IN VITRO ANTI PROLIFERATIVE, ANTI OXIDANT AND PHYTOCHEMICAL SCREENING OF A SIDDHA DRUG CHITTIRAMOOLA KULIGAI (CMK)

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ABSTRACT
Siddha medicines are time tested formulations and having abundant anticancerous drugs cited in many classical references. Chittiramoola Kuligai (CMK) is a known drug and it is indicated for Chronic ulcers, Cancerous conditions of urogenital regions, chest regions and eight types of Gunmams (acute and chronic abdomen). The CMK is tested against HeLa cell lines which represents cervical cancer. The cell viability is applied through MTT assay technique. The different concentrations of the CMK (10 µg/ml, 50 µg/ml, 100 µg/ml) were tested. The IC 50 value was 83 µg/ml. The anti oxidant activity via different methodologies Nitric oxide and DPPH assay method were applied. The CMK showed good anti oxidant activity by lesser concentrations. The phyto chemical analysis revealed the presence of Alkaloid, Flavanoids and Triterpinoids. These phytochemicals components may be helpful in anticancerous mechanisms. Finally the drug can be taken as choice of drug to treat cervical cancer patients and the clinical evidences must be documented.

Key words: Siddha, Chittiramoola Kuligai, MTT assay.

INTRODUCTION
Siddha Medicines are wide spreadly indicated for many cancerous conditions in many classical references. This age old system describes in detail starting from diagnosis to treatment by its own way of principles. Chittiramoola Kuligai (CMK) is one of the familiar drugs among Siddha physicians prescribed internally for various types of cancerous
conditions including cervical cancer \[^1\]. The alarming rate of incidence of cervical cancer is getting high signaled by WHO report 2008. The projected rise over will be of 13.1 million in 2030\[^2\]. In India as evidenced by NCRP 2001 report, 1, 30,000 patients are suffering and death rate is also high \[^3\]. Ensuring efficacious drugs to treat cervical cancer is need of the present scenario. Trust worthy Siddha medicines are being validated to ensure its efficacy as said in the classical reference and to bring back the hope for focused keen research through scientific studies. The Siddha drug *Chittiramoola Kuligai (CMK)* was considered into account for *In vitro* MTT assay and was performed on HeLa cell lines as it represents human cervical cancer.

**MATERIALS & METHODS**

*Chittiramoola Kuligai (CMK)* is prepared as per the text book reference \[^4\]. All the chemicals used in the present study were of analytical grade and purchased from a reputed laboratory. The alcoholic extracts at different concentrations were tested for percentage of cell viability against HeLa cell lines. The extract also subjected for Anti oxidant property by some of the specified methods. The same has been tested for phytochemical analysis for assessing its qualitative nature.

**Cell lines and culture conditions**

HeLa cell lines which are representing for human cervical carcinoma were purchased from NCCS Pune was maintained in Dulbecco’s modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (HIMEDIA) for 2 minutes and passaged to T flasks in complete aseptic conditions.. Extracts were added to grown cells at a concentration of 10 µg, 50µg and 100µg from a stock of 10mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

**Preparation of *Chittiramoola Kuligai (CMK)* extract**

The extracts are prepared by refluxing in ethanol for 72 hours followed by solvent recovery using rotary evaporator. The extracts are resuspended in 1% DMSO in a final concentration of 10mg/ml.
Figure 1: Effect of CMK on HeLa cell lines at different concentrations

1. Cell viability assay

Cell viability was determined by MTT assay performed according to a standard method. MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (HIMEDIA) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The cell culture suspension was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was
transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank.

\[
\text{Viability} = \frac{\text{OD of Test}}{\text{OD of Control}} \times 100
\]

Where OD = Optical density

The data were presented as percent of viable cells (%).

**Total Cell concentration by Dye exclusion**

Cells are loaded such that \(5 \times 10^6\) cells /ml final density and grown to 60% con-fluency and was assessed by trypan blue cell exclusion assay. \(^{[6]}\)

**Observation of Cell morphological variations:** It is viewed through phase contrast microscope and variations in morphology and photographs were taken (Figure 1).

**Statistical analysis**

The IC50 (median inhibition concentration) is the concentration of toxic compound that reduces the biological activity by 50 %. The IC50 value was obtained from the MTT assay and calculated using non-linear regression analysis in Microsoft Excel software. The value was expressed as a geometric mean. Differences were considered to be statistically significant when \(p < 0.05\) and \(p < 0.01\).

![Figure 2: Percentage of cell viability at different concentrations of CMK on HeLa cell lines.](image)

**Figure 2:** Percentage of cell viability at different concentrations of CMK on HeLa cell lines.
2. Determination of Anti oxidant activity

Anti oxidant activity of *Chittiramoola Kuligai (CMK)* is performed by applying assays of Nitric oxide scavenging activity and DPPH method. The results obtained are considered for further discussion.

a. Nitric Oxide Scavenging activity\[^7,8,9\]

Nitric oxide (NO.) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite the possible beneficial effects of NO, its contribution to oxidative damage is also reported. This is due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potential oxidant that can decompose to produce OH and NO. The procedure is based on the principle that, sodium nitro prusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO may lead to tissue damage. Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL\(^{-1}\)) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (250-2500µg mL\(^{-1}\)) prepared in methanol and incubated at 25°C for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl...
ethylene diaminedihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard.

**Calculation**

\[
\% \text{ scavenging/Inhibition} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}}\right] \times 100
\]

**Fig.4 Nitric Oxide Scavenging activity**

b) **DPPH Assay (2, 2-diphenyl -1-picrylhydrazyl)**

The radical scavenging activity of different extracts was determined by using DPPH assay according to the standard method followed. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

**Principle**

1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as, \(\text{DPPH} + [\text{H-A}] \rightarrow \text{DPPH-H} + (\text{A})\) Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.
Reagent Preparation

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Procedure

Different volumes (1.25-10µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

Calculation

\[
\% \text{ inhibition} = \frac{\text{control} - \text{test}}{\text{control}} \times 100
\]

3. Phytochemical Analysis\textsuperscript{[11, 12]}

Phytochemicals, chemical compounds that occur naturally in plants (phyto means "plant" in Greek), are responsible for color and biological properties. The term is generally used to refer to those chemicals that may have biological significance but are not established as essential nutrients. The following tests are used for the analysis of phytochemicals as described by a standard method. The following tests are used for the analysis of phytochemicals present in the alcoholic extract of the tested drug. Some of the tests are done based on the standard procedure to assess the presence of alkaloid, flavanoid, phenols, glycosides, terpinoids, saponins and tannins. The inferences were listed in table 1.

a) Test for Alkaloids

Dragandroff’s test
8g of Bi (No₃)₃. 5H₂O was dissolved in 20 ml HNO₃ and 2.72g of potassium iodide in 50 ml H₂O. These were mixed and allowed to stand. When KNO₃ crystals out, the supernatant was discarded off and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na₂CO₃ followed by extraction of the liberated base with ether. To 0.5ml of alcoholic solution of extract added to 2.0 ml of HCl. To this acidic medium 1.0 ml of reagent was added. An orange red precipitate produced immediately indicates the presence of alkaloids.

b) Test for Flavanoids

Shinoda’s test
In a test tube containing 0.5 ml of alcoholic extract 5-10 drops of dilute HCl and a small piece of ZnCl₂ or Mg were added and the solution was boiled for few minutes. In the presence of flavanoids reddish pink or dirty brown color was produced.

d) Test for Phenol

Ferric chloride test
To 2 ml of alcoholic solution of extract, 2 ml of distilled water followed by drops of 10% aqueous solution of FeCl₃ solution were added. Formation of blue or green indicates the presence of phenols.

e) Test for Glycosides
A small amount of alcoholic extract was dissolved in 1 ml of H₂O and the aqueous NaOH solution was added. Formation of yellow color indicates the presence of glycosides.

f) Test for Steroids

Salkowski test
To 2ml of chloroform extract 1ml of concentrated H₂SO₄ was added carefully along the sides of the test tube in the presence of sterols a red color was produced in the chloroform layer.

g) Test for Tannins

Ferric chloride test
To 1 -2 ml of aqueous extract, few drops of 5% aqueous ferric chloride solution was added. A bluish black colour, which disappears in addition of a few ml of sulfuric acid, formation of yellowish brown precipitate.
Table 1. Phytochemical Analysis of CMK

<table>
<thead>
<tr>
<th>S.No</th>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>Orange red precipitate was found</td>
<td>Presence of alkaloid (++)</td>
</tr>
<tr>
<td>2.</td>
<td>Flavanoids</td>
<td>Pink colour was formed</td>
<td>Presence of Flavanoid (+)</td>
</tr>
<tr>
<td>3.</td>
<td>Phenols</td>
<td>No characteristic change was observed</td>
<td>Absence of phenol(-)</td>
</tr>
<tr>
<td>4.</td>
<td>Glycosides</td>
<td>No characteristic change was observed</td>
<td>Absence of glycosides(-)</td>
</tr>
<tr>
<td>5.</td>
<td>Steroids</td>
<td>Red colour was formed in the chloroform layer</td>
<td>Presence of terpenoids (++)</td>
</tr>
<tr>
<td>6.</td>
<td>Tannins</td>
<td>No characteristic change was observed</td>
<td>Absence of tannins (-)</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The results showed that there was a concentration dependent cytotoxic effect of crude extract of Chittiramoola Kuligai (CMK). At the concentration increased from 10 to 100 µg/ml, percentage of inhibition increased from 61.10% to 54.93%. At a concentration of 100 µg/ml there was a decrease in cell viability (Figure 2). The IC50 value was obtained at 83 µg/ml (Figure 3). The antioxidant activity observed in different methodologies showed that Chittiramoola Kuligai (CMK) is having significant anti-oxidant activity (Figure 4 & 5). The total cell count of HeLa cells was decreasing with increase in concentration of the Chittiramoola Kuligai (CMK) extract indicating an inhibitory effect on the cancer cell line.

Phytochemical analysis revealed that the presence of alkaloid, flavanoids and terpinoids (Table 1).

Flavonoids might induce mechanisms that affect cancer cells and inhibit tumor invasion. They exhibit broad pharmacological functions such as anticancer, anticarcinogenic, antiviral, antioxidant, antithrombogenic, and antiatherogenic activities. In recent years, flavonoids and their synthetic analogues have been intensely investigated in the treatment of ovarian, breast, cervical, pancreatic, and prostate cancer. Plant phenolics appear to have both preventive and treatment potential in combating cancer.

Antioxidants are slow down the oxidative damage of our body. Antioxidants act as a free radical scavengers. Preventing and repairing damages. Health problems such as Heart diseases, cancer and degenerative disorders are all exacerbated by oxidative damage. The Antioxidant activity of the drug Chittiramoola Kuligai (CMK) was tested by Nitric oxide scavenging activity and DPPH assay.
The results revealed that *Chittiramoola Kuligai (CMK)* has anti proliferative effect on HeLa cell lines in a concentration dependent manner (Fig.1, Fig.2 & Fig.3). Decrease in cell viability was observed on different concentration levels but significant decrease in cell viability was found in 100µg/ml concentration.

CONCLUSION

The current study enumerates anti proliferative effect of the drug *Chittiramoola Kuligai (CMK)* at dose dependent manner which is indicated in the classical reference. It leads hope for administering this drug for cervical cancer. This maneuver is easy to administer orally and moreover cost effective. This prelim study will initiate more research in the field of anti proliferative drugs.

REFERENCES

