Purpose: Impressed by the exceptional anticancer activity of cinnamon, the present study was conducted to elucidate the anticancer potential of essential oil of Cinnamon (EOC).

Methods: EOC was tested against various cell lines (FaDu, Detroit-562 and SCC-25) of head and neck squamous cell carcinoma (HNSCC) using MTT assay. The Hep-2 cell xenograft model was used to assess the positive bio-activity of EOC. EGFR-TK inhibitory assay was also carried out to explain the possible mechanism of action of EOC. Moreover, to rationalise the key contacts responsible for attenuating EGFR, the major component of EOC, i.e., trans-cinnamaldehyde, as identified by GC-MS analysis, was subjected to molecular docking experiments with the catalytic domain of EGFR protein model.

Results: EOC exhibited significant anticancer activity with percent inhibition 66.12, 87.32, and 99.34%, against FaDu, Detroit-562 and SCC-25, respectively. Moreover, EOC reduced the tumor burden to 43.5% in Hep-2 cell xenograft model along with 89% inhibition of EGFR-TK activity in the EGFR-TK inhibitory assay. Docking experiments showed that trans-cinnamaldehyde was proficiently fitted into the inner groove of the active site of EGFR by making close inter-atomic contacts with the key catalytic residues Val702, Ala719, Lys721, Leu764, Thr766 and Leu820 and with inhibition constant $K_i = 77.93 \mu M$.

Conclusion: EOC exhibits significant anticancer activity against HNSCC cells in vitro. The mechanism underlying its anticancer action was attributed to the suppression of EGFR-TK. It also significantly suppressed the tumor growth in Hep-2 cell xenograft model.

Key words: anticancer activity, EGFR-TKI activity, essential oil cinnamon, head & neck cancer, squamous cell carcinoma

Introduction

Despite significant advances in systemic therapies, radiotherapy, and surgical techniques, cancer is incurable in many cases [1] and represents the second leading cause of morbidity and mortality after the cardiovascular diseases. Out of existing subtypes of cancer, HNSCC ranks 8th as the most widespread cause of morbidity and mortality across the globe [2]. Its incidence is approximately 600,000 cases per year [3]. This cancer type refers to carcinoma arising in the mucosal surfaces of the oral cavity, oropharynx, larynx and hypopharynx of which 90% are HNSCC [4]. The current treatment options involve multimodality approaches that include surgery, γ-irradiation, and chemotherapy, depending on the site, size and the stage of the lesions [5]. However, the 5-year survival of patients with HNSCC is about 40-50% despite recent therapeutic advances [6,7]. Molecular-targeted therapies, based on molecular findings of the last 50 years, are one of the most...
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Promising gateways to the development of new strategies in cancer therapeutics [8,9].

A novel therapeutic approach has been to target the EGFR-TK, which is often mutated and/or overexpressed in many tumors and regulates proliferation, apoptosis, angiogenesis, tumor invasiveness, and distant metastases [10,11]. In HNSCC, either overexpression or mutation of EGFR is found in 80-100% of the patients, and both are associated with poor prognosis and decreased survival [12]. Therefore, it has been expected that treatment with EGFR inhibitors could offer numerous advantages, including selective inhibition of tumor growth and side-effects [13] (Figure 1).

Natural products are important sources of anticancer lead molecules and currently over 60% of anticancer agents come from natural sources [14], including vinca alkaloids, taxanes, podophyllotoxin and its derivative, camptothecin and its derivatives, anthracyclines and others [15]. More surprisingly, half of entire anticancer armamentarium approved internationally comes either from natural products or their derivatives [16]. Therefore, newer anticancer agents were developed on the basis of knowledge gained from small molecules or macromolecules that exist in nature.

The present study was undertaken to assess the antiproliferative activity of essential oil derived from Cinnamon against HNSCC along with a VEGFR-TKs inhibition study.

**Methods**

The bark of *Cinnamon cassia* spp used in this study was purchased from a local market. All solvents and chemical used were of analytical grade.

**Extraction of cinnamon oil using hydrodistillation**

EOC was extracted by using hydrodistillation with a Clevenger type apparatus. About 50 g of precisely weighed cinnamon bark powder were put into 500 mL distillation flask with few porcelain chips and 350 mL of distilled water was poured into the flask. The mixture was kept immersed for 1 h, followed by heating at 100 °C for 6 hrs. Then, the distillate was transferred to a 250 mL conical flask and the volatile compounds were extracted using dichloromethane from the water phase (three times). The resultant oil was then dehydrated with anhydrous sodium sulphate for 30 min and filtered through a filter paper. The cinnamon oil thus obtained was concentrated and stored at 4 °C for further analyses.

**GC-MS analysis**

The determination of the active component of volatile compound in essential oil of the plant was performed on varian 4000 GC/MS/MS model (Varian Inc, USA). Column CP8944 30 mm x 0.25 mm x 0.39 mm was used for separation. The oven temperature was raised from 40 °C to 250 °C at a constant heating rate of 5 °C min−1. The active compound in essential oil was identified using the given software.

**Cell lines and culture conditions**

Human squamous cell carcinoma cell lines FaDu, Detroit-562 and SCC-25 were obtained from the American Type Culture Collection (Manassas, Va, USA) and were used to analyse the anticancer potential of EOC. FaDu and Detroit-562 cells were grown in Eagles’ Minimum Essential Medium (EMEM, Sigma, St.Louis, MO, USA) and SCC-25 cells were grown in 1:1 mixture of DMEM and Ham’s F12 medium. Both mediums were supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan City, UT, USA) and 100 U/ml penicillin G (Sigma, St.Louis, MO, USA). Human laryngeal cancer line Hep-2 was used for in vivo experiments and cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin (Sigma, St.Louis, MO, USA). Cultures were maintained in a 5% CO2 humidified atmosphere a 37 °C.

**MTT assay**

*In vitro* cytotoxicity was determined using standard MTT assay with protocol appropriate for the individual test system [17]. The three cancer cell lines FaDu, Detroit-562 and SCC-25, were cultured in EMEM.
medium supplemented with 10% FBS, 1% glutamine and 50 mM/ml gentamycin sulfate in an incubator in humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 hrs at 37°C and in 5% CO₂. The cell lines were exposed to various concentration of EOC.

The DMSO-treated cells served as control. Cells were then treated with MTT reagent (20 µl/well) for 4 hrs at 37°C and then DMSO (200 µl) was added to each well to dissolve the formazan crystals. The experiment was performed in triplicate. Cell survival was calculated as the percentage of MTT inhibition (percent growth inhibition=100-[mean optical density/OD of each individual test group/mean OD of each control group] x 100).

**EGFR-TK inhibition assay**

An aliquot (12.5 µL) of solutions (H₂O/DMSO: 99%/1%, v/v) containing EOC (0.5 mg/ml) was added to 12.5 µL DTT/kinase buffer (pH 7.5, Cell Signaling Technology, Beverly, MA, USA) containing 100 ng of EGFR kinase (Sigma), and then incubated at room temperature (25 °C) for 5 min, followed by addition of 25 µL solutions containing 12.5 µL PTP1B (Tyr66) biotinylated peptide (CST), 1 µL ATP (CST) and 11.5 µL distilled water (D2O). The resulting mixture was left for reaction at 37 °C for 1 h, and then 50 µL stop buffer were added (50 mM EDTA, pH 8, CST) to stop the reaction. Next, 25 µL/well of each enzymatic reaction mixture and 75 µL/well of D2O were transferred to a 96-well streptavidin-coated plate and incubated at 37 °C for 1 h. Following three washings with PBS/T, 100 µL of primary antibody (Phospho-Tyrosine Mouse mAb; Cell Signaling Technology, Beverly, MA, USA), 1:1000 in PBS/T with 1.5% bovine serum albumin (BSA) were added to each well and the plate was incubated at 37 °C for another 1 h. The plate was washed again three times with PBS/T, and then 100 µL of secondary antibody (Jackson’s Immuno Research, West Grove, PA, USA, HRP-labeled Goat AntiMouse IgG, 1:1000 in PBS/T with 1.5% BSA) were added to each well for 1 h of incubation at 37 °C, followed by three washing with PBS/T. Finally, 100 µL of tetramethylbenzidine (TMB) substrate system were added to each well and the plate was incubated at 37 °C for 15 min, and then the reaction was stopped by addition of 100 mL of 1 M H₂SO₄, and the plate was read on the ELISA plate reader at 450 nm and 650 nm (SpectraMax M5 Molecular Devices Corporation, Sunnyvale, CA, USA).

**Animals**

Fifteen male nude mice (3-month old, weighing 20±2 g) were procured from the Central Animal House of Fugian Medical University, China, and kept at controlled conditions (temperature: 23 ± 2°C; light-dark cycle: 7 am to 7 pm). Mice had free access to a standard laboratory diet and water till the completion of the experiment. All animals were killed by cervical dislocation method. The research was approved by the Institutional Animal Care and Use Committee.

**Antitumor activity in the nude mouse tumour xenograft model**

All animal studies were carried out in accordance with the “Guide for the Care and Use of Laboratory Animals.” Hep-2 cells (5x10⁶ cells per 0.2 ml in PBS), were injected subcutaneously into the mice right armpit. Tumor size was measured every other day in two dimensions using caliper, and tumor volume was calculated using the formula ab²/2, where ‘a’ is the larger diameter and ‘b’ is the smaller dimension. When tumors reached about 150 mm³, animals were randomly divided into 3 groups (5 animals/group). Nude mice in the first group were injected with 5% DMSO into the caudal vein for 22 consecutive days (one injection per day). The mice in the cisplatin group were treated with 100 mg/m² of cisplatin injected into the caudal vein on the first day, followed by 21 days of normal saline administration, whereas mice in the second group were injected with EOC (50 mg/kg/day) dissolved in 5% DMSO into the caudal vein for 22 consecutive days (one injection per day). The animals were sacrificed by cervical dislocation, and the tumors were excised and weighed. The drug effects were expressed as the percent inhibition of control.

**Tumor inhibition rate**

The inhibitory effect on tumor growth was evaluated by the tumor inhibition rate. Twenty-two days after administration, rats were anesthetized with ethyl ether and killed, and the whole body and tumor were weighed immediately. The inhibition rates of solid tumor growth were calculated according to the formula: inhibition rate (%) = (1-mean weight of tumor in the drug-treated groups/mean weight of tumor in the NS group) x 100 %.

**Docking study**

Docking calculations were carried out using DockingServer [18]. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined.

Docking calculations were carried out on EGFR-TK protein model (1m17.pdb). Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [19]. Affinity (grid) maps of 60 × 60 × 60 Å grid points
and 0.375 Å spacing were generated using the Autogrid program [19]. AutoDock parameter set and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [20]. Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

Results

GC-MS analysis

Analysis was performed by comparing the levels of trans-cinnamaldehyde (Sigma, USA) as known standard for the quality control of composition of EOC in each experiment. About 9 major volatile compounds, including alcohols, aldehydes, alkenes, carboxylic acid, ether, ester and ketone were detected in the EOC. The trans-cinnamaldehyde was confirmed to be the major component with the highest percent area of 86.92 %.

In vitro anticancer activity

As shown in Table 1, EOC exerted dose-dependent inhibition in all the three tested cancer cell lines. It is noteworthy mentioning that, against the FaDu cell line, the maximum inhibitory activity was achieved at 10% v/v. However, on decreasing the test concentration to half, no significant decline in activity was observed. In the next case against Detroit–562 cells, EOC showed considerable inhibitory activity and cell viability was reduced in a dose-dependent manner. It is noteworthy to mention that, at 2.5% v/v concentration, approximately only one-third of the total cells stayed viable and the rest were inhibited by the prominent antiproliferative activity of the oil. However, on increasing the concentration to three-fold, i.e., 10% v/v, EOC showed almost complete reduction in cell viability against the SCC-25 cells. On comparing the inhibition data, it was clearly established that viability of the entire set of HSNCC cells was reduced considerably by EOC and more significantly against the SCC-25 (Table 1, Figure 2).

EGFR-TK inhibition

Due to involvement of EGFR in the process of cancer progression, its inhibition offers advantages compared with other targeted therapies, therefore, the EOC was tested to determine its inhibition against EGFR. In the inhibition assay, EOC exhibited 89% inhibition of EGFR-TK (Table 2).

Antitumor effect of EOC on Hep-2 xenograft model

Twenty-two days after the administration of EOC, the mean tumor weights in the treatment groups decreased significantly compared with those in the control group (p<0.05). In the cisplatin group and the EOC group, the tumor inhibition rates were 52% and 38.5%, respectively. The difference in tumor inhibition was significant between the cisplatin group and the EOC group (p<0.05). It was clearly corroborated from the in vivo study that the drug treatment in the nude mouse model utilizing Hep-2 cells produced a significant reduction in tumor burden, as shown in Figure 3.

Docking study

To confirm the specific binding affinity of trans-cinnamaldehyde, a major constituent of EOC responsible for pharmacological activity, it was docked in the active pocket of EGFR-TK. In the docking study, as seen from Table 3, the trans-cinnamaldehyde was snugly and deeply fitted into the solvent filled cavity of the EGFR active site confirmed by the excellent interaction surface, Free Energy of Binding, vdW + Hbond + desolv Energy and total intermolecular energy. The trans-cinnamaldehyde excellently inhibited

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Concentration in v/v</th>
<th>FaDu</th>
<th>Detroit-562</th>
<th>SCC-25</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.625</td>
<td>12.31</td>
<td>18.25</td>
<td>15.73</td>
</tr>
<tr>
<td>2</td>
<td>1.25</td>
<td>20.23</td>
<td>32.45</td>
<td>54.56</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>41.35</td>
<td>44.86</td>
<td>74.25</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>60.54</td>
<td>73.66</td>
<td>89.45</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>66.12</td>
<td>87.32</td>
<td>99.54</td>
</tr>
</tbody>
</table>
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On interacting with EGFR-TK domain, trans-cinnamaldehyde exhibited formation of H-bonds and hydrophobic interaction with key catalytic residues, like Val702, Ala719, Lys721, Leu764, Thr766 and Leu820 (Figure 4).

Discussion

Cinnamon comes from the inner bark of several tropical evergreen trees from the genus Cinnamomum and Lauraceae family and is used in sweet and savoury foods [21]. There are two main varieties of cinnamon: *Cinnamomum verum*, sometimes known as *C.zeylanicum* Nees or *Laurus cinnamomum* L which are native in India and Sri Lanka, and *Cinnamomum cassia* Blume, also known as *C.aromaticum* Nees, which is native in China, Indonesia, Laos, and Vietnam [22]. The extracts of cinnamon contain several active components such as essential oils (cinnamic aldehyde and cinnamyl aldehyde), tannin, mucus and carbohydrates [23]. They have various biological functions including antioxidant, antimicrobial, antiinflammatory, antidiabetic effects, and anti-tumor activities [24]. Cinnamon extracts exert anticancer effect via attenuating NF-kB, AP1 [24] and inhibit angiogenesis by blocking vascular en-

<table>
<thead>
<tr>
<th>Entry</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential oil of Cinnamon</td>
<td>89%</td>
</tr>
</tbody>
</table>

Table 2. Percentage of inhibition of essential oil of Cinnamon against EGFR

![Figure 2. Comparative bar-chart depicting anticancer activity of EOC (p<0.05).](image)

![Figure 3. Antitumor effect of essential oil of Cinnamon.](image)
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The extract of cinnamon exhibits potent antiproliferative effect in vitro and induces active death of tumor cells by upregulating proapoptotic molecules. It also inhibits NF-κB and AP1 activity and their target genes such as Bcl-2, BcL-xL and survivin [26]. In GC-MS, the level of trans-cinnamaldehyde was highly expressed and identified as a major component with the percent highest area of 86.92%. The results of our study were in concordance with a previous study [26]. Results of the anticancer activity clearly showed that viability of the entire set of HSNCC cells was reduced considerably by EOC and more significantly against the SCC-25 cells. The study suggests that the anticancer activity of EOC was directly linked with its ability to inhibit EGFR-TKs. Generally, head and neck cancers (HNC) are associated with enhanced expression and activity of EGFR and the EGFR downstream signalling pathway in HNSCC leads to activation of RAS/RAF/MEK/ERK, PI3-K/ AKT, STAT, PLC/PKC, EGFR nuclear signalling and the Src pathways. It further leads to cell proliferation, angiogenesis and enhanced transcription [27]. Thus, EGFR has a more selective role and could be a preferred target for anticancer agents. It has been found that EOC exhibits considerable inhibition of the enzymatic activity of EGFR-TKs. It also reduced the tumor burden of nude mouse model utilizing Hep-2 cells. In order to better understand the key structural requirements to arrest the catalytic pathways of EGFR-TKs, a molecular docking study was conducted. Molecular docking is a very powerful tool to determine the preferred orientation of one molecule to a second when bound to each other to form a stable complex. It is mainly used to define the orientation of ligand into the protein of choice for inhibition, which translates into pharmacological activity [28].

Table 3. Docking analysis of trans-cinnamaldehyde

<table>
<thead>
<tr>
<th>Drug</th>
<th>Est. free energy of binding</th>
<th>Est. inhibition constant, Ki</th>
<th>vdW + Hbond + desolv Energy</th>
<th>Electrostatic energy</th>
<th>Total intermolec. energy</th>
<th>Interact. surface</th>
<th>Interact. residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-cinnamaldehyde</td>
<td>-4.24 kcal/mol</td>
<td>775.93 µM</td>
<td>-4.80 kcal/mol</td>
<td>-0.03 kcal/mol</td>
<td>-4.83 kcal/mol</td>
<td>457.95</td>
<td>Val702, Ala719, Lys721, Leu764, Leu768, Thr766, Leu820</td>
</tr>
</tbody>
</table>

Figure 4. Docked pose of trans-cinnamaldehyde (shown in red) interacting with neighboring residues (shown in green) in the catalytic domain of EGFR-TK.
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After the pharmacological activity will be achieved. The affinity of ligand to the protein will be computed based on various mathematical descriptors or scoring parameters, e.g. free energy of binding, PLP1, PLP2, Ludi1, Ludi2, Ludi3, Inhibition constant, electrostatic energy, intermolecular energy etc. It was shown that trans-cinnamaldehyde was easily fitted in the deep cleft of the active cavity of the EGFR-TKs, revealing its mechanism of action as anticancer agent.

Conclusion

The essential oil of Cinnamon exhibited significant anticancer activity against HNSCC cells in vitro. The mechanism underlying its anticancer action was attributed to the suppression of EGFR-TK. It also significantly suppressed the tumor regression in Hep-2 xenograft model. However, much more work has to be done on the mechanism of action of essential oil of Cinnamon.

References

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