

Evaluation of *In vitro* Cytotoxic Activity of Ethanolic Extract of *Symplocos racemosa* Roxb

Vijayabaskaran M*, Yuvaraja KR, Saravanakumar M, Abhenaya K

Natural Products Research laboratory, J. K. K. Nataraja College of Pharmacy, Komarapalayam, Namakkal, Tamil Nadu, India

ABSTRACT

Traditional plants are valuable source of novel cytotoxic agents and are still playing greater role in health care. Development of new therapeutic drugs needs assay systems to evaluate the toxicity potential of the drugs early in the drug discovery process. *In vitro* cell-based cytotoxicity assay is an easy and cost effective tool for hit ranking and lead optimization at the early stage of drug discovery. The present study is intended to investigate the *in vitro* cytotoxic study of the bark of *Symplocos racemosa* Roxb (Family: *Symplocaceae*). The Ethanolic Extract of *Symplocos racemosa* (EESR) revealed the presence of carbohydrates, glycosides, alkaloids, terpenoids and phenolic compounds. In pharmacological screening the cytotoxic activity of EESR using 3 human cancer cell lines [i.e. Breast Cancer (MCF7), Colon Cancer (HT29), Liver Cancer (HepG2)] were evaluated with MTT assay method. The result of EESR showed potent cytotoxic effect on HT29 cell line, moderate in MCF7 cell line and less cytotoxic effect on the HepG2 cell line.

Keywords: *Symplocos racemosa*, MTT assay, Cell Lines, Cytotoxicity.

INTRODUCTION

Nature has provided an excellent storehouse of remedies to cure all the ailments of mankind. In ancient days, almost all the medicines used were from natural sources, particularly from plants. Plants continue to be an important source of new drugs even today. The importance of botanical, chemical and pharmacological evaluation of plant-derived agents used in the treatment of human ailments has been increasingly recognized in the last decades. Herbal remedies are widely used for the treatment and prevention of various diseases and often contain highly active multitude of chemical compounds. Modern research is now focusing greater attention on the generation of scientific validation of herbal drugs based on their folklore claim. In this modern era, a large Indian population still relies on the traditional system of medicine, which is mostly plant based. *Symplocos racemosa* Roxb, a plant belonging to the Family: *Symplocaceae* is a small evergreen tree with stems up to 6m height and 15 cm diameter. Bark dark grey, rough. Blaze 7.5-13 mm. [1] Shortly fibrous, pale yellow, finely mottled with pale orange brown. The plant grows throughout North East India, very commonly in the plains and lower hills of Bengal, Assam and dry forests of Chota Nagpur plateau. The medicinal attributes of *Symplocos racemosa* have been known since a long time.

A number of pharmacological properties have been reported the plant includes human recombinant nucleotide pyrophosphates phosphodiesterase-1 inhibition [2], gonadotropin release in immature female rats [3], thymidine lipoygenase inhibition [4] phosphorylase-inhibition [5], wound healing activity [6], anti- fibrinolytic activity [7], skin topical agents, preventing skin aging and skin whitening agents. In indigenous system of medicine it has been used as anticancer agent. [8] The present study aims the evaluation of *in vitro* cytotoxic activity of ethanolic extract of *symplocos racemosa* Roxb.

MATERIALS AND METHODS

Collection and Preparation of Plant Materials

The plant *Symplocos racemosa* (Family: *Symplocaceae*) was collected from Kolli Hills at Namakkal District, Tamilnadu, India. It was authenticated by the Dr. V. Sathyanathan, Taxonomist, Epoch Pharma and Research Labs Pvt. Ltd. Chennai, and its voucher specimens were deposited in the Herbarium for further reference. After proper identification, the bark of *Symplocos racemosa* was dried under shade and then coarse powdered with a mechanical grinder. The coarse powder of bark stored in an airtight container for further use. The dried coarse powder material of bark (250 g) was extracted with ethanol (95 %) for 72 h in Soxhlet apparatus. The extract was made solvent free distillation process under reduced pressure and the resulting semisolid residue was vacuum dried using rotary flash evaporator. [9] The yield of

*Corresponding author: Mr. M. Vijayabaskaran,
Natural Products Research laboratory, J. K. K. Nataraja
College of Pharmacy, Komarapalayam, Namakkal, Tamil
Nadu, India; E-mail: vijayabass@gmail.com

the ethanolic extract was 18.28 % w/w and it was used for the study of *in vitro* cytotoxic activity.

Chemicals

RPMI-1640 media, Penicillin-G, Streptomycin, Amphotericin-B, Phosphate buffered saline (PBS), Ethylenediamine tetra-acetic acid (EDTA), Trypan blue, SDS lysis buffer, MTT (3 - (4, 5 - dimethyl thiazol - 2yl) - 2, 5 - diphenyl tetrazolium bromide) all were purchased in Himedia, Mumbai, India. Fetal bovine serum (FBS), Trypsin 0.25 % were purchased in Gibco's, USA. Dimethyl sulphoxide (DMSO) was purchased in Merck India Ltd, Mumbai, India.

Equipments

CO₂ incubator, Laminar air flow cabin, Refrigerated centrifuge, ELISA-reader (for MTP), Deep freezer, Ultrasonic bath (Transonic [460/H]), Vacuum pump (Zenith [model: PDF-2-2.5]), Pipettes, Culture plates, Centrifuge tubes, Eppendorf tubes, Aerosol resistant tips, Flat-bottomed 96-well MTP, tissue culture grade.

Preparation of antibiotic solutions

Penicillin-G

0.61 g (one vial) of penicillin-G was weighed and dissolved in 1 ml of sterile phosphate buffered saline (PBS). Contents were stirred for 5 minutes. The contents were sterilized with syringe by passing through 0.22-micron filter aliquot of 5 ml fractions in 15 ml storage vials. Stored at -20°C until use.

Streptomycin sulfate

10 g (one vial) of streptomycin sulfate was weighed and dissolved in 10 ml of sterile PBS. 250 mg of Amphotericin-B was weighed and dissolved in 10 ml of sterile PBS. ^[10]

Preparation of Dulbecco's PBS

9.6 g (one vial) of D-PBS was suspended in 800 ml triple distilled water and mixed until dissolved. It was then autoclaved at 121°C (15 lbf) for 15 minutes. After cooling to room temperature aseptically, sterile 100ml CaCl₂ (1 mg/ml) solution and 100 ml MgCl₂ (1 mg/ml) solution were added and mixed. ^[11]

Preparation of Lysis buffer

15 g of NaCl was weighed and dissolved in 1:1 mixture of dimethylformamide and distilled water. The final pH was adjusted to 3, with O-phosphoric acid.

Preparation of RPMI-1640 Medium

10.39 g (one vial) of RPMI-1640 was weighed in a sterile conical flask. The contents were dissolved in 900 ml of Milli-Q (triple distilled) water by stirring and the antibiotic solutions were added (100 U/ml Penicillin-G Sodium, 50 µg/ml of streptomycin and 2 µg/ml of Amphotericin-B) Once all constituents of the medium were completely added, the pH was adjusted to 7.2 to 7.5 with 0.1 N HCl. The volume was made up to one liter with triple distilled water. Contents were transferred to the Millipore filtration kettle supported by 0.22 microns membrane filter, kept in laminar flow hood that was connected to the outlet by negative pressure pump and filtered. Pressure was adjusted so that the flow rate of medium from the filtration unit is 10-12 min/L i.e., 100 ml/min. 5 ml of the sample is taken in T₂₅ tissue culture flask; kept for 24 hours in CO₂ incubator for sterility check. 100 ml of sterile Fetal Bovine Serum was added to 900 ml medium. Reconstituted medium was checked for sterility by transferring 5ml of medium into a T₂₅ tissue culture flask and incubated for 24 hours in CO₂ incubator. ^[12]

Trypsinization

The media was aspirated from each flask, being sure to change pipettes between cell lines to prevent cross contamination. Monolayer was rinsed with 5-10 ml PBS to remove traces of serum and the rinsing solution was aspirated. 1 ml of 0.25 % trypsin-EDTA was added to each flask and spread evenly over cell monolayer and depending on cell type, the flask was either placed in hood or in incubator for 2-5 mins. The flask was gently 'tapped' for dislodging the cells. Then the cells were resuspended in 8 ml of the medium containing serum to stop the action of the trypsin. Gentle pipetting was carried out up and down for breaking up the clumps. Cell suspension was transferred to a properly labelled 15 ml centrifuge tube. The tubes were centrifuged at 1000 rpm for 5 mins. The pellet was resuspended in 5-10 ml of medium depending upon the size of the pellet or cell number.

MTT assay

MTT measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a microtiterplate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. This method involves culturing the cells in a 96-well microtiterplate, and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilised and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP. ^[13]

Cell lines

MCF7 - Breast cancer, HT29 - Colon Cancer, HepG2 - Liver Cancer.

Method

0.1 ml of the cell suspension (containing 5×10⁵ cells /100 µl) and 0.1 ml of the test solution (6.125 -100 µg/ml) in DMSO such that the final concentration of DMSO in media is less than 1 %) were added to the 96 well plates and kept in carbon dioxide incubator with 5 % CO₂, at 37°C for 72 hours. Blank contains only cell suspension and control wells contain 1 % DMSO and cell suspension. After 72 hours, 20 µl of MTT was added and kept in carbon dioxide incubator for 2 hours followed by 80 µl of lysis buffer (15 % SLS in 1:1 DMF and water). The plate was covered with aluminum foil to protect it from light. Then the 96 well plates are kept in rotary shaker for 8 hours. After 8 hours, the 96 well plates were processed on ELISA reader for absorption at 562 nm. The readings were averaged and viability of the test samples was compared with DMSO control. The percentage growth inhibition was calculated using the following formula

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of Individual Test Group} / \text{Mean OD of Control Group}}{\text{Group}} \times 100$$

Scientific strategies for the *in vitro* evaluation of natural products with biological activity have changed in the past few years. One recent development is the highly automated bioassay screening based on colorimetric methods that quantify the proliferation of cell cultures. Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by MTT assay, a non-radioactive, fast and economical assay widely

Table 1: Cytotoxic effect of test compound on Human cancer cell lines

Sample	HT29			HepG2			MCF7			Mean
	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀ (µg/ml)
EESR	80	96	>100	>100	>100	>100	75	>100	>100	85

Average of 3 determinations.

GI₅₀: Drug concentration inhibiting 50% cellular growth following 72 hours of drug exposure.

TGI: Total cellular growth inhibition.

LC₅₀: Concentration of drug resulting in a 50% reduction at the end of drug treatment as compared to that at the beginning.

used to quantify cell viability and proliferation.

MTT is a yellow water soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. MTT is cleaved by all living, metabolically active cells but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogenous cell population.

In the present study the cytotoxic activity of EESR using three human cancer cell lines [i.e. Breast cancer (MCF7), Colon cancer (HT 29), Liver cancer (HepG2)] were evaluated with MTT assay. The cells were treated for 72 hrs with various concentration of ethanol extract (5-100 µg/ml), the relative cell survival progressively decreased in a dose dependent manner.

The GI₅₀ of the EESR was found to be 80, 100 and 75 µg/ml on HT 29, HepG2 and MCF7, cell lines respectively. Among the tested cell lines, EESR was more selective cytotoxic against MCF7 cell line (Table 1).

The TGI of EESR was found to be 96, 100 & 100 µg/ml on HT29, HepG2 and MCF7 cell lines respectively. Among the tested cell lines, EESR was more selective cytotoxic against HT29 cell line.

The LC₅₀ of the EESR was found to be >100 µg/ml for all these three cell lines.

CONCLUSION

The present investigation revealed that the ethanolic extract of bark of *Symplocos racemosa* showed potent Cytotoxic activity against MCF7 cell line, moderate activity against HT29 cell line and less activity on HepG2 cell line. Further investigation is under progress to evaluate the *in vivo* anticancer activity and to determine the exact phytoconstituents responsible for cytotoxic activity.

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