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Research Article

EFFECTS OF THEAFLAVINS ON GASTRIC CANCER CELL LINE

SURMI ROY[1], ARPITA SAHA[1], ANANYA CHATTERJEE[1], SIRSHENDU CHATTERJEE3, SANTASREE MAZUMDER[2], SANDIP K BANDYOPADHYAY[1]*

[1]Department of Biochemistry, KPC Medical College and Hospital, Kolkata, West Bengal, India. [2]Department of Biochemistry, University of Calcutta, Kolkata, West Bengal, India. 3. Department of Biotechnology, Techno India University, Kolkata, India. Email: skb.teaboard@gmail.com

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ABSTRACT

Objective: The objective of this study was to evaluate the anticarcinogenic effects of theaflavins (TFs), component of black tea, on human gastric cancer cell line.

Materials and Methods: Cell viability was assessed by WST-1 reagent. Acridine orange (AO) and ethidium bromide (EB) fluorescence staining method were used to detect apoptotic cells, if any, followed by flow cytometric detection. To evaluate the molecular basis for understanding the apoptotic pathway, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were measured. Pro- and anti-apoptotic protein and gene expressions were detected by western blot and real-time techniques.

Results: TFs-treated AGS cell indicated apoptosis-mediated cell death. The presence of TFs induced the generation of ROS and which in turn changes the MMP with the elevated level of cyt c, activation of caspase-9, caspase-3, and PARP cleavage. Our study also showed that TFs treatment altered the Bax/Bcl2 ratio and upregulated the expression of p53.

Conclusion: All these observations lead to the fact that TFs developed potential chemopreventive effects through the induction of apoptosis through mitochondria-mediated death cascade in the human gastric cancer cell.

Keywords: AGS, Apoptosis, Bax, Bcl-2, Mitochondrial membrane potential, Reactive oxygen species, Theaflavins.

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INTRODUCTION

Stomach cancer, or gastric cancer, arising from the inner layer of the stomach is the second leading cause of cancer-related death just behind lung cancer [1,2]. Growth factors, cytokines, and angiogenic factors have been proposed as molecular factors of gastric cancer development [3]. A series of factors may be involved in the development of gastric cancer; however, according to various studies, it is clear that chronic inflammation plays a crucial role in this malignancy [4]. It could be expected that anticancer therapies have these factors as targets. However, many of the drugs so far studied are contraindicated, and there are many studies highlighted certain drug-related problems in cancer patients [5]. However, natural compounds that reduce both inflammation and angiogenesis could be used as anticancer therapeutic agents because of their less cytotoxicity on the normal cell. It has also been reported that phytochemicals derived from tea can induce apoptosis in various cancer cells [6]. Since chronic inflammation is the key feature of gastric cancer development, the ability of black tea to reduce inflammation is the main aspect of chemopreventive therapy in gastric cancer. From the different studies, it is also clear that along with the anti-inflammatory effect through inhibiting the NF-κB transcription factor, black tea plays an important role also in the healing of indomethacin-induced gastric ulcer [7,8].

Black tea from the young leaves of *Camellia sinensis* is one of the most popular non-alcoholic beverages consumed worldwide [9]. The extended oxidation of black tea increases the concentrations of two polyphenols - theaflavins (TFs) and thearubigins [10,11] which have the antioxidant activity and their biological activities may be relevant to cancer prevention. TFs are major polyphenolic components present in black tea and are composed of theaflavin (TF1), theaflavin-3-gallate (TF2a), theaflavin-3'-gallate (TF2b), and theaflavin-3,3'-digallate (TF3) [10]. It has also been reported that catechin, a component of green tea and black tea polyphenols, inhibits the tumor growth [12]. Numerous studies support the chemopreventive effect of black tea in the development of lung cancer, prostate cancer, breast cancer, and ovarian cancer [13-16]. On the basis of these reports we have selected Theaflavins, the major polyphenol of the black tea as experimental compound for our recent study. Our previous work with black tea component in indomethacin-induced gastric ulcer model [8] highlighted their role in ulcer healing. Since gastric cancer is also caused by the chronic inflammation in ulcer margin, the aim of our study is to evaluate the therapeutic efficacy of Theaflavins in gastric cancer model.

MATERIALS AND METHODS

Materials

Theaflavins (≥80% theaflavins), WST1 reagent, DCFDA (2',7'-dichlorofluorescein diacetate), AO, and EtBr were acquired from Sigma, JC-1 kit from BD Biosciences, Foetal Bovine Serum (FBS), 0.25% trypsin-EDTA and F12K media were purchased from Gibco. Antibiotic penicillin-streptomycin was purchased from MP Bio. AnnexineV-PI kit was obtained from BD Pharmingen. Primary antibodies (Bcl2, Bax, Caspase-3, Caspase-9, cyt c, p53, and PARP) and polyclonal secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Molecular Grade BSA, Tween-20, NBT/BCIP, Tris-HCl, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNA plus solution from MP Bio and cDNA synthesis kit purchased from Invitrogen were was procured.

Culture of cell lines

AGS (human gastric cancer cell line) cell line was obtained from National Center for Cell Science (NCCS), Pune, India. The cells were cultured in F12K media at pH 7.4, supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin-G, 100 µg/ml-streptomycin). The cells were incubated at 37°C in a

humidified atmosphere containing 5% CO_2 inside a CO_2 incubator. Cells were passed weekly, and cells from passages 6 to 8 were used for experiments. Cells were subcultured by treatment with Trypsin-EDTA solution in phosphate buffer saline (PBS).

WST-1 assay

The antiproliferative assay was carried out using the reagent WST-1. The cells (2×10^6 cells in 100 µl medium/well) were plated in 96-well plates. The cells were then treated with different concentrations of Theaflavins for 24 h and 48 h, respectively. Untreated cells were used as controls. At the end of the treatments, each well was treated with WST-1 solution (10 µl), and after incubation for 2 h, the absorption at 450 nm was read with a microplate reader (BIOTEK; ELX 800, USA). The inhibition of cell proliferation by TFs was evaluated by calculating the IC_{50} value.

Determination cellular morphology by fluorescence microscopy

Apoptotic cells were analyzed by fluorescence microscope using Acridine Orange and Ethidium Bromide as fluorescent probes. Photographs were taken at random locations under the fluorescent microscope. Untreated cells were used as a control.

Detection of apoptosis by flow cytometry

Cells were treated with different concentration of TFs for 48 h**,** and apoptosis was measured using Annexin V Alexa fluor 488 and PI Apoptosis Detection Kit (BD Pharmingen). AGS cells were harvested, and PI (5 µl) and Annexin V Alexa Fluor 488 (5 µl) were added directly to the binding buffer, 100 µl initially, incubated for 15 min at room temperature in dark, added 400 µl of binding buffer to each sample as per kit protocol, and then analyzed on flowcytometer (equipped with 488 nm argon laser light source; 515 nm bandpass filter for Alexa fluor 488 and 623 nm bandpass filter for PI **-** fluorescence) using Cellquest software. A total of 10,000 events were acquired**,** and the cells were properly gated for analysis.

Measurement of intracellular reactive oxygen species (ROS) level

AGS cells were seeded into 96-well plate (2×10^6 cells) for overnight. Cells were treated with TFs for 48 h and then collected by trypsinization; 200 µl of cell suspension containing 2×10^6 cells/ml was added to 800 µl of PBS and was incubated with DCFDA at 10 µM concentration for 15 min. H_2O_2 (25 µM) and TFs were added in the culture, and the incubation was continued for an additional 20 min at 37°C. The production of intracellular H_2O_2 was measured using fluorometer (excitation at 365 nm and emission at 430 nm) as per the standard protocol [17].

Measurement of mitochondrial membrane potential (MMP)

The loss of MMP is a hallmark of apoptosis.

It is an early event preceding phosphatidylserine externalization and coinciding with caspase activation. MMP was measured using JC-1 kit following the protocol submitted by the supplier. The MMP detection kit uses a unique fluorescent cationic dye, JC-1 (excitation at 488 nm and emission at 525 nm), to signal the loss of MMP. Cells were harvested by trypsinization at 48 h of treatment with different concentrations of TFs. Then, mitochondrial permeability transition was determined by staining of the cells with JC-1, as described below. Briefly, equal numbers of cells (2×10^6) were incubated with JC-1 at 2.5 µg/ml in 1 ml PBS for 30 min at 37°C with moderate shaking. Cells were then centrifuged at 300 g at 4°C for 5 min, washed twice with ice-cold PBS, and finally resuspended in 200 µl PBS. Mitochondrial permeability transition was subsequently quantified on a spectrofluorometer, and data were given in the ratio of 590/530.

Protein expression analysis by western blot

Western blot analysis was done to determine the expressions of different proteins. The concentration of protein was evaluated using Bradford reagent. Equal amounts of proteins from the cells were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After transfer, the nonspecific sites of the membrane were blocked with 5% skimmed milk in 0.1% Tween-20 in 20 mM TBS (**pH** 7.5), and the membrane was incubated with human-specific primary polyclonal antibodies (Bax, Bcl2, PARP, caspase-3, caspase-9, and β-actin) for overnight at 4°C. After washing with TBS containing 0.1% Tween 20, the membrane was incubated with AP-conjugated secondary antibodies. Therefore**,** the membrane was treated with BCIP/NBT at **pH** 8.8 for 15–20 **min**. Colored bands were observed in the membrane due to the chromogenic reaction.

Gene expression study by RT PCR

After extracting total RNA with RNA plus solution (MP Bio) and checking its integrity by electrophoresis, the cDNA was synthesized from purified total RNA using a commercial kit (Invitrogen). Expressions of genes were detected using designed primers, such as Bax (Forward 5′ - TGA ACT GGA CAA CAA CAT GGA GC -3′, Reverse 5′ - GGT CTT GGA TCC AGA CAA ACA GC -3′), Bcl-2 (Forward 5′ - GGA GGA TTG TGG CCT TCT TTG AG -3′, Reverse 5′ - TAT GCA CCC AGA GTG ATG CAG GC -3′), BCL-XL (Forward 5′ - GTA AAC TGG GGT CGC ATT GT -3′, Reverse 5′ - TGG ATC CAA GGC TCT AGG TG -3′), Bad (Forward 5′ - AGG GCT GAC CCA GAT TCC -3′, Reverse 5′ - GTG ACG CAA CGG TTA AAC CT -3′), Caspase 3 (Forward 5′ - TTC AGA GGG GAT CGT TGT AGA AGT C-3′, Reverse 5′ - CAA GCT TGT CGG CAT ACT GTT TCA G-3′), and GAPDH (Forward 5′ - GGA GCC AAA AGG GTC ATC ATC -3′, Reverse 5′- AGA GGC AGG GAT GAT GTT CTG -3′) etc [Metabion (Germany)].

Statistical analysis

Statistical analysis of the data was done using the software like GraphPad InStat. Each experiment was repeated at least 3 times.

RESULTS

Effects of TFs on AGS cell viability

The antiproliferative effects of TFs were examined and evaluated on AGS cell line. TFs exerted cytotoxicity against AGS cell line, as judged by phase contrast microscopy and WST-1 cell viability assay. The antiproliferative effects of TFs were carried out in a time and dose-dependent manner to compare the relative IC_{50} value. There was no significant reduction in cell viability till 24 h treatment. However, a significant reduction in cell viability was evident at 48 h of TFs treatment at their varying concentrations as shown in (Fig. 1). Data analysis of cytotoxicity assay revealed that IC_{50} of TFs was 70 µg/ml for 48 h of treatment. In case of NIH-3T3 for TFs treatment, no significant reduction in cell viability was observed even at <280 µg/ml concentration. After treatment with different concentrations of TFs, the morphological alteration was observed in the treated cells compared to the control cells in a dose-dependent manner (Fig. 2).

Analysis of cellular morphology indicating apoptosis

To confirm whether the reduced cell viability was due to apoptosis, Acridine Orange and Ethidium Bromide fluorescence staining was used to detect apoptotic cells. Using fluorescence microscopy, uniform normal green cells were observed in the control group, whereas an

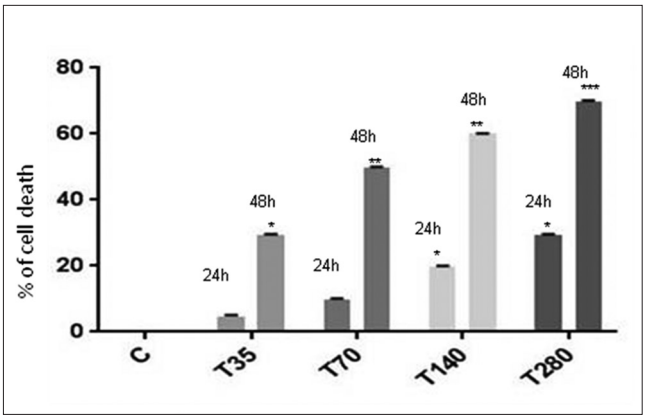

Fig. 1: Effects of theaflavins on the viability of AGS cells in dose-dependent manner

increased number of dark orange or red cells with increased TFs concentrations were detected (Fig. 3).

Effect of TFs on detection of apoptosis

Cell death may occur in different pathways - these may be necrosis or apoptosis.

Our flow cytometric data revealed that, in comparison with control untreated AGS, TFs -treated AGS cells indicated the apoptosis-mediated cell death. Different concentrations of TFs induced apoptosis by 16.2% early apoptotic and 27% late apoptotic phase, whereas cell treated with higher concentration of TFs showed the late apoptotic phase which was 36.6% (Fig. 4).

Effect of TFs treatment on intracellular ROS level

There is a prevalent notion that conveys intracellular ROS as signaling intermediates, which are involved in signal transduction pathways of apoptosis. Hence, furthermore, we investigated whether TFs treatment resulted in the generation of ROS in the gastric cancer cell. It has been found that a significant increase of intracellular ROS level was detected after treatment with TFs in AGS cell (Fig. 5).

Effect of TFs treatment on MMP

Loss of MTP is associated with mitochondrial dysfunction leading to apoptosis. Consequently, we next evaluated the effect of TFs on MMP. Our results showed that these TFs significantly ($p<0.001$) decreased MMP in AGS cells (Fig. 6). CCCP was used as a mitochondrial-uncoupling agent.

Effect on expression of mRNA

It was found that TFs upregulated the mRNA level expression of proapoptotic proteins such as Bax, Bad gradually in dose-dependent manner, whereas mRNA expression levels of antiapoptotic members such as Bcl-xL and Bcl-2 were found to be inhibited after TFs treatment in comparison to the control cell. It was also found that TFs treatment induced apoptosis in the gastric cancer cell through caspase 3 activation (Fig. 7). The gene expression level of caspase 3 was found to be upregulated in dose-dependent manner compared to the control cell.

Effect on expression of pro- and anti-apoptotic proteins

TFs induced activation of proapoptotic protein Bax and suppressed the antiapoptotic Bcl-2 expression (Fig. 8). Bax was found to be downregulated in control cell while its expression got enhanced on TFs-treated cells in dose-dependent manner. Conversely, Bcl2, an antiapoptotic protein was found to be overexpressed in a control cell. Its expression was downregulated on TFs-treated cells in dose-dependent manner. Results of western blotting of the above proteins revealed a comparison in their expression between the control and treated cells. It was found that TFs-treated cells pronounced the proapoptotic effect of Bax and antiapoptotic effect of Bcl2 in comparison to control cells. TFs treatment also upregulated p53 expression, which finally enhanced cyt c activation. It was found that TFs treatment induced apoptosis through

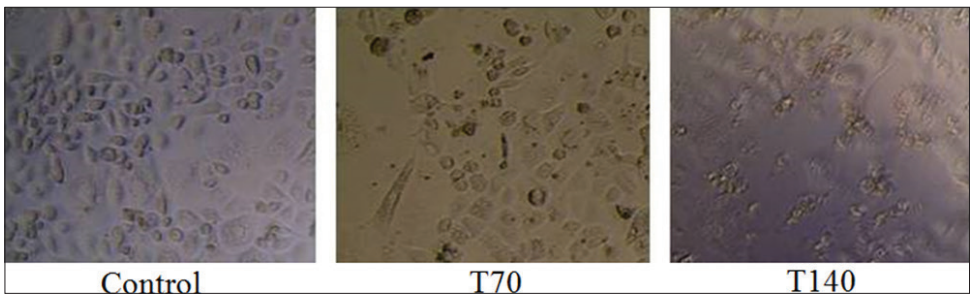

Fig. 2: Effect of theaflavins on AGS (phase contrast microscopy)

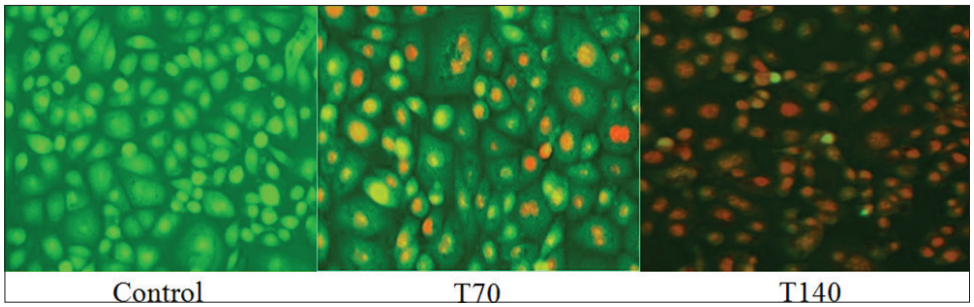

Fig. 3: Analysis of cellular morphology by fluorescence microscopy

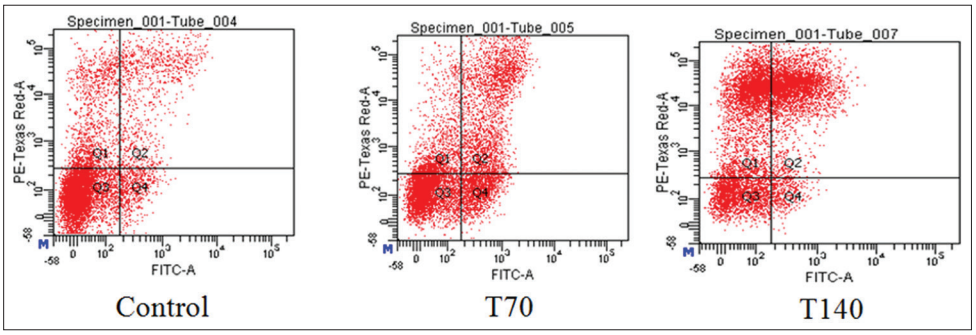

Fig. 4: Effect of theaflavins on detection of apoptosis

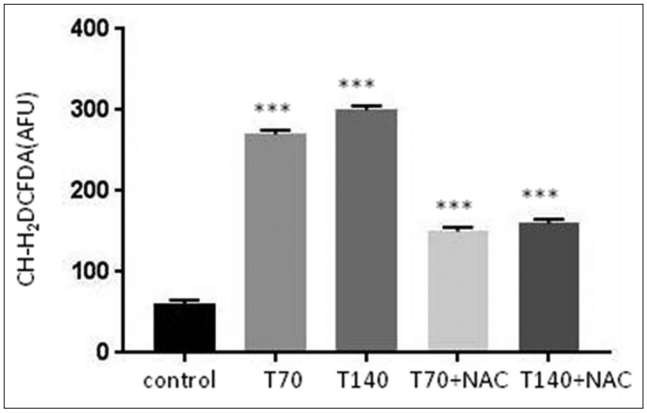

Fig. 5: Effects of theaflavins treatment intracellular reactive oxygen species level

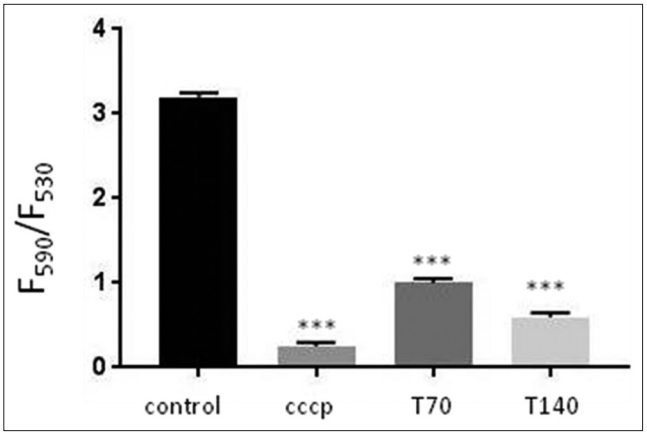

Fig. 6: Effect of theaflavins treatment on mitochondrial membrane potential

caspase activation and PARP cleavage. Western blot results showed that expression levels of caspase 3 and caspase 9 were elevated in dose-dependent manner compared to the control cells. TFs treatment also resulted in cleavage of PARP which is the characteristic hallmark of apoptosis (Fig. 8).

Cells were treated with different concentrations (0, 35, 70, 140, 280 µg/ml) of TF grown in 96-well plate for 24 h and 48 h. Inhibitory effects of TFs were determined by WST assay. Each value is expressed as mean±standard deviation (SD)(n=5). * P < 0.05, **P < 0.01 and ***P < 0.001 are the TFs treated groups compared with the control (0 µg/ml).

Negative control group (normal cells): The cells showed only green fluorescence, Experimental group (early apoptotic cells): Nucleus showed yellow-green fluorescence by AO staining, and (late apoptotic cells): The nucleus of cell showed orange fluorescence by EB staining.

TFs induced apoptosis in AGS cells. AGS cells were treated with 70 µg/ml and 140 µg/ml TFs and stained with Annexin V Alexa Fluor 488/PI, then analyzed by FACS.

Estimation of Intracellular ROS was done in AGS cells after treatment with different concentrations (0, 70, 140 µg/ml) of TFs. Fluorescence intensity was analyzed by fluorometer. Each value is expressed as mean ± SD (n = 3), P<0.001.

Cells were treated with different concentrations (0, 70, and 140 µg/ml) of TF induced cells which were labeled with JC-1 probe, and the altered MMP was measured by fluorometer. Mean of experiments done in triplicate; ±SD (n=3), p<0.001.

In experimental group, Bcl-2 and Bcl-xL expressions were decreased significantly compared with the control group; Bax and Bad expressions were increased in comparison with control. Activation of caspase 3 was also found in TFs-treated AGS cells.

Our observation revealed that the expression of the proapoptotic protein Bax has been upregulated in AGS cells treated with IC_{50} dose

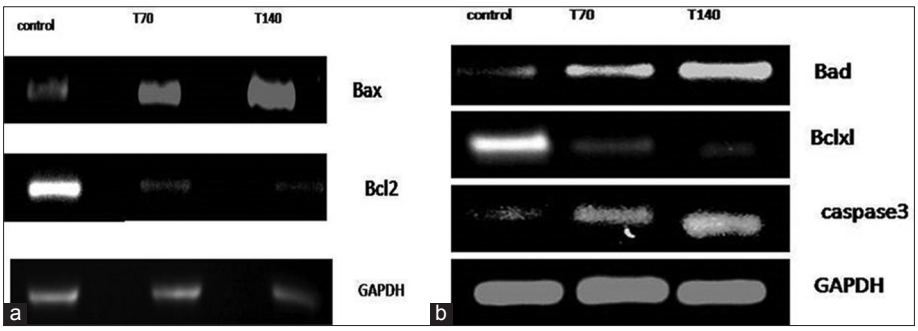

Fig. 7: (a and b) The study of gene expression on AGS cell line

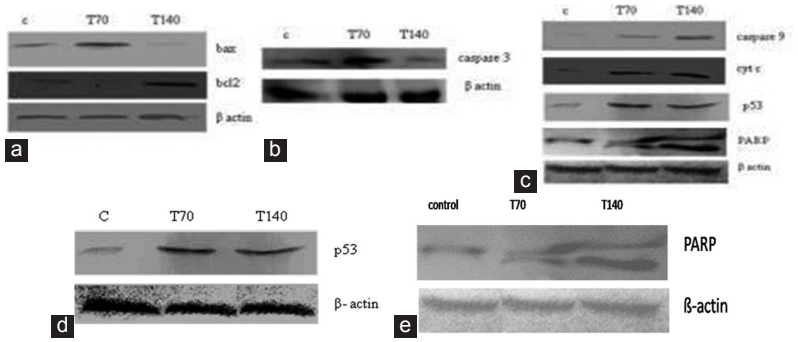

Fig. 8: (a-e) Western blot showing the effect of theaflavins on different protein expressions

of TFs, whereas the expression of antiapoptotic protein Bcl2 has been downregulated in treated cells followed by p53 activation. TFs treatment elevated the level of cytochrome c, activated downstream caspase 9, and caspase 3 leading to the cleavage of PARP.

DISCUSSION

Our study demonstrated that the induction of apoptosis in AGS cell by the TFs treatment might considered as a protective mechanism against development and progression of cancer. Thus, to evaluate the molecular mechanism of apoptosis *in vitro* and the probable signaling cascade of chemoprevention induced by TFs in gastric cancer cellsis our subject of study. TFs exhibited antiproliferative activity in a dose-dependent manner in the gastric cancer cell (Fig. 1). Apoptosis is mediated by anti- and pro-apoptotic effectors that include a large number of proteins. Therefore, to attain the perception of mechanisms, which control apoptosis, we observed the effects of TFs on antiapoptotic and proapoptotic proteins of the Bcl2 family. Bcl2 is an antiapoptotic protein, which acts as a suppressor of apoptosis [18], whereas the proapoptotic protein Bax, having predomination over Bc12, promotes apoptosis [19]. In the present study, we have found that different concentrations of TFs treatment in AGS cells resulted in a reduction of Bcl2 protein expression, whereas increased the expression of Bax protein (Fig. 8). This observation indicates that the increased ratio of Bax: Bcl2 proteins may be responsible to induce apoptosis in AGS cell. It was also found that there might be a relationship between p53 activation and the upregulation of Bax in TFs-treated AGS cells. In the induction of apoptosis, the change in MMP plays a significant role [20]. The mitochondrial membrane permeability is limited, and this is essential for the generation of the MMP [21]. The release of cytochrome C and the activation of subsequent downstream caspases are triggered by permeabilization of this membrane [22,23]. It was also found that there might be a relationship between p53 activation and the upregulation of Bax in TFs treated AGS cells. In case cancer development loss of p53 function is prevalent, so this protein is an ideal candidate for cancer therapy [24]. From our data we have found that Theaflavins treatment increased p53 activity in gastric cancer cell line (Fig. 8). Intracellular ROS are known as the signaling intermediates and the generation of ROS may trigger apoptosis [25]. As shown in the Fig. 5, in contrast to the control the fluorescence intensity in Theaflavins treated cell is significantly higher. In our study, it was observed that treatment of Theaflavins induced MMP changes (Fig. 6) with the elevated level of Cytochrome c (Fig. 8). As shown in the Fig. 8 exposure of AGS cells to Theaflavins results in the activation of initiator caspase-9 and executor caspase-3 which plays an important role in proteolytic cleavage of PARP, suggesting that Theaflavins could induce apoptosis in AGS cells through the mitochondrial pathway.

In our study, it was observed that treatment of TFs induced MMP changes with the elevated level of cytochrome C, activation of downstream caspase 9, caspase 3 leading to cleavage of PARP. TFs treatment also increased intracellular ROS level inducing apoptosis in AGS cell.

CONCLUSION

Our study indicates that TFs induce apoptosis through mitochondria-mediated pathway and involvement of pro-and anti-apoptotic proteins, thus providing a molecular basis for understanding the chemopreventive effect of TFs that might be ideal candidates to induce effective apoptosis in human gastric cancer cells. It can be concluded that TFs with the antiproliferative activities may be useful for developing potential chemopreventive agent against gastric carcinogenesis. These observations will add a new light in the field of developing therapeutic strategies for the treatment of gastric cancer in the near future.

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