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Bioavailability Enhancement of Silybin Through Carbon-23 Acid Derivatization

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Abstract

Silybin is an isomer derived from the seed of *Silybum marianum*, a biennial metropolitan plant that is grown naturally in Iraqi Kurdistan Region, it shows antioxidant and anticancer activity however; its poor water solubility encounters to low bioavailability which limits its use in clinical practice. To improve water solubility thereby bioavailability, a chemical derivatization (carboxylic acid) on C-23 ($C_{27}H_{24}O_{12}$) was performed. Silybin derivative on C-23 as ether function improves solubility through increasing the ability to donate unshared electron pairs like Lewis base. The elongated carbon chain 25 as carboxylic acid increases the polarity of our derivative in water as well as the acidity. Our derivative was prepared by Williamson method and showed improvement of water solubility in comparison to silybin; this will lead to enhance the bioavailability and consequently the activity of the drug. Structure elucidation confirmed by FTIR, ^{13}C-NMR and mass spectroscopy. The solubility assessment was performed according to Beer's-Lambert law.

Aim: Enhance water solubility and bioavailability of silybin through acid derivatization.

Introduction

Silybum marianum (Milk Thistle) L. (Gaertn.) (Asteraceae), is a biennial herb that grows wild worldwide ***[1, 2]***, it has been used in herbal medicine for long ***[3, 4]***. Phytochemical studies on the plant showed that the seed retinas the highest concentrations of the flavonolignans diastereoisomers which retain the biologic actions ***[5, 6]***.

Silybin isomers (silybin A and B) (*figure: 1*) have proven medicinal importance *in vitro* and *in vivo* ***[7, 8]***, many studies reveled significant antihyperglycemic, antioxidant, anti-inflammatory and antihepatotoxic actions in regard ***[4, 9]***. Among the therapeutic properties credited to silybin, is anticancer activity ***[10]***.

Recently, the effect of silybin was evaluated as a significant inhibitor of breast cancer cell invasion and metastasis through suppressing Wnt co-receptor LPR6 expression in human ***[11]***, other mechanisms proposed inhibition of metastasis through "regulation epithelial-to-mesenchymal transition, proteases activation, adhesion, motility, invasiveness as well as supportive tumor-microenvironment component" on cancer cell-lines ***[12]***, however; low water solubility decreases the absorption of the drug from gastrointestinal tract and create difficulties during pharmaceutical formulations ***[8, 13]***. Structure

modification was made on silybin to increase water solubility and activity ***[14, 15]***. Comparative anticancer activity on cell lines between the parent drug and structurally modified one showed enhanced activities ***[16]***. Natural products provide lead molecules that are difficult to be produced by chemical synthesis, therefore; researchers are focusing on modulation of the chemical structure rather than finding new templates which may consume more time and efforts ***[17, 28]***.

Silybin A and B are diastereoisomers that composed of a flavone part and a cinnamic acid derivative (lignan) (*figure: 1*), despite numerous phenolic hydroxyl units. The most active part of the compound is the alcoholic ethyl group which is liable for addition reactions because of the mesomerism without affecting the biological activity ***[16, 29]***.

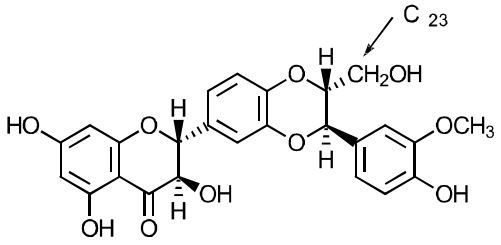

Figure-1: Chemical formula of Silybin A.

Materials and Methods

A. Materials

Silybin was obtained from Sigma, containing Silibinin ≥ 98% (HPLC) grade (CAS No. 2288870-6). Chloroacetic acid purchased from Fluka (purity ≥ 99% (T). Sodium hydroxide from Sigma-Aldrich as ACS reagent ≥ 97% pellets. Condenser from Aldrich Pyrex® Liebig condenser with top and bottom joints jacket L 200mm, Joint: ST/NS 19/38, round flask (Single Neck Flasks) from PYREX Brand 4320 round-bottom flask; 250mL Magnetic stirrer (IKA® RCT basic IKAMAG™ safety control universal hot plate magnetic stirrer, 20L, 1500rpm, 115V, 1/cs. pH–meter pH/Cond 340i/SET (WTW Wissenschaftlich-Technische Werkstatten, 82362 Weilheim, Germany), Shaker : type 3017, No. 10365906 I/Germany,

Shaking Water Bath: type BS-11, No. K109072/Korea, UV-Vis 1700 Pharma Spec. S/N: A11024403239 LP/Japan, shaker, FT-IR 8400S (P/N 206-72400) from Shimadzu/Japan. ^{13}C-NMR from Bruker Ascend 850 MHz, model: Ascend 850, Cryoprobe with 4 channels (13C) plus Z-gradient by (Shraddha Anlytical Services-Mumbai), HPLC-MS (Waters 2545-USA) Quaternary Gradient module equipped with system fluidics organizer (Waters-SFO) coupled with SQ detector in positive ionization mode at range from m/z = 200-900 with 2 scan/min. The compound was identified by comparison to the retention time and MS data of authentic standard.

B. Method

Acid derivatization for silybin

3.0gm (6.21mmole) silybin was dissolved in 15ml sodium hydroxide (30%) solution and refluxed for one hour until clear solution obtained, followed by addition of 3ml aqueous solution of 4.5gm chloroacetic acid (47.6mmole) in drop wise manner through the condenser to the boiling solution over a period of 10 minutes. Refluxing was continued for another 10 minutes and the reaction mixture was cooled to room temperature then diluted with 25ml distilled water and the solution was acidified with 10% hydrochloric acid in drop wise manner. The pH was adjusted to (pH4) by using pH paper and pH-meter, then cooled in ice bath until brown precipitate product is obtained. Recrystallization was performed to obtain solid crystals from distilled water ***[18]***.

Structure elucidation and characterization were performed through interpretations of FT-IR and ^{13}C-NMR in DMSO and mass spectrum screening for both parent and new derivative drug.

HPLC/MS Analysis using isocratic running solution 95% methanol and 5% H_2O, analytical column XSelect C18 size 4.6 x 150mm, and UV lambda range 190nm - 800nm using Photodiod array detector

(Water 2998) each 0.2 sec scanned. Mass scan (m/z) 200-900 is using SQ mass detector each 0.2 sec scanned, 10 microliter for injection was used.

Solubility assessment was carried out using the same method adopted by Higuchi ***[19, 27]***. A stock solution of the new derivative was prepared and went under 5 serial dilutions (3 of each) and set for continuous shaking under constant temperature ($25^{O}C$) on a shaker for 72 hours followed by filtration and determined by UV-spectrometer at λ_{max}=550nm. Calculation of the solubility was done according to Beer-Lambert Law ***[19]***. The procedure was repeated three times for reliability and precision ***[20]***.

A plot of absorbance versus concentration for five dilutions was made, the correlation of slope at different concentrations were obtained. An excess amount of the acid derivative (0.25g/dl) was added to distilled water to obtain a saturated solution for determination of solubility of the new compound. The saturated solution was fixed on a continuous shaker for 24 hours. Filtration was performed for each fraction using Watmann No.1 under vacuum. The filtrate was analyzed spectrophotometrically at λ_{max}=550nm. The procedure was performed at constant temperature ($25^{o}C$).

Results and Discussion

Silybin molecule contains several hydroxyl groups as phenolic and alcoholic. Phenolic groups are less active than alcoholic group due to mesomeric process ***[21, 29]***. The hydroxyl group on C-23 is more active and available to enter nucleophilic substitution reaction. Furthermore *α*-halocarboxylic acid which holds as well nucleophile (halogen) is good leaving group and ready to form ether function ***[18]***. The simplest way to synthesize ether derivative of silybin as new derivative is Williamson reaction (*figure: 2*). Formation of silybin alcoholate on C-23 in alkaline medium ***[18, 22]***.

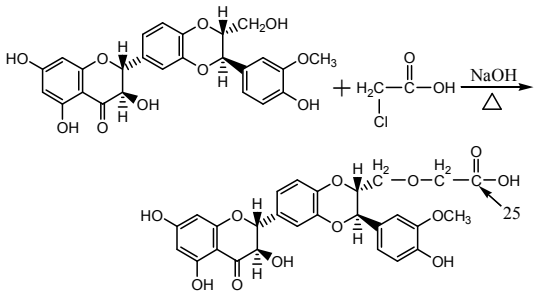

Figure-2: Ether derivatization of Silybin.

The identifications of our desired silybin derivative by FTIR, ^{13}C-NMR as well as MS-spectrum showed the following data:

Table-1: FTIR spectrum analysis.

v, Observed (cm^{-1})	**Function group assignment**
2500-3413.55	*More possible: O-H stretching of carboxylic acid*
3030	*C-H stretching of aromatic ring*
2890-2924.95	*C-H stretching of alkanes*
1740	*As weak shoulder more possible of C=O stretching of carboxylic acids*
1705	*As shoulder more possible of C=O stretching of aromatic ketones*
1639.01	*C=C stretching of aromatic ring in mesomeric form due to O-H group*
1618.70	*C=C stretching of aromatic rings*
1508.07	*C-H stretching of aromatic ring*
1463.68	*C-H bending of CH_2 group*
1383.85-1440	*More possible of O-H bending of carboxylic acids*
1423.73	*More possible of O-H bending of phenol*
1274.66	*C-O stretching of carboxylic acids*
1030.74	*C-O stretching of phenols*

618.00 *More possible C-H (OOP) of aromatic rings*

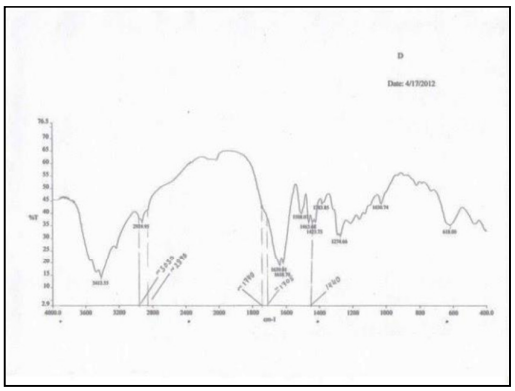

Figure-3: IR-spectrum.

While ^{13}C-NMR shows a good interest peaks at 173.988ppm and 55.771ppm for carboxylic acid and ether function groups.

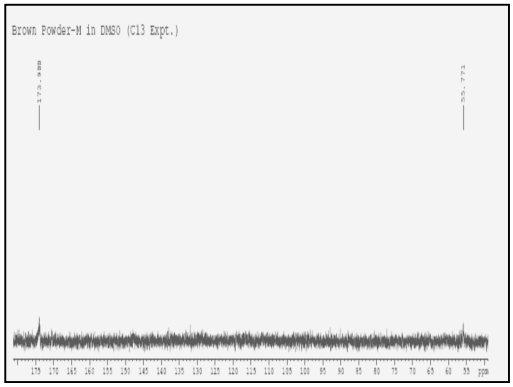

Figure-4: 13C-NMR.

Furthermore, the mass spectrum of our silybin derivative is identical to etherate with elongate chain to C-25 holding carboxylic acid, showing molecular ion expressing an important peak (M+ =540), which detect molecular formula and degree of unsaturation by using rule of thirteen (540/13= 41+7/13) $C_{41}H_{48}$, then by replacing each oxygen atom by CH4 ($C_{41}H_{48}$ – (12 X CH4) = $C_{29}O_{12}$) and again replace two carbon atoms by 24H to get the desired molecular formula $C_{27}H_{24}O_{12}$ corresponds to our silybin derivative and the degree of unsaturation (R+D =27-12+1=16), as the same of our silybin derivative structure. The distinguished fragments in the higher diagnostic α and β cleavages occurs on ether side (*figures: 5, 6*) are giving those distinguished ions at m/z = 525, 509 and 451 ***[23]*** and the β cleavage especially shows 14aum lower as α-methylene of carboxylic acid (495 – 481 = 14) ***[24]***.

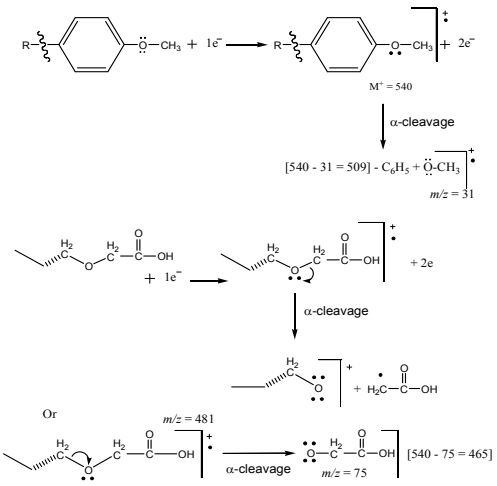

Figure-5: Fragmentation of aryl ether function group.

There is also an important fragment that appears to be characteristic at m/z = 495. This is identified as function of carboxylic acid, (540 – 495 = 45) (*figure: 7*).

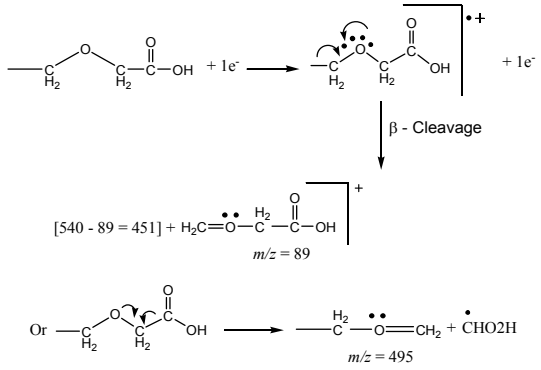

Figure-6: Fragmentation of ether function group.

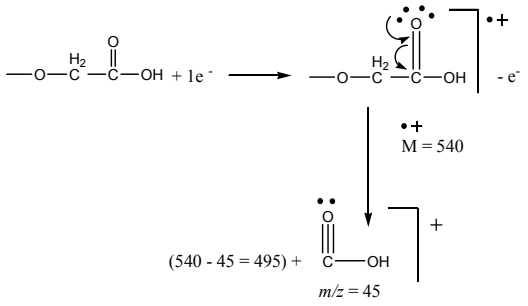

Figure-7: Fragmentation of carboxylic acid function group.

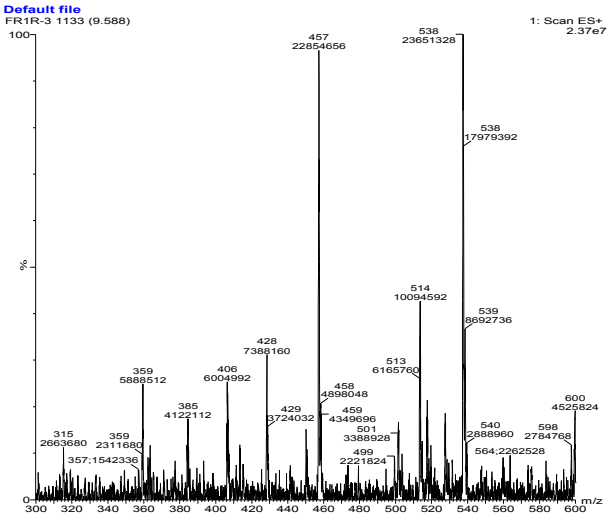

Figure-8: Mass spectrum of silybin derivative.

Creation of ether

Silybin on C-23 as aliphatic etherate with elongate chain to C-25 holding carboxylic acid function increasing the strength of polarity of our derivative throughout ether-oxygen as rich center for donation of electrons as well as the function of carboxylic acid (acetic acid), which is considered as strong polar and lower pH, both together going to improve the solubility of our derivative in water.

Measurement of solubility

Table-2 bellow shows the results for absorbance of five samples of acid derivative, while (*figure: 9*) shows the plot of the absorbance versus concentration for acid derivative in which correlation parameter R^2 = 0.9727 which is highly acceptable, this indicates accordance to Beer-Lambert law ***[19]***. The mean for the absorbance was 0.3412 and the standard deviation (SD) for each absorbance was calculated.

Table-2: Results of average absorbance for correspondent concentrations of the acid derivative.

Sample No.	Absorbance (A) ± (SD)	Con. g/ml
1	*0 ± (0.0)*	*0*
2	*0.107 ± (0.000577)*	*0.0013*
3	*0.286 ± (0.003215)*	*0.0025*
4	*0.358 ± (0.005132)*	*0.005*
5	*0.955 ± (0.010017)*	*0.01*

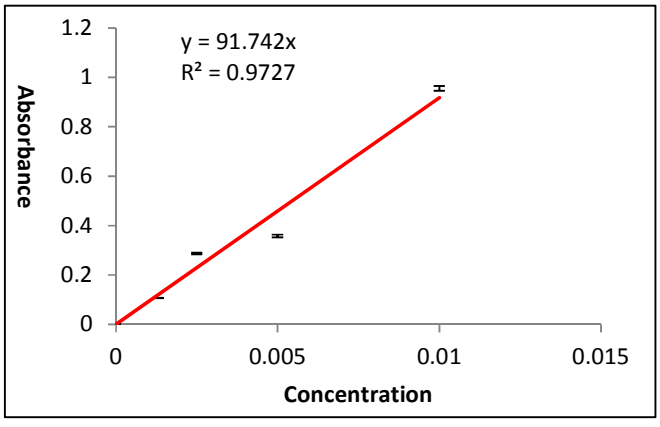

Figure-9: Calibration plot.

Results show an increase in the absorbance with increasing concentration. The plot according to Beer's Law showed significant correlation between absorbance and concentration.

Absorbance of the saturated solution of the acid derivative in water at λ_{max}= 550nm was (1.6). Calculation of the concentration of the solution done using the obtained straight line equation of the calibration curve and the result was (0.102g/ml) compared to very low water solubility of silybin ***[25, 26]***.

The results of solubility showed significant improvement of water solubility in the favor of the acid derivative compound over the silybin which will impart to enhance the bioavailability of the new derivative.

Conclusions

In summary, C-23 as aliphatic etherate with elongate chain to C-25 holding carboxylic acid function as silybin derivative were designed and synthesized according to Williamson reaction. The derivative compound has shown a significant improvement in water solubility through increasing the polarity and decreasing the pH of the compound.

Recommendations

Further structure derivatization is required, assessment of the toxicity of the new compound as well as evaluation of the biological actions are required.

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