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Supplementary Article

SYNTHESIS, CHARACTERIZATION, ANTI-MICROBIAL, ANTI-CANCER, AND ANTI-OXIDANT ACTIVITY OF NOVEL 1-(NAPHTHALEIN 2-YL OXY)(PHENYL)(METHYL) THIOUREA MANNICH BASE AND ITS METAL COMPLEXES

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Cell line and culture

A459cell lines was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), andstreptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Reagents

MEM was purchased from Hi Media Laboratories Fetal bovine serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

In vitro assay for Cytotoxicity activity (MTT assay)

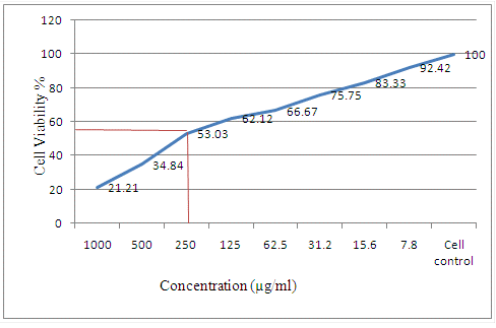
The Cytotoxicity of samples on A549 was determined by the MTT assay(*Mosmann et al*.,1983). Cells (1 × 10^5/well) were plated in 1ml of medium/well in 24-well plates (Costar Corning, Rochester,NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide cells(MTT) phosphate- buffered saline solution was added. After4h incubation, 0.04M HCl/ isopropanol was added. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability(IC50) was determined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of A549 was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%.

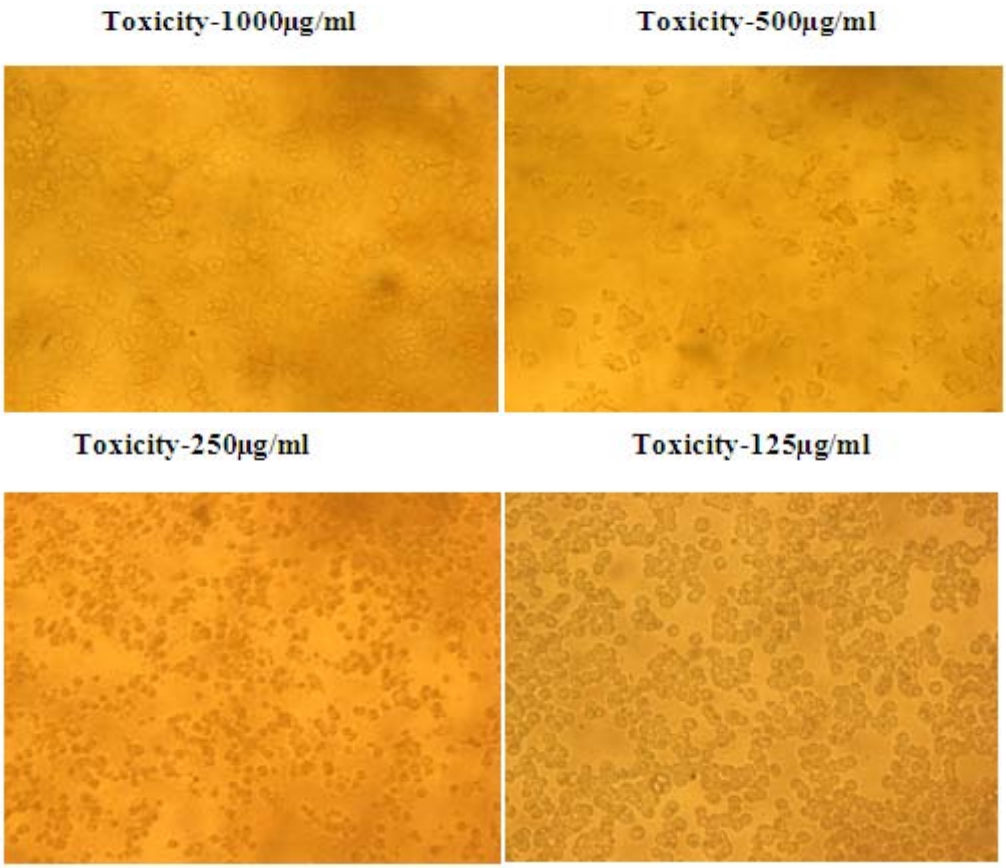
Table: anti Cancer effect of sample on A549 cell line

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.14	21.21
2	500	1:1	0.23	34.84
3	250	1:2	0.35	53.03
4	125	1:4	0.41	62.12
5	62.5	1:8	0.44	66.67
6	31.2	1:16	0.50	75.75
7	15.6	1:32	0.55	83.33
8	7.8	1:64	0.61	92.42
9	Cell control	-	0.66	100

MTT ASSAY



Normal A549 cancer cell line



PROCEDURE

Materials required in mem:

- Monolayer culture bottle of vero cell line
- 5ml,10ml serological pipette
- Minimal essential media (MEM) with 10%,2% foetal calf serum
- TPVG (Trypsin, PBS, versene, glucose)
- Discarding jar, inverted microscope, dessicator
- Gloves, spirit, cotton, label pad, marker pen

Materials required in cytotoxicity assay:

- Monolayer culture in log phase
- Drug extract (different concentrations)
- MEM without FCS
- 0.4µ filter
- 5ml sterile storage vial
- Tissue paper, spirit, cotton, marker pen and gloves
- Micropipette and tips
- Discarding jar

Minimal Essential Media Preparation

Media is defined as a complex source of nutritional supplementation vital for the growth proliferation and maintenance of cells in vitro

The MEM vial is dissolved in the pre sterilized Millipore distilled water and mixed well, closed and sterilized at 15lbs 121ºc for 15mins. Allow ingredients in the quantity, depending on the concentration of foetal calf serum (2% or 10%) mix well by shaking. Take care avoid spills pass CO_2 using sterile pipette, Shake the bottle, check pH and adjust to 7.2 to 7.4. The MEM bottles are kept for 2 days at 37ºc and checked for sterility, pH drop and floating particles they are then transferred to the refrigerator.

Ingredients	10% Growth media	2%Growth media	Maintance media without Fcs
MEM	857 ml	937 ml	957 ml
Penicillin and streptomycin	1ml	1ml	1ml
Phenol red	1ml	1ml	1ml
Amphotericin B	1ml	1ml	1ml
3% L-glutamine	10ml	10ml	10ml
Foetal calf serum	100ml	20ml	nil
7.5%NaHCo3	30ml	30ml	30ml
Total volume	1000ml	1000ml	1000ml

Preparation of ingredient

Penicillin and streptomycin: (concentration 100IU of penicillin and 100 µg 0f streptomycin**)**

Dissolve both antibiotics in sterile Millipore distilled water, so as to give a final concentration 100 IU of penicillin and 100µg of streptomycin/ml. Mix well and distribute in 1ml aliquots. Store at -20ºC check sterility.

Fungizone (amphotericin B)(20µg/ml):

Dissolve in sterile Millipore distilled water so as to give a final concentration of 20µg/ml and distribute in 1ml aliquots in vials. Store at -20ºc. Check sterility before using.

L-glutamine (3%)

Weigh 3g of l-glutamine accurately and dissolve in 100ml sterile Millipore distilled water and mix well. Filter through Millipore membrane filter 0.22µ and distribute in 5ml aliquots in vials. Store at -20ºc. Check sterility.

7.5% sodium-bi-carbonate

Weigh requisite quantity of sodium-bi-carbonate (to give 7.5% solution) accurately and dissolve in 100ml of sterile Millipore distilled water. Filter through what man filter paper No.4, distribute into bottles and at 121ºc, 15lbs, 15mins. Cool and store at +4ºc.

Foetal calf serum

Bring FCS at room temperature. Inactivated at 56ºc in water bath for½ hour and cool at room temperature. If floating particles are seen filter through Seitz filter. Distribute in 100ml, 50ml, and 20ml quantities in sterile bottles. Store at -20ºc.

Trypsin, PBS, versene, glucose solution: (TPVG)

2% trypsin: 100ml

Weigh 2g of trypsin accurately; dissolve in 100 ml sterile Millipore distilled water with magnetic stirrer for ½ hour. Filter through membrane filter. Store at -20ºc

0.2%EDTA (versene)

Weigh 200mg of EDTA accurately. Dissolve in 100 ml of sterile Millipore distilled water. Autoclave at 15lbs/15mins.

10%glucose -100ml

Weigh 1g of glucose accurately. Dissolve in 100 ml of sterile Millipore distilled water and filter through whatmann filter paper and autoclave at 15lbs/15mins.

TPVG-100ml

PBS - 840ml

2%trypsin -50ml

0.2%EDTA -100ml

10%glucose -5ml

Penicillin & streptomycin -5ml

Mix all ingredients and adjust the pH to 7.4 with 0.1 N HCl or 0.1 N NaOH. Distribute in 100 ml aliquots. Store at -20ºc.

SUBCULTURING AND MAINTENANCE OF CELL LINE:

- Bring the medium and TPVG to room temperature.
- Observe the tissue culture bottles for growth, cell degeneration, pH and turbidity.
- Select the bottles for splitting.
- The following procedure is followed in sequence.
- Wipe the mouth of the bottle with cotton soaked in spirit.
- Remove the growth medium using 10ml pipette.
- Then the cells in the bottle where gently rinsed with MEM without FCS. The dead cells and FCS are washed out and then discard the medium.
- 4-5ml of TPVG was added over the cells. Allow TPVG to act for 3-5 minutes and it was pipette out.
- And incubate at 37ºc for 3-5 minutes. The cells becomes individual and its present as suspension.
- Add 5ml of 10% MEM with FCS by using serological pipette.
- Gently give passaging by using serological pipette and add 20ml of MEM and homogenize.

"Seeding of cells"

After homogenize take 4-5ml pour in to 24 well plates. In each well add 1ml of the suspension to the 24 well plates and kept in a dessicator in 5% CO_2 atmosphere. After 2 days incubation observe the cells in inverted microscope. If the cells became 80% confluent. Then it is used for the Cytotoxicity studies.

Cytotoxicity assay

In order to study the antitumor activity of a drug, it is important to determine the cytotoxic concentration of the drug. Cytotoxicity tests define the upper limit of the extract concentration, which is non-toxic to the cell line. The concentration nontoxic to the cells is chosen for antitumor assay.

Drug dilution

Stock drug concentration

1. 5 ml of extract was prepared giving a concentration of 10mg/ml.

2. 500µl of MEM without FCS was taken in about 9 eppendroff tubes.

3. Then 500µl of the working concentration was added to the first eppendroff tube then 500µl of the volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the drug.

Sampling

- 48hr monolayer culture of Vero cell line at a concentration of one lakh /ml /well (10 cells / ml / well) was seeded in 24 well titer plate.
- The plates were microscopically examined for confluent monolayer, turbidity and toxicity.
- The growth medium (MEM) was removed using pipette. Care was taken so that the tip of the pipette did not touch the cell sheet.
- The cell monolayer was washed twice with MEM without FCS.
- To the washed cell sheet, 1ml of the medium (without FCS) containing defined concentration of the drug was added..
- Then each dilution of the drug ranges from 1:1 to 1:256 and they were added to the respective wells of the 24 well titer plates.
- To the cell control wells add 1ml MEM (w/o) FCS control.

The plates were incubated at 37ºc in 5% CO_2 environment and observed for Cytotoxicity using inverted microscope

MTT ASSAY

MTT assay is a calorimetric assay for measuring cell viability, for cellular proliferation and activation. It is also used to determine the cytotoxicity of potential medical agents and other toxic materials.

PRINCIPLE

MTT was first described by Mosmann in 1983. Yellow MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazol) is reduced to purple formazan in the mitochondria of the living cells. A solubilization solution is added to dissolve the insoluble purple formazan product into a colored solution The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600nm) by a spectrophotometer.

The reduction takes place only when mitochondrial dehydrogenase enzyme is active and therefore conversion is directly related to number of viable cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, effectiveness of the agent is causing death of cells can be deduced

After the addition of the drug, cell death and cell viability was estimated. The result is confirmed by additional metabolic intervention experiment such as MTT assay.

Procedure:

- After incubation, remove the medium from the wells for MTT assay.
- In each well wash with MEM (w/o) FCS. And add 200µl of MTT concentration of (5mg/ml).
- And incubate for 6-7hrs in 5% CO_2 incubator.
- After incubation 1ml of DMSO was added in each well and mix by pipette and leave for 45sec
- And it shows the purple color formation.
- The suspension is transferred in to the cuvette of spectrophotometer and O.D values is read at 595nm
- Graph is plotted using the O.D at Y-axis and concentration of the extract in X-axis.

RESULTS AND DISCUSSION

Morphological changes

In the present study, cytotoxic effect of sample on cancer cell line, A549 was investigated under different concentrations. The sample produced significant morphological alterations on A549 cancer cell line in culture. Under normal growth conditions(control) these cells were regular in shape and size and adhere to substratum (Figure 1a). After treatment with 1000µg of sample, the cells become irregular in shape and size and causes some changes on the cell surface associated with adhere of the substratum. After treatment with 250µg of sample, the cells become spherical in shape and size with alter nuclear cytoplasm ratio (Figure 1c). This has indicated that sample render some changes on the cell surface associated with the adherence of the substratum (Kim et al., 1999). Most of the cells had relatively flattened appearance with long multiple cytoplasmic processes forming cross bridges with neighbouring cells (Figure 1d).

Inhibition of hepatic cancer line by sample

The sample extracts suppress the proliferation of A549 cell lines (Figure 2).

Cell viability decreased in a dose dependent manners. The result indicated that increasing concentration of sample from 125µg/ml to 1000µg/ml, the percentage of growth dilution of A549 cells increased progressively from 92.42% to 21.21%(Table 1).

Finally we reported that the sample cause significant growth. Inhibition of human hepatic cancer cell line at 1000µg/ml concentration. am et al., (2001) reported that sample was able to inhibit tumor cells by apoptosis mediated cell inhibiton.

CONCLUSION

The low activity may be due to the less percentage of the active compounds present in the sample extract. The bioactivity directed fractionation can be made to increase the effectiveness of the extract towards the cancerous cell lines by enriching the active chemical components. In the present study, sample showed a strong anticancer activity against hepatic cancer cell line.