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Immunomodulatory and Apoptotic Effect of Cinnamaldehyde in HepG2 Cells

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Abstract: Hepatocellular carcinoma (HCC), associated with various clinicopathological features such as genetic mutations and viral infections, is the fifth most common cancer worldwide. In Asia and Africa, the incidence of HCC is the highest. Half of all cases of HCC are associated with hepatitis B viral infection, with a further 25% associated with the hepatitis C virus. The most widely used drug against liver cancer is Doxorubicin as a single agent or in combination with other 9 chemotherapeutics like Cisplatin. Since the normal hepatocytes are affected by the subsisting conventional chemotherapeutic drugs, the outcomes remain considerably low. Therefore, the field is longing for the discovery of new therapeutic agents without hepatotoxicity or with low hepatotoxicity. Recent studies discovered that an α, β-unsaturated aromatic aldehyde has anti-inflammatory, antiproliferative, and anti-apoptotic against the HepG2 cell line. This α, β-unsaturated aromatic aldehyde is cinnamaldehyde, an extensive component that is present in cinnamon essential oil and is also used as a flavoring agent in food, beverages, and perfume industries. Results showed that the cinnamaldehyde decreased the proliferation of HepG2 cells in a dose-dependent manner (MTT assay). We observed a significant increase in the levels of IL-1 β and a decrease in the levels of IL-10 after Cinnamaldehyde treatment. Cinnamaldehyde also increased the Caspase-3 activity in HepG2 cells significantly. The present study showed that cinnamaldehyde has strong potential as an anti-tumor agent against hepatocellular carcinoma cells.

Keywords: cancer; cinnamaldehyde; apoptosis; interleukin; carcinoma.

1. Introduction

The uncontrolled proliferation, growth, and spread of the abnormal cells result in a malignant condition known as cancer. It involves the development of normal cells or precancerous lesions into malignant tumors. Cancerous deaths have been affecting adults throughout the world. For instance, the USA has encountered such deaths of one in four people [1], while the international agency for cancer research in India reported the deaths of 635,000

cancer patients in 2008 [2]. Despite the technological advancements in the medical field, the survival rates remain poor due to the inability to detect and treat the cascade of diseases. However, this condition has led to therapeutic strategies without compromising the minimization of side effects [3–6]. Anticancer medications that target critical biological processes, including deoxyribonucleic acid (DNA), topoisomerases, microtubules, and histone deacetylases in cancer cells, may also have a comparable effect on healthy cells [7,8]. These are referred to as "side effects" when a drug's inhibitory activity extends to more normal cells than malignant ones [9–13]. Simple side effects, including neutropenia, anemia, mucositis, colitis, and diarrhea, may also occur, as more serious ones include mitotic spindle arrest and critical cell signaling processes [14–16]. The most frequent negative side effects of anticancer medications are nausea and vomiting [17].

Hepatocellular Carcinoma has been a widely known common morbid malignancy in the United States of America and worldwide [18]. The incidence of new cases in the United States has been around 8500 to 11,500 annually. However, the statistics show a rapid increase since the mid-1980s [19]. The annual occurrence rate of hepatocellular carcinoma in one lakh individuals in India varies from 0.2 to 2.2 in females and 0.7 to 7.5 in males, while 70 percent of the cases exist in the advanced stage [20].

An active ingredient, cinnamaldehyde (CA), with high antioxidant properties, has been the source of the characteristic aroma of cinnamon, used as a natural spice in homes and food industries [21–23]. The bark of the cinnamon tree consists of 1% to 4% CA concentration, and the cinnamon oil accounts for an abundance of 65% to 75%. Though its usage was limited to providing aroma candles and flavoring agents, its higher potential has been proven by possessing antibacterial, antifungal, and antiviral properties [24]. It has also been demonstrated to possess anticancer activities in vivo and in vitro for rectal, oral, and lung cancers [25–29]. The main aim of the present study is to investigate the apoptotic and immunomodulatory consequences of CA on the cancer cell line, HepG2.

2. Materials and Methods

2.1. Chemicals and cell line.

Human Hepatocellular Carcinoma (HepG2) obtained from NCCS, Pune, was used for the experiment. Ethanol, crystal violet, dimethyl sulfoxide, penicillin, streptomycin, DMEM medium, cinnamaldehyde, caspase activity assay kit, trypsin, and thiazolyl tetrazolium (MTT) were purchased from Sigma Aldrich. The other chemicals and materials used in the experiment were cell culture grade.

2.2. Cell culture.

HepG2, cells were routinely cultured in DMEM with supplementation of penicillin (10,000µg/ml) and streptomycin (10,000µg/ml). Cells were incubated in a CO_2 (5%) incubator at 37°C. The medium was replaced every 2nd or 3rd day, and when the cells reached the confluency of 90%, they were trypsinized using 0.25% Trypsin-EDTA before passaging them.

2.3. MTT assay.

Cells were (1×10^5) seeded in 96 well plates, and CA was used in the concentration range of 5 – 35 µM for 24-72 h. After the incubation, 0.1 mg/mL MTT (100 µL) was added

and incubated for 4 hours in a CO_2 incubator at 37°C before adding dimethyl sulfoxide (DMSO). The absorbance was recorded at 570 nm in an ELISA reader. Cell proliferation was calculated as the percentage of untreated (control) cells.

2.4. qRT-PCR.

The total RNA was isolated from the cell culture, and first-strand synthesis was made following the manufacturer's protocol. The primers listed in Table 1 were used for real-time expression profiling.

Table 1. Primers used for the real-time expression profiling.

Gene	Forward	Reverse
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
IL-1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG

2.5. Detection of caspase activity.

The caspase activity was determined by using a colorimetric assay kit. The cells (2 x 10^5 cells/well) were seeded in a 96-well plate, allowed to attach, and treated with CA for 72 hours. Following the incubation, the cells were washed, centrifuged for 5 minutes at 600xg for 10 min, and washed twice with ice-cold PBS. Cells were lysed with 50 µl of cell lysis buffer on ice for 5 min with sonication and micro centrifuged to remove the pellet. The cells were then centrifuged at 4°C for 15 minutes at 16000 rpm. To the supernatant in the 96 well-plate 50 µl of 1 x assay buffer, 10 µl of caspase inhibitor was added 5 µl of (Ac- DEVD-pNA) for caspase-3 was added. After incubation for 90 minutes at 37◦C, 5 µl of specific caspase 3 substrates was added, and the caspase activity was calculated. ZVAD, a pan-caspase inhibitor, was pretreated to cells to inhibit caspase activation.

2.6. Statistical analysis.

ONE-WAY ANOVA analyzed the results with multiple comparisons. The mean ±SD was shown as error bars of three independent experiments. Values (*p<0.05),(**p<;0.01),(***p<0.001) were considered statistically significant.

3. Results and Discussion

3.1. Cinnamaldehyde decreases liver cancer cell proliferation.

Cancer cells were treated with increasing concentrations of cinnamaldehyde (5- 35 µM). The structure of cinnamaldehyde is shown in Figure 1.

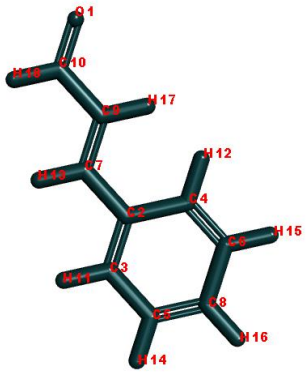

Figure 1. Molecular structure of cinnamaldehyde (Pubchem ID: 637511).

The cells were treated at 24, 48, and 72 h. Treatment of the cancer hepatocytes reduced the cell proliferation (Figure 2) of cells in a dose and time-dependent manner. The results of the cell proliferation assay are shown in Figure 2. The IC_{50} of the drug was found to be 16.36, 12.57, and 11.12 µM for 24 h, 48 h, and 72 h, respectively.

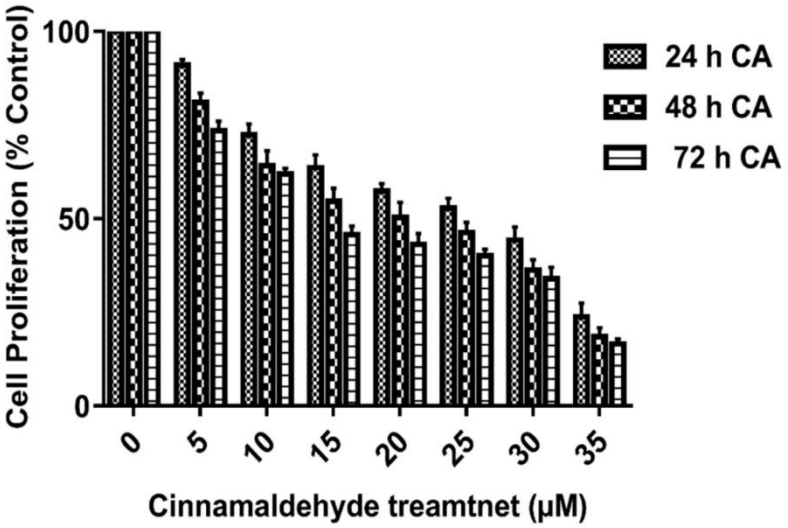

Figure 2. MTT assay of HepG2 cell line treated with cinnamaldehyde for up to 72 h.

3.2. Cinnamaldehyde treatment activates caspase-3 in cancer cells.

The treatment with the drug in the cells for 72 h showed increased caspase-3 activity (Figure 3). To confirm the drug mediates it, the pan-caspase inhibitor zVAD was pretreated to cells, and such treatment inhibited the caspase-3 activation. These results demonstrate that the activation of caspase mediates the drug's action.

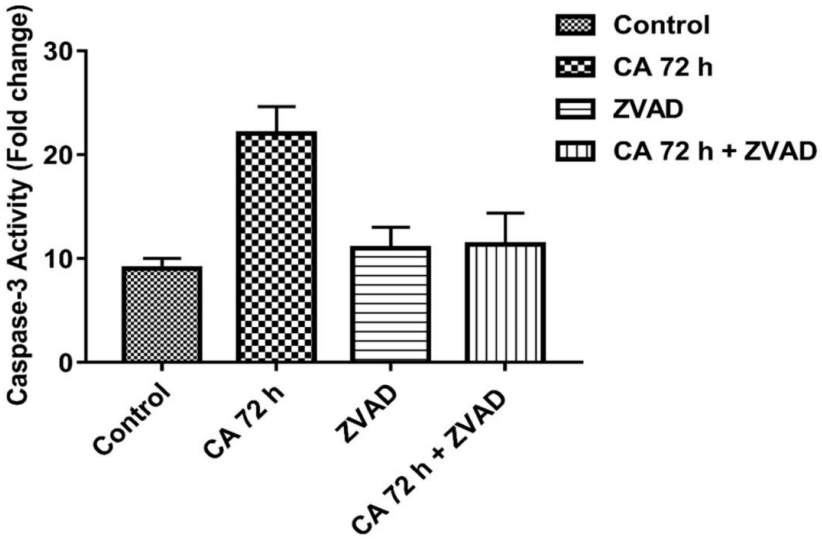

Figure 3. Caspase-3 activity after treatment with the drug.

3.3. Cinnamaldehyde treatment downregulates interleukin gene expression.

The mRNA expression of immune-modulatory IL-10 (Figure 4) was found to be decreased within 48 and 72 h of treatment with a significant change in the decrease of the mRNA expression. At 24 h of drug treatment, there was no significant reduction in the mRNA IL-10 expression. On the other hand, the mRNA expression profile of IL-1β (Figure 4) showed an increase in the expression levels in a dose-dependent manner. The highest increase was seen

after 72 h of treatment with cinnamaldehyde. Similar to the mRNA expression of IL-10, the 24 h expression was not significant.

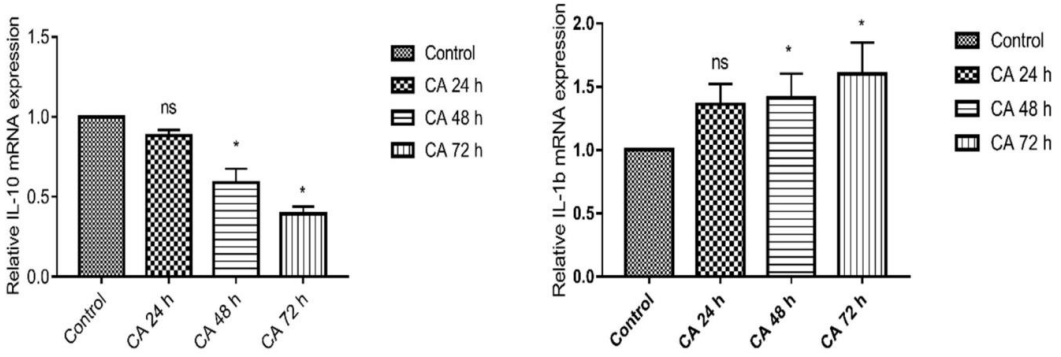

Figure 4. mRNA (IL-10 and IL-1β) expression of cinnamaldehyde treated cells.

4. Discussion

To alleviate the cancer burden, the best approach is an early therapeutic intervention. For a very long time, the human ailment has been treated by traditional Indian medicine. It has been shown that traditional Indian medicine-based therapies may stabilize tumors and prevent tumorigenesis, metastasis, recurrence, and pain. CA's tumor inhibitory and apoptotic nature (Figure 1) has been illustrated and has attained great attention for its anticancer properties [30]. This study aimed to explore the antiproliferative effect of CA on HepG2 cells. In this study, it has been observed that there was a decrease in the proliferation of HepG2 cells upon the addition of CA in a dose-dependent manner (Figure 2). The results agree with previous results [26,31].

The inflammatory responses are the key step to inducing anti-tumor immunity. It is known that HepG2 cells express mRNAs of certain cytokines like TNF-α, IL-4, IL-5, IL-7, IL-10, and IL-11 [32]. HepG2 cells contain IL-1 receptors with a similar affinity towards IL-1alpha and IL-1β [33]. In the present study, the levels of interleukin 1 beta, a proinflammatory marker, were elevated than the initial levels in cancer cell lines with the addition of CA. The results are in concordance with the results produced by [34]. It was observed that HepG2 cells stimulated with IL-1 (Figure 4) showed upregulation of various proteins involved in translation, post-translational/post-transcriptional modifications machinery, cellular metabolism, cell cycle machinery, activity modulators, and trafficking/motor proteins [35]. The results demonstrate that the elevation of IL-1β may have a role in the translational modification of cancer cells; therefore, the inhibition resulted in the antiproliferative effect observed in the present study.

On the other hand, it was observed that the treatment of HepG2 cells with CA has decreased the anti-inflammatory marker IL10 (Figure 4). This result is in concordance with [36]. This study also indicates that the treatment of CA to HepG2 cells increased the levels of Caspase-3 activity (Figure 3) in concordance with the results of Ka *et al*., 2003 and Deveci *et al*., 2019 [34, 37]. Thus, CA is an apoptotic agent and can induce cell death in liver cancer cell lines.

5. Conclusions

In conclusion, the present study results demonstrate that cinnamaldehyde could decrease proliferation by modulating inflammatory markers and promoting apoptosis in HepG2 cells, thus elucidating another facet of its anti-tumor properties.

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Not Applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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