IN VITRO EVALUATION OF ANTI-CANCER ACTIVITY OF SELECTED MEDICINAL PLANTS AGAINST LUNG CANCER

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ABSTRACT

The use of organic substances to inhibit carcinogenesis is a rapidly evolving aspect of cancer research. In present investigation the methanolic extract of Trachyspermum copticum, Coriandrum sativum, Curcuma longa, Trigonella foenum graecum and PHF (poly Herbal Formulation) was evaluated. The results obtained indicate that presences of all major phytochemicals except glycosides. The maximum antioxidant activity was reported on PHF 17.07µg/ml. Cytotoxic activity of extracts, PHF and calu6 cell lines shows 87.93 IC₅₀ value and maximum of 76.68% inhibition for concentration of 320µg/ml. The cell cycle studies on calu6 cells and human erythrocytes, PHF shows the best results with 39.25 % gated for G2M phase. In general, it was observed that the extract of PHF was found to be more effective, the results of the present findings may be useful for the discovery of novel anticancer and antioxidant agents from the plant origin.

KEYWORDS: Trachyspermum copticum, Coriandrum sativum, Curcuma longa, Trigonella foenum graecum, Calu6.

INTRODUCTION

Cancer is an endemic class of disease in both urbanized and developing countries, occurs due to various changes in the DNA sequence within cell. The origin of cancer is diverse, complex
and merely partially understood, some of them are physical, environmental, metabolic, chemical and genetic factors, which play an indirect or direct role in the stimulation of cancers\textsuperscript{[1]} About 10 million novel cases are diagnosed and over 6 million deaths occur worldwide annually because of cancer.\textsuperscript{[2]} The risk of developing cancer generally increases with age group,\textsuperscript{[3]} The chances of surviving the disease vary greatly by the type, location of the cancer and the stages of disease. Chemotherapy is plant derived phytochemicals has appeared as an accessible and promising approach to cancer control and management.\textsuperscript{[4]} Because of high death rate and serious side effects of radiation therapy and chemotherapy, many cancer patients seek alternative and complementary methods of treatment. Recent researches revolve round the urgency to evolve suitable chemotherapy consistent with new discovery in cell biology for the treatment of cancer with no toxic effect on the healthy cells. Current trends various therapies are available for the treatment of cancer such as, radiotherapy, chemotherapy etc. Besides these expensive therapies phytotherapy plays a significant role for the treatment of the cancer. Since medieval times, plants are the main source of medicines for the treatment of diseases. Despite the consequences of the availability of a wealth of synthetic drugs, plants remain even in the 21st century an integral part of the health care in different countries, especially the developing ones. In the late 90’s, the WHO stated that a big percentage of the world’s population depends on plant based therapies to cover the needs of the primary health care (WHO 1999) \textsuperscript{[5]} In this study, we determined the effect of \textit{Trachyspermum copticum}, and PHF induced apoptosis in lung cancer cells Calu6. The results of our anti-proliferative and cell cycle studies demonstrated that extracts caused a significant decrease in cell proliferation, cell growth inhibition, and induced cell cycle arrest in lung cancer cells.

MATERIALS AND METHOD

Plant Materials

Healthy plant parts of these medicinal plants \textit{Trachyspermum copticum}, \textit{Coriandrum sativum}, \textit{Curcuma longa} and \textit{Trigonella foenum graecum} were collected in month of September 2014 from local market located at Yeswantpur, Bangalore, Karnataka state. The PHF1 consist of the above plants in equal ratio, the plant material were washed thoroughly 2-3 times with running tap water and once with sterile distilled water, air dried at room temperature on a sterile blotter and used for preparation of extract.\textsuperscript{[6]}
Solvent Extraction
The air-dried and powdered plant materials (10g of each) were extracted with 250 ml methanol (CH$_3$OH) by Soxhlet apparatus at 60 °C for 8 hours \cite{7,8}. The crude extracts were filtered and the filtrate evaporated using a rotary evaporator. The dissolved constituents were further dried under pressurized vacuum conditions. Stock solutions were prepared by dissolving the dried residue in Dimethylsulphoxide (DMSO). Extract solutions were stored at -20°C until use. The working concentration of the test sample i.e. 0, 10, 20, 40, 80, 160 and 320µg/ml were prepared from the stock.

Cell Culture
The current study involves Calu6 cancer cell line, obtained from American Tissue Culture Collection (ATCC). The cell line was grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37 °C in a 5% CO$_2$ humidified incubator.

Phytochemical evaluation \cite{9}
Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the phytoconstituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1989).

Isolation of human erythrocytes
Five ml of blood was collected from healthy persons, tube containing 5.4 mg EDTA to prevent coagulation and centrifuged at 1000rpm for 10 min at 4 °C. Plasma was removed and white buffer layer was pipette with care. The erythrocytes were washed with 1X PBS, pH 7.4. Washed erythrocytes were stored at 4°C and issued within 6h for the haemolysis assay.

Haemolysis Assay
Take 50µl of 10 dilution (100µl of Erythrocytes suspension: 900µl of 1X PBS) of erythrocytes suspension into 2ml of eppendorf tube and was incubated with 100µl of various concentration of plant extracts (0, 2, 4, 8, 16, 32, 64 and 128 µg/ml) at 37°C water bath for 60-90 min. 100µl of 1% SDS as positive controls and 100µl of 1X PBS as negative control and then adjusted the volume of reaction mixture to 1ml by adding 850µl of 1X PBS. Ultimately centrifuged at 300 rpm for 5 min and the result were measured at 540nm by Tecan.
micro plate reader \(^{[10-11]}\). The haemolysis caused by 100 µl of 1% SDS was taken as 100% haemolysis; and the percentage of haemolysis was calculated by the equation

\[
\% \text{haemolysis} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100
\]

**DPPH Assay**

Antioxidant activity (DPPH free radical scavenging activity) of the extract was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by modified method.\(^{[12]}\) 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Aldrich. The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as standard in 1-100 µg/ml solution. 0.002% of DPPH was prepared in methanol and 1 ml of this solution was mixed with 1 ml of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using UV Spectrophotometer. Methanol (1 ml) with DPPH solution (0.002%, 1 ml) was used as blank. The optical density was recorded and % inhibition was calculated using the formula given.\(^{[13]}\)

Percent (\%) inhibition of DPPH activity = (A-B/A) 100

Where A = optical density of the blank and B = optical density of the sample.

**Cytotoxic activity (MTT assay)**

The cytotoxic assay detects the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase to blue insoluble formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. Briefly 5.0 X 10\(^3\) cells of Calu6 were plated in triplicate in 96 well plates with DMEM and incubated for 24 hrs at 37°C. Plant extracts were tested as working standard in serum free DMEM media and incubated for 24 hr in CO\(_2\) incubator at 37°C. After incubation with plant extracts, the media was removed from the wells and added 100 µl/well of the MTT reagent and incubated for 3-4 hrs. After incubation, the MTT reagent was removed before adding 100µl DMSO to each well and gently shaken. Plant extracts treated cells were compared to untreated cell control wells. Measure the absorbance at 590nm using a Tecan microplate reader. The percentage inhibition was determined using the formula.

\[
\% \text{Inhibition} = 100-(\text{optical density of sample/optical density of control}) \times 100.
\]
IC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell.[14-17]

**Clonogenic assay**

The Cells were seeded in a 6 well plate and incubated for 24h in 5% CO2 at 37°C. The cells were treated with two concentrations of *Curcuma longa* and *Trachyspermum copticum* plant extracts and a control with no treatment. The media was removed after 24h and fresh media along FBS was added, and incubated for 3 weeks. The media was removed and washed with PBS and were fixed with 1ml of 4% PFA: 6% Glutaraldehyde: Acetic acid: Methanol solution for 30 min. Later stained with crystal violet for 40min and washed off with distilled water and allowed to dry. Colonies containing more than 50 cells were counted.

**Statistical Analysis**

Using GraphPad Prism 5 (Graphpad, SanDiego, CA, USA) software, IC50 values for DPPH radical scavenging activity of test compounds are computed from a nonlinear regression analysis (curvefit) based on sigmoidal dose response curve (variable).

**RESULTS AND DISCUSSIONS**

Totally five crude methanolic extracts of *Trachyspermum copticum*, *Coriandrum sativum*, *Curcuma longa* and *Trigonella foenum graecum*, were analysed by various qualitative phytochemical tests to determine the active constituents. Based on Phytochemical analysis provide an understanding of the biological activities which a particular plant will have as shown in Table 1.

<table>
<thead>
<tr>
<th>Sl.no</th>
<th>General tests</th>
<th><em>Trachyspermum copticum</em></th>
<th><em>Coriandrum sativum</em></th>
<th><em>Curcuma longa</em></th>
<th><em>Trigonella foenum-graecum</em></th>
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<tbody>
<tr>
<td>1</td>
<td>Carbohydrate</td>
<td></td>
<td></td>
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<td></td>
<td>A. Molisch’s test</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td></td>
<td>B. Benedict’s test</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>2</td>
<td>Proteins</td>
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<td></td>
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<td>A. Xanthroproteic test</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>B. Ninhydrin test</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>3</td>
<td>Alkaloids</td>
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<td></td>
<td>C. Mayer’s test</td>
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Haemolysis assay the main objective is to investigate the cytotoxic activity on human erythrocytes. Haemolytic activity of the plant extracts is showed in graph 1 as per the results obtained PHF mixture in 1:1:1:1 ratio shows maximum inhibition % at the maximum concentration of 320µg/ml. When the samples were taken alone it shows maximum % inhibition at maximum concentration. And all the samples show minimum inhibition for lowest concentration of crude extracts.

![Graph 1: Haemolytic activity of selected plant extracts](image)

The antioxidant activity of the different plant extracts carried out using DPPH assay, PHF exhibited the highest scavenging activity, with an IC50 value of 15.72µg/ml compared of the
IC$_{50}$ value of the standard Quercitin was found to be 12.28µg/ml. Graph 3 represent the scavenging activity of the standard antioxidant and the Plant extracts respectively.

![DPPH assay graph](image)

**Table 2: Effect of plant extracts on calu6 cell lines.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc. µg/ml</th>
<th>OD 590 nm</th>
<th>% Inhibition</th>
<th>IC$_{50}$</th>
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<tr>
<td><strong>PHF1</strong></td>
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<tr>
<td>Control</td>
<td>0.3457</td>
<td>0.00</td>
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<td>320</td>
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<td>0.1695</td>
<td>50.97</td>
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</tbody>
</table>

Graph 2: Comparison of the scavenging activity of the methanolic extracts of the selected plants.

The MTT assay is used to determine the anti-cancer activity of the plant extracts on living cells. Table 2 shows the percentage of inhibition and graphically represented in the Graph 3.
The Calu6 cells treated with 160 and 320 µg/ml of PHF, Curcuma longa, and Trachyspermum copticum extract for 24 h showed significant inhibition of colony forming capability. Overall, result suggests that these plant extracts have inhibited the growth of lung cancer cells by preventing the formation of colony and thereby inducing apoptosis as shown figure 1 and graph 4.
Figure 1: growth of lung cancer cells

Graph 4: colony forming abilities of cell lines
CONCLUSION
The acquired data, totally the extract of mentioned medicinal plants can be considered as a source for potential anticancer and antioxidant agents. Also, the selected plants can be supplementary exploited for the discovery of novel anticancer/anti oxidant agents. The plants materials were extracted using methanol as solvent and 7-14% extraction was reported including PHF. The antioxidant activity of all the samples and PHF was conducted successfully and maximum activity was reported on PHF direct (17.07µg/ml). The second major objective that is cytotoxic activity of these plants and PHF and calu6 cell lines and 87.93 IC\textsubscript{50} value and maximum of 76.68%inhibition was reported for concentration of 320µg/ml Survival and colony forming abilities of the cell lines in the presence of crude plant extractors and all the plant extracts reported null survival of colonies. Future studies to identify induction of apoptosis, molecular mechanism of genes expression that are associated with lung cancer has to be elucidated. Nevertheless, the present findings may also supplement and strengthen the process of standardization and validation of herbal drugs containing active ingredients derived from the selected medicinal plants.

REFERENCES


