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Inhibition of HepG-2 Cells (Liver Cancer Cell Line) Viability by 3-Hydroxypyridine-2-Carboxaldehyde N(4)-Methylthiosemicarbazone

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ABSTRACT

Thiosemicarbazone have the antiviral, antibacterial, antifungal, and anticancer effects. 3-OH-Me-TSC inhibited the cell viability of HepG-2 cells by CV assay in a concentration dependent manner (control, 1µM, 3µM, 10µM, 30µM, and 100µM) with IC50 value of 9.587622µM. Further colony formation assay demonstrated that 3-OH-Me-TSC inhibits colony number and size of HepG-2. Wound healing assay exhibited that 3-OH-Me-TSC inhibit the migration of HepG-2 cells. DAPI staining showed that 3-OH-Me-TSC inhibited proliferation of HepG-2 cells in 30µM and 100µM concentrations respectively. 3-OH-Me-TSC inhibited VEGF, p38 alpha, C-JUN, BECN-1, ERK, NF-KB, in HepG-2 cells. We found that 3-OH-Me-TSC inhibit proliferation of HepG-2 cells by inhibiting MAPK signaling pathway, 3-OH-Me-TSC can be developed as future chemotherapeutic agent for treatment of hepatocellular carcinoma after the evaluation of this compounds in more cancer cells an *in vivo* model.

Keywords- Thiosemicarbazone, viability, HepG-2, antiviral, antibacterial, antifungal, and anticancer, wound healing assay, colony formation assay, CV assay, JC-1 staining, western blot, 3-Hydroxypyridine-2-Carboxaldehyde N(4)-Methylthiosemicarbazone.

I. INTRODUCTION TO THIOSEMICARBAZONE SYNTHETIC COMPOUNDS

Thiosemicarbazone are Schiff based ligands, which have earned a huge importance in last decade, as a potential drug candidate. One of the best characteristic of thiosemicarbazone is that it has a good tendency to bind with metals, which makes thiosemicarbazone effective for using it as antibacterial, antiviral, antioxidant, and as antiprotozoal agents.[5]

Thiosemicarbazone are synthesized by condensation reaction between primary amines and aldehydes or ketones. In the fifties the scientist has shown its effect on leprosy disease and in sixties their antiviral effect has been appeared. Recent researches have proved that different kind of thiosemicarbazone have different effects on different pathways of the different cells, such as triapine (3-aminopyridine-2-carboxaldehyde) have direct effect on inhibition of tumors as there are other kinds of thiosemicarbazone which bound with different kind of metal, which inhibits different pathways in the cell.[4,5]

The interaction of thiosemicarbazone with metal ions leads to structure in which the metal ion binds to hydrophilic part and the hydrophobic part remains free outside, this characteristic makes the entry of thiosemicarbazone easy inside the cell through cell membrane. The other characteristic of metals is that; most of the mammalian cell lines are sensitive to different metal ions, so nowadays these different kind of metals are used for inhibiting different pathways in the cell for therapeutic approaches against different disease.4 Recently scientists demonstrated that these derivatives of thiosemicarbazone have shown to be interesting and attractive for development of anti-cancer compounds because of their chemical reactivity.[4, 10]

II. MECHANISM OF ACTION OF THIOSEMICARBAZONE

Most of these anticancer compounds inhibit different pathways in different cell lines such as cell cycle arrest, ROS generation, TOPO isomerase II inhibition, and Ribonucleotide reductase inhibition that I am going to explain each pathway briefly.[4,5]

1. Topo isomerase II inhibition

Topo isomerase II is a nuclear enzyme in eukaryotic cells it has the activity of decatenation of DNA coils, which possess one helix from another.5 which passess one helix on another for prevention from supercoiling during DNA replication. TopoII is necessary for DNA synthesis and cellular divisions. In most of the cancerous cells there is increase in TopoII; hence it became an important target for cancer therapy researchers.

2. Ribonucleotide diphosphat reductase inhibition.

Ribonucleotide reductase is an iron dependent enzyme which promotes reduction of ribose to deeoxyribose through a free radical mechanism in most of

the cancerous cells there is also increase in number of this enzyme which have a strong correlation with tumor growth. Recently scientist have demonstrated that by targeting RR with different kind of thiosemicarbazone derivative such as 5-HP (5-Hydroxy-2-formyl pyridine).[4] The positive result in animal models have shown that by exposing RR to these compounds, the compound inhibits tyrosyl group of RR in which this reaction is reversible. Finally the inhibition of RR leads to blockage in S phase of cell cycle which finally leads to programed cell death (apoptosis).

3. ROS generation

The redox metal complexes ((cu-cl)2(p2)(clo4) and cucl2(dca)(clo4)) of thiosemicarbazone can act as ROS generators, as the level of ROS increases in cancerous cells so the cancerous cells up regulates the anti-oxidant proteins for balancing the level of ROS.In most of the researches it have been shown that cu based thiosemicarbazone compound do the oxidative cleavage of DNA in the presence of meracptopropionic acid as reducing agent by gel electrophoresis using supercoiled PUC18.5 Both complexes have been shown to produce single and double stranded DNA break which leads to ROS generation which causes apoptosis in the cell.

III. MATERIALS AND METHODS

DPBS, FBS, Trypsin, DMEM, were purchased from high media laboratories pvt. limited, DMSO, TEMED, Tween-20 were obtained from AMRESCO, antibiotics (10,000 units/ml penicillin and 10mg/ml streptomycin in 0.9% normal saline, purchased from HIMEDIA), Rabbit polyclonal antibody NFKB was purchased from Rockland antibodies, Rabbit polyclonal C-JUN was purchased from Santa Cruz biotechnology, Mouse monoclonal IgG1 Beta-actin, goat anti-rabbit IgG-HRP was purchased was purchased from santa cruz Biotechnology, Goat anti-mouse IgG were obtained from sigma life science,3-OH-Me-TSC were purchased from Tribhuvan University, central department of chemistry, HepG-2 cell lines were purchased from NCCS, Pune.

Cell culture condition

HepG-2 cells were cultured in T25 flask, T75 flask, 10cm petridish for routine subculture, at 37 ċ in 5% CO_2, 95% O_2 in commercially available DMEM (10,000 units/ml penicillin and 10mg/ml streptomycin in 0.9% normal saline, purchased from HIMEDIA), 10% standard Fetal Bovine serum, amino acids (L-Glutamine), glucose (4.5 grams of glucose per liter), Vitamins (folic acid, nicotinamide, riboflavin, B12), and salts (sodium bicarbonate and sodium pyruvate). 500 ml of DMEM complete media contains 50ml FBS and 100X (5ml) antibiotic. For all experiments cells were grown to 85% confluency and subjected to no more than 20 cell passages. All cells were detached by using Trypsin and seeded in 6 well plates for the extraction of whole cell proteins.

Cell culture

The cells were washed with 1X DPBS and, were detached by using trypsin followed by observing under the compound microscope to detect whether the cells are detached or not, then the cells were neutralized by adding DMEM and collected for centrifugation at (800-1000)rpm at 4Ċ. supernatant was discarded and cell suspensions was prepared by re suspending the cell's pellet in a fresh medium and re suspension was taken according to confluency for experiments (by cell counting) or culture. Then the cells were stored at 37Ċ, 95% O_2, 5% CO_2, in T25, T75, and 10cm dish. And then after every 24 hours the confluency of cells was observed by electron microscope. And then cells were used for experiments according to need.[27]

Crystal violet assay

5000 HepG2 cells were seeded to 96 well plates and stored at 37Ċ, 95% O_2, 5% CO_2, and 200 ul DMEM media/well and after 24 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours , media were discarded from the wells and the viable adherent cells were stained by adding 20µl/well 0.5% crystal violet dissolved in 50% methanol and rotated in shaker for 30 minutes at room temperature. And then the CV was discarded and wells were washed 3 times by adding 250 µl of mili-Q water, crystal violet was dissolved by adding 10ul/well 50% methanol and rotated in shaker for 20 minutes. Absorbance was taken at 562nm and cell viabilities were expressed as ratios versus untreated controls cells.

Cell cycle assay

250,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and after 24 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, and then we detached the cells by adding 250µl of trypsin and then we neutralized the wells by adding media 1:3 ratio, collected the cells in 6 different 15ml falcon tube, centrifuged at 900rpm, for 5 minutes at 4Ċ, after that we discarded the supernatant and washed the cells by adding 500ul of DPBS in each falcon tube and re-suspended the cells, after that we again centrifuged at 900rpm, for 5 minutes at 4Ċ, after that we discarded the supernatant, then the supernatant were discarded and the cells were fixed by adding 3ml ice cold 80% ethanol while vortexing the falcon tube and stored for 72 hours in -20C (we can store for as long that we want and then we can process it for staining).

PI staining

The fixed cells in falcon tube were centrifuged at 4000rpm for 5 minutes in 4Ċ, then supernatant were discarded and cells were resuspended by adding 500ul of

ice cold DPBS, again cells were centrifuged at 4000rpm for 5 minutes in 4Ċ, then supernatant were discarded and stained with propodium iodide (970µl DPBS, 10µl PI, 20µl RNASE A for two samples), and stored for 30 minutes at room temperature, and then the samples were further analyzed by FACS machine and results were taken.[28]

Wound healing assay

1000,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and 2ml DMEM media/well and after 24 hour, when the wells were completely full of cells we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with 1µM, 3µM, 10µM, 30µM, and 100µM, then wound were created in the middle of each well and imaging was done from wound area by fluorescence microscopy at 0 hour, 24 hour, and 72 hour for showing the migration of cells in every treated and control.

Colony formation assay

1000 HepG-2 cells were seeded in 6 well plate and were stored at 37Ċ, 95% O_2, 5% CO_2, and 2ml DMEM media/well and after 72 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, and after every 72 hours media were changed and treated again, and after 15 days we had the control well full of cells, then media was discarded and the cells were stained by adding 500µl of 5% crystal violet for 30 minutes and then imaging was done with GEL-DOC.

Dapi staining

250,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2 and 2ml DMEM media/well, and after 24 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with, 10µM, 30µM, and one without treatment (control), and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, then 0.5% of DAPI stain dissolved in 500µl of DPBS were added, and finally imaging were done by fluorescent microscopy for observing the cell proliferation in different treated concentration and control.

Jc-1 staining

200,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2 and 2ml DMEM media/well, and after 24 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with, 10µM, 30µM, 100µM and one without treatment (control), and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, then we added 0.5% of JC-1 stain dissolved in 500ul of DPBS,[29] And finally imaging was done by fluorescent microscopy for observing the apoptosis in different treated concentration and control.

Western blots

300,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and after 24 hour we had treated them with thiosemicarbazone-3-OH-Me synthetic compound with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, then the cells lysate were taken by adding 60-80 µl of SDS (lysate buffer) and were scraped with scrapper, then lysate were collected and transferred to micro centrifuge tube, then sonication were done three times 10 seconds gap between each time at 30% amplitude, then the MCT tubes were centrifuged at 16000 rpm for 20 minutes at 4Ċ, then we collected the supernatant which contained the proteins of the cells and estimation of proteins in each treated and untreated tubes by BCA assay.

BCA assay

1ul of protein sample were loaded in duplicate for each treatment concentration in 96 well plate and then we added 200ul/ well of BCA reagents (196ul reagent A and 4ul of reagent B), and rotated in shaker for 30 mints at 37Ċ, then OD were taken at 572 by spectrophotometry machine and then we calculated the concentration of protein for 30ug/µl and we mixed them with loading dye, and loaded to 8% 10%, 12%, 15% SDS gel for detecting different proteins having different molecular weight.

SDS-PAGE

RESOLVING GEL				
Acrylamide percentage	8%	10%	12%	15%
Distilled water	5.2ml	4.6ml	3.8ml	3.2ml
Acrylamide/Bis Acrylamide (30%/0.8%)w/v	2ml	2.6ml	3.4ml	4ml
Tris PH=8.8	2.6ml	2.6ml	2.6ml	2.6ml
10% w/v SDS	0.1ml	0.1ml	0.1ml	0.1ml
10%w/v ammonium per sulfate (APS)	0.1ml	0.1ml	0.1ml	0.1ml
TEMED	0.01ml	0.01ml	0.01ml	0.01 ml

All the reagents were mixed and then loaded in to the SDS-glasses, after solidification stacking gel were added.

STACKING GEL	
H_2O	4ml
Acrylamide/Bis Acrylamide(30%/0.8%)w/v	630ml
Tris PH=6.8	830ml
10% w/v SDS	50µl
10%w/v ammonium per sulfate (APS)	50µl
TEMED	6µl

REAGENTS AND BUFFER PREPARATION	
WEIGHT	MATERIALS
30.3G	TRIS BASE
144G	GLYCINE
10G	SDS

SDS-PAGE 2X SAMPLE PREPARATION

VOLUME	MATERIALS
3.55ml	MQ water
1.25ml	0.5 TRIS HCL, PH=6.8
2ml	10% w/v SDS
0.2ml	0.5% Bromophenol blue

50ul of B-mercaptoethanol was added to 950ul of sample buffer before use.

1.5M TRIS, PH-8.8 (stock buffer for separating gels) for 500ml

Dissolve 90.825g Tris base in around 400ml of MQ water. Adjust the PH to 8.8 with concentrated HCL. Bring up the volume to 500ml with MQ water.

0.5M TRIS, PH-6.8 (stock buffer for stacking gels) for 500ml

Dissolve 60.67g Tris base in around 400ml of MQ water. Adjust the PH to 6.8 with concentrated HCL. Bring up the volume to 500ul with MQ water.

10% APS (ammonium per sulfate)

10% APS prepared fresh for each run (i.e. 0.05%g APS+0.45ml MQ water).

10% SDS (sodium dodecyl sulfate)

50g of SDS was taken, and then volume was make up by 500ml of MQ water. 32

SDS PAGE Transfer Buffer

WEIGHT/VOLUME	MATERIALS
3.03g	TRIS BASE
1404g	GLYCINE
200ml	METHANOL

Gel making

First we prepared the gel caster and all equipment's were washed gently using water. Again all equipment was cleaned with 70% ethanol and deionized water and wiped with the help of blotting paper. Plates were fit in gel caster with tight knots and checked the leakage used by 70% ethanol than decant the alcohol and dry paper. Then we started preparing the resolving gel and added the TEMED when everything were ready, and then we loaded the resolving gel and kept empty space for stacking gel and filled that space with 70% ethanol, when the resolving gel have become solidified, then we discarded the ethanol and loaded the stacking gel, immediately the comb were inserted in both the plates and it was left for 30 minutes for solidification.

Preparation of sample for loading

According to absorbance by mass spectrometry we calculated the loading of sample for 30ug/µl as following:

Absorbance at 562			
CONCENTRATION	SAMPLE 1	SAMPLE2	AVERAGE
CONTROL	0.411	0.454	0.459
1µM	0.349	0.34	0.3445
3 µM	0.393	0.383	0.388
10 µM	0.353	0.351	0.353
30 µM	0.319	0.342	0.3305
100 µM	0.336	0.319	0.3275

CONCENTRATION	30µg/µl	LOADING DYE	LOAD
5	6	2 µl	8 µl
3.1375	9.561752988	3.187251 µl	12.749µl
4.225	7.100591716	2.366864 µl	9.467456 µl
3.35	8.955223881	2.985075 µl	11.9403 µl
2.7875	10.76233184	3.587444 µl	14.34978 µl
2.7125	11.05990783	3.686636 µl	14.74654 µl

IV. RUNNING OF SAMPLES

Samples were taken from -80Ċ and loaded according to the table above, and after mixing with loading dye we incubated the samples at 96Ċ, electrophoresis vessels was prepared to run the protein samples over it and get containing plates was fixed in both sides of the vessel. and then we load the samples in to the gel followed by protein ladder, and 1X running buffer were filled till the mark, and we run the gel at 90V for one hour and then 120 volt for 1.5-2 hours.

When the samples reached to bottom of the gel then we stopped running the samples, protein of different molecular weight run in to different destination as low molecular weight protein transfers faster and high molecular weight slower, as proteins are the polar molecules moves from negative pole to the positive pole of the electric field.

The PVDF membrane were cut in the appropriate size and dipped 5 minutes in to the methanol for saturation. Then protein band transfer was done to PVDF from the SDS-GEL instead of nitrocellulose because, the former was highly hydrophobic, and so it can easily bind the protein. And in the meantime blotting pad were soaked in the transfer buffer (1X). To transfer

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the gel sandwich, frame was made by putting sponge, the blotting pad, and then the gel. The PVDF membrane, again blotting pad, then the sponge was put in the order and fit (sandwich) in the cassette and placed in to the tank. Ice pack was kept inside the transfer tank, and also some ice 34 around the tank which makes the tank to remain cold, otherwise current will increase due to evaporation of ethanol from transfer buffer as a result ban will not come properly. Transfer was started after covering the lid of transfer tank; current was set for 1-1.5 hour at constant 100 voltages.

PRIMARY ANTIBODY	MOLECULAR WEIGHT	SUPPLIED BY
ERK	42 KDA	SANTA CRUIZ
Becn-1	52 KDA	CLOUD CLONE
VEGF	42 KDA	ABCAM
B-CATENINE	95 KDA	SANTA CRUIZ
B-ACTIN	43 KDA	SIGMA LIFE SCIENCE
C-JUN	42 KDA	CLOUD CLONE
BAX	20 KDA	SANTA CRUIZ
BCL-XL	22 KDA	SANTA CRUIZ
P-ERK	45-46 KDA	SANTA CRUIZ
P38 alphha	41 KDA	SANTA CRUIZ
NFKB	65 KDA	CELL SIGNALING
C-MYC	55 KDA	SANTA CRUIZ
PROCASPASE9	44-46 KDA	CLOUD CLONE

Secondary antibody	Supplied by
HRP CONJUGATED ANTI-RABBIT IgG	SIGMA LIFE SCIENCE
HRP CONJUGATED ANTI-MOUSE IgG	SIGMA LIFE SCIENCE
HRP CONJUGATED ANTI-GOAT IgG	SIGMA LIFE SCIENCE

Developing blots by ECL

After transfer, the PVDF membrane was taken out using forceps and kept in to 5% skimmed milk in 1X TBST blocking reagent for one and half hour on rocker. Blocking was done to prevent from nonspecific bindings. After blocking the blot kept in primary antibody overnight and then the blot was washed three times with 1X TBST, and then secondary antibody (anti-mouse IgG, anti-Goat IgG, anti-rabbit IgG) was added and kept on rocker for 1-2 hours at room temperature, then again the blot were washed 3 times with 1X TBST, and then developing were done in dark room, by adding 1:1 of ECL kit (purchased from BIO-RAD) reagent A and reagent B, added on the PVDF paper on the area of band that we expect that the protein is present according to molecular weight, and then by binding of ECL to secondary antibody the bands give fluorescence, then we keep the PVDF on cassette and keep the X-RAY film on the PVDF paper and close the cassette for the expected time that each proteins band comes, then the X-RAY films were removed after a certain of time and then dipped in to the developer-water-fixer respectively, and finally we were detecting the bands in the X-RAY film, all these steps were done in dark.

V. RESULTS & DISCUSSIONS

1. 3-OH-Me-TSC inhibits cell viability of HepG-2 cell lines by CV assay.

5000 HepG2 cells were seeded to 96 well plates and stored at 37C, 95% O_2, 5% CO_2, and 200ul DMEM media/well and after 24 hours cells were treated with 3-OH-Me-TSC with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, from the absorbance by mass-spectrometry it was found that as much the drug concentration increases the viability of HepG-2 cells decreases, and IC50 value was calculated the results showed that in a **9.587622µM** concentration there is 50% of the cells which become none viable. P-Value=6.99013E-08 was also calculated and showed that 10µM, 30µM, 100µM concentrations are more significant in decreasing the viability of HepG-2 cells as shown in Figure 1.

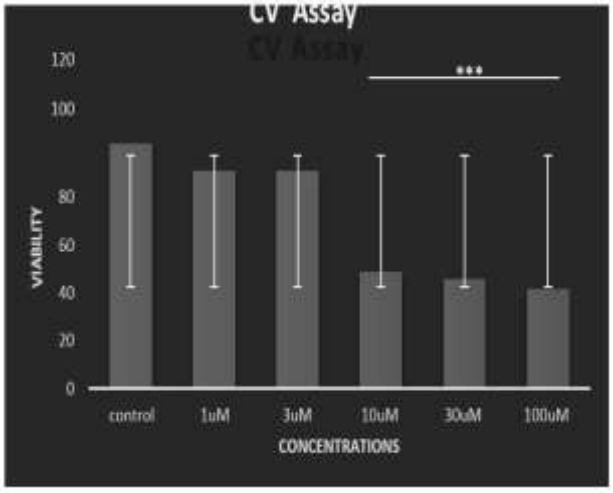

Figure 1: Cell viability assay performed to determine and screen the effect of 3-OH-Me-TSC on HepG-2 cells. 6000 cell were seeded in 96 well plates and then treated with 3-OH-Me-TSC for 72 hour. Viability of HepG-2 cells is declined with increase in concentration of 3-OH-Me-TSC; IC50=9.587622µM.

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2. Inhibition of HepG-2 cell migration by 3-OH-Me-TSC

1000,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and 2ml DMEM media/well and after 24 hour, when the wells were completely full of cells they were treated with 3-OH-Me-TSC and migration of cells were observed at control, 30µM, and 100µM, then wound were created in middle of each well, after 48 hours. Imaging was done from wound area by fluorescence microscopy at 0 hour, 24 hour, 48 hour, and 72 hour for showing the migration of cells in every treated and control, the image demonstrates that there is a complete inhibition of migration in 100µM concentration and partial inhibition in 30µM concentration and in control there is migration of cells in wound area, so finally we can say that 3-OH-Me-TSC inhibits migration of HepG-2 cells as shown In Figure 2.

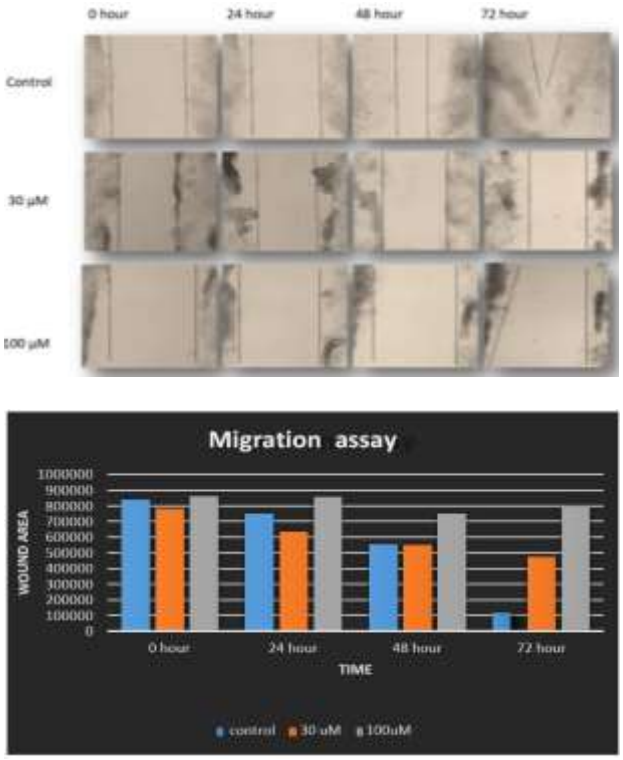

Figure 2: Shows the inhibition of migration of HepG-2 by 3-OH-Me-TSC synthetic compound by wound healing assay, as per images we can see that there is complete inhibition of migration in treated wells and in control we can see the migration of cells, so it demonstrates that the compound have effect on inhibition of HepG-2 cells migration.

3. Inhibition of HepG-2 cell proliferation by 3-OH-Me-TSC by Dapi staining

250,000 cells/well were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2 and 2ml DMEM media/well, and after 24 hour we had treated them with 3-OH-Me-TSC compound with, 10µM, 30µM, and one without treatment (control), and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, then we added 0.5% of DAPI stain dissolved in 500µl of DPBS, finally imaging were done by fluorescent-microscopy for observing the HepG-2 cells proliferation in different treated concentration and control, the images in here demonstrates that 3-OH-Me-TSC inhibits HepG-2 cells proliferation in a concentration dependent manner as shown in Figure 3.

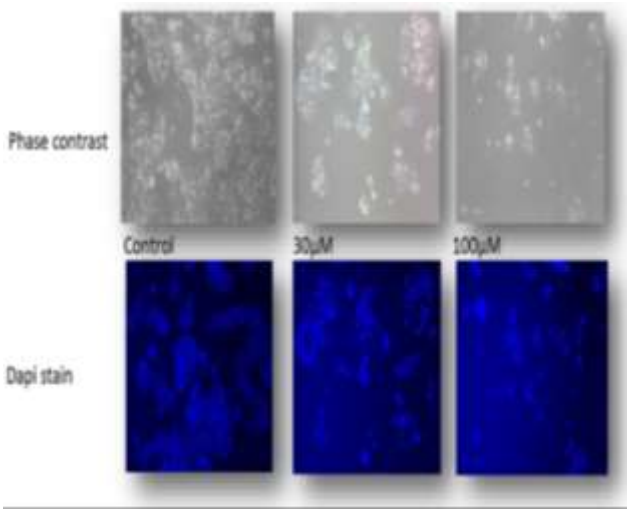

Figure 3: Shows the effect of 3-OH-MeTSC on inhibition of HepG-2 cell proliferation by phase contrast and fluorescence imaging stained with DAPI stain. As from the figure we can see that 3-OH-MeTSC inhibits HepG-2 cell proliferation at 10µM, and 30µM concentrations.

4. Cell cycle assay

250,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and after 24 hour HepG-2 cells were treated with 3-OH-Me-TSC with 1µM, 3µM, 10µM, 30µM, and 100µM, concentrations and were again incubated in same condition for 72 hours, then after that the cells were fixed with 80% ice-cold ethanol, and stored for 72 hours in -20Ċ, then it was stained with PI stain and further analyzed in flow cytometer, the result showed that all the cells are damaged even in control and treated, but it also showed that in treated the cells are more damaged than control, it might be because HepG-2 colonies were not separated and fixed in colonies, and stained in colonies with PI, and when the colony of cells were brought to detector the detector of flow cytometer were not able to identify the condition of whole colony, so it was showing that the cells are damaged, and no proper peak was coming for G1M and S phase in all the treated and control wells, this experiment were repeated 4 times, in all of them it was showing that all the cells are Damaged as shown in Figure 4.

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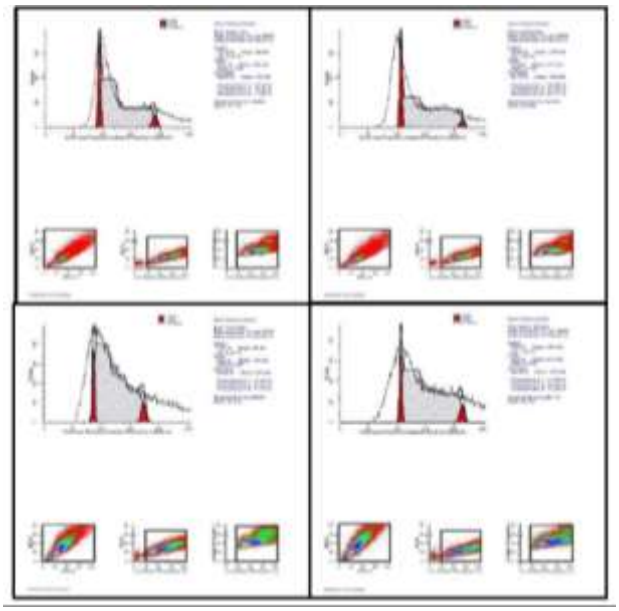

Figure 4: Demonstrates results of cell cycle assay for analysing the effect of 3-OH-Me-TSC on cell cycle of HepG-2 cells at control, 10µM, 30µM, and 100µM concentrations respectively.

5. Inhibition of colony formation in HepG-2 cells by 3-OH-Me-TSC

1000 HepG-2 cells were seeded in 6 well plate and were stored at 37Ċ, 95% O_2, 5% CO_2, and 2ml DMEM media/well and after 72 hour we had treated them with 3-OH-Me-TSC with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, and after every 72 the media was changed and cells were treated again, after 15 days we had the control well full of cells, then media was discarded and the cells were stained by adding 500µl/well of 5% crystal violet and incubated for 30 minutes at room temperature and then imaging was done with GEL-DOC. Finally from the image there is partial inhibition of colony formation in 10µM treated well, and complete inhibition in 30µM and 100µM treated wells. So it shows that 3-OH-Me-TSC inhibits colony formation in a concentration dependent manner.

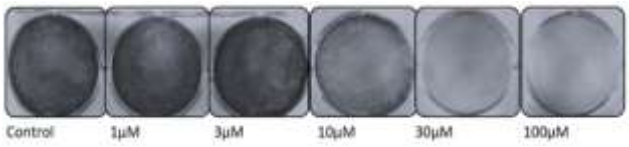

Figure 5: Demonstrates the complete inhibition of HepG-2 colony formation (1000cells/well for 15 days and the media were changed after every 72 hours) by 3-OH-Me-TSC at 30µM, 100µM, and partial inhibition at 10µM concentrations. So finally the results shows the inhibition of HepG-2 colony formation by 3-OH-Me- TSC in a concentration dependent manner.

6. Detection of HepG-2 cell apoptosis by 3-OH-Me-TSC, by JC-1 staining

200,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2 and 2ml DMEM media/well, and after 24 hour HepG-2 cells were treated with 3-OH-Me-TSC with 10µM, 30µM, 100µM and one without treatment (control), and were again incubated in same condition for 72 hours, after that we discarded the media, washed the wells by adding 500ul of DPBS, then 0.5% of JC-1 stain dissolved in 500ul of DPBS, was added to each wells, and finally imaging were done by fluorescent microscopy for observing the apoptosis by staining the mitochondrial membrane potential by JC-1 in different treated concentrations and control. The result showed that in control there were increase in proliferation of cells and made huge colonies while in treated there were inhibition of colony formation and proliferation so in treated there were small colonies that's why the control were not stained properly because of the huge colonies and did not showed the proper result that we have to compare with treated one, while in treated there were small Colonies and stained properly and showed the apoptosis as shown in Figure 6.

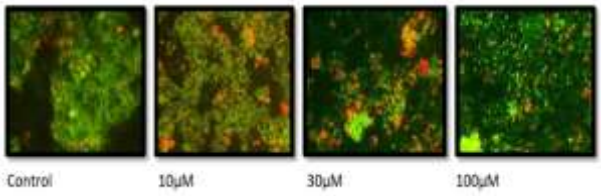

Figure 6: shows HepG-2 cells stained with JC-1 stain for the purpose of observing the effect of 3-OH-Me-TSC on apoptosis by detecting the mitochondrial membrane potential by JC-1 in HepG-2 cells, unfortunately because of the huge colonies in control we were not able to stain the cells properly, and get the proper result for it.

7. Inhibition of signaling pathways in HepG-2 cells by 3-OH-Me-TSC detected by western Blots

300,000 cells were seeded in 6 well plates and were stored at 37Ċ, 95% O_2, 5% CO_2, and after 24 hour we had treated them with 3-OH-Me-TSC with 1µM, 3µM, 10µM, 30µM, and 100µM, and were again incubated in same condition for 72 hours, after media were discarded, washed the wells by adding 500µl of DPBS, then the cells lysate were taken by adding 60-80µl of SDS (lysate buffer) and were scraped with scrapper, then lysate were collected and transferred to micro centrifuge tube, then sonication were done three times, 10 seconds gap between each time at 30% amplitude, then the MCT tubes were centrifuged at 16000 rpm for 20 minutes at 4Ċ, then we collected the supernatant which contained the proteins of the cells and estimation of proteins were done in each treated and untreated tubes by BCA assay. Further BCA assay were performed and from immuno-blotting there have shown inhibition of signaling

pathways in a concentration dependent manner, and finally as the results demonstrates there is inhibition in proliferation, growth, inflammation, angiogenesis, autophagy, and etc. all of these pathways are important for a cancerous cell to survive and migrate. From the result we can say that 3-OH-Me-TSC is an Inhibitor of oncogenic signaling in HepG-2 cells as shown in Figure 10.

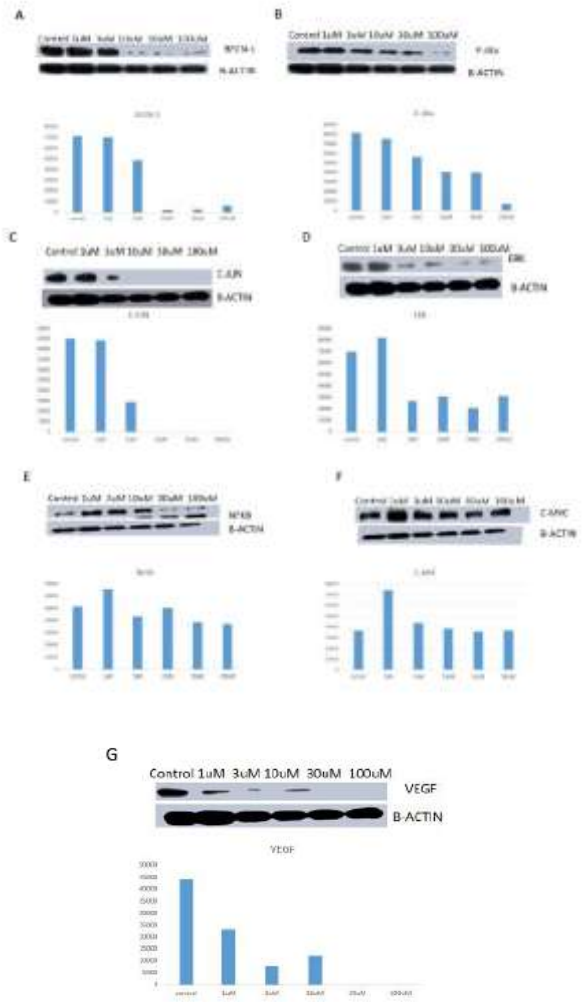

Figure 7: Demonstrates western blots of oncogenic signals in treated and control concentrations. A, shows the inhibition BECN1 protein by 3-OH-Me-TSC in 10µM, 30µM, and 100µM concentrations which is responsible for autophagy process in cells. B, shows the inhibition of P38 alpha protein at 100µM concentration, which is responsible for growth and differentiation of the cell. C, shows the inhibition of C-JUN at 10µM, 30µM, 100µM, and partial decrease at 3µM, which is responsible for proliferation of cell. D, shows the down regulation of ERK protein at 3µM, 10µM, 30µM, and 100µM concentrations which are responsible for survival of the cells. E, shows down regulation of NFKB at 30, and 100µM concentrations which is responsible for activation of inflammation which disables immune cells from attacking. F, shows the C-MYC protein blots in which I didn't found any specific effect by drug. G, shows down regulation of VEGF protein at 3, 10, 30, 100µM concentrations and partial inhibition in 1µM concentration, which is responsible for angiogenesis in the cell.

VI. DISCUSSION

The study clearly demonstrates that different concentration of 3, hydroxy-methyl thiosemicarbazone suppressed the proliferation of HepG-2 cell lines, as per CV assay we found that 3-OH-Me-TSC inhibits HepG-2 cell viability in a concentration dependent manner, with IC50 value of **9.587622 µM,** further wound healing assay showed that 3-OH-Me-TSC inhibits migration of HepG-2 cell lines so it shows that the drug is more effective as today the main problem with cancer is its migration as recent research have proved that after chemotherapy there is excess release of oncogenic signals by exosomes from the cancerous cells which remain alive after chemotherapy26 it proves that the main problem with cancer is its migration even after chemotherapy they increase in migration. Further colony formation assay proved that 3-OH-Me- TSC inhibits colony formation of HepG-2 cell lines, as the doubling time for HepG-2 cells are 48 hours and they grow in colonies and whenever the cells were seeded in low amount the cells were coming in stress so for that the cells were seeded 1000cells/well in six well plate. Further the dapi staining were done for observing the effect of 3-OH-Me-TSC on proliferation of HepG-2 cells, the dapi images and phase contrast by fluorescent microscopy shows that there is an excess decrease In cell proliferation due to increase in 3-OH-Me-TSC concentration. Further Jc-1 stain were performed for detecting the potential of mitochondrial membrane channels and its relation with apoptosis as JC-1 stain did not showed proper result or any proper effect in apoptosis, the problem might be due to colonies, the stain didn't entered in to the cell and mitochondrial membrane was not stained. Finally western blots were performed for detecting the amount of oncogenic signals reduced by 3-OH-Me-TSC in HepG-2 cells at different concentrations, and it showed that there is inhibition of angiogenesis, autophagy, growth, survival, proliferation, inflammation, and etc. signals as per my idea the best mechanism to inhibit cancerous cells viability by drug is to inhibits its colony formation and autophagy signaling pathway, as the autophagy signaling pathways made the treatment of HCC difficult and these two characteristics were inhibited by 3-OH-Me-TSC in HepG-2 cells.

VII. CONCLUSION

Finally from the study we found that 3-OH-Me-TSC inhibits HepG-2 cells viability, proliferation, growth, survival, migration, autophagy, angiogenesis, colony formation, as per studies the best effect which is done by 3-OH-Me-TSC is that it inhibits autophagy

pathway in liver cancer cell lines which helps in inhibition of HepG-2 cell viability, as most of the drugs are troughed out by the process of autophagy in liver cells and makes treatment of liver disease harder. So finally we can say that 3-OH-Me-TSC is a multitask drug for treatment of liver cancer.

FUTUR PERSPECTIVES OF THIOSEMICARBAZONE

Thiosemicarbazone is an effective drug in treatment of cancer, as we have explained earlier that it has good tendency to bind with metals these metals have effect on specific pathways in the cell. But we have to also should keep in mind that there are specific derivative for inhibition of specific pathway for specific cancer type. For instance the treatment of liver cancer is effective if we use the kind of thiosemicarbazone which generates ROS for destruction of cancerous cells, as HepG2 cell line is growing in colonies and it is difficult to detect the cell cycle pathway by FACS or maybe it is difficult to cure the liver cancer by inhibiting other pathways such as Ribonucleotide reductase inhibition or TOPOII. In recent years most of the researchers are working on its side effect and working on its specificity to target only the cancer cells in the body.

In future it can be a good drug for targeting different kind of cancer and diseases but we should work on its side effects and specificity to cancer cells, to be more effective for the survival of the Patients maybe one of the best way for specificity of thiosemicarbazone to target only cancerous cells is to bind them with Nano particles and target only the cancerous cells.

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ABBREVIATIONS

Hepatocellular Carcinoma (HCC)
3-Hydroxypyridine-2-Carboxaldehyde N(4)-Methylthiosemicarbazone (3-OH-me-TSC)
AFB1 (Aflatoxin B1)
Diabetes Mellitus (DM)
None Alcoholic Fatty Liver Disease (NAFLD)
Growth factor (GF)
Reactive Oxygen Species (ROS)
Topo Isomerase 2 (TOPOII)
Ribonucleotide Reductase (RR)
Dellbeco's phosphate buffer saline (DPBS)
Fetal Bovine Serum (FBS)
Dellbeco's Modified Eagle Media (DMEM)
Mili-Q water (MQ)
Propidium Iodide (PI)
Micro centrifuge Tube (MCT)
Sodium Dodecyl sulfate-polyacrylamide Gel (SDS-PAGE)
Vascular Epithelial Growth Factor (VEGF)
Nuclear Factor Kappa B (NFKB)
Micro Molar (μM)
Micro Liter (μl) 7
Horseradish Peroxidase (HRP)
Enhanced Chemiluminescence (ECL)
Poly Vinylidene di fluoride (PVDF)
Ammonium per Sulfate (APS)
Di methyl sulfoxide

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