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RESEARCH ARTICLE

APOPTOTIC INDUCTION BY LEAF EXTRACTS OF *Barringtonia acutangula* L. AND *Stereospermum colias* L. IN COLO320 CELLS

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ABSTRACT

In the present scenario where herbal and eco-friendly substances are being preferred over their synthetic counterparts, the present study is a contribution to the ongoing oncology research with a similar aim. *Barringtonia acutangula* L. and *Stereospermum colais* L. plants were selected based on their medicinal properties, which are well documented. The plants were subjected to a series of assays to evaluate its anti-cancer potentials. The preliminary screening by NO assay and MTT assay indicated the free radical scavenging and anti-cancer activity of the plants in the methanol and ethyl acetate extracts of *S. colais* and ethyl acetate extract of *B. acutangula* against Colon cancer cell lines Colo320. Further, DNA fragmentation assay attributed the cytotoxicity of the plant extracts to apoptosis. The CASPASE assays proved the activation of apoptosis in the cells treated with the plant extracts. Hence, it is suggested that both *B. acutangula* and *S. colais* have anti-cancer potentials. The present research suggests the use of these two medicinal plants as a therapeutic agent to treat the deadly disease of colorectal cancer.

INTRODUCTION

Throughout history plants have been used by human beings for medicinal purposes and even in modern times have formed the basis of many pharmaceuticals in use (Schmidt *et al.,* 2008). The complex secondary metabolites produced by plants have found various therapeutic uses in medicine from time immemorial. The early history of modern medicine contains descriptions of plant-derived phytochemicals, many of which are still in use. Some examples are the discovery of cardiotonics in foxglove, salicylic acid in willow bark, and morphine in poppies (Mohammed Rahmatullah *et al.,* 2009).
Barringtonia acutangula L. (Samudraphal,Indian Oak), an important medicinal plant of India has been studied for its phytochemical and pharmacognostic biomarkers in both leaves and bark (Daniel *et al.*, 2011). This plant has been traditionally used for the cure and treatment of many ailments like diarrhoea, antihelmintic, hemolytic disease (various diseases of blood), abdominal colic, malaria and diabetes. Free radical scavenging activity and antioxidant property of the hydroalcoholic extract of plant in invitro models showed dose dependent antioxidant activity (Sahoo, 2008). *Stereospermum colais* L. is one of the 10 plants that are employed in compounding of ayurvedic formulations, known as Dasamoola. Each of the plants is endowed with incredible medicinal properties and they act synergistically in combination (Linga Rao and Savithramma, 2011). This plant has been studied extensively for its antibacterial activity and other medicinal properties (Vijaya Bharathi *et al*., 2010).

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Colon cancer, a cancer that starts in the large intestine or the rectum, is the fourth most commonly diagnosed cancer in the world. It is estimated that worldwide, in 2008, 1.23 million new cases of colon cancer were clinically diagnosed, and that it killed 608,000 people (Ferlay *et al*., 2010). The present study involves the analysis of these two tropical medicinal plants *B. acutangula* and *S. colais* for their efficacy against human colon cancer cell lines.

MATERIALS AND METHODS

Sample Collection

The leaf materials of the plants *S. colais* and *B. acutangula* (Voucher number BRT-P/005 and BRT-P/004) were collected from Anna Hospital Campus, Arumbakkam, Chennai, Tamil Nadu and Kovilanchery, Chennai, Tamil Nadu India respectively. The herbarium specimen was identified by Prof. Narasimhan of Botany Department, Madras Christian College, Chennai. The leaves were separated from other parts, washed, cleaned and dried for further use.

Chemicals

Rosewell Park Memorial Institute 1640 (RPMI 1640) medium, Trypsin Phosphate Versene Glucose (TPVG) and Fetal Bovine Serum (FBS) were purchased from HiMedia, India. Dimethyl sulfoxide (DMSO), Penicillin and Streptomycin was bought from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture plastic wares were from Nunc Co. (Denmark). All other chemicals and solvents were of laboratory grade.

Cell lines

Human Colon carcinoma cell lines Colo320 (Quinn *et al*, 1979) were purchased from the National Centre for Cell Sciences (Pune, India). The cells were cultured in RPMI 1640 containing L- Glutamine and 25 mM 4- [2- hydroxyethyl] –1– piperazineethanesulfonic acid (HEPES), Penicillin (100µg/mL), Streptomycin (50µg/mL) and 10% foetal bovine serum. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Extract Preparation

The plant materials (leaves of *S. colais* L. and *B. acutangula* L.) were air-dried at room temperature (26°C) for 2 weeks, after which it was ground to a uniform powder. The extracts of the leaf samples were prepared in a sequential procedure by soaking 100 g of dried powder in 900 ml of different solvents (Ethyl acetate, chloroform and methanol) for 48 h. At the end of each respective extraction, the extracts were filtered using Whatman filter paper. The filtrate was concentrated under reduced pressure in vacuum at 40°C for 25 min using a rotary evaporator.

Nitric Oxide Assay (NO Assay)

The nitric oxide assay was performed as described previously with slight modification (Yang *et al*., 2009). After pre-incubation of Colo320 cells (1.5×10^5 cells/mL) with LPS (1µg/ml) for 24h, the plant extracts (50µg/ml, 100 µg/ml, 200 µg/ml) were added and incubated for 48h. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µl of cell culture medium was mixed with 100 µl of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader (Tecan, Switzerland). Fresh culture medium was used as a blank in every experiment.

MTT Assay

The cytotoxicity of the plant extracts against Colo320 cells were analysed using the MTT assay (Mossman, 1983). This method is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. In brief, approximately 5x10³cells/well (cell line) were seeded into 96 well plate, 100µl of RPMI 1640 medium was added and incubated at 37ºC. After 24hours, the medium was discarded and fresh medium was added with different concentration of plant extracts (50 µg/ml, 100 µg/ml, 200 µg/ml). The plates were incubated for 48h at 37ºC in a CO_2 incubator. After the incubation period, medium was discarded and 100µl fresh medium was added with 10µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT 5mg/ml). After incubation at 37ºC in a CO_2 incubator for 4h, the medium was discarded and 200µl of DMSO was added to dissolve the formazon crystals. Then the absorbance was read in a microplate reader at 570nm and cell survival was calculated by the following formula.

Viability % = (Test OD/Control OD) X 100

Cytotoxocity % = 100 – viability%

The positive control used was Cyclo-90 (Cyclophosphamide) at a concentration of 90µg/ml.

DNA Fragmentation assay

Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 base pairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis. (Alexei G.*et.al.*, 1994) Cells were seeded in 24 well plate and kept in CO_2 incubator. Cells were treated by extracts in three different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml) for 48 h. At the end of incubation period, the cells were centrifuged for 1000rpm for 3mins at 14°C. The pellet was resuspended in a lysis buffer (10 mM Tris-HCI, pH 8.0, 10 mM NaCl, I0 mM EDTA, 20mg/ml Proteinase K, 10% SDS), and incubated at 37°C. The DNA was extracted by phenol-chloroform method, precipitated overnight in -20°C ethanol containing 0.3 M final concentration sodium acetate. The pellet was dissolved in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). DNA samples were electrophoretically separated on 1.8 % agarose gel containing ethidium bromide (0.4µg/mL). DNA was visualized by a UV (302 nm) transilluminator. (Emmy P. Rogakou *et al*, 2000; Gavrieli *et al*, 1992) Quercitin treatment was used as positive control, while untreated cells were used as control.

CASPASE 3 Assay

Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Caspases activities were determined by chromogenic assays using caspase-3 activation kits according to the manufacturer's protocol (Calbiochem, Merck). After treating with plant extracts, the cells were lysed using Lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 100mM EDTA). Lysates were centrifuged at 10,000rpm for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by the Lowry's method (Lowry, 1951) using BSA as a standard. 100µg protein (cellular extracts) was diluted in 50 µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5 µl of the 4mM p-nitroanilide (pNA) substrates, DEVD--pNA (caspase-3 activity) for 2 h at 37°C. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured at 405nm in a microtiter plate reader. Relative caspase-3 activity was calculated as a ratio of the absorbance of treated cells to untreated cells. Camptothecin (1µM) was used as positive control.

RESULTS AND DISCUSSION

B.acutangula and *S.colais* plant leaf extracts in ethyl acetate, methanol and chloroform were tested for their cytotoxicity activity against human colon cancer cell lines Colo320 using various assays. The Griess reaction, a spectrophotometric determination for nitrite, was carried out to establish the nitrite levels in conditioned medium of Colo320 cells treated with the plant extracts. Methanolic and ethyl acetate extracts of *S.colais* and ethyl acetate extract of *B.acutangula* showed the

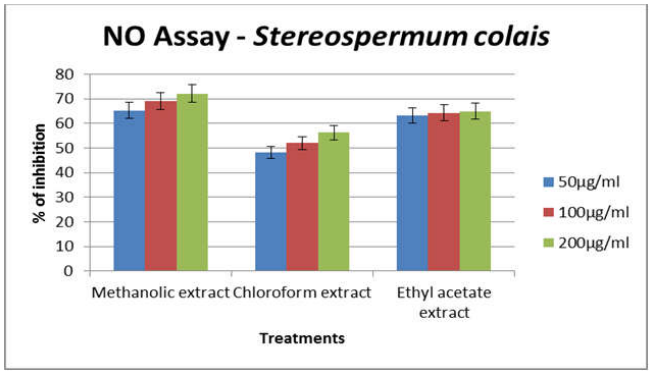

Fig. 1. NO inhibition by leaf extracts of *Stereospermum colais*

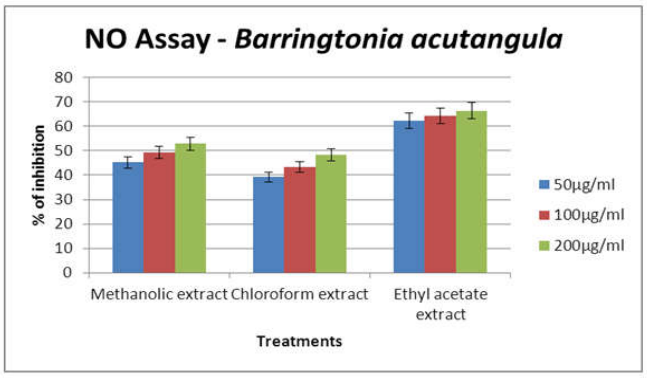

Fig. 2. NO inhibition by leaf extracts of *Barringtonia acutangula*

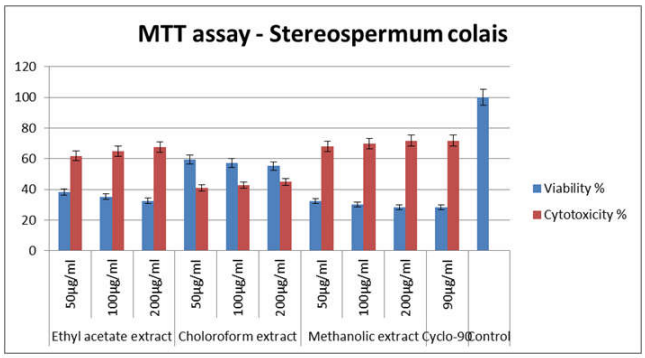

Fig. 3. Invitro cytotoxicity of Leaf extracts of *Stereospermum colais* on Colo320 cell lines.

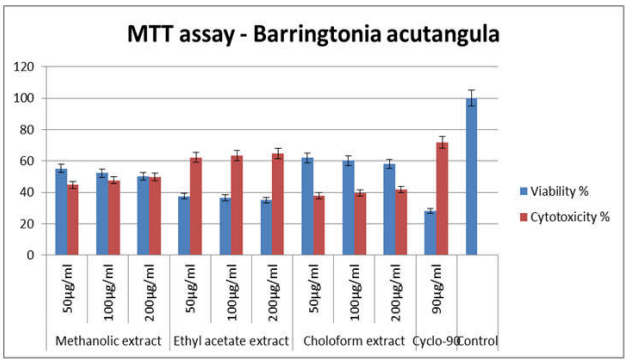

Fig. 4. Invitro cytotoxicity of Leaf extracts of *Barringtonia acutangula* on Colo320 cell lines

highest free radical scavenging activity at 72.1%, 64.9% and 66.3% respectively, as shown in Fig. 1 and 2. MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme

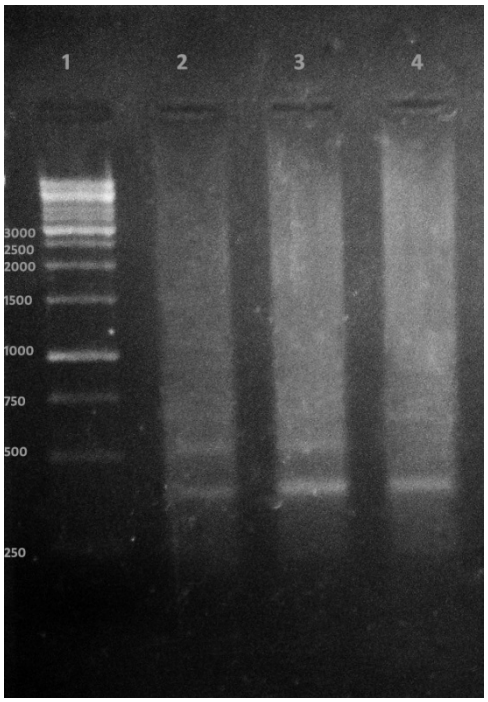

Fig. 5 . DNA Fragmentation assay

Lane 1 – 1kb DNA Ladder, Lane 2 – *S. colais* ethyl acetate extract, Lane 3 – *S. colais* methanolic extract, Lane 4 – *B. acutangula* ethyl acetate extract.

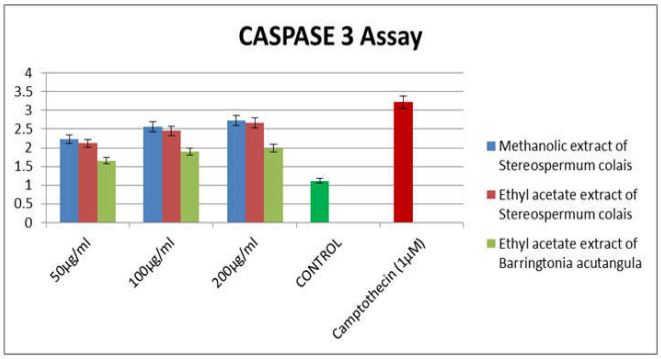

Fig. 6. Caspase-3 levels in Colo320 cell lines treated with Leaf extracts of *Barringtonia acutangula* and *Stereospermum colais*

from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells (Wu LC *et.al*., 2006). Addition of a detergent results in the liberation of the crystals, which are solubilised. The color can be spectrophotometrically measured at 570nm. The level of the colored formazan products is directly proportional to the number of surviving cells (Huang SS *et.al*, 2008). There is a concentration dependent increase in the percentage of cytotoxicity of the extracts as shown in Fig. 3 and 4. The highest cytotoxicity of 71.7% was recorded in the methanolic extract of *S.colais*.

Apoptotic DNA fragmentation is a key feature of apoptosis, characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of approximately 180 base pairs (bp) and multiples thereof (Wylie, 1980). It is often analyzed using total genomic DNA extraction and agarose gel electrophoresis to demonstrate a "ladder" pattern at ~180-BP intervals. Necrosis, on the other hand, is usually

characterized by random DNA fragmentation which forms a "smear" on agarose gels. DNA fragmentation assay was performed for the ethyl acetate and methanol extracts of *S.colais* and ethyl acetate extract of *B. acutangula* based on the preliminary assays. The results showed apoptotic induction in the cancer cells as a result of the treatment with the extracts (Fig. 5).

The intrinsic and extrinsic apoptotic pathways converge to CASPASE-3, a protease which cleaves the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease becomes active leading to nuclear apoptosis. The upstream caspases that converge to CASPASE-3 are CASPASE-9 and CASPASE-8 in the intrinsic and extrinsic pathways, respectively. CASPASES also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation, DNA fragmentation, and membrane blebbing (Irene M. Ghobrial *et al*., 2005). CASPASE 3 levels were observed to have been upregulated in the cells treated with the extracts (Fig. 6). This confirmed the activation of apoptosis in all the treatments, with the maximum upregulation being observed in the methanolic extract of *S.colais*.

CONCLUSION

Plant based therapy is a preferred route to discover a panacea for the deadly illness. The current study is undertaken with a similar aim. *B. acutangula* and *S. colais* plants were selected based on their medicinal properties, which are well documented (Vijaya Bharathi *et al.,* 2010, S. Sahoo *et al.*, 2008). The plants were subjected to a series of assays to analyse their anti-cancer properties. The preliminary screening by NO assay and MTT assay indicated the free-radical scavenging and cytotoxicity of the plant in the methanol and ethyl acetate extracts of *S.colais* and ethyl acetate extract of *B.acutangula* against Colo320 cells. Further, DNA fragmentation assay attributed the cytotoxicity of the plant extracts to apoptosis. The CASPASE-3 assays proved the activation of apoptosis in the cells treated with Ethyl acetate and methanol extract of *S.colais* and the ethyl acetate extract of *B.acutangula*.

Hence, it is suggested that both *Barringtonia acutangula* and *Stereospermum colais* have anti-cancer potential. The present research suggests the use of these two medicinal plants as a therapeutic agent to treat the deadly disease of human colon cancer. Further investigations are under process to purify and characterise the bioactive constituents present in the plant extracts, followed by an *in silico* analysis of the compounds against potential drug targets involved in cancer pathway.

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