A PRELIMINARY INVESTIGATION ON THE CYTOTOXIC AND ANTIPROLIFERATIVE EFFECT OF RASAKARPOORA KULIGAI (RSK)

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ABSTRACT
Cancer has long ranked as the most common diseases causing death worldwide in the view of the general public and many health professionals. About 70% of cervical cancers occur in developing countries. In low income countries, it is the most common cause of cancer death. The chemotherapy is the standard method of treatment for cancer patients. Rasa karpoora kuligai is one of the Siddha herbomineral preparation mentioned to treat carcinoma. The effect of Rasa karpoora kuligai against HeLa cell lines analyzed through MTT assay, Trypan blue exclusion assay, Dual staining assay, Nuclear morphological assay, Flowcytometry and Gene expression study. Result of the present study suggested Rasakarpooarakuligai have antioxidant property and potent anticancer activity against HeLa cell lines.

KEYWORDS: Rasakarpooarakuligai, HeLa cell lines.

INTRODUCTION
Cancer has long ranked as the most common diseases causing death worldwide in the view of the general public and many health professionals. According to the World Health Organization, Physicians currently diagnose 10 million new cases of cancer each year. Statistical trends indicate that this number will double by 2020 (Mignogna et al. 2004). The cervical adeno carcinoma is second gynecological carcinoma. Now a day the incidence of cervical carcinoma decreased in developed countries (like USA) after the invention of Pap smear study. But cervical adeno carcinoma is common in developing countries due to low
level of awareness and examination. The chemotherapy is the standard method of treatment for carcinoma patients. In Siddha system of Medicine various types of Herbal compounds, Herbomineral compounds & Metallic drug preparations mentioned to treat carcinoma. Rasakarpoora Kuligai\(^1\) is one of the Siddha metallic drug preparation mentioned to treat carcinoma (i.e. cervical carcinoma, vaginal carcinoma & carcinoma of the penis). However there is no contemporary study reference for global understanding of anticancer effect of Rasakarpoorakuligai. The present study aimed to carrying out the cytotoxic and antiproliferative effects of Rasakarpoora kuligai against the human cervix carcinoma cell-HeLa by invitro method.

**MATERIALS AND METHODS**

**RASAKARPOORAKULIGAI PREPARATION\(^1\)**

a. **Ingredients**

1. Calomel (Mercury subchloride)
2. Garlic (Allium sativum)
3. Pepper (Piper nigrum)
4. Betel leaf (Piper betel)

b. **Identification & Authentication**

Calomel was purchased from K. Ramaswamy Chetty Shop, Dealers in Country drugs, 177, Rasappa chettystreet, Park town, Chennai-600003. The calomel was identified and authenticated in the Department of Geology, University of Madras, Maraimalai campus, Chennai-600025.

Pepper, Garlic and bettle leaf (Vetrilai and Kammaru vetrilai) are purchased from local market shop - Tambaram, Chennai. Pepper, Garlic, Betel leaf (Vetrilai and Kammaru vetrilai) are botanically identified and authenticated by Department of Medicinal Botany, National Institute of Siddha, Tambaram sanatorium, Chennai-600047.

c. **Purification**

1. Calomel: 53 gms of bettle leafs and pepper had taken each and grind well with water (required quantity) until paste like consistency (i.e. karkam). The paste was mixed with 6.18 liter of water and poured into a mud pot. And 200 gms of calomel tied in the cloth and immersed in the medicated water containing mud pot by Thula Iyanthiram process. It was heated with mild fire until reaching the 1/4\(^{th}\) of initial quantity.\(^1\)
2. Garlic: The skin of the garlic was peeled and then used.\cite{2}
3. Pepper: Pepper was fried for emanating the flavor.\cite{2}
4. Betel Leaf: Betel leaf was purified by removal of petiole & mid rib.\cite{2}

d. Medicine preparation procedure\cite{1}
Purified Calomel (5 varagan-21gms), Garlic (20 varagan- 84gms), Pepper (30 varagan-126gms) and Betel leaves (40 varagan- 168gms) were grinded with required quantity of betel leaf juice for 15hr (5 saamam). After that the pills was made like Solanum tuberosum size (sundai alavu: 0.798gms) and then dried in sun light. Finally the pills were stored in closed air tight container.

PRELIMINARY PHYTOCHEMICAL AND PRIMARY, SECONDARY METABOLITE ANALYSIS

Preliminary phytochemical and Primary, secondary metabolites analysis of Rasakarpooora kuligai by following standard protocols.

Extraction
The test substance - RSK were coarsely powdered and subjected to cold maceration using 70% methanol. The extracts were concentrated under vacuum, dried and used for Chemoprofiling.

Chemoprofiling
Preliminary Phytochemical Analysis
i. Detection of phenol
To a small amount of test substance, a few drops of 1:10 diluted Folin’s phenol reagent and few drops of 10% sodium hydroxide were added. The formation of bluish green colour indicates the presence of phenol.

ii. Detection of tannins
To a small amount of test substance, a few drops of 1:10 diluted Folin’s phenol reagent and few drops of sodium carbonate (dissolved in water) were added. The appearance of bluish green colour indicates the presence of tannins.

iii. Detection of saponins
A small amount of test substance was shaken well with few ml of water in the test tube. Copious lather formation shows the presence of saponins.
iv. Detection of flavones
Shinoda test: small amount of the test substance was dissolved in alcohol. A few magnesium turnings and a few drops of concentrated hydrochloric acid were added to the tube and boiled for few minutes. The appearance of red colour indicates the presence of flavones.

v. Detection of alkaloids
A small amount of the test substance was dissolved in water and mixed with few drops of concentrated hydrochloric acid. To this acidic medium, 1ml of dragendorff’s reagent was added. The appearance of red precipitate indicates the presence of alkaloids.

vi. Detection of Carbohydrates
a. Fehling’s Test
A small amount of the test substance was dissolved in water, equal volume of Fehling’s A and B reagents were added and heated. Appearance of red colour indicates the presence of carbohydrates.

b. Benedict’s test
small portion of the test substance was dissolved in water, few drops of Benedict’s reagent was added and heated. Appearance of red colour indicates the presence of carbohydrates.

vii. Detection of triterpenoids
Salkowski test
The test substance was warmed with tin granules and thionyl chloride. Pink colour formation indicates the presence of triterpenoids.

viii. Detection of glycosides
The test substance was mixed with a little anthrone on a watch glass. 1 drop of concentrated sulphuric acid was added and warmed gently over water bath. Dark green colouration indicates the presence of glycosides.

ix. Detection of anthraquinones
5 ml of the test substance was hydrolyzed with diluted sulphuric acid and 1ml of diluted ammonia was added. Appearance of pink colour indicates the presence of anthraquinones.
x. Detection of quinones
The test substance was mixed with 1ml of 10% sodium hydroxide and shaken vigorously. Blue coloration indicates the presence of quinones.

Primary Metabolites
The primary metabolites like carbohydrates, total lipids and protein contents were quantified by following standard methods.

1. Carbohydrate
1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 1ml of supernatant with duplicates in two other test tubes.
3. Make up the volume to 1ml with water in all test tubes. A tube with 1ml of water serves as the blank.
4. 4 ml of Anthrone reagent was added and heated for eight minutes in water bath and cooled.
5. The green color developed was read at 630 nm.
6. A standard graph of glucose was plotted, from which the carbohydrate content of the extract was determined.

2. Total protein
1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 0.1ml and 0.2ml of the sample extract in two other test tubes.
3. The volume of all the test tubes was made to 1ml with distilled water. A tube with 1ml of water serves as the blank.
4. 5ml of the reagent C was added to each tube including the blank. Mixed well and allowed to stand for 10min.
5. 0.5 ml of reagent D was added mixed well and incubated at room temp in the dark for 30 min.
6. Blue color was developed.
7. The Colour intensity was read at 660nm
8. A standard graph of protein was plotted, from which the protein content of the extract was determined.
3. **Total lipids**
   1. Pipette out 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the working standard into a series of test tubes.
   2. Pipette out 0.1ml of the supernatant with duplicates in two other test tubes.
   3. The volume was made upto 1 ml with working FeCl₃ acetic acid reagent (0.05%).
   4. To this 4 ml of FeCl₃ acetic acid regent was added & kept at room temperature for 10 min. to this 3ml of Con Sulphuric acid was added.
   5. The tubes were kept at ice cold condition for 20 mts.
   6. Pink Colour was formed.
   7. The color intensity was read at 540 nm.
   8. A standard graph of cholesterol was plotted from which the lipid content of the extract was determined.

**Secondary Metabolites**

1. **Total Flavonoids**

   Total flavonoid content was determined using aluminium chloride colorimetric method as described by Chang et al., (2002). The test substance was diluted in water to yield final concentrations of 125, 250, 500 and 1000 µg/250µl and the volume was made up to 2.5ml with water. 0.1ml of 10% aluminum chloride, 0.1ml of 1M sodium acetate and 2.8ml of distilled water were added to all the tubes. All the above reagents excluding the test substance served as blank. The tubes were incubated at room temperature for 30 min. The absorbance was measured at 415nm in Thermo Scientific multiskan spectrophotometer, USA. A calibration curve was plotted using quercetine as standard (Concentration range- 20-100µg).

2. **Total Tannin**

   Total tannin content was determined as per the method proposed by Schanderl et al., 1970. The test substance concentrations of 125, 250, 500 and 1000 µg/250µl were prepared in water. 0.2 ml of the sample was made up to 0.5ml with water. 0.25 ml of Folin’s phenol reagent and 2.5 ml of 1% sodium carbonate were pipetted into all the tubes. The tubes were incubated for 5 minutes at room temperature. 0.25 ml of Folin’s phenol and 2.5 ml of 1% sodium carbonate serves as blank. The blue colour developed was measured at 640 nm using Thermo Scientific multiskan spectrophotometer, USA. A calibration curve was plotted using gallic acid as standard (Concentration range- 20-100µg).
3. Total Phenols
Total phenols were determined by Folin’s Ciocalteau reagent (McDonald et al., 2001).\(^5\) 0.25 ml of the test substance (125, 250, 500 and 1000 μg) or working standard (standard phenolic compound), 1.25 ml of 1:10 diluted Folin’s Ciocalteau reagent and 1 ml of 7.5% Na\(_2\)CO\(_3\) were added to the tubes. The mixture was allowed to stand for 30 min at 37°C and the total phenols were determined using Thermo Scientific multisikan spectrophotometer, USA at 765 nm. A calibration curve was plotted using gallic acid as standard (Concentration range - 20-100μg).

4. Total reducing capacity
Reducing capacity of the drug was evaluated by the method of Oyaizu. M, 1986.\(^6\) To the test sample, a drop of thiourea (10%) and 0.25 ml of 2% Dinitro phenyl hydrazine (in 9N H\(_2\)SO\(_4\)) were added and incubated at 37°C for 3 h. After incubation, 1.25 ml of 85% H\(_2\)SO\(_4\) was added under ice-cold condition and kept at room temperature for 30 min. The absorbance was measured at 540 nm against a blank in Thermo Scientific multisikan spectrophotometer, USA. The total antioxidant activity was expressed as mg equivalents of vitamin C/g substance.

5. Total antioxidant capacity
The total antioxidant activity was evaluated by the method of Prieto et al., 1999.\(^7\) An aliquot of sample / vitamin E (equivalent to 500 μg) was combined with reagent solution (0.6M Sulphuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate). In case of blank, methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 60-90 min. Samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against the blank in Thermo Scientific multisikan spectrophotometer, USA. The total antioxidant activity was expressed as mg equivalents of vitamin E/g substance.

**Development of HPTLC fingerprint of Rasakarpoora kuligai**

**Sample Preparation**
100 mg of RSK was weighed and dissolved in 70% methanol to get a concentration of 10mg/ml concentration; this is then used for injection.

**Chromatographic Conditions**
Stationary Phase: Silica gel 60 F 254
Mobile Phase: TOL: EtAc: GAA: FA (20:45:20:5)
Scanning Wavelength: 254 nm
HEAVY METALS ANALYSIS
Presence of heavy metals like Lead, Cadmium, Mercury and Arsenic in RSK was analyzed by using Atomic absorption spectrophotometer.

PESTICIDES CONTENT ANALYSIS
Herbal formulations contain pesticide residues which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administration of fumigants during storage. Since many medicinal preparations of plant origin are taken over long periods of time, limits for pesticide residues should be established following the recommendations of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) which have already been established for food and animal feed. These recommendations include the analytical methodology for the assessment of specific pesticide residues.

Pesticide content in Rasakarpoora kuli gai was determined by GC-MS and LC/MS/MS.

INVITRO ANTIOXIDANT POTENTIAL OF RASAKARPOORA KULIGAI (RSK)

DPPH Radical Scavenging Assay
DPPH radical scavenging assay was performed as described by Koleva II et al., (2002).[8] About 10µL each concentration (1.5-1000µg/ml) of sample was added to 190µL ethanolic DPPH (150µM) solution. After vortexing, the mixture was incubated for 30min at 37°C. Control blank contains solvent without extract. The decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was measured at 517 nm in Thermo Scientific multiskan spectrophotometer, USA and the percentage inhibition was calculated using the formula

\[
\text{Percentage inhibition} = \left(\frac{\text{control} - \text{test}}{\text{control}}\right) \times 100
\]

Superoxide Radical Scavenging Assay
To 0.25ml of sodium pyrophosphate buffer (0.025M), 0.025ml of PMS (186µM) and 0.075ml of NBT (300µM), 0.1ml of the sample was added. The reaction was triggered by the addition of 0.075ml of NADH (780µM). After incubation at 30°C for 90 seconds, the reaction was terminated by addition of 0.25ml glacial acetic acid. The reaction mixture was stirred
vigorously and shaken with 2.0ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. n-butanol alone served as blank. The colour intensity of the chromogen was read at 560nm in Thermo Scientific multiskan spectrophotometer, USA (Kakkar P.et al., 1984). The percentage of superoxide radical scavenging activity was calculated using the formula

\[
\text{Percentage inhibition} = \frac{(\text{control} - \text{test})}{\text{control}} \times 100
\]

**Nitric Oxide Radical Scavenging Assay**

Aqueous sodium nitroprusside at physiological pH generates nitric oxide (NO) spontaneously, which interacts with oxygen to produce nitrite. Nitrite, thus formed can be estimated using Greiss reagent. The reaction mixture contains 0.5ml of the sample (in various concentrations - 1.95-1000 µg/ml) and 1.25ml of sodium nitroprusside (5mM) in phosphate buffer which in turn is allowed to react with 1.25ml Greiss reagent after the incubation period (150min at 37°C). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546nm (Green et al., 1982)\[^9\] in Thermo Scientific multiskan spectrophotometer, USA. The percentage of nitric oxide radical scavenging activity was calculated using the formula

\[
\text{Percentage inhibition} = \frac{(\text{control} - \text{test})}{\text{control}} \times 100
\]

**Lipid Peroxidation Inhibitory Assay**

10% liver homogenate was prepared using ice-cold KCl (0.15M) in a Teflon tissue homogenizer. In the control system, to 1 ml of tissue homogenate, the lipid peroxidation was initiated by the addition of 0.1ml of FeSO\(_4\) (25 µM), 0.1ml of ascorbate (100µM) and 0.1ml of KH\(_2\)PO\(_4\) (10mM) and the volume was made up to 3ml with distilled water and incubated at 37°C for 1h. After incubation, 1ml of 5% TCA and 1ml of 0.8% TBA were added to the reaction mixture and the tubes were boiled for 30min in a boiling water bath. Tubes were then centrifuged at 3500rpm for 10min. In the test system, homogenate was incubated with sample (different concentrations levels- 1.95-1000µg/ml). The extent of inhibition of lipid peroxidation was determined in terms of thiobarbituric acid reactive substances (TBARS) level by measuring the optical density at 532 nm in Thermo Scientific multiskan spectrophotometer, USA (Ohkawa et al., 1979).\[^10\] The percentage inhibition of lipid peroxidation was calculated using the formula

\[
\text{Percentage inhibition} = \frac{(\text{control} - \text{test})}{\text{control}} \times 100
\]
Hydroxyl Radical Scavenging Assay

The deoxyribose method was used for determining the scavenging effect on hydroxyl radicals as described by Halliwell, et., al.(1987). The reaction mixtures contained ascorbic acid (100µM), FeCl₃ (200µM), EDTA (1.04mM), H₂O₂ (1mM), deoxyribose (28 mM) with different concentrations of the test substance in a final volume of 1 ml in potassium phosphate buffer (10 mM, pH 7.4). It was incubated at 37°C for 1 h and then 0.5ml of 5.0% TCA (w/v in water) and 0.5ml of 0.8% thiobarbituric acid (TBA) (w/v in 0.5N Hcl) were added. The mixture was heated in a boiling water bath for 5 min. It was cooled and absorbance was taken at 535 nm. 0.8% TBA serves as blank whereas the reaction mixture without extract serves as control. All tests were performed in duplicate and the graph was plotted. IC₅₀ value (the concentration required to scavenge 50% hydroxyl free radical) was calculated. All analyses were run in duplicate and mean values were calculated. Percentage of inhibition was calculated using the formula:

\[
\text{Percentage inhibition} = \left(\frac{\text{control} - \text{test}}{\text{control}}\right) \times 100
\]

H₂O₂ Radical Scavenging Assay

H₂O₂ radical scavenging assay was used for determining the scavenging effect on H₂O₂ radicals as described by Asru K Sinha (1987). The reaction mixture contained H₂O₂ (2mM) with different concentrations of the test extracts in a final volume of 1 ml in phosphate buffer (10 mM, pH 7.4). It was incubated at 37°C for 5 min and then Dichromate Acetic Acid reagent (5% Potassium dichromate in water, Glacial Acetic Acid mixed in 1:3 ratio) was added and absorbance was taken at 570 nm. 2ml Dichromate Acetic acid reagent acts as blank whereas the reaction mixture without extract acts as control. All tests were performed in duplicate and the graph was plotted. IC₅₀ value (the concentration required to scavenge 50% H₂O₂ free radical) was calculated. All analyses were run in duplicate and mean values were calculated. Percentage of inhibition was calculated using the formula:

\[
\text{Percentage inhibition} = \left(\frac{\text{control} - \text{test}}{\text{control}}\right) \times 100
\]

ANTI CANCER EFFECT OF RASAKARPOORA KULIGAI AGAINST HeLa CELL LINES BY INVITRO STUDY

CELL PREPARATION AND CULTURING

HeLa cell line was procured from National Centre for Cell Science (NCCS), Pune, India with the passage number of 15. Cells were maintained in Dulbecco’s Minimum Essential Media
(DMEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C. Cells were grown in 75 cm² culture flask and after a few passages, cells were seeded for experiments. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using 0.25% Trypsin-EDTA solution.

**CELL PROLIFERATION ASSAY (OR) MTT ASSAY**

Proliferation of HeLa cells was assessed by MTT assay (Safadi et al., 2003). The proliferation test is based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. Cells were plated in 96-well plate at a concentration of 5 × 10⁴ cells/well 24 h after plating. After 24 h of cells incubation, the medium was replaced with 100 μl medium containing test drug at different concentrations (2 – 1024 μg/well) and incubated for 24 h. Untreated cells served as control and received only 0.1% DMSO in which the drug was prepared. At the end of treatment period, media from control and drug-treated cells was discarded and 20 μl of MTT (5 mg/ml PBS) was added to each well. Cells were then incubated for 4 h at 37°C in CO₂ incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 200 μl of DMSO and mixed effectively by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570 nm. Optical density of each sample was compared with control optical density and graphs were plotted.

**ASSESSMENT OF CELL VIABILITY BY TRYPAN BLUE EXCLUSION ASSAY**

HeLa cells were seeded in 96-well plates at a density of 5 × 10⁴ cells/well. After 24 h, 100 μl of culture medium with test drug (concentration ranging from 2 – 1024 μg/well) was added to the wells. Untreated cells served as control and received only 0.1% DMSO. Cells were harvested after 24 h and washed with PBS, followed by centrifugation at 2500 g for 5 min. The cell pellet was re-suspended in 1 ml of fresh culture medium, cell suspension and trypan blue (4 mg/ml) were mixed in the ratio 1:1 and incubated for 5 min at 37°C. The total number of viable cells was estimated using a hemocytometer chamber (Yumei Fan et al., 2013). Percentage of viable cells were calculated by the formula,

\[
\text{Percentage viable cells} = \left[1.00 - \left(\frac{\text{Number of trypan blue stained cells}}{\text{Total cells}}\right)\right] \times 100.
\]

**ETHIDIUM BROMIDE/ACRIDINE ORANGE (DUAL STAINING) ASSAY**

Ethidium bromide/acridine orange staining was carried out by the method of Gohelet al., 1999. HeLa cells were plated at a density of 1×10⁴ in 6-well plates. They were allowed to
grow at 37°C in a humidified CO₂ incubator until they were 70–80% confluent. Then cells were treated with 25µg/ml and 50µg/ml (selected based on the IC₅₀ concentration) of the test drug for 24h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and drug treated were mixed with 100µl of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells was counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

ASSESSMENT OF NUCLEAR MORPHOLOGY AFTER PROPIDIDIUM IODIDE STAINING (OR) NUCLEAR MORPHOLOGICAL ASSAY
Propidium iodide staining was carried out by the method of Chandramohan et al 2007.[14] HeLa cells were plated at a density of 1 × 10⁴ in 48-well plates. They were allowed to grow at 37°C in a humidified CO₂ incubator until they are 70–80% confluent. Then cells were treated with 25µg/ml and 50µg/ml of test drug for 24 h. Culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature, before fixing in methanol: acetic acid (3:1 v/v) for 10 min, and stained with 50µg/ml Propidium iodide for 20min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1 ×10³ cells were counted for assessing apoptotic cell death.

FLOW CYTOMETRY
To investigate the effect of the test drug on the cell cycle distribution, HeLa cells (1×10⁵ cells/ml) were treated with 25µg/ml and 50µg/ml cultured for 24h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and fixed in 70% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40µg/ml propidium iodide (PI) and 0.1mg/ml RNase A followed by shaking at37°C for 30min. The stained cells were analyzed with flow cytometer (Becton-Dickinson San Jose, CA, USA) and the data were consequently calculated (Tuet al., 2004).[15]

Statistical analysis
Data were expressed as mean ± S.E.M and analysed by Tukey’s test to determine the significance of differences between groups. A p value lower than 0.05, 0.01 and 0.001 was considered to be significant.
GENE EXPRESSION (mRNA)
Reverse transcriptase (RT) - PCR was performed to determine the level of mRNA expression. Briefly, cells were homogenized with 500μl TRIzol and the tubes were incubated for 10min and centrifuged at 1000 rpm for 5min. 200μl of chloroform was added to the supernatant, allowed to incubate for 5min at room temperature and centrifuged at 12000rcf for 20min. Then 500μl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000rcf for 15min following the incubation period of 10min. The supernatant was decanted carefully; the pellet was washed three times with 75% ethanol, centrifuged at 12000rcf for 15min and the pellet was allowed to air dry. The pellet was resuspended in 20μl of RNase free water and stored in -80°C until use. The isolated RNA was allowed to undergo reverse transcription and polymerization reaction to get cDNA using PCR master cycler gradient. PCR products were electrophoresed at 80V for 30min using 1.0% agarose gel with ethidium bromide stain and quantified (Bio1D software) using gel documentation unit, Vilber Lourmart, France. The following sequence was performed for each PCR reaction: 42°C for 30s, 94°C for 5min (1 cycle); 94°C for 1min, β-actin (55.4°C), Bax (58.8°C), Bcl-2 (56.7°C) and 72°C for 1 min (with 35 cycles); and a final extension phase at 72°C for 5 mins.

<table>
<thead>
<tr>
<th>Gene Marker</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>5’-CGGGAGATCGTGATGAAGT-3’</td>
<td>5’-CCACCGAACTCAAAGAAGG-3’</td>
</tr>
<tr>
<td>Bax</td>
<td>5’-GAGTGTCCTCCGGCGCAATTG-3’</td>
<td>5’-TGGTGAGCGAGCGGCTGAG-3’</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>5’-AATTCAGGGAGCGGCTAGT-3’</td>
<td>5’-GCTTGTCGCGCTACAGTTTC-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-GACATGGAGAAATCTGGCA-3’</td>
<td>5’-AATGTACGCACGGATTTCCC-3’</td>
</tr>
</tbody>
</table>

Statistical analysis
Data were expressed as mean ± standard error mean (SEM). Mean difference between groups were analysed by one way ANOVA followed by Tukey’s multiple comparison as posthoc test using Graph Pad Prism 5.0 (San Diego, USA) software. p value ≤ 0.05 was considered significant.

RESULTS
Preliminary phyto-chemical analysis
Preliminary phytochemical analysis of RSK was performed and the results were shown in the Table 1. Methanolic extract of RSK showed the presence of phenol, alkaloids and tannins contents whereas, sugars, flavones, glycosides, saponins, anthraquinones, quinines and proteins were found to be absent.
Table 1: Qualitative preliminary phytoconstituents in RSK

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenol</td>
<td>++</td>
</tr>
<tr>
<td>2.</td>
<td>Sugars</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Flavones</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Glycosides</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Anthraquinones</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>Quinones</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>Protein</td>
<td>-</td>
</tr>
</tbody>
</table>

+, ++ and +++ indicates low, mid and high levels, respectively; - indicates absence.

Primary and secondary metabolites

Plant synthesizes a wide variety of chemical compounds was sorted by their chemical class, biosynthetic origin and functional groups into primary and secondary metabolites. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic compounds, steroids etc (Akindele et al., 2007).[16]

Primary metabolites

Primary metabolite contents such as carbohydrates, total protein and lipids were analyzed in RSK and the results were shown in Table 2. RSK was found to posses’ high carbohydrate content (27.87%) when compared to total proteins and lipids (4.66 and 0.17 %, respectively).

Table 2: Primary metabolite content in RSK

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test subs.</th>
<th>Carbohydrate (% w/w)</th>
<th>Total protein (% w/w)</th>
<th>Total lipids (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RSK</td>
<td>27.87</td>
<td>4.66</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Secondary metabolites

Secondary metabolite contents such as phenol, tannin and flavonoids contents, vitamin C and E equivalents were measured in RSK and the results were shown in Table 3. The phenol, tannin and flavonoids content in RSK were found to be 93.32±5.7, 25.63±0.49, 19.70±1.92 mg/g extract, respectively. Vitamin C and E equivalent was found to be 115.92±2.93 and 2.68±0.19 mg equivalent/g, respectively.
Table 3: Secondary metabolite contents in RSK

<table>
<thead>
<tr>
<th>S. No</th>
<th>Secondary metabolite</th>
<th>Content</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenol</td>
<td>93.32±5.73</td>
<td>mg phenol/g extract</td>
</tr>
<tr>
<td>2.</td>
<td>Tannin</td>
<td>25.63±0.49</td>
<td>mg tannin/g extract</td>
</tr>
<tr>
<td>3.</td>
<td>Flavonoids</td>
<td>19.70±1.92</td>
<td>mg Flavonoids/g extract</td>
</tr>
<tr>
<td>4.</td>
<td>Vitamin C equivalent</td>
<td>115.92±2.93</td>
<td>mg equivalent /g vitamin C</td>
</tr>
<tr>
<td>5.</td>
<td>Vitamin E equivalent</td>
<td>2.68±0.19</td>
<td>mg equivalent /g vitamin E</td>
</tr>
</tbody>
</table>

**Figure 1: Primary metabolite content in RSK**

**Figure 2: Secondary metabolite contents in RSK**

**HPTLC analysis**

HPTLC fingerprint of RSK shows seven peaks at Rf values 0.14, 0.28, 0.43, 0.51, 0.76, 0.87 & 0.94. The peak at Rf value of 0.94 is found to have greater area (2175.3) among the observed peaks. The peaks at this Rf values in this chromatography condition may be tannins / polyphenols.
Finger print of RSK at 254 nm in HPTLC

<table>
<thead>
<tr>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Height (%)</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.8</td>
<td>0.14</td>
<td>12.5</td>
<td>6.44</td>
<td>0.15</td>
<td>11.6</td>
<td>350.4</td>
<td>3.45</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>7.8</td>
<td>0.28</td>
<td>40.1</td>
<td>20.68</td>
<td>0.33</td>
<td>10.6</td>
<td>3074.8</td>
<td>30.30</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>15.6</td>
<td>0.43</td>
<td>24.8</td>
<td>12.77</td>
<td>0.48</td>
<td>14.4</td>
<td>1641.2</td>
<td>16.17</td>
</tr>
<tr>
<td>4</td>
<td>0.48</td>
<td>14.7</td>
<td>0.51</td>
<td>16.2</td>
<td>8.37</td>
<td>0.56</td>
<td>0.0</td>
<td>673.3</td>
<td>6.63</td>
</tr>
<tr>
<td>5</td>
<td>0.72</td>
<td>6.9</td>
<td>0.76</td>
<td>23.7</td>
<td>12.22</td>
<td>0.77</td>
<td>22.6</td>
<td>602.2</td>
<td>5.93</td>
</tr>
<tr>
<td>6</td>
<td>0.84</td>
<td>25.0</td>
<td>0.87</td>
<td>39.6</td>
<td>20.44</td>
<td>0.90</td>
<td>22.1</td>
<td>1632.2</td>
<td>16.08</td>
</tr>
<tr>
<td>7</td>
<td>0.90</td>
<td>22.2</td>
<td>0.94</td>
<td>37.0</td>
<td>19.10</td>
<td>1.01</td>
<td>0.4</td>
<td>2175.3</td>
<td>21.43</td>
</tr>
</tbody>
</table>

Photo document of RSK in HPTLC

Heavy metal content

Heavy metals such as lead, Cadmium, Mercury and Arsenic contents in RSK were analyzed using atomic absorption spectrophotometer (AAS). The results were shown in Table 4.
### Table 4: Heavy metal content in RSK

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Heavy metals</th>
<th>Permissible Limit (AYUSH)</th>
<th>RSK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lead</td>
<td>10 ppm</td>
<td>2.05</td>
</tr>
<tr>
<td>2</td>
<td>Cadmium</td>
<td>0.3 ppm</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Mercury</td>
<td>1 ppm</td>
<td>0.75</td>
</tr>
<tr>
<td>4</td>
<td>Arsenic</td>
<td>3 ppm</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not detected.

### Pesticide residue content

Pesticides like organochlorine, organophosphorous and pyrethroids compounds were analyzed in RSK and the results were shown in the Table 5. Pesticide contents were not detected in RSK.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Pesticide Residues</th>
<th>AYUSH Limits (mg/kg)</th>
<th>RSK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Organochlorine compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alachlor</td>
<td>0.02</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Aldrin and Dieldrin</td>
<td>0.05</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>α- BHC, β- BHC, γ- BHC, γ'- BHC, Butachlor</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Chlordane (cis&amp; trans)</td>
<td>0.05</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Chlorothalonil</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>, o,p’DDT, p’-DDE, o,p’-DDE, p’-DDE, o,p’-DDD, p,p’-DDD</td>
<td>1.00</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Dicofol</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Endosulfan-Alpha, Endosulfan-Beta, Endosulfan-Sulphate</td>
<td>3.0</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Endrin, Endrin aldehyde, Endrin ketone,</td>
<td>0.05</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Epoxide</td>
<td>0.05</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Heptachlor, Heptachlor</td>
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</tr>
<tr>
<td></td>
<td>Methoxychlor</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td>2</td>
<td><strong>Organophosphorous compounds</strong></td>
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<td>Acephate</td>
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<tr>
<td></td>
<td>Diazinon</td>
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<td></td>
<td>Diclorvos</td>
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<tr>
<td></td>
<td>Dimethoate</td>
<td>-</td>
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<td></td>
<td>Ethion</td>
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<td>Not detected</td>
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<td></td>
<td>Etrimphos</td>
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</tr>
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<td></td>
<td>Iprobenphos</td>
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<td>Malaton</td>
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<td></td>
<td>Malathion</td>
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<td></td>
<td>Methamidaphos</td>
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<tr>
<td></td>
<td>Methyl paraxon</td>
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<tr>
<td></td>
<td>Monocrotophos</td>
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<tr>
<td></td>
<td>Omethoate</td>
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<tr>
<td></td>
<td>Parathion ethyl</td>
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</tr>
<tr>
<td>Chemical</td>
<td>Concentration</td>
<td>Detection</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------</td>
<td>-----------</td>
<td></td>
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<tr>
<td>Parathion methyl</td>
<td>0.2</td>
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<tr>
<td>Pencanozole</td>
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</tr>
<tr>
<td>Phorate</td>
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<td></td>
</tr>
<tr>
<td>Phoratesulphone,</td>
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<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>Phosalone,</td>
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<td></td>
</tr>
<tr>
<td>Phoratesulphoxide</td>
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<td>Not detected</td>
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<tr>
<td>Phosalone</td>
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<tr>
<td>Phoratesulfone</td>
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</tr>
<tr>
<td>Phosphamidone</td>
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<td></td>
</tr>
<tr>
<td>Profenofos</td>
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<td>Chlorpyrifos</td>
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<td>Chlorpyrifos-methyl</td>
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<td>4-Bromo-2-Chlorophenol</td>
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<tr>
<td>Chlorfenvinphos (cis &amp; trans)</td>
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<tr>
<td>Penitrothion</td>
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<tr>
<td><strong>Pyrethroids compounds</strong></td>
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</tr>
<tr>
<td>Permethrin</td>
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<tr>
<td>Cyfluthrin (I &amp; II)</td>
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<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>Cypermethrin (I, II, III &amp; IV)</td>
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<td>Not detected</td>
<td></td>
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<tr>
<td>Deltamethrin</td>
<td>0.5</td>
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<td></td>
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<tr>
<td>Fenvelarate</td>
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</tr>
<tr>
<td>Etofenprox</td>
<td></td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>Lambda cyhalothrin</td>
<td></td>
<td>Not detected</td>
<td></td>
</tr>
</tbody>
</table>

**INVITRO ANTIOXIDANT POTENTIAL OF RASAKARPOORAKULIGAI**

In vitro antioxidant potential of RSK was performed using standard methods.

**DPPH radical scavenging activity of RSK**

DPPH radical scavenging activity of RSK was concentration dependent. The IC₅₀ of 224.40µg/0.01ml. The results were represented in Figure 3.

**Superoxide radical scavenging activity of RSK**

The result of Super Oxide scavenging activity of RSK was represented in Figure 4. RSK was found to be an effective superoxide radical’s scavenger. The IC₅₀ of 3.86µg/0.1ml.

**Nitric oxide radical scavenging activity**

Nitric oxide radical scavenging activity of RSK was represented in Figure 5. RSK was found to be a potent nitric oxide scavenger. The IC₅₀ of 0.02µg/0.5ml.

**Lipid peroxidation inhibitory activity**

Effect of RSK on non-enzymatic peroxidation of lipids when incubated with ferrous sulphate is shown in Figure 6. RSK shows potential lipid peroxidation inhibitory effect with an IC₅₀ of 1.73 µg/ml.
**OH⁻ radical scavenging activity**

OH⁻ radical scavenging activity of RSK was represented in Figure 7. RSK shows potential OH⁻ radical scavenging effect with an IC₅₀ of 15.47 µg/0.1ml.

**H₂O₂ radical scavenging activity**

H₂O₂ radical scavenging activity of RSK was represented in Figure 8. RSK shows effective H₂O₂ radical scavenger. The IC₅₀ of 1390 µg/0.1ml.

---

**Figure 3: DPPH radical scavenging activity of RSK**

**Figure 4: Superoxide radical scavenging activity of RSK**
Figure 5: Nitric oxide radical scavenging activity of RSK

Figure 6: Lipid peroxide radical scavenging activity of RSK

Figure 7: Hydroxyl radical scavenging activity of RSK
Figure 8: Hydrogen peroxide radical scavenging activity of RSK

ANTICANCER EFFECT OF RASAKAPOORA KULIGAI AGAINST HeLa cells

MTT assay

HeLa cells were treated with various concentrations (2 - 1024µg) of test drug and subjected to MTT assay. As shown in Fig 9. Treatment of HeLa cells with the RSK resulted in significant dose – dependent reduction in cell growth ranging from 5.36±0.42 to 74.53±0.10 after 24h. The IC\textsubscript{50} value was found to be 56.06µg.

Assessment of cell viability by Trypan blue exclusion assay

HeLa cells were treated with RSK at varying concentrations (2 - 1024µg) to perform trypan blue exclusion assay. This study suggest RSK exhibited dose – dependent reduction in cell viability with an IC\textsubscript{50} value of 55.08µg (Fig 10).
Acridine Orange/Ethidium Bromide assay

Acridine Orange/Ethidium Bromide (AO/EB) was done to evaluate the type of cell death induced by RSK in HeLa cells; the morphological variations after double staining were investigated. Live cells stained with AO emitted green fluorescence. Early apoptotic cells had fragmented DNA which exhibited intense green coloured nuclei. Late apoptotic and necrotic cells DNA were fragmented and stained orange and red. From the data it was clear that with increasing concentration of drug, the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with 25µg/ml and 50µg/ml of drug was significantly increased (p< 0.001) to 36% and 63% respectively. The results are shown in Fig11 and Fig12.

Fig 11: Percentage of number of apoptotic cells in HeLa cells after 25µg/ml and 50µg/ml of RSK treatment for 24h.
Fig 12: Induction of apoptosis by the various concentration of RSK in HeLa cells

Nuclear morphology assay
Apoptosis was further confirmed by analysing the nuclear morphology of RSK treated HeLa cells. Nuclear morphology was evaluated with membrane-permeable PI stain. The treated cells contained more apoptotic nuclei when compared to untreated cells. There was characteristic nuclear fragmentation of nuclei in treated HeLa cells whereas the untreated control cells did not show any nuclear fragmentation. The apoptotic cells displayed characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. The percentage of apoptotic nuclei after treatment with 25µg/ml and 50µg/ml of RSK increased significantly (p<0.001) to 36% and 61%, respectively. And the results are shown in Fig13 and Fig14.

Fig13: Showing the percentage of apoptotic nuclei after treatment with RSK
The effect of the RSK was studied on the cell cycle phases of HeLa cells. After 24h of RSK drug treatment, stability in the phases of cell cycle is generally noticed and compared with the control cell line which is without any treatment. From the result it is observed that RSK is able to induce cell cycle arrest at S phase. The S phase of control group shows 4.95 of cells whereas the RSK low dose (25µg) shows 18.01% RSK high dose (50µg) shows 20.56% of cells respectively. Similarly a mild increase in G2/M phase of both low and high dose was also observed. An increased cell population in the S and G2/M with a simultaneous decrease in the G0/G1 phase (71.63% in 25µg and 62.71% in 50µg compared to control of 90.56%) compared to the untreated cells suggest that the RSK inhibited the cell cycle progression in S phase and subjected the cells to apoptosis. The results are shown in Fig15 and Fig16.

Fig 14: Shows the nuclear localization of HeLa cells after treatment with RSK by Propidium iodide staining

Flowcytometry analysis

Fig 15: Percentage of cell population in cell cycle (HeLa cells) after treatment with RSK in various concentrations
Fig: 16. Cell cycle analysis of HeLa cells after treatment with RSK
Gene expression

mRNA expression of Bax, Caspase 3 were significantly (P<0.01) up-regulated and BcL-2 was down-regulated (P<0.01) in RSK treatment, compared to un-treated control. The effect of RSK on mRNA expression of Bax, Caspase 3 and Bcl-2 show in Figure 17 and Fig 18. In our study, we observed that treatment with RSK ameliorated Bcl-2 and up-regulated Bax expression which might have promoted apoptotic responses in tumour cells. Increased Caspase 3 activation in RSK treatment might be through regulation of Bax and Bcl-2 ratios. From the data, it is suggested that RSK may be effective as an anti cancer agent at least partly by regulating the intrinsic apoptotic pathway.

![Lane 1 - Untreated Control, Lane 2 – RSK treated.](image)

**Fig 17. Effect of RSK on mRNA expression of Bax, Caspase 3 and Bcl-2**

![Graph showing relative density of apoptotic markers](image)

**Fig 18 : Effect of RSK on mRNA expression of Bax, Caspase 3 and Bcl-2**

DISCUSSION

Preliminary phytochemical analysis of methanolic extract of RSK showed the presence of Phenol, Alkaloids and Tannins. The presence of phenol in RSK was significant (Qualitatively
++). Phenols are very important plant constituents. There is a highly positive relationship between total phenols and antioxidant activity of many plant species, because of the scavenging ability of their hydroxyl groups (Vinson et al., 1998).\cite{17,18} It was also reported that phenolic compounds are effective hydrogen donors, making them very good antioxidant (yen et al., 1993).\cite{17,19} It was suggested that Polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans. Tannin also responsible for antioxidant activity in preventing a number of diseases through free radical scavenging activity. These fact supports that RSK has antioxidant action.

The presence of Tannins/Polyphenols are also confirmed by HPTLC analysis. Primary metabolite analysis of RSK showed that high carbohydrate content when compared to total protein and lipids. Secondary metabolites Phenol, Tannin and Flavonoids content in RSK were found to be 93.32±5.7, 25.63±0.49, 19.70±1.92 mg/g extract, respectively. Vitamin C and E equivalent was found to be 115.92±2.93 and 2.68±0.19 mg equivalent/g, respectively. Phenolic compounds possess antioxidant properties, which in turn aids them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Pietta, 2000).\cite{20} Tannins have a characteristic feature of metal chelation and also act through their redox property and hydrogen donating potential.\cite{21,22} Flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties.\cite{23} The presence of phenol in RSK was comparatively high than Tannin and Flavinoids. Vitamin C is considered to be a most important antioxidant in extracellular fluids and has many cellular activities of an antioxidant nature as well. Vitamin C likely to be involved in the detoxification of free radicals. In studies with human plasma lipids, it was shown that Vitamin C was for more effective in inhibiting lipid peroxidation (Estuo et.al., 1995).\cite{24} Vitamin C is a strong biological antioxidant besides Vitamin E. Vitamin E act as a peroxyl radical scavenger, preventing the propagation of free radical in tissues by reacting with them to form a tocopheryl radical, which will then be reduced by a hydrogen donor (such as Vitamin C)\cite{25} and thus return to its reduced state. The free radicals are implicated in the development of Cancer, Heart diseases and Aging. Supplementation of the diet with Vitamin C, Vitamin E and β carotene has been found to decrease the incidence of chronic diseases such as Cancer and coronary heart diseases. The secondary metabolite content analysis of RSK suggest that the presence of phytoconstituents such as Phenols, Flavonoids, Tannin and Vitamin C, Vitamin E in RSK may be responsible for antioxidant activity in preventing a number of diseases (i.e, Cancer, Heart diseases and Aging) through free radical scavenging activity.
Free radicals are the species that contain unpaired electrons. The oxygen radical electrons, the oxygen radicals such as super oxide radical (O$_2^-$), hydroxyl radical (OH) and non-free radical species, such as H$_2$O$_2$and singlet oxygen (1/2 O$_2$) are various forms of activated oxygen (Gulin et al., 2002$^{[26]}$; Yildirim et al., 2000$^{[27]}$) generated in redox species process. In the biological system, they are trapped and destroyed by specific enzymes such as super oxide dismutase, catalase and glutathione peroxidase. Over production of free radicals, together with A, C and E avitaminosis and a reduced level of the above mentioned enzymes is considered to be the causative factors for oxidative stress (Ellaim-Wojtaszek et al., 2003)$^{[28]}$. These oxygen radicals may induce some oxidative damage to biomolecules such as protiens, Lipids and DNA (Kellog & Fridovich., 1975$^{[29]}$; Lai & Piette., 1997$^{[30]}$; Wiseman & Halliwell., 1996$^{[31]}$), thus accelerating aging, cancer, cardiovascular diseases, neurogenerative diseases and inflammation (Ames., 1983$^{[32]}$; Stadtman., 1992$^{[33]}$; Sun.,1990$^{[34]}$).

Antioxidants in biological system have multiple functions which include protection from oxidative damage and in the major signalling pathways of cells. The major action of antioxidant in cells is to prevent damage caused by the active oxygen species (ROS)$^{[35]}$. Antioxidants – Vitamin C, Vitamin E and β carotene, play a beneficial role in the prevention of several chronic disorders (Diplock et al., 1998)$^{[36]}$. Flavonoids, Tannins, Anthocyanin’s and other Phenolic constituents from plant origin are reported as potential antioxidants (Salah et al., 1995; Saskia et al., 1996)$^{[37,38]}$. Therefore to analyze the invitro antioxidant activity of RSK was important to prove it act against cancer.

Interference of DPPH (stable free radical) with antioxidants, results in transfer of electrons or hydrogen atoms to DPPH, thus neutralizing free radical nature (Naik et al., 2003)$^{[39]}$. Results from the current study suggest that RSK exhibited potent [IC$_{50}$ of 224.40 µg/0.01 ml] DPPH radical scavenging activity.

The superoxide radical (O$_2^-$) is a highly toxic species which is generated by numerous biological and photochemical reactions. RSK was found to be an effective [IC$_{50}$ of 3.86µg/0.1ml] superoxide radical’s scavenger.

Reactive oxygen species (ROS) like O$_2^-$ reacts with nitric oxide (NO) and give rise to various other reactive nitrogen species (RNS) such as NO$_2$, N$_2$O$_4$, Peroxynitrite. Both ROS and RNS together induce damages various cellular molecules (Pacifici and Davies, 1991)$^{[40]}$. RSK was found to be a potent [IC$_{50}$ of 0.02µg/0.5ml] nitric oxide scavenger.
The normal cellular physiology depends on the intactness of the plasma membrane and any damage including oxidative stress modulates signal transduction pathways that may subsequently affect various downstream processes (Santana et al., 1998). RSK shows potential lipid peroxidation inhibitory effect with an IC$_{50}$ of 1.73 µg/ml.

The main oxygen species responsible for oxidative stress in biological system are hydrogen peroxide (H$_2$O$_2$), the free radical superoxide anion (O$_2^-$) and the hydroxyl radical (OH$^-$). H$_2$O$_2$ and OH$^-$ radical scavenging activity of RSK suggested that RSK exhibited H$_2$O$_2$ and OH$^-$ radical scavenging activity [IC$_{50}$ of 1390 and 15.47 µg/0.1ml, respectively].

Heavy metal content of RSK was analysed by Atomic absorption spectrophotometer. Lead and Mercury in RSK was found to be well within the limits recommended in the AYUSH guidelines and Cadmium, Arsenic not detected. The results suggest that RSK is safety for medication.

Pesticides like organochlorine, organophosphorous and pyrethroids compounds were analysed in RSK and the results shows Pesticides contents were not detected in RSK.

The effect of RSK against HeLa cell lines analysed by various assays. The proliferation of HeLa cells was assessed by MTT assay. The results suggests that treatment of HeLa cells with the RSK resulted in significantly reduce the cell growth ranging from 5.36±0.42 to 74.53±0.10 in dose dependent manner after 24h. The IC$_{50}$ value was found to be 56.06µg. The MTT suggest that RSK act as cytotoxic agent against HeLa cells.

Assessment of cell viability by Trypan blue exclusion assay shows RSK exhibited dose – dependent reduction in cell viability with an IC$_{50}$ value of 55.08µg.

To evaluate the type of cell death induced by RSK in HeLa cells was analyzed by Acridine Orange/Ethidium Bromide (AO/EB) assay. From the data it was clear that with increasing concentration of drug, the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with 25µg/ml and 50µg/ml of drug was significantly increased from 36% and 63% (p< 0.001) respectively. Finally the result suggests that RSK induce apoptosis of HeLa cells and increases apoptosis in various concentrations.

The percentage of apoptotic nuclei and antiproliferative action of RSK was measured by Propidium Iodide – Nuclear Fragmentation assay. The results suggest that RSK treated HeLa
cells shows nuclear fragmentation of nuclei and apoptotic cells shows characteristic features of condensed nuclear chromatic and formation of membrane blebs. From the data it was clear that with increasing concentration of drug, the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with 25µg/ml and 50µg/ml of RSK drug was significantly increased (p< 0.001) to 36% and 63% respectively whereas the untreated control did not show any nuclear fragmentation.

The effect of RSK on the cell cycle distribution was investigated by Flow cytometry. The cells that are constantly replicating constitute the growth fraction of the tumour. Some anticancer drugs act at particular phases on the cell cycle such as Phase specific agents, cycle specific agents and cycle non specific agents. Many cytotoxic drugs act at different points in the cell cycle.[42] Cell proliferation is characterized by four distinct phases: G1 phase, S phase, G2 phase and M phase. Non-proliferative or “resting cells” are referred to as G0 phase. In the G1 phase, cells commit to enter the cell-cycle; it is the first growth phase of a cell. In the G1 phase the cell increases in size preparing for cell division. S phase is the phase where the DNA in the cell gets replicated. After S phase, cells enter the G2 phase, where repair might occur along with preparation for mitosis in M phase. In the Mitosis phase, chromatids and daughter cells separate. After M phase, the cells can enter G1 (or) G0, a non-proliferative phase. The effect of the RSK was studied on the cell cycle phases of HeLa cells suggested that RSK inhibited the cell cycle progression in S phase and subjected the cells to apoptosis. These facts suggest RSK may act as phase specific agent in Cancer therapy.

To determine the level of mRNA expression of Bax and Caspase -3 and Bcl2 in HeLa cells after RSK treatment was performed by RT-PCR. Bcl2 family proteins are over expressed in tumour cells which in turn induces resistance against cancer therapy. Bax, a pro-apoptotic member of Bcl2 family proteins acts as promoters of apoptosis and homeostasis between Bcl2 and Bax expression plays critical role in determining the susceptibility of melanoma cells to apoptosis (Irene MG et al., 2005).[43] In our study, we observed that treatment with RSK ameliorated Bcl2 and up-regulated Bax expression which might have promoted apoptotic responses in tumour cells. The final pathway leading to execution of apoptosis is activation of series of Caspases and proteases. The intrinsic and extrinsic pathways converge to activation of Caspase 3 leading to apoptosis (Reed JC et al., 1997).[44] Impaired Cytochrome C releases and thereby losses of Caspase -3 activation are implicated in increased resistance of melanoma cells to apoptosis (Monika R et al., 2001).[45] Mitochondrial release of Cytochrome
C is regulated by Bax: Bcl2 ratio. Increased Caspase 3 activation in RSK treatment might be through regulation of Bax and Bcl2 ratios. From the data, it is suggested that RSK effective as an anti-cancer agent at least partly by regulating the intrinsic apoptotic pathway.

CONCLUSION
Invitro antioxidant potential of Rasakarpoorakuligai was performed by using standard methods suggest RSK has potent antioxidant activity.

The anticancer activity of Rasakarpoorakuligai against HeLa cell lines suggest RSK shows Antiproliferative, Cytotoxic and Apoptotic action.

In cell cycle analysis against HeLa cells RSK shows inhibited the cell cycle progression in S phase and subjected the cells to apoptosis. These facts suggest RSK act as phase specific agent.

Through gene expression study of Rasakarpoorakuligai has effective an anticancer agent partly by regulating the Intrinsic apoptotic pathway.

According to these studies reveals that Rasakarpoora kuligai has potent anticancer activity. Finally the author suggest further invivo studies are needed to prove the anticancer activity of Rasakarpoora kuligai.

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