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Synthesis, Characterization and Preliminary Cytotoxic Activity Study of New 5 - Fluorouracil Conjugate with Pyrrolidine Dithiocarbamate as A Mutual Anticancer Prodrug

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Abstract

5-Fluorouracil (5-FU)s one of the commonly used chemotherapy drugs in anticancer therapy; unfortunately treatment with 5-FU solely has many drawbacks low lipophilicity, low permeability, low molecular weight, and its relatively poor plasma protein binding; also a brief half-life, therefore frequent administration is required to maintain the optimal therapeutic plasma level which in addition to its poor selectivity, drug resistance and limited penetration to cancer cells; leads to increased incidence of side-effects to healthy cells/tissues and low response rates. In order to minimize these drawbacks; 5-FU was chemically conjugated with pyrrolidine dithiocarbamate (PDTC) in a mutual prodrug moiety (S-*(9H-purin-6-yl) 3-((pyrrolidine-1-carbonothioyl)thio)propanethioate*) "compound [IV]" with (chloroacetic acid) and (chloroethanol) being the linkers ;synthesized prodrug and intermediates were characterized and identified using FTIR ,^{1}H NMR and all the results shown good agreements with the proposed chemical structures of the synthesized compounds. ; *in-vitro* preliminary cytotoxicity study was conducted for compound [IV] and 5-FU on CAL 51 and B16V cell lines ,results showed enhanced cytotoxic effects for [IV] over 5-FU.

Keywords: 5-Fluoruracil, Prodrug, PDTC, Cytotoxicity.

تحضير وتشخيص وتقييم اولي للفعالية ضد السرطان لمقترن مستحدث للـ٥- فروروبوراسيل مع بايروليدين ثنائي ثايوكارباميت كمقدم دوائي ذو فعالية مزدوجة ضد السرطان

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الخلاصة

٥-فلوروبوراسيل من الادوية الشائعة الاستعمال في العلاجات المستعملة ضد مرض السرطان , من المؤسف ان استعمال ٥-فلوروبوراسيل منفردا فيه مساوء عديده تشمل: انخفاض الفته للدهون ,نفوذيته المنخفضة ,وزنه الجزيئي الصغير و قابليته الضعيفة على الارتباط ببروتينات البلازما وكذلك عمر النصف القصير مما يتطلب تكرار جرعاته لادامة تركيز بلازمي فعال, الامر الذي بالاضافة الى سوء انتقائيته السمية والمقاومة الدوائية له ونفاذيته المحدودة الى الخلايا السرطانية ,يؤدي الى زيادة نسب حدوث الاعراض الجانبية عند الخلايا والانسجة الطبيعية وانخفاض معدلات الاستجابة له؛ لاجل تحجيم هذه المساوئ تم اقران ٥-فلوروبوراسيل مع (بايرولدين ثنائي ثايوكاربامیت) كمقدم دوائي ذو فعالية مزدوجة يدعى (S(9H purin 6 yl)3((pyrrolidine 1 carbonothioyl)thio)propanethioate) المسمى مركب رقم ٤ واستعمل (١-كلورو-حامض الخليك) و(٢-كلورو-ايثانول) ك روابط بينهما ؛تم تشخيص المركب النهائي والمركبات الوسطية بواسطة طيف الاشعة تحت الحمراء و الرنين النووي المغناطيسي واظهرت النتائج مطابقة للهيكلية الكيميائية المقترحة ؛ اجريت دراسة مختبرية اولية للسمية الخلوية للعقار المصنع و ٥-فلوروبوراسيل على الخطوط السرطانية : (سرطان الثدي البشري) و (سرطان الجلد الميلانومي للفأر) ,الدراسة اظهرت تحسن السمية الخلوية للعقار ٤ مقارنة بال ٥-فلوروبوراسيل.

الكلمات المفتاحية:٥-فلوروبوراسيل, مقدم دوائي, بايروليدين ثنائي ثايوكارباميت و سميّة خلوية.

Introduction

Most anticancer chemotherapies activate Nuclearfactor kappa B(NF-KB), so does Gamma irradiation, activation of NF-κB leads to resistance to apoptosis induced by chemotherapy or irradiation, a state of antiapoptosis take place which eventually leads to resistance to anticancer treatment, here comes the role of NF-κB blockers as chemopreventive agents which if administered in combination with chemotherapeutic agents or gamma irradiation give synergism[(1)]. PDTC A novel stable dithiocarbamate with antioxidant [(2,3)] and potent NF-κB inhibitory effect . [(4–6)] where NF-κB inhibition is independent of its antioxidant property [(7)].

5-Fluorouracil (5-FU) the Fluorinated analogue of uracil, one of the commonly used chemotherapy drugs in anticancer therapy, for a variety of human malignancies breast cancer, head, and neck cancer [(8)], skin cancer,[(9)] colorectal and other gastrointestinal cancers [(10)] . also ovarian and liver cancers [(11)] . Treatment with 5-FU by itself has many

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drawbacks; high water solubility, low permeability, low molecular weight, and its relatively poor plasma protein binding; also a brief half-life of about 10–20 min requires frequent administration to maintain the optimal therapeutic plasma level [12–14] .Properties like poor selectivity, drug resistance and limited penetration to cancer cells; not only lead to low response rates but also have potentially serious side-effects to healthy cells/tissues. Among 5-FU's known side effects are myelosuppression, mucositis, nausea, emesis hand-foot-syndrome,[15,16] neurotoxic effects (cerebellar syndrome and encephalopathy) [17].

Prodrug strategies may be the most advanced research area that enables the safe delivery of cytotoxic drugs to neoplasm tissues and activate it specifically by over-expressed enzymes at the cancer microenvironment leading to the controllable drug release in target sites, researches also showed that there are higher concentrations of esterase in cancerous cells than in normal ones and rational design of ester prodrugs is the increased bioavailability of drugs with high polarity making ester drugs good targets for prodrug research [18,19] . When used in combination, PDTC act as an enhancer of 5-fluorouracil-induced growth inhibition of colon carcinoma cells [19]. This work aims to synthesize a novel mutual prodrug of PDTC and 5-FU linked by an ester linkage and evaluates the synthesized compound for *in vitro* cytotoxic activity.

Materials and Methods

Materials

5-Fluorouracil (5-FU) was purchased from Baoji Guokang Biotechnology/China, all the chemicals and solvents used in synthesis were of analytical grade and used without further purification. Reactions' progress and the purity of compounds were monitored and confirmed by thin-layer chromatography (TLC), using Silica gel GF254 (type 60) pre-coated Aluminium sheets, Merck (Germany) exposed to UV-254nm light, The chromatograms were eluted with ethyl acetate/methanol (1:1) for [I] and [III], ethyl acetate/n-Hexane (4:1)for [IV]; Melting points were determined using Stuart SMP30 melting point apparatus with one-end sealed capillary method, and are uncorrected. Fourier-Transform Infrared spectroscopy (FTIR), were recorded using Specac quest ATR (diamond)-UK on (IRAffinity-1) spectrophotometer, Shimadzu, Japan at Baghdad university/college of pharmacy. ^{1}H NMR was recorded on (NMReady-60 spectrometer by Nanalysis Corp. 60 MHz, Canada) at Central laboratory service /college of education for pure sciences / Ibn AL-Haithem /University of Baghdad. The chemical shift was expressed as (δ=ppm) and coupling constants in Hz, Methanol-$_{d6}$ or DMSO-$_{d6}$ were used as solvents.

Methods of chemical synthesis

Synthesis of (pyrrolidine-1-carbodithioate triethylammonium) [I][20]

In a 250 ml beaker a stirred solution of pyrrolidine (Pyr) (5 g, 0.07 mole) and triethylamine (TEA)(9.7 mL, 0.07 mole)in 70 ml dry ethanol was cooled on an ice bath to -5° C, carbon disulfide (CS_2) (5 ml,0.084mole) was added from a dropping funnel slowly (over a period of 30 mins) stirring was maintained for additional 1h at room temperature after CS_2 dropping has stopped then the precipitate filtered and recrystallized from ethanol/ether (1:2) to give compound I as light beige powder the. The % yield: 87% m.p.=130 °C (D) , R_f= 0.8. IR(υ= cm^{-1}):2300-2966 broad N-H of tert. amine, , 2966 C-H str. of CH_3 2858,2704 C-H str. of CH_2,1454 CH_2 scissor vibration,1373,1323 C=S str. of dithiocarbamate;999 and 937 C-S str.

^{1}HNMR(60 MHz, Methanol-$_{d6}$, δ= ppm):0.9-1.35(9H,m,CH_3(TEA's)); 1.5-2 (6H,m,CH_2(Pyr 's)); 2.8-3.24(4H,m,CH_2(Pyr's)); 3.43-4.15 (4H,m,CH_2(TEA's)).

Synthesis of 2-hydroxyethyl pyrrolidine-1-carbodithioat e[II] [21]

To a stirred solution of compound [I] (2g,0.008mole) dissolved in 41 ml acetone, chloroethanol (0.504ml,0.008 mole) was added, and the reaction mixture was stirred for 18 hrs. at ambient temperature (30°C) after which time filtered in the freezer and the precipitate was washed with cold acetone dropped on the filter paper and the filtrate was devoted from its solvent and the resulting oil re-dissolved in chloroform, extracted with brine sol. and the organic layer was dried with $MgSO_4$ and used directly in the next reaction as the product was a volatile oil . Physical properties: oil coffee brown in color with greenish hue IR (υ= cm^{-1}):3600-3200 broad (O-H) str.,2970,2870 C-H str. of CH_2; 1435,1222,1161 C=S str. of dithiocarbamate; 999 C-S str.

Synthesis of 2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetic acid(5-FU acetic acid)Compound [III] [22]

In a 100 ml flat bottom quick-fit boiling flask mixed 5-FU (2g , 0.0153 mole) with 15ml freshly prepared 10% KOH solution mixing continued at room temperature until 5-FU completely dissolved where chloroacetic acid (2.18g , 0.0153mole) dissolved in 5 ml D.W was added and stirring continued for 30 mins (at room temperature) thereafter the pH of the mixture was adjusted to pH10 by the addition of 10% KOH and the reaction was put under reflux for 2 hr. then cooled to room temperature, acidified with concentrated (35-38%) HCl to pH=5 and left to precipitate at 4°C (for 2hr) slowly, the precipitate got filtered away and the filtrate was acidified once again with concentrated HCl to pH = 2.2 ,cooled at 4°C to slowly give the

product, Recrystallized by dissolving in (5%) $NaHCO_3$ and precipitation by concentrated HCl addition to give compound IV as white transparent short needle-like crystals. Percent % yield= 62%, m.p. =275-276°C (ref=276-277°C). [23];R_f=0,7. IR(υ= cm^{-1}): 3200-2800 (broad) COO−H stretching (H bonded) ; 3186 (N-H)str.of imide; 3055C (C-H)str ; 2970,2846 Asym. and sym. C−H stretching of (CH2);1735,1685,1662 are (C=O)of COOH overlapped with (C=O) str in pyrimidine ring, 1141 (C-F). ^{1}HNMR (60 MHz,DMSO-$_{d6}$,δ= ppm): 4.31(1H,s,CH_2) ; 7.99(1H,d,CH); (11.79,s,N-H);11.87 (1H,s,C=OOH).

Synthesis of *((pyrrolidine-1-carbonothioyl)thio)methyl 2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate*
Compound[IV][22]

To a stirred solution of compound [III] (1g, 0.0053 mole) in 30 ml dry acetone cooled on an ice bath, Dicyclohexyl carbodiimide (DCC) (1.23g, 0.006 mole) was added and stirring continued for 30 min. Then reaction cooled and compound **[II]** (0.5g, 0.0026 mole)was added gradually, the reaction mixture was left to stir in cold condition under temp= -10 to -20°C (average of -15°C) for three consecutive days and on the fourth day stopped, filtered ,dried completely from solvent then re-dissolved in Ethyl acetate and cooled for 2hrs then filtered again and washed successively with: 0.1N HCl (50ml x3), DW(50ml x3), $NaHCO_3$ 5% (50 ml x3) and finally brine sol. (50ml x2), the organic layer was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to give a reddish-dark brown oil that was triturated with petroleum ether and recrystallized with Ethyl acetate/n-Hexane (1:3) to give compound IV as light brown powder .Percent yield yield=52% , m.p.=128 °C (D) ,R_f=0.75. IR(υ= cm^{-1}):3263(N-H) str. of imide;2931,2850Asym. & sym (C-H) str of CH_2 ; 1701 (C=O) of ester;1728,1658 (C=O)str. of uracil; 1338,1157 (C=S) of thiocarbamate.^{1}HNMR (60 MHz, DMSO-$_{d6}$,δ= ppm): 1.5-2.15 (4H,m,CH_2 (Pyr)); 3.4-4(2H,m,CH_2); 4-4.6 (4H,m,CH_2) two signals combined ; 8.06 (1H,m,C-H); 11.8 (1H,s,N-H).

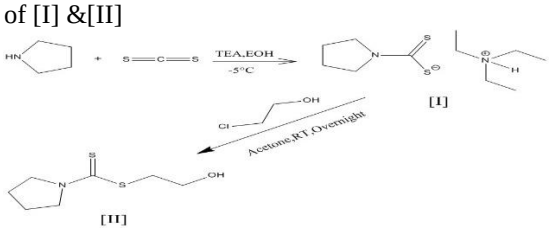
Cytotoxicity Study

A preliminary *in vitro* cytotoxicity assay (Cell Viability Assay on Cancer Cell Line) for the compounds ([IV], and 5-FU(as a standard) has been carried out at the Iraqi Centre for Cancer and Medical Genetic Research (ICCMGR). Both CAL-51 (human breast cancer) and B16V (mouse melanoma) cell lines were maintained using Rosswell Park Memorial Institute (RPMI)-1640 media supplemented with 20% fetal calf serum and seeded on micro-titration (96- well plates at a concentration of 1×104 cells/well) and incubated at 37 °C until the cells became confluent monolayer and various concentrations of tested compounds ([IV] and 5-FU) were added in (12.5,25,50, and100 mcg/ml) prepared by serial two-fold dilutions using maintenance media from stock solution of test sample in triplicate form of each concentration. The negative control wells contained only the cells with culture media, then the 96-well cell culture plate incubated at 37° C in an incubator supplemented with 5% CO2 gas for 72 hrs. [24]The cytotoxic activity of compounds was evaluated by Crystal violet assay [25], the optical density of each well was measured by using ELISA (Enzyme Linked Immuno Sorbent Assay) reader at a transmitting wavelength on 492 nm. The inhibition rate of cell growth (the Inhibition rate %(IR%)) was calculated as (A-B)/A×100, where A is the optical density of untreated wells (control conc.=zero), and B is the optical density of treated wells [26,27] .Data of *in vitro* cytotoxicity were subjected to unpaired t-test to compare the IR% Mean values and standard error of the mean (SEM) were calculated , using Microsoft excel; also a non-linear regression analysis to obtain dose-response curves and IC50values (using AAT Bioquest Quest Graph).

Results and Discussion

Chemistry

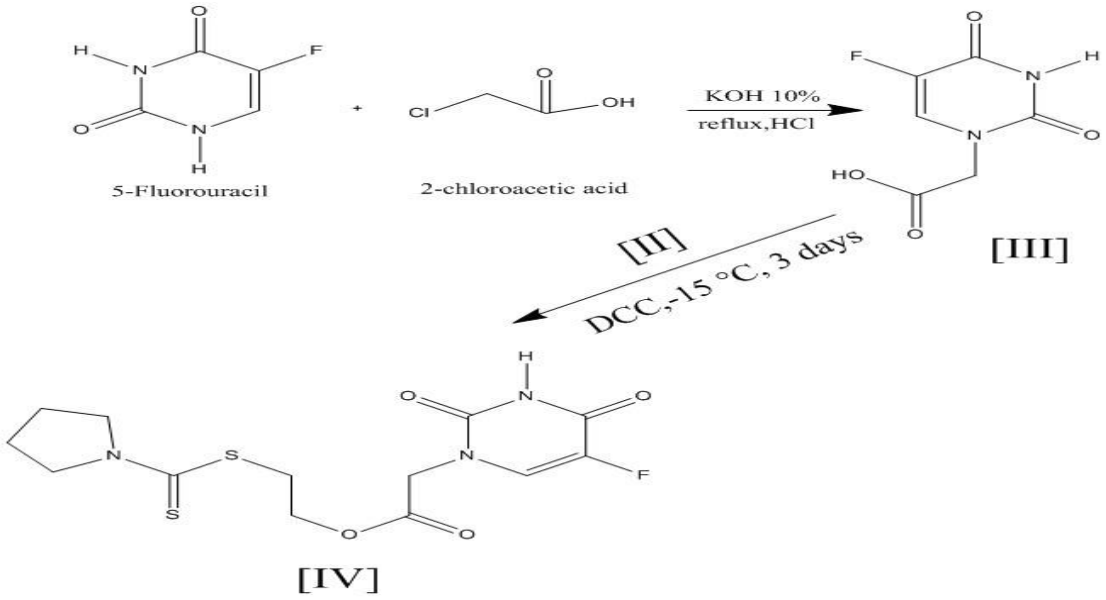
Compound [I] was prepared by reacting carbon disulfide with pyrrolidine in the presence of (TEA) as a base, the reaction is exothermic so was carried out over an ice bath .[28] Then the reaction of comp. [I] with 2-chloroethanol using acetone as a solvent took overnight to give comp [II] that was characterized by the appearance of broad O-H stretching vib. between 3600-3200 and disappearance of broad NH str vib.of TEA at 2300-2966 ,as shown in **scheme 1** shows path of synthesis of [I] &[II]



Scheme 1.Synthesis of compound [I] and [II]

To synthesize compound [III] , N1 of 5-FU was subjected to alkylation with Chloro acetic acid using KOH as a base to capture the chloride (Scheme 2),[III] structure was confirmed by appearance of 1735 cm^{-1} which is (C=O) stretching of carboxyl in FTIR along with O-H proton signal at δ=11.87 in ^{1}HNMR. **(Scheme 2)**

Coupling of compounds [II] and [III] was through the catalysis of DCC[29], it took 3 days and continuous cooling to give the final product [IV] FTR showed disappearance of the broad O – H str. of compound [III] and the appearance of C= S str. of thiocarbamate at 1338 and 1157 cm^{-1} .Alao O – H signal in proton NMR was not observed with appearance of new proton signals for the PDTC indicating ester formation **(Scheme 2).**



Scheme 2 .Synthesis of compounds [III] and [IV]

Cytotoxicity study

Results are represented in tables 1 and 2, the inhibition rate percentage (IR %), and in figures 1 and 2 as histograms of IR% versus concentration; the estimated IC50 values for 5-FU and [IV] are illustrated in table 3.

Figures 3 and 4 exhibit visual comparison between cancer cells before and after treatment with compound [IV].

Table 1. Cytotoxic effect (cell viability assay) on CAL-51 cell line of 5-FU & compound [IV] by crystal violet assay method [25].

Cell line		Concentration			
CAL51		12.5 mcg/ml	25 mcg/ml	50 mcg/ml	100 mcg/ml
5-FU	I.R.%	35.9	34.56	36.35	37.4
	P- value (compared to control)	8.474E-05 S	1.229E-05 S	6.605E-11 S	1.40022E-09 S
[IV]	I.R.%	16.1357	4.68567	19.4429	60.139
	P- value (compared to control)	0.0647192 NS	0.61836 NS	0.03287 S	3.4937E-05 S
	p-value (compared to 5-FU)	0.03375 S	0.01857 S	0.05167 S	0.003274 S

(p<0.05) considered significant, S=statistically significant, NS= non significant.

Table 2. Cytotoxic effect of 5-FU and compound [IV] on B16V cell line by crystal violet assay method[25]

Cell line		Concentration			
B16V		12.5mcg/ml	25mcg/ml	50mcg/ml	100 mcg/ml
5-FU	I.R.%	14.999	-23.450	-11.322	-70.630
	P- value (compared to control)	0.001549 S	0.0399 S	0.4786 NS	0.00311 S
Cpd [IV]	I.R.%	7.771956	50.60804	27.25844	40.91145
	P- value (compared to control)	0.75201 NS	0.005268 S	0.060048 NS	0.011975 S
	p-value (compared to 5-FU)	0.76965 NS	0.000355 S	0.0666 S	7.85E-05 S

(p<0.05) considered significant, S=statistically significant, NS= non significant, (Negative values indicate increased proliferation)

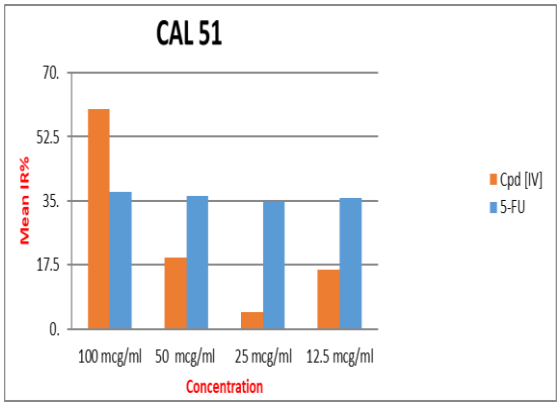


Figure 1. Inhibition rate %(%IR) versus different concentrations of compound [IV] and 5-FU, on CAL-51 cell lines at 72 hrs.

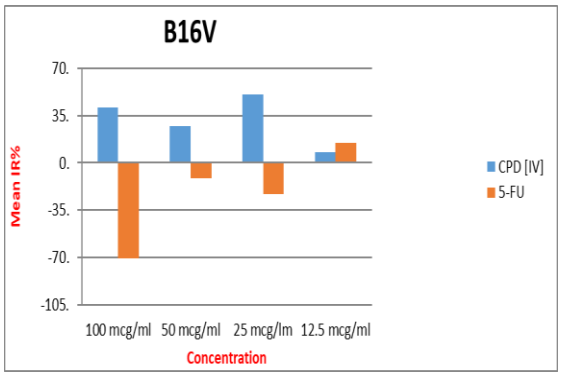


Figure 2. Inhibition rate% (%IR) versus different concentrations of compound [IV] and 5-FU, on B16V cell lines at 72 hrs. (Negative values indicate increased proliferation).

Table 3. IC50 values in mcg/ml for 5-FU and comp. [IV].

	CAL51	B16V
5-FU	50.914	13.503
[IV]	56.108	10.502

As illustrated in table 1 and figure 1 on CAL-51, The percent inhibition rate of 5-FU showed moderate but consistent cytotoxicity whereas compound IV gave significantly higher cytotoxicity (60%) at conc. =100 mcg/ml. B16V cells resisted 5-FU as the I.R% flipped to negative most of 5-FU's concentrations, the opposite case is seen for compound IV that showed moderate but superior activity when compared to 5-FU's on the same cell line. Compound IV gave significant IR % at conc.= 25and 100mcg/ml see table 2 and figure 2 .

Compound IV has superiority over 5-FU considering the IC-50 (Table 3).

Since most of the prodrug's weight is not 5-FU (100mcg of prodrug contains 36 mcg 5-FU) even though the comparisons are not entirely fair for compound IV regarding the 5-FU quantity provided, the drug made a good impression of enhanced cytotoxic effect ,this enhancement could be explained by the increased lipophilicity and eventually improved cellular penetration , also PDTC's NF-κB inhibiting properties may aid in decreasing esistance to 5-FU by stimulating apoptosis [1] .

Added cytotoxic effects are probably due to the direct anticancer action of pyrrolidine dithiocarbamate , through its antioxidant, antiproliferative and NF-κB inhibiting nature ; [4–6]This dual effect of the prodrug is mostly pronounced when it was found killing B16V cells that were resisted by 5-FU alone, figure 2 .

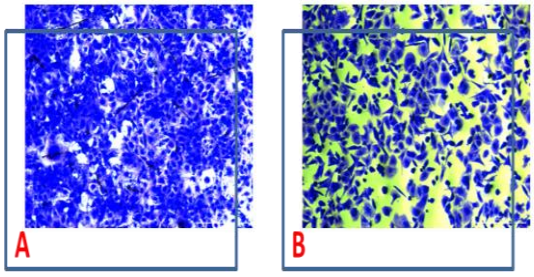


Figure 3. Cells of CAL-51; A: Effect of control. B: Effect of 100mcg/ml of compound [IV] at 72 hrs.

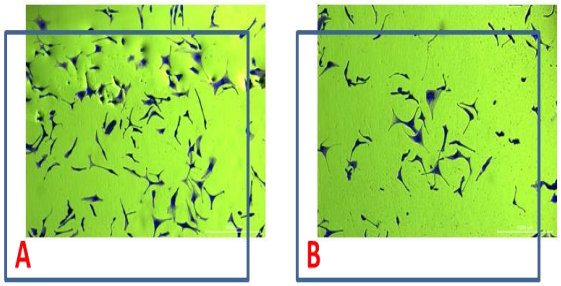


Figure 4. Cells of B16V ; A: Effect of control. B: Effect of 100mcg/ml of compound [IV] at 72 hrs.

Conclusion

The procedure for the synthesis of the target compound was achieved successfully; characterization of the structures was done by FTIR spectroscopy and ^{1}H NMR for the final and intermediate compounds, purity confirmed by R_f values and melting points.

Preliminary anticancer activity of the final product [IV] shows that it gave considerable cytotoxic activity against two types of cancer cells, and an improvement in cytotoxicity compared to the parent drug 5-FU.

Acknowledgments

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