Anticancer and anti-inflammatory activities of some dietary cucurbits

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In this study, we investigated few dietary cucurbits for anticancer activity by monitoring cytotoxic (MTT and LDH assays), apoptotic (caspase-3 and annexin-V assays), and also their anti-inflammatory effects by IL-8 cytokine assay. Aqua-alcoholic (50:50) whole extracts of cucurbits \( Lagenaria siceraria \) (Ls), \( Luffa cylindrica \) (Lc) and \( Cucurbita pepo \) (Cp) were evaluated in colon cancer cells (HT-29 and HCT-15) and were compared with isolated biomolecule, cucurbitacin-B (Cbit-B). MTT and LDH assays revealed that the cucurbit extracts and Cbit-B, in a concentration dependent manner, decreased the viability of HT-29 and HCT-15 cells substantially. The viability of lymphocytes was, however, only marginally decreased, yielding a potential advantage over the tumor cells. Caspase-3 assay revealed maximum apoptosis with Ls while annexin V assay demonstrated maximum efficacy of Lc in this context. These cucurbits have also shown decreased secretion of IL-8, thereby revealing their anti-inflammatory capability. The results have demonstrated the therapeutic potential of dietary cucurbits in inhibiting cancer and inflammatory cytokine.

Keywords: Annexin V assay, Apoptosis, Cancer, Cell viability, Caspase-3 assay, \( Cucurbita pepo \), Cucurbitacin-B, IL-8 cytokines, Inflammation, \( Lagenaria siceraria \), \( Luffa cylindrica \), Lymphocytes, Tumor cells

The inflammatory reactions are inevitable during pathogenic invasions, toxic effects of pollutants and even oncogenic progression\(^1\). Chronic inflammation often leads to increased risk of cancers\(^2,3\). Several evidences support the view that gut microbiota (probiotics) is involved in the mitigation of cancer\(^4\) and inflammation\(^5\). The effects of the prebiotics, probiotics and symbiotics on the metabolic profile of enteric microbes and metabolome have been demonstrated by many workers\(^6,7\). The mechanisms by which prebiotics and probiotics may inhibit cancer include anti-inflammatory\(^8\), anticancer\(^9\), pro-apoptotic\(^9\) and autophagic\(^11\) activities of probiotics.

In the intestinal lumen the host tissue and secretions, the enteric microbes (mainly commensals/probiotics) and dietary components (prebiotics) interact intensively to generate the ‘metabolome’. Absorption of various components from metabolome through intestinal mucosa provide important biomolecules to various systems of the body for carrying out many specific and generalized reactions. The bioactive molecules from metabolome may also modulate the tumor microenvironment directly and also through the probiotics\(^4\).

Prebiotics which modify the microbiota by increasing the population of commensals have been viewed for many implications\(^12\). We have earlier reported that cucurbits \( [Cucurbita pepo \) (Cp), \( Lagenaria siceraria \) (Ls) and \( Luffa cylindrica \) (Lc) stimulated the growth of probiotics\(^13\). Also, the cucurbit fruits are known to contain a number of bioactive molecules\(^14\) like cucurbitacin, flavonoids, quercetin, phytosteroids, which induce apoptosis\(^15,16\). Earlier studies have shown different bioactive molecules present in dietary cucurbits may evoke anti-neoplastic and apoptotic action\(^17\). Further, the regulation of apoptosis plays an important role in the survival of tumor cells\(^18\). The tumor cells which are able to avoid receiving the apoptotic signals, survive the effect of tumorcidal drugs.

In the present study, we tested the hypothesis that dietary cucurbits (Cp, Ls and Lc), due to the presence of different kinds of bioactive molecules may reduce inflammation and viability of cancer cells and increase apoptosis and, thus manifest anticancer activity.

Materials and Methods

Chemicals—DMEM, Fetal bovine serum (FBS) and Trypsin were procured from M/S Sigma Aldrich, MTT and Cucurbitacin B (Cbit-B)hydrate (C8499) from M/s. Himedia, India, LDH assay kit from M/s Biovision (USA), Annexin V and Propidium iodide from M/s. Cayman (USA)
**Extract preparation**—Fresh cucurbit fruits (Cp, Ls and Lc) procured from the local market were washed thoroughly with clean water two times followed by distilled water several times. Equal quantity of each plant material (100 g/100 mL) was homogenized separately using absolute ethanol and triple distilled water (50:50, v/v) as solvent and kept for 24 h at room temperature. The homogenate was centrifuged at 5000 rpm and filtered through a fine strainer having a spread of muslin cloth. The filtrate was further passed through membrane filter (0.22 μ) and concentrated using rota-vapor and stored at 4 °C in air-tight bottles.

**Animal cell culture**—The human carcinoma cells (HT-29 and HCT-15), procured from National Centre for Cell Sciences, Pune were grown in DMEM containing 10% FBS and penicillin/streptomycin (100 µg/mL) at 37 °C in CO₂ incubator. After every 48 h, cells were sub-cultured.

**Preparation of peripheral blood mononuclear cells (PBMCs)**—Blood samples from healthy volunteers were collected into 15 mL heparinized tubes and processed for isolation of PBMCs following the method described by Song et al.19. Cell viability was determined by the trypan-blue dye exclusion method.

**Assays for bioactivities**—For cell viability, MTT assay was conducted following the method described by Shahneh et al.20. LDH-cytotoxicity assay kit (catalog no. K311-400; BioVision) was used for cytotoxic studies21 according to the manufacturer’s protocol. Caspase-3 and annexin V assays were performed for apoptosis. Caspase-3/CPP32 colorimetric assay kit was used to evaluate caspase-3 activity following the protocol described by the Biovision Research products, USA and referred by Konishi et al.22. Apoptosis by annexin-V assay was determined by using the method described by Schiller et al.23.

To assess inflammatory response, IL-8 cytokines secretion was measured by the commercially available kit (IL-8 Elisa Kit, Assay Pro, USA) following its protocol.

The cucurbit extracts and Cbit-B were added to different wells at different concentrations for evaluation of their anti-proliferative and cytotoxic effects. Different agents were administered to the wells, and after 6 h it was observed for various parameters as described above. The equitoxic concentrations (Inhibition concentrations, IC₅₀) were evaluated for each cucurbit extract and Cbit-B on the basis of MTT data. These concentrations only were used for apoptotic studies.

For stimulation of IL-8 production, cells were stressed with H₂O₂ for 30 min, and thereafter treated with each cucurbit extract and Cbit-B for 1 h. The production of IL-8 was quantitatively monitored at 450 nm by Microplate Reader.

**Statistical analysis**—Results were presented in the form of Mean Standard Deviation (MSD). Student’s t test was used to analyze the effect of control and treated cells. P <0.05 was considered statistically significant.

**Results and Discussion**

**Cell viability**

**MTT assay**—MTT assay, based on the metabolic activity of the viable cells, was done to study the adverse effect of the cucurbit extracts. The extracts of Cp, Ls and Lc were found to decrease the viability of HT-29 and HCT-15 cells in a concentration dependent manner (Fig. 1A & B). Compared to the control (100 % viability), the test samples (50 µg/mL of an extract of Cp, Ls and Lc extracts) significantly

![Fig. 1](image-url)
(P <0.05) decreased the viability of HT-29 cells by 92, 85 and 84 %, respectively. Higher quantity (150 µg/mL) of a cucurbit extract reduced the viability to about 40% only. Beyond this concentration, the cucurbit extracts did not render any significant decrease in the viability. May be that beyond this concentration the bioavailability of bioactive molecules present in the extracts did not improve further. Expectedly, the cucurbitacin (Cbit-B), a characteristic compound isolated from the family cucurbitaceae, also decreased the viability of both HT-29 and HCT-15 cells in a concentration dependent manner. However, the effect of Cbit-B was almost two times higher than that of the cucurbit extracts (Fig. 1A and B).

The IC_{50} for different agents implying 50 % survival was calculated from the data obtained through MTT assay (Table 1). IC_{50} of Lc was most effective in reducing viability of HT-29 and HCT-15 cells (IC_{50} being 147 ± 4.5 and 167 ± 3.0 µg/mL). It indicates that Lc may have higher concentration of bioactive molecules as compared to Ls and Cp. Earlier workers have demonstrated the presence of bioactive molecules such as flavonoids, alkaloids and saponins, etc., cucurbit extracts and also documented their cytotoxic and anti-tumor effects\(^\text{24-25}\).

Also, we compared the effect of cucurbit extract on human lymphocytes and cancer cells. Increasing concentrations of different cucurbit extracts and Cbit-B had little effect on the viability of lymphocytes as compared to the cancer cells (Fig. 2). The increase in the concentration of Ls and Lc from 50 to 200 µg/mL brought down the viability of lymphocytes from 94 to 81 % and 91 to 80 %, respectively. Cp was less toxic against lymphocytes and showed only 97 to 90 % decrease in the viability. Increased concentration of Cbit-B from 50 to 100 µg/mL decreased the viability of lymphocytes from 91 to 85%. However, this decrease was much less as compared to the cancer cells. This differential effect against cancer cells and lymphocytes may be attributed to the cellular receptors present on normal and cancer cells. However, the mechanism of differential action of these cucurbit extracts deserves further investigation.

LDH assay—The assay depends on a soluble cytosolic enzyme lactate dehydrogenase (LDH), present in the cells. On cell death, LDH is released into the culture medium and its concentration is directly proportional to the frequency of cell death. To confirm the reliability of MTT assay the effect of cucurbit extracts on the viability of HT-29 and HCT-15 cells were also evaluated using LDH assay.

Increased concentrations of Cp, Ls, Lc and Cbit-B enhanced the toxicity in a progressive manner (Fig. 3A & B). Ls extract, at a concentration of 50 µg/mL was more toxic (11%) than equivalent concentrations of Lc (9%) and Cp (4%) against HT-29 cells. At higher concentrations (200 µg/mL), Lc exhibited maximum toxicity (71%) followed by Ls (63%) and Cp (57%). Similar pattern was revealed by the cucurbit extracts and Cbit-B against HCT-15 cells.

Table-1—IC_{50} values of different cucurbit extracts and Cbit-B evaluated against colon cancer cell lines (HT-29 and HCT-15) by MTT assay*

<table>
<thead>
<tr>
<th>Different agents</th>
<th>IC_{50} Values (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HT-29 cells</td>
</tr>
<tr>
<td>Cbit-B</td>
<td>87 ± 0.9</td>
</tr>
<tr>
<td>Lagenaria siceraria</td>
<td>170 ± 5.0</td>
</tr>
<tr>
<td>Lagenaria cylindrica</td>
<td>147 ± 4.5</td>
</tr>
<tr>
<td>Cucurbita pepo</td>
<td>181 ± 1.9</td>
</tr>
</tbody>
</table>

*The data used for computing IC_{50} values has been presented in Fig.1A and 1B.

Fig. 2—Effect of cucurbit extracts and Cbit-B on the viability of lymphocytes evaluated by MTT assay. The control group had no treatment and its viability was considered as 100 %. The effect of various agents on the viability was evaluated as percentage of the control. Each bar was a mean of three experiments; top of the bar displayed the Mean ± SD. Significance (*P <0.05) was compared with control (Student’s t test).

Apoptotic activity

Caspase-3 assay—The caspase-cascade system play a vital role in the induction, transduction and amplification of intracellular apoptotic signals\(^\text{26-27}\). Initiator caspases (Caspase- 8, 9 and 10) activated by extrinsic or intrinsic factors in turn activate effector caspases, such as 3, 6 and 7 that cleave the cellular proteins. This generates typical morphological changes in cells undergoing apoptosis\(^\text{28}\). Fig. 4 shows the...
caspase-3 activity as measured spectrophotometrically in both HT-29 and HCT-15 cells here in this study.

The cucurbit extracts increased the frequency of apoptosis in both HT-29 and HCT-15 cells (Fig. 4). Ls, Lc, Cp and Cbit-B at IC_{50} increased the frequency of apoptosis over the control value to 30, 12, 16 and 14 %, respectively in HT-29, and 33, 20, 10 and 26 %, respectively in HCT-15 cells. The Ls treatment to HT-29 and HCT-15 cells exhibited higher incidence of apoptosis as compared to Lc, Cp and cucurbitacin. The effect of Ls have surpassed the effect of the reference compound, Cbit-B. This higher incidence could be attributed to the presence of different types of cucurbitacins (like Cbit-C, Cbit-C, Cbit-E, etc) other than the Cbit-B in Ls. These compounds may synergistically enlarge the magnitude of effects than the one manifested by Cbit-B alone.

Annexin V staining assay—Annexin V/Propidium iodide staining method detects the early stage of apoptosis. This method is based on externalization of phosphatidylserine, a plasma membrane phospholipid, from the inner to the outer leaflet of the plasma membrane. Annexin V binds to phosphatidylserine, exposed on the cell surface, and thus, detects the apoptotic cells. In the control group, the frequency of apoptotic cells was about 9 and 4 % in HT-29 and HCT-15 cells, respectively (Table 2). By this method, the frequency of apoptosis in HT-29 cells was found maximum in the presence of Lc (27 %) compared to Ls (18 %) and Cp (21 %). Similarly, in HCT-15 cells, maximum apoptosis was observed in Lc (29 %) than Ls (28 %) and Cp (27 %). Cbit-B rendered 25 % and 23 % apoptosis in HT-29 and HCT-15 cells, respectively. The differences in apoptotic frequency between the HT-29 and HCT-15 cells may be attributed to the differences in the sensitivity of cells for these cucurbits.

Anti-inflammatory activity

IL-8 cytokine assay—During this study, IL-8 assay was carried out in HT-29 cells. IL-8 which is a pro-inflammatory cytokine, attracts the neutrophils and other granulocytes and directs them to move towards the site of infection. The effect of the cucurbit extracts on IL-8 production has been shown in Fig. 5. Increasing concentration of Cp extract from 50 to 200 µg/mL decreased the production of IL-8 cytokine from 61 to 44% against the control value (100%). Similar increase in the concentration of Lc and Ls extracts brought down the IL-8 production from 80 to 48% and 81 to 53%, respectively against control (Fig. 5). Cbit-B at equivalent concentration was less effective than the cucurbit extracts. It reduced the IL-8 production from 85 to 60 %.
The significance of pro-inflammatory cytokine IL-8 is an important benchmark for evaluation of tissue inflammation. Many chronic diseases like cancer owe their genesis in the chronic inflammation in same tissue(s). The scaling down of inflammation by the cucurbit extracts could be useful in mitigating the oncogenic activity. Even, acute inflammation which is a useful activity to begin with, may in many cases transform into chronic diseases. Slowing down of inflammation may again prove useful in reducing chronic diseases like Crohn’s disease, IBS and colon cancer.

The above study based on HT-29 and HCT-15 cells (colon cancer cells) indicates that the cucurbit extracts viz., Lagenaria siceraria, Luffa cylindrica and Cucurbita pepo could serve as a potential source of anticancer and anti-inflammatory agents.

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**Table-2—Effect of cucurbits and Chit-B (at IC₅₀) on the frequency of apoptotic and dead cells evaluated by annexin V staining method**

<table>
<thead>
<tr>
<th>Different agents</th>
<th>HT-29 cells (%)</th>
<th>HCT-15 cells (%)</th>
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<tbody>
<tr>
<td></td>
<td>Dead cells</td>
<td>Apoptotic cells</td>
</tr>
<tr>
<td>Control</td>
<td>9 ± 1.6</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>Ls</td>
<td>47 ± 2.5*</td>
<td>18 ± 2.0*</td>
</tr>
<tr>
<td>Lc</td>
<td>43 ± 2.1*</td>
<td>27 ± 2.0*</td>
</tr>
<tr>
<td>Cp</td>
<td>52 ± 2.0*</td>
<td>21 ± 1.0*</td>
</tr>
<tr>
<td>Chit-B</td>
<td>41 ± 1.0*</td>
<td>25 ± 1.2*</td>
</tr>
</tbody>
</table>

Different cells (dead and apoptotic) were counted under fluorescence microscope (40 × 10 X) and presented as percentage of the total cells. The apoptotic cells were identified by annexin V staining and dead cells which included apoptotic cells also were identified using propidium iodide staining. Each value is the mean of three experiments and was displayed as Mean ± SD. Significance at *P < .05 was compared with control (student’s t test).

**Fig. 5—Anti-inflammatory effects of cucurbits and Chit-B by quantifying of cytokine IL-8 secretion by HT-29 cells.** The control group had H₂O₂ treatment and the IL-8 secretion was considered as 100 %. The effect of the samples on the IL-8 production was evaluated as percentage of the control. Each bar was a mean of three experiments; top of the bar displayed the Mean ± SD. Significance ( *P <0.05) was compared with control (Student’s t test).

**References**


