## 5. Anticancer activity of prepared extracts using *in-vitro* cytotoxic assay

### 5. 1. Instruments and equipments

Fluorescence inverted microscope (Leica Micro Systems, Germany)

Biosafety cabinet class-II (EscoMicro Pte. Ltd., Singapore)

Cytotoxic safety cabinet (Esco Micro Pte. Ltd., Singapore)

$CO_2$ incubator (C. M. Scientific, UK)

Deep freezer (Dairei Co. Ltd., Japan)

ELISA plate reader (Thermo Fisher Scientific, USA)

Micropipettes (Eppendorff, India)

RO water system (Merck Life Science Pvt. Ltd., India)

Electronic water bath (Biotech and Sceintific Industries, India)

### 5.2. Chemicals, reagents and materials

Trypsin 1X gamma irradiated, DMSO cell culture grade, penicillin and streptomycin solution stabilized were procured from Sigma Aldrich, USA, amphotericin B, dulbeco's phosphate buffer saline (DPBS) without $Ca^+$ and $Mg^+$, fluid thioglycolate media (TGM), tryptone soya broth (TSB) were obtained from Himedia Laboratories, India. EDTA and sodium carbonate were procured from S. D. Fine Chem Ltd., India. Dulbeco's modified eagle medium (DMEM) low glucose with glutamine was procured from MP Biomedicals India Pvt. Ltd., India, fetal bovine serum (FBS) was obtained from Quadditive Manufacturing solutions, Greece. Anticancer drugs doxorubicin was used as the standard obtained from Medox Biotech India Pvt. Ltd., India. Cell proliferation kit for MTT dye was obtained from AMRESCO, USA.

### 5.3. *In-vitro* cytotoxic assay - MTT assay

Assay is based on ability of mitochondrial dehydrogenase enzyme present in viable cells to cleave the tetrazolium rings of the pale yellow MTT dye and form dark purple formazan crystals which are largely impermeable to cell membranes, results in its accumulation in the cells (Figure 1.2) (Subhasree, 2009).

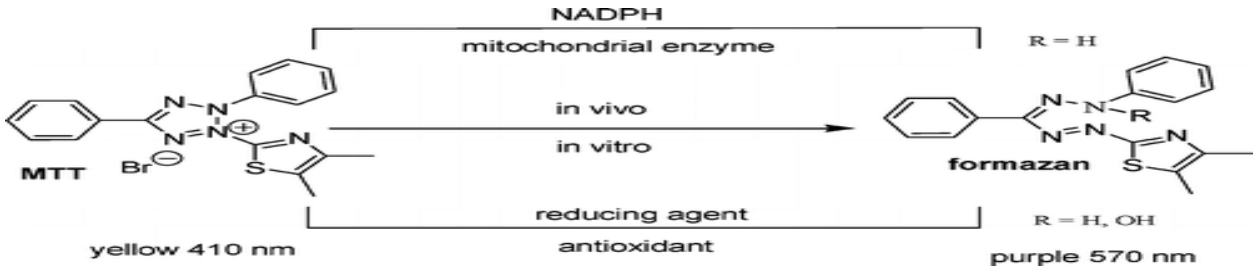


**Figure 5.1: Principle reaction behind MTT assay**

### 5.3.1. Applications of MTT assay

- ✓ Analysis of cytotoxic and cytostatic compounds such as anticancer drugs and other pharmaceuticals
- ✓ Measurement of cell proliferation in response to growth factors, cytokines, mitogens and nutrients
- ✓ Assessment of growth inhibitory antibodies and physiological mediators (Wilson, 2000)

## 5.4. Experimental work

### 5.4.1. Cell lines and culture medium

Hep-3B (human hepatoma cell) and BRL-3A (normal rat liver cell) cell lines were used in the experiment obtained from National Centre for Cell Science (NCCS), Pune. The cells were matained in DMEM, supplemented with 10% FBS, penicillin (100IU/mL), streptomycin (100µg/mL) and amphotericin-B (5µg/mL) in a humidified atmosphere of 5% $CO_2$ at 37°C.

Characterization of cell line is essential not only when deriving new lines, but also when a cell line is obtained from a cell bank or other laboratory. Cultures were examined under an inverted phase microscope before starting of experiments and frequent assessments are made for the viability of the cell population throughout the experimental periods.

Stock cells of these cell lines were cultured in DMEM, supplemented with 10% FBS (fetal bovine serum). Along with media cells were also supplemented with penicillin, streptomycin and amphotericin-B, in a humidified atmosphere of 5% $CO_2$ at 37 °C until confluence reached. The cells were dissociated with 0.2 % trypsin, 0.02% EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 $cm^2$ tissue culture flasks, than in 75 $cm^2$ and finally in 150 $cm^2$ tissue culture flask and all cytotoxicity experiments were carried out in 96 microtitre well plates. $2 \times 10^4$ cells/well was added in to each well of 96 well-plates. It was calculated as follow.

**Calculation for number of cells in 96 well plates:**

For this we need to calculate for no. of cells required for 100 wells ≈ 96 well, No. of cells / well × 100

$= 2 \times 10^4 \times 100$

$= 2 \times 10^6$ cells / plate

Total volume of media for 100 wells

= volume of media / well × 100

= 100 µl × 100

= 10 mL

Therefore, a total of $2 \times 10^6$ cells in 10 mL of medium, then aliquot the required volume of cell suspension in to each well.

**5.4.2. Characterization of cell lines and culture media**

The characterization of cell lines was performed for the detection of microbial growth and cross contamination. Detection carried out using special media like fluid thioglycollate media (TGM) and tryptone soya broth (TSB) and observed using gram staining. Contamination by bacteria, yeast or fungi was detected by an increasing in the turbidity of the medium and/or by decreasing in pH. Cells were inspected daily for presence or absence of microbial growth.

**5.4.3. Preparation of media**

**5.4.3.1. Preparation of DMEM**

Nine g of DMEM powder was added in 1 L of distilled water and then it was stirred continuously until clear solution obtain. To this, $NaHCO_3$ was added to maintain pH 7.0-7.2 and then solution was filtered using membrane filtration assembly. It was sterilized using autoclave and stored in reservoir bottle in refrigerator at 4 $^oC$ (Freshney, 2005).

**5.4.3.2. Preparation of the trypsin solution**

Five mL of trypsin solution was pipette out in to 50 mL falcon centrifuge tube containing 45 mL of PBS using 10 mL pipette.

**5.4.4. Preparation of standard and test solutions**

**5.4.4.1. Preparation of stock solution of test compounds**

The stock solutions of test compounds were prepared at concentration of 1mg/mL using DMSO and diluted with culture medium to achieve working concentration of 1000 µg/mL.

**5.4.4.2. Dilution of test Compounds**

For this, initially 100 µl of complete media was added in to well number 1-9. Well number 10 contained 150 µl test substance only, from that 50 µl was pipette out and added in to well no. 9 which already contain 100 µl of complete media, which lead to 1:3 dilution of test sample. Same procedure was repeated 9 times in order to get final concentration of test sample up to 0.005 µm which is shown in Table 5.1.

**Table 5.1: Dilution of test compound used in the assay (1:3)**

Well no. 1-9 contain complete media 100 µl

| Well No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample dilution | 50 µL from well no 2 | 50 µL from well no 3 | 50 µL from well no 4 | 50 µL from well no 5 | 50 µL from well no 6 | 50 µL from well no 7 | 50 µL from well no 8 | 50 µL from well no 9 | 50 µL from well no 10 | 150 µL test sample |
| Final concentration µg/mL | 0.05 | 0.15 | 0.46 | 1.37 | 4.12 | 12.35 | 37.04 | 111.1 | 333.3 | 1000 |

**5.4.4.3. Reference standard stock solution**

Standard stock solution was prepared to achieve the concentration 1000 µg/mL. Stock solution was further diluted using DMSO to obtain the dilutions of solutions.

**5.5. Design of experiment**

Cell lines in exponential growth phase were washed, trypsinized and re-suspended in complete culture media. Cells were seeded at $2 \times 10^4$ cells / well in 96 well microtitre plate and incubated for 24 h during which a partial monolayer forms. The cells were then exposed to various concentrations of the test compounds (as indicated in plate assignment) and standard doxorubicin. Control wells were received only maintenance medium. The plates were incubated at 37 °C in a humidified incubator with 5 % $CO_2$, 75 % relative humidity for a period of 24 h. Morphological changes of drug treated cells were examined using an inverted microscope at different time intervals and compared with the cells serving as control. At the end of 24 h, cellular viability was determined using MTT assay.

**5.5.1. *In-vitro* assay for cytotoxic activity (MTT assay)**

Both normal and cancer cells were pre incubated at a concentration of $2 \times 10^6$ cells/mL in culture medium for 3 h at 37°C and 6.5 % $CO_2$, 75 % relative Humidity. Cells were seeded at a concentration of $5 \times 10^4$ cells/well in 100 µl culture medium and various concentration of compound (1000 µg/mL-0.05 µg/mL) were added into microplates (tissue culture grade, 96 wells, flat bottom). Cell cultures were incubated for 24 h at 37 °C and 6.5% $CO_2$. 10 µl of MTT labeling mixture was added and incubated for 4 h. 100 µl of DMSO was added to each well and incubate for overnight. Absorbance of the samples was measured using a microplate (ELISA) reader at wavelength 570nm. Three independent experiments were performed. The effect of the ESME of *S. racemosa* on the viability of normal liver and hepatoma cells were as the % of viability using following formula. % viability = ($A_{570}$ of treated cells – $A_{570}$ of blank cells) / ($A_{570}$

of controlled cells – $A_{570}$ of blank cells) × 100. Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula % cytotoxicity = 100 – % cell viability (Cory et al., 1991).

**5.5.1.1. Data Interpretation**

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

After 24 h, the cytotoxicity data was evaluated by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95 % confidence limit and $R^2$ were used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the formazan by 50 % ($IC_{50}$). Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

**% viability = $(A_T - A_B) / (A_C - A_B) \times 100$ … … … (1)**

Where, $A_T$ = Absorbance of treated cells (drug)

$A_B$ = Absorbance of blank (only media)

$A_C$ = Absorbance of control (untreated)

**%cytotoxicity = 100 – % cell survival … … … (2)**

**5.5.1.2. Determination of $IC_{50}$ value**

According to the FDA, $IC_{50}$ represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In our study, $IC_{50}$ is a concentration of drug at which 50 % of cell population die.

For primary screening, a threshold of 50 % cell growth inhibition as a cut off for compound toxicity against cell lines was used. $IC_{50}$ values were determined from plot of dose response curve between log of compound concentration and percentage cell growth inhibition. Graph was plotted by keeping log concentration of drug on X axis and % cell growth inhibition or % cytotoxicity Y axis. $IC_{50}$ values were estimated as a concentration of drug at 50 % position on Y axis. The relationship should be sigmoidal, log concentration of the drug on the X axis and 'response / measurement' of the Y axis. The prism web site has some good guides for this. So, we have used this software.

$IC_{50}$ values were calculated using the nonlinear regression program origin. The average of two

(duplicates manner) were taken in determination. $IC_{50}$ value has been derived using curve fitting methods with graph pad prism statistical software (Ver. 5.02).

## 5.6. Results and discussion

### 5.6.1. Characterization of cell lines and culture media

Characterization of cell lines was performed for detection of microbial growth and cross contamination. Cell lines used in experiments were free from any kind of microbial or fungal contamination (Table 5.2), which is essential in order to continue the screening experiments.

Cross contamination of cell line was tested by direct observation of particular cell line under inverted phase microscope and population doubling time (PDT) for specific cell line was determined. From viability study and PDT, we concluded that the cell lines procured from NCCS, Pune were initially free from cross contamination.

To prevent the cross contamination of cell lines during our experiments work, separate pipettes and plastic tips were used for individual cell line. Along with that, particular cell line was used at the time under class–II bio safety cabinet. These were proving to be valid steps to prevent cross contamination of cell lines throughout the experiment.

**Table 5.2: Characterization of cell lines**

| Cell line | %viability | | PDT(h) | Microbial contamination | Cross contamination | pH |
|---|---|---|---|---|---|---|
| | Stock | After | | | | |
| Hep-3B | 60.46 | 81.8 | 35.12 | No | No | 7.5 |
| BRL-3A | 61.40 | 87.30 | 28.6 | No | No | 7.0 |

Culture media were also tested for microbial contaminations. To prevent microbial contamination, 2.5 % Amphotericin B (0.025 µg/ml) was supplemented to media which act as working concentration. Bacterial contamination was prevented by addition of 1 % of Antibiotic, 100 X (10000 U/ml Penicillin G, 10000 µg/ml Streptomycin) in to culture media. All subculturing activities were done under class-II bio safety cabinet (Esco, Singapore).

### 5.6.2. *In-vitro* assay for cytotoxic activity (MTT assay)

All the fourteen prepared extracts and fractions were tested for the *in-vitro* anticancer activity using MTT assay on Hep3B cell. $IC_{50}$ values (µg/ml) were calculated and shown in Table 5.3. The results revealed that ethyl acetate soluble fraction of methanol extract (ESME) showed the

potent anticancer activity ($IC_{50}$ value 32.55 µg/ml) as compared to standard drug doxorubicin. Graphical representation and the dose response curve of the assay were shown in Figure 5.1-5.4.

**Table 5.3: *In-vitro* anticancer activity of standard and samples on Hep3B cell**

| **Name of extract** | EtOH extract (Hot) | MeOH extract (Cold) | **MeOH extract (Hot)** | Water extract (Hot) | Hexane extract | EtOACfr of Hexane extract | CHCl3 fraction of Hexane extract | n-butanol fraction of Hexane extract |
|---|---|---|---|---|---|---|---|---|
| **$IC_{50}$ (µg/mL)** | 578.9 | 288.6 | **73.91** | 642.7 | 75.55 | 63.45 | >1000 | 111.3 |

| **Name of extract** | Water fraction of Hexane extract | Hexane fraction of MeOH extract | **EtOAC fraction of MeOH extract** | CHCl3 fraction of MeOH extract | n-butanol fraction of MeOH extract | Water fraction of MeOH extract | **Std. Doxorubicin** |
|---|---|---|---|---|---|---|---|
| **$IC_{50}$ (µg/mL)** | >1000 | 62.86 | **32.55** | 122.6 | 97.65 | >1000 | **55.43** |

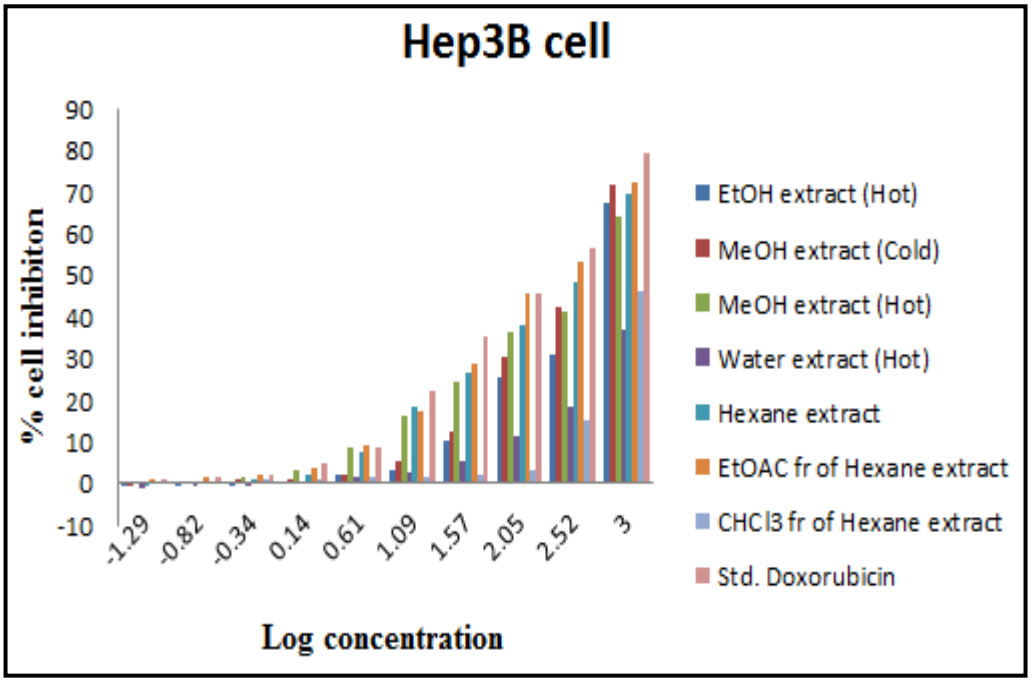


**Figure 5.2: Cytotoxicity assay of standard and samples on Hep3B cell**

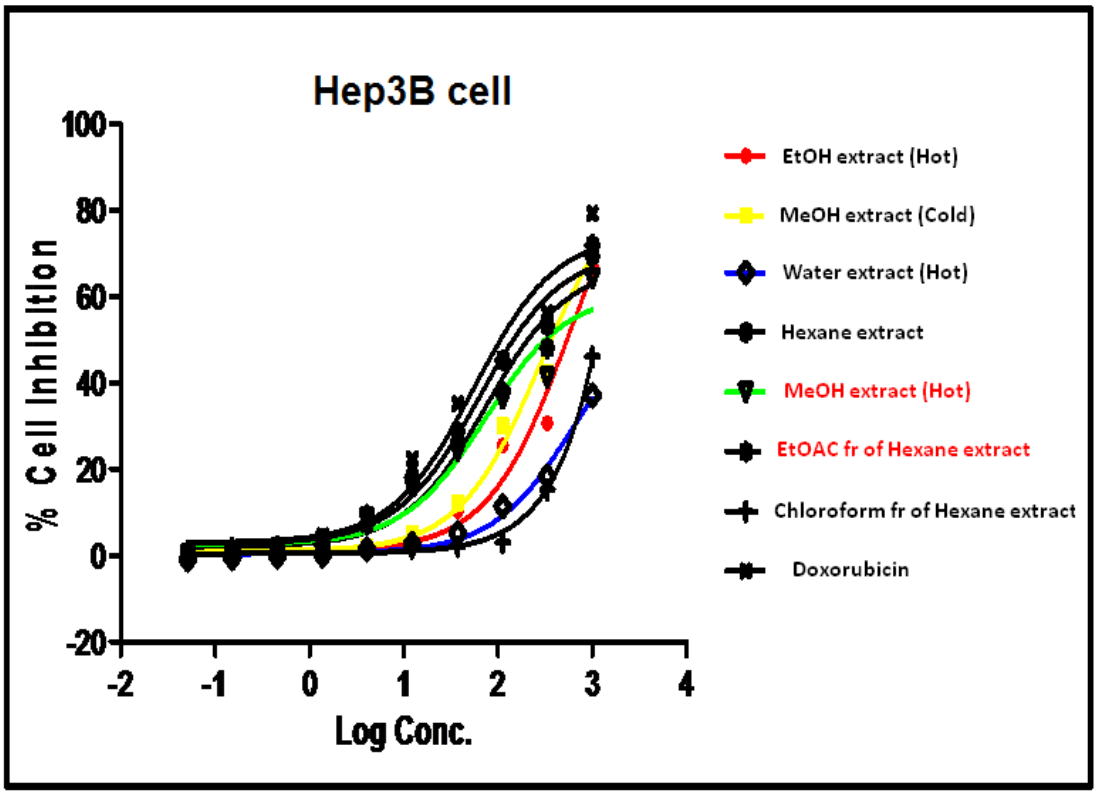


**Figure 5.3: Dose response curve of standard and samples on Hep3B cell**

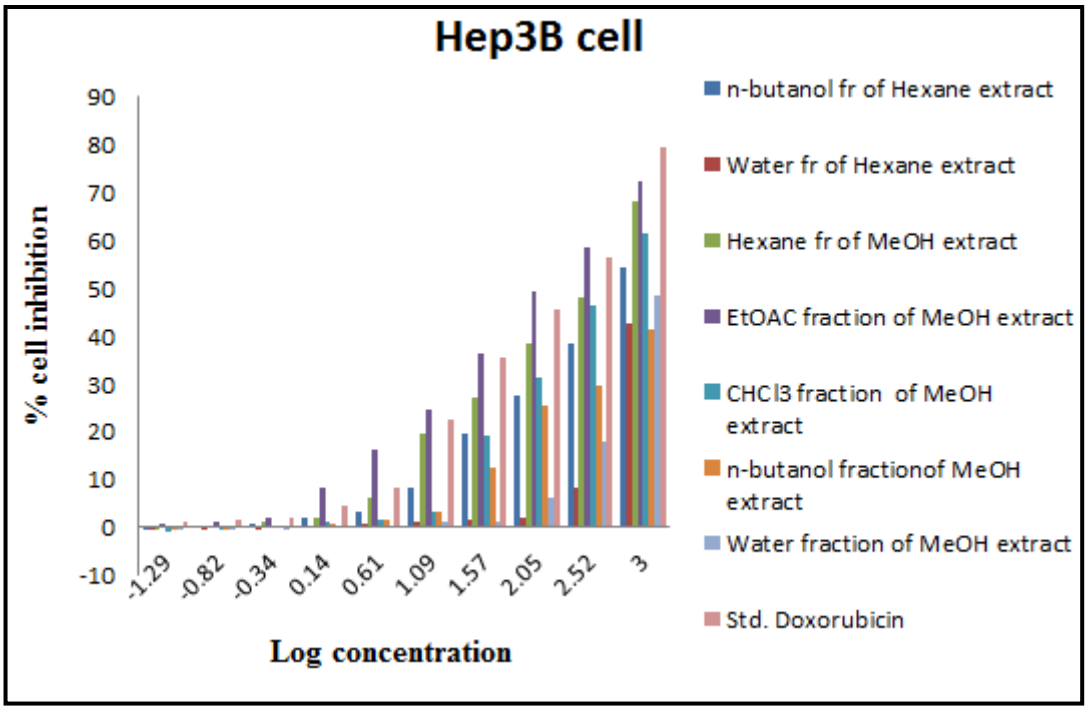


**Figure 5.4: Cytotoxicity assay of standard and samples on Hep3B cell**

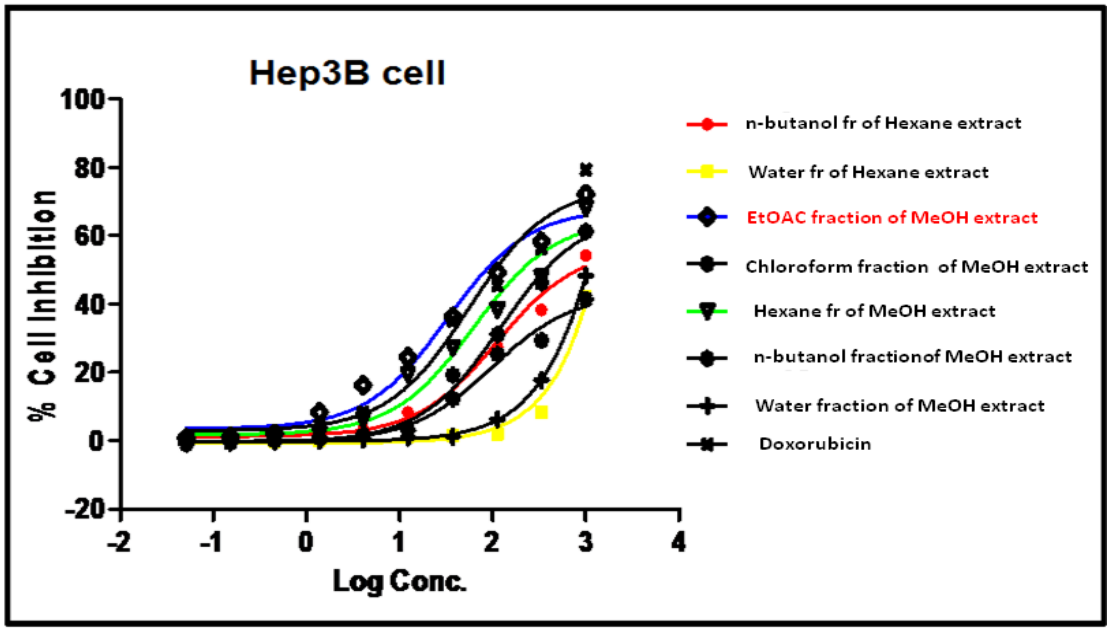


**Figure 5.5: Dose response curve of standard and samples on Hep3B cell**

## 5.7. Conclusion

Among all the prepared extracts tested for the *in-vitro* anticancer activity using MTT assay on Hep3B (rat hepatoma) cell, ethyl acetate soluble fraction of methanol extract (ESME) was found to be potent cytotoxic ($IC_{50}$ value 32.55 µg/ml) as compared to standard drug doxorubicin. In addition methanol extract, hexane extract, hexane fraction of methanol extract and n-butanol fraction of methanol extract showed the moderate anticancer activity against Hep3B cell. This anticancer activity may be obtained due to the various phytoconstituents present in the plant. Literature review indicates the presence of phenolics, steroids and triterpenoids in the ethyl acetate soluble fraction of methanol extract; which may attribute the anticancer activity.

## 5.8. References

Cory, A. H., Owen, T. C., Barltrop, J. A., Cory, J. G., 1991. "Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture". Cancer communications 3(7), 207-12.

Freshney, I. R., 2005. Culture of animal cells, Amanual of basic technique. 5th ed., Wiley-Liss, 508.

Subhasree, B., Baskar, R., Keerthana, R. L., Susan R. L., Rajasekaran, P. 2009. Evaluation of anti-oxidant potential in selected green leafy vegetables. Food chemistry 115, 1213-20.

Wilson, A. P., 2000. Cytotoxicity and Viability Assays in Animal Cell Culture: A Practical Approach,Vol. 1, 3rd ed. Oxford University Press, New Delhi.