EVALUATION OF IN VITRO CYTOTOXIC ACTIVITY OF PETROLEUM ETHER, METHANOL AND AQUEOUS EXTRACTS OF INDIAN BAY LEAF, CINNAMOMUM TAMALA (BUCH.- HAM.) T. NEES & EBERM. ON CANCER CELLS

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ABSTRACT

Cinnamomum tamala (CT) (Family- Lauracea) is widely consumed condiment and commonly known as tejpat. In present study, in vitro cytotoxic activity of three different extracts of leaves of CT was evaluated against human peripheral blood mononuclear cells and three human cancer cell lines (Lung: A549, Breast: MCF-7 and Colon: COLO 205) by Sulforhodamine B assay. Crude petroleum ether and methanol extracts were obtained by soxhlet extraction technique and aqueous extract by Cold maceration technique. Hemolytic activity of extracts was also evaluated. All three crude extracts lack cytotoxic effects on normal human cells. Whereas, different degrees of cytotoxic activity on cancer cells by these extracts were observed. Methanol extract; rich in phenols, flavonols and flavonoids; was found to be significantly more active and potent against all the cancer cell lines as compared to pet. ether and aqueous extract of CT. Lack of hemolytic activity suggests that cytotoxic activity of CT is may be due to apoptosis rather than membrane destabilization. The present study suggests novel potential antitumor activity of the Indian spice CT and need for further identification of bioactive compounds along with mechanism finding studies.

Keywords –Cinnamomum tamala, Sulforhodamine B, Tumor Cell Line, Antitumor Drug Screening Assays, Plant Extracts.
1. INTRODUCTION

Leaves and bark of Cinnamomum tamala (CT) (Buch.-Ham.) T. Nees and Eberm. (family- Lauracea) are widely used as spice throughout the world. It is commonly known as tejpat, tejpatta, tamalpatra, Indian cassia or Indian bay leaf. Its’ a medium sized evergreen tree 2-10 m tall and grows wild in the tropical and subtropical Himalayas, the Khasi hills, the nilgiri hills and at the foot of the Sikkim Himalayas. ‘Ayurveda’ describes the use of its leaves in the treatment of anorexia, bladder disorders, diarrhea and nausea [1]. Cinnamomum tamala has been shown to have mycotoxic, antimalarial [2], antimicrobial [3], hypoglycemic, hypolipidemic, antidiabetic [4], antioxidant [5], gastroprotective [6], anti-diarrheal [7] and anti-inflammatory [8] properties. Crude extract of plant and essential oil have shown brine shrimp cytotoxic activity [9,10]. Brine shrimp cytotoxicity test has been used as a bioassay for toxic substances [11] and it can be extrapolated for cell-line toxicity and antitumor activity. Hence present study was planned to evaluate cytotoxic activity of CT against normal human cells as well as human cancer cell lines.

In 2010, around 5,56,400 deaths occurred in India due to cancer. In age group of 30-69; oral, stomach and lung cancer were most fatal cancers for men; whereas; for women most prominent cancers were cervical, stomach, breast and oral cancers [12]. Apart from the disease itself, cancer patients also suffer from side effects of existing chemotherapeutic treatment [13]. Present scenario demands need for new alternative medicine with absolutely or nearly no adverse effects. Here by in vitro assay technique, we are trying to evaluate capacity of various extracts of CT to inhibit the growth or reduce the survival of cancer cells that can be correlated with their in vivo potency as an antitumor agent. Freshly isolated peripheral blood mononuclear cells (PBMC) served as normal human cells for assessing cytotoxic/ proliferative potential of test extracts.

2. MATERIALS & METHODS

2.1 Extraction of Cinnamomum tamala (leaves)

Leaves were purchased from local market, Trivendrum and authenticated (Voucher specimen No. 2010/02) by Dr. Ganesh Ayyar, Botany Dept., Ruia College, Mumbai. Powdered dried leaves were extracted by Soxhlet extraction with petroleum ether (60-80° C) followed by methanol to get petroleum ether (P.E-CT) and successive methanol extract (M.E.-CT) of CT. Both extracts were concentrated in rotary evaporator. Solvents evaporated completely from extracts till dryness, ensuring no traces of solvents remained in extract. Aqueous extract
(A.E.-CT) was prepared by cold maceration technique with intermittent shaking of dried leaf powder with distilled water. Extract was filtered, concentrated and dried.

2.2 Phytochemical investigations
Extracts were tested for presence of reducing sugars, steroids, saponins, flavonoids, tannins, phenolic compounds and gums using standard tests [14].

2.2.1 Total Phenolic Content
Phenolic content of M.E.-CT and A.E.-CT was estimated from Gallic acid calibration curve as described by Sharma et al [15]. 1ml of 1:1 diluted Folin and Ciocalteu’s reagent was added to 100 µl of various concentration of gallic acid (2-10 µg/ml) and 100 µl of 10g/L test extracts. After 5min incubation, 7% Na₂CO₃ was added to each test tube, followed by 90min incubation. Absorbance was taken at 750nm using a UV-Visible spectrophotometer (SHIMADZU UV-1650PC) and compared to a gallic acid calibration curve. The total phenolic content is expressed as gallic acid equivalent (GAE) in milligrams per gram of dry extract. The data are presented as the average of triplicate analyses.

2.2.2 Total Flavonol Content
Total Flavonol Content was estimated as rutin equivalents and expressed as mg rutin acid/g of extract. Rutin calibration curve was derived using 0.5-0.015 mg/ml Rutin in ethanol (total volume 2ml). To each test tube, 2ml of 20 g/L Aluminium trichloride in ethanol and 6 ml of 50 g/L sodium acetate were added. After incubation for 2.5 hrs at 20° C, absorbance of each tube was noted at 440nm. For test sample, 2ml of 10g/L extract in ethanol was taken instead of rutin [16].

2.2.3 Total Flavonoid Content
Total flavonoid content of successive methanol and aqueous extracts of CT was found out by method described by Tunalier [16]. To 1ml of various concentration of Rutin and test sample (10g/L), 1ml of 20 g/L Aluminium trichloride in ethanol was added and volume was made up to 25 ml with ethanol. Absorbance was taken at 415 nm after incubation at 25° C for 40 min. Blank samples were prepared from 1mL plant extract and one drop acetic acid, and diluted to 25 mL. Total flavonoid content was expressed as mg rutin acid/g of extract.
Statistical Analysis- Data were expressed as mean ± SD of triplicate determinations. Linear regression analysis was used to compare absorbance of test extracts with respective standard calibration curve.

2.3 Cell culture and harvesting
Cell lines MCF-7 (Human Breast Adenocarcinoma), COLO-205 (Human colorectal adenocarcinoma) and A549 (Human Lung carcinoma) were procured from National Centre for Cell Science, Pune, India. MCF-7 was cultured in Eagle’s Minimal Essential Medium (MEM) with Earle’s salts, L-Glutamine, 1mM sodium pyruvate, NEAA and 1.5 gm/L Sodium bicarbonate. RPMI-1640 with L-Glutamine, 25 mM Hepes buffer, 1 mM sodium pyruvate, 4.5g/L glucose and 1.5 g/L sodium bicarbonate was used for maintaining COLO-205 cell line. A549 cell line was cultured in Ham's F-12K (Kaighn's) Medium with L-Glutamine and 1.5gm/l sodium bicarbonate. Media were supplemented with FBS (10%) and antibiotic Gentamycin; Gentycin® by Piramal Health Care (40µg/ml). Cell lines were maintained in Nunc T-25 flasks at 37 °C in 5% CO2 environment (Nuaire). Cells were harvested from 80% confluent culture by trypsinization. MEM, RPMI-1640, F 12-K media, fetal bovine serum (FBS) and Trypsin Phosphate Versene Glucose (TPVG) were purchased from Himedia®.

2.3.1 Cytotoxicity assay
Sulpharhodamine B (SRB) Assay was performed to check the cytotoxic potential of plant test extracts. The monolayer cell culture was trypsinized and the cell suspension was prepared using complete medium containing 10% fetal bovine serum. To each well of the 96 well microtitre plate (Tarsons), 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when cells were adhered to plate surface, the supernatant was flicked off. Extract was first dissolved in DMSO (cell culture grade), sterilized using 0.22 micron filter (Pall) and stock of strength 2000 µg/ml was prepared in sterile nutrient media. 100µl of different test extracts concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 48 hours (72 hours for MCF-7) in 5% CO2 incubator and microscopic examination was carried out and observations recorded every 24 hours. After incubation time, SRB assay was performed by standard procedure\textsuperscript{17, 18}. 50µl of 50% trichloroacetic acid (Himedia) was added to each well. After incubation at 4°C for one hour, plates were washed four times with tap water and air dried. 96 well plates were stained with 100µl 0.04% SRB (Sigma Aldrich) and kept in dark for 30 minutes at room temperature. The
excess of dye was removed by washing four times with 1% acetic acid. 100µl of 10mM Tris base (Himedia®) (pH 10.4) was added to each well of air dried plates. The absorbance was measured using microplate reader (Biotek ELX 800 MS) at a wavelength of 540nm. Suitable blanks and positive controls were also included. Assays were performed in triplicate. Percentage of cell viability was counted as follows.

\[
\text{Percent cell viable} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100
\]

2.3 Human PBMCs

Peripheral blood mononuclear (lymphocytes) cells were collected from fresh heparinized blood of healthy volunteers (22-30 years old) by venopuncture and separated by density gradient centrifugation. 2.5 ml of HiSep™ LSM 1077 (HiMedia) was aseptically transferred to a 15 ml sterile centrifuge tube (Tarsons Products Pvt. Ltd., India) and carefully over layered with 7.5 ml 1:1 diluted blood. It is followed by centrifugation at 1000 rpm for 30 min at room temperature. The lymphocyte layer along with separation medium was carefully collected in phosphate-buffered saline and washed twice. Cells were re-suspended in RPMI-1640 medium. 10,000 PBMCs were added per well. Drug dilutions were added after 24 hr incubation. SRB assay is performed as described above after incubation of 24 hrs with drug.

2.4 In vitro Hemolytic Assay

Fresh human blood sample (heparinized) is diluted 1:1 with sterile phosphate buffered saline (PBS pH 7.4). Red blood cells (RBCs) were separated by centrifugation (1000g for 5 min) and resuspended in PBS. This procedure was repeated three times. A final 2% RBCs suspension (v/v) was prepared by suspending RBCs in PBS. For positive control 100µl sterile Distilled water was used. Test extracts were serially diluted in PBS. 100ul of 2% RBCs suspension were added to all wells of the plate. This procedure resulted in eight dilutions of ranging from 7.8125–1000.0 µg/ml of extracts. PBS and RBCs alone served as the 0% haemolysis negative control. Experiment was performed in triplicate. Plates were incubated for 3 h at RT. Supernatent were collected from each well after centrifugation at 1000 RPM for 5mins and the liberated hemoglobin was measured by taking optical density at 450 nm using Microplate Reader (Biotek Instrument )[^19].

Statistical Analysis

All the experiments were performed in triplicate. IC\textsubscript{50} (Mean ± SD) were calculated by non-linear regression analysis using GraphPad Prism version 5.00 for Windows, GraphPad
Software, San Diego California USA. Statistical significance with P value < 0.05 was determined by One-way ANOVA with Dunnett’s post test as compared to control cells using the same software.

3. RESULTS
3.1 Phytochemical Investigations

Soxhlet extraction is very efficient method and utilizes less solvent as compared to other methods of extraction. As stated in table 3.1, reducing sugars, saponins, flavonoids, tannins and phenolic compounds were found to be present in both M.E.-CT & A.E.-CT. Aqueous extract also showed presence of gums & mucilage in abundance. Wax, steroids, terpenes were found to be present in P.E.-CT.

Table 3.1 Preliminary investigations of phytochemical constituents present in different extracts of Cinnamomum tamala.

<table>
<thead>
<tr>
<th>Phytochemical Test/ reagent</th>
<th>M.E.-CT</th>
<th>P.E.-CT</th>
<th>A.E.- CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CARBOHYDRATES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molish’s test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Test for reducing sugar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Fehling’s Test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>b. Benedict’s Test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Test for Monosaccharides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barfoed’s Test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Test for Hexose Sugars</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tollen’s phloroglucinol test for galactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cobalt chloride test for glucose and/or fructose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Iodine test for Starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Test for Gums</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Test for Mucilage</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GLYCO SIDES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molish’s Test after Hydolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PROTEINS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Biuret Test (General Test)**

**FIXED OILS AND FATS**
Filter paper spot test

**SAPONINS**
Foam Test

**STEROIDs**
a. Salkowski reaction
b. Liebermann-Burchard Reaction

**FLAVONOIDS**
a. Shinoda Test
b. Lead Acetate Test
c. Sodium Hydroxide Test

**ALKALOIDS**
a. Dragendorff’s Test
b. Hager’s Test

**TANNINS AND PHENOLIC COMPOUNDS**
a. 5% FeCl₃ Test
b. Lead Acetate Test

(+ ) indicates present, (- ) indicates absent

Results of total phenolics, flavonol and flavonoid content in M.E.-CT and A.E.-CT are summerised in Table 3.2.

**Table 3.2: Content of total phenols, total flavanoids and total flavanols in methanol and aqueous extract of CT**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Total phenolsᵃ</th>
<th>Total flavanoidsᵇ</th>
<th>Total flavanolsᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.E.-CT</td>
<td>124.66 ± 6.06</td>
<td>119.49 ± 5.82</td>
<td>36.81 ± 0.48</td>
</tr>
<tr>
<td>A.E.-CT</td>
<td>27.87 ± 1.96</td>
<td>5.57 ± 0.98</td>
<td>-</td>
</tr>
</tbody>
</table>

Each value is expressed as mean± S.D (n=3)

ᵃ Total phenols expressed as gallic acid equivalents: mg of gallic acid per gm of extract;
ᵇ Total flavanoids expressed as rutin equivalents: mg of rutin per gm of extract;
ᶜ Total flavanols expressed as rutin equivalents: mg of rutin per gram of extract.
Flavanols were found to be absent in A.E.-CT whereas M.E.-CT showed higher phenolic and flavonoid content than A.E.-CT.

3.2 In vitro cytotoxicity activity against human cancer cell lines

In vitro cytotoxicity of different extracts of CT was evaluated on three cancer cell lines of different tissue origins viz, Breast, Lung, Colon. (Figure 3.1, 3.2 & 3.3) Growth inhibition in dose dependent manner was observed for M.E.-CT and P.E.-CT on all the cell lines.

![Cytotoxic Profile of Methanol Extract of Cinnamomum tamala](image)

**Figure 3.1** Percent cell viable Vs Concentration of Methanol extract of *Cinnamomum tamala* (M.E.-CT) depicting its cytotoxic activity in dose dependent manner on all three cancer cell lines.

![Cytotoxic Profile of Petroleum ether Extract of Cinnamomum tamala](image)

**Figure 3.2** Percent cell viable Vs Concentration of Petroleum ether extract of *Cinnamomum tamala* (P.E.-CT) showing dose dependent cytotoxic activity.
A.E.-CT has no significant inhibitory action on any cell lines. Doxorubicin was used as positive control.

![Cytotoxic Profile of Aqueous Extract of Cinnamomum tamala](image)

**Figure 3.3** Percent cell viable Vs Concentration of Aqueous extract of *Cinnamomum tamala* (A.E.-CT) indicating lack of significant cell killing ability.

IC$_{50}$ values were calculated and shown in Table 3.3. M.E.-CT was found to be most active amongst all the three extracts especially colorectal cancer cell line (COLO 205) with IC$_{50}$ value 115.73 ± 14.12 µg/ml. 5-Flurouracil was kept as positive control for COLO 205 cell line *in vitro* cytotoxicity study.

**Table 3.3:** IC$_{50}$ values of Methanol, Petroleum ether and Aqueous extracts of *Cinnamomum tamala* for Human cancer cell lines

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>M.E.-CT</td>
<td>154.53 ± 31.96 *</td>
</tr>
<tr>
<td>P.E.-CT</td>
<td>479.17 ± 7.24 *</td>
</tr>
<tr>
<td>A.E.-CT</td>
<td>&gt; 2000</td>
</tr>
</tbody>
</table>

Values are shown in mean ± S.D. (n=3)

Statistical significance with *P* value < 0.05 was determined by One-way ANOVA with Dunnett’s post test as compared to media control.
3.4 Effect on Human Peripheral Blood Mononuclear Cells

None of the extracts have inhibitory activity on normal PBMCs. Slight proliferative action on PBMCs was observed for successive methanol extract as shown in Figure 3.4. Doxorubicin was used as positive control for in vitro cytotoxic activity on lymphocytes with IC$_{50}$ value $21.08 \pm 2.89 \mu$g/ml***. Statistical significance with P value < 0.05 was determined by One-way ANOVA with Dunnett’s post test as compared to control cells.

![Figure 3.4](image)

Figure 3.4 Percent cell viable Vs Concentration of Aqueous, Petroleum ether and Methanol extracts of *Cinnamomum tamala* depicting no cell killing effect on normal human PBMC’s.

3.5 Hemolytic activity

In vitro hemolytic activity on erythrocytes by extracts obtained from leaves of CT was performed (Table 3.4). The total hemolysis was obtained using 100 µL of sterile D/W after three hours of incubation at room temperature. The IC50 and 95% confidence interval (95% CI) were obtained by non-linear regression analyses. The test extracts did not possess any hemolytic activity against RBCs.

### Table 3.4: In vitro hemolytic activity of various extracts obtained from leaves of CT human erythrocytes.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Human Erythrocytes IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.E.-CT</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>P.E.-CT</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>A.E.-CT</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
4. DISCUSSION

Discovery and development of new anticancer drug that has good efficacy and none of the side effects of present chemotherapeutic drugs can be cumbersome and expensive. Need for time saving, low cost, high throughput drug efficacy testing system has given rise to *in vitro* cytotoxicity testing model on human cancer cell lines. Amongst several cancer cell line panels that are available today NCI60 (National Cancer Institute-60) remains most powerful. This cell line panel comprises of 60 cell lines belonging to 8 organ sources. Other cell line panels are JFCR39 (Japanese Foundation for Cancer Research- 39 cell lines) and CMT1000 (Center for Molecular Therapeutics1000), which currently consists of 1200 human cancer cell lines \(^{20,21}\). Three cell lines were chosen for present study A549 (Lung adenocarcinoma), COLO 205 (colorectal adenocarcinoma) and MCF 7 (Breast adenocarcinoma) belonged to NCI-60 panel.

Phytochemicals are known to kill cancer cells through various mechanisms. Three extracts of leaves of *Cinnamomum tamala* were prepared using solvents of different polarities, hence, they differ in their phytochemical constitution. Methanol and water are both polar solvents. Yet it has been observed that cold maceration with water did not successfully extract active principles. It can be further confirmed by absence of flavonols. Flavonols such as kaempferol, quercetin are well documented for their *in vitro* cytotoxic activity against various human cancer cell lines \(^{22}\). These might be the reasons behind difference in level of activity between A.E.-CT and M.E.-CT. In spite of good cytotoxic activity, petroleum ether extract has solubility issues due to presences of waxes etc. Successive Methanol extract of CT was found to be most active and potent on all three cell lines.

In present study, M.E.-CT was found to be more inhibitory to colorectal cancer cells with \(IC_{50} = 115.73 \pm 14.12** \, \mu g/ml\) as compared to MCF-7 and A549. As all three cell lines are from different source organs, they have different set of receptors and growth requirements. This justifies cell line specific activity of phytochemicals. As M.E.-CT has no hemolytic activity on RBCs, we can predict that membrane destabilization or cell lysis may not be cause of cytotoxic activity of the extracts \(^{19}\). Further studies are essential to understand mechanism of cell killing.

Doxorubicin, positive control used in present study, is a broad spectrum antitumor antibiotic that acts by intercalating into DNA, thereby altering DNA structure, replication, and topoisomerase II function \(^{13}\). As its targets are also presents in normal cells, prolonged
administration of Doxorubicin causes myelosuppression, hepatotoxicity, nephrotoxicity, skin toxicity and Cardiomyopathy [23]. Thus dose limitation or co administration of immunostimulant becomes necessary for patients welfare. In such circumstances, alternative medicine therapy with effective anticancer activity sans toxicity on normal cells will act as a boon. Methanol extract of CT can be a promising candidate as it has significant cytotoxic activity against cancer cells, at the same time, no harmful effects on normal human cells. Human peripheral blood mononuclear cells were chosen as source of normal cells because myelosuppression is one of major ill-effects of current chemotherapy drugs. None of the extracts have significant cytotoxic activity on Human PBMC’s even at highest concentrations. By preliminary phytochemical studies we had been able to identify group of compounds present in M.E.-CT. But it’s a crude extract and contains cocktail of phytoconstituents necessitating need for further bioactivity guided fractionation.

5. CONCLUSIONS
In addition to being commonly used spice and flavouring agent, *Cinnamomum tamala* or tejpat has many health benefits. Here, in present study, novel *in vitro* cytotoxic activity of CT has been determined. CT is found to be selectively cytotoxic to human cancer cells, thus emphasizing its potential antitumor activity. M.E.-CT showed rich flavonoid and flavonol content and high cancer cell inhibitory activity especially on colon cancer cell line. At the same time, M.E.-CT was observed to be harmless for normal human cells and slight proliferative activity on human peripheral blood mononuclear cells. M.E.-CT has no hemolytic activity that suggests M.E. - CT is killing cells not by membrane destabilization or lysis. Study concludes, bioactive compounds present in M.E.-CT should be studied further for their potential to be used as alternative or complementary medicine for cancer treatment.

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**Conflict of Interest:** None.
REFERENCES


