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SYNTHESIS AND ANTICANCER SCREENING OF SOME NOVEL SUBSTITUTED IMIDAZOLE DERIVATIVES

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ABSTRACT

The two synthetic compounds 15b and 15h is tested for their ability to prevent cervical cancer using HeLa cell line. The study shows that both 15b and 15h compounds might be useful to develop an anti-cervical cancer drug and possess potential applications in the treatment of cervical cancer. The 15h compound treatment at 100 µg/ml has significantly arrested at 62.21 % in G2M phase of cell cycle, compare to untreated cells (9.46 %). At 50 µg/ml, 15h compound has also showed good arrest at 42.89 %. This result suggests 15h compound has more effectively arrested cells at the G2 phase of the cell cycle compare to 15b. Thus both 15b and 15h compounds show more significant cell cycle arrest at G2M phase and at a same time it could induce the apoptosis in HeLa cells. These compounds can be taken up for further studies for drug development.

KEYWORDS: HeLa cell line, Imidazole derivative, FACS.

INTRODUCTION

Cancer is a dreadful disease in which a cell or a group of cells display uncontrolled growth, invasion and sometimes metastasis. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor.[1] The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology. In 2004, worldwide cancer caused 13% of all deaths. The leading causes were lung cancer, stomach cancer, colo-rectal cancer, liver cancer and breast cancer,[2, 3] Cancers are primarily an environmental disease with 90-95% of cases due to lifestyle and environmental factors and 5-10% due to genetics. Common environmental factors leading to cancer death include: tobacco, diet, obesity, infections,

radon exposure, radiation, stress, lack of physical activity and environmental pollutants.[4] These environmental factors cause abnormalities in the genetic material of cells,[5] Cancer affects people at all ages with the risk for most types increasing with age.The traditional anticancer drugs are the basis for the new drug development for cancer in which imidazole is an important moiety. Imidazole is a heterocyclic ring containing basically 3C and 2N atom present in 1st and 3rd positions.[6] The substitution on different positions gives a number of compounds of interest. Thus, imidazole compounds have been an interesting source for researchers for more than a century. Structure activity relationships were reduced from biological results and will be used in further design of new active compound. Presently a number of drugs are used in the treatment of the cancer, but majority of them were produced controlled effect on the cancer cell. By application of these drugs the disease can be controlled. Imidazole and its derivatives are reported to be physiologically and pharmacologically active and find applications in the treatment of several diseases. In the drug discovery the imidazole is the most important synthetic strategy. Many imidazoles are reported as pharmacological agents like Azomycine, Clotrimazole, Miconazole, Ergothionine, Clonidine and Moxonidine. One of the most important applications of imidazole derivatives is their used as material for treatment of denture stomatities and in cancer.[7,8] Mostly synthetic compounds which are biologically active have five membered nitrogen containing.[6]

Imidazoles are generally well known as anticancer agents. These are heterocyclic compounds containing 5-membered planar ring, soluble in water and other polar solvents. Imidazoles are of two equivalent tautomeric forms because of hydrogen atom which is located on either of the two nitrogen atoms. They are amphoteric and therefore can function as both an acid and base. Imidazoles are aromatic compounds because of the presence of a sextet of p-electrons, consisting of a pair of electrons from the protonated nitrogen atom and one from each of the remaining four atoms of the ring.[1] Ozkay et al. studied 18 novel imidazole-(benz)azole and imidazole piperazine derivatives The structures of the compounds were confirmed by IR, HNMR and EI-MS spectral data. Most of the compounds, showed greater activity against carcinogenic cell lines.[9] In the present study synthesis of compounds 15b and 15h were carried out for testing their anti cancer activity using MTT and FACS *in vitro* analytical assay.

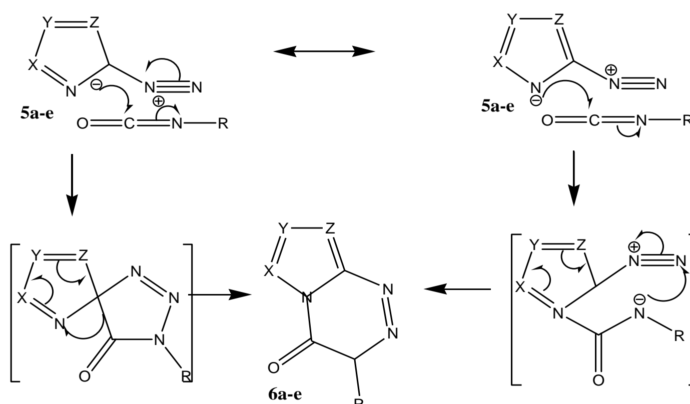
MATERIALS AND METHODS

Synthesis of compound

The novel chemical derivatives were synthesised namely 15b and 15h of Imidazole, for evaluation of anti cancer activity in cervical cancer cells. The new ring system imidazo[2,1-d][1,2,3,5]tetrazine-4(3H)-one ,were obtained from moderate to excellent yields by reaction of 5-(propylthio)-2-diazo-2H-benzimidazole with alkyl- or – aryl-isocynates in non hydroxylic solvent at room temperature.

Synthesis of imidazotetrazinone.

The mechanism of this regiospecific net 1, 7- cycloaddition is not completely clarified. A fully concerted [4n+2] mechanism, formerly proposed.An ionic pathway might be involved initial nucleophilic attack at the isocyanates carbon to give dipolar intermediate which spontaneously undergoes ring closure (1,3-cycloaddut) or a [3+2] cycloaddition leading to a spiro compound which by a sigmatropic rearrangement affords the bicyclic system.



a. X=N, Y=Z=C (pyrazole)

b. X=Y=N, Z=C (triazole)

c. X=N, Y=Z=C-benzofused (indazole)

d. X=Z=CR, Y=N (imidazole)

e. Z=N, X=Y=C-benzofused (benzimidazole)

Mechanism of cycloaddition

The synthesis of the new ring system benzimidazo[2,1-d][1,2,3,5]tetrazine-4(3H)one 15 by reaction of the key intermediate 5-propylthio-2-diazo-2H-benzimidazole 13 with alkyl- or aryl- isocyanates and the antiproliferative activity of these nine derivatives are tested invitro against various human cancer cell lines.

Synthesis of benzimidazotetrazinones (15a-i)

The 5-propylthio-2-diazo-2H-benzimidazole 13was obtained, in nearly preparative yield, by diazotization of the corresponding amine 12and subsequent neutralization. The reaction is carried out in acetic acid with stoichiometric amount of sodium nitrite under atmospheric nitrogen in the dark by addition of aqueous sodium carbonate. The strict control of the temperature at -10˚c to 0°C during diazotization and neutralization is crucial in obtaining high yield. The structure of the 5-propylthio-2-diazo-2H-benzimidazole 13was confirmed by IR spectra showed a sharp and strong band at IR (KBr ʋmax cm-1):2192.10 (N2+).[10]

Cell Culture

The current study involves Hela cancer cell line, obtained from American Tissue Culture Collection (ATCC). The cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin. Cells were incubated at 37 ^{o}C in a5% CO_2 humidified incubator.

Cytotoxic activity (MTT assay)

The cytotoxic assay detects the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase to blue insoluble formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. Briefly 5.0 X 10 cells of Hela were plated in triplicate in 96 well plates with DMEM and incubated for 24 hrs at 37 ^{0}C. Plant extracts were tested as working standard in serum free DMEM media and incubated for 24 hr in CO2 incubator at 37°C. After incubation with plant extracts, the media was removed from the wells and added 100 µl/well of the MTT reagent and incubated for 3-4 hrs. After incubation, the MTT reagent was removed before adding 100µl DMSO to each well and gently shaken. Plant extracts treated cells were compared to untreated cell control wells. Measure the absorbance at 590nm using a Tecan microplate reader. The percentage inhibition was determined using the formula.

% Inhibition = 100-(optical density of sample/optical density of control) × 100.

IC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell.[11-14]

Clonogenic assay

The Cells were seeded in a 6 well plate and incubated for 24h in 5% Co_2 at 37°C. The cells were treated with compound and a control with no treatment. The media was removed after

24h and fresh media along FBS was added, and incubated for 3 weeks. The media was removed and washed with PBS and were fixed with 1ml of 4% PFA: 6% Glutaraldehyde: Acetic acid: Methanol solution for 30 min. Later stained with crystal violet for 40min and washed off with distilled water and allowed to dry. Colonies containing more than 50 cells were counted.

Flow Cytometry Analysis

Is a laser based biophysical technology employed in cell sorting, counting and biomarker detection via suspending cells in a stream of fluid. 1 x 10^6 cells were cultured in 6 well plate containing 2ml of complete DMEM media, after 24hour of incubation, cells are treated with 100 and 200µg/ml compound; 20µM Colchicine as positive control and 1% DMSO as control in 1ml/well of DMEM media was used and incubate for 24 hour. After treatment, cells were collected and pelleted cells at 1500rpm for 5minutes at room temperature and discard the supernatant. Cells pellet was fixed overnight at 4°C in a 2 ml of fixing solution containing 20% PBS in 70% ethanol. Centrifuge at 4000rpm for 10min at room temperature and discard the supernatant and washed with cold 1XPBS. Cells were incubated for 15min at room temperature in 500µl of propidium iodide (PI) solution containing 0.05mg/ml PI and 0.05mg/ml RNase A in PBS. The percentage of cells in various stages of cell cycle in compounds treated and un-treated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA).

Statistical Analysis

Using GraphPad Prism 5 (Graphpad, SanDiego, CA, USA) software, IC_{50} values for DPPH radical scavenging activity of test compounds are computed from a nonlinear regression analysis (curvefit) based on sigmoidal dose response curve (variable).

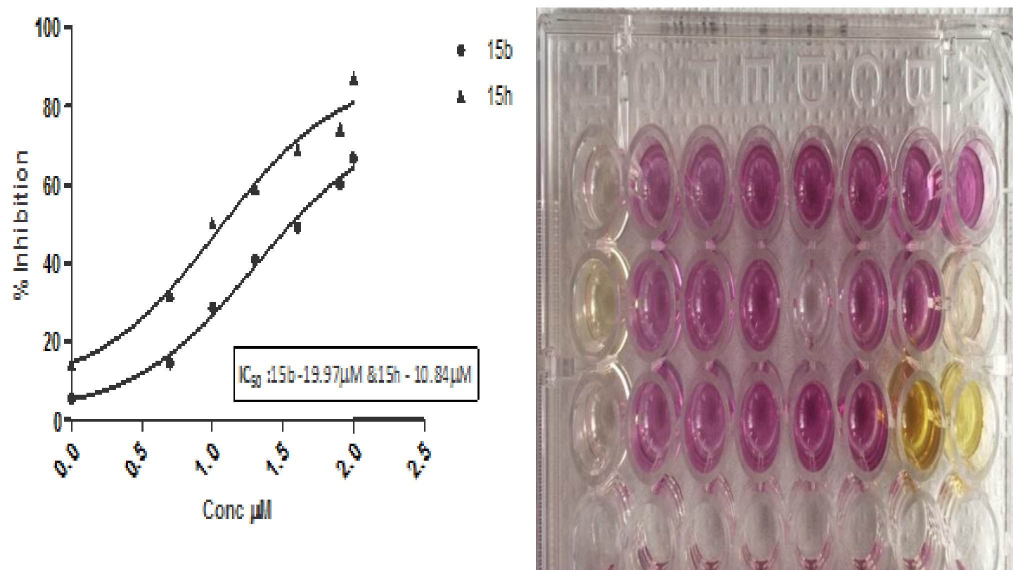
RESULTS AND DISCUSSIONS

The benzimidazolo-tetrazines **15a-i** were prepared in moderate to good yields (40-80%) by reaction of the 5-propylthio-2-diazo-2H-benzimidazole with stoichiometric amounts of the proper isocyanates **14a-i** in DCM at room temperature for 24-48 hrs. The structure of the newly synthesized compounds was elucidated by their ^{1}H NMR, LCMS and IR spectral data analysis. In the IR spectra, the band due to the carbonyl group of the tetrazine moiety, which is present in all studies compounds were observed at about 1720 cm^{-1} - 1748 cm^{-1},respectively (Table1). The structures of all derivatives **15a-i** were confirmed by spectroscopic data.

Table 1 Percentage yield of Benzimidazotetrazinones 15a-i

Entry	Isocyanate	R	Product	Yield (%)	Mp(˚C)
1	14a	C_6H_5	15a	80	233.0–234.0
2	14b	CH_2-C_6H_5	15b	65	150.5-152.5
3	14c	*o*-C_2H_5-C_6H_4	15c	60	139.5–140.5
4	14d	*o*-Cl-C_6H_4	15d	75	234.5–236.5
5	14e	*m*-Cl-C_6H_4	15e	79	248.5–250.5
6	14f	*p*-Cl-C_6H_4	15f	68	275.5–276.5
7	14g	*n*-butyl	15g	57	178.0–180.0
8	14h	*Iso*-propyl	15h	50	142.5-144.5
9	14i	*c*-Hexyl	15i	72	184.5–186.5

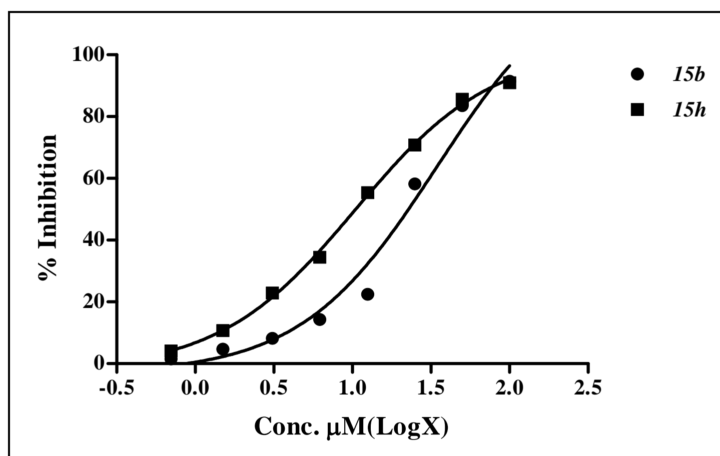
The synthesized nine benzimidazotetrazinones derivatives (1**5a-i**), were selected for *in-vitro* disease-oriented antitumor screenings against the four human tumor cell lines, including non-small lung, cervical, prostate and breast cancers cell lines by the MTT assay. The potential cytotoxicities of all compounds were determined by measuring the percentage of cancers cells death/inhibition. The selective compounds were tested in various concentrations of 0, 1, 5, 10, 20, 40, 80,100 µM against human cancer cell lines (ATCC) viz., H1975 (non small lung), PC3 (Prostate), HCT116 (Cervical) & MCF7 (Breast) to determine IC_{50} values using Non-linear regression by Graph pad Prism 6. The benzimidazotetrazine derivatives 15a, 15c, 15d, 15e & 15f showed no significant activity, while 15b, 15g, 15h, 15i showed antitumor activity against H1975, PC3, Hela& MCF7. The IC_{50} values of all tested compounds, 15b showed 19.97µM, 9.29µM, 37.63µM and 16.95µM against H1975, PC3, MCF7 and Hela respectively. The compound 15h showed IC_{50} 10.84µM, 14.91µM, and 38.91µM against H1975, MCF7 and Hela respectively. The compound 15g & 15i showed IC_{50} values were 8.84µM & 36.13µM against MCF7 respectively.



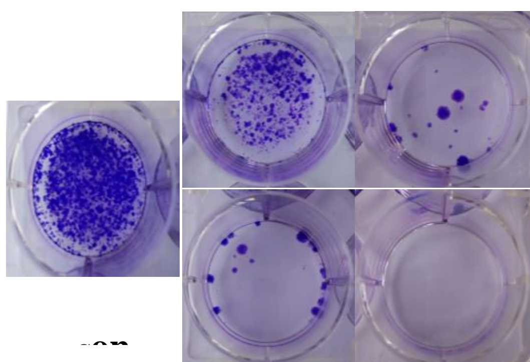
Graph: 1 Antiproliferative activity of 15h Figure:1 The effect of 15b and 15h compound on inhibitio and 15b compound on human cervical cancer Hela cells of human cervical cancer hela cells.

Table 2: Determination of IC50 of 15b and 15h on hela cells

Samples	Conc. □□	OD 590 nm	% Inhibition	IC 50
	Control	0.6451	0.00	
15b	0.7	0.6353	1.52	34.7
	1.5	0.6145	4.74	
	3.1	0.5921	8.22	
	6.2	0.5529	14.29	
	12.5	0.5005	22.42	
	25	0.2697	58.19	
	50	0.1062	83.54	
	100	0.0551	91.46	
15h	0.7	0.6186	4.11	10.44
	1.5	0.5762	10.68	
	3.1	0.4974	22.90	
	6.2	0.4227	34.48	
	12.5	0.2877	55.40	
	25	0.1883	70.81	
	50	0.0926	85.65	
	100	0.0583	90.96	



Graph.2- Compound 15b and 15h showed IC50 values of 34.7 and 10.44uM respectively



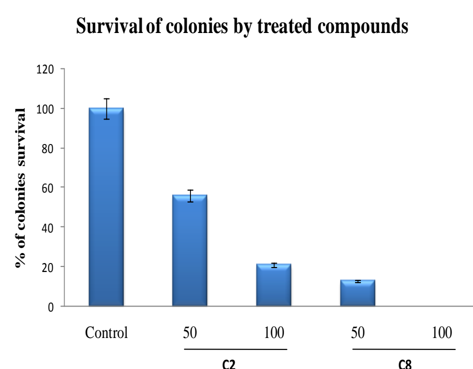
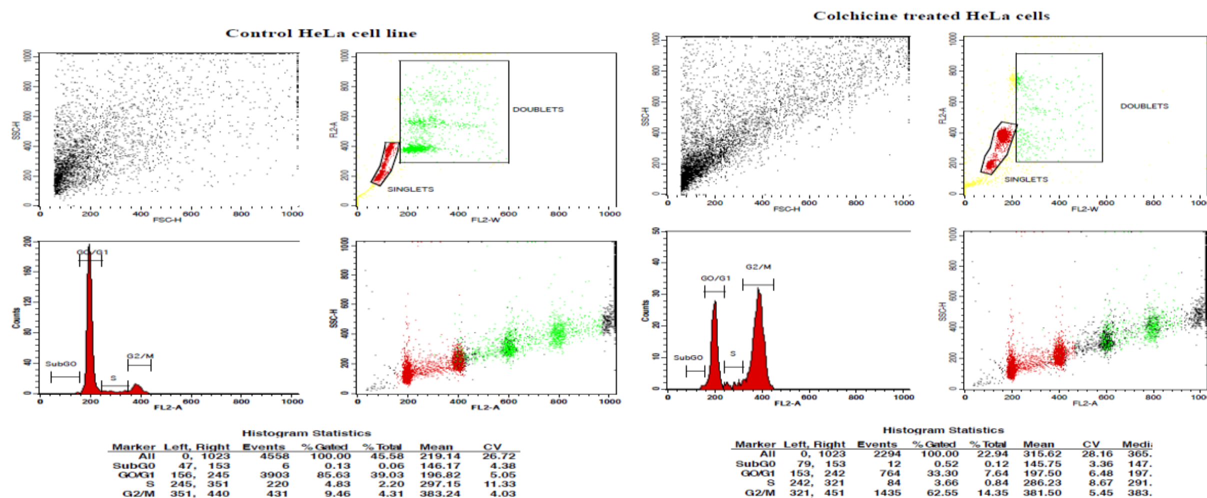


Figure:2. Clonogenic assay of 15h and 15b compounds bytreated compoundson Hela cells

Graph.3. Survival of colonies

The HeLa cells treated with 50 and 100 µM/ml of 15b compound for 24 h showed around 50% of inhibition at 50 µM/ml and around 20 % of inhibition at 100 µM/ml colonies forming ability. However the compound 15h has showed inhibition of 15% at 50 µM/ml and 100% inhibition at 100 µM/ml of colony forming capability. This result suggests that 15h has more significant colony forming inhibition abilities compare to 15b, which also at higher concentration showed inhibition. Overall, these results show that both 15h and 15b have showed inhibition of the growth of cervical cancer cells by preventing the formation of colony and there by inducing apoptosis.



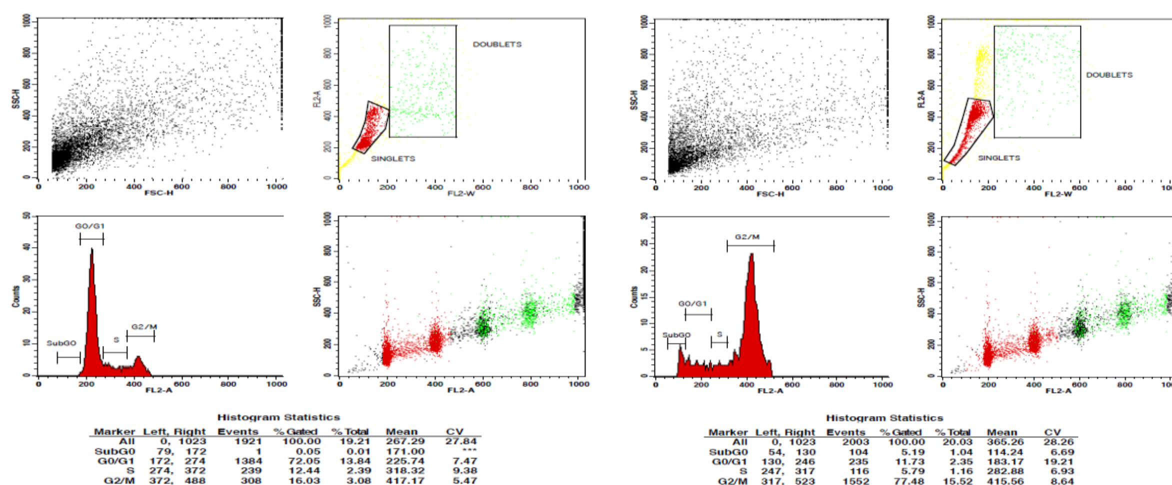
Histogram Statistics (Control)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	4558	100.00	45.58	219.14	26.72
SubG0	47, 153	6	0.13	0.06	146.17	4.38
GO/G1	156, 245	3903	85.63	39.03	196.82	5.05
S	245, 351	220	4.83	2.20	297.15	11.33
G2/M	351, 440	431	9.46	4.31	383.24	4.03

Histogram Statistics (Colchicine)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Medi:
All	0, 1023	2294	100.00	22.94	315.62	28.16	365.
SubG0	79, 153	12	0.52	0.12	145.75	3.36	147.
GO/G1	153, 242	764	33.30	7.64	197.50	6.48	197.
S	242, 321	84	3.66	0.84	286.23	8.67	291.
G2/M	321, 451	1435	62.55	14.35	381.50	5.45	383.

Graph: 4 and 5. Flow Cytometry plots of HeLa cells treated with 1 % DMSO as Control and 20 µM of Colchicine.



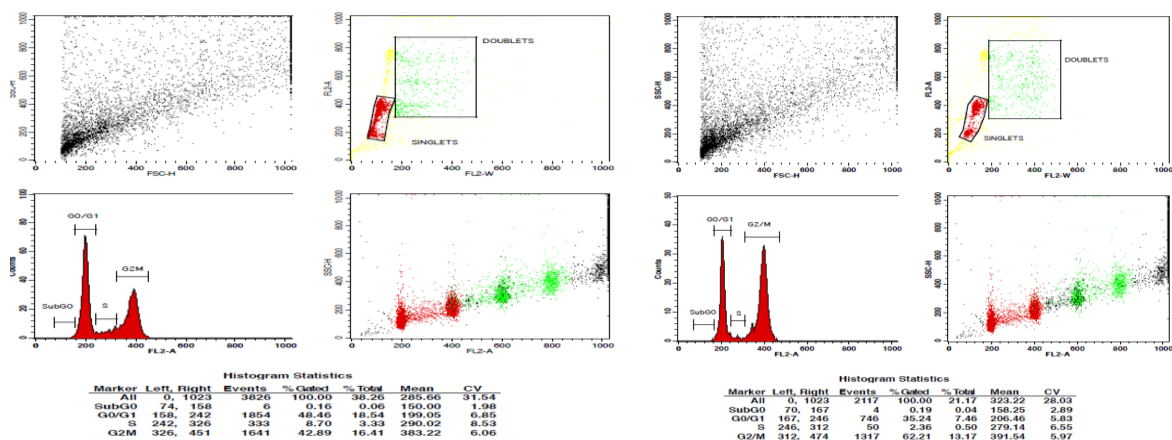
Histogram Statistics (50 µg/ml 15b)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	1921	100.00	19.21	267.29	27.84
SubG0	79, 172	1	0.05	0.01	171.00	***
G0/G1	172, 274	1384	72.05	13.84	225.74	7.47
S	274, 372	239	12.44	2.39	318.32	9.38
G2/M	372, 488	308	16.03	3.08	417.17	5.47

Histogram Statistics (100 µg/ml 15b)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	2003	100.00	20.03	365.26	28.26
SubG0	54, 130	104	5.19	1.04	114.24	6.69
G0/G1	130, 246	235	11.73	2.35	183.17	19.21
S	247, 317	116	5.79	1.16	282.88	6.93
G2/M	317, 523	1552	77.48	15.52	415.56	8.64

Graph: 6 and 7. Flow Cytometry plots of HeLa cells treated with 50 µg/ml of 15b compound and 100 µg/ml of 15b compound.



Histogram Statistics (50 µg/ml 15h)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	3826	100.00	38.26	285.66	31.54
SubG0	74, 158	6	0.16	0.06	150.00	1.98
G0/G1	158, 242	1854	48.46	18.54	199.05	6.85
S	242, 326	333	8.70	3.33	290.02	8.53
G2M	326, 451	1641	42.89	16.41	383.22	6.06

Histogram Statistics (100 µg/ml 15h)

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	2117	100.00	21.17	323.22	28.03
SubG0	70, 167	4	0.19	0.04	158.25	2.89
G0/G1	167, 246	746	35.24	7.46	206.46	5.83
S	246, 312	50	2.36	0.50	279.14	6.55
G2/M	312, 474	1317	62.21	13.17	391.54	5.97

Graph: 8 and 9. Flow Cytometry plots of HeLa cells treated with 50 µg/ml of 15h compound and 100 µg/ml of 15h compound

The effect of 15band 15h test extract on cell cycle in HeLa cells as analysed by flow cytometry are depicted in figures below, they are; Fig. 4. Control, Fig. 5. Colchicine 20µM treated, Fig. 6. 15b50 µg/ml treated, Fig. 7. 15b100 µg/ml treated, Fig. 8. 15h 50 µg/ml treated and Fig. 9. 15h 100 µg/ml treated compound. The 15b compound treatment at 50 and 100 µg/ml has arrested at 16.03 and 77.48 % in G2M phase of cell cycle, respectively, compare to untreated cells (9.46 %). Colchicine has showed arrest at 62.55 % of cell cycle arrest at G2M phase.

CONCLUSION

It is concluded that, the synthesised compound can be consider as the resource of potential anti-cancer activity and analysed the phase at which the cell gets arrested which leads to apoptosis, Also, the selected derivatives can be further exploited for the discovery of new anticancer agents. Nevertheless, the present findings may strengthen the process of standardization and confirmation of new compound drugs synthesised.

REFERENCES

1. http://en.wikipedia.org/wiki/Cancer Epidemiology.
2. WHO (February 2006). "Cancer". World Health Organization. http://www.who.int/mediacentre/factsheets/fs297/en/. Retrieved 2007-06-25
3. Reuter-American Cancer Society. Report sees 7.6 million global 2007 cancer deaths, 2007.
4. P. Anand, A.B. Kunnumakara, et al., Pharm.Res., 2008; 25: 2097–2116.
5. W. Kenneth, V. Bert; *The genetic basis of human cancer*, McGraw-Hill, Medical Pub. Division, 2005.
6. C. D. Lednicer, L. A. Mitscher; *Inorganic Chemistry of Drug Synthesis*, Wiley Interscience NewYork, 1997.
7. U. Ucucu, N. Gundogdu, I. Isikadog, IL. Pharmaco., 2001; 56: 285-290.
8. R. W. Al-Azzawi, A master thesis, University of Baghdad, 2007.
9. Y. Ozkay, I. Iskar, Z. Incesu, G. E. Akalin, Euro. J. Med. Chem., 2010, 1-9. Article in Press.
10. Anand sreenivasan, yogisha, Ramesh, 2014. *Synthesis and Evaluation for anticancer activity of imidazo [2, 1-d][1, 2, 3, 5] tetrazine-4(3H)-one, a new class of imidazotetrazinone heteroanalogues*.

11. Mehboob Hoque, Sandeep Dave, Pawan Gupta, Mohammed Saleemuddin. *Oleic Acid May Be the Key Contributor in the BAMLET Induced Erythrocyte Hemolysis and Tumoricidal Action*. Plosone., 2013; 8(9): e68390.
12. Braca A, Sortino C, Politi M. *Anti-oxidant activity of flavonoids from Licania licaniaeflora*. J Ethnopharmacol., 2002; 79: 379-381.
13. Bors W, Saran M, Elstner EF. Screening for plant antioxidants. In: Linskens HF, Jackson JF. editors. *Modern Methods of Plant Analysis Plant Toxin Analysis* New Series, Vol 13. Springer, Berlin; 1992: p. 277-295
14. Ramzi A Mothana, Ulrike Lindequist, Renate Gruenert and Patrick J Bednarski. "*Studies of the in vitro anticancer, antimicrobial and antioxidantpotentials of selected Yemeni medicinal plants from the island Soqotra*. BMC Complement Alt Med., 2009; 9: 7.