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IMIDAZOLIDIN DERIVATIVE A NOVEL ANTI COLORECTAL CANCER AGENT

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ABSTRACT : The research includes the preparation of new heterocyclic imidazolodine-4-one derivative ,starting from 2-hydroxy-5-((4-nitrophenyl)diazenyl)benzaldehyde (F1). This work involved the synthesis and identification of imidazolodine-2-one derivative (F2) from the Schiff base derivative(F1). These compounds were characterized by Fourier-transform infrared spectroscopy (FTIR) and the Proton nuclear magnetic resonance (^{1}H-NMR) spectrum. The biological activity of the Imidazolidine derivative was also investigated by the thiazolyl blue tetrazolium bromide (MTT) cytotoxicity test on the LS -174T colorectal adenocarcinoma cells ,the African green monkey kidney (VERO) cells and the assessment of the concentration that inhibits the growth of 50% of cell viability (IC_{50}). Doxorubicin (DOX) and 5-Fluorouracil (5-FU) were also used for comparative goals. The results showed that Imidazolidine derivative was effectively and significantly inhibited the cell proliferation ($p<0.00001$) by decreasing the viability of LS -174T cells at different concentrations, which involved 1, 10, 50, 100, 250, 500 and 1000 µg/ml, with a half maximal inhibitory concentration (IC_{50}) of 54.406µg/ml. The DOX and 5-FU IC_{50} values were 63.140 µg/ml and 55.006 µg/ml, respectively. We concluded that the Imidazolidine derivative has an anticancer effect against the LS -174T colorectal carcinoma cell line.

Key words : MTT, LS-174T colorectal cancer, Imidazolidine, Thiadiazole, Schiff base.

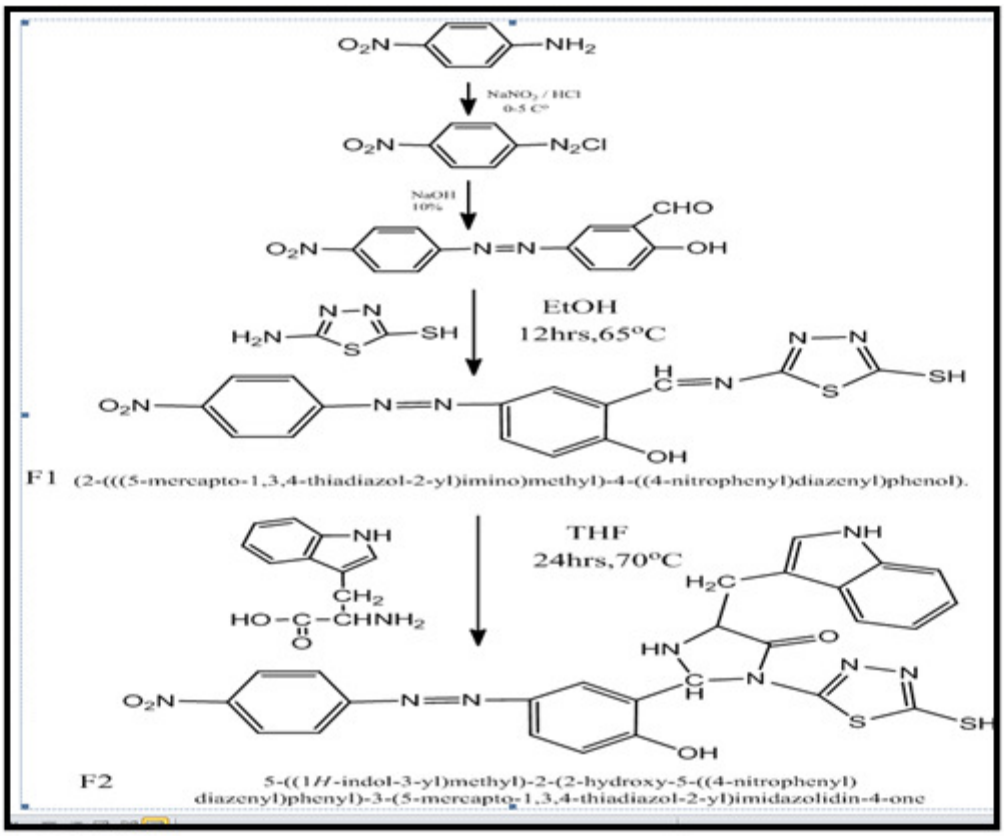
INTRODUCTION

Cancer causes cells to divide uncontrollably. This can result in tumors, damage to the immune system, and other impairment that can be fatal. Cancer cells do not die at the natural point in a cell's life cycle. Colon cancer is a world-wide health problem and the second-most dangerous type of cancer, affecting both men and women (Kuppusamy *et al*, 2014). Colorectal cancer is a cancer that starts in the colon or the rectum. These cancers can also be named colon cancer or rectal cancer, depending on where they start. Colon cancer and rectal cancer are often grouped together because they have many features in common. Cancer starts when cells in the body start to grow out of control. Cells in nearly any part of the body can become cancer, and can spread to other areas of the body, In the United States, colorectal cancer is the third leading cause of deaths among men and women, and the second most common cause of cancer deaths when men and women are combined. It is expected to cause 51,020 deaths during 2019. Mortality rate (deaths per 100,000 people per year) due to colon and rectum Cancer has fallen in both men and women for decades (Ernst *et al*, 2016). Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths in the world (Very *et al*, 2018). Many studies have used many chemical and pharmaceutical compounds to treat cancer, including colon cancer, lung cancer, uterine cancer and breast cancer, and compared the results with medicines approved by the International Health Organizations (Kelaby *et al*, 2016; Aditya *et al*, 2017; Very *et al*, 2018; Gomha *et al*, 2018; Mohammed *et al*, 2018).

MATERIALS AND METHODS

The chemical synthesis of Schiff base (2-((E)-((5-mercapto-1,3,4-thiadiazol-2-yl)imino)methyl)-4-((4-nitrophenyl)diazenyl)phenol) (F1) (Awaad and Al-Rammahi, 2018)

A mixture of (4-nitrophenyl)diazenyl)benzaldehyde (0.5164 gm, 0.002 mol),with 5-amino-1,3,4-thiadiazole-2-thiol (0.2664 gm, 0.002 mol) was heated in a water bath at 65°C with stirring for 10 hrs., then allowed to cool to room temperature. The solid product was filtered, and recrystallized from ethanol as orange-red powder (F1) whose chemical formula is ($C_{15}H_{10}N_6O_3S_2$). M.Wt. (386.41): (0.547gm, 70,85%, m.p. 167-169 °C). The



Scheme 1 : The Synthesis of the Imidazolidine derivatives (F1 and F2).

reaction was followed by disappearance of (N—H_2) and (C = O) absorption bands at (3500 – 3200) and (1700) cm^{-1}, respectively and the appearance of (N = C) absorption band at (1660) cm^{-1}. The Schiff base (F1) is identified by its table (1),the FTIR spectrum in Figure (1) , and table (2)that reveal the ^{1}H NMR spectrum and it is also shown in Fig. 2.

The synthesis of 5-((1H-indol-3-yl)methyl)-2-(2-hydroxy-5-((4-nitrophenyl)diazenyl) phenyl)-3-(5-mercapto-1,3,4-thiadiazol-2-yl)imidazolidin-4-one. (F2) (Alrammahi and Abood, 2017)

A mixture of (2-((E)-((5-mercapto-1,3,4-thiadiazol-2-yl)imino)methyl)-4-((4-nitrophenyl)diazenyl)phenol) (F1), (0.386 gm, 0.001 mol), with amino acid (treptophane) (0.2042 gm, 0.001 mol) in THF 50 ml , was stirred at (70 °C) for (20 hrs.). Then it was allowed to the reaction mixture to cool to room temperature and the solid product (F2) was filtered, and recrystallized from ethanol as red powder whose chemical formula is ($C_{26}H_{20}N_8O_4S_2$), M.Wt(572.62): 95 gm, 65.78%, m.p. 172-173 °C). The reaction was followed by the disappearance of (C = N) absorption bands at (1660)cm^{-1} and appearance of (N-C = O) absorption band at (1612) cm-1 and NH imidazolic and indolic group (3419)cm^{-1} .The imidazolidine derivative (F2) is identified by its table (1),the FTIR spectrum in Fig. 3 and Table 2. The ^{1}H NMR spectrum also shown in Fig. 4.

Note: The FTIR spectra were obtained with the KBr disk Shimatzu spectrometer(400-8000)cm^{-1}. ^{1}H-NMR spectra were obtained at DXR 500 MHz spectrophotometer, in DMSO-6d solvent, University of Tehran, Iran. The purity determination is done by TLC on silica gel (benzene: methanol 1:1) as a solvent.

Cytotoxicity assay

The anticancer activity of Imidazolidine derivative, Doxorubicin (DOX) and 5- Fluorouracil (5-FU) against LS-174T colorectal adenocarcinoma cell Line was evaluated by MTT assay (Bahuguna *et al*, 2017), in the Department of Clinical and Laboratory Sciences, Faculty of Pharmacy, University of Kufa.

The cell line was placed in 96 well plates at a concentration of 1.0×10^5 cells / ml. After incubation at 37°C for 24 to 48 hours, the monolayer was confluent of LS-174 or / and complete Vero cells (80%-100%). Various concentrations (0, 1, 10, 50, 100, 250, 500 and 1,000 µg/ml) of microtiter compound and (1, 10, 100, 250, 500 and 1000 µg/ml) of the microtiter DOX and 5-FU drugs were added to wells Cultured at a final volume of 100 µl in each well except the control cells in triplicate.

After 24 hours of incubation at 37°C in 5% CO_2, 35 micro wells were laid off and transferred to the biosafety

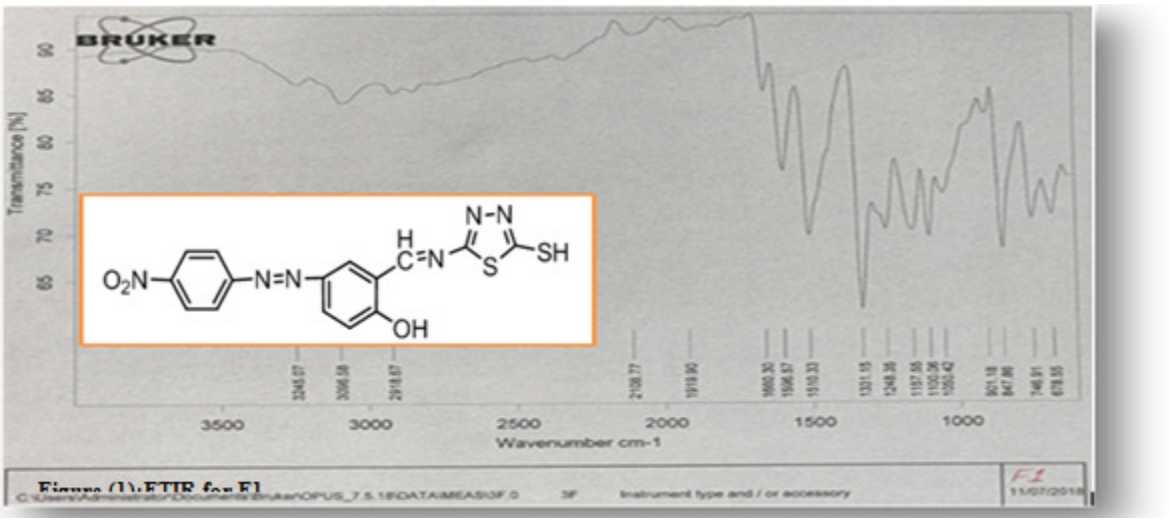


Fig. 1 : FTIR for F1.

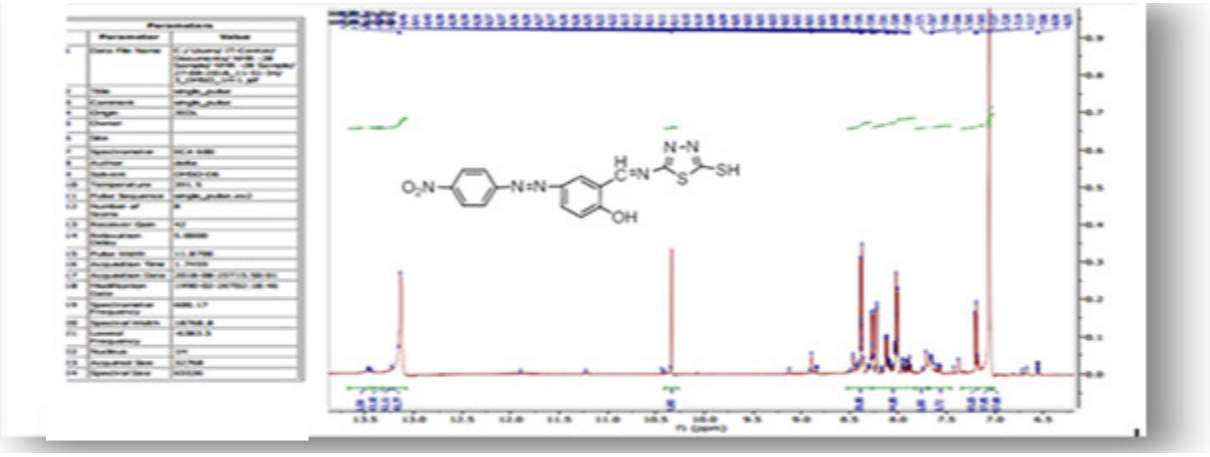


Fig. 2 : ^{1}HNMR for F1.

cabinet by sterile environments to avoid any contamination. All well media used was eliminated and the LS-174T -cell monolayer was washed 3 times by the PBS solution to remove any residual amount of standard anticancer compounds or the drugs used that may interact with MTT reagents. A volume of 100 µl of maintenance media was then added to all wells that included drug-treated cells, untreated cells and empty wells, and an MTT reagent (20 µl) was added to each well. After 4 hours of incubation at 37°C and 5% carbon dioxide, the Formazan molecules were formed as an enzymatic mitochondrial process for LS-174T and Vero cells that were not feasible, the dead or viral effected cells didn't form formazan particles because their mitochondria organelles were disrupted. The formazan was solubilized by adding diluted dimethylsulfoxide DMSO (1:1) in isopropanol on each well including blank wells. The absorbance was read at 490 nm with a reference wavelength of 630 nm by an ELISA reader. This protocol of MTT assay measurement was mentioned by many investigations (Lili *et al*, 2004; Accardo *et al*, 2014). Mean blank absorption was being subscribed from other samples and controls wells absorptions.

Statistical analysis

Mean blank absorption reading was subscribed from other samples and controls absorptions. The inhibitory concentration was fitted by blotting the inhibition percentage versus log of concentration of other used compound. % inhibition was calculated by the following formula: (Cytotoxicity = A-B/A × 100, where A and B are the optical density of control and the optical density of test after 24 hrs.). While the 50% of maximum inhibition (= Y in the formula of IC_{50} calculation) was calculated according as follows: 50% of maximum inhibition=Max GI % - 50% × (Max GI%- min GI%) (Al-kelaby *et al*, 2016). Statistical analyses were performed using SPSS 21.0 for windows. Inc. Data were expressed as Mean ± SEM , Two-tailed T test unless otherwise stated by ANOVA test. In all tests, $P<0.05$ was considered statistically significant. R^2 was calculated by Pearson Correlation Coefficient (Lili *et al*, 2004; Mao *et al*, 2016).

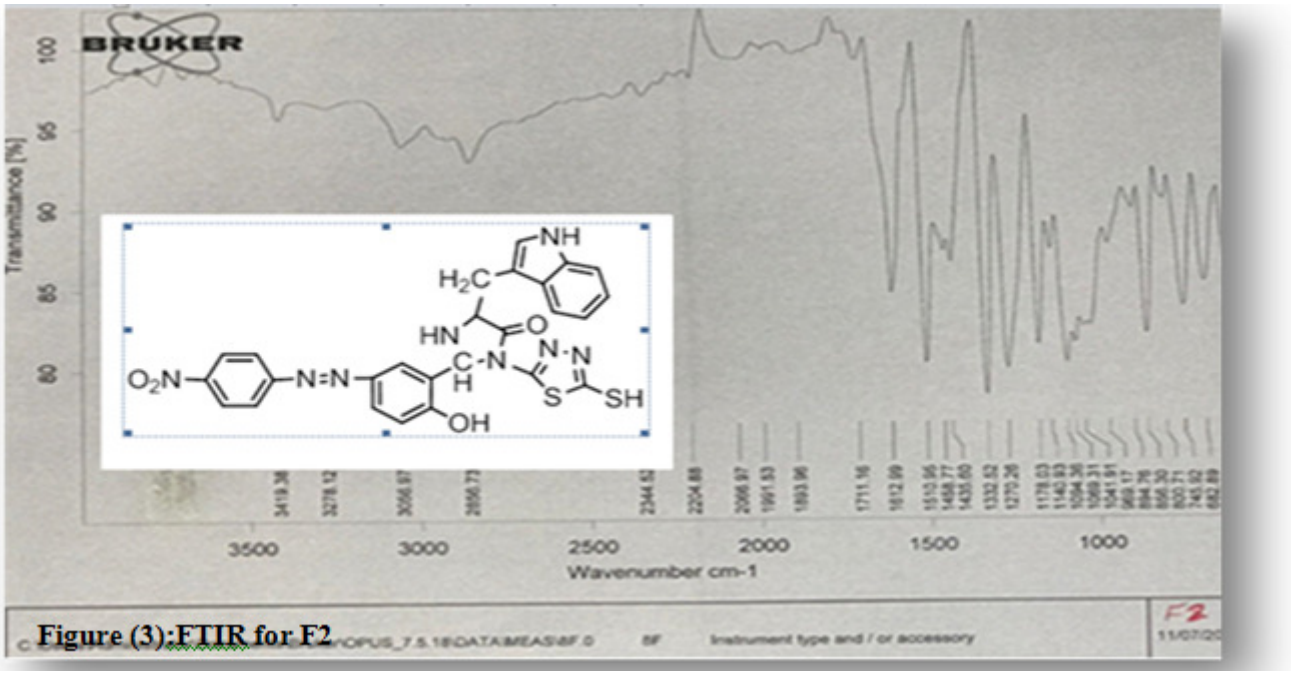


Fig. 3 : FTIR for F2.

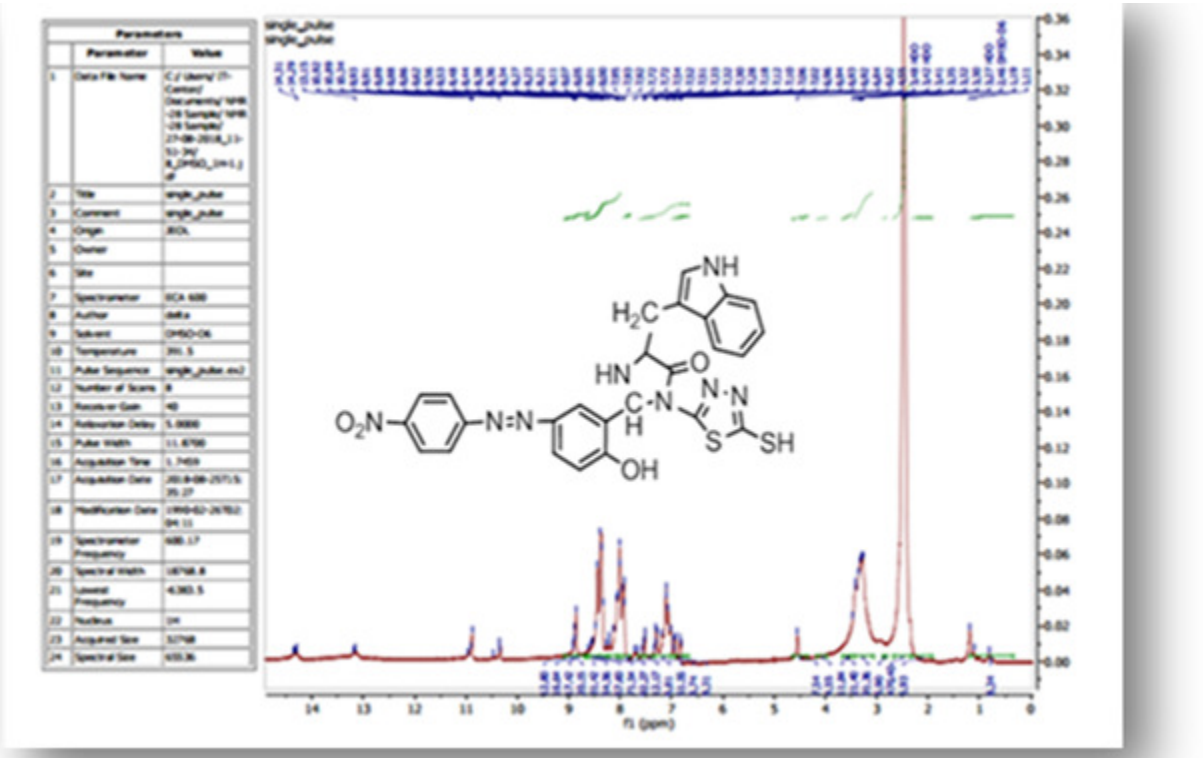


Fig. 4 : ^{1}HNMR for F2.

RESULTS

The Synthesis of the Imidazolidine derivative

This work involved synthesis and identification of imidazolodine-2-one derivative (F2) from the Schiff base derivative (F1). These compounds were characterized by the Fourier-transform infrared spectroscopy (FTIR) and the Proton nuclear magnetic resonance (1H-NMR) spectrum. Results are shown in Tables 1 and 2. The

Table 1 : FTIR spectrum of F1 and F2 (Bands t=Cm^{-1}).

No. of com.	Bands t=Cm^{-1}
F1	OH=3245, $CH_{(aromatic)}$ = 3096, $CH_{(aldehyde)}$ = 2918, SH = 2108, (C=N)=1660, (N=N)=1510, NO_2=1331
F2	NH=3419,OH=3278, $CH_{(aromatic)}$ = 3056, $CH_{(aldehyde)}$ = 2856, SH = 2066, (C=O) =1612 (N=N) =1510, NO_2=1332

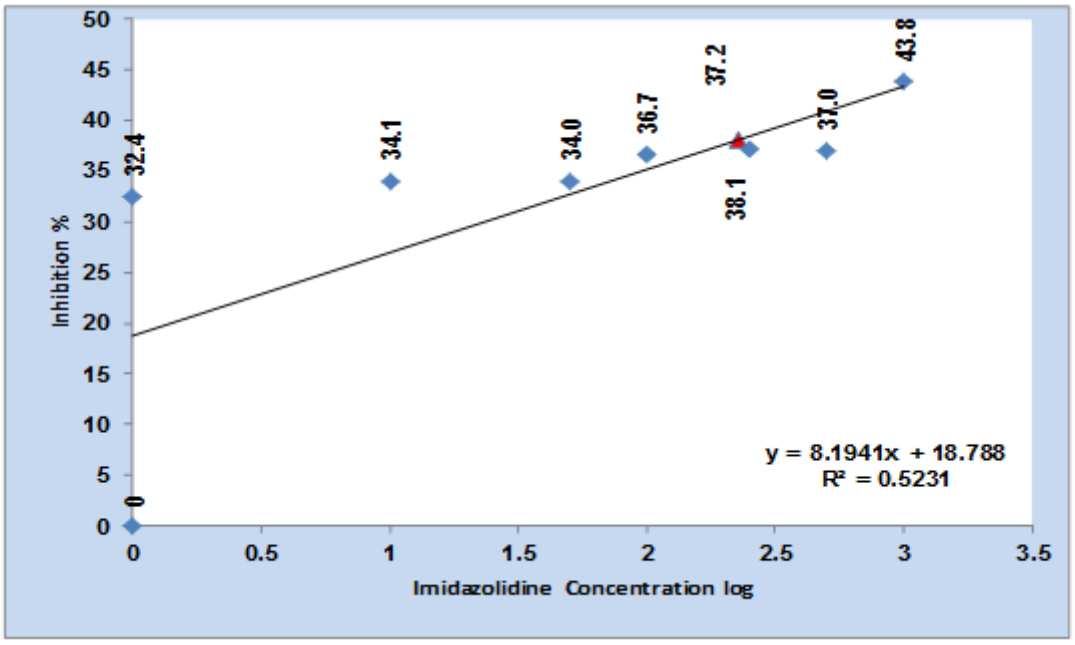


Fig. 5 : The Cytotoxic effect of Imidazolidine on VERO cells presented by plotting of conce.log vs GI% values.

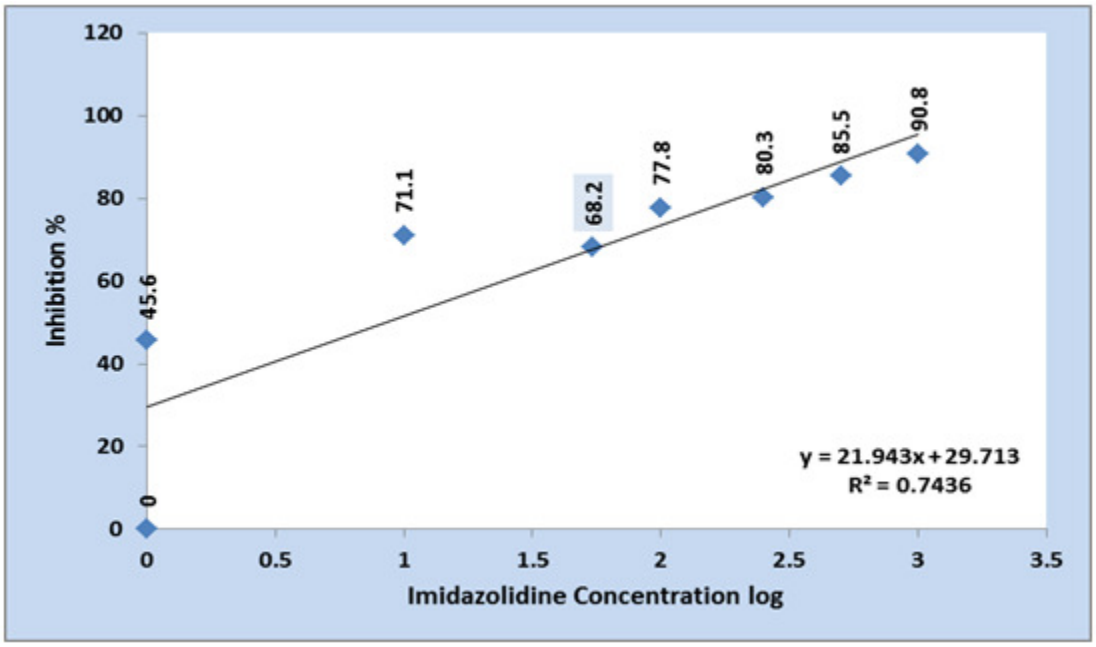


Fig. 6 : The anticancer activity of Imidazolidine on LS-174T cells presented by plotting of conce. log vs GI% values.

Table 2 : ^{1}H NMR spectrum of F1 and F2.

No	^{1}H-NMR spectrum signals
F1	ä 13.13(s,SH ä10,34(s,H benzilic)), ä 8.89-7.67(m,7H aromatic), ä 7.37(s,OH), ä3.30s, HDO, ä2.46s, DMSO
F2	ä 14.31(s,SH), ä 13.15(s,NH indolic), ä 10,92 (s H benzilic), ä10, 34(s,NH totomirism N=C-SH)), ä 8.93-7.02(m,7H aromatic), ä7.02 H indolic, ä 6,96(s,OH), ä3.48s HDO, ä2.46s, DMSO

Imidazolidine derivative chemical formula is ($C_{26}H_{20}N_8O_4S_2$), Molecular weight:(572.62). Physical appearance: red powder, Yield%: 65.78%; m. p. : (172-173)°C and RF value: 83% (in benzene: methanol 1:1).

Vero Cell and LS-174T cells culture was examined under inverted microscope after 48-72 hrs. Pure confluent monolayers were seen as shown in Fig. 13(a and b). Significant differences between control (untreated) LS-174T cells by MTT cytotoxicity assay as compared with those treated with different concentrations of the Imidazolidine compound ($P<0.0001$) were noticed (Fig. 13e×j).

The Imidazolidine effect on Vero Cell and LS-174T

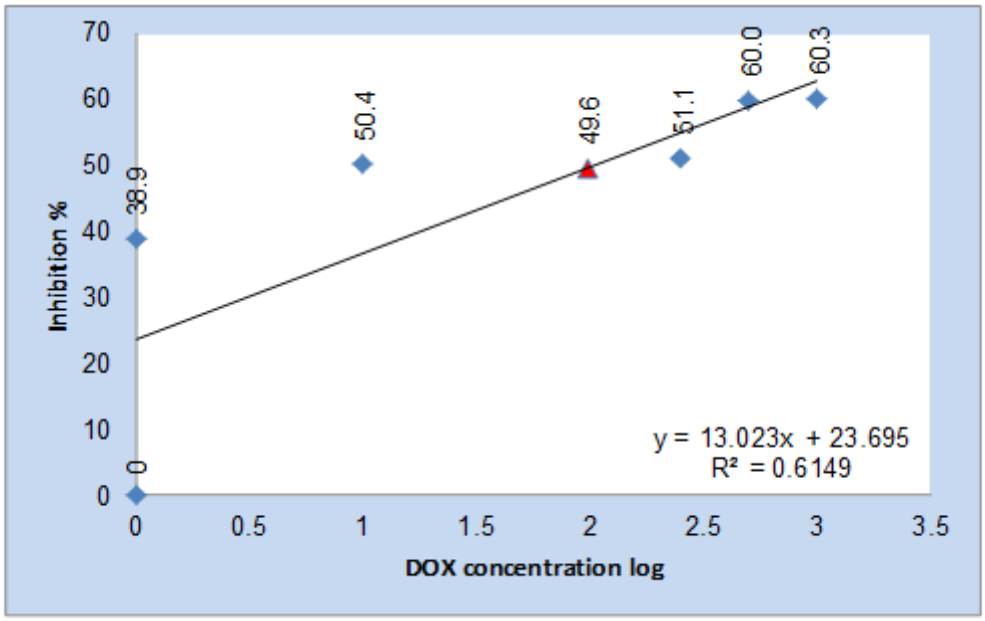


Fig. 7 : The Cytotoxic effect of DOX on VERO cells presented by plotting of conce.log vs GI% values.

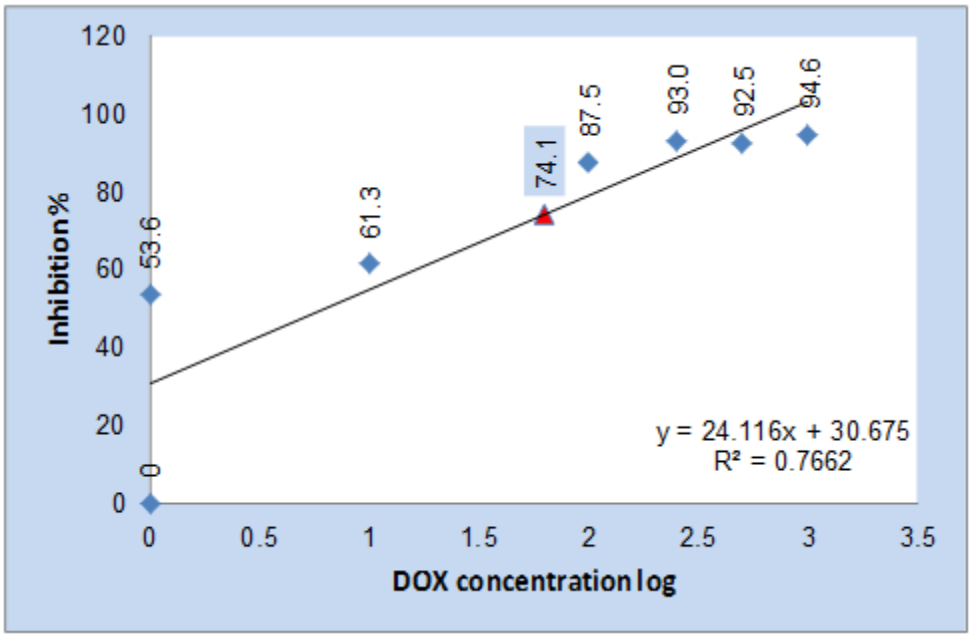


Fig. 8 : The anticancer activity of DOX on LS-174T cells presented by plotting of conce.log vs GI% values.

Table 3 : The Imidazolidine effect on Vero Cell and LS-174T cells, presented by Mean±SEM of optical density at 490nm.

Imidazolidine Dose (ìg/ml)	N	Effect on VERO cells	GI% on VERO cells	Effect on LS-174T cells	GI% on LS-174T cells
		Mean±SEM		Mean±SEM	
0	3	.99333±.041062	0	.63600±.019975	0
1	3	.67100±.000000	32.4	.34567±.026585	45.6
10	3	.65467±.054137	34.1	.18367±.058405	71.1
50	3	.65500±.020000	34.0	.18700±.029366	70.6
100	3	.62900±.002000	36.7	.14133±.017150	77.8
250	3	.62367±.039295	37.2	.12533±.005364	80.3
500	3	.62567±.039767	37.0	.09233±.002404	85.5
1000	3	.55800±.004000	43.8	.05833±.001333	90.8

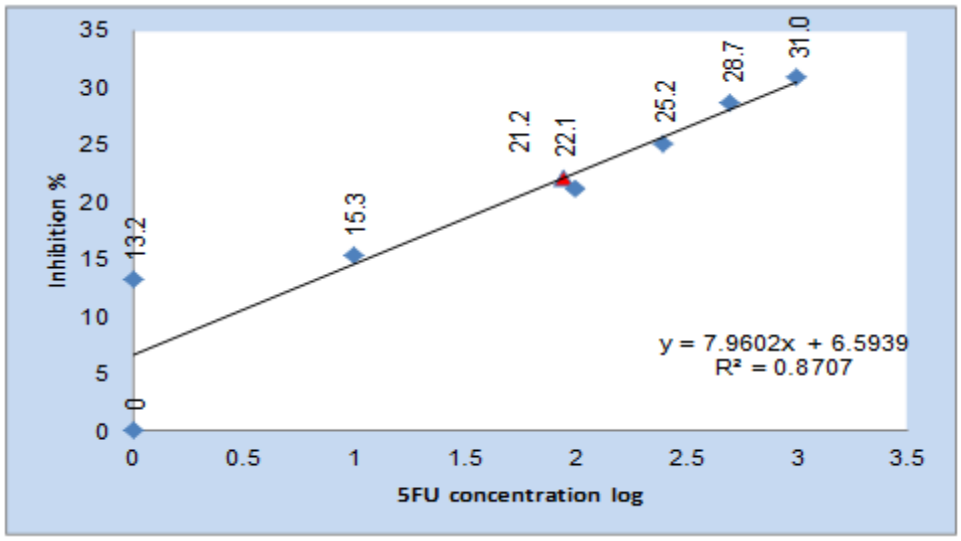


Fig. 9 : The Cytotoxic effect of 5-FU on VERO cells presented by plotting of conce.log vs GI% values.

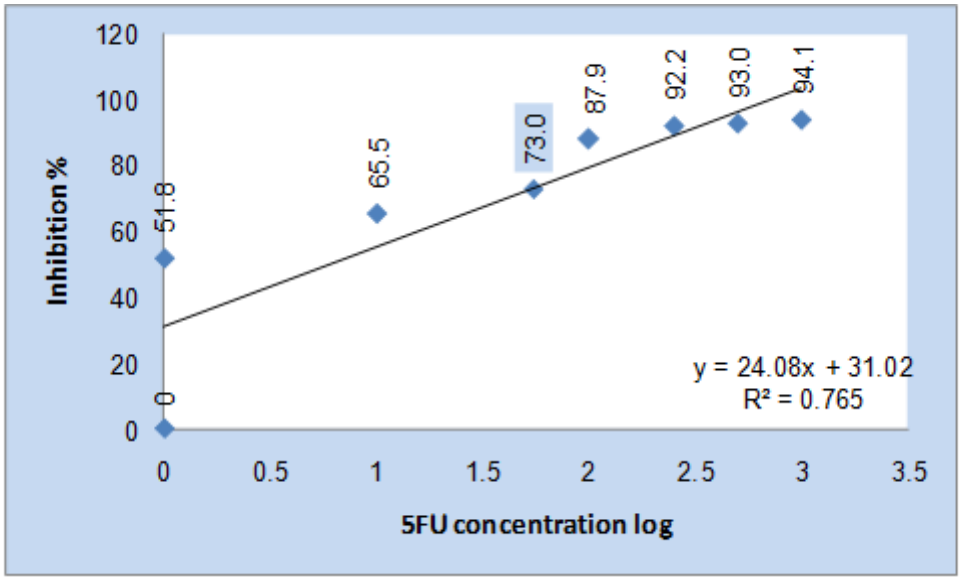


Fig. 10 : The anticancer activity of 5-FU on LS-174T cells presented by plotting of conce.log vs GI% values.

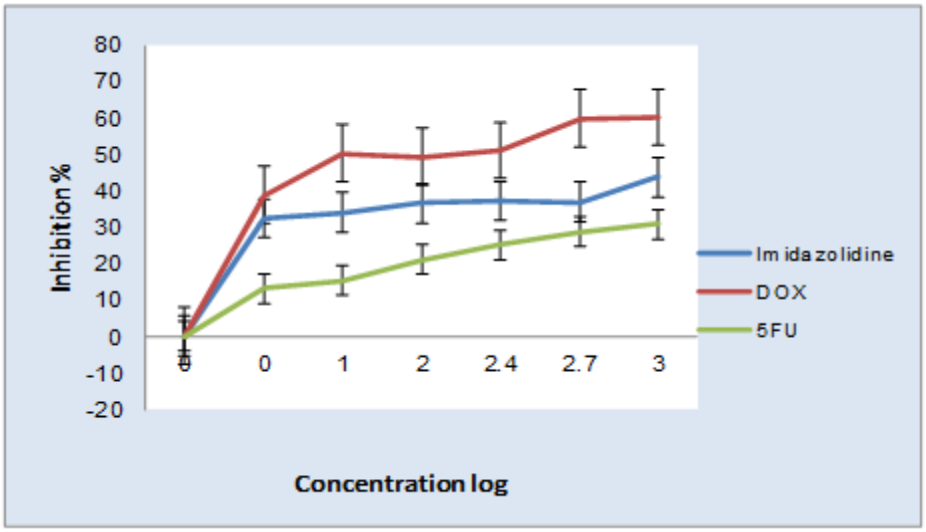


Fig. 11 : The Cytotoxic effect of the Imidazolidine derivative as compared with DOX and 5FU presented by plotting of concentration log versus GI% and SD values.

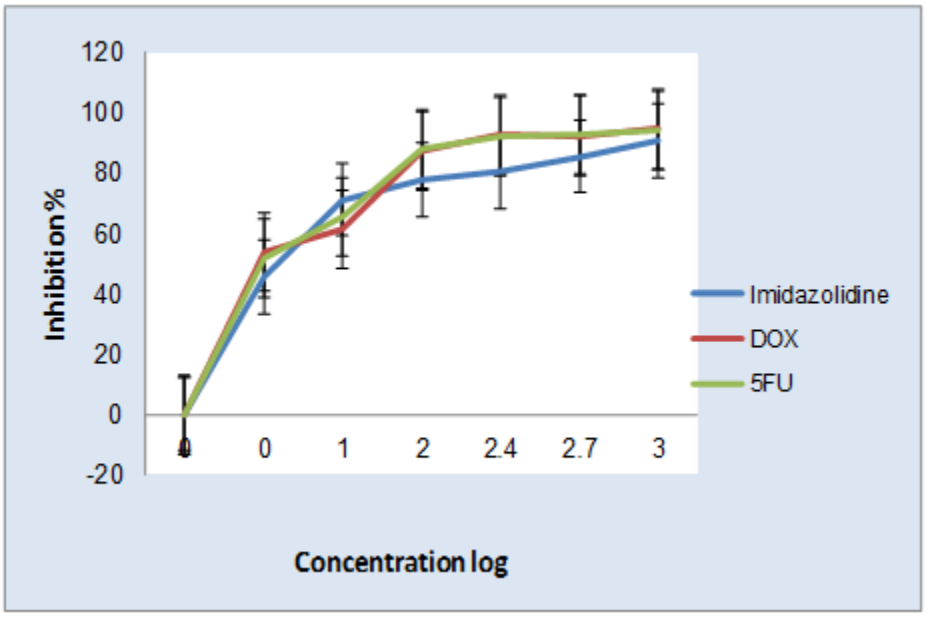


Fig. 12 : The anticancer activity of the Imidazolidine derivative as compared with DOX and 5FU presented by plotting of concentration log versus GI% and SD values.

Table 4 : The DOX effect on Vero Cell and LS-174T cells, presented by Mean±SEM of optical density at 490nm.

DOX Dose (ìg/ml)	N	Effect on VERO cells	GI% on VERO cells	Effect on LS-174T cells	GI% on LS-174T cells
		Mean±SEM		Mean±SEM	
0	3	.45333±.001453	0	.63600±.019975	0
1	3	.27667±.031540	38.9	.29500±.032083	53.6
10	3	.22467±.011319	50.4	.24633±.072953	61.3
100	3	.22900±.009815	49.4	.07967±.005783	87.5
250	3	.22133±.010745	51.1	.04467±.002186	93.0
500	3	.18133±.027546	60.0	.04800±.010693	92.5
1000	3	.18000±.037749	60.3	.03433±.003480	94.6

Table 5 : The 5-FU effect on Vero Cell and LS-174T cells, presented by Mean±SEM of optical density at 490nm.

5-FU Dose (ìg/ml)	N	Effect on VERO cells	GI% on VERO cells	Effect on LS-174T cells	GI% on LS-174T cells
		Mean±SEM		Mean±SEM	
0	3	.45333±.001453	0	.63600±.019975	0
1	3	.39333±.043333	13.2	.30633±.061904	51.8
10	3	.38367±.016667	15.3	.21933±.034744	65.5
100	3	.35700±.031000	21.2	.07700±.036000	87.9
250	3	.33900±.013000	25.2	.04967±.004333	92.2
500	3	.32300±.023000	28.7	.04467±.000667	93.0
1000	3	.31267±.012667	31.0	.03767±.002667	94.1

Table 6 : The Cytotoxicity of the Imidazolidine, DOX and 5FU on VERO and LS-174T cells presented by IC_{50} and R^2 values.

Dose/effect	Imidazolidine		DOX		5-FU	
	on Vero Cell	on LS-174T cells	on Vero Cell	on LS-174T cells	on Vero Cell	on LS-174T cells
Log IC50	2.3568	1.7356	1.99185	1.800	1.9474	1.740
IC50 (ìg/ml)	227.41426	54.406	98.14228	63.140	88.6117	55.006
**R^2	0.5231	0.7436	0.6149	0.7662	0.8694	0.7653

** R^2 = correlation coefficient of concentration log Vs growth inhibition %; DOX=Doxorubicin; IC_{50}= Half maximal inhibitory concentration; 5FU=5-Flourouracil.

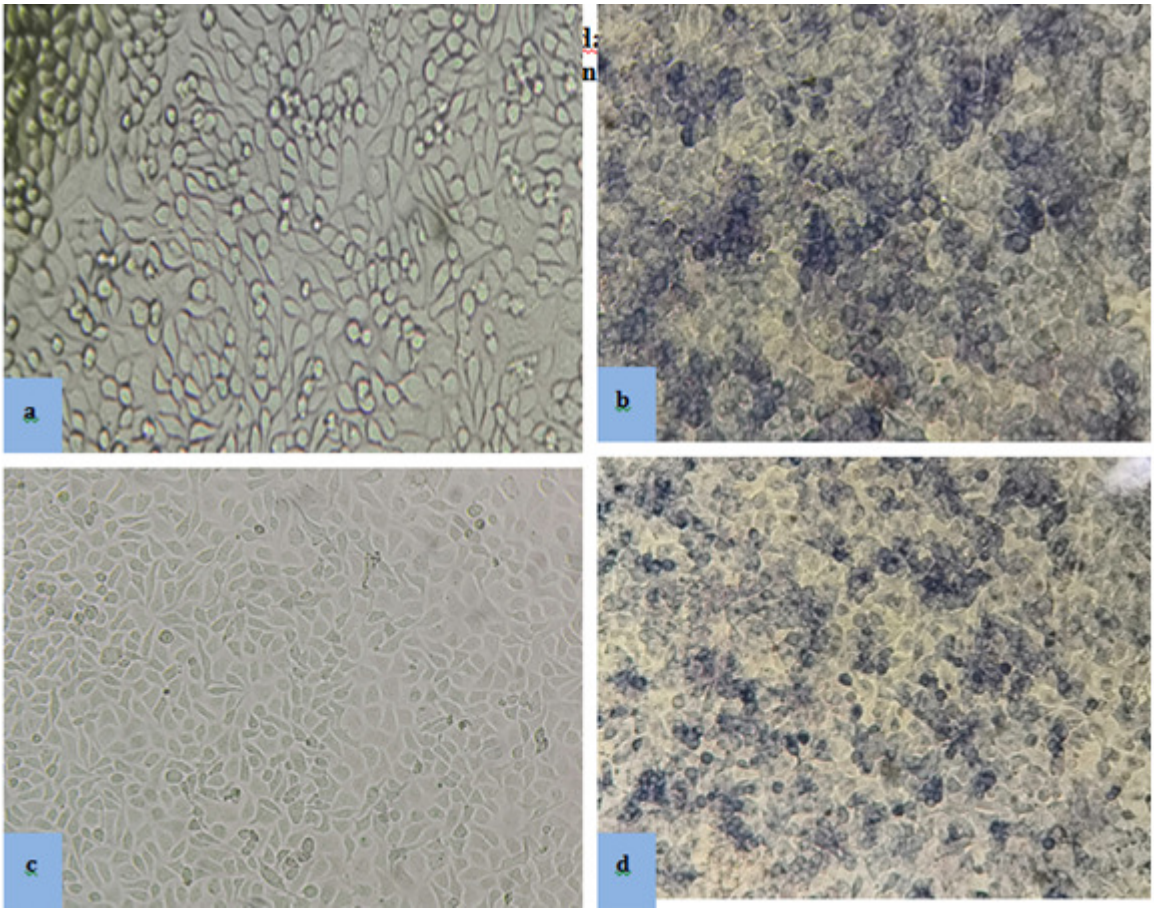


Fig. 13 : A- The LS 174T Cell Line before treatment with the Imidazolidine derivative or addition of MTT reagent, B- The untreated LS-174T cell monolayer after addition of MTT reagent, C& D :The untreated VERO cells before and after MTT addition, respectively.

cells was shown in Table 3 and Figs. 5 and 6, respectively with values of growth inhibition %. The inhibitory activity of the Imidazolidine compound was also compared with the untreated control group and the statistically significance factor was less than 0.000 for all concentrations with the positive correlation coefficient ($R^2 = 0.7436$) and IC_{50} value of 54.406 ìg/ml, while the IC_{50} value on VERO cells was equal to 227.41426 ìg/ml. The conventional anticancer drugs showed IC50 value of 63.140 ìg/ml and 55.006 ìg/ml for DOX and MTX on LS-174T cells respectively, with positive spearman correlation coefficient of 0.7662 and 0.7653 for the corresponding drugs respectively as shown in Table 6.

Doxorubicin effect on Vero Cell and LS-174T cells, presented by Mean±SEM of optical density at 490nm was shown in Table 4 and Figs. 7 and 8, respectively with values of growth inhibition %. The conventional anticancer drug DOX showed IC_{50} value of 98.14228ìg/ml on Vero Cells with positive spearman correlation coefficient of 0.6149.

The conventional anticancer drug 5-FU effect on Vero Cell and LS-174T cells presented by Mean±SEM of optical density at 490nm was shown in Table 5 and Figs. 9 &10, respectively with values of growth inhibition %. 5-FU showed IC_{50} value of 88.6117ìg/ml on Vero Cells, with positive spearman correlation coefficient of 0.8694.

The cytotoxic effect of Imidazolidine derivative as compared with DOX and 5-FU presented by the plotting of concentration log versus GI% and SD values on Vero Cell and LS-174T cells was shown in Figs. 11 and 12, respectively. These results revealed nicely declining curve fit was shown via plotting the GI% versus the different concentrations "log" of each compound (s) (Fig. 11). Different concentrations of the Imidazolidine derivative revealed the potency of growth inhibition % against LS-174T cells monolayer.

DISCUSSION

Results showed that the Imidazolidine derivative may have the anticancer potency against LS-174T Cell Line that has dose dependency, which is somewhat equal to DOX (R Squared calculated by plotting the GI% response Vs the concentration log of the Imidazolidine derivative, DOX and 5-FU were 0.7436, 0.7662 and 0.7653, respectively and IC_{50} of 54.406, 63.140 and 55.006,

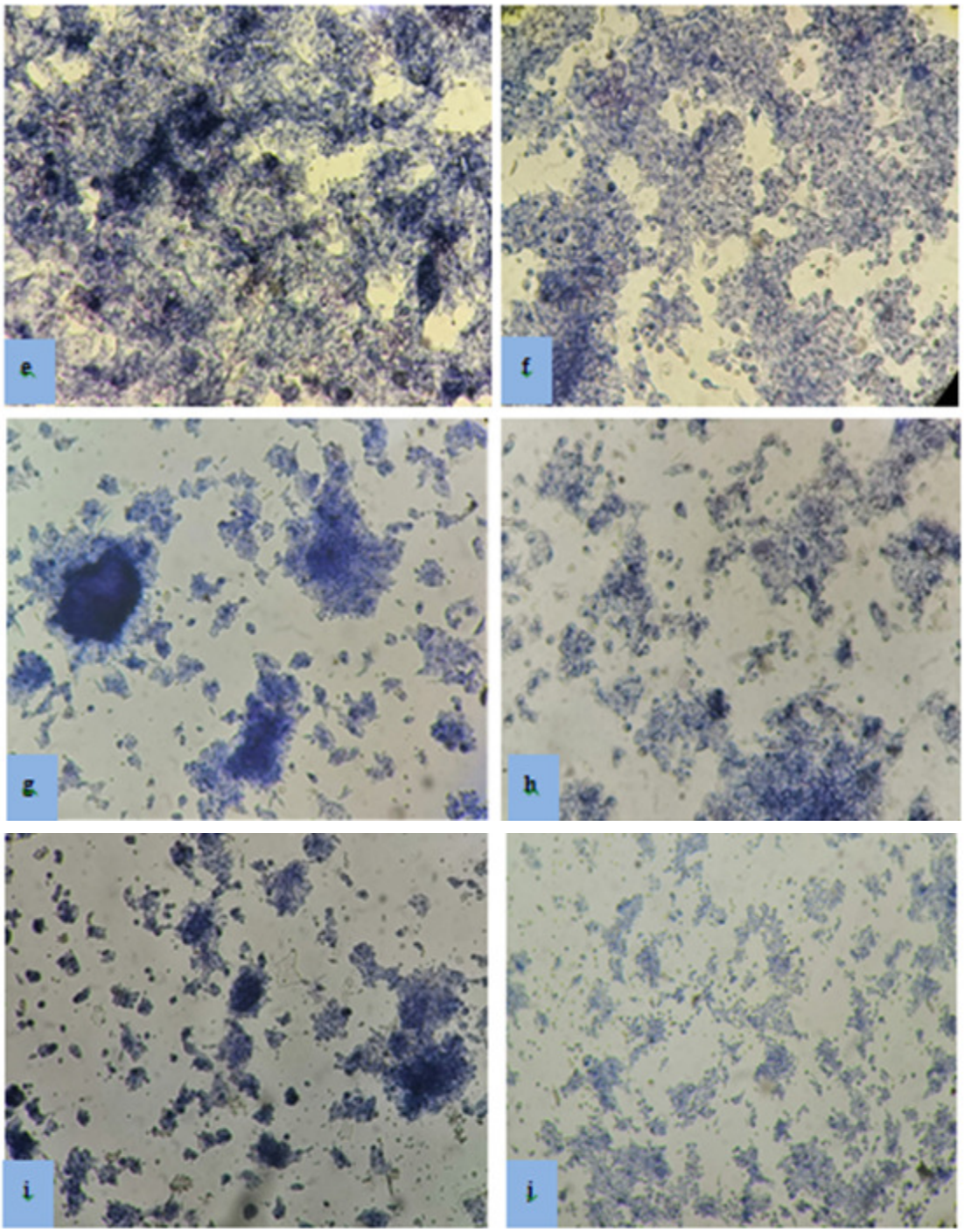


Fig. 14 : E-j: The LS-174T Cell Line monolayer after treatment with 1 µg/ml, 10µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml of the Imidazolidine derivative, respectively.

respectively. This fact gives us a hope to use the newly designed Imidazolidine derivative compound as an alternative anticancer in the near future and reflects the advantage of this compound. LS-174T Cell Line is one of the types of cancer cell lines in the colon that are important in therapeutic studies and the effect of toxicity of drugs for their rapid growth in vitro. Human colon cancer cells LS-174T cultured in a culture medium of substance on VERO Cells in a humidified incubator with an atmosphere of 5% CO_2 at 37° C. Viable cells in the samples were measured using the MTT method (Strobel *et al*, 1990; Zhang *et al*, 2004; Apolin *et al*, 2012; Zhang *et al*, 2019, Matos-Rochaa *et al*, 2019).

The assay of MTT, tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the MTT assay is a colorimetric test to assess cell proliferation based on a metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes reflect the

number of viable cells. These enzymes are able to reduce the pigment of MTT (yellow) to formazan insoluble and have a purple (dark brown) color. As the living cells are colored in violet and the sick or dead cells do not change the color, this means that the therapeutic compound is having an effect by killing the largest amount of abnormal cells (Anthony *et al*, 2008; Wuhan Fine Biotech, 2015; Al-Kelaby *et al*, 2016; Tachon *et al*, 2019). Other reports mentioned that the Imidazolidine has been reported as a broad-spectrum anticancer agent, showing much lower activity colorectal cancer agent (Apolin *et al*, 2012; £ukasz *et al*, 2014; Matos-Rochaa *et al*, 2019; Elhady, 2018; Alkahtani *et al*, 2019).

The Imidazolidine effect on Vero Cell and LS-174T cells was shown in tables (3-6). This compound showed IC50 value on VERO cells equal to 227.41426 ìg/ml, while The conventional anticancer drug DOX and 5-FU showed IC50 values of 98.14228ìg/ml and 88.6117ìg/ml on Vero Cells, respectively. These results reflect the safety of the Imidazolidine as compared with DOX and 5-FU. The compound of 1,3,4-Thiadiazole nucleus exhibited remarkable pharmacological activities. Literature indicates that compounds having 1,3,4-Thiadiazole nucleus have a wide range of pharmacological activities that include antibacterial, antifungal, antitubercular, antiviral, antileishmanial, anti-inflammatory, analgesic, CNS depressant, anticonvulsant, anticancer, antioxidant, antidiabetic, molluscicidal, antihypertensive and diuretic. The present review provides a broad view of the antimicrobial activity possessed by compounds having a 1,3,4- Thiadiazole nucleus (Suddhasatwa *et al*, 2014; Khalilullah *et al*, 2014;Rahman *et al*, 2014; AL-Rammahi *et al*, 2015).

The Indol and Tryptophan. Many other studies have examined the effect of tryptophan as an inhibitor of colon cancer and its vital mechanism to reduce the spread of cancer cells and tumors. These studies have shown that tryptophan is highly selective in inhibiting colon cancer cells with the least amount of treatments used (Crotti *et al*, 2017; Zhang *et al*, 2019). The 5-FU is regarded as the first chemotherapy against colorectal cancer (CRC); orally (Capecitabine) or intravenous 5-fluorouracil (5-FU). The best course of treatment is through the method of clinical practice. Evidence of practice comparisons is important in considering the net benefit of alternative chemotherapy regimens, given the expected differences in survival associated with compliance and age of patients treated in real life versus controlled trial settings .The FOLFOX system was the most commonly used treatment regimen. The method of using 5-FU was highly dependent on the patient's age ($P < 0.001$), tumor stage ($P < 0.001$) and location ($P < 0.001$) (Al-kelaby *et al*, 2016; Onozawa *et al*, 2016; Zhang *et al*, 2017; Pardini *et al*, 2017; Dai *et al*, 2018).

The Doxorubicin (DOX) was approved for medical use in the United States in 1974. It is listed on the World Health Organization's list of essential drugs as the most effective and safest drugs needed in the health system. There is much research to compare DOX with new compounds that have inhibitory activity against cancers, especially colon cancer. Doxorubicin is the most commonly used drug for the therapy of leukemia, lymphoma and breast cancer, but not for CRC. Although, DOX has been shown to be a better adjuvant chemotherapy drug for CRC at advanced stages (Ernst *et al*, 2016; Dalmark *et al*, 2009; Sonowal *et al*, 2017).

Chemotherapeutic agents and radiation are the most current cancer treatments that rely on that work in the body represented by killing rapidly dividing cells. The main drawback of the conventional chemotherapy is the adverse effects on the body as it lacks delivering selective and specific action to the cancer cells. Thus, damage extends to the surrounding normal healthy tissues or rapidly dividing healthy cells such as the cells of bone marrow, gastrointestinal tract, hair follicles, causing issues like cardiac, hepatic, pulmonary, gastrointestinal and renal toxicities (Thorgeirsson *et al*, 2002).

The study illustrates of toxicity tests for Imidazolidine prepared on LS-175T human colon cancer cells and normal cells of the African green monkey epithelial cells (Vero). It was studied and compared with (5-FU and DOX). The results recorded in this study were highly selective in killing cancer cells. It is extremely safe with normal cells so that it does not target healthy cells. The Imidazolidine derivative is therefore a new treatment for colon cancer with a high sensitivity and selectivity, approximately equal to (5-FU) used in the treatment of colon cancer, using MTT as a pigment to differentiate between living cells and killed cells (IC_{50}) Inhibition% for the Imidazolidine.

The Imidazolidine derivative may be used as effective agent against colon cancer in future.

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