Design, Synthesis, Characterization and Preliminary Anticancer Study for Methotrexate Silibinin Conjugates

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Abstract

The spectrum of clinical efficacy of Methotrexate (MTX) is broad in that MTX is used in the treatment of certain cancers, severe psoriasis and rheumatoid arthritis.Various mechanisms by which cancer cells grown in tissue culture become resistant to anticancer drugs. The use of multiple drugs with different mechanisms of entry into cells and different cellular targets allows for effective chemotherapy and high cure rates. In an efforts to develop effective strategies that increase the therapeutic potential of anticancer drugs with less systemic toxicity ,are being directed towards the investigation of dietary supplements and other phytotherapeutic agents for their synergistic efficacy in combination with anticancer drugs. A promising approach to improve the cancer cell selectivity of methotrexate is the chemical transformation into reversible derivatives which convert the conjugate to the parent drug by virtue of enzyme within cancer tissue. The present study includes the synthesis of two derivatives of methotrexate which are :-Schiff base methotrexate-silibinin conjugate (compound 5), and Methotrexate-silibinin conjugate (compound 6).The synthesis of the target compounds was accomplished following multistep reaction procedures. The chemical reactions were followed up and purity of the products was checked by TLC. The structures of the final compounds and their intermediates were characterized and identified by their melting points, infrared spectroscopy, ^{1}H-NMR and elemental microanalysis(C H N S).The anticancer activity of these compounds was investigated by HEP-2 cell line(Larynx carcinoma), which showed that compounds 5 and 6 have the higher activity than methotrexate or silibinin alone.These are promising data for the discovery of new anticancer agents in future. These compounds may deliver the parent drug selectively into the cancer cells to be hydrolyzed by enzymes that are elevated in tumor tissues compared with normal tissues .

Keywords: Methotrexate, Silibinin, Cancer treatment resistance, Folate receptor, Cancer cell targeting.

تصميم وتصنيع وتشخيص ودراسة اولية لمضادية السرطان لمقترن ميثوتركسيت – سليبينين

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الخلاصة

تتميز مادة المثوتريكسيت بطيف من الفعالية السريرية وذلك لاستعمالها في علاج انواع من امراض السرطان،والصدفية والتهاب المفاصل الروماتيدي. هناك عدة اليات التي بموجبها تنمو الخلايا السرطانية في الزراعة النسيجية والتي تكون مقاومة للادوية المضادة للسرطان. ان استعمال ادوية متعددة مع اليات مختلفة للدخول الى الخلايا واهداف خلوية مختلفة يسمح لعلاج كيميائي مؤثر ومعدلات شفاء عالية ولغرض تطوير ستراتيجيات فعالة فان جهودا تبذل لزيادة الفعالية العلاجية للادوية المضادة للسرطان مع اقل سمية من خلال توجيهها ناحية التحري عن المكملات الغذائية وعوامل الادوية النباتية (الاعشاب الطبية)لتصبح ذات فعالية اكبر بالاتحاد مع الادوية المضادة للسرطان.ومن الاهداف الواعدة لتحسين انتقائية الخلايا السرطانية تجاه عقار الميثوتريكسيت هو التحول الكيميائي للمقدمات الدوائية الى العقار الام (الاصل) بحكم الانزيم الموجود في النسيج السرطاني. تتضمن الدراسة الحالية تخليق مشتقين للمثوتريكسيت وهما :

-قاعدة شف ل مقترن المثوتريكسيت –سليبنين (مركب ٥).

- مقترن ميثوتريكسيت –سليبنين (مركب ٦).

تم تحضير المركبات التي يهدف اليها البحث باتباع طرائق متعددة الخطوات وتم التاكد من نقاوة هذه المركبات وذلك باستعمال تقنية كروماتوغرافياالصفائح الرقيقة.كذلك تم تشخيص وتوصيف النتواتج النهائية وموادها الوسطية من خلال تعيين درجات انصهارها واطياف الاشعة تحت الحمراء وطيف الرنين النووي المغناطيسي للبرتون،والتحليل الدقيق للعناصر.

لقد اثبتت دراسة الفعالية المضادة للسرطان لهذه المركبات باستخدام الخلية السرطانية ال هب٢ - وهي خلايا سرطان الحنجرة والتي اظهرت بان المركب ٥ والمركب ٦يمتلكون فعالية اعلى من الميثوتريكسيت والسليبنين و هذه النتائج تعتبر واعدة لاكتشاف مضادات سرطانية جديدة في المستقبل. ووفقا لهذه النتائج المبينة اعلاه يتضح بان هذه المركبات لها القدرة على ايصال الادوية بانتقائية للخلايا السرطانية وبالية تحرير تتضمن تاثير الانزيمات الموجودة بمستوى عالي في الانسجة السرطانية مقارنة مع الانسجة الطبيعية .

الكلمات المفتاحية:ميثوتركسيت ،سيلبنين،مقاومة علاج السرطان،مستقبلات الفوليت،استهداف الخلايا السرطانية

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Introduction

The design of cancer chemotherapy has become increasingly sophisticated,yet there is no cancer treatment that is 100% effective against disseminated cancer. Resistance to treatment with anticancer drugs results from a variety of factors including individual variations in patients and somatic cell genetic differences in tumors, even those from the same tissue of origin[1]. Researchers have been tabulating the various mechanisms by which cancer cells grown in tissue culture become resistant to anticancer drugs. Some of these mechanisms, such as loss of a cell surface receptor or transporter for a drug, specific metabolism of a drug, or alteration by mutation of the specific target of a drug, all of which occur for antifolates such as methotrexate[2]. MTX competitively inhibits dihydrofolate reductase[3] . Folate receptor a (FRa) is a membrane-bound protein with high affinity for binding and transporting physiologic levels of folate into cells. Folate is a basic component of cell metabolism and DNA synthesis and repair, and rapidly dividing cancer cells have an increased requirement for folate to maintain DNA synthesis, an observation supported by the wide spread use of antifolates in cancer chemotherapy[4]. Endocytosis is the general mechanism of (FRa) mediated folate uptake. One specialized route, potocytosis , was proposed from the observation that(FRa) recycled between an acid-resistant (intracellular) and acid-sensitive (extracellular) pool. Proposed that (FRa)was concentrated in clusters in invaginations of the cell membrane surface called caveolae, whereby the membrane would transiently close and internalize the folate-bound receptor complex. Increased acidification of the internal compartment would dissociate folate from the receptor and move it across the membrane into the cytoplasm of the cell using the energy generated by the acidic gradient[5]. Folate receptor-targeted cancer therapies constitute a promising treatment for the approximately one third of human cancers that over express the folate receptor (FR). However, the potencies of all folate-receptor targeted therapies depend on the rate of folate-linked drug conjugate binding to the cancer cell surface, the dose of folate conjugate that will saturate tumor cell surface FR in vivo, the rate of FR internalization, un loading,and recycling back to the tumor cell surface for another round of conjugate uptake, and the residence time of the folate conjugate before its metabolism or release from the cell[6].Linkage of a drug to folic acid can generate a molecular "Trojan Horse" that can enable tumor-specific delivery of both imaging and therapeutic agents into cancer cells. Molecules that have been targeted to tumors by conjugation to folic acid include radiopharmaceuticals [7] , enzymes for pro-drug activation [8] ,nanoparticles [9] , peptides, toxic proteins [10] , immunologically potent haptens [11] , antisense oligonucleotides [12] , chemotherapeutic agent , gene therapy vectors [13] , viruses[14] ,and polymeric drug carriers and liposomes [15],for example Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance[16]and Conjugation of methotrexate with 5-Fluorouracil[17] . In an effort to develop effective strategies that increase the therapeutic potential of anticancer drugs with less systemic toxicity, more strategies are being directed towards the investigation of dietary supplements and other phytotherapeutic agents [18].Silibinin has shown promising chemo preventive and anticancer effects in various in vitro and in vivo studies[19] .Several studies have shown that silibinin and silymarin have anticancer activity against breast, skin, androgen-dependent and independent prostate, cervical, bladder, hepatocellular,colon, ovarian, and lung cancer cells in culture and several in vivo animal cancer model systems[20]. The efficacy of silibinin in inhibiting the growth of different cancer lines is quite different. Such differences in the potency of the drug in arresting cell growth may be due to differences in the experimental conditions used, the cell type and potential carcinogenicity of the cell lines[21]. In view of this observation, two derivatives of methotrexate are designed, synthesized and characterized.

Experimental Section

General

Chemicals used in the synthesis were of analytical grade(methotrexate and p-nitro phenyl chloroformate from(Hongmao,china)and silibinin(Tolbiac S.R.L ,Argentina). The melting points of the compounds and their intermediates (uncorrected) were determined by capillary tube method on Barnstead Electrothermal (USA) and FT-IR spectra were recorded on FT-IR spectrophotometer Shimadzu (Japan) at the college of pharmacy /University of Baghdad. The CHNS analysis was carried out using Euro-vector EA3000A(Italy).Ascending thin layer chromatography(TLC)was run on silica gel 60 F_{254} pre-coated aluminum sheets, Merck (Germany) to check the purity and the reactions progress, ^{1}H-NMR analysis was performed on 1H-NMR spectroscopy (Shimadzu,Japan) at University of AL-bayt,Jordan, and the

chromatograms were eluted by three solvent systems:

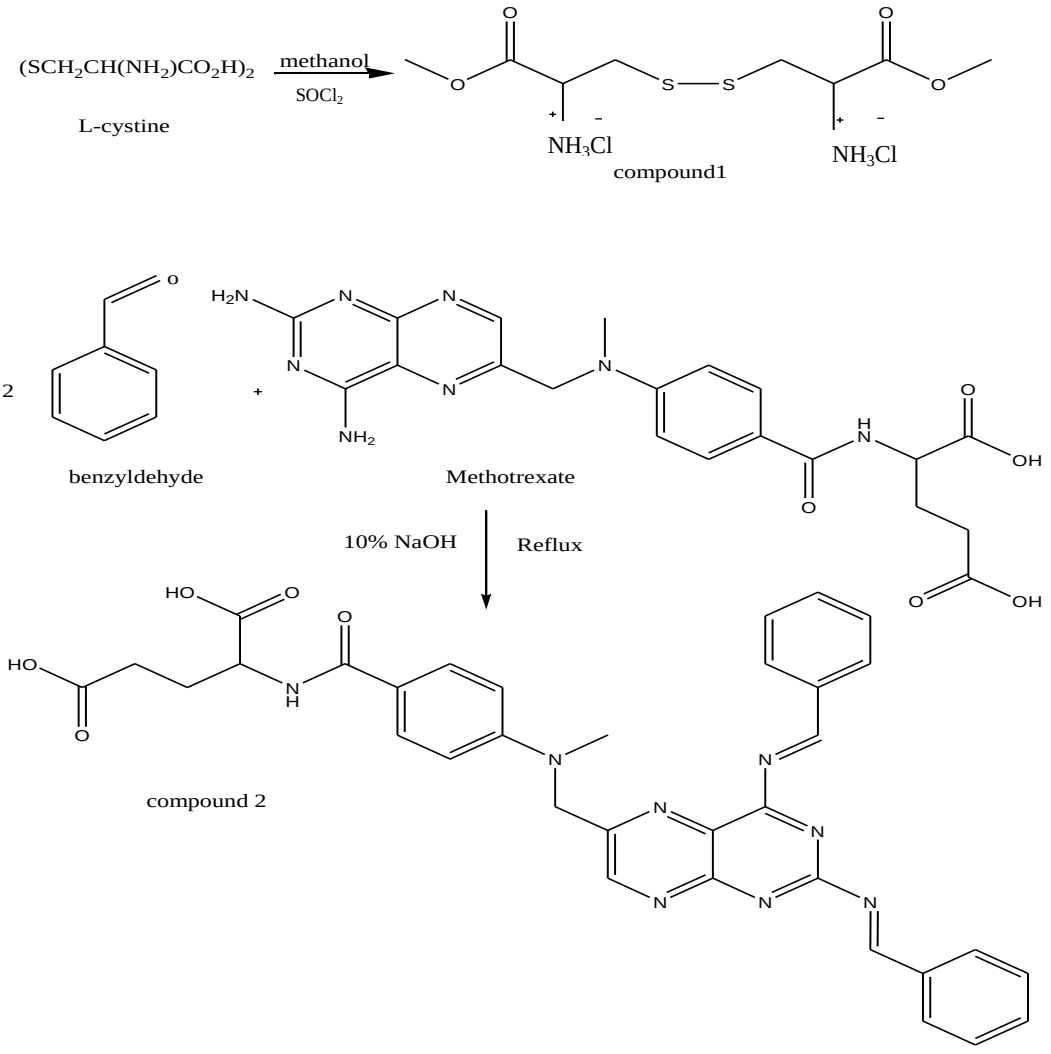
A-Aceton:Chloroform (3:1).

B- Aceton:Chloroform :Acetic acid(3:1: 0.5) (22).

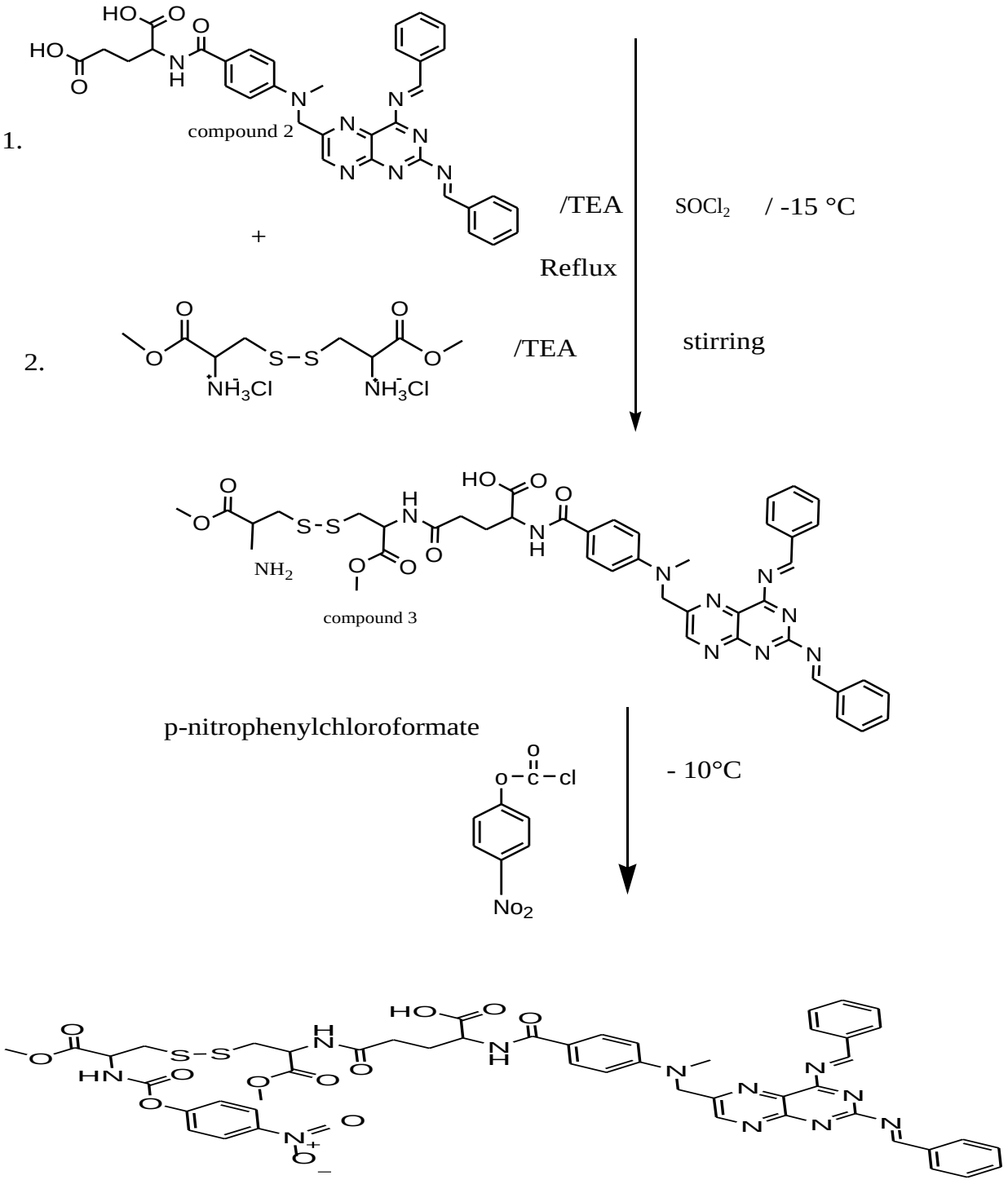
The evaluation of cytotoxic activity was done at Al-Naharin biotechnology research center using Rosswell Park Memorial Institute (RPMI-1640)which consists from HEPES buffer and L-glutamin(Sodium bicarbonate ,Streptomycin ,Benzyl penicillin G,Mycostatin ,Fetal calf serum) .

Chemistry

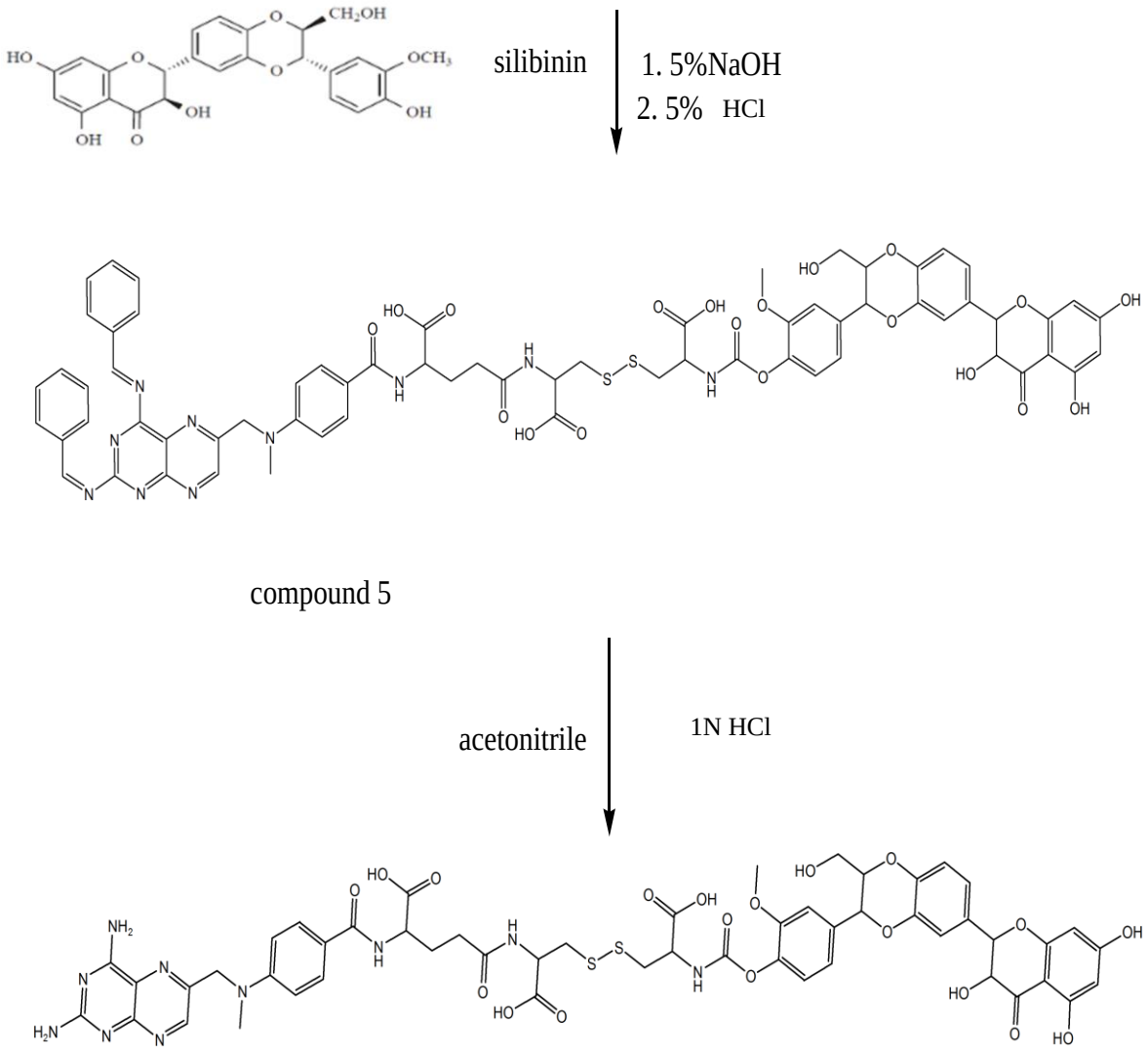
The synthetic procedures for the designed target compounds 5 and 6 are illustrated in schemes (1 and 2) . The starting material for the generation of the target compounds 5 and 6 was methotrexate which was refluxed with 2 equivalent benzaldehyde in the presence of 10% sodium hydroxide to give the imines derivative of methotrexate (compound 2). Compound 2 was converted to acylchloride derivative by useing thionyl chloride and triethylamine in which one carboxylate group was blocked. The acylchloride derivative was allowed to react with NH_2 group of dimethyl ester derivative of cystine in the presence of triethylamine to give the imine-methotrexate cystine derivative (compound 3) which was reacted with para-nitrophenylchloroformate to give compound 4. Then (compound 4) was stirred with silibinin in the presence of sodium hydroxide to give imine-methotrexate silibinin conjugate (compound 5).The methotrexate silibinin conjugate (compound 6)was obtained by hydrolysis of compound 5.The physical properties of the synthesized compounds are showed in table (1). The structures of the final compounds were characterized by elemental microanalysis (table2) .Infrared spectroscopy (table3) and ^{1}H-NMR done for compound 5(table4).



Scheme (1): Synthesis of compounds 1and 2.


1.
compound 2
/TEA
$SOCl_2$
/ -15 °C
+
Reflux
2.
/TEA
stirring
compound 3
p-nitrophenylchloroformate
- 10°C

Compound 4



Scheme(2): Synthesis of compounds 3 – 6 .

Chemical Methods:

Synthesis of 3,3'-disulfanediylbis(1-methoxy-1-oxopropan-2-aminium) chloride.(compound 1).

A suspension of cystine (41.6mmole,10g) in (150 ml)of absolute methanol,was cooled to -15 °C then thionyl chloride(99.8 mmole,7.4 ml) was added drop wise ,(the temperature should be keep below -10 °C) and the reaction mixture was left at 40°C for 1hr,then reflux for 4 hr and left at room temperture over night,the excess solvent was evaporated to dryness under vacuum;recrystallize the product from methanol-diethyl ether and collected as HCl salt [23] .

Synthesis of 2 - (4 - (((2 , 4 -bis (benzylideneamino) pteridin - 6 -yl) methyl) (methyl) amino) benzamido) pentanedioic acid.(compound2).

In 500 ml round bottom flask attached with a reflux condenser, mixture of benzaldehyde (44 mmole,4.47 ml), methotrexate (11mmole,5 g), and 10% sodium hydroxide (22mmole,0.88 g). Add 200 ml of absolute ethanol to the mixture and the final mixture is refluxed for 3 hours, cooled and acidified to pH 5with 5% HCl . The mixture was filtered,the product was washed with cold ethanol and dried [24].

Synthesis of 4 - [2 - (2 - Amino - 2 - methoxycarbonyl - ethyldisulfanyl) - 1 - methoxycarbonyl - ethylcarbamoyl] - 2 - (4 - { [2 , 4 - bis - (benzylidene - amino) - pteridin - 6 - ylmethyl-methyl - amino } - benzoylamino) - buty ric acid .(compound 3).

A suspension of compound 2 (7.93mmol,5 g) and triethylamine (7.93mmole ,1.10ml) in acetonitrile (50ml),was coolded down to-15°C then thionyl chloride (7.93mmol,0.59ml) was added slowly, and the mixture was refluxed for 4hr at(60-70)°C with continuous stirring.Then evaporate the excess solvent by using rotary evaporater .The viscous liquid was slowly added drop wise into beaker contain compound 1(7.93mmol,2.7g) and triethylamine (15.86mmol, 2.2ml) dissolved in acetonitrile(50ml) for 1 hr in an ice bath ,then stirring over night at room temperature. The obtained suspension was filtered and the filterate was washed with distilled water (20ml),dried over anhydrous magnesium sulfate and the solvent was evaporated by vacuum. The obtained compound was recrystallized from diethyl ether-petroleum ether(4:6)to give compound 3.[25]

Synthesis of 2-(4-(((2,4-bis(benzylideneamino)pteridin-6-yl)methyl)(methyl) amino)benzamido)-5-((1-methoxy-3-((3-methoxy-2-(((4-nitrophenoxy)carbonyl) amino)-3-oxopropyldisulfanyl)-1-oxopropan-2-yl)amino)-5-oxopentanoic acid.(compound 4).

To a stirred solution of compound 3 (2.17 mmol,2 g) in toluene(100ml) at -10°C was added triethylamine (2.17 mmol,0.61ml). After 5 min, a cooled mixture of para-nitrophenylchloroformate (2.17 mmol,0.443 g) in toluene (5 ml)was added drop wise . The mixture was stirred for 2 hr at - 10°C and for 15 hr at room temperature, filtered,and evaporated to dryness under reduced pressure.The residual oil was crystallized twice from diethyl ether (150ml) to give a yellow powder [26] .

Synthesis of15-(4-(((4-benzylideneamino)-2-((Z)-benzylidene amino) pteridin - 6 -yl) methyl) (methyl) amino)phenyl) - 1 - (4-(3-(hydroxylmethyl)-7-(3,5,7-trihydroxy-4-oxochroman - 2 - yl) -2 , 3 dihydrobenzo [b][1,4]dioxin-2-yl)-2-methoxyphenoxy)-1,10, 15-trioxo-5,6-dithia-2,9,14-triazapentadecane-3,8,13-tricarboxylic acid .(compound 5) .

To a mixture of silibinin(0.477mmol,0.23 g) and compound 4 (0.477 mmol ,0.5 g)in 30ml distalled water ,add 5%NaoH drop wise until the solution become basic at pH 10 and left stirred for 48hr.The mixture was then acidified with hydrochloric acid 5% drop wise to give a precipitate.This precipitate was washed with distalled water thoroughly ,then with ethanol and dried in oven at 50°C,to provide compound 5.[27-29]

Synthesis of15-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino) phenyl)-1-(4-(3-(hydroxymethyl) -7 - (3 , 5 , 7 - trihydroxy -4-oxochroman - 2 – yl) - 2 , 3 -dihydrobenzo [b][1,4]dioxin-2-yl)-2-methoxyphenoxy)-1 , 10 , 15 – trioxo - 5,6-dithia-2,9,14-triaza penta decane-3,8,13-tricarboxylic acid. (compound 6).

Compound (5) (0.36 mmole, 0.5 g) was dissolved in 2.5 ml of acetonitrile. The pH of the solution was adjusted to 2 with 1N HCl and it was stirred for 1hr at (0-5) °C. The precipitate was filtered,washed with ether and dried to give compound 6 [30] .

Cell culture and culture conditions

HEP- 2 cell line(passage number(75) were isolated from human epidermoid larynx carcinoma . This type of Cells were maintained in RPMI-1640 medium with 30% bovine calf serum and 10% DMSO , then the cell treated with trypsin/versin mixture in order to pursue subculture process .

Evaluation of cytotoxic activity

The in vitro cytotoxic activity (cell viability assay) of these compounds(MTX,silibinin, compounds 5,6) were evaluated by Neutral red dye assay; a nonradioactive ,fast assay widely used to quantify cell viability and proliferation . A set of 3 concentrations (0.5,1,2) µg/ml was made for each product and the exposure time of the assay was 24 hours,48hours. The cytotoxicity assay done according to a reported method [31] where the cell exposed to different concentrations of different compounds (0.5,1,2) µg /ml respectively, each compound was added to the cell in triplicate form of each concentration ,RPMI media and cell added as positive control ,only cells incubated with culture media represented the negative control.

Results and Discussion

The prepared compounds were characterized by means of physical properties, FTIR,CHNS analysis and ^{1}H-NMR.The results are illustrated in table 1,2,3 and4,respectively .

Mechanism of action

The compounds(5 and 6) can be considered as prodrugs, when inter the cancer cell can be hydrolysed by reductase enzyme which elevated in cancer cell and lead to hydrolysis of disulfide, and production of two moieties [32] .

Table(1): Physical data of the synthesized compounds.

Compound	Physical appearance	%Yield	Melting point observed (^oC)	R_f value
1	White powder	59.8	105-107	A= 0.65 B=0.4
2	Dark Yellow powder	90.9	185 (decomposed)	A=0.166 B=0.2
3	Yellow powder	52.2	115-118	A=0.71 B=0.78
4	Yellow powder	54.28	145-148	A=0.85 B=0.82
5	Yellow powder	30	203 (decomposed)	A=0.72 B=0.78
6	Yellow powder	58.6	170 (decomposed)	A=0.78 B=0.9

Table (2): Elemental microanalysis of the final compounds.

Compound	Molecular weight	Empirical formula	Elemental microanalysis %		
5	1360	$C_{66}H_{60}N_{10}O_{19}S_2$	C	58.2	58.15
			H	4.41	4.649
			N	10.29	10.688
			S	4.7	4.48
6	1184	$C_{52}H_{52}N_{10}O_{19}S_2$	C	52.7	51.984
			H	4.38	4.582
			N	11.8	12.141
			S	5.4	5.192

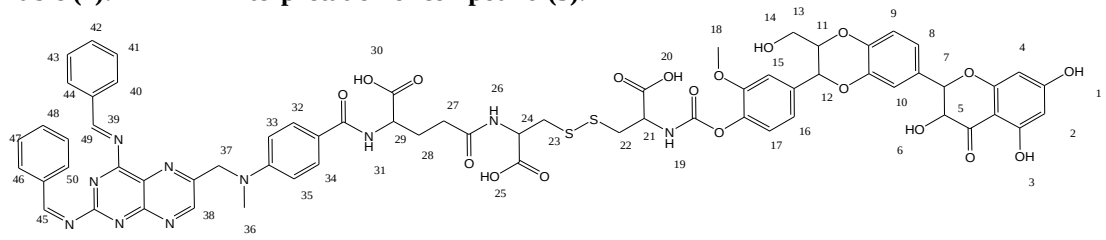
Table (3): The characteristic IR bands of synthesized compounds(1-6).

Compound	Characteristic I.R. bands (cm^{-1})
1	(3404-2800Broad band N-H stretching vibration of NH_3Cl,(1745C=O stretching vibration of ester).
2	(3346N-H stretching vibration of amide), (1668C=O stretching of carboxylic acid),(1655N-H bending of amide),(1600C=N stretching of imine
3	(3346N-H stretching vibration of amide), (1745C=O stretching vibration of ester),(1643C=O stretching vibration of amide),(1602C=N stretching of imine).
4	(3331N-H stretching vibration of amide),(1745 C=O stretching vibration of ester),(1716C=O stretching vibration of carbamate),(1643C=O stretching vibration of amide),(1604C=N stretching of imine),(1521 Asymmetric Stretching vibration of NO_2),,(1346Symmetric stretching vibration of NO_2).
5	(3354N-H stretching vibration of amide),(1735C=O stretching vibration of COOH),(1637C=O stretching vibration of amide),(1604C=N stretching of imine).
6	(3346N-H stretching vibration of amide) (1735C=O stretching vibration of COOH),(1637C=O stretching vibration of amide).

^{1}H-NMR analysis:

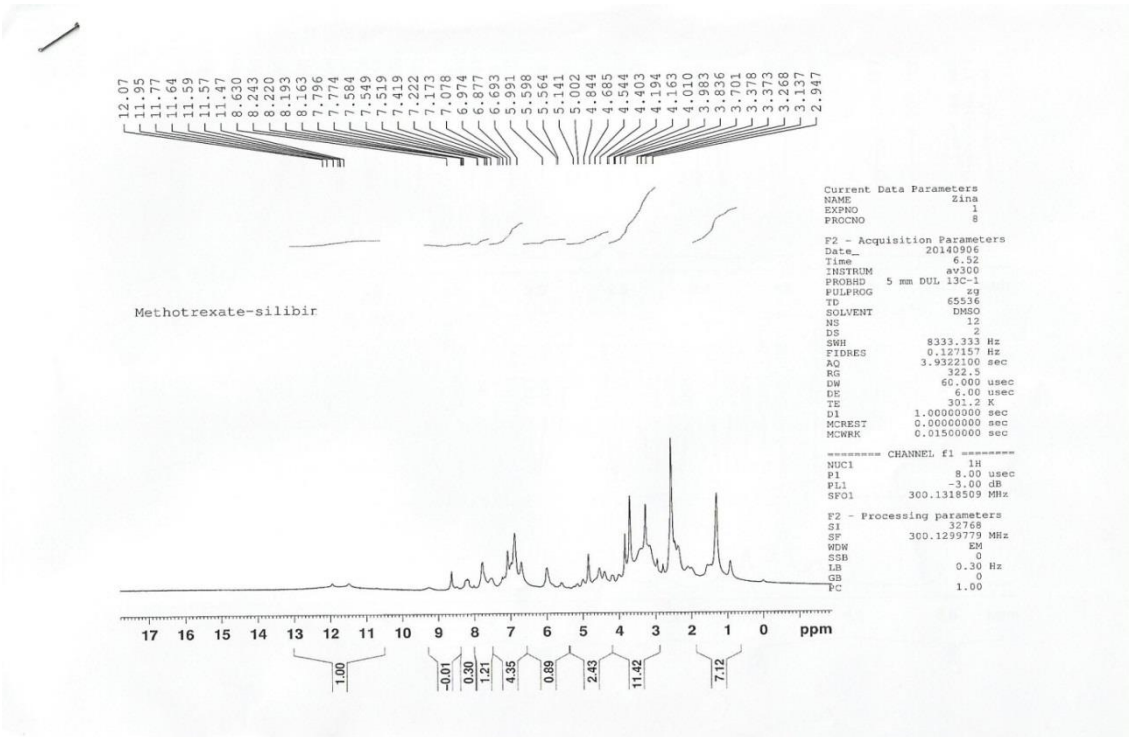
^{1}H-NMR analysis was performed on ^{1}H-NMR spectroscopy (Shimadzu,Japan) at University of AL AL-bayt,Jordan,using Dimethyl sulfoxide(DMSO) as a solvent and the results are listed on table (4).

Table (4): ^{1}H-NMR interpretation of compound (5).



H	Chemical shift (ppm) δ	Functional group
1,3	5.56	Aromatic OH
2,4	5.99	1-benzen
6	2.94	Alcoholic OH
5,7	5.59	Methine
8,9,10,16	6.87	1-benzen
15,17	7.17	1-benzen
13	4	Methylene
14	3.7	Alcoholic OH
18	3.83	Methyl
19,26	8.16	Secondary amide
20,25,30	11.47	Carboxylic acid
21,24,29	4.68	Methine
22,23	2.94	Methylene
32,34	7.58	1-benzen
33,35	6.97	1-benzen
36	3.13	Methyl
37	4.54	Methylene
38	8.63	2-pyrazine
39,45	8.42	Ar.protons, Benzylidenimin
40,44,46,50	7.79	Ar.protons, Benzylidenimin
41,42,43,45,47,48,49	7.51	Ar.protons, Benzylidenimin

^{1}H-NMR spectrum of compound 5



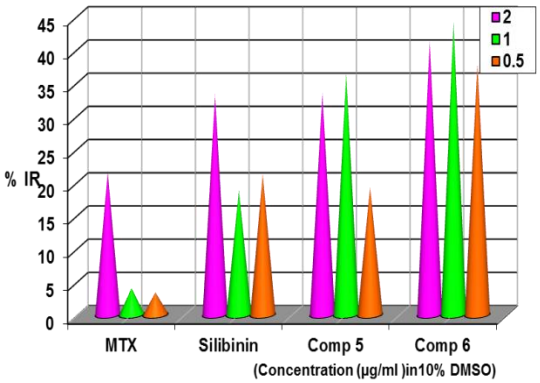
Results of cytotoxicity assay

The cytotoxic study was done on HEP- 2 cell line(passage number(75) were isolated from human epidermoid larynx carcinoma

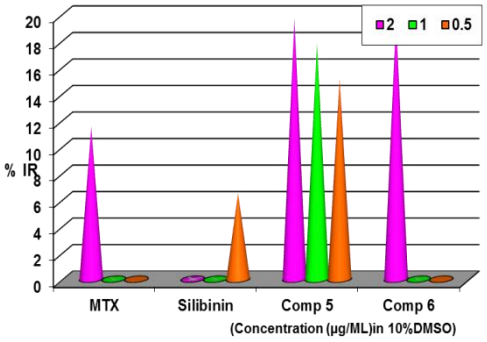
Inhibition Rate%

$$= \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\textit{Absorbance of test}} \times 100$$

The inhibition rate percent (I.R. %)[33] or inhibitory concentration percent (IC %) was estimated, and the result was varied among samples as shown in Figure (1,2).



Figure(1):The cytotoxic activity represented by inhibition rate percent of compounds MTX,silibinin,compound 5,6 at different concentrations (0.5 ,1,2) µg/ml in 24 hrs time of exposure .



Figure(2): The cytotoxic activity represented by inhibition rate percent of compounds MTX,silibinin,compound 5,6 at different concentrations(2,1,0.5) µg/ml in 48 hrs time of exposure.

One cell lines was studied (HEP-2) at two times of exposure 24 and 48hr , using a 3 fold dilutions to get concentrations from (2 to 0.5) µg / ml of our compounds. The produced effect was explained as the following:-

The cytotoxic effect of MTX on HEP – 2 (passage NO.75), fig.1 revealed that the high concentration 2 µg/ml gave a significantly high inhibition rate of cells while being low gradually with low concentration 0.5 µg/ml during 24 hr of exposure(fig.1) , while during 48hr the cytotoxic effect high at high concentration 2 µg/ml (figure 2) and proliferation develop during lo w concentration due to develop resistant of cell cancer to MTX.

The cytotoxic effect of silibinin gave high inhibition rate (33.1%) at high concentration 2 µg/ml and low with low concentration 0.5 µg/ml during 24 hr of exposure (fig.1) while low inhibition rate at low concentration 0.5 µg/ml and proliferation develop as the concentration increase((1,2) µg/ml) during 48 hr of exposure (figure 2).

The compound 5 shows high inhibition rate at high concentration 2 µg/ml , in both times and decrease as the concentration decreases(fig.2) , but shows higher inhibition rate at 1µg/ml during 24hr of exposure (fig 1).

The compound 6 also shows high inhibition rate (41.2%) at high concentration 2 µg/ml and at 1µg/ml shows higher inhibition rate 44.2% during 24 hr(fig.1), while the inhibition rate decrease during 48 hr(fig.2)and proliferation was develop.

An explanation for this behaviour ,that in the design of cell culture experiment , it was important to be aware of growth state of the culture, as well as the quantitative characteristic of cell stain or cell line. Culture will vary significantly in many of their properties between exponential growth and stationary phase[34].

Also the differences in HEP-2 responding to word different treatment might indicate a presence or absence of specific cellular receptor in each type of cell line, making the cell interact at same concentration in different manners. Moreover the metabolic pathways in response to each treatment differed from one cell line to another .This fact was mentioned in different studies which investigated at different plant extracts in treating several types of cell line[35,36].

HEP-2 resistance might be explained by over expression phenomena through genes responsible for binding- receptor blockage that prevent the cytotoxic effect of any treatment[37].From the result we can conclude that the activity of compounds 5,6 better than the activity of MTX or silibinin alone,and these may be attributed to the reduction in the resistance development.

Conclusions

1. The synthetic procedure for the designed target compounds was successfully achieved and the structural formula for the synthetic compound was characterized using FT-IR spectroscopy, elemental microanalysis, melting points and Rf values and ^{1}H-NMR analysis done for compound 5.

2.The activity of compounds 5 and 6 were studied as anticancer drugs.They were more

effective drugs compared with MTX and silibinin.

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