Evaluation of alternative chemotherapeutic agent from *Ruellia tuberosa* for hepatocellular carcinoma using HepG2 cell lines

B Arirudran\(^a\), Vijayalakshmi Krishnamurthy\(^b*\), A Saraswathy\(^a\)

\(^a\)Captain Srinivasa Murti Drug Research Institute for Ayurveda (CCRAS), Anna Hospital Campus, Arumbakkam, Chennai, Tamil Nadu, India

\(^b\)Department of Biochemistry, Bharathi Women's College, Affiliated to University of Madras Chennai, Tamil Nadu, India

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**Corresponding Author:**
Krishnamurthy V.*
Associate Professor
Email: viji42research@yahoo.co.in
Phone: +919884418074

Arirudran B.
Research Scholar
Email: arirudran_1975@yahoo.com
Phone: +919841560205

**Saraswathy A.**
Director
Email: saraswathy20002004@gmail.com
Phone: +919444456710

**Abstract**
Background: Cancer is one of the most life-threatening diseases with more than 100 different types. Cancer has become an important issue in medicine as it is a major cause of death in both the developed and developing countries and it is now well thought-out as secondary to that of myocardial infarction. Objective: Plants have a special place in the treatment of cancer. It is estimated that plant derived compounds one or the other constitute more than 50% of anticancer agents. The objective of the research is to evaluate the anticancer potential of *R. tuberosa* using HepG2 cell line. Method: The present communication attempts to study the cell viability, cytotoxic activity, apoptosis, flow cytometry analysis and DNA fragmentation analysis on HepG2 cells treated with ethyl acetate and ethanolic extracts of *R. tuberosa*. Result: The ethanolic extract of *R. tuberosa* was found to posses better anticancer potential than ethyl acetate extract of *R. tuberosa*. Conclusion: The results provide useful information about antiproliferative, cytotoxic and apoptotic activity of *R. tuberosa*. It is suggested that *R. tuberosa* could be a new alternative chemotherapeutic agent for liver cancer.

**Citation:**

1. Introduction

Hepatocellular carcinoma is the 5th most frequent solid tumor worldwide, whose etiology includes viral hepatitis, Afliatoxin B1, alcohol abuse and some metabolic diseases (Chen et al., 1997). Due to lack of effective drugs, expensive cost for chemotherapeutic agents and side effects of anticancer drugs, cancer can be a cause of death (Newman et al., 2003). A great majority of human cancers (about 80%-90%) are attributable to environmental factors (Benjamin et al., 1990). Therefore, there is an ongoing search for...
better control and preventive methods in order to reduce cancer mortality and related side effects. Many investigations are now being carried out to discover naturally occurring compounds, which can suppress or prevent the process of carcinogenesis (Wargovich et al., 1988; Thapliyal et al., 2002). Plants have a special place in the treatment of cancer. It is estimated that plant derived compounds one or the other constitute more than 50% of anticancer agents (Nipun et al., 2011). In addition Asian medicinal herbs are used as treatment for various ailments including malignancies (Yadav et al., 1989). In folk medicine, R. tuberosa (Figure 1.1) has been used as anti-diabetic, antipyretic, analgesic, anti hypertensive, thirst quenching, antidotal agent and this plant was traditionally used for reducing toxicity and healing urine tract inflammation (Chiu, 1995). R. tuberosa has antimicrobial activity for both Gram positive and Gram negative bacteria (Arrirudran et al., 2011). In vitro human liver cell model could be a potent tool for predictive studies on drug toxicity and metabolism in the pharmaceutical industry. In general, in vitro test systems represent the first phase of the evaluation procedure, in which the cells are isolated from various tissues or cell lines are cultured and studied (Ventura, 2005; Shimazawa et al., 2005). The purpose of this in vitro study was to investigate the molecular mechanisms of ethyl acetate and ethanolic extracts of R. tuberosa and their preventive effects on liver cancer cells to provide a scientific rationale for using R. tuberosa as a chemopreventive agent against liver cancer.

**Figure 1: Ruellia tuberosa L.**

2. Materials and Methods

It was planned to study the anticancer potential of ethanolic and ethyl acetate extract of R. tuberosa using HepG2 cell line. The anticancer activity was evaluated by MTT assay, cell cycle analysis using Flow cytometry, caspase 3 and 9 activity and gene expression of p53 and NFkB.

### 2.1 Chemicals

DMEM, TPVG and FBS were obtained from HiMedia (India). MTT, HEPES, Cyclophosphamide, Camptothecin, Propidium iodide, DMSO, Penicillin, Streptomycin and L-Glutamine were bought from Sigma Chemical Co. (St. Louis, MO, USA). Caspase 3 and 9 estimation kit was purchased from Merck, Germany. Cell culture plastic werees were from Nunc Co. (Denmark). All other chemicals used were of analytical grade.

### 2.2 Preparation of plant extract

Fresh plant materials were collected from Tamilnadu and identified by the Flora of Presidency of Madras (Gamble, 1967; Brain, 1975). Plant material identification, authentication and dried specimen (No:00628) was deposited in museum of CSMDRIA Chennai. The plant extracts were prepared as described by earlier method with little modification (Aqil, 2007). The whole plant of R. tuberosa was collected and allowed to dry at room temperature in the laboratory for a period of 2 weeks. The dried plant material was coarsely powdered and weighed. Hundred grams of dry plant material was soaked in 1lit of ethyl acetate/ethanol for 48hrs with intermediate shaking. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). Then the filtrate was concentrated by distillation over boiling water bath and the last trace of solvent was removed under vacuum. Extraction was repeated with 97% methanol and concentrated under reduced pressure (T< 40° C) and then stored in dry clean bottle at 4° C for further use. One gram ethyl acetate and ethanolic extracts of R. tuberosa whole plant was reconstituted by using 10% solution dimethyl-sulfoxide at a stock concentration of 100mg/ml and the final concentrations of 100µg/ml, 250µg/ml 500µg/ml and 1000µg/ml were used for further studies.

### 2.3 Media preparation

Dulbecco’s modified eagle’s medium was prepared as per manufacturer’s protocol (Hi Media, India) filtered and sterilized. The other components were added to the media to prepare a complete media that include fetal bovine serum, sodium bicarbonate and antibiotics.

### 2.4 Cell lines

HepG2 cell lines were purchased from the National Centre for Cell Sciences, Pune, India. The cells were cultured in Dulbecco’s modified eagle’s medium containing L-Glutamine and 25mM 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% CO₂.

2.5 Maintenance of cell lines
Cell culture flasks were selected by confluence by observing under inverted microscope. For maintaining the cell lines, sub culturing was done. It was done to facilitate the cell growth by removing the cells from the medium and introducing them into a new fresh medium. Mostly, enzymatic methods were used for cell maintenance. The enzyme used here was TPVG. Growth medium was removed from the flasks and the cells were incubated at 37°C after adding the enzyme. Initially all cells get detached from the surface. The cells were suspended in 5 ml of the medium. The suspension was aspirated a few times to break cell clumps. Cell line, date of seeding and passage number was marked on the bottom of the T-flask. 5 ml of cell suspension was transferred to fresh T-flasks.

2.6 Experimental design
Group I as control, Group II and III are ethyl acetate and ethanolic extract of R. tuberosa. The cells were incubated at 37°C in a controlled humidified atmosphere of 5% CO₂ and 95% air for 48hr and then the following experiments were carried out for the above three groups.

2.7 Culturing of cells
Human liver carcinoma cell lines (HepG2) were obtained from NCCS, Pune, India and it was cultured in Dulbecco’s modified eagle’s medium according to a method described previously (Kang et al., 2004). All cultured cells were maintained in the logarithmic phase of growth in the medium supplemented with 10% fetal calf serum, 100 IU/ml of penicillin, 100mg/ml of streptomycin, 1% of nonessential amino acid (L. glutamine) and the stock was maintained at -20°C. The cells were seeded at a density of 5x10⁵/well and cultured using 25cm2 flasks (Nunc, Denmark), in a 5% CO₂ incubator (Sanyo, Japan) and incubated overnight at 37°C and allowed to attach to form a mono layer.

2.8 MTT Assay
In vitro cell culture test method is a very sensitive assessment for testing the toxicity of plant extracts and their degradation products. This method is very effective and generally accepted for bio-safety testing. In this in vitro test methods after exposure of cells to the plant extracts, microscopical evaluation of cell morphology, cell viability and cytotoxicity was determined by various method, such as MTT assay (Kang et al., 2004). This method is based on the ability of live cells but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. Approximately 5x10⁵ of HepG2 cells / well (cell line) were seeded into 96 well plates; 100µl of medium was added and incubated at 37°C. After 24hr, the medium was discarded and fresh medium was added with ethyl acetate and ethanolic extract of R. tuberosa in the graded concentration of 250µg/ml, 500µg/ml and 1000µg/ml for 12 – 24hr. The cell viability and proliferation were monitored. The plates were incubated for 48h at 37°C in a CO₂ incubator. After the incubation period, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After incubation at 37°C in a CO₂ incubator for 4hr, the medium was discarded and 200µl of dimethyl sulfoxide was added to dissolve the formazan crystals. Then the absorbance was read in a microplate reader at 570nm and cell survival was calculated by the following formula. Cell Viability % = (Test OD/Control OD) x 100. Cell Cytotoxicity % = 100 - viability %. The cyclophosphamide (cyclo-90) at a concentration of 90µg was used as positive control.

2.9 Light microscopic studies
Light microscopic examination of the cells was performed to observe the morphological changes after the treatment with ethyl acetate and ethanolic extract of R. tuberosa for 48hr. Then HepG2 cells were grown in 35mm sterile petri plates treated with plant extract at the concentration of 60 and 100µg/ml. Cells were then fixed for 5min with 10% methanol/PBS and morphological changes were observed under inverted microscope.

2.10 DNA Fragmentation assay
Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into 180-200 base pairs and can be visualized as an ‘oligosomal ladder’ by standard agarose gel electrophoresis (Alexei et al., 1994). HepG2 cells were seeded in 24 wells plate and kept in CO₂ incubator. Cells were treated by the ethyl acetate and ethanolic extracts of R. tuberosa R. tuberosa in three different concentrations of 100µg/ml, 250µg/ml and 500µg/ml and kept for 48hr. At the end of incubation period, the cells were centrifuged at 1000rpm for 3min at 14°C. The pellet was re-suspended in lysis buffer (10mM Tris-HCl, pH 8.0, 10mM Sodium chloride, 10mM EDTA, 20mg/ml proteinase K,
10% SDS), and incubated at 37°C. The DNA was extracted by phenol-chloroform method, precipitated and kept for overnight at 20°C in ethanol containing 0.3M concentration sodium acetate. The pellet was dissolved in TE buffer (0.1M Tris-HCl, pH 8.0, 10mM EDTA). DNA samples were electrophoretically separated on 1.8% agarose gel containing ethidium bromide (0.4µg/ml). DNA was visualized by a UV (302nm) transilluminator (Gavreilli et al., 1992). Quercetin treatment was used as positive control, while untreated cells were used as control.

2.11 Cell cycle analysis by Flow Cytometry
Flow cytometry analysis and cell apoptosis was assayed by the propidium iodide staining method (Takada et al., 2001). Approximately 5x10^6 cells (HepG2) / well were seeded into micro titre plate; 300µl of medium was added and incubated at 37°C. After 24hr, the medium was discarded and fresh medium was added with appropriate concentration of ethyl acetate and ethanolic extract of R. tuberosa. The plates were incubated for 48hr at 37°C in a CO2 incubator. Cells were pelleted by centrifugation at 500 rpm at 4°C and washed with phosphate-buffered saline of pH 7.4. Cells were harvested by trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in phosphate buffer), washed with phosphate buffer twice, and fixed in 80% ice-cold ethanol at 4°C. The cells were then stained with 0.4 ml of (40mg/ml) propidium iodide for 30min at 37°C. The cells were then harvested by trypsin-EDTA solution and fixed in 80% ice-cold ethanol at 4°C for 30min, followed by incubation with 100µg/ml RNase for 30min at 37°C. The cells were then washed with phosphate buffer twice, and fixed in 80% ice-cold ethanol at 4°C for 30min, followed by incubation with 100µg/ml RNase for 30min at 37°C. The cells were then stained with 0.4 ml of (40mg/ml) propidium iodide for 15min at room temperature and subjected to flow cytometric analysis using a FAC Scan flow cytometer (Becton-Dickinson Immunocytometry Systems USA, San Jose, CA). Approximately 1x10^6 counts were made for each sample. The percentage of distribution of cell apoptosis was calculated by CELL Quest software. Distribution of cells in G1, S and G2 phases was determined. Data was acquired using a flow cytometer after 30min of incubation. Cells were harvested after treatment with different concentrations (100-1000µg/ml), cell lysates were prepared by incubating 2x10^6 cells/ml in cell lysis buffer for 10min on ice. Lysates were centrifuged at 10,000 rpm for 1min. The supernatants (cytosolic extract) were collected and protein concentration was determined (Compton, 1985) using bovine serum albumin as a standard. 100-200µg protein (cellular extracts) was diluted in 50µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5µl of the 4mM pNA substrates of DEVD-ala-pNA (caspase-3 activity) and LEHD-pNA (caspase-9 activity) at 37°C for 1-2hr. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405nm in a microtiter plate reader (Wang et al., 2005). Relative caspase 3 and 9 activity was calculated as a ratio of the absorbance of ethanolic extract of R. tuberosa treated cells to untreated cells. The absorbance of pNA from every sample was compared with the uninduced controls and values were expressed as µmol/L of pNA per microgram of cytosolic protein (µmol/L per microgram).

2.12 Caspase 3 and caspase 9 assay
Apoptosis in mammalian cells is initiated by activation of the caspase family of cysteine proteases. This assay quantifies caspase-3 activation in vitro by measuring the cleavage of caspase-3 substrate DEVD-ala-pNA and caspase-9 substrate LEHD-pNA to free pNA, which emits fluorescence. This free pNA chromophore is measured using a microtiter plate reader. Caspases activities were determined by colorimetric assays using caspase-3 activation kits according to the manufacturer’s protocol. Wash the well with 1 ml PBS at room temperature. After treatment with designated concentrations of extract (100-1000µg/ml), cell lysates were prepared by incubating 2x10^6 cells/ml in cell lysis buffer for 10min on ice. Lysates were centrifuged at 10,000 rpm for 1min. The supernatants (cytosolic extract) were collected and protein concentration was determined (Compton, 1985) using bovine serum albumin as a standard. 100-200µg protein (cellular extracts) was diluted in 50µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5µl of the 4mM pNA substrates of DEVD-ala-pNA (caspase-3 activity) and LEHD-pNA (caspase-9 activity) at 37°C for 1-2hr. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405nm in a microtiter plate reader (Wang et al., 2005). Relative caspase 3 and 9 activity was calculated as a ratio of the absorbance of ethanolic extract of R. tuberosa treated cells to untreated cells. The absorbance of pNA from every sample was compared with the uninduced controls and values were expressed as µmol/L of pNA per microgram of cytosolic protein (µmol/L per microgram).

2.13 Reverse transcriptase PCR
RT-PCR was performed to analyze the gene expression during apoptosis, as a result of the extract treatment on the cancer cells. Cells were harvested after treatment with different concentrations (100µg/ml, 250µg/ml, and 500µg/ml) of the extracts. Total RNA was isolated and cDNA was synthesized according to the manufacturer’s protocol (Sigma Aldrich, USA). Using this cDNA as template, PCR was carried out with p53 and NFkB gene specific primers.
2.14 Positive control for RT reaction
GAPDH is one of the most commonly used housekeeping genes used in comparisons of gene expression data (Robert et al., 2005). GAPDH was simultaneously amplified along with the respective genes to confirm uniformity of RNA concentration taken for the expression studies.

2.15 Total RNA isolation
Total RNA from cell lines was isolated using ONE STEP-RNA solution (phenol and guanidine isothiocyanate) (Chomczynski, 1987). During this procedure, all glassware and plastic-ware were treated by incubating them overnight in 0.01% DEPC water (RNase-free) to decrease or reduce the risk of RNA being degraded by RNases (Narumi, 1987). Cultured HepG2 cells (5-10x10^6) were pelleted by centrifugation at 1000 rpm; 5min using 1 ml of ONE STEP-RNA reagent. Cell lysis was performed by repeated pipetting. Homogenized samples were incubated at 15°C to 30°C for 5min to permit the complete dissociation of nucleoprotein complexes; 0.2 ml of chloroform per 1ml of ONE STEP-RNA reagent to the sample. Tubes were shaken vigorously by hand for 15sec and incubated at 15°C to 30°C for about 2 to 3min and then the samples were centrifuged at 12,000 rpm for 15min at 2°C to 8°C. The mixture separated into two phase, lower phenol-chloroform inter-phase of cloudy white and upper colorless aqueous phase. The RNA remains exclusively in 60% volume of upper aqueous phase of ONE STEP-RNA reagent used for homogenization. RNA was precipitated from the aqueous phase by mixing it with isopropyl alcohol. Samples were incubated at 15°C to 30°C for 10min and centrifuged at 12,000 rpm for 10min at 2°C to 8°C. Supernatant was removed and the gel-like RNA pellet at the bottom was washed once with 75% ethanol by centrifuging at 7,500 rpm for 5min at 2°C to 8°C. RNA pellet was dried by vacuum-dry for 5 to 10min and finally dissolved in DEPC treated water and stored in -20°C.

2.16 cDNA preparation
After RNA isolation, RNA was immediately reverse transcribed with Easy Script Plus™ Reverse Transcriptase. For RT-PCR reaction, 1-2µg of RNA was used corresponding to 1-10µl of total RNA isolate. RNA isolated from fresh tissue samples was reverse transcribed, where oligo-dT was used as a primer, into a 1.5 ml eppendorf PCR tube, 1-2µg of RNA, 2µl of oligo-dT (stock was 10µM) was added and the total volume was made up to 12.5µl with DEPC treated water. The tube was incubated at 65°C for 5min and chilled on ice. Then, 4µl of 5X reverse transcriptase buffer (final concentration 1X), 2µl of 2mMdNTP mix (final concentration 0.2mM) and 0.5µl of RNase inhibitor (40U/µl) were added in the indicated order. After incubating at 42°C for 5min, 1µl of Easy Script Reverse Transcriptase (200 U/µl) was added. The reaction was carried out at 42°C for 50min. Finally, the tube was heated up to 70°C for 10min and chilled on ice. The samples were stored at -20°C until further use.

2.17 The Polymerase Chain Reaction (PCR)
The cDNA obtained was amplified by PCR. Gene specific PCR was used to amplify p53 and NFκB separately. A constitutively expressed gene, namely GAPDH, was chosen in order to assess the quality of PCR.

Sequence of the primer used in the study

```plaintext
GAPDH Forward
TCGCTGGTACATCAAATCCAC
GAPDH Reverse
TCTCAGGTCAACGTTCACAT
NFκB Forward
TCGCTGGTACATCAAATCCAC
NFκB Reverse
TCTCAGGTCAACGTTCACAT
p53 Forward
TCGCTGGTACATCAAATCCAC
p53 Reverse
TCTCAGGTCAACGTTCACAT
```

PCR reaction conditions for GAPDH, NFκB and p53

2.18 Agarose Gel Electrophoresis of PCR Products
In a total volume of 25 ml, 1.5% agarose and 1X TAE buffer were prepared and poured onto
a gel tray. PCR product was mixed with the loading dye. The mixture was loaded to each well along with 1kb ladder as a reference. The gel was run at 50V for 90min and visualized.

2.19 Expression fold calculation
Expression ratio was derived by analyzing the gel photos in software - Image J. Expression ratio was obtained using the formula.

3. Justification of Research

There is no literature available for the anticancer potential of ethanolic and ethyl acetate extract of R. tuberosa, so work was planned to study its anticancer potential in *in vitro* condition using HepG2 cell line.

4. Results and Discussion

The most preponderant form of programmed cell death is apoptosis, which is also known as type-1 cell death (Jaattela, 2004). In cancer therapy, tumor growth can be suppressed by activating the apoptotic machinery in the cell (Lowe, 2000; Bold et al., 1997). Many malignant cells, however, have been shown to be unable to regulate the genes that control apoptosis, rendering them resistant to the induction of apoptosis by a variety of stimuli, including chemotherapeutic drugs and radiotherapy (Reed, 1997). In the present study, *R. tuberosa* exerted antiproliferative activity on the growth of HepG2 cells by apoptosis, which was confirmed by DNA fragmentation and flow cytometry analysis.

Figure 3.1: MTT assay and cell viability of ethyl acetate and ethanolic extract of *R. tuberosa* (whole plant) on (HepG2) cell lines

The figure 3.1 shows HepG2 cells when treated with ethyl acetate extract of *R. tuberosa* at 250µg, 500µg and 1000µg concentrations. It shows 45.77%, 42.33% and 31.86% of viability, similarly the HepG2 cells when treated with ethanolic extract of *R. tuberosa* at 250µg, 500µg and 1000µg concentration shows 48.22%, 41.89% and 27.76% of cell viability. This result shows that the viability of HepG2 cell is decreased proportionately with increase in the concentration for ethanolic extract than ethyl acetate extract of *R. tuberosa*. The viability of HepG2 cells were significantly inhibited at 1000µg for both the ethyl acetate and ethanolic extract of *R. tuberosa* within 12 - 24hr, when compared with standard cyclophosphamide (cyclo-90µg). This result emphasizes that *R. tuberosa* remarkably inhibits the cell proliferation in dose dependent manner and which is specific toward the tumor cell (HepG2). Flavonoids are known as nature’s tender drugs and have long been recognized to possess various biological activities including antimicrobial, antiviral, anti-inflammatory, immunomodulatory, antithrombotic, anti-allergic, anticancer and anti-proliferative activities (Havsteen, 1983). The ability of antiproliferation and anticancer activity of ethanolic extract is significantly higher than ethyl acetate extract of *R. tuberosa* and it may be due to presence of high amounts of flavonoids. This results obtained confirmed that *R. tuberosa* may have potential antiproliferation property towards the tumor cell (HepG2).

Figure 3.2: MTT assay and toxicity of ethyl acetate and ethanolic extract of *R. tuberosa* (whole plant) on (HepG2) cell lines

The figure 3.2 shows that HepG2 cells when treated with ethyl acetate extract of *R. tuberosa* at 250µg, 500µg and 1000µg concentration shows 54.23%, 57.67% and 68.14% toxicity, similarly the HepG2 cells when treated with ethanolic extract of *R. tuberosa* at 250µg, 500µg and 1000µg shows 51.78%, 58.11% and 72.24% of toxicity. This result shows that the toxicity of HepG2 cell is
increased proportionately with the increase in the concentration of ethanolic extract than ethyl acetate extract of *R. tuberosa*. It was found that 1000µg of ethyl acetate and ethanolic extract of *R. tuberosa* (whole plant) was found to be more toxic for HepG2 when compared to the standard cyclophosphamide. The toxicity of the ethanolic extract was almost comparable to cyclophosphamide. This cytotoxicity activity of *R. tuberosa* for HepG2 cells line may be due to presence of cirismarin (Chwan-Fwu Lin et al., 2006).

**Plate 3.1:** Effect of ethyl acetate and ethanolic extract of *R. tuberosa* on HepG2 cell lines

The plate 3.1 (a,b,c,d,e,f,g,h) shows that ethyl acetate and ethanolic extract of *R. tuberosa* exhibit no sign of any negative effects at all concentrations tested. When the results from the plate 3.1 was co-related with the figure 3.1 and 3.2 it was found that the viability of HepG2 cells were decreased by 45.77% and 48.22% at the concentration of 250µg/ml for ethyl acetate and ethanolic extract of *R. tuberosa*. The cell viability was decreased by about 60% at 500µg/ml. The result obtained emphasise that the viability of HepG2 cells were decreased with increase in the concentrations of ethyl acetate and ethanolic extract of *R. tuberosa*. Cancer develops when the balance between cell proliferation and cell death is disturbed, and aberrant cell proliferation leads to tumor growth. Apoptosis is a form of physiological cell death is essential to normal tissue development and homeostasis (Vaux, 1999). After receiving an apoptotic death stimulus, cells first enters a signaling phase followed by the final degradation phase, in which apoptosis is identifiable by chromatin condensation, cell shrinkage, caspase activation, membrane lipid rearrangement, DNA fragmentation, and cell fragmentation, through the formation of apoptotic bodies (Jacobson et al., 1997). Apoptosis and its related signaling pathways have a profound effect on the progression of cancer (Lowe, 2000). Induction of apoptosis is therefore a highly desirable goal of preventive strategies for cancer control (Reed, 2005). Many studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide and teniposide (Kolfschoten et al., 2002) and also by plant extracts (Abdullah et al., 2010). Hence the result shows that ethyl acetate and ethanolic extract of *R. tuberosa* comprise rich amount of phyto chemicals like triterpenoids, phenols, flavonoids, coumarins, tannins and alkaloids they may act as chemotherapeutic agents which may induce apoptosis in HepG2 cell lines.

**Figure 3.3:** Effect of ethanolic extract of *Ruellia tuberosa* L. on Caspase 3 and 9 in HepG2 cell lines

Concentration of ethanolic extract from *R. tuberosa* at 100µg/ml to 1000µg/ml, the activities of caspase 3 had increased than caspase 9 treated cells in time and concentration dependent, when compared with untreated control cells. Camptothecin was used as standard. The apoptotic pathway induced by different concentrations of ethanolic extract of *R.
The results show that ethyl acetate and ethanolic extract of *R. tuberosa* has more ability to inhibit the proliferation of HepG2 cells. To confirm that *R. tuberosa* treated cell death is via apoptosis the extent of DNA fragmentation was analysed. The DNA fragmentation assay is a typical hallmark of apoptotic cell death. The figure 3.4 shows that detection of apoptosis in cultured cells relies heavily on techniques involved the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. DNA extracted from HepG2 cells treated with different concentrations of ethyl acetate and ethanolic extract of *R. tuberosa* for 24hr were subjected to agarose gel electrophoresis revealed a progressive increase in the 180-200bp ladder fragments, such a pattern corresponds to inter nucleosomal cleavage which is the characteristic of apoptosis. A typical DNA ladder was observed, with bands separated by multiples of 200bp. The lane 3, 4 shows ethyl acetate and lane 5, 6 shows ethanolic extracts of *R. tuberosa* induces apoptosis in HepG2 cells resulting in the degradation of chromosomal DNA in to small oligonucleosomal fragments, which results in fragmentation of DNA. But in case of untreated HepG2 cell there was no fragmentation of DNA (Lane 2) seen. Control cells did not exhibit any such DNA fragmentation. Hence the DNA fragmentation analysis confirmed that extract of *R. tuberosa* exerted good antiproliferative and induced apoptotic activity on the growth of HepG2 cells.

In cancer therapy, tumor growth can be suppressed by activating the apoptotic machinery in the cell. Many malignant cells however, are unable to regulate the genes that control apoptosis, rendering them resistant to the induction of apoptosis by a variety of stimuli, including chemotherapeutic drugs and radiotherapy (Reed, 1997). Apoptosis
characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into inter-nucleosomal fragments of 180bp and their multiples. DNA cleavage during apoptosis occurs at sites between nucleosomes, protein-containing structures that occur in chromatin at 200bp intervals. This DNA fragmentation was often analyzed by using agarose gel electrophoresis to demonstrate a ladder pattern at 200bp intervals. Necrosis, on the other hand, was characterized by random DNA fragmentation which forms a smear on agarose gels. This indicates the presence of DNA fragments the size of single nucleosomes and oligonucleosomes (Duke et al., 1983) which are considered to be characteristic biochemical markers for apoptotic cells (Wyllie, 1980). The extent of DNA fragmentation (i.e. intensity and number of bands) is dose dependent. The result of the DNA fragmentation analysis emphasizes that DNA laddering was observed in all the ethyl acetate and ethanolic extract of *R. tuberosa* treatment for HepG2 cells in a dose dependent manner, in which DNA from control cells showed intact band depicting no degradation and the DNA from the treated cells showed degradation in agarose gel electrophoresis, reflecting the genotoxicity of the extracts.

Figures 3.5: (a, b, c) demonstrate the results of the cell cycle arrest analysis carried out by using flow cytometry technique. After an exposure to the ethyl acetate and alcohol extracts for 48hrs, HepG2 cells were pelleted and after treatment with fixative, were subjected to FACS analysis using propidium iodide dye. The results showed the arrest of the cancer cells at the sub G₀ phase

**Figure 3.5(a):** Flow cytometry analysis of control, drug 1 and drug 2 treated HepG2 cells with propidium iodide dye.

Control = untreated, Drug 1 = ethyl acetate extract and Drug 2 = ethanolic extract of *R. tuberosa*
Cell cycle is a coordinated process involving a number of molecular players. There are two important stages with two intermediate preparatory phases. They are G₁, S, G₂ and M. The S phase represents the synthesis phase, where DNA synthesis occurs and M phase denotes mitosis. Hence, during these phases, the cellular DNA content varies and measuring the DNA content gives an indication of the cells in the respective phases. Propidium iodide is an analog of ethidium bromide, which intercalate with DNA and RNA. This binding markedly enhances the fluorescence of the dye. In cells incubated with propidium iodide dissolved in an isotonic solution, dye enters into the cell and it is prevented by the cell membrane and laser-excited fluorescence is seen only in nuclei of dead cells. Thus, propidium iodide dissolved in isotonic saline can be used for rapid fluorometric quantization of dead cells in a population (Krishnan, 1975). Flow cytometer is high throughput equipment capable of quantifying the DNA content by measuring the propidium iodide bound. Cell cycle analysis is a useful technique for studying the contribution of an oncogene in response to growth stimuli, for identifying cell cycle phase specific inhibitors. There is substantial evidence that alteration in the cellular and molecular pathways that control the cell cycle and apoptosis may change the sensitivity and resistance to anticancer agents (Pezzuto, 1997). In the cell cycle analysis the number of cells counts were calculated and presented in figures 3.5(a,b,c). It shows that there was a significant difference observed in sub G₀/G₁ and G₀/G₁ for treated and untreated HepG2 cells at 24hr.

The figure 3.5 (b) shows that the % of cell counts for (sub G₀/G₁) phase was found to be 21.63% and 31.61% for ethyl acetate and ethanolic extract of *R. tuberosa* treated HepG2 cells when compared with non treated cells (7.75%). The % of cell counts for (G₀/G₁) phase was found to be 44.50% and 21.38% for ethyl acetate and ethanolic extract of V treated for HepG2 cells when compared with non treated cells 71.88%. The % of cell counts for (S) phase was found to be 4.35% and 4.48% for ethyl acetate and ethanolic extract of *R. tuberosa* treated for HepG2 cells when compared with non treated cells (6.27%). The % of cell counts for (G₂/M) phase was found to be 2.11% and 0.72% for ethyl acetate and ethanolic extract of *R. tuberosa* treated HepG2 cells when compared with non treated cells 5.03% respectively. As shown in figures 3.5 (c), cells under normal conditions (untreated) showed the expected pattern for continuing growing cells by which the highest peak in the region of G₁ phase and a small and low peak in G₂/M phase.

Decreased cell population observed was due to normal process for the cells to go apoptosis. Cell cycle progression and apoptosis are two pivotal signaling mechanisms used to maintain homeostasis in healthy tissues. Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G₀/G₁, S, or G₂/M phase and then induce apoptotic cell death (Kessel and Luo, 2000). Many reports indicate that cell cycle arrest leads to cell growth inhibition or apoptosis. Cell cycle arrest is a
good marker for chemopreventive or antitumor activity of chemicals or drugs (Chang, 1999). It was found that the % of HepG2 cells count were decreased in G$_2$/M, S and G$_0$/M phase for ethanolic extract of *R. tuberosa*. This result emphasize that *R. tuberosa* cause DNA damage and thereby arrested the cell cycle at sub G$_0$ state and induce apoptotic cell death. This cell cycle arrest is confirmed and shows that *R. tuberosa* has good antitumor activity. Apigenin, a common dietary flavonoid abundantly present in fruits and vegetables also reported to induce cell cycle arrest of HepG2 cells at G$_2$/M phase through p53 dependent pathway (Chiang et al., 2005). The leaves contained only traces of apigenin and luteolin, while the flowers contain malvidin-3, 5-diglucoside in appreciable quantity. The flower buds contained the maximum proportion of flavonoids yielding about 3% of apigenin-7-O-glucuronide: the other flavones were identified as apigenin-7-glucoside, apigenin-7-O-rutinoside and luteolin-7-O-glucoside (Harborne, 1967). In the present cell cycle analysis figures 4.5 (a,b,c) shows that the HepG2 cells at G$_0$/M phase was arrested. This apoptotic activity of *R. tuberosa* may be due to the presence of apigenin.

**Plate 3.2:** Expression of GAPDH

Full length cDNA when subjected to amplification using GAPDH primers (using Eppendorf Personnel Master cycler, Germany) yielded a band at a size of 400bp.

The plate 3.2 shows the GAPDH mRNA expression. GAPDH is housekeeping genes simultaneously amplified along with the respective genes to compare and confirm the uniformity of RNA concentration taken for the gene expression studies. The plate 3.3 and figure 3.6 shows the p53 gene expression. Full length cDNA when subjected to amplification using p53 primers yielded a band at a size of 500bp. p53 mRNA levels in ethanolic extract of *R. tuberosa* treated samples were 4.75 fold more than control sample. The plate 3.4 and figure 3.7 shows the NFkB gene expression. Full length cDNA when subjected to amplification using NFkB primers yielded a band at a size of 600bp. NFkB mRNA levels in ethanolic extract of *R. tuberosa* treated samples were 0.76 fold less than control samples.

**Plate 3.3:** Expression of p53

Full length cDNA when subjected to amplification using p53 primers (using Eppendorf Personnel Master cycler, Germany) yielded a band at a size of 500bp.

**Figure 3.6:** Graphical representation and expression profile for p53

Graph was obtained using a statistical package SPSSv16. Expression ratio was derived by analyzing the gel photos in software – Image J. Expression ratio was obtained using the formula. Target Gene expression/ internal control X 100

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Expression ratio of p53 mRNA</th>
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</thead>
<tbody>
<tr>
<td>UNTREATED</td>
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</tr>
<tr>
<td>TREATED</td>
<td>4.75</td>
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</table>

p53 mRNA levels in the treated samples were 4.75 fold more than control samples.
The plate 3.3 and figure 3.6 shows that up-regulation of the p53 gene expression in ethanolic extract of R. tuberosa treated for HepG2 cells confirmed the pro-apoptotic activity. The plate 3.4 and figure 3.7 shows that down-regulation of the NFkB gene revealed the pro-apoptotic activity of the ethanolic extracts of R. tuberosa on the HepG2 cells. p53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein encoded by the TP53 gene in humans. Apoptosis is an important phenomenon in cytotoxicity induced by anticancer drugs. The execution of apoptosis or programmed cell death (Evan, 2001) is associated with characteristic morphological and biochemical changes mediated by a series of gene regulation and cell-signaling pathways. Apoptosis can be initiated via two alternative signaling pathways: the death receptor-mediated extrinsic apoptotic pathway and the mitochondrion-mediated intrinsic apoptotic pathway (Reuter et al., 2008). Mitochondria play critical roles in the regulation of various apoptotic processes including drug-induced apoptosis (Cory, 2002). Alterations of the p53 gene are the most frequent genetic change in human cancers. It is estimated that about 50% of all human malignancies contain mutations of this gene (Bellärmý et al., 1997). The p53 tumor-suppressor gene has been implicated in the regulation of many critical cellular functions, including cell cycle arrest and apoptosis (Agarwal et al., 1995). The p53 is crucial in multi cellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor that is involved in preventing cancer. As such, p53 has been described as the guardian of the genome because of its role in conserving stability by preventing genome mutation (Read, 1999). The up regulation of p53 may be due to cell cycle arrest and apoptosis and the down-regulation of NFkB is due to the activated p53. The data also suggest that the inhibition of cell cycle progression might be associated with alterations of p53 gene. This result confirmed that the elevation of p53 expression may play an important role in sub G0/G1, G0/G1 and G2/M cell cycle arrest and apoptosis in HepG2 cells.

The plate 3.4 and figure 3.7 shows that down-regulation of the NFkB gene revealed the pro-apoptotic activity of the ethanolic extracts of R. tuberosa on the HepG2 cells. NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NFkB plays a key role in regulating the immune response to infection (kappa light chains are critical components of immunoglobulins). Incorrect regulation of NFkB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NFkB has also been implicated in processes of synaptic plasticity and memory (Albensi, 2000).

Plate 3.4: Expression of NFkB

![Image](image_url)

Full length cDNA when subjected to amplification using NFkB primers (using Eppendorf Personnel Master cycler, Germany) yielded a band at a size of 600bp.

Figure 3.7: Graphical representation and expression profile for NFkB

Graph was obtained using a statistical package SPSSv16. Expression ratio was derived by analyzing the gel photos in software – Image J. Expression ratio was obtained using the formula. Target Gene expression/internal control x 100

<table>
<thead>
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<th>SAMPLE</th>
<th>Expression ratio of NFkB mRNA</th>
</tr>
</thead>
<tbody>
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<td>1</td>
</tr>
<tr>
<td>TREATED</td>
<td>0.76</td>
</tr>
</tbody>
</table>

NFkB mRNA levels in the treated samples were 0.76 fold less than control samples.
NFκB is widely used by eukaryotic cells as a regulator of genes that control the cell proliferation and cell survival. As such, many different types of human tumors have misregulated by NFκB that is, NFκB is constitutively active. Active NFκB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. Defects in NFκB results in increased the susceptibility to apoptosis leading to increased cell death. It is because NFκB regulates anti-apoptotic genes especially the TRAF1 and TRAF2 and, therefore, checks the activities of the caspase family of enzymes, which are central to most apoptotic processes. In tumor cells, NFκB is active either due to mutations in genes encoding the NFκB transcription factors themselves or in genes that control NFκB activity (such as IkB genes); in addition, some tumor cells secrete factors that cause NFκB to become active. Blocking NFκB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus, NFκB is the subject of much active research among pharmaceutical companies as a target for anti-cancer therapy (Escarcega et al., 2007). Nuclear factor kB (NFκB) transactivates numerous genes involved in the regulation of cell proliferation and cell death (Joyce et al., 2001). This result shows that down-regulation of the NFκB gene increased the susceptibility to apoptosis leading to increased cell death.

Research Highlights

This research work emphasizes the cell viability, cytotoxic activity, apoptosis, caspase activity and DNA fragmentation and flow cytometry analysis of human Hepato cellular carcinoma cells treated with ethyl acetate and ethanolic extract of R. tuberosa whole plant in vitro model. The ethanolic extract of R. tuberosa had better anticancer potential than ethyl acetate extract.

Limitations

The ethanolic extract of R. tuberosa may be subjected to GC-MS analysis to identify the volatile compound present in it which has the anticancer potential and thereby identify the lead molecule.

Recommendations

Apart from this cell line studies, the animal studies can also be done to further elucidate the pathway / mechanism by which the ethanolic / ethyl acetate extracts of R. tuberosa could exact into anticancer potential in in-vivo system.

Funding and Policy

None

Justification

This research work has identified a prominent alternative hepatoprotective against for safe, cost effective and eco-friendly novel innovative anticancer drug from plant source.

Author’s Contribution

B. Arirudran executed this research work, Dr. K Vijayalakshmi and Dr. A. Saraswathy prepared and designed this concept and parameters.

Competing Interests

None

Summary and Conclusion

Ethanolic and ethyl acetate extract of R. tuberosa has showed very good antiproliferative, cytotoxic effect on HepG2 cells. The caspase activation triggered by the ethanolic extract of R. tuberosa at a concentration of 1000µg/ml induces apoptosis in HepG2 resulting in cell death and degradation of chromosomal DNA into small oligonucleosomal fragments, which results in fragmentation of DNA. These results suggest that R. tuberosa can be a promising anti-cancer therapeutic agent for hepatocellular carcinoma and prevents non-tumor tissues from sustaining chemotherapy-induced damage. The % of HepG2 cells counts were decreased in G0/G1, S and G2/M phase and this result shows that, ethyl acetate and ethanolic extract of R. tuberosa inhibit HepG2 cells growth at G2/M phase and prevent HepG2 cells which undergo mitosis process. This result emphasize that R. tuberosa cause DNA damage and thereby arrest the cell cycle at sub G0 state and induce apoptotic cell death. This cell cycle arrest confirmed that R. tuberosa has good antitumor activity. Up regulation of the p53 gene expression and down regulation of the NFκB gene expression in treated cells confirmed the pro-apoptotic activity of the ethanolic extracts of R. tuberosa on the cancer cells thereby highlighting the apoptotic role. These interpretations proved
that cell death occurred by increased nuclear condensation and DNA fragmentation caused by ethyl acetate and ethanolic extract through apoptosis. Overall, these findings indicate that ethyl acetate and ethanolic extract from R. tuberosa whole plant was found to be non-toxic to normal cells and probably points to the anti-proliferative, cytotoxic and apoptotic activity than ethyl acetate extract. Therefore it is recommended that the whole plant extract of R. tuberosa has anticancer activity against liver cancer. It could be a new alternative chemotherapeutic agent for human hepatocellular carcinoma after further clinical studies.

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