EVALUATION OF ANTICANCER EFFECT OF THIOAMIDES SYNTHESIZED FROM PYRAZOLE ALDEHYDES USING DMSO AGAINST HUMAN LIVER CANCER CELL LINE (HepG2)

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Abstract

The aim of the present study to investigate the anticancer activity of thioamide against human hepatoma cell line (HepG2). In this present examination, based on the outcome of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl2H-tetrazolium bromide (MTT) assay, 12.5 to 200µg/mL were chosen for further *in-vitro* studies. The cell growth inhibition of the Methanethione tested against HepG2 cell line at different concentrations (12.5, 25, 50, 100 and 200µg/ml). The results of the study observed that the concentrations increases there is an increase in the cell growth inhibition but is found to be lowest growth inhibition was 7.02 % at 12.5 µg/ml and highest growth inhibition was 79.38% at 200 µg/ml while standard (Doxorubicin) was 87.65% at 5µg/ml. Methanethione has anticancer potential than with IC_{50} of 102.03µg/ml. The results of this study provided evidence of anticancer or cytotoxic activity of Methanethione *in vitro*. These findings emphasize promoting increased use as an essential means to prevent or even to treat cancer. However, further research is essential for exploration of *in vivo* molecular mechanisms on anticancer activity of Methanethione.

Keywords: Anticancer, Cytotoxicity, HepG 2 cell line, Thioamides.

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Introduction

Cancer is a multifactorial disease that results from the mutation of certain genes that regulate cell function upon exposure to specific environmental factors. It is characterized by out-of-control cell growth leading to expansive masses of abnormal cells that infiltrate and damage nearby normal tissues. According to the World Health

Organization (2012), cancer is the second leading cause of death globally and accounted for 7.4 million deaths in 2004 and will continue to increase with an expected 11.5 million deaths in 2030. Liver cancer is one of the cancers with a high mortality rate worldwide, and it is challenging to cure. The highest incidence of liver cancer is in Asia and Africa. More than 75% of liver cancer incidents were reported from Asian countries. Epidemics of liver cancers are increased around the world including India and the USA (Venook *et al*., 2010). Although significant progress has been made in cancer treatment, adverse side effects and drug resistance remain serious problems. Therefore, the search is still on for safe and effective chemotherapeutic agents for cancer treatment.

Recent reviews insisted the importance of one-pot multicomponent strategy for many new organic reactions for novel drug-like compounds (Nandi et al., 2022; Pathare et al., 2022). Multicomponent reactions (MCRs) have played an essential role in biologically relevant advances in the pharmaceutical, medical, agricultural and chemical industries (Bovonsombata et al., 2017). Moreover, MCRs impart high-quantum yields, simple protocols, involving reactants of lower cost and incorporating green methodologies when compared with multi-step procedure. The key advantages of MCRs are faster reaction rates, more selectivity, a simple workup technique, atom economy, and the use of green solvents. Thioamides have fascinated recognition of scientific society from an expert with its vast-range of pharmaceutical activities. For the treatment of tuberculosis, ethionamide and prothionamide are the very excellent examples of thioamide drugs (Wang et al., 2007; Tan et al., 2017). Keeping in view, the present study to investigate the anticancer activity of Methanethione against human hepatoma cell line (HepG2).

MATERIALS AND METHODS

Synthesis:

The reaction was carried out by mixing pyrazole aldehyde (2 mmol), precipitated sulphur (8 mmol), pyrrolidine (2 mmol) in the ratio of 1:4:1 using DMSO as solvent at 100ºC without using catalyst in an oil bath with constant stirring. The progression of the reaction was observed by TLC. The whole mixture was poured into an ice water with continuous stirring after the completion of the reaction. Then precipitated product was procured by normal filtration, washing with

distilled water and dried. The synthesized compound further characterized and identified as Methanethione (Helen perci *et al*., 2023).

In vitro cytotoxic effect determination by MTT assay

Cytotoxic assay was evaluated by the MTT reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (Mosmann, 1983; Igarashi et al., 2001). HepG2 (Human liver cancer cell line) was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Dulbecco's Modified Eagle Medium) (Sigma aldrich, USA). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of Methanethione was weighed and dissolved in 1mL of DMEM using a cyclomixer. The test solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Cytotoxicity Evaluation:

After 24 hours the growth medium was removed, freshly prepared each compounds in 5% DMEM were five times serially diluted by two fold dilution (200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non-treated control cells were also maintained. Standard drug Doxorubicin (5µg/ml) used in this study.

Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm. % Cytotoxicity using the following formulas: % Cytotoxicity = 100 - [Abs (Test) /Abs (control)] x100. % Cell Viability = [Abs (Test) /Abs (control)] x100.

Statistical analysis

The amount of Methanethione needed to inhibit the cancer growth by 50%, IC_{50}, was graphically determined by a linear regression method using Ms- Windows based graphpad Instat (version 3) software. Results were expressed as graphically.

RESULTS AND DISCUSSION

Cancer is the most distressing and life threatening disease that enforces severe death worldwide. Mortality is still unacceptably high despite many therapeutic advances. Nowadays, there are four standard methods for the treatment of cancer: surgery, chemotherapy, radiation therapy, and immunotherapy (Chabner and Roberts, 2005). The most common option used for treatment of cancer is chemotherapy. The aim of the present study to investigate the anticancer activity of thioamide against human hepatoma cell line (HepG2).

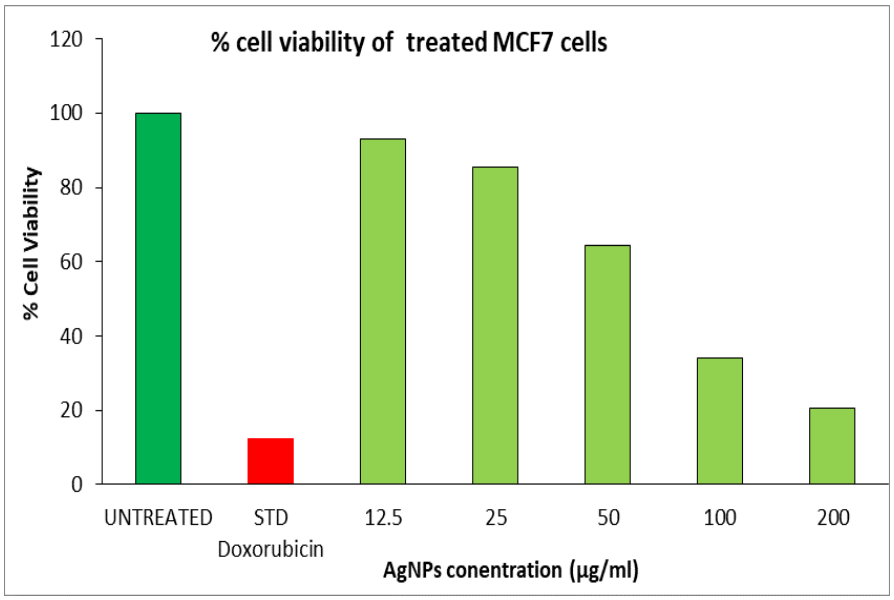
The number of animals used in research has increased with the advancement of research and development in medical technology. Every year, millions of experimental animals are used all over the world. The pain, distress and death experienced by the animals during scientific experiments have been a debating issue for a long time. Besides the major concern of ethics, there are few more disadvantages of animal experimentation like requirement of skilled manpower, time consuming protocols and high cost. Various alternatives to animal testing were proposed to overcome the drawbacks associated with animal experiments and avoid the unethical procedures. A strategy of 3 Rs (i.e. reduction, refinement and replacement) is being applied for laboratory use of animals. Different methods and alternative organisms are applied to implement this strategy. These methods provide an alternative means for the drug and chemical testing, up to some levels (Sonali et al., 2015).

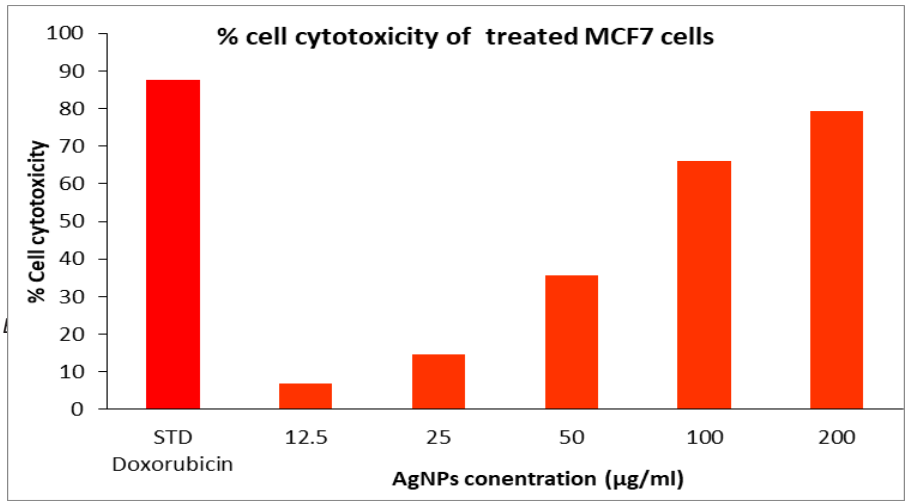
Use of *in vitro* cell and tissue cultures which involves growth of cells outside the body in laboratory environment can be an important alternative for animal experiments. The cells and tissues from the liver, kidney, brain, skin etc. are removed from an animal and can be kept outside the body, in suitable growth medium, for few days to several months or even for few years. *In vitro* culture of animal/human cells includes their isolation from each other and growing as a monolayer over the surface of culture plates/flasks. Cellular components like membrane fragments, cellular enzymes can also be used. Various types of cultures like cell culture, callus culture, tissue culture and organ culture are used for various purposes. Benefits associated with techniques are, easy to follow, less time consuming and are less expensive. These methodologies are routinely used for preliminary screening of potential drug molecules/chemicals to check their toxicity and efficacy (Shay and Wright, 2000; Steinhoff et al., 2000).

The cell growth inhibition of the Methanethione tested against HepG2 cell line at different concentrations (12.5, 25, 50, 100 and 200µg/ml). The results of the study observed that the concentrations increases there is an increase in the cell growth inhibition but is found to be lowest growth inhibition was 7.02 % at 12.5 µg/ml and highest growth inhibition was 79.38% at 200 µg/ml while standard (Doxorubicin) was 87.65% at 5µg/ml. The IC_{50} value was 102.03µg/ml (Table 1 and Fig 1).

Table 1 Cell growth inhibition and cytotoxicity of Methanethione on HepG2 cell line by MTT assay

S.No.	Concentrations (µg/ml)	Absorbance (Optical density)	Cell growth inhibition (%)	Cell Viability (%)
1	12.5	0.775	**7.02**	92.98
2	25	0.713	14.51	85.49
3	50	0.537	35.56	64.44
4	100	0.283	66.01	33.99
5	200	0.172	**79.38**	20.62
Standard (Doxorubicin) (5µg/ml)		0.103	87.65	12.35
Cell Control		0.834	100	0
Half Inhibition Concentration (IC_{50})				**102.03µg/ml**





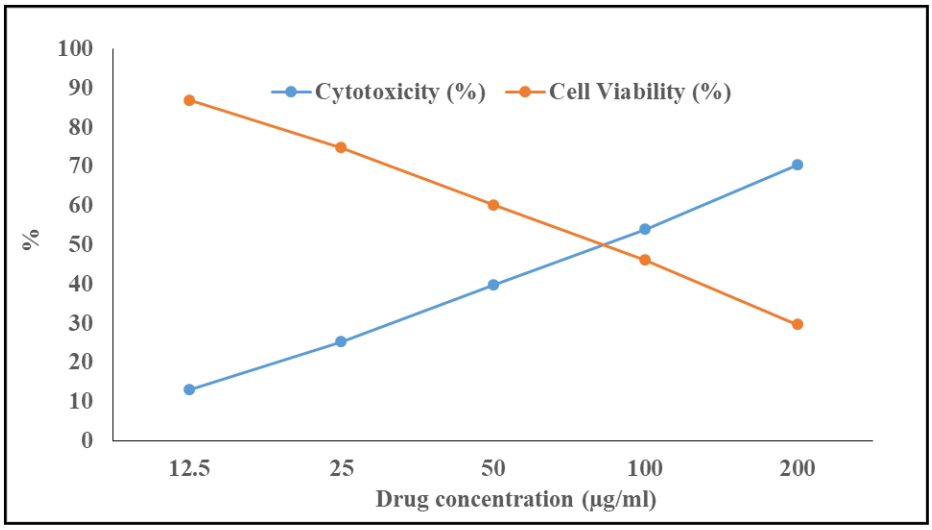


Fig. 1: Percentage of cell viability and cytotoxicity of Methanethione on HepG2 cell line by MTT assay

Morphological examination

Figure 2 shows the morphological changes that occurred HepG2cells upon treatment with different concentrations of Methanethione. Increasing the Methanethione concentration (upto200µg/mL) resulted in a drastic change in the morphological characteristics of the tested cell line, which was proportional to the applied concentration. Cells started to shrink and lose their capacity to adhere to the surface of the cultivation plate. Moreover, at the highest applied concentration, cells appeared rounded and were completely floated in comparison to the control morphology. The anticancer activity by inducing cell toxicity and apoptosis (Arrow mark) (Morphology change) in HepG2 cells. However the untreated cells appeared normal and were confluent. The results of cytotoxicity study (MTT assay) suggested that Methanethione showed toxic in nature after the treatment period of 72hrs with IC_{50} value of 102.03µg/ml

Research has shown that there are three types of programmed cell death, apoptosis, autophagy and non-lysosomal cell death (Li et al., 2018). Apoptosis is considered as an important component of various processes including normal cell homeostasis, immune system development, and chemical-induced cell death (Zhao et al., 2018). A number of studies have been reported that some chemopreventive agents exert their anticancer activity by regulating apoptosis, thereby leading to potential for new drugs that could be useful for anticancer treatment (Yuan et al., 2014). Apoptosis is a hereditarily controlled reaction of a characteristic cell framework that is required for a basic harmony between cell expansion and cell demise in typical improvement and support of homeostasis of a living being. Numerous chemotherapeutic specialists smoother the development of changed or dangerous cells by actuating apoptosis (Green, 2017).

An enlistment of tumour cell apoptosis is one of the productive focuses for medicating advancement and has become a significant concentration in the investigation of disease treatment. Along these lines, the light and fluorescence minuscule and DNA fracture investigation was concentrated to decide the nearness of apoptosis in Methanethione -treated HepG2 cells. Indicated the morphological changes of control and Methanethione -treated HepG2 cells at the concentration of 12.5 to 200µg/mL for 72 hours of treatment. In Methanethione treated HepG2 cells, the obliteration of monolayer was observed. It is not observed in HepG2 cells without Methanethione treatment. Control HepG2 demonstrated the growing and adjusted morphology of the cells with dense chromatin and their film. Methanethione -treated cells appeared rounded and were completely floated in comparison with control. Dynamic auxiliary adjustments and a decrease of HepG2 cell populaces were seen in directly proportional to the concentrations of Methanethione declared that the microscopic image of control and Methanethione treated HepG2 cells for 72 hours.

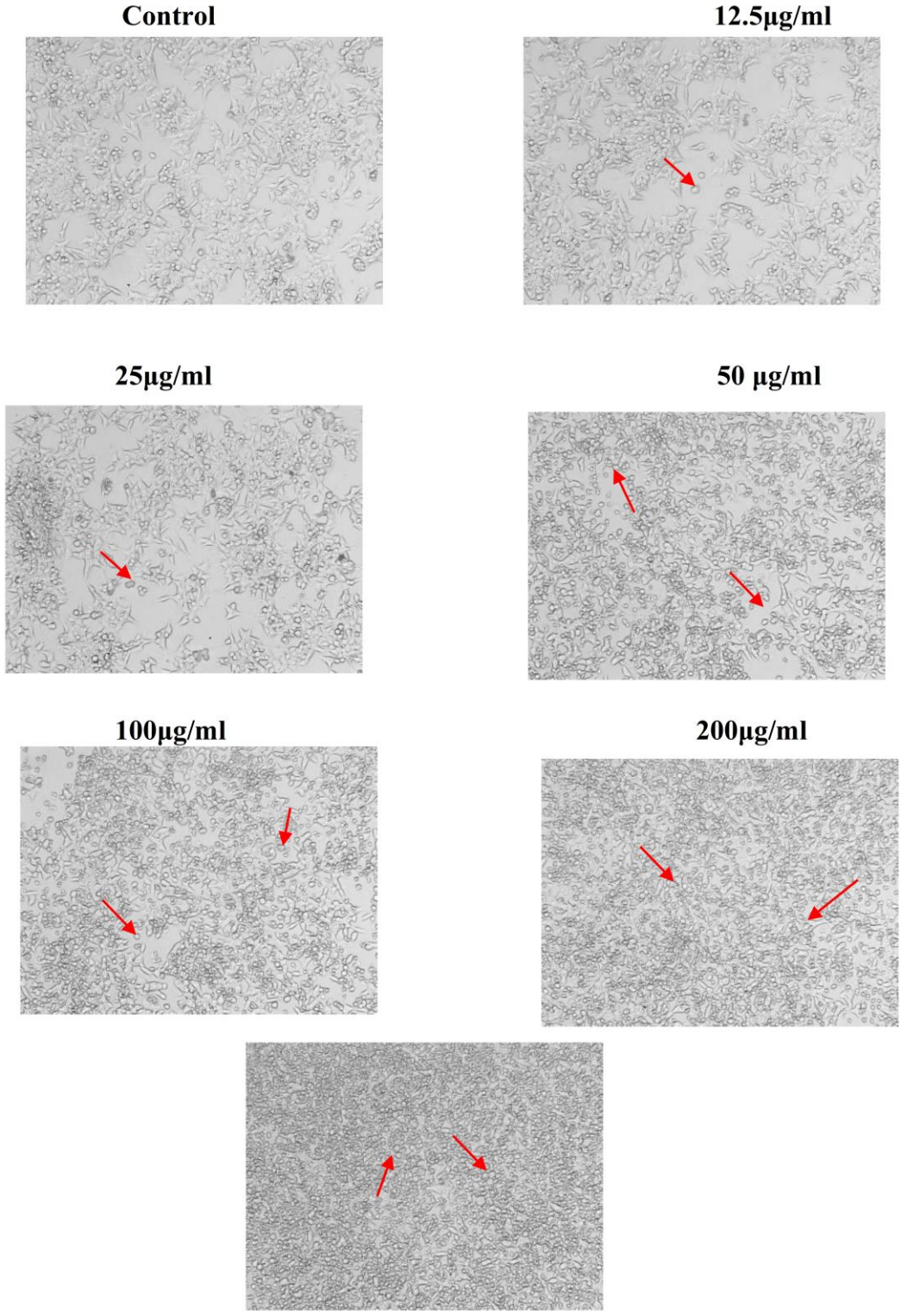


Figure 2 Photomicrograph of HepG2 cell line on different concentrations of Methanethione treatment as determined by MTT assay (Arrow mark indicate representative apoptotic cells).

In conclusion, this study has provided evidence of anticancer or cytotoxic activity of Methanethione *in vitro*. The Methanethione was tested for cytotoxic activity in HepG2 (Liver cancer) cell line by MTT assay. The result shows that Methanethione has anticancer potential than with IC_{50} of 102.03µg/ml. These findings emphasize promoting increased use as an essential means to prevent or even to treat cancer. However, further research is essential for exploration of *in vivo* molecular mechanisms on anticancer activity of Methanethione.

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