Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 707 - 716

Research Article **Medicinal Chemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

COMPARISON OF THE MTT AND ALAMAR BLUE ASSAY FOR IN VITRO ANTI CANCER ACTIVITY BY TESTING OF VARIOUS CHALCONE AND THIOSEMICARBAZONE DERIVATIVES

HITESH D PATEL*[1], ANURAG D ZAVERI[2], DILIP N ZAVERI[2], SAAVANI SHAH[1] AND ANKITA SOLANKI[1]

[1]Department of Chemistry, Gujarat University, Gujarat Ahmedabad
[2]Biocare Research (I) Pvt. Ltd., Ahmedabad, Gujarat, India

ABSTRACT

The MTT assay was compared with the Alamar Blue assay for in vitro anti cancer activity by testing of various Thiosemicarbazone and Chalcone derivatives on various human tumor cell lines. The Alamar Blue assay provided a better linearity with a cell number and a higher sensitivity, and its activity was not cell line dependent. In contrast to the MTT assay, Alamar Blue assay showed the convenience in successive observations and more accurate results. In our case, with thiosemicarbazone and chalcone derivatives two variables, sensitivity and colorimetric linearity with cell number were studied.

KEYWORDS: **MTT assay, Alamar Blue assay, Anticancer activity, Comparison**

HITESH D PATEL
Department of Chemistry, Gujarat University, Gujarat Ahmedabad

Corresponding author

INTRODUCTION

Several comparisons of various tests and methods are always necessary, with the development of new technology. It becomes mandatory to compare old methods / techniques with the new one because the sensitivity and specificity matters in experiment carried out in a laboratory. Cell viability studies are generally conducted for two purposes. One of them is to study the effect of cancer drug on tumor cell lines as well as normal cell lines and another is to study the toxic effect of any compound / agent, on different types of normal cell lines. In our study we tried to compare MTT and Alamar Blue methods used for cell viability studies. Cisplatin was used as conventional drug to compare the positive results and fifteen other compounds were obtained from Department of Chemistry, Gujarat University, Ahmedabad, Gujarat (India) to check its performance on three cell lines, CCRF CEM, SKMEL 28 and MDA MB 435S.

MATERIALS AND METHODS

Cell lines and culture

The human melanoma cell line SK-MEL 28 and MDA-MB 435S and leukemia cell line CCRF CEM were obtained from NCCS (National Center for Cell Science), Pune. Cell lines were maintained at 37^{o}C in 5% CO_2 as subconfluent monolayers in 75 cm^2 culture flasks (Himedia) and were subcultured once in a week while medium was changed every three days with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% filter sterilized fetal bovine serum (FBS) (Himedia) and 1mmol/l L-glutamine . During experiments 50µl gentamycin was added to the culture medium. Passage levels were in the range of 5 to 20 after receipt of the cell lines from NCCS, Pune.

Drugs

Cisplatin (Sun pharmaceutical) was stored as a -4.3 X 10^{-5} M stock solution in 0.9% methanol (HPLC grade). Other derivatives (B_1 to B_{15}) supplied by the Department of Chemistry were aliquoted in the same manner and dilutions ranging between $\log_{10}$ -4.3 M to $\log_{10}$ -8.3 M concentration solutions were prepared and stored at 2-8^{o}C till use. Drugs were brought to room temperature prior to use. B_1 to B_{10} compounds are Chalcone derivatives where as B_{11} to B_{15} are thiosemicarbazone derivatives details are indicated in table 1 and 2 below.

Table 1
Details about chalcone derivatives

Sr. No.	Code at Biocare	R_1	R_2	X_1	X_2	X_3	X_4	X_5	Mol. Formula (Mol.Weight)
1	B_1	OCH_3	OCH_3	H	H	$N(CH_3)_2$	H	H	$C_{19}H_{21}NO_3$ (311.37)
2	B_2	OCH_3	OCH_3	H	H	NO_2	H	H	$C_{17}H_{15}NO_5$ (313.3)
3	B_3	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	H	$C_{20}H_{22}O_6$ (358.39)
4	B_4	OCH_3	H	H	H	$N(CH_3)_2$	H	H	$C_{18}H_{19}NO_2$ (281.35)
5	B_5	OCH_3	H	H	H	NO_2	H	H	$C_{16}H_{13}NO_4$ (283.28)
6	B_6	OCH_3	H	H	H	OH	H	H	$C_{16}H_{14}O_3$ (254.28)
7	B_7	OCH_3	H	H	OCH_3	OCH_3	H	H	$C_{19}H_{20}O_5$ (328.36)
8	B_8	OCH_3	H	OCH_3	H	H	H	OCH_3	$C_{19}H_{20}O_5$ (328.36)
9	B_9	OCH_3	H	H	OCH_3	OCH_3	OCH_3	H	$C_{18}H_{18}O_4$ (298.12)
10	B_{10}	OCH_3	H	OCH_3	OCH_3	OCH_3	H	H	$C_{18}H_{18}O_4$ (298.12)

(281.35)

Table 2
Details about thiosemicarbazone derivatives

No.	R	Molecular Formula (M.wt.)
S_1	H	$C_{10}H_{10}ClN_5S$ (267.738 g)
S_2	C_6H_5	$C_{16}H_{14}ClN_5S$ (343.834 g)
S_3	C_2H_5	$C_{12}H_{14}ClN_5S$ (295.791 g)
S_4	C_4H_9	$C_{14}H_{18}ClN_5S$ (323.844 g)
S_5	C_6H_{11}	$C_{16}H_{20}ClN_5S$ (349.882 g)

MTT assay

The MTT is an established method for studying the efficacy of any therapeutic agents in drug screening cytostatic potential of anticancer compounds in toxicological testing. We used the method described by the manufacturer of the kit used during the assay (Himedia, EZcount^{TM} cell assay kit). In brief, cell were harvested from the exponential phase when the confluency of the cultured flask reaches >85%. Cells were harvested using trypsinization, counted and viability check was carried out using neubauer chamber and plated in 96-well flat-bottomed microtitter plates (Thermo) (100µl suspension per plate) Optimal seeding densities for each cell line were determined to assure exponential growth during a 3-day assay, which was equivalent to absorbance readings not exceeding a value of 1.8. Experiments were carried out in triplicate. Following plating and 24 hour recovery to allow the cells to attach to the plate surface and resume to exponential growth, 100µl drug was added to the wells. 72 h after drug addition, 20µl MTT (final concentration, 0.5

mg/ml was added to the wells. After 2h at 37^{o}C, the medium was removed (in adherent cell line only where as in suspension the culture medium was allowed to remain in the wells) and the formed formazan crystals were dissolved with equal amount of dissolving buffer provided along with the kit. The optical density was measured at 570 nm using an ELISA plate reader (Eldex, Mumbai).

Alamar Blue assay

The Alamar Blue assay was performed essentially according to the method of kit manufacturer (Himedia). The method of plating and incubation was identical to the MTT assay till the end of incubation. The dissolving solution was not added to the wells as it is not required in the Alamar Blue assay. The optical densities were directly measured at 570 nm keeping 600 nm as a reference wavelength.

Data handling

Absorbance of ELISA plate was read on Eldex ELISA plate reader, Mumbai (India). Data were printed and were analyzed using the following method.

MTT

We subtracted mean 570 nm absorbance value of control wells from the mean 570 nm absorbance values of corresponding experimental wells.

Alamar Blue

Subtract the absorbance values of medium control from absorbance of value of only medium.

Equations for calculations

A. $A_{(\lambda H)}$ = Absorbance of Alamar blue in medium – Absorbance of only medium

$A_{(\lambda L)}$ = Absorbance of Alamar blue in medium – Absorbance of only medium

Where,

$A_{(\lambda H)}$ = Absorbance of oxidized form at higher wavelength

$A_{(\lambda L)}$ = Absorbance of oxidized form at lower wavelength

B. Calculation of Correction Factor (CF):

Correction Factor (CF): $= \frac{A_{(\lambda H)}}{A_{(\lambda L)}}$

C. Calculation for percentage reduction of Alamar blue with different wavelength:

Percentage reduction of Alamar blue = $\{A_{(\lambda L)} – [A_{(\lambda H)} \times CF] \times 100\}$

RESULTS & DISCUSSION

To determine the sensitivity cells were plated in the equal seeding densities. After 4-h incubation, the MTT and Alamar Blue assays were performed. In the Alamar Blue assay, there was a linear relation between plated cell number and OD at 570 nm keeping 600 nm as a reference wavelength. The MTT assay did not provide a linear relation with cell number (not at higher cell densities). At the lower cell densities (<50 000 cells / well) no significance difference with the blanks could be observed in the MTT assay, but when using the optimal wavelength for the Alamar Blue assay 12500 cells/well could be measured reliably (Figure 1). The graph of the same 96-well plate shows that the limit of detection of Alamar Blue can reach 50 cells per well with linearity to 1,000 cells per well by using an 18 h incubation. The horizontal line represents the optical density, calculated as three times the mean of the “Vehicle control” (Figure 2).

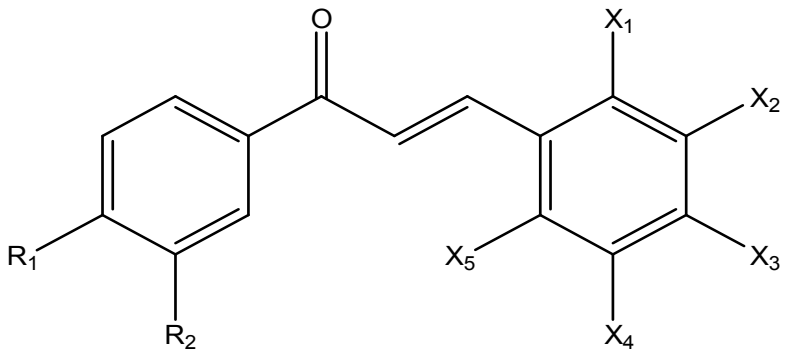


Figure 1
General structure of Chalcone

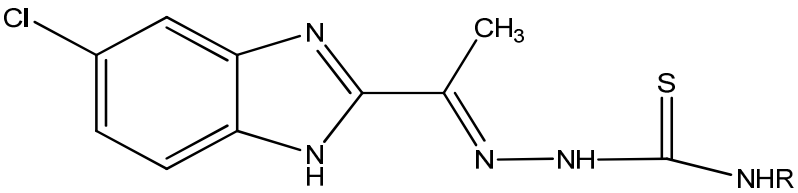


Figure 2
General structure of thiosemicarbazone

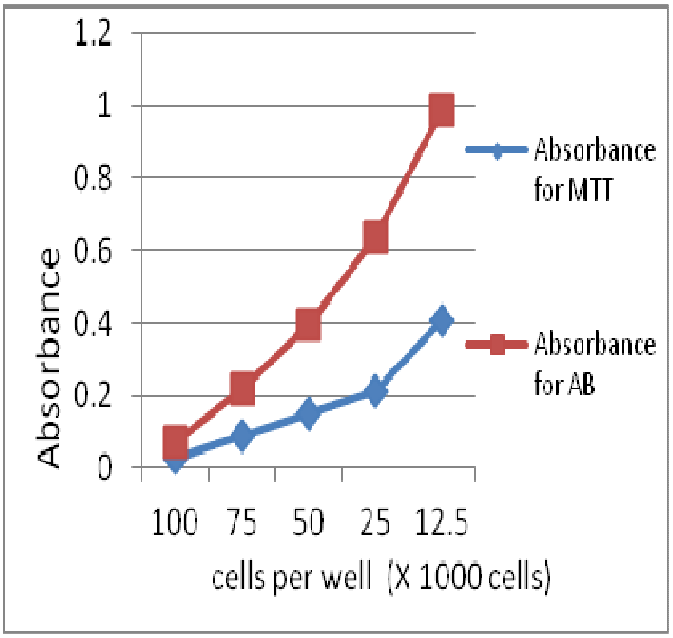


Figure 3
Relation between planted cell number and Absorbance in the MTT and Alamar Blue Assays

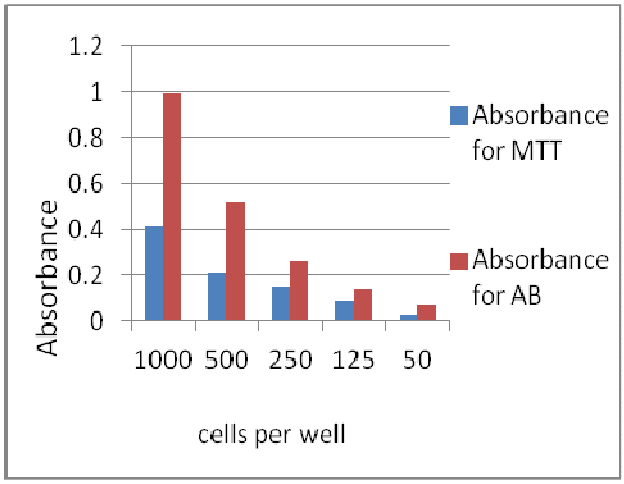


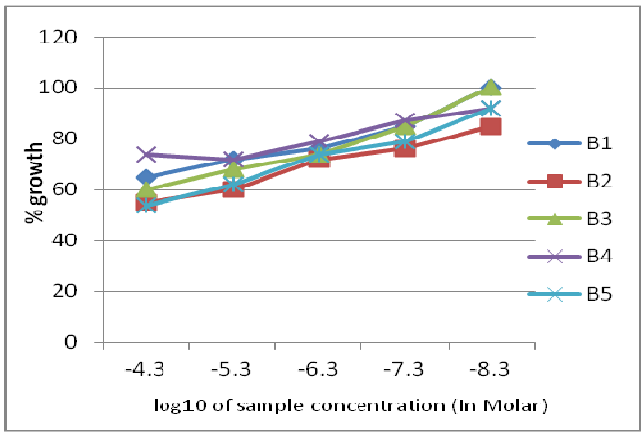
Figure 4
Comparison of limit of detection of cell numbers against absorbance for MTT and Alamar Blue Assays

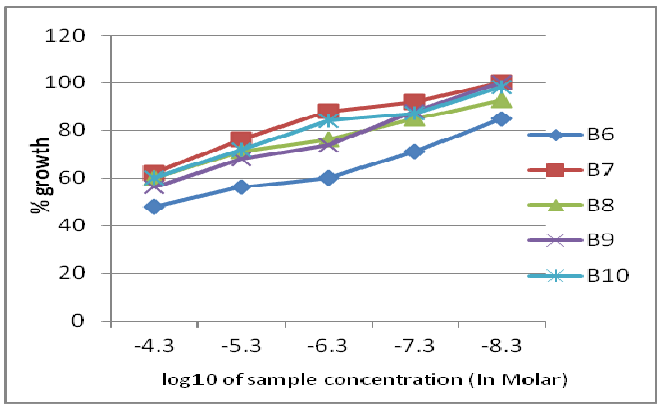
MTT assay was first described by Mosmann "as described by Mosmann [1]" and later on it was improved by Nicks and Otto "as described by Nicks and Otto [2]". It is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of cellular mitochondrial dehydrogenase enzyme in living cells to reduce yellow water-soluble substrate 3-(4,5-dimethylthiazol- 2yl)-2,5-diphenyl tetrazolium bromide [MTT] into dark blue / purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cell lines. The MTT assay has greater applicability in detection of cells which are not dividing but are still metabolically active. It can, therefore be used to distinguish between proliferation and cell activation. An additional advantage to the assay is that it can be used in suspended or monolayer cell preparations. In our study we used both types of cell lines which includes suspended cell line (CCRF CEM) and adherent cell line (SK-MEL 28, MDA MB 435S). Compared to this Alamar Blue assay (Resazurin assay) makes use of Resazurin [7-hydroxy-10oxido-phenoxazin-10ium-3one] dye which was used by many workers to access bacterial or yeast contamination in various biological fluids and milk "as described by Erb. and Ehlers [3]". This assay is also used to measure semen quality, corneal viability, mitochondrial function etc…"as described by Wang et al. [4], Perrot et al. [5] and Zhang et al. [6]". Resazurin is a redox dye which exhibits colorimetric and fluorometric change according to the cellular metabolic activity "as described by Fields et. al. [7]". As this assay is based on ability of viable, metabolically active cells to reduce dye resazurin to resorufin and di-hydro-resorufin this reduction occurs due to the intra cellular activity where the oxidized form of resazurin is entering to cytosol and then it is converted to reduced form by various mitochondrial enzymes by accepting electrons from NADPH, FADH, FMNH, NADH additionally it also accepts electrons from numerous cytochromes "as described by Al-Nasiry et al. [8] and O'Brien et al. [9]". This reduction is related to growth and causes of resazurin to convert the oxidized blue form [non fluorescence] to reduce red form [fluorescent form]. The fluorescent signal can be monitored using 530-560 nm excitation wavelength and 590 nm emission wavelength, while absorbance can be monitored at 540 and 630 nm wavelength. In our study we used Alamar Blue where we used 570 and 630 nm wavelength filters for observation of optical density. ODs were corrected after calculating correction factor. While testing fifteen different

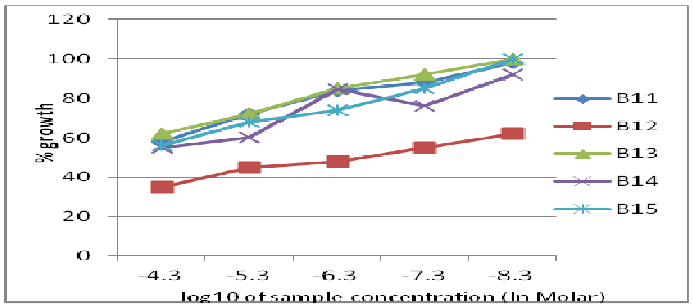
chemically synthesized moieties we found that P score was below three for all molecules tested by both methods on all three cell lines. However, we found that molecule B_{12} (S_2) and B_8 (A_7) are showing consistent results in both methods. NCI-DTP US in vitro testing results for CCRF CEM, MDA MB 435S and SK-MEL 28 for chemical B_{12} (S_2) are similar with our results "as described by Hitesh D Patel et al. [10]".

For CCRF CEM cell line

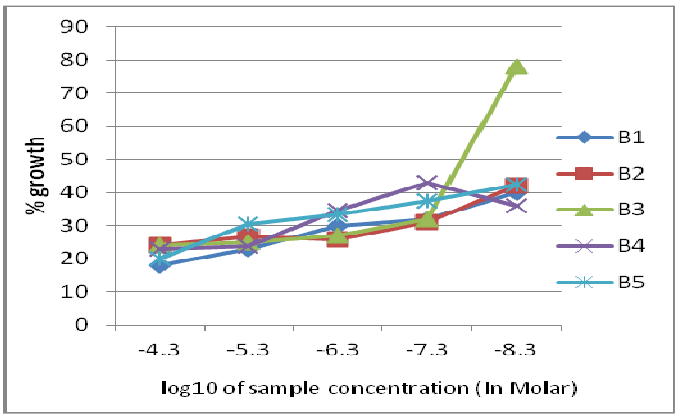
MTT Assay results

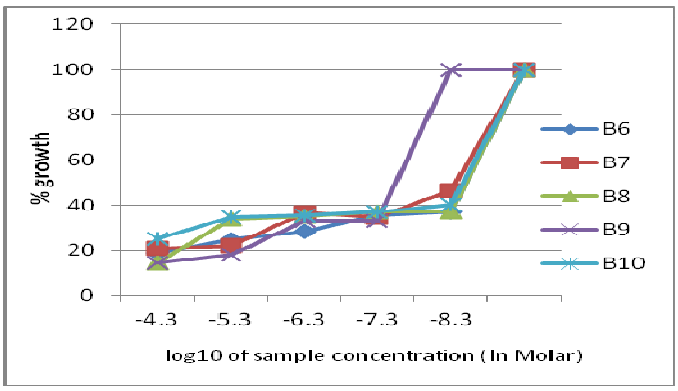


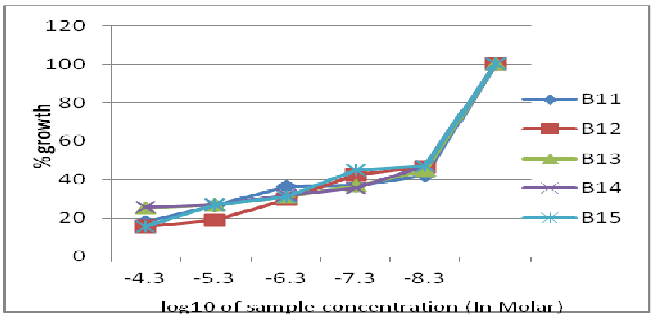




Alamar Blue Assay Results

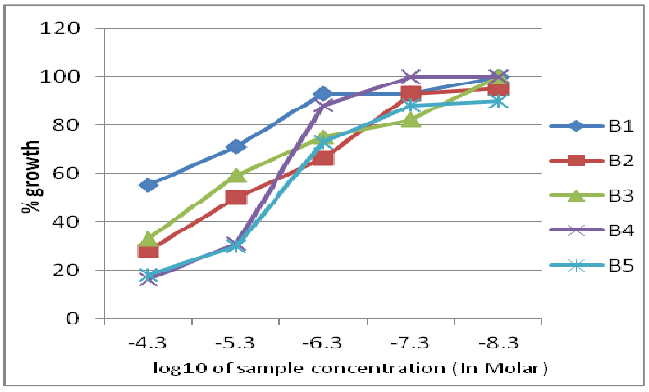


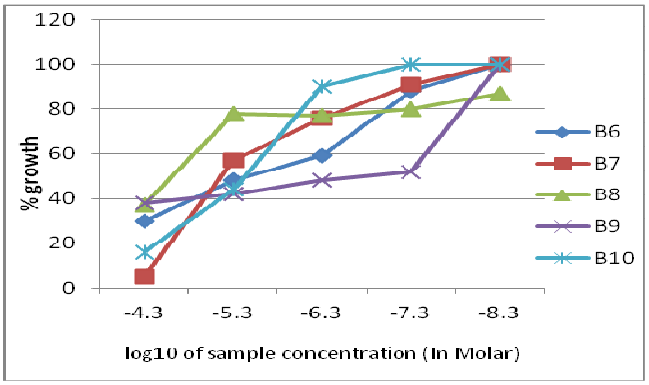


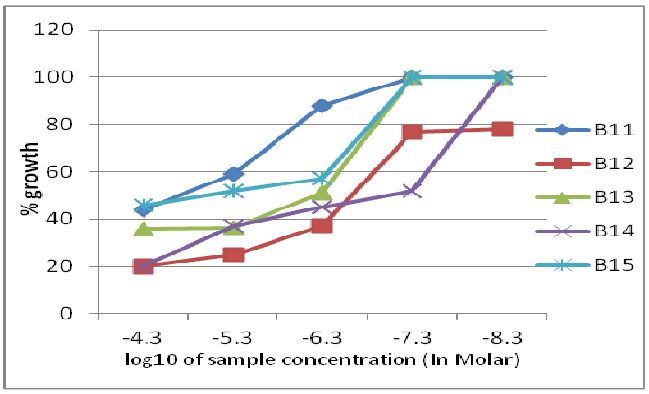


For SK-MEL 28 cell line

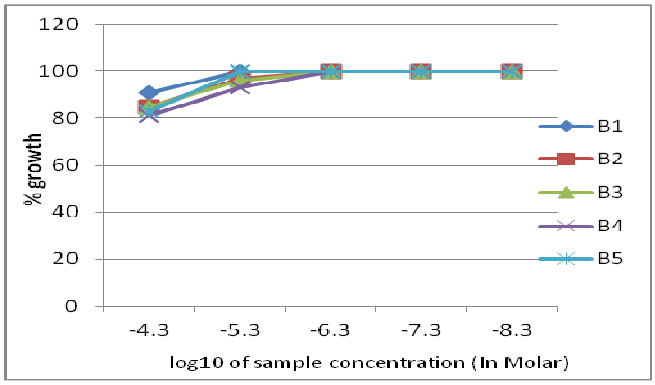
MTT Assay results

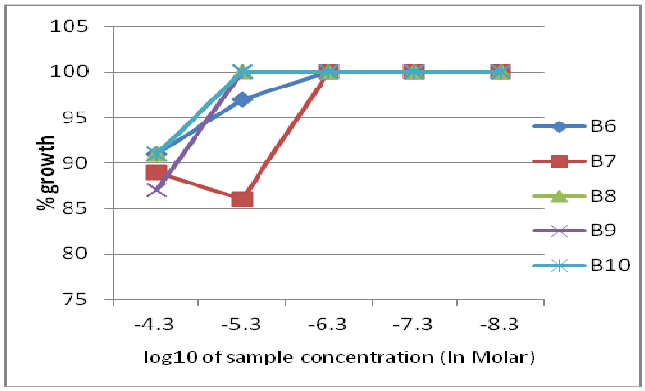


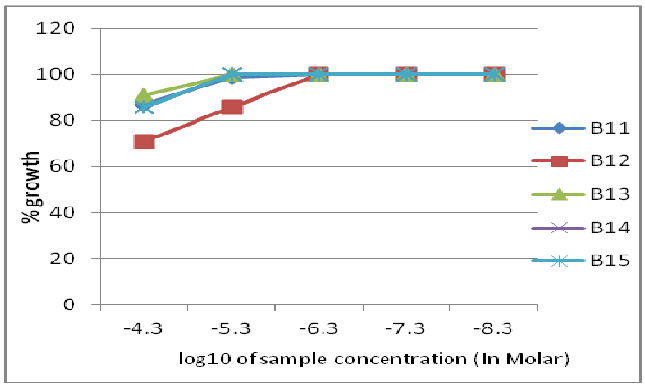




Alamar Blue Assay Results

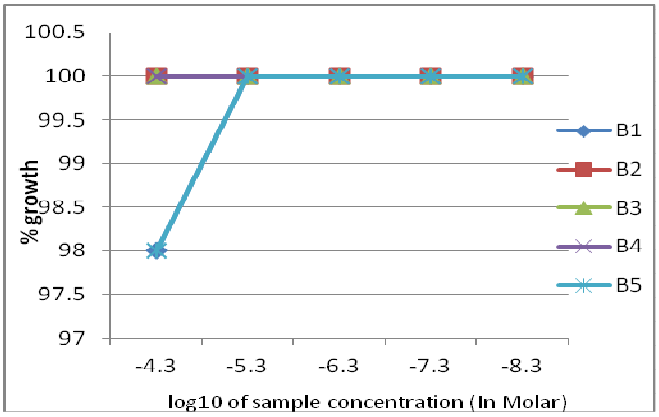




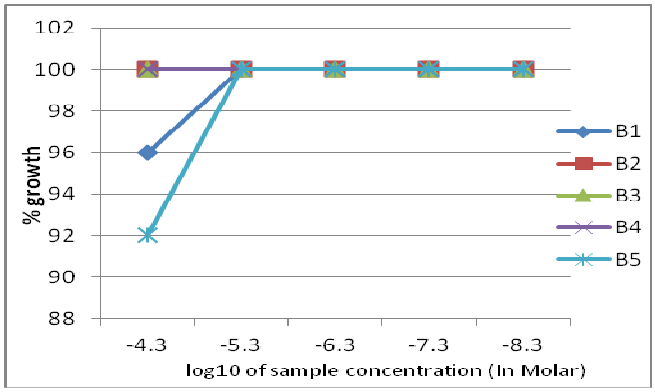


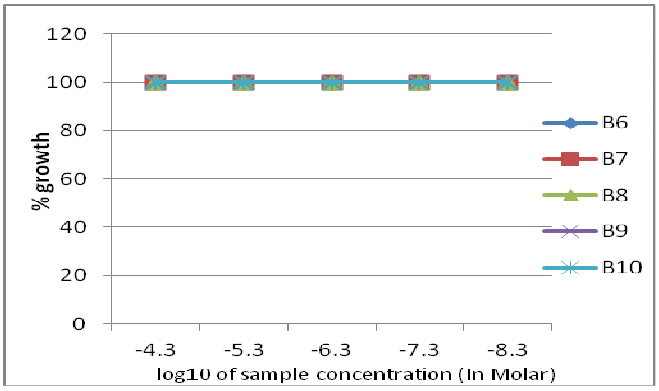
For MDA-MB 435S cell line

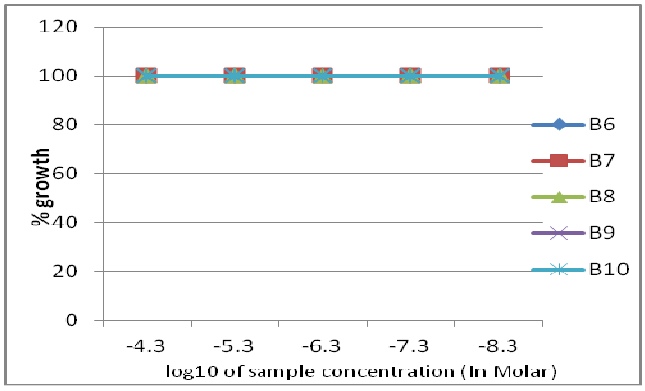
MTT Assay results

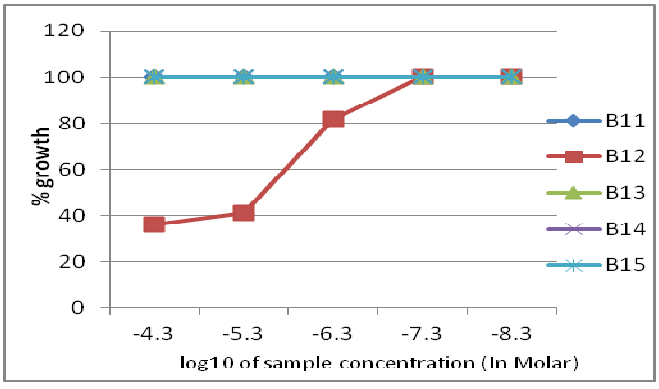


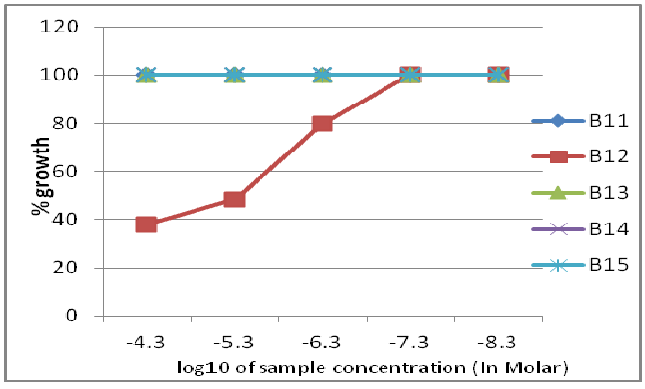
Alamar Blue Assay Results











CONCLUSION

We can conclude from the present study that Alamar Blue is better assay while the assayer requires consecutive evaluation of cell activity against various toxic substances. However, MTT assay is also recommended by various workers for the cell viability assessment but we found break in linearity at some points when compared with the Alamar Blue assay for different molecules. However, MTT remains thoroughly understood method whose mechanism is well understood. Thus MTT may be helpful for the molecules whose mode of action is not known. It is observed that MTT and Alamar Blue assays are not equivalent when an effective molecule behaves as an inducer or inhibitor for various metabolic enzymes. One has to be conscious while interpreting data and variation between replicates should be considered. P value found with the data obtained was within the normal range. Out of all fifteen new chemical entities we found that B_{12} (S_2) and B_8 (A_7) are candidates which require further detailed investigation.

ACKNOWLEDGEMENT

We would like to thank Department of Chemistry, Gujarat University, Ahmedabad, Gujarat for providing all the necessary facilities. We are also thankful to Biocare Research (I) Pvt. Ltd., Gujarat, Ahmedabad.

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