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Research article

Tea flavonoid theaflavin induces apoptosis in hepatocellular carcinoma cells through improving ROS level

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ABSTRACT

Flavonoids, a class of normal polyphenolic mixes, restrain cell cycle movement and instigate apoptosis. This examination was performed to explore the anticancer effect of theaflavin, a natural flavonoid found in the leaves of tea plant *Camellia sinensis*. Although this molecule was found to inhibit several cancer cells, the specific anticancer action in liver cancer remains unexplored, especially in human hepatocellular carcinoma (HepG2) cells. Henceforth, the present study was designed to elucidate the anticancer activity in HepG2 cells, level of reactive oxygen species (ROS) in the cancer cells and tumour cell apoptosis. The action of theaflavin in provocation apoptosis was explored through the improved ROS by MTT assay and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) method. As per the results obtained from the MTT assay, theaflavin had cell hindrance effect on HepG2 cells. The IC_{50} estimation of theaflavin to hindered cell development at 25, 50 and 75 µM concentration and instigating apoptosis through ROS improvement. The progressions in mitochondrial morphology, portion conditionally that diminished cell expansion, were seen in various concentrations of the drug treatment. In this manner, theaflavin might be useful as a chemotherapeutic agent for the treatment of liver cancer.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most pervasive cancers and positions the third reason for cancer-related demise, causing roughly 2.0 million passing around the world per year (Asrani et al., 2019). The currently accessible modalities to treat HCC incorporate careful evacuation of affected territories followed by radiotherapy or chemotherapy with 30% five-year endurance rate which shows the restricted adequacy of cancer modalities against HCC (Balogh et al., 2016). Albeit careful resection is as yet the best option for early-staged HCC treatment, most HCC patients are frequently as of now at transitional or propelled stages upon clinical analysis. Besides, HCC is profoundly repeated inclined after remedial hepatectomy. In such case, directed treatment drugs, for example, engineered mixes are the essential decision despite its lack of ability of totally stifling HCC improvement just as drug resistance (Duan et al., 2019). The rate of HCC has consistently expanded in Western nations over the previous decade and the worldwide occurrence of HCC is anticipated to keep on ascending throughout the following scarcely any years. HCC has the third-most elevated pace of cancer-related mortalities and in this manner concentrates into the compelling treatment of HCC are basic.

At present, the primary treatment systems for patients with HCC comprise of careful resection, liver transplantation and neighbourhood removal treatments (Byam et al., 2013). Countless the patients determined to have propelled stage HCC may just be treated with palliative consideration, as there is no accessible compelling palliative chemotherapy. Thusly, it is helpful to grow more compelling new drugs. In the terminal stage of HCC, chemotherapy treatment isn't routinely utilized as it is chemorefractory and due to antagonistic occasions (AEs). Various examinations have announced 10-20% reaction rates for chemotherapeutic specialists in HCC. Be that as it may, chemotherapeutic specialists have indicated their restricted utilization because of poison levels.

A few polyphenolic mixes are known as cancer chemopreventive specialists (Niedzwiecki et al., 2016). Flavonoids are a class of normal polyphenolic mixes, pervasively happening and generally expended auxiliary metabolites of plants and have significant pharmacological properties (Yonekura-Sakakibara et al., 2019). They are accounted for having antiviral activity (Zakaryan et al., 2017), antiparasitic (Salem et al., 2011) and anticancer (Kopustinskiene et al., 2020) exercises. Flavonoids smother cancer cell multiplication, capture cell cycle movement, and actuate apoptosis (Sakagami et al., 2000).

Theaflavin (Fig. 1) is accounted for to have hostile to inflammatory or potentially against unfavourably susceptible exercises, antibacterial and antineoplastic exercises. It is accounted for that theaflavin of extricating has cell reinforcement action on receptive oxygen species in human leucocytes and β-glucosidase-subordinate freedom of theaflavin from artichoke separates represses hepatic cholesterol biosynthesis. Theaflavin additionally restrains the development of an assortment of cancer cells including oesophagal squamous carcinoma cells and pancreatic, gastric and prostate (Angst et al., 2013) cancer. In this assessment, we intended to investigate the action of theaflavin on HepG-2 through ROS.

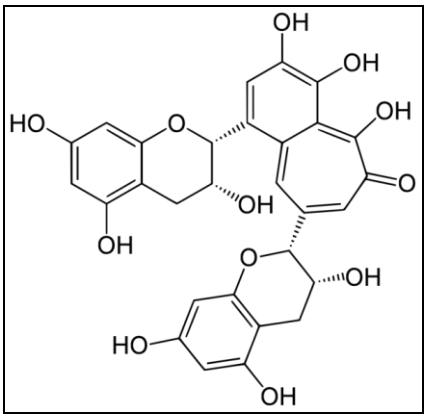

Fig. 1. Chemical structure of Theaflavin

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagles Medium (DMEM), Phosphate Buffered Saline (PBS), fetal bovine serum (FBS), 0.25% trypsin EDTA, antibiotics (penicillin, streptomycin), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-diacetyl dichloro-fluorescein (DCFH-DA), Ethidium Bromide (EtBr), Rhodamine 123, Acridine Orange (AO), Hoechst 33342 stain were obtained from Hi-media Lab Ltd., Mumbai, India. Theaflavin was provided by Dr BAA Abdul (Ton Duc Thang University, Ho Chi Minh, Vietnam) as a kind gift for the present study.

Cell culture

The Human Laryngeal carcinoma (HepG2) cell line was purchased from NCCS, Pune, India. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) and maintained at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air incubation. Theaflavin freshly dissolved in 1% dimethyl sulfoxide (DMSO) before drug treatment.

Cell proliferation assay

The effect of theaflavin on the cell proliferation of HepG-2 cells was determined by MTT assay based on the detection of mitochondrial dehydrogenase activity in healthy cells following the method of. HepG2 cells were seeded in 96-well plates at a density of $5x10^3$cells/well in a final volume of 100 ml with DMEM and incubated up to 24 h. The cells were treated with different concentration of theaflavin. After 24 h, the cells were incubated with 100 ml of MTT solution (1 mg/ml) for 2 h at 37°C. The MTT solution was removed and added 100ml of DMSO to dissolve the formazan crystals. The plate was read at 570 nm in a Readwell touch, ELISA plate reader (Robonic, India).

Measurement of intracellular ROS generation

Intracellular ROS was measured by using a non-fluorescent probe, DCFH-DA that can freely penetrate into the intracellular matrix of cells where it is oxidized by ROS to fluorescent dichlorofluorescein (DCF). Thus, the fluorescence intensity is directly proportional to the amount of ROS generation. The cells were seeded ($1x10^6$ cells/well) in 6-well plate, treated with theaflavin at different concentrations and kept in a CO_2 incubator for 24 h. After 24 h incubation, 1 ml of cells was incubated with 100 ml of DCFH-DA for 10 min at 37 °C. Fluorescent intensity was measured with excitation and emission filters set at 485 and 530 nm, respectively (Shimadzu RF-5301 PC spectrofluorometer). The results were articulated as the percentage increase with fluorescence intensity.

Statistical analysis

Data are expressed as mean ± standard error (SE) for a minimum of three independent determinations in triplicate for every experimental point. Data were analyzed using SPSS Statistics software. For all the measurements, one-way analysis of variance followed by Duncan's new multiple range tests ($p \leq 0.05$) was used to assess the statistical significance of the difference between control and treated groups.

RESULTS

Effect of theaflavin on cell cytotoxicity

Table 1 shows the cytotoxic effect of theaflavin on HepG2 cells determined by MTT assay. Cells were treated with different concentrations of theaflavin (5-200 µM) for 24 h incubation, which revealed a dose-dependent inhibition of cell proliferation. Maximum cell death was observed at 150 µM concentrations. Hence, the IC_{50} of theaflavin against HepG2 cells at 75 µM apparent from growth inhibition curve, we selected 25, 50 and 75 µM doses of theaflavin for further studies.

Table1. The inhibitory concentration of theaflavin against HepG2 cells using MTT assay

Replication	Control	25 µg/ml	50 µg/ml	75 µg/ml
1	72.4 ± 6.9	36.5 ± 4.4	21.7 ± 2.1	19.5 ± 1.6
2	78.0 ± 7.4	34.0 ± 4.2	28.4 ± 2.4	18.5 ± 1.3
3	74.5 ± 7.1	25.0 ± 3.4	21.0 ± 1.9	17.2 ± 1.2

Data are expressed as Mean ± SE.

Effect on intracellular ROS generation

The intracellular ROS generation was measured by DCFH-DA staining. The levels of ROS generation in control and theaflavin-treated cells are given in Fig. 2. HepG2 cells were treated with different concentration of theaflavin (25, 50 and 75 µM) shows significantly increased levels of ROS generation which indicating extreme green fluorescence intensity as compared to untreated control cells.

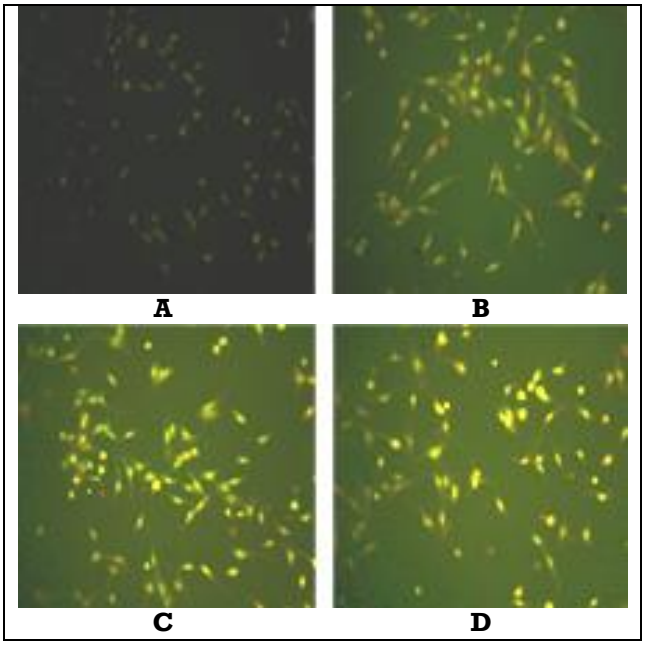

Fig. 2. Effect of theaflavin on intracellular ROS generation in HepG2 cells using DCFH-DA staining. **A**: Control cell shows weak fluorescence; **B**: Treatment with 25 µg/ml shows mild fluorescence; **C**: Treatment with 50 µg/ml shows moderate fluorescence; **D**: Treatment with 75 µg/ml shows enhanced fluorescence indicating highest ROS generation.

DISCUSSION

As of now accessible chemotherapeutic medications stay inadequate to treat hepatocellular carcinoma because of improvement of auxiliary medication opposition and serious hepatotoxicity (Lohitesh et al., 2018). Ongoing proof from different examinations has demonstrated that liver cancer cells create medicate obstruction chiefly through concealment ROS. In this way, elective helpful specialists that improve actuation of ROS based apoptosis in cancer cells are profoundly alluring.

ROS, including O_2^-, H_2O_2 and OH^- are results of vigorous breath in eukaryotic cells. ROS have a cytotoxic impact and are additionally intracellular sign transduction and quality articulation guideline particles, with key jobs in the guideline of cell development, endurance and apoptosis (Jeong and Joo, 2016). The mitochondria of a eukaryotic cell are the primary site for the creation of ROS, and a lot of ROS might be delivered during the time spent oxidative phosphorylation. The effect of receptive oxygen on the mitochondria brings about the scattering of the mitochondrial layer potential, the arrival of cytochrome c and causes the mitochondrial film porousness progress pore (PTP) (Fleury et al., 2002). An assortment of proapoptotic signals, including physical harm, radiation, chemotherapy, excitatory amino acids and passing ligands, may cause an expansion in cell endogenous or exogenous ROS, or change the redox harmony, and these signs may trigger cell apoptosis. When an apoptotic pathway is enacted, an expansion in the declaration of ROS may quicken the apoptosis procedure (Aggarwal et al., 2019). In this way, the arrival of ROS and the procedure of apoptosis are commonly influenced.

It has become a built-up actuality since cancer cells contain higher oxidative pressure contrasted with ordinary cells which assumes a significant job in cancer cell proliferation, endurance and medication obstruction (Nikolaou et al., 2018). Present-day research has demonstrated that this biochemical property of cancer cells can be abused for helpful advantages. Theaflavin is plant-inferred bioactive particles which have for quite some time been utilized to treat fiery infections in customary Indian meds. Present-day research has demonstrated that theaflavin is an intense inducer of responsive oxidative pressure (Mao et al., 2017). Which notwithstanding contain ketone, has been

appeared to prompt apoptosis and ROS age in different cancer cells (Perillo et al., 2020). Be that as it may, the useful connection between ROS age and different flagging falls significant for apoptosis has not been completely settled. The current study was conducted to know that whether theaflavin can initiate oxidative pressure and restrain the proliferation of HepG2 liver cancer cells. We found that theaflavin could viably restrain development and instigate oxidative pressure intervened apoptosis in HepG2 cells.

Oxidative pressure is brought about by expanded intracellular ROS age and diminished movement of cells' antioxidant barrier framework (Birben et al., 2012). The current outcomes exhibited that theaflavin instigates HepG2 cells to altogether deliver ROS, including OH- and H_2O_2, in the cytoplasm and mitochondria of the cell, to a comparative level as the positive control, contrasted and the negative control ($P<0.05$). In the current investigation, the particular tests focusing on differential-localized ROS recognized that ROS segments stayed in the cytoplasm and mitochondria of the cells; notwithstanding, the principle area for the creation of ROS was seen to be in the mitochondria. The ROS created in the mitochondria may straightforwardly diffuse to the cytoplasm because of the expanding penetrability of the mitochondrial layer (Webster, 2012). This might be because of the accompanying potential clarifications.

Apoptosis, incited by antitumor medications, may have an alternate apoptosis reaction contrasted with non-chemotherapy-induced apoptosis due with cell cycle affectability (Crowe and Yoon, 2003). The porousness of the film of the HepG2 cells remains moderately high when presented to antitumor medications, showing an expected chance of an intracellular example of dispersion as ROS enters the cell. Different discoveries have shown that the development of tumour cells might be restrained when they are pretreated with chemotherapeutics (Gao et al., 2014). Ensuing reproduction by ROS may accordingly bring about tumour shedding and necrosis, which has a lower fluorescence signal.

Taking everything into account, theaflavin actuates HepG2 cells to deliver ROS and builds the articulation and action of the apoptosis-associated cell passing, which initiate downstream flagging pathways, in the end prompting cell apoptosis. In this manner, theaflavin is a likely anticancer medication, which may have a clinical application in future.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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