

## EXPERIMENTAL STUDY

# Caffeic acid suppresses HT-29 cell death induced by H<sub>2</sub>O<sub>2</sub> via oxidative stress and apoptosis

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**ABSTRACT**

**AIM:** The purpose of this research was to examine the suppressing effect of caffeic acid (CA) on colon cancer cells triggered by an overdose of H<sub>2</sub>O<sub>2</sub>, and molecular mechanisms involved.

**METHOD:** This study examines cell proliferation, measurement of ROS and lipid peroxidation (LPO) levels, total antioxidant status (TAS) level, catalase (CAT) activity, TUNEL assay for calculating the apoptotic index, immunohistochemical staining for caspase-3 proteins, and qRT-PCR for measuring mRNA levels of apoptotic and anti-apoptotic genes.

**RESULTS:** In this study, CA considerably suppressed HT-29 cell death induced by cytotoxicity achieved by and overdose of H<sub>2</sub>O<sub>2</sub>. Additionally, inducing cells with H<sub>2</sub>O<sub>2</sub> caused a rise in ROS and LPO levels, decrease in TAS level and CAT activity whereas pre-treatment of cells with CA reversed these effects. Additionally, a considerable increase was observed in the expression of *Bax*, *cas-3*, *cas-8*, *cyt c*, *p53* at mRNA levels after H<sub>2</sub>O<sub>2</sub> treatment, however, pre-treatment with CA considerably decreased H<sub>2</sub>O<sub>2</sub>-induced upregulation of these genes.

**CONCLUSION:** In light of all these findings, the antioxidant use should be paid attention to as it could decrease the level of ROS, and in turn decrease the apoptotic cell death which is an unwanted situation in the setting of cancer remedy. Overall, these data revealed that CA can suppress apoptosis in HT-29 cells triggered by an overdose of H<sub>2</sub>O<sub>2</sub> (Fig. 5, Ref. 37). Text in PDF [www.elis.sk](http://www.elis.sk)

**KEY WORDS:** oxidative stress, HT-29, caffeic acid, *cyt c*, *cas-8*; apoptosis.

**Introduction**

Colon cancer is among the primary reasons of cancer-associated mortality on earth and one of the most commonly diagnosed cancers (Wu et al, 2016). In comparison to healthy cells, cancer cells are under oxidative stress linked with increased production of reactive oxygen species (ROS), which is crucial for several biological events in healthy cells (Toyokuni et al, 1995). Any aberrations in redox equilibrium may be associated with human diseases including cancers (Wang and Yi, 2008). Oxidative injury with excessive ROS production has been shown to be participating in several biological events, some of which include cell viability, and apoptotic and necrotic pathways (Masgras et al, 2012). The involvement of oxidative injury has been examined in the treatment of carcinogenesis and drug developments for decades (Sosa et al, 2013, Gupta et al, 2009). A sufficient amount of ROS can cause mitochondrial impairment which has a key role at the beginning of apoptosis (Ravindran et al, 2011). Among ROS, H<sub>2</sub>O<sub>2</sub>

is particularly interesting because it is the most stable ROS (Li and Jackson, 2002), it penetrates cell membranes (Waldron and Rozenguert, 2000), and acts as both extra- and inter-cellular messengers resulting in modification of expression of some proteins (Knapp and Klan, 2000).

ROS and mitochondria have a key role in inducing the apoptotic pathway under both pathologic and physiologic circumstances. Evidence from recent studies suggests that mitochondria are not only the source but also a target of ROS. The release of cytochrome c from mitochondria activates the caspases, and it seems to be primarily intermediated through ROS action (Simon et al, 2000). The apoptotic pathway includes *Bcl-2* and the *p53* gene family, while involving pro-survival and pro-apoptotic genes. Furthermore, one of the main apoptotic pathways is the mitochondrial pathway that is accompanied by a rise in cytochrome c levels that stimulates apoptosis, and leads to the activation of caspase-3. Therefore, the level of active caspase-3 increases and triggers apoptosis through the mitochondrial pathway (Tartik et al, 2016)

The process of ROS protection contains the antioxidant mechanism that supports the mitigation of oxidative injuries. During oxidative stress, ROS and lipid peroxidation (LPO) increase and free radicals generated through cellular respiration cause an impairment in lipids, proteins, and DNA, while hastening the disease risk (Darendelioglu et al, 2017; Frisard and Ravussin, 2006). Antioxidants protect different cellular compounds from injury and

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death (Das and Roychoudhury 2014). Polyphenols are reported to be antioxidants that are beneficial to human health (Sato et al, 2011). The consumption of coffee that contains various types of antioxidants, for example chlorogenic and caffeic acids (CA) was shown in many studies to decrease the risk associated with cardiovascular disease in addition to some cancer types including gastric and colon cancers (Schmit et al, 2016, Xie et al, 2016).

The suppression of ROS by using diverse types of antioxidants could be an effective methodology in preventing the oxidative stress-triggered cellular death (Choi et al, 2010). However, as demonstrated in previous research, the use of antioxidants as a supplement in cancer treatment might not be always beneficiary (Tartik et al, 2016, Fu et al, 2014). The main aim of this research was to investigate the effect of CA on HT-29 cells stimulated with and overdose of H<sub>2</sub>O<sub>2</sub>, and molecular mechanisms underlying its effect.

**Material and methods**

*Cell culture*

Colon cancer cells (HT-29; ATCC HTB-38™) were gratefully received from Uludag University as a gift and seeded in DMEM including 10 % FBS and 1 % antibiotics. The cells were grown in humidified incubator with 5 % CO<sub>2</sub> (Tartik et al, 2016).

*Experimental groups*

The colon cancer cells were separated in three groups. In control, HT-29 cells were grown in complete medium. In H<sub>2</sub>O<sub>2</sub> group, the cells were grown in the presence of 0.8 mM H<sub>2</sub>O<sub>2</sub> for 12 h. In CA+H<sub>2</sub>O<sub>2</sub> pre-treatment group, HT-29 cells were pre-incubated with CA (0.05 and 0.1 mM) for 3 h, and 0.8 mM H<sub>2</sub>O<sub>2</sub> was added for 12 h.

*Cell viability assay*

The cytotoxic effect on HT-29 cells was assessed in H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>+CA and control groups by water-soluble tetrazolium-1

(WST-1) cell proliferation assay kit following the instructions supplied with the kit as described in Darendelioglu et al (2017).

*Intracellular ROS assay*

Cellular ROS generations were analysed as described in Darendelioglu et al (2017).

*Lipid peroxidation (LPO) assay*

LPO production was examined as described by Ohkawa et al (1979).

*Catalase (CAT) activity and total antioxidant status (TAS)*

The experiments were carried out following the protocols supplied with the catalase assay kit (Elabscience) and Human TAS ELISA kit (SunRed).

*TUNEL assay*

The assay was performed following the procedure supplied with the ApopTag kit (Milipore) as described in Tartik et al (2016).

*QRT-PCR analysis*

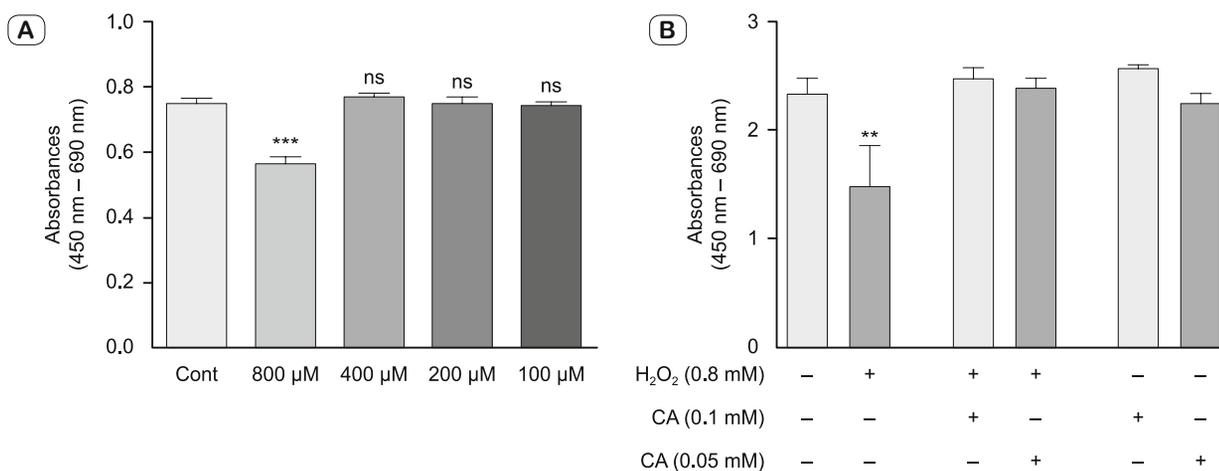
The expression levels of the genes studied within this research were investigated by quantitative real-time gene expression assay kit (Jena Bioscience). The expression levels of *p53*, *Bax*, *Bcl-2*, *cytochrome c*, *cas-8* and *cas-3* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene were analysed as explained in Tartik et al (2016).

*Caspase-3 staining*

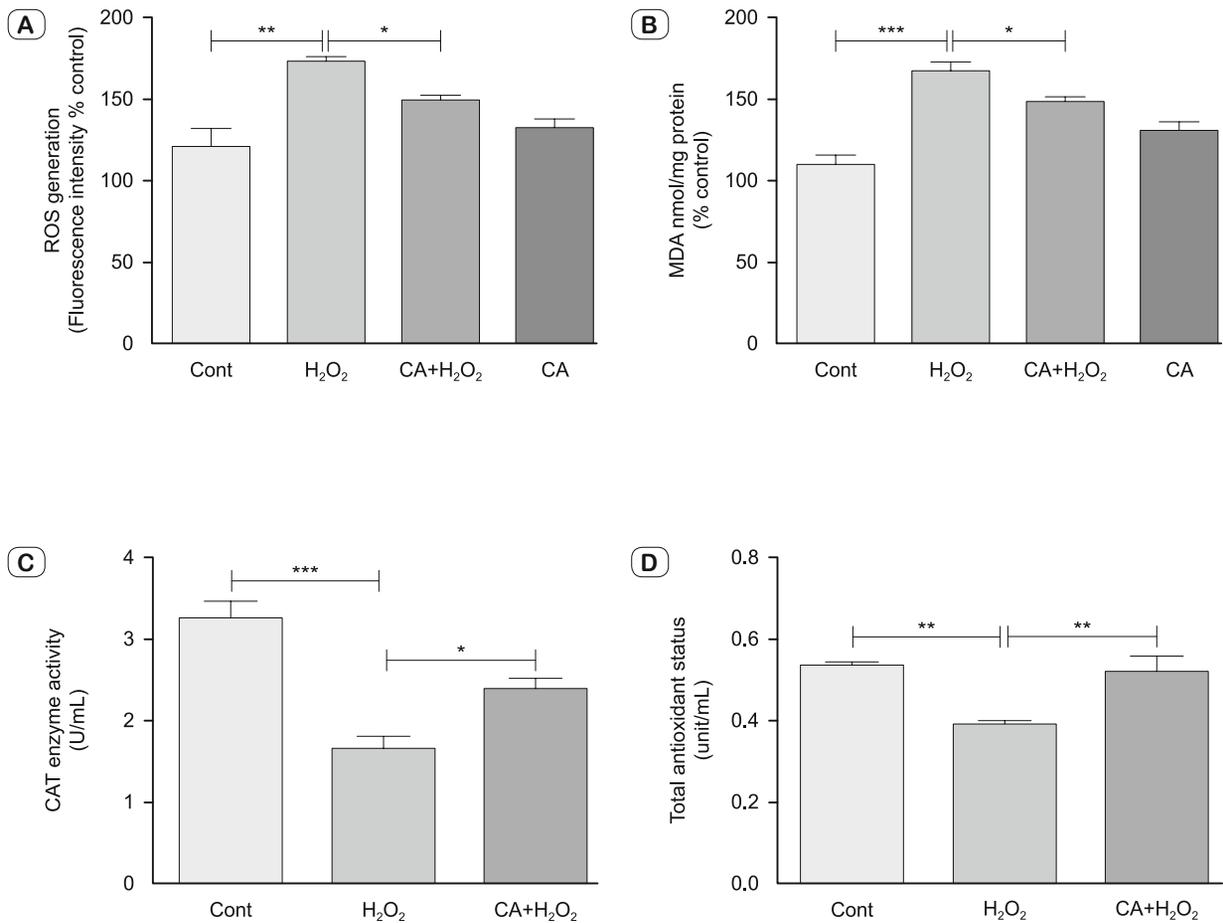
Immuno-histochemical staining experiments were carried out by using UltraVision LP Large Volume Detection System (Santa Cruz Biotechnology); kit protocol as described in Tartik et al (2016).

*Statistics*

All experiments were repeated at least three times. Statistical analysis and comparable data groups were assessed using Graph-



**Fig. 1.** Effects of H<sub>2</sub>O<sub>2</sub> (A) and CA+H<sub>2</sub>O<sub>2</sub> on HT-29 cell proliferation (B). Experimental data are presented as mean ± SEM (n ≥ 3). \*\*\* p < 0.001 points out statistically significant differences between control and other groups; ns: not significant.



**Fig. 2.** The effect of CA pre-treatment on H<sub>2</sub>O<sub>2</sub>-induced ROS generation (A), MDA levels (B), CAT activity (C), and TAS (D) in HT-29 cells. Experimental data are given as mean  $\pm$  SEM (n  $\geq$  3). \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05 point out statistically significant differences between control and other groups

Pad Prism 5 by one-way ANOVA Newman-Keuls post-hoc test; p < 0.05 was considered significant.

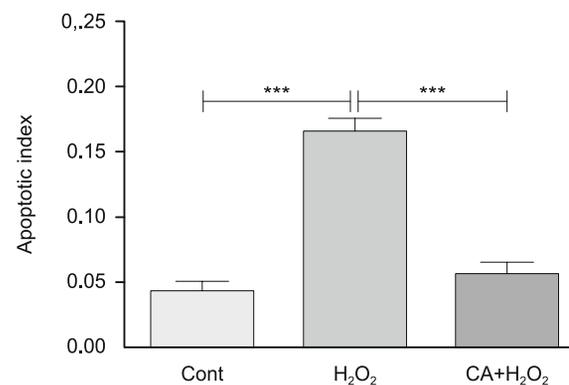
## Results

### Cell proliferation assay

HT-29 cells were treated with H<sub>2</sub>O<sub>2</sub> (0.1–0.8 mM) for 12h and 24h and cell cytotoxicity was evaluated by WST 1 assay. As given in Figure 1A, 0.8 mM of H<sub>2</sub>O<sub>2</sub> induced a cell survival that was significantly lowered in a time-dependent manner. The treatment with 0.8 mM of H<sub>2</sub>O<sub>2</sub> significantly decreased cell numbers in comparison to control cells when the cells were exposed to H<sub>2</sub>O<sub>2</sub> for 12 h. For that reason, the treatment with 0.8 mM H<sub>2</sub>O<sub>2</sub> for 12 h was used to trigger HT-29 cell damage in the following experiments. As shown in Figure 1B, 0.1 mM and 0.05 mM CA treatment increased cell viability by protecting cells from H<sub>2</sub>O<sub>2</sub>-induced damage.

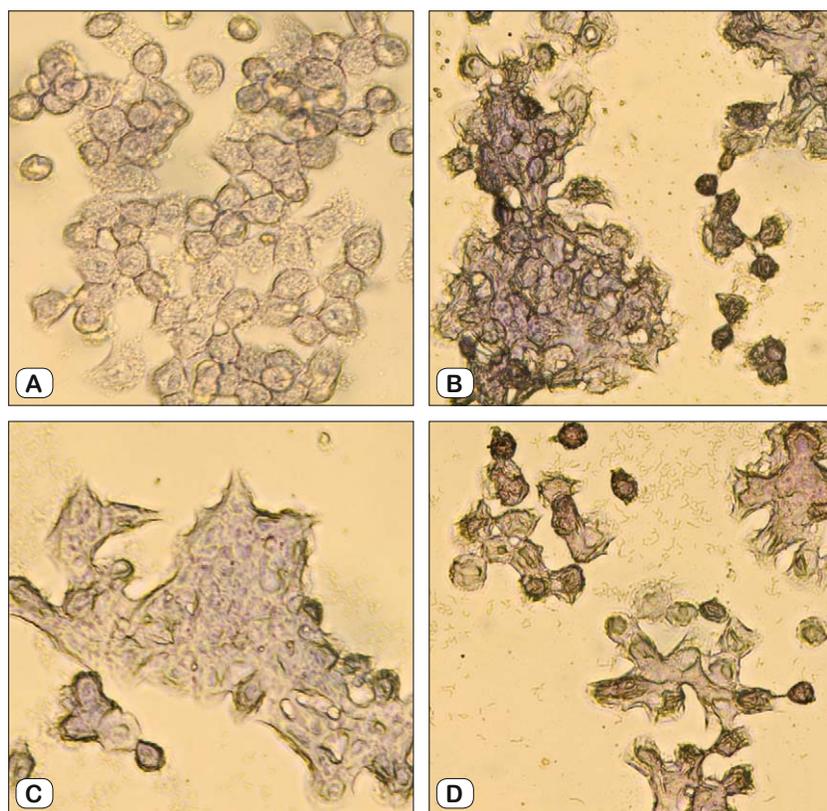
### Detection of intracellular ROS, LPO, CAT activity and TAS level

H<sub>2</sub>O<sub>2</sub> provoked cellular ROS production and affected the associated cellular metabolic reactions. Herein, the effects of CA treatment on H<sub>2</sub>O<sub>2</sub>-triggered ROS production in HT-29 cells were studied.



**Fig. 3.** The effect of H<sub>2</sub>O<sub>2</sub>, and CA+H<sub>2</sub>O<sub>2</sub> treatments on apoptosis in HT-29 as compared to control group. AI was calculated according to TUNEL assay instructions. Experimental data are given as mean  $\pm$  SEM (n  $\geq$  3). \*\*\* p < 0.001 points out statistically significant differences between control and other groups.

The results revealed that the treatment led to a remarkable rise in ROS production, however, 0.1 mM CA pre-treatment significantly reduced ROS production (Fig. 2A). Moreover, the MDA level was



**Fig. 4.** The effect of  $H_2O_2$  (B) and CA+ $H_2O_2$  (C) treatments on apoptosis-related active caspase-3 in HT-29 cells as compared to control group (A). Caspase-3 expressing cells are characteristic by their dark brown colouring.

meaningfully increased in  $H_2O_2$ -treated cells as compared with the control group, while CA pre-treatment could reduce the MDA production (Fig. 2B). The CAT activity was remarkably decreased in  $H_2O_2$ -exposed colon cancer cells as compared with the control group, while CA pre-treatment increased the CAT activity (Fig. 2C). Similarly, TAS was noticeably reduced in  $H_2O_2$ -triggered HT-29 cells, however, CA pre-treatment reversed this effect (Fig. 2D).

#### TUNEL Assay

The effect of CA on preventing apoptosis provoked by  $H_2O_2$  in HT-29 cells was also evaluated. As detected by TUNEL assay,  $H_2O_2$  induced apoptosis brought about by breaks in DNA strand and increases in AI. Pre-treatment with CA significantly inhibited the  $H_2O_2$ -induced apoptosis (Fig. 3).

#### Immunohistochemical staining for caspase-3

The results of examination by means of IHC staining demonstrated that the expression of caspase-3, the apoptosis marker, increased in  $H_2O_2$ -treated HT-29 cells while this effect was reversed by pre-treating the cells with CA (Fig. 4).

#### QRT-PCR analysis

Gene expressions of *Bax*, *Bcl-2*, *p53*, *cyt c*, *cas-8* and *cas-3* were studied to find out whether  $H_2O_2$  treatment and CA pre-

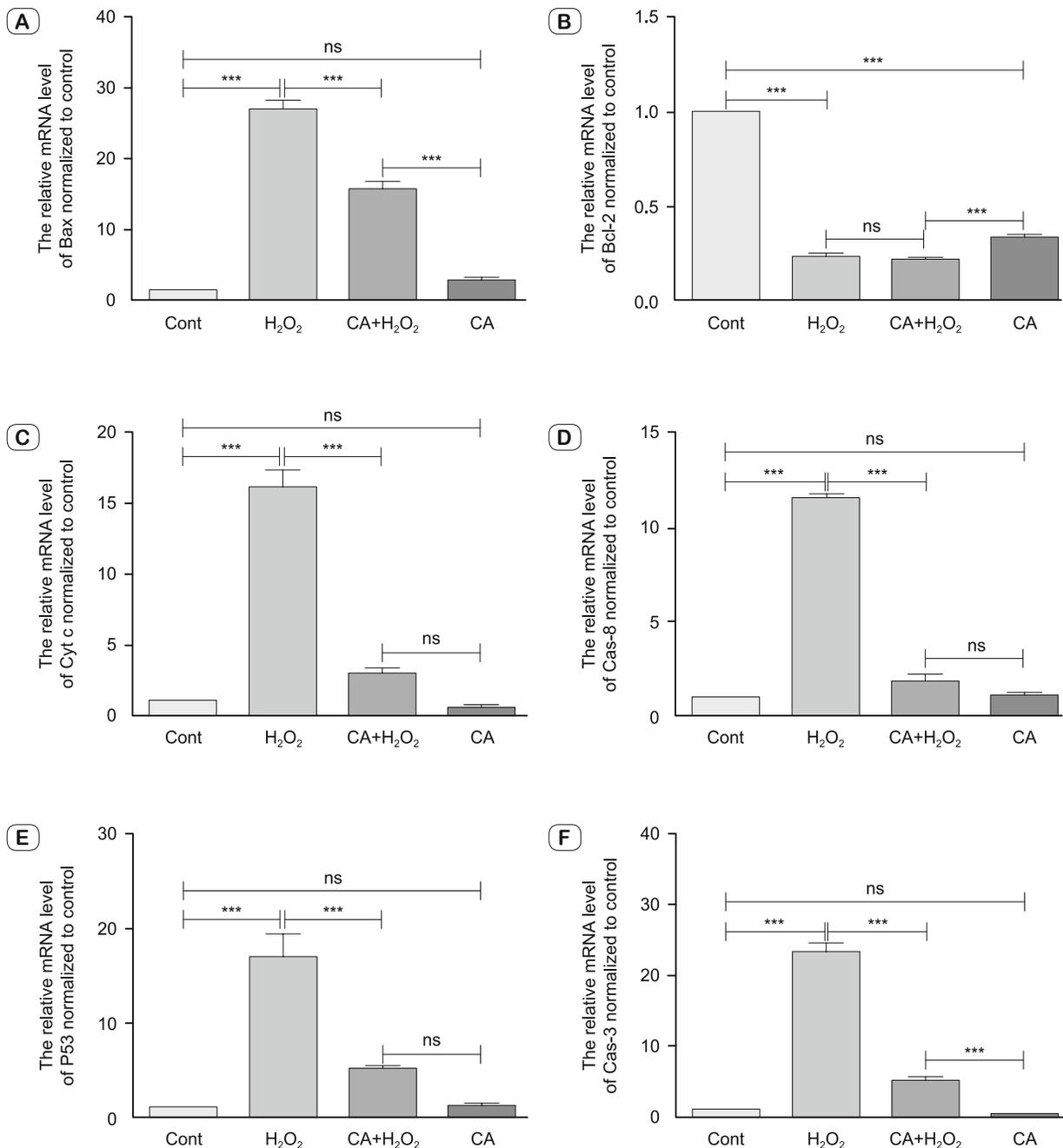
treatment result in a change in the level of expression of these apoptotic and anti-apoptotic genes. The results suggested that  $H_2O_2$  provoked apoptosis by upregulating the expression levels of apoptotic genes while downregulating the anti-apoptotic gene (*Bcl-2*). Pre-treatment with CA remarkably inhibited the  $H_2O_2$ -triggered apoptosis (Figs 5 A–F).

#### Discussion

Several studies have indicated that oxidative injury is a crucial mediator of cellular injury in a number of cancers (Reuter et al, 2010). Oxidative stress induces ROS inclusive of  $H_2O_2$  and superoxide ions which cause dysfunction in the mitochondrial function leading to cellular death (Reuter et al, 2010). Eradication of ROS by using various antioxidants could be an efficient approach in preventing oxidative stress-provoked cellular death (Choi et al, 2010). However, as shown in previous studies, the use of antioxidants as a supplement in cancer treatment might not always be beneficial (Fu et al, 2014, Tartik et al, 2016, Duval et al, 2019). The focus of present study was to examine the effect of CA on HT-29 cells treated with an overdose of  $H_2O_2$ , and molecular mechanisms underlying its effect.

$H_2O_2$  has been comprehensively utilised to induce oxidative injury both *in vivo* and *in vitro* (Sato et al, 1996, Ben Saad et al, 2019). The exposure of cells to  $H_2O_2$  damages the cellular metabolic pathways while ROS have harmful effects on proteins and lipids within cell membranes. In previous studies, CA was reported to demonstrate hepato-protective activity through multiple mechanisms (Janbaz et al, 2004). The protective effects of some other antioxidants were also shown to be essential in pathogenetic mechanisms intermediated by ROS, and support comprehension of the apparent suppression of colon cancer (Rosignoli et al, 2001). In this study, it was confirmed that exposing the colon cancer cells to  $H_2O_2$  caused a decrease in cell proliferation in a concentration-dependent and timely manner (Fig. 1A). However, pre-treating the cells with 50 and 100  $\mu$ M of CA, reduced the cell viability loss (Fig. 1B) and indicates that CA considerably suppressed HT-29 cell death from cytotoxicity induced by the overdose of  $H_2O_2$ .

Oxidative injury could influence cell viability, differentiation and survival by triggering cell signalling pathways (Reuter et al, 2010).  $H_2O_2$ -provoked oxidative injury was reported to cause dysfunction in mitochondria (Frisard and Ravussin, 2006). Former researches have demonstrated that phenolics and flavonoids possess strong free radical scavenging capacity and prevent ROS generation in a concentration-dependent manner (Baydas et al, 2007, Kamiya et al, 2012). It was also reported that an antioxi-



**Fig. 5.** The effect of H<sub>2</sub>O<sub>2</sub> and CA+H<sub>2</sub>O<sub>2</sub> on level of apoptosis-related gene expressions in HT-29. Cells were treated as stated in experimental groups. The mRNA levels of pro-apoptotic *Bax* (A), anti-apoptotic *Bcl-2* (B), apoptotic *cyt c* (C), *cas-8* (D), *p53* (E) and *cas-3* (F) were measured by using QRT-PCR analysis. Experimental values are expressed as mean  $\pm$  SEM ( $n \geq 3$ ). \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$  indicate statistically significant differences between control and other groups; ns: not significant.

dant-rich bee product, propolis, has a potent free-radical scavenging activity, and obstructs LPO, thus defending the cells against low-density lipoprotein-induced oxidative injury resulting in the apoptotic pathway (Fang et al, 2014). As a subject of this research, it was shown that H<sub>2</sub>O<sub>2</sub> significantly increased both cellular ROS and LPO levels while CA pre-treatment reversed their levels in

H<sub>2</sub>O<sub>2</sub>-exposed HT-29 cells. On the other hand, in this study, the TAS level and CAT activity were significantly reduced in H<sub>2</sub>O<sub>2</sub>-treated HT-29 cells as compared with the control group, while CA pre-treatment increased their activity (Fig. 2 C, D).

Several indications of apoptosis used in this research provide a better understanding and a more comprehensive picture of the

influence of CA on the viability of H<sub>2</sub>O<sub>2</sub>-treated HT-29 cells. The AI was evaluated by TUNEL assay and it was shown that H<sub>2</sub>O<sub>2</sub> exposure remarkably increased AI in HT-29 cells. Notably, pre-treatment with CA decreased the increase in AI in H<sub>2</sub>O<sub>2</sub>-exposed HT-29 cells. Present results are in agreement with previous studies proposing that oxidative stress is possibly playing an important role in H<sub>2</sub>O<sub>2</sub>-induced apoptosis (Jin and Wang 2019). In another study, the apoptotic index and caspase-3 expression of Hcy-treated MCF-7 cells were significantly decreased by co-treatment with antioxidant propolis (Tartik et al, 2016). Overall, these data revealed the capability of CA to prevent ROS generation and oxidative injury which plays a key role in H<sub>2</sub>O<sub>2</sub>-triggered apoptosis. Within this study, it was also confirmed that H<sub>2</sub>O<sub>2</sub> exposure induced cell death by activating caspase-3, and this was decreased by CA pre-treatment. These data demonstrated that CA acted as an anti-apoptotic compound by altering ROS, LPO, and TAS levels as well as CAT activity under the condition of H<sub>2</sub>O<sub>2</sub> overdose.

Extreme ROS generation causes cell death that could be either apoptotic or necrotic. In this study, the role of CA in the setting of H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis was also studied. The research has demonstrated that the members of caspase enzymes participate in regulating the apoptotic pathway (Tartik et al, 2016). The equilibrium between anti- and pro-apoptotic genes might be crucial to the survival of cells. A noteworthy observation in this study was that of the upregulation of the expression of *Bax*, *cas-3*, *cas-8*, *cyt c*, *p53* and downregulation of *Bcl-2* at mRNA levels after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 5 A–F). Pre-treatment with CA considerably reversed the effect of H<sub>2</sub>O<sub>2</sub> on these genes. Overall, these data revealed that CA can suppress HT-29 cell death due to apoptosis triggered by H<sub>2</sub>O<sub>2</sub> overdose, while alteration in the expression of apoptotic genes may suggest an anti-apoptotic role of CA in H<sub>2</sub>O<sub>2</sub>-treated colon cancer cells.

Even though, preceding researches have indicated the efficacy of CA in inhibiting the viability of cancer cells (Cai et al, 2004, Ishida et al, 2018, Min et al, 2018), this study has pointed out the influences of CA in H<sub>2</sub>O<sub>2</sub>-treated HT-29 cells probably to go back to the mismatching results in cancer studies. Davison et al, (2013) and Park (2013) independently reported a controversial information on the influence of antioxidant supplement on cancerous cells which was in conflict with the outdated notion that antioxidants ought to serve as a remedy that would decrease the risk for cancer development by scavenging cancer-leading radicals. In another study, the use of antioxidants simultaneously with a chemotherapeutic drug 5-FU treatment was shown to decrease apoptosis in colon cancer cells (Fu et al, 2014). Additionally, the treatment of different cancer cells such as Huh-7, HepG2, and PLC-PRF-5 with sorafenib and diclofenac significantly increased cancer cell death compared to sorafenib or diclofenac alone. On the other hand, antioxidants such as N-acetyl-cysteine and ascorbic acid, reversed the deleterious effects of diclofenac and sorafenib co-therapy, indicating that the production of toxic levels of oxidative damage was responsible for cellular death (Duval et al, 2019). The novel significant data of this study demonstrated that the antioxidant intake can repair cancer cells in a similar way as they defend healthy cells by decreasing the level ROS and stimulating the activity of enzymatic antioxidants. This entire information cannot lead to a proposal

that antioxidant supplementation can obviously safely stop the progression of tumorigenesis in accord with conventional wisdom.

## Conclusion

In case of cancer progression, the drugs used against cancer or overproduction of endogenous compounds such H<sub>2</sub>O<sub>2</sub> induce apoptotic cell death due to excessive production of ROS in cancer cells. In nutshell, the results obviously present that the exposure of HT-29 cells to H<sub>2</sub>O<sub>2</sub> resulted in oxidative stress via decreasing the CAT and TAS activities, increasing the ROS and LPO levels, increasing the expression level of *cas-3*, *cas-8*, *bax*, *cytochrome c* and *p53*, and decreasing the mRNA level of anti-apoptotic *Bcl-2*. The pre-treatment of HT-29 cells with CA reversed the apoptosis-inducing effect of H<sub>2</sub>O<sub>2</sub> essentially via mitigating the oxidative stress and apoptosis. In light of all these findings, it could be concluded that the use of antioxidants should be paid attention to as it could decrease the level of ROS and in turn decrease the apoptotic cell death which is an unwanted situation in the setting of cancer remedy. These findings highlight the importance of studying the detrimental effect of CA on cells treated with an overdose of H<sub>2</sub>O<sub>2</sub>.

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