JOURNAL OF CANCER THERAPEUTICS AND IMMUNOTHERAPY
Jan-Mar 2025, VOL. 2, ISSUE 1, pp. 1-6
https://doi.org/10.61577/jctai.2025.100001

RESEAPRO JOURNALS

ORIGINAL

OPEN ACCESS

Anti-Proliferative action and cytotoxic effect on chloroform extract of Shorea robusta plant resin on Cancer cell lines

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ABSTRACT

Present study investigates anti-proliferative and cytotoxic effect of chloroform extract of Shorea robusta plant resin on murine and human cancer cell lines. The plant resin powder sample was collected after air dried and extracted by cold percolation method in chloroform and column fractionate the chemical constituents into individual fractions. The confirmation of compounds present with different solvents by using preparative and analytical Thin Layer Chromatography (TLC) methods. Cytotoxic effects of chloroform extract of S. robusta plant resin were analyzed through various of morphological changes like Trypan Blue exclusion assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and4',6-di-amidino-2-phenylindole (DAPI) Test. Apoptotic or necrotic cell death by Acridine Orange/Ethidium Bromide (AO/EtBr) double staining was investigated. We found that the half-maximal inhibitory concentration (IC50) value of S. robusta resin was 163.94, 120.57, 144.89, 189.54. Hence extract of (70%) chloroform of S. robusta resin sample was used for its anti-inflammatory and anti-proliferative actions, which induced apoptosis in cancer cell lines.

KEYWORDS

Shorea robusta; Anti-proliferative; Cytotoxicity; Chloroform; Resin; Cancer

ARTICLE HISTORY

Received 21 February 2025;
Revised 03 March 2024;
Accepted 27 March 2024

Introduction

S. robusta (Sal), a huge deciduous tree belonging to the Dipterocarpaceae family, is highly valued for its medicinal, cultural, and economic importance. Different parts of this tree have been traditionally used in indigenous medicine systems, including Ayurveda, to treat a wide range of ailments, from diarrhoea and diabetes to bacterial infections. The presence of diverse phytochemicals, such as Asiatic acid, triterpenic acid, tannic acid, and phenols, contributes to its reported antimicrobial properties [1]. Globally, flowering plants represent a rich source of potential medicinal compounds [2]. The Dipterocarpaceae family, particularly the Shorea genus (Meranti), is notable for its diverse secondary metabolites. Shorea species, primarily found in Southeast Asia, have been extensively studied, revealing a variety of compounds, including coumarins, terpenoids, and oligostilbenoids [1]. Oligostilbenoids, a class of polyphenolic stilbenes, have demonstrated an extensive range of biological activities, with antioxidant, chemo preventive, anti-inflammatory, and antimicrobial effects [3]. S. robusta itself has been the subject of several studies exploring its traditional uses, photochemistry, and pharmacological properties. Its bark, in particular, has a long history of use in traditional medicine [4]. Phytochemical analyses have identified a variety of constituents, including terpenoids, flavonoids, carbohydrates, lignans, phenols, and sterols. These compounds are thought to contribute to the diverse pharmacological actions reported for S. robusta, including anti-inflammatory, anti-obesity, antimicrobial, wound healing and immunomodulatory effects. This study consolidates the available literature on S. robusta, examining its traditional uses, phytochemistry, and pharmacological activities to highlight its therapeutic potential and guide future research directions.

S. robusta and related species have demonstrated a range of promising pharmacological activities [5]. S. robusta leaf extracts exhibit significant anti-inflammatory effects in various rat models, including carrageen an-induced paw edema and cotton pellet granuloma, with some extracts surpassing the activity of standard drugs like diclofenac sodium [6]. These effects are linked with the presence of various phytochemicals, including flavonoids, tannins, and phenols. Furthermore, S. robusta resin displays gastro protective properties, mitigating ethanol and pyloric ligation-induced gastric ulcers in rats by stabilizing antioxidant markers and reducing gastric acid secretion [7]. Beyond S. robusta, S. roxburghii has yielded stilbenoids and dihydroisocoumarins with hepatoprotective action against D-galactosamine/lipopolysaccharide-induced liver injury in mice, potentially through modulating macrophage activation and TNF-α sensitivity. Specific compounds like alpha and beta amyrin, isolated from S. robusta, have shown potential against hepatocellular carcinoma in molecular docking studies [8]. Additionally, S. robusta resin promotes wound healing in rats by increasing hydroxyproline content and tensile strength [9]. Finally, various stilbenoids and related compounds from Shorea species have demonstrated diverse cytotoxic activities against different cell lines, with some, like (-) hopeaphenol, exhibiting potent cytotoxicity against specific cancer cell lines [10]. These diverse biological activities highlight the potential of Shorea species as a source of valuable medicinal compounds.

Materials and Methods

Collection and extraction of the samples

The plant samples are being collected from the vicinity of the

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North Odisha and the surrounding areas of the town Baripada (Figure 1 and Figure 2) and approximate amount of around 1kg of resin samples of S. robusta was collected and air dried in dark for around 2 weeks. The air-dried samples are then grinded with the help of mechanical grinder and the resultant powder was then packed in the zip lock polythene bags to keep them moisture free and stored at cold temperature for further use.

Figure 1. Sampling site with S. robusta plant

Figure 2. Plant S. robusta with resins

Extraction of crude plant extract

By using cold percolation method, the air-dried resin sample was powdered with different solvents of increasing order of polarity, starting with a highly non-polar solvents such as petroleum ether followed by chloroform water, ethyl acetate, methanol and ethanol. The plant samples are weighed to 20gm and are poured into the wide mouth bottles of volume 500ml. Then prepare 150ml Petroleum ether, Chloroform, Ethyl acetate, Methanol, Ethanol, as a solvent then aqueous was added to in five steps each step comprising of one solvent and was allowed to stand for 4hours. After that the solvents were filtered and allowed to stand for another 24hours for the reduction of the solvent. After evaporation of the solvent, the residue was transferred to 100ml reagent bottles. These are labelled as the crude extracts and were tagged with the name of the plant's samples and the various solvents totaling to a volume of 45ml at least. From the water extract, 20ml of extracts has been taken for column fractionation and 5ml stored at deep freeze for the Cytotoxicity test.

Separation of compound by column fractionation

Principle

In the solvent there is a specific solubility in every compound of a mixture and a specific affinity to be adsorbed by the solid adsorbent. It is used in column chromatography.

Preparation of column

Column size 90cm X 2.5cm used as an adsorbent of silica gel mesh 60-120,100-200 made into a homogenous suspension by shaking with petroleum ether is used (Figure 3). The lowermost of the column was plugged with little cotton to prevent the adsorbent pass out, then the silica gel suspension was poured into the column.

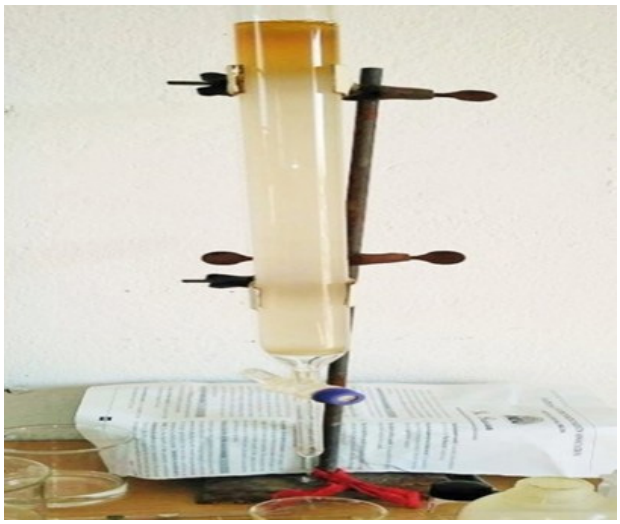

Figure 3. Column chromatography

The extracts of Shorea combined with chloroform were subjected to chromatography over (100-200 mesh) silica gel, then polarity increases with solvents was eluted with column. These are Chloroform from 20-100%, like Chloroform 80%, 60%, 40%, 20% and Ethyl acetate 20%, 40%, 60%, 80% etc and Methanol 40%,60% and 100% respectively.

Thin layer chromatography (TLC) profile of the crude extracts

Thin Layer Chromatography is an effective technique used for identification and the separation of chemical constituents of an extract. In the early 70's the Pharmacopoeias increasingly employed this technique for assessing the quality and purity of compounds of both synthetic and natural origin.

Solvent systems used in TLC

Various solvent system used in different ratios such as solvent system I [Chloroform: Acetonitrile: Acetic acid (40:30:30)], solvent system II [Hexane: Ethyl acetate: Methanol (40:30:30)], solvent system II [Hexane: Chloroform: Ethanol (50:25:25)], solvent system IV [Hexane: Chloroform: Ethanol (60:20:20)].

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After pre-saturation with mobile phase for 20 min development were used. For the detection of the bands on the TLC plates are dried and sprayed freshly prepared iodine reagents were used. The movement of the active compound was expressed by its retention factor (Rf), values were calculated for different samples.

$$R_f = \frac{Distance\ Travelled\ by\ solute}{Distance\ travelled\ by\ solvent\ front\ of\ TLC}$$

Analysis

Various methods are adopted to visualize the spots because of the chemicals are colourless and separated with iodine vapors are a common unspecific colour reagent. The TLC plate is dipped into the specific colour reagents and sprinkled on top of the plate like Potassium permanganate.

Application of extracts for separation method

The various diluted extracts spotted on a TLC plate 2cm above its bottom using capillary tube and solutions for application were between 0.1 - 1% strength. The starting point was equally sized and spots with diameter ranging from 2-5mm. The extracts with petroleum ether, n-hexane, chloroform, acetone, alcohol and aqueous extracts were subjected to Thin Layer Chromatography (TLC) using different solvent systems and observed for characteristic spots under UV light and Iodine chamber.

Culture of cancer cell lines (PBMC)

The cell lines of normal hepatocarcinoma were obtained from National Centre for Cell Science (NCCS), Pune, cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% (FBS)+1% antibiotic Penicillin-Streptomycin-Neomycin (PSN) solution, incubated at humidified temperature37°C with 5% Carbon dioxide. After 75-80% confluent, cell was harvested with 0.025% trypsin and 0.52mM Ethylene diamine tetra acetic acid (EDTA) in Phosphate Buffered Saline (PBS) and a day before the start of experimentation were seeded at density to allow them to re-equilibrate. All experiments were used (DMEM,) as a basal medium for supplementing the growth of different cells with 10% FBS,1% antibiotic and solution of Antimycotic respectively [11].

Detection of cell morphology

The crude plant extract was dissolved dimethyl sulfoxide (DMSO) and mixed thoroughly by using a vortex rotator and one minute centrifuged at 1000rpm in a table centrifuge. The prepared drugs then were added to normal liver cell line cultured in the 96 well plates in duplicates for 24 and 48 hours. The morphological changes take place after the incubation the grown in 96-well tissue culture plates and observed with an Inverted Phase Contrast Microscope.

Assessment of cell cytotoxicity

DAPI staining

To assess nuclear damage cells treated with crude extracts and after the treatment, cells are harvested, washed thoroughly with PBS and fixed at room temperature with for 2hours with 3.7% paraformaldehyde, then stained the fixed cells with 10µg/ml of 4',6'-diamidino-2-phenylindole (DAPI) with excitation at 359nm and emission at 461nmk was observed under Fluorescence Microscope [12].

Acridine Orange / Ethidium Bromide (AO/EtB) staining

The conventional (AO/EtB) staining process was followed to distinguish the live, apoptotic and necrotic cells. These are treated with crude extracts were stained with (50µg/ml) of Acridine orange and Ethidium bromide was analyzed under fluorescent microscope. Photographs were taken by the help of digital camera.

Anticancer assays

In vitro anti-cancer screening

In the field of cancer, there are two types of in vitro assays such as molecular and cellular assays. Molecular assays are very much particular as directed at a single subcellular target. They are particular importance with specific mechanism is of interest in case of drug discovery program, binding and inhibition assays can be used to discover new compounds. Because of their specific activity such type of assays results in a low hit rate from a large number of diverse samples screened. More than one mechanism a battery of such screens is often used in conjunction to detect working conditions of compounds [13]. Cellular assays are also two types that is cytotoxicity & morphological assays.

The use of both types of assays in conjunction with one another, the initial assays of cytotoxicity giving a large number of positive leads, then further screened by biochemical assays to select compounds acting through mechanisms of interest. One must be taking care of employing antagonistic screens such as the positive leads of one assay should be further tested in another assay which is working by an unrelated mechanism.

Cell growth and cytotoxicity assays

There are four important types of non-radioactive cell growth and cytotoxicity assays [14] are colony/cell counts, macromolecular dye binding, metabolic impairment and membrane integrity, Sulforhodamine B (SRB) assay etc. It is a bright pink amino xanthene dye found under mildly acidic conditions and binds to basic amino acid residues of Trichloroacetic acid (TCA) fixed proteins. The end-point is stable and does not have to be measured within any fixed period of time. After proper stained and air dried, plates can be kept for months before solubilization and reading. For large scale drug screening, this assay has proven very useful.

MTT assay

The measurement of cell viability and proliferation forms the numerous in vitro assays of a cell population's response to external factors [15]. Now a days examine of cell proliferation is widely accepted and reliable to the reduction of tetrazolium salts. The yellow tetrazolium MTT is reduced by metabolically active cells, by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. It can measure the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis and reduction in cell viability. The MTT reagent yields low background absorbance values in the absence

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of cells. The linear relationship between cell number and signal produced is established in case of each cell type and allowing an accurate quantification of changes in the rate of cell proliferation. The number of assay steps is as follows:

1st Day: One flask (T-25) with 5ml of complete media to trypsinized cells then centrifuge for 5mins, in a sterile 15ml falcon tube at 500rpm in the swinging bucked rotor. Media removed and resuspend cells to 1.0ml with complete media then count and record cells per ml with aseptically. Dilute the cells to 75,000 cells per ml and use complete media to dilute cells. At last, add 100µl of cells (7500 total cells) and incubate overnight.

2nd Day: Treat cells on second day with against inhibitor or drug. Final volume should be 100µl per well.

3rd Day: Add 20µl of 5 mg/ml MTT to each well should be done aseptically. Include one set of wells with MTT but no cells (control). Incubate for 3.5 hours at 37 in culture hood and remove media carefully without disturb cells. Add 150µ l MTT solvent, cover with tinfoil and agitate cells on orbital shaker for 15 min then read absorbance at 590nm with 620nm reference filter.

Results and Discussion

The extracts that are being done by are being treated with blood cells. The cells exhibit cell death i.e. apoptosis of the cell happen due to cytotoxicity of the drugs that are being treated to them the main drug range where drug concentration obtained from 5 to 40µg/ml shows low level cytotoxicity, whereas drug level 50 to 100µg/ml shows medium level cytotoxicity, and with drug level more than 100 µg/ml shows full cell population. The level of cytotoxicity and main characteristics can be observed from the staining, where the formation apoptotic body and cell blabbing takes place. The cytotoxic activity of S. robusta was determined by using TC1 cell lines. The results indicate the presence of polyphenols and aqueous resin extract had shown (Figure 4-8) dose depend effect on TC1 cell line proliferation, but minimal inhibitory effect was observed [16,17].

Growth inhibitory effect of Chloroform SR on various cancer cells

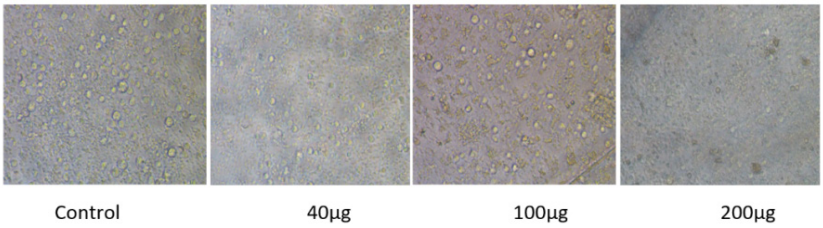

Figure 4. PBMC

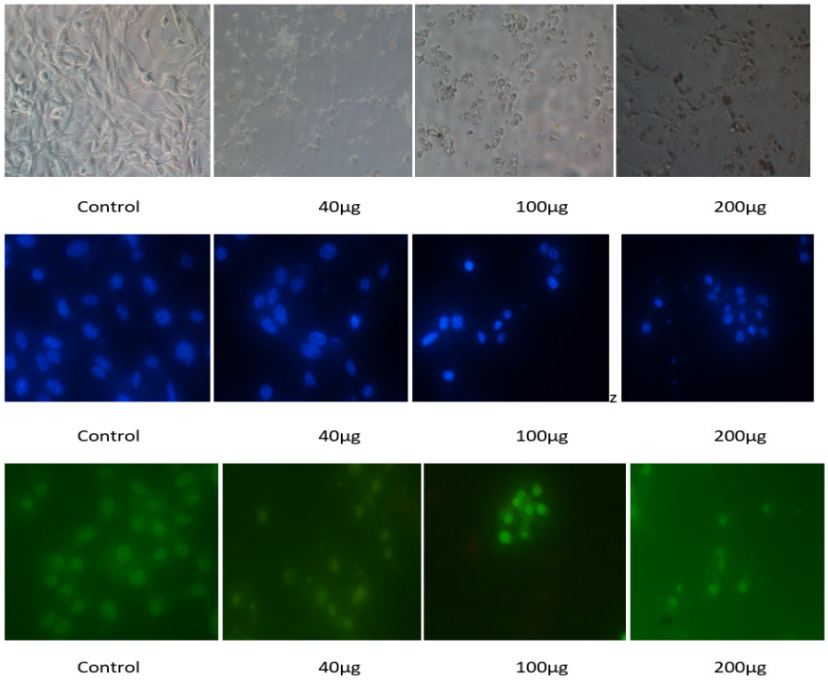

Figure 5. TC1 Drug

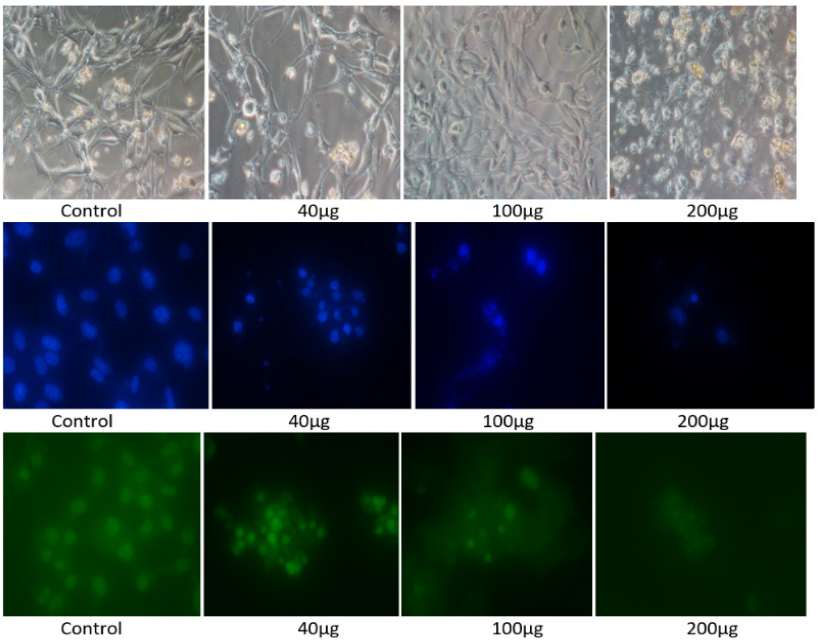

Figure 6. Drug: 106

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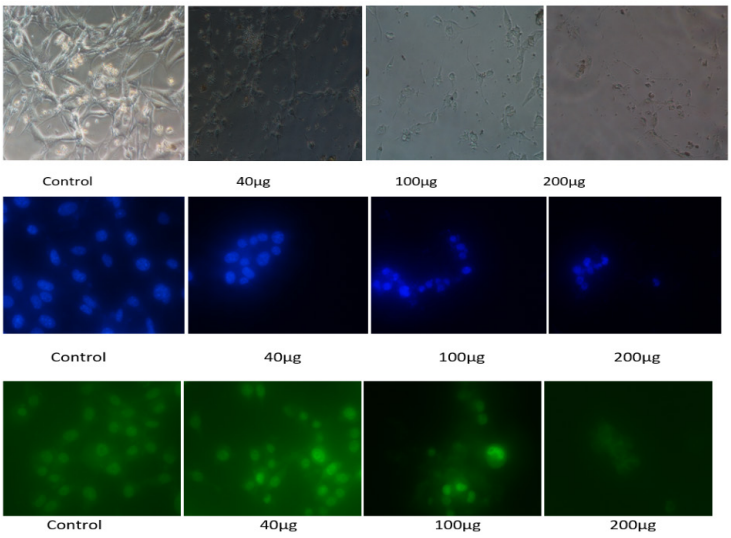

Figure 7. Drug: 107

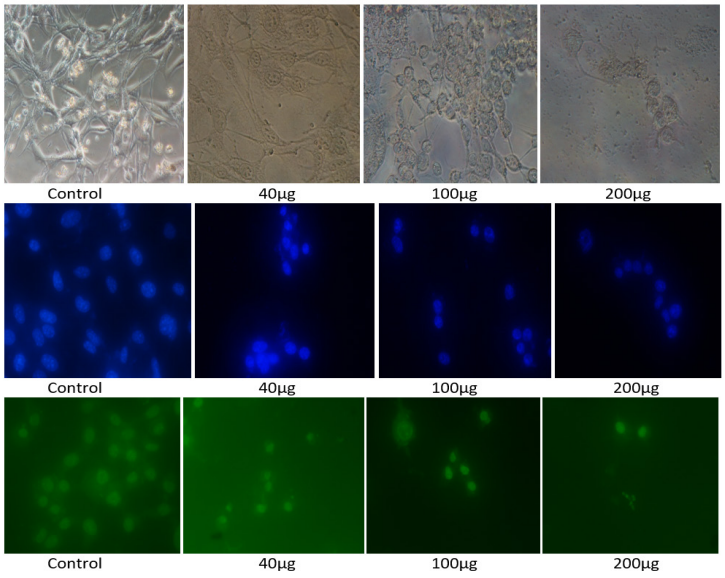

Figure 8. Drug: 112

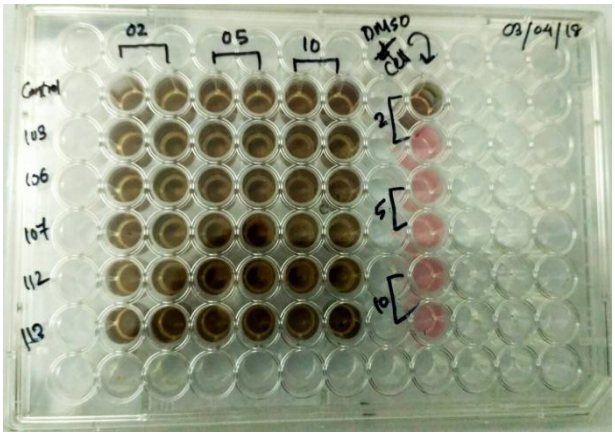

Figure 9. MTT Assay in 96 well plate

and blood cell on (A) Blood cells (Figure 9) TC1. Cells were treated with different concentrations like 0 µg, 40µg, 100µg and 200µg of the CSR for 24hours and viability was measured by using MTT assay (Figure 10 and 11). The data were represented as mean ± SD from triplicate independent experiments. Morphological and nuclear changes in (A) Blood cell and (B) TC1 treatment for 24 hours. After treatment morphological, nuclear changes seen under light microscope and fluorescence microscopy after DAPI and AO/EtBr staining respectively. The chloroform extract of S. robusta plant resin inhibit proliferation in cancer cell and may induce apoptotic cell death. The extracts of the sample may be used to determine bioactive natural products which may be serve as a basic source for the development of new drugs for therapy of different diseases like skin allergies, diarrhea, and dysentery. Apoptotic alterations like cytoplasmic blabbing, chromatin condensation, DNA fragmentation with irregularity in shape in case

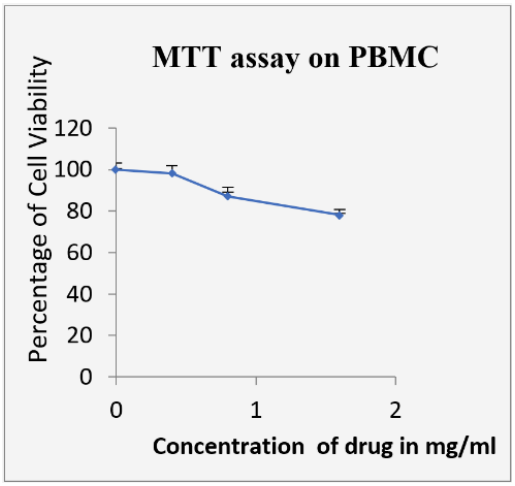

Figure 10. Blood cells treated with different Conc. with cell viability

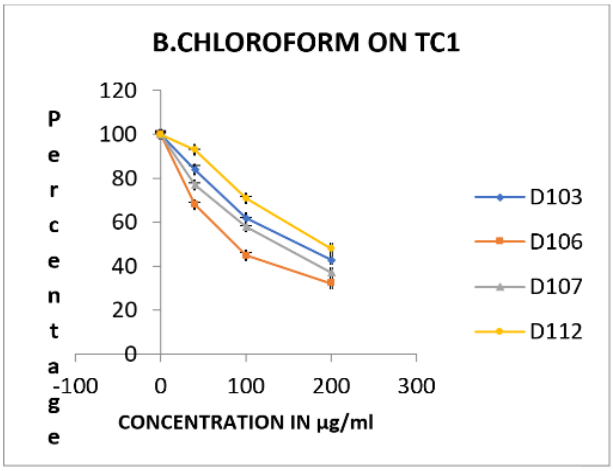

Figure 11. TC1.Cells were treated with different concentrations (0, 40, 100 and 200µg) of the CSR for 24 hour & viability was measured by MTT assay

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of morphological analysis were detected by TC1 cell lines and significantly different from the Peripheral Blood Mononuclear Cell (PBMC) normal cell line.

Conclusion

The present results suggest that the chloroform extract of S. robusta plant resin might be feasible source for biologically active anticancer substances for therapeutic use. The result of initial screening work, aimed at the identification of active components present in these extracts and further studies elucidation of their mechanisms as cancer therapeutics are warranted. It also provides us to support the uses of the plants in traditional medicine hence various toxicological analysis of the active compounds is necessary, to assess step by step its tolerance in the human body after administered. The current study may be concluded that resin of S. robusta have a stronger and broader spectrum of antimicrobial activity against a number of pathogenic microorganisms.

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