.

**Spectrometry**

**IR-spectrum analysis (figure 7):**

Sample results for the combined fractions sample (mixture of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol fractions) that include silymarin mixture (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin) under IR-(KBr) spectrum analysis showed absorption bands as listed in (Table 3/Figure 4). The absorption spectrum frequencies at (3207, 3257, 3338, 3431, 3545, 3607, 3688) $cm^{-1}$ are for O-H stretching of phenolic and alcoholic hydroxyl groups, these bands represent multi hydroxyl groups with different electronic environment [19]. Frequencies at (3151, 3113, 3080, 3066) $cm^{-1}$ are for (C-H) stretching of aromatic rings and cyclic compounds (figure 1).

Frequencies at (2999, 2926, 2893, 2845) $cm^{-1}$ are (C-H) stretching for methyl and methylene group [19].

Frequency at (1747) $cm^{-1}$, represents unique band for carbonyl (C=O) (ketone)

group, which presents just one carbonyl group in mixture [19]. Absorption bands at (1580, 1610) $cm^{-1}$ represents (C=C) stretching for aromatic compounds. Different bands represent more than one aromatic ring with different electronic environment and substituent [19]. Absorption band at (1147) $cm^{-1}$ is for stretching (C-O) of ether group [19, 24].

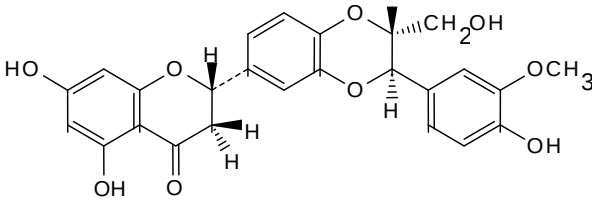


**Fig. 1** The structural formula of silybin with highlights of the functional groups

The different silymarin isomers have same flavone nucleus that show same absorption band frequencies as they have similar electronic environment (10, 18, 59, 62). The flavone nucleus contains (three phenolic hydroxyls, two aromatic rings, one heterocyclic compound, and one carbonyl group) functional groups that should give IR-absorption band spectra in regions (3650-3584 $cm^{-1}$ for hydroxyl group of alcohols and phenols) [19], aromatic rings and cyclic compounds give absorption at (3100-2990 $cm^{-1}$) [19], while carbonyl group gives absorption at (1870-1540 $cm^{-1}$) [19]. The different IR-spectrum absorption bands between silymarin isomers will be for the substitution on the lignan part (alcoholic hydroxyl, methyl, and ether groups) of the molecule and the position of lignan on the flavone nucleus as shown (Table 3) [19].

Results for IR-spectrum analysis also showed below close identical absorption bands for the similar flavone nucleus and different bands for the substitution on the lignan moiety.

In the study conducted by Kim *et. al*. [15] on the complete isolation and characterization of silymarin constituents showed similar absorption spectrum bands for silymarin components that confirm IR-spectrum results, (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin) according to (Table 3).

Comparing our IR-spectrum results with those obtained by Kim *et. al.* [15], all the functional groups appeared with comparable absorption bands for each functional group. However the presence of more than one absorption band for a function group represents presence of a mixture (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin).

**UV/VIS-spectrum analysis:**

Our results for UV-spectrum analysis for ethyl acetate fractions (8, 9, and 10) showed absorption band with three frequencies (λmax) at (288, 230, and 215) nm (Figure 6), same frequencies were obtained with the standard silybin compound (Figure 6) as a confirmation results. UV-spectrum analysis for silymarin mixture by Kim *et. al.* in 2003 also showed same frequencies. Such results indicate the presence of the same compounds in the silymarin mixture [12, 18].

**Preparative HPLC analysis (qualitative):**

Preparative HPLC analysis for the drug sample fractions showed retention times on HPLC chromatogram listed in Table (2). These correspond to silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and taxifolin [15, 24], as each compound listed to its correspondent retention time.

However all fractions (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol) showed the presence of silymarin components, yet each fraction reserved certain peaks that correspond to one or more

component [15]. The highest percentage detected in the ethyl acetate fraction (dielectric constant = 6.0) [10] for silybin A (Retention time 35.983 and 13.55% area) (table 2), results are confirmed with those obtained Kim *et. al.* (figure 5) [15]. More polar components of silymarin complex (isosilychristin) were isolated with more polar solvents like *n*-butanol (dielectric constant 17.8) (Table 2).

The *n*-hexane (dielectric constant = 1.9) [10] fraction extracted some parts of the less polar silybin A and B, as they have been washed out by three volumes of *n*-hexane (high quantity) [10, 15] while chloroform (dielectric constant = 4.81) extracted both polar and non-polar flavonolignans [10]. The pharmacologically active silybin A and B were obtained mainly with ethyl acetate fraction [like dissolve like] [7, 15], however; taxifolin which is polar compound was isolated by chloroform [10] which supposed to extract both polar and nonpolar compounds [10].

Silydianin did not appear in the HPLC analysis; this may be due to low percentage in the seed sample [19, 24].

In a study conducted by Kroll *et. al.* [7] separation of the components of silymarin using preparative HPLC is shown, however retention times (17.027 for silybin A/ 17.523 for silybin B/ 18.635 for isosilybin A, 19.025 for isosilybin B/ 9.847 for taxifolin/ 12.047 for isosilychristin/ 12.805 for silychristin/ and 13.836 for silydianin) were different from our result due to the difference in the circumstances [different mobile phase (methanol : water 30:70 to 70:30), time (over 20 minutes) and columns (Meta Therm column heater)] were used, as shown in (Figure 4).

**Preparative TLC:**

Preparative TLC results for the ethyl acetate fraction (samples 8 and 9) showed five separated compounds for sample 8 and three separated compounds for sample 9 each with different $R_f$-values indicating presence of eight different isomers [8].

**Conclusions:**

- The result of this research indicate that *Silybum marianum* (L.) which grows indigenously in Kurdistan Region- Iraq, contains the same active components (silybins) which have been demonstrated in other researches conducted on the same plant grown in other parts of the world.
- This research has brought to light a neglected cosmopolitan plant in Kurdistan Region- Iraq into the forefront of medical sciences, being one of the best therapeutic agents as antihepatotoxic.

**Recommendations:**

- As a result of this research, this plant (indigenous *Silybum marianum*) could be conveyed into cultivation practices as additional cash crop for farmers in future when demands on this material increase either by international or domestic pharmaceutical industries.
- This line of research opens future possibilities in using this active principle (silymarin) in treatment protocols of other illnesses among which has been suggested, a research program for its anticancer, and antidiabetic. We might add as anti-inflammatory for the possible use of this extract for the treatment of rheumatoid arthritis.
- Although we were concerned with the flavonolignans as the active components, but the plant contains high content of fixed oils (25%) which is considered among high oil yielding corps. The total oil contains vitamin-E which is could also be considered another avenue for further study.

**List of figures and tables:**

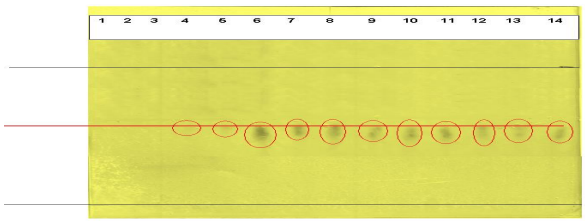

**Fig. 2** TLC chromatogram for ethyl acetate sub-fraction

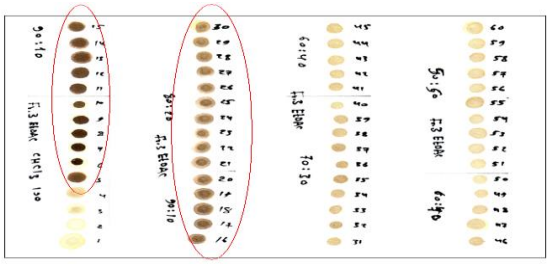

**Fig. 3** ferric chloride test for ethyl acetate sub-fraction

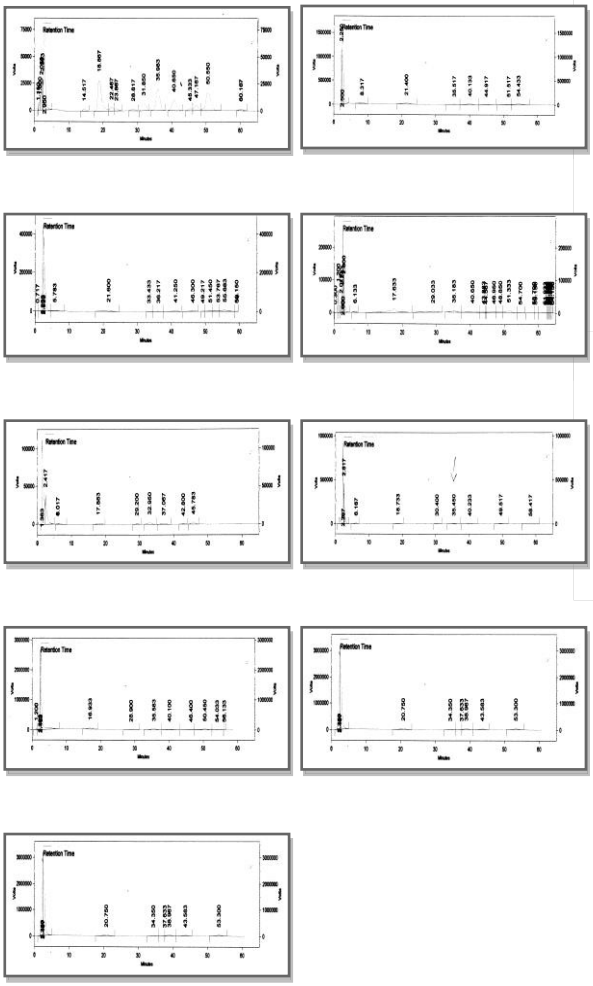

**Fig.4** HPLC analysis chart all column fractions of silymarin isomers

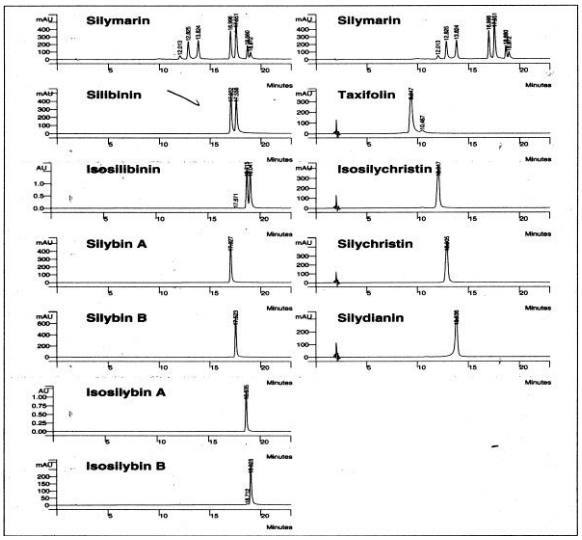


**Fig. 5** HPLC analysis chart for silymarin isomers, Kim *et. al.* [15]

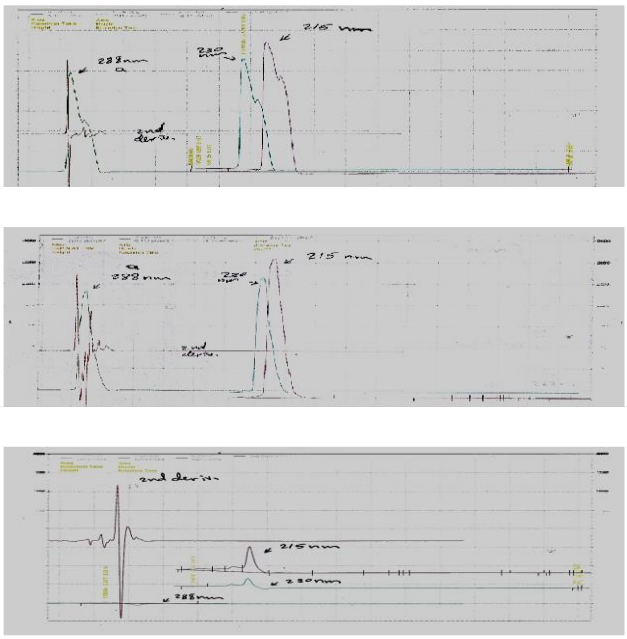

**Fig. 6** UV-spectrum for standard silybin compound)/ethyl acetate sub-fractions (8,9, and 10)

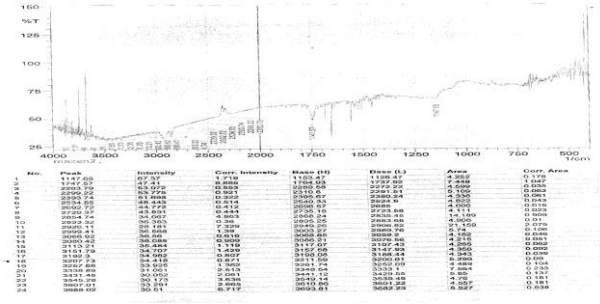

**Fig. 7** IR-spectrum for silymarin mixture

**Table 3:** IR-spectrum results

| **IR-spectrum $cm^{-1}$ (Kim *et. al*).[15]** | | | | | **IR-spectrum $cm^{-1}$ (Our results)** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3565,3456 3424,3423 ,3422,341 6,3403 | 3262 3191 | 3086 | 2941, 2940, 2939 2888 1745 | 1641,1640 ,1639,163 8,1616 1595,1592 1166,1165 ,1163,116 2,1155,11 38 | 3688 3607 3545 3431 | 3338 3257 3207 3151 | 3113 3080 3066 2999 | 2926 2893 2845 1747 | 1610 1580 1147 |
| **Compound** | **IR-spectrum $cm^{-1}$** | | | | **Compound** | **IR-spectrum $cm^{-1}$** | | | |
| Silybin A | 34 56 31 91 30 86 16 38 | 15 92 15 10 14 67 14 35 | 1365 1279 1237 | 1188 1165 1082 | Silybin B | 3565 3422 1639 | 1595 1510 1470 | 1435 1361 1277 | 1182 1162 1086 |
| **Compound** | **IR-spectrum $cm^{-1}$** | | | | **Compound** | **IR-spectrum $cm^{-1}$** | | | |
| Isosilybin A | 34 24 29 39 16 40 | 15 95 15 10 14 68 | 1365 1271 1163 | 1085 1027 | Isosilybin B | 3423 2940 2888 | 1640 1595 1510 | 1466 1444 1275 | 1163 1086 1030 |
| **Compound** | **IR-spectrum $cm^{-1}$** | | | | **Compound** | **IR-spectrum $cm^{-1}$** | | | |
| Silydianin | 34 60 29 58 17 45 | 16 41 15 16 14 68 | 1275 1155 | 1080 1033 | Isosilychristin | 3403 2941 1640 | 1515 1495 1361 | 1271 1162 | 1084 1031 |
| **Compound** | **IR-spectrum $cm^{-1}$** | | | | | | | | |
| Taxifolin | 3416 3262 1640 1616 | 1507 1475 1371 1266 | | 1166 1138 1083 | | | | | |

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