

POTENT *IN VITRO* ANTICANCER ACTIVITY OF *SYMPLOCOS RACEMOSA* BARK

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Objective. Assessment of anti-tumor activity of *Symplocos racemosa* Roxb. (Symplococaceae) plant extracts. *Methods:* Plant bark was procured, identified and verified. Chloroform, butanol and ethyl acetate extracts were prepared and their cytotoxic activity determined using the XTT salt based cytotoxicity assay in 96-micro plate format against one leukaemia and one cervical cancer cell line. Cyclophosphamide was used as positive control. Absorbance was measured after 48 h at 450 nm. *Results.* Total 3 extracts evaluated, butanolic and ethyl acetate extracts demonstrated strong cytotoxic potential overall. Cell proliferation assay showed dose dependent inhibition of cell growth. Butanolic extract was found cytotoxic against HL 60 (Human leukemia cell line, IC₅₀ = 27183 ng/ml), HeLa (Human cervix cancer cell line, IC₅₀ = 22861 ng/ml), Ethyl acetate extract was found to be less cytotoxic against HL 60 (IC₅₀ = 117084 ng/ml), HeLa (IC₅₀ = 137151 ng/ml). Chloroform extract displayed no cytotoxicity against both cell lines. *Conclusion:* *S. racemosa* shows potential anticancer activity.

Key words: HL60, HeLa, cell line, plant based drug.

INTRODUCTION

Cancer is the second leading cause of death in the world (Madhusudan, 2005). Throughout history and across the world, the plant kingdom has provided a variety of medicines for cancer treatment. In modern times, plants have been a source of analgesics, anti-inflammatories, antiasthmatics, antiarrhythmic agents, antihypertensives, antimicrobial agents known to be numerous. Currently over 60% of the drugs are derived in one or other way from natural source including plant, marine organism and micro-organism (Newman, 2003). There are worldwide efforts to discover anticancer agents from plants.

Symplocos racemosa is a traditional Ayurvedic medicine. The plant, plant extract and isolated salireposides have been extensively investigated in several laboratories for their menstrual regulatory effects and a number of reports are available confirming their action. In addition, researchers have evaluated the anti-bacterial effect of *S. racemosa* extracts against acne inducing bacteria (Kumar, 2007).

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Symplocos racemosa is proven to contain the dithiadiazetidins namely symplolate, as well as linoleic acid, oleic acid, salireposide, symplocoside, beta-sito-glycoside, symponoside, symplioside, symploveroside, benzoylsalireposide and salireposide (Abbasi, 2004), (Abbasi, 2005), (Ahmed, 2007). No cytotoxicity of *S. racemosa* has been reported.

MATERIALS AND METHODS

Reagents. Trypan blue, Triton X100, DMSO cell culture grade, Sodium bicarbonate, Antibiotic/ Antimycotic solution, 100X (10 000 U/ml Penicillin G, 10,000 µg/ml Streptomycin, 25 µg/ml Amphotericin B), EDTA, DPBS/modified 1X (Dulbecco's phosphate buffer saline without Ca⁺ & Mg⁺), 0.25% Trypsin 1X, Cell proliferation kit (XTT) 2 500 tests, Cyclophosphamide monohydrate as a control, HBSS – 1X (Hank's Balanced Salt solution).

Media. DMEM (Dulbecco's Modified Eagles medium, low glucose with glutamine), FBS (Fetal Bovine Serum, South American origin, 500 ml), SFM HEK-293 (Serum Free Media, 500ml).

Cell Lines HeLa (Human cervix carcinoma cell line), HL60 (Human leukemia cell line), Both cell lines were purchased from NCCS, Pune Job. No. 669-670.

Plant Collection. Ethnobotanical literature was reviewed and plant bark was procured from Lallubhai Vrajlal Gandhi, Ahmedabad, Gujarat, India and authenticated by Dr. Ritesh Vaidya, Bioscience Department, Ganpat University.

Aqueous Extract Preparation. Bark was shed, dried and ground by simple grinding machine to visually fine powder. No particle size analysis was done. The fine powder of *S. racemosa* (500 g) was exhaustively extracted with 1.5 l methanol for 24 h at room temperature. After 24 h, the methanol was evaporated and dried residue was subjected to successive partition with chloroform, butanol and ethyl acetate, respectively. All three layers were collected individually and were subjected to evaporation and drying. Extracts were dissolved in 2% Dimethyl Sulphoxide (DMSO) solution. 10 serial dilutions were prepared from each of the extracts ranging from 100 µg/ml to 0.005 µg/ml.

Preparation of Media Dmem (1 × Liquid Medium With Fbs & Antibiotic) 5% less distilled water was measured out than the total volume of medium desired. Powdered medium was added to water with gentle stirring. 3.7 g of NaHCO₃ was added per litre of medium. Diluted to a desired volume with water and stirred until dissolved. pH of medium was adjusted to 0.2–0.3 below desired final working pH. After pH adjustment, was kept in a closed container and sterilized immediately by membrane filtration. DMEM, Antibiotic, and FBS (Fetal bovine Serum) were prepared. 10% FBS and 5 ml antibiotics were added into the DMEM. (Freshney, 2005).

Subculture of Cell Lines Cultures were viewed using an inverted phase contrast microscope. Centrifugation for subculture was not performed unless the pH of the medium was acidic which indicated the cells overgrown and were not recovered. Cells were centrifuged at 150g for 5 minutes, re-seeded at a slightly higher cell density and 10-20% of conditioned medium added to the fresh media. A small sample of the cells from the cell suspension was taken. Cells/ml was calculated and the desired number of cells re-seeded into freshly prepared flasks without centrifugation just by diluting the cells (Freshney, 2005).

XTT Assay XTT assay was employed to assess cell proliferation. Viable cells were seeded into 96-well microtitre plates at 5×10^4 cells/well in DMEM (Dulbecco's modified Eagle's medium) supplemented with FBS (fetal bovine serum), 100units/ml penicillin, 100 μ g/ml streptomycin and cultured in a humidified atmosphere of 5% CO₂ and 95 air at 37° C. 180 μ l of cell suspension was cultured with 20 μ l of various concentrations of all three extracts (0.005-100 μ g/mL) dissolved in 2% DMSO solution. Control cells were incubated in culture medium only. Wells containing only media were considered as a blank. All extract dilution doses were tested in duplicates.

The cell proliferation is based on the ability of the mitochondrial succinate-tetrazolium reductase system to convert yellow tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4 methoxy-6-nitro) benzene sulfonic acid hydrate) to orange formazan dye. The test denotes the survival cells after toxic exposure. 50 μ l of XTT mixture was added to each well. After 48h incubation at 37° C temperature and 5% CO₂, the absorbance of soluble formazan product produced by viable cells was measured at 450nm using ELISA plate reader (Thermo, USA). Reference wavelength used was 650 nm.

Percentage inhibition of the extract against all cell line was calculated using the following formula.

$$\% \text{ cell survival} = \frac{(\text{At} - \text{Ab})}{(\text{Ac} - \text{Ab})} \times 100$$

At = Absorbance of Test,

Ab = Absorbance of Blank (Media),

Ac = Absorbance of control (cells)

% cell inhibition = 100 - % cell survival

The effects of extracts were expressed by IC₅₀ values calculated from dose response curves.

RESULTS AND DISCUSSION

The effects of chloroform, butanol and ethyl acetate extracts of *Symplocos racemosa* bark (test) and cyclophosphamide (control) on the growth of HeLa and

HL60 cell lines were examined by the XTT assay. Dose response curves constructed between the range 0.005–100 $\mu\text{g/ml}$ and 0.005–100 μM for *S. racemosa* extracts and cyclophosphamide (control) respectively, express a decreasing number of viable cells with increasing concentration of extract as well as of cyclophosphamide. Calculation of IC_{50} and R^2 value was done using graphs generated from Microsoft Excel 2003 edition (Figs. 1–3). The susceptibility of cells to the extract and cyclophosphamide was characterized by IC_{50} values (Table 1). Results indicate that the cytotoxic effect strengthens with increase in the concentration of extract.

Table 1

IC_{50} values of various extracts of *Symplocos racemosa* and cyclophosphamide calculated from dose response curve on Figs. 1–3 respectively

IC_{50}	HL-60	HeLa
Butanol Extract (ng/ml)	27183	22861
R^2	0.9856	0.9753
Ethyl acetate Extract (ng/ml)	117084	137151
R^2	0.9916	0.8808
Cyclophosphamide (nM/ml)	602143	30492
R^2	0.9633	0.9817

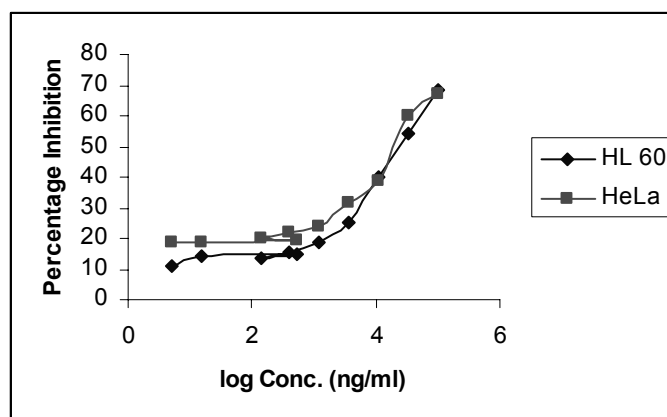


Fig. 1. XTT cytotoxicity dose response curve of butanol extract of *Symplocos racemosa* against HL60 and HeLa cell lines (48 h).

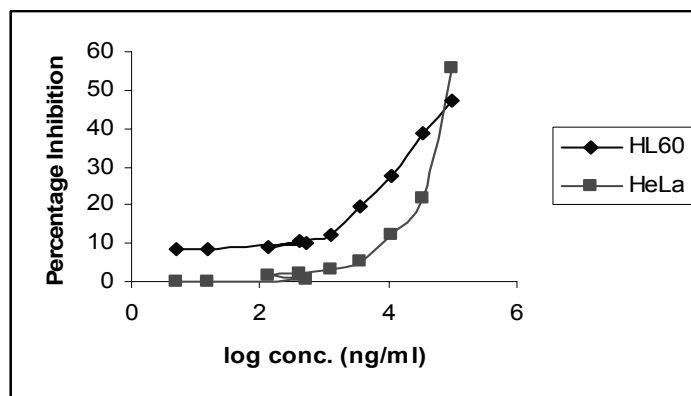


Fig. 2. XTT cytotoxicity dose response curve of ethyl acetate extract of *Symplocos racemosa* against HL60 and HeLa cell lines (48 h).

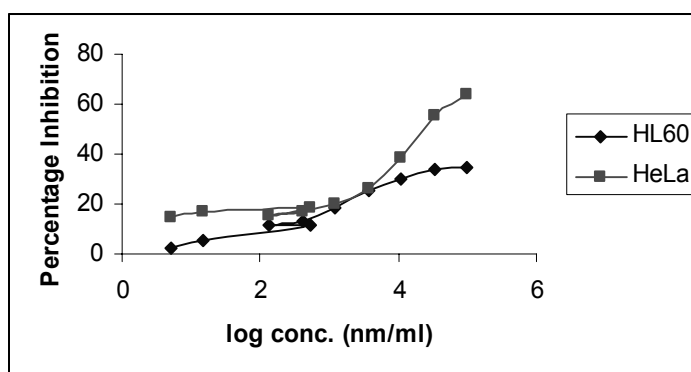


Fig. 3. XTT cytotoxicity dose response curve of cyclophosphamide against HL60 and HeLa cell lines (48 h).

The highest cytotoxicity of butanol extract was found against HeLa cell line, which is more potent than that of cyclophosphamide, which shows that the extract was proven more active against the HeLa than the cyclophosphamide, while in case of ethyl acetate extract the highest cytotoxicity was found against HL60 cell line.

Butanolic extract also exerts the high cytotoxicity against HL60, which is higher than cyclophosphamide cytotoxicity against this cell line. Ethyl acetate extract was also found to be toxic against HeLa cell line. Chloroform extract was found to be non-toxic against both cell lines. The reason behind such cytotoxicity of butanol and ethyl acetate can be the presence of certain phytochemicals such as phenolic glycosides or dithiadiazetidins derivatives. The mechanism behind cytotoxicity can be apoptosis as revealed by structure activity relationship of the phytochemicals present.

CONCLUSIONS

Different cytotoxicity of *Symplocos racemosa* extracts against both cancerous cell lines evaluated is based on their chemical composition and relative content of biologically active substance. The literature data prove the presence of phenolic glycosides, salireposide and benzoyl salireposide, which are found to be present in ethyl acetate extract. The butanol extract has not been explored much to confirm phytochemicals present in it. There are also lots of literature data proving the different pharmacological activity of *Symplocos racemosa* extract, e.g. gonadotropin releasing, antioxidant, antiarthritic and antibacterial (Choudhary, 2004), (Bhutani, 2004) (Kumar, 2007).

Further studies are required to determine intracellular pathway(s) involved in the mechanism of cytotoxicity. This study provides only basic data. Further studies with isolated active substances from extract of analyzed plant should be undertaken.

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