Pharmacogn. Mag.
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ORIGINAL ARTICLE

Carthami flos Induces Apoptosis by Activating Caspases and Regulating Mitogen-Activated Protein Kinase and Reactive Oxygen Species Signaling Pathways in AGS Human Gastric Cancer Cells

Song Ee Han, Min Ji Kwon, Jeong Nam Kim, Eun Yeong Lim[1,2], Yun Tai Kim[1,2], Byung Joo Kim

Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan, Gyeongsangnam-do, [1]Division of Food Functionality, Korea Food Research Institute, Wanju, [2]Department of Food Biotechnology, Korea University of Science and Technology, Daejeon, Republic of Korea

Submitted: 14-Apr-2020 Revised: 29-May-2020 Accepted: 13-Aug-2020 Published: 12-Jul-2021

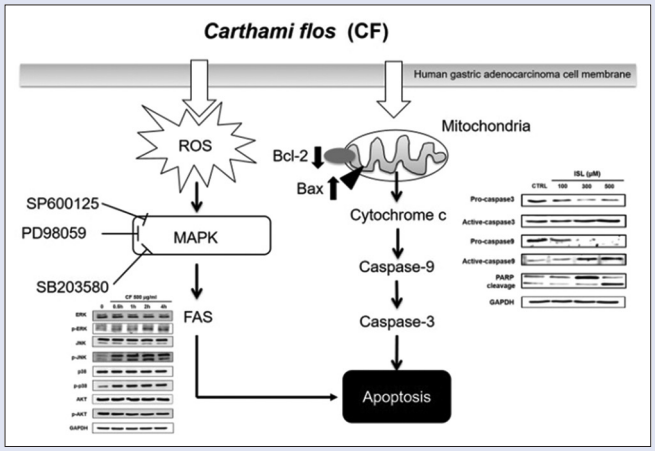
ABSTRACT

Background: *Carthami flos* (CF) is a traditional medicine used to treat various diseases, especially cancer therapy. **Objectives:** The mechanisms of cell death induction by CF were investigated in AGS human gastric adenocarcinoma cells. **Materials and Methods:** Cell viability assay, cell cycle analysis, caspase activity assay, western blotting, and reactive oxygen species (ROS) assay were used to check the anti-cancer effects of CF on AGS cells. **Results:** Treatment with CF (100500 μg/mL) inhibited AGS cell proliferation and increased the ratio of the subG1 phase of the cell cycle. CF induced cell death was associated with a reduction in Bcl-2 and an increase in Bax levels. Moreover, expression of the apoptosismediating surface antigen (FAS) was increased. CF activated caspase-3 and -9. In addition, it regulated the activation of mitogen-activated protein kinases and increased intracellular ROS generation. **Conclusion:** These findings suggest that CF induced apoptosis in AGS cells and could thus, serve as a novel anticancer agent to promote apoptosis of gastric cancer cells.

Key words: AGS, apoptosis, *Carthami flos,* gastric cancer, proliferation

SUMMARY

- Rutin and isoquercitrin were found in the high-performance liquid chromatography chromatogram of *Carthami flos* (CF) at retention times of 26.8 and 27.7 min, respectively [Figure 1]
- CF inhibited AGS survival and treatment with CF for 24 h resulted in a dose-dependent reduction in cellular viability [Figure 2]
- Sub-G1 phase ratios were increased by CF [Figure 3] and Bcl-2 was down-regulated by CF, whereas Bax was upregulated. In addition, the expressions of Fas and FasL were both up-regulated by CF [Figure 4]
- CF treatment dose-dependently increased the activities of caspase -3 and -9 and z-VAD-fmk significantly suppressed CF-induced AGS apoptosis [Figure 5]
- Co-treatment with CF and SP600125, PD98059 or SB203580 inhibited cell death [Figure 6] and the phosphorylation of Mitogen-activated protein kinases, including ERK, JNK and p38, increased after treating cells with CF [Figure 7]
- CF significantly and dose-dependently increased reactive oxygen species levels in AGS cells [Figure 8].



Abbreviations used: CF: *Carthami flos*; MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species; hERG: Human ether à-go-go-related gene.

Correspondence:

Prof. Byung Joo Kim,
Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Beomeori, Mulgeum-eup, Yangsan, Gyeongsangnam-do, 50612, Republic of Korea.
E-mail: vision@pusan.ac.kr
DOI: 10.4103/pm.pm_127_20

Access this article online
Website: www.phcog.com
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INTRODUCTION

Apoptosis (programmed cell death) is one of the most extensively studied topics, as unraveling the mechanisms regulating this process are promising for understanding the pathology of various diseases and designing new treatment strategies.[1] The molecules involved in apoptosis also play important roles in homeostasis maintenance and cancer development.[2-4] Inhibition of apoptosis is a prominent hallmark of tumors[5,6] and cancer cells generally arise due to impaired apoptosis, which promotes tumor development and metastasis.[7,8] Therefore, the regulation of apoptosis through intracellular signaling pathways that control cell growth and survival is a fundamental target of cancer therapies.[9]

Nearly 1 million new patients of gastric cancer are diagnosed with gastric cancer each year.[10,11] In the West, the incidence of

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Cite this article as: Han SE, Kwon MJ, Kim JN, Lim EY, Kim YT, Kim BJ. *Carthami flos* induces apoptosis by activating caspases and regulating mitogen-activated protein kinase and reactive oxygen species signaling pathways in AGS human gastric cancer cells. Phcog Mag 2021;17:307-14.

gastric cancer increases steadily, with nearly 25,000 people being diagnosed annually in the United States.[10] Surgical treatment is the only cure available now, although adjuvant chemoradiotherapy can improve patient outcomes.[12] Indeed, recent evidences support the importance of chemotherapy to improve patient prognosis. Furthermore, new biological therapies are emerging for gastric cancer treatment.[12] Nevertheless, novel, more effective antitumor agents are urgently required.

Carthami flos (CF) is a traditional medicine used to treat various diseases.[13] Previous studies have shown that CF extracts exert protective effects on many tissues, including anti-thrombotic and anti-inflammatory activities.[14-17] CF has been used as an adjuvant in cancer therapy and pharmacological investigations have proven its antitumor activity.[14,18,19] Caspases and the mitogen-activated protein kinase (MAPK) are involved in CF-induced apoptosis.[20] Reactive oxygen species (ROS) also have been reported to involve in CF-induced apoptosis.[21] However, studies on the anticancer efficacy of CF and the underlying molecular mechanisms are lacking. In the present study, we explored the mechanisms responsible for CF-induced apoptosis of gastric cancer cells.

MATERIALS AND METHODS

Sample preparation

The CF extract was obtained from the Korea Plant Extract Bank (Ochang, Chungbuk, Korea). The CF extract was prepared from safflower florets by extraction with 95% ethanol at 45°C for 3 days, followed by filtering and evaporation to dryness at 45°C. Then, the CF extract was resuspended in methanol and diluted in water at a final concentration of 2 mg/mL.

High-performance liquid chromatography analysis

For high-performance liquid chromatography (HPLC) analysis, a Jasco HPLC system (Jasco, Hachioji, Tokyo) was used. Reverse-phase HPLC assays were performed by gradient elution using a mobile phase comprising A, 0.5% formic acid and B, acetonitrile: Methanol (80:20 v/v), as follows: B 5%–35% over 30 min; B 35% to 90% over 6 min; and B 90%–5% over 1 min. Standard calibration curves for rutin and isoquercitrin were linear (r^2 >0.999**) in the range of 20–100 μg/mL. Analyses were performed three times. Rutin and isoquercitrin were identified in the HPLC chromatogram of CF extracts at retention times of 26.8 and 27.7 min, respectively; the concentrations of rutin and isoquercitrin were 13.57 ± 0.06 and 7.73 ± 0.23 mg/g, respectively [Figure 1].

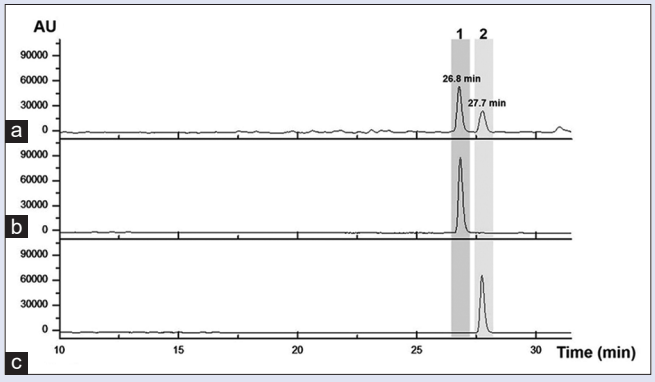

Figure 1: High-performance liquid chromatography chromatogram of *Carthami flos* extract (a) and standard compounds (b: Rutin and c: Isoquercitrin) at a wavelength of 280 nm

3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and cell counting kit-8 assay

The human gastric adenocarcinoma cell line (AGS) was cultured in RPMI-1640 medium and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cell counting kit-8 (CCK-8) assay.

Measurement of cell cycle

AGS cells were treated with ethyl alcohol and treated at 4°C overnight before incubating them. A fluorescence-activated cell sorter was used to check at λ = 488 nm.

Western blot analysis

Antibodies against ERK (#9102), pERK (#9106), JNK (#9252), pJNK (#9251), p38 (#9212), pp38 (#9216), AKT (#9272) and pAKT (#9271) were purchased from Cell Signaling Technology (Danvers, MA, USA), whereas antibodies against Bcl-2 (#sc-783), Bax (#sc-493), caspase-3 (#sc-7148), caspase-9 (#sc-7885), PARP (#sc-7150), β-actin (#sc-47778) and GAPDH (#sc-32233) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All procedures were performed following standard methods.

Reverse transcription polymerase chain reaction analysis

The methods used in the previous study were used exactly as they were.[22]

Caspase assay

Assays were performed using caspase-3 and-9 assay kits (BioMol, Plymouth, PA, USA). Cells were resuspended in lysis buffer and the cell pellets were incubated with caspase substrates at 37°C. Absorbance of the samples was measured at 405 nm.

Measurement of reactive oxygen species levels

ROS generation was evaluated by FACS at excitation/emission wavelengths of 488/525 nm, respectively.

Statistical analysis

One-way ANOVA was performed for comparison of data from multiple groups using Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and Origin 8.0 (OriginLab Corporation, Northampton, MA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Apoptotic effects of *Carthami flos* in AGS cells

MTT assays were performed to investigate whether AGS cell growth was inhibited by CF treatment for 24 h. Treatment with CF (100, 200, 300, 400, and 500 μg/mL) inhibited AGS survival by 71.0% ± 2.5%, 61.3% ± 2.9%, 52.8% ± 3.2%, 43.2% ± 4.8%, and 16.3% ± 1.3%, respectively [Figure 2a]. Moreover, the survival of AGS cells was evaluated through the CCK-8 assay method. CF treatment (100, 200, 300, 400, and 500 μg/mL) induced a reduction in cellular viability by 54.5% ± 1.4%, 43.8% ± 1.6%, 40.4% ± 1.6%, 32.2% ± 1.1%, and 27.3% ± 0.9%, respectively [Figure 2b]. In addition, to determine whether CF-induced apoptosis, cell cycle analysis was conducted by flow cytometry. Sub-G1 phase ratios were increased following CF treatment by 7.5% ± 1.9% at 100 μg/mL, 14.7% ± 1.9% at 200 μg/mL, 19.8% ± 1.7% at 300 μg/mL, 28.3% ± 2.1% at 400 μg/mL and 29.1% ± 2.3% at 500 μg/mL, compared to untreated cells [Figure 3a and b]. Altogether, these

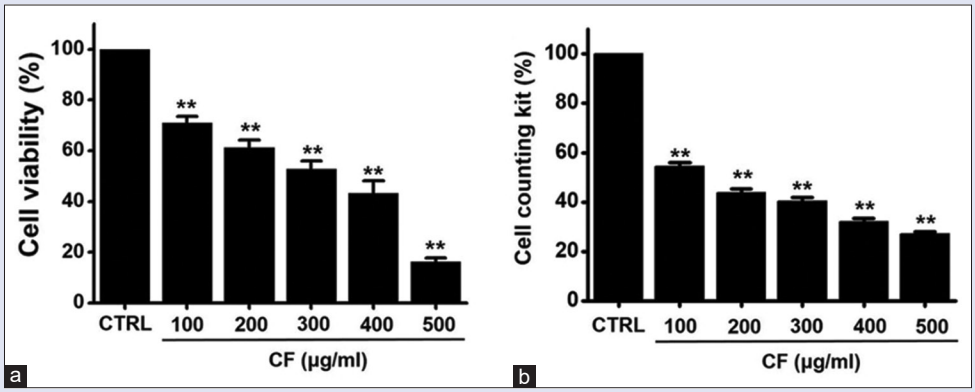

Figure 2: Effect of CF on AGS cell viability. Cell viability was assessed with the (a) 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and (b) cell counting kit-8 (cell counting kit-8) assay. CF treatment for 24 h reduced cell viability. Results are presented as the means ± standard error means. **$P < 0.01$ versus untreated cells. CF: *Carthami flos*; CTRL: Control

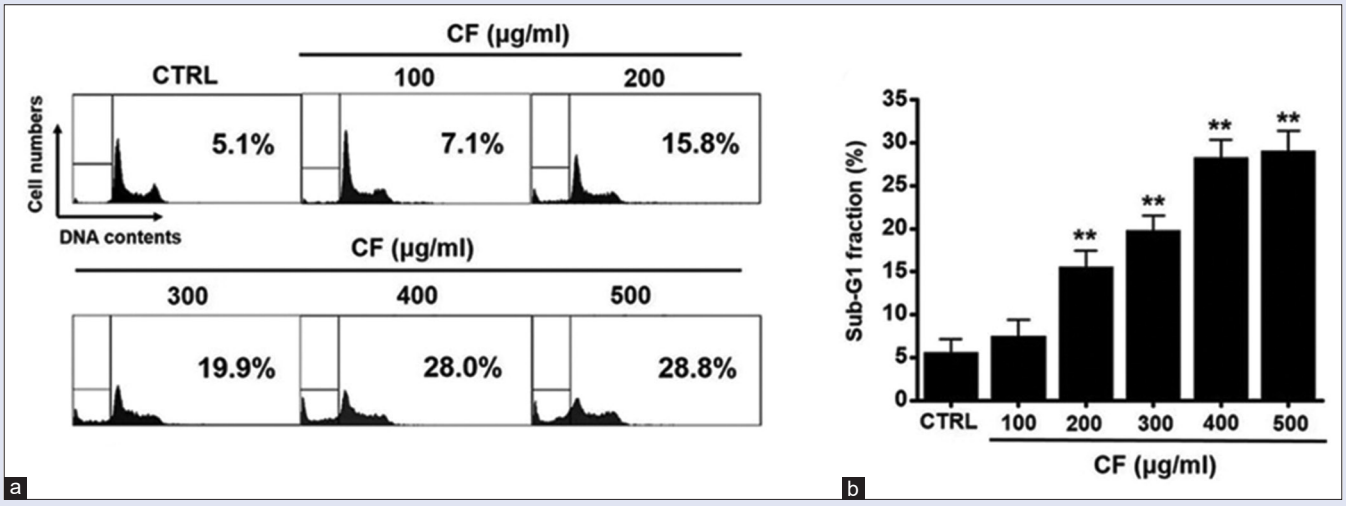

Figure 3: Effect of CF on the apoptosis of AGS cells. (a) Sub G1 cell fractions were check by flow cytometery. (b) They were expressed as percentages. Results are presented as the means ± standard error means. **$P < 0.01$ versus untreated cells. CF: *Carthami flos*; CTRL: Control

results indicate that CF inhibits AGS proliferation and that these effects may have been associated with apoptosis.

Carthami flos-induced the mitochondrion-dependent apoptosis pathway in AGS cells

To check the regulation by Bcl-2 and Bax, we conducted western blotting experiments. Following CF treatment, Bcl-2 levels were decreased, whereas Bax levels were increased [Figure 4a-c]. In addition, we examined the effect of CF on the Fas/FasL system, which plays a key role in death receptor-induced apoptosis. The expression of these molecules increased after treatment with CF [Figure 4d]. These results indicate that CF-induced apoptosis was linked with the activation of the mitochondrial pathway in AGS cells.

Caspase activation following *Carthami flos* treatment of AGS cells

Caspases are important mediators in the intrinsic and extrinsic apoptosis pathways. These pathways induce the activation of cytoplasmic endonucleases and degrade cleave various substrates, including PARP.[23] Western blotting indicated that CF treatment increased the caspase-3 and -9 activities and decreased the levels of pro-caspase-3 and-9 [Figure 5a]. In addition, western blotting revealed that CF increased PARP protein levels. CF treatment also increased the caspase-3 and -9 activities, whereas pretreatment with z-VAD-fmk (a broad-spectrum caspase inhibitor) suppressed their activities [Figure 5b]. Furthermore, when AGS cells were treated with CF and z-VAD-fmk at the same time, z-VAD-fmk inhibited CF-induced apoptosis [Figure 5c]. These results indicate that CF-induced apoptosis involved the activation of caspases in AGS cells.

Carthami flos -mediated regulation of the mitogen-activated protein kinase signaling pathways in AGS cells

To check the effect of CF on the MAPK pathways, the viability of cells co-treated with various concentrations of CF and JNK (SP600125), ERK (PD98059) or p38 (SB203580) inhibitors was evaluated using the MTT assay. When cells were treated with CF (100, 200, 300, 400, and 500 μg/mL) and SP600125 together, cell death was inhibited by 85.2% ± 3.4%, 72.5% ± 1.9%, 69.1% ± 3.0%, 61.9 ± 2.9, and 52.6% ± 2.1%, respectively [Figure 6a]. Treatment with CF (100, 200, 300, 400, and 500 μg/mL) and PD98059 inhibited cell death by 86.2% ± 2.8%, 78.9% ± 4.2%, 74.6% ± 4.3%, 66.7% ± 4.9%, and 54.5% ± 0.7%, respectively

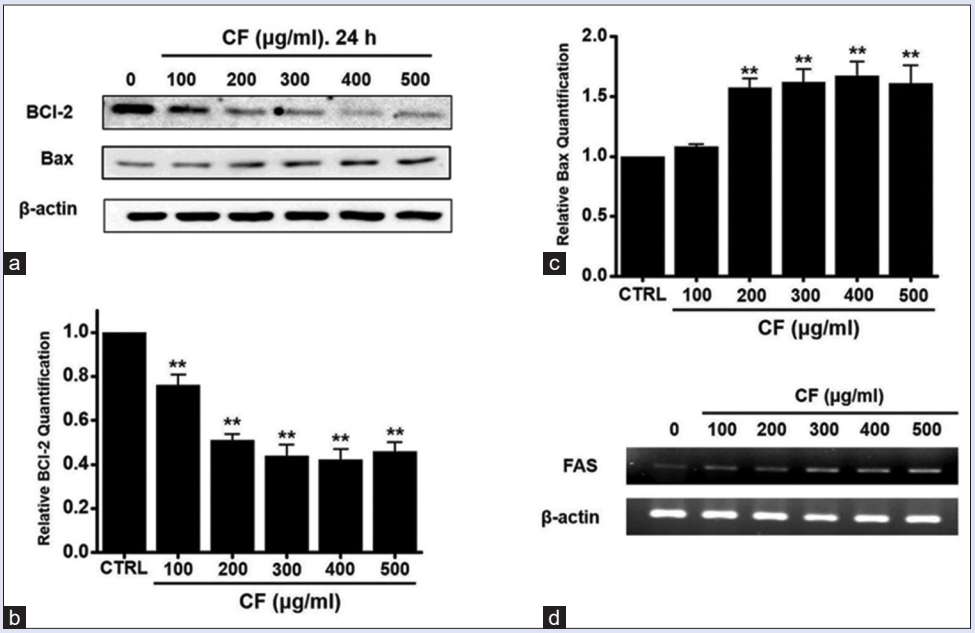

Figure 4: Effects of CF on Bcl-2, Bax and Fas levels in AGS cells. (a) Bcl-2 levels were gradually reduced by CF, whereas Bax levels were gradually increased, as shown by western blotting. (b) Bcl-2 and (c) Bax protein levels were normalized to those of βactin. (d) Fas and FasL expression levels were both upregulated by CF, as shown by reverse transcription polymerase chain reaction. Results are presented as the means ± standard error means. ***P* < 0.01 versus untreated controls. β-Actin was used as the loading control. CF: *Carthami flos*; CTRL: Control

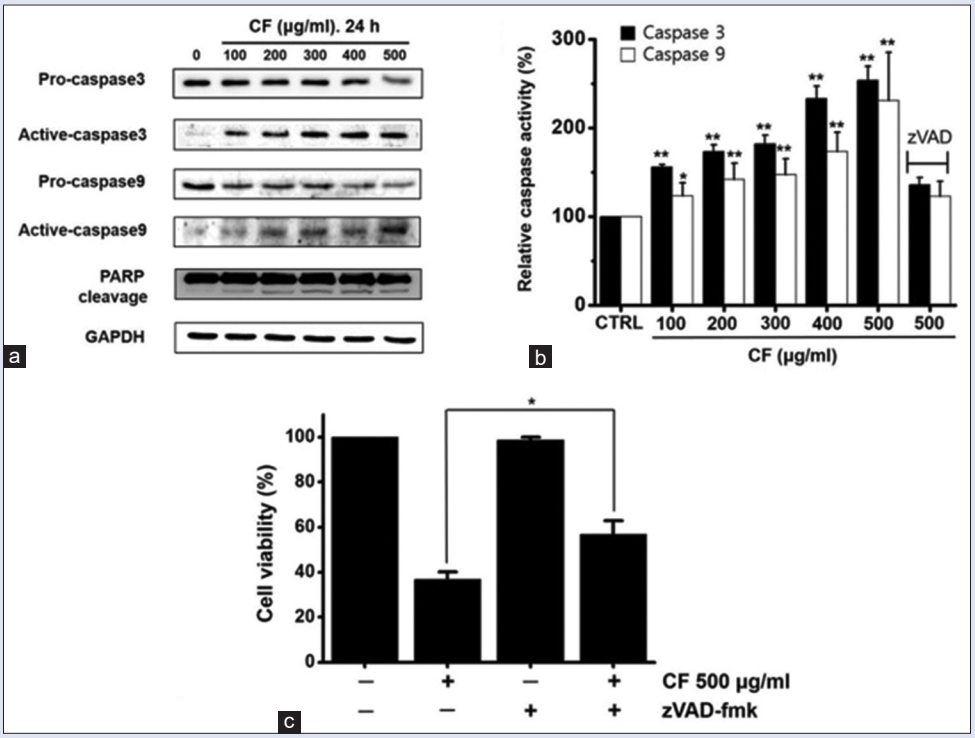

Figure 5: Activation of caspases and degradation of PARP [poly (ADP-ribose) polymerase] protein in AGS cells following CF treatment. (a) Changes in caspase-3 and-9 and PARP cleavage activity were investigated by western blotting. (b) AGS cells were lysed and these lysates were assayed for caspase-3 and -9 activities. (c) Pretreatment with z-VAD-fmk significantly suppressed CF-induced apoptosis of AGS cells. Results are presented as the means ± standard error means. **P* < 0.05, ***P* < 0.01 versus untreated controls. GAPDH was used as the loading control. CF, *Carthami flos*; CTRL, control

[Figure 6b]. Similarly, when cells were treated with CF (100, 200, 300, 400, or 500 μg/mL) and SB203580 together, cell death was inhibited by 90.3% ± 6.7%, 86.0% ± 6.6%, 73.0% ± 7.1%, 56.7% ± 6.8%, and 49.3% ± 5.1%, respectively [Figure 6c]. To further examine the regulation of these signaling pathways, we performed western blotting to investigate whether CF could induce the phosphorylation of MAPK

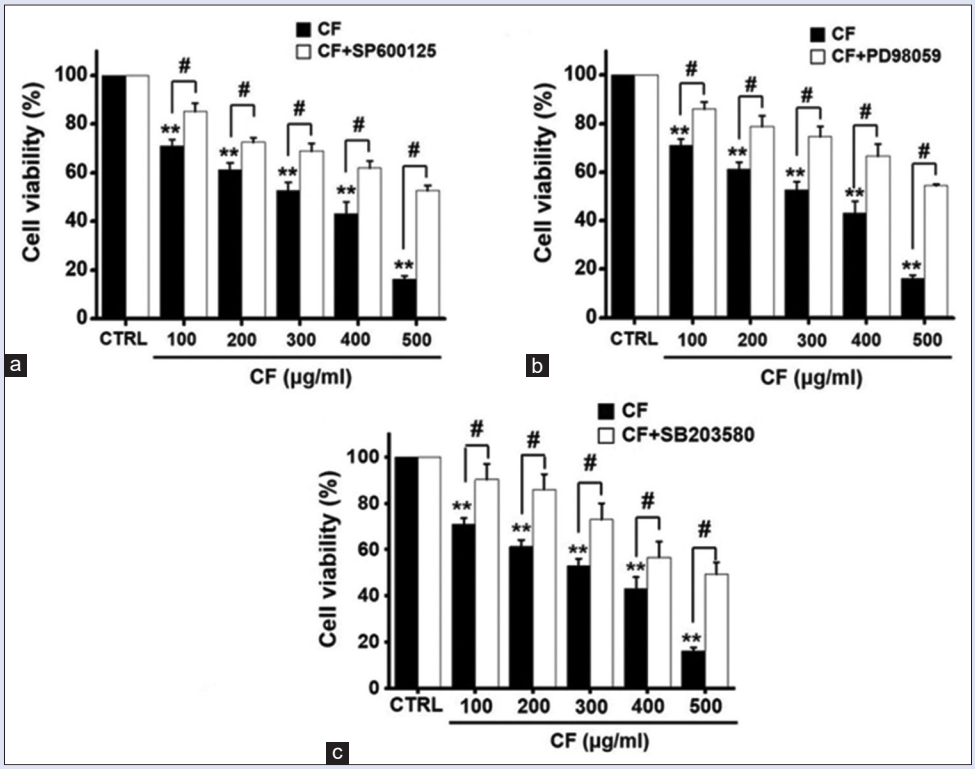

Figure 6: Effects of MAPK pathway inhibitors on the activity of CF in AGS cells. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay was used to evaluate cell viability after co-treating AGS cells with CF and (a) SP600125, (b) PD98059, or (c) SB203580 for 24 h. Results are presented as the means ± standard error means. ***P* < 0.01 versus untreated controls. #*P* < 0.05 for comparisons between the treatments. CF: *Carthami flos*; CTRL: Control

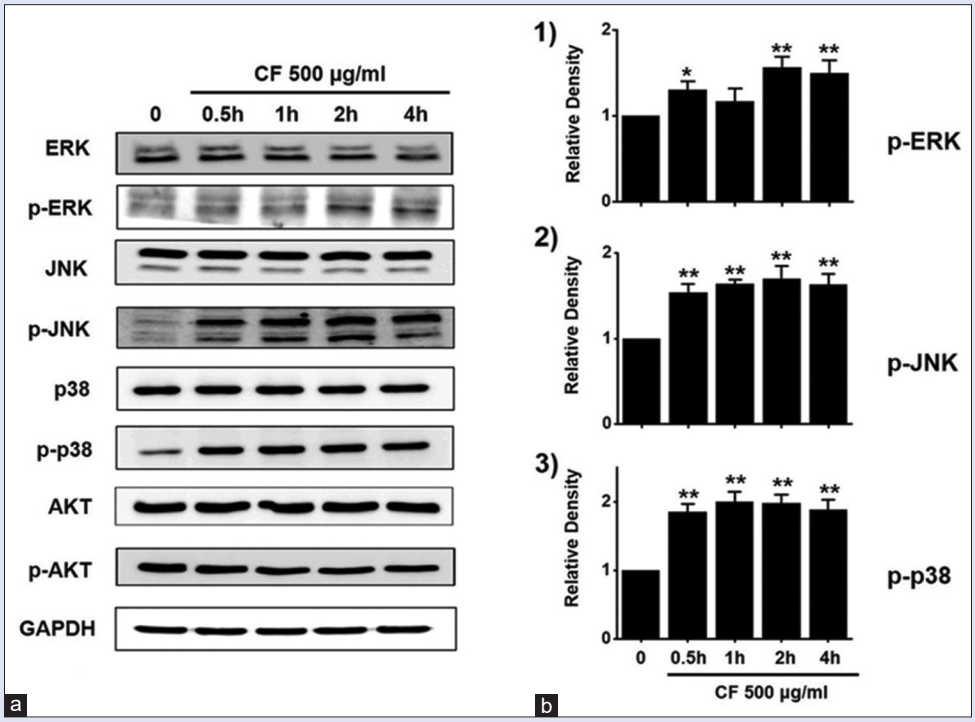

Figure 7: MAPK pathways in AGS cells following CF treatment. (a) Phosphorylation of ERK, JNK, p38 and AKT was evaluated by western blotting. (b) Phosphorylated ERK, JNK and p38 are presented as band densities relative to GAPDH density. Results are presented as the means ± standard error means. **P* < 0.05, ***P* < 0.01 versus untreated controls. CF: *Carthami flos*; CTRL: Control; ERK: Extracellular signal-regulated kinase; JNK: cJun Nterminal kinase

proteins. The phosphorylation of MAPKs, including ERK, JNK and p38, increased following treatment with CF (500 μg/mL) for 30 min and this was sustained for 4 h [Figure 7b]. In contrast, AKT phosphorylation did not increase, but rather, slightly decreased [Figure 7a]. These results indicate that CF inhibited AGS cell proliferation by modulating the MAPK signaling pathways and thus, favored apoptosis.

Carthami flos increased intracellular reactive oxygen species generation in AGS cells

We investigated whether CF increased ROS generation in AGS cells. To investigate whether ROS generation was related to CF-induced apoptosis, we used DCF-DA (a fluorescent dye). As shown in Figure 8a, flow cytometry showed that treatment with CF increased the levels of ROS in AGS cells [Figure 8b].

DISCUSSION

Traditional medicine often involves formulations containing combinations of herbs and medicinal herbs and their active principals are gaining recognition as useful complementary cancer treatments.[24] CF extracts have been widely used in traditional medicine, as they are known to be effective treatments for various diseases, including cancer.[14-19] The oil extracted from CF is reported to contain alkane-6,8-diols, which have anticancer properties.[25] Until now, CF has been used as an adjunct in cancer treatment, but its function has not yet been ascertained. CF regulates the expression of genes involved in the Fas and Bcl-2 pathways to exert antiproliferative effects in hepatic stellate cells.[26] Zhang *et al.*[19] reported that CF facilitated apoptosis due to an increase in Bax expression and a decrease in Bcl-2 expression in SMMC-7721 cells. In addition, Chang *et al.*[18] proved the anticancer properties of CF-treated dendritic cell vaccines in mice, indicating that CF favored polarization toward Th1 cytokine production and an increase in the number of cytotoxic T lymphocytes. The present study demonstrates that CF can induce apoptosis in AGS gastric cancer cells. Rutin and isoquercitrin were identified in the HPLC chromatogram of CF extracts; the setting of these standard components (rutin and isoquercitrin) during CF testing is a necessary component of the future industrialization of functional materials. In addition, in the future, we will conduct an *in vitro* efficacy test on CF extracts and their standard components.

Apoptosis is considered a programmed cell death[27,28] and is one of the homeostatic mechanisms utilized during development and aging. Apoptosis can also act as a defensive mechanism during immune response or following cell damage due to diseases or in response to harmful substances.[29] There are two important apoptosis mechanisms: The intracellular mitochondrial and the extracellular death receptor pathways. However, recent research suggests that these two pathways are linked and affect each other.[30] The intrinsic signaling pathway initiates apoptosis and releases mitochondrial signals that act directly on intracellular targets.[31,32] In contrast, the extrinsic signaling pathway causes apoptosis following interaction of membrane receptors (Fas, DR4, and DR5). Several pro- and anti-apoptotic proteins and death receptors are involved upstream and downstream.[33] In the present study, CF markedly increased the activation of caspase-3 and -9, significantly decreased Bcl-2 levels and increased Bax levels in a dose-dependent manner. Caspases are an important part of apoptosis and many studies have shown that apoptosis can be caused by a caspase-dependent or independent mechanism.[34,35] In the present study, CF stimulated the active forms of caspase-3 and -9 and decreased the levels of pro-caspase-3 and-9. Moreover, CF-mediated increases in caspase-3 and -9 activities were inhibited by z-VAD-fmk. These observations indicate that CF-induced AGS cell apoptosis resulted from caspase-dependent death receptor and mitochondrial pathways in AGS cells.

Membrane proteins are responsible for intra- and inter-cellular signaling and contribute to the pathophysiological characteristics of different cancers.[36] TRPM7 (transient receptor potential melastatin type 7) channel expression is vital for gastric adenocarcinoma cell survival.[37,38] In addition, this channel plays important roles in gastric cancer cell migration, invasion and growth.[39] hERG1 (human ether à-go-go-related gene 1) is aberrantly expressed in human gastric cancer and is a candidate bimolecular panel prognostic marker of gastric cancer.[40] The effect of CF treatment on ion channel regulation is not completely understood. CF acts efficiently as a Ca^{2+} channel inhibitor, leading to antiulcerogenic effects[41] and a major active component hydroxysafflor yellow A of CF, is also known to inhibit Ca^{2+} influx from cardiac cells and reduce their contractile force. This function is thought to be caused by the BK_{Ca} and K_{ATP} channels.[16] Therefore, it should be determined whether these ion channels are involved in the inhibition of gastric cancer cell growth following CF treatment.

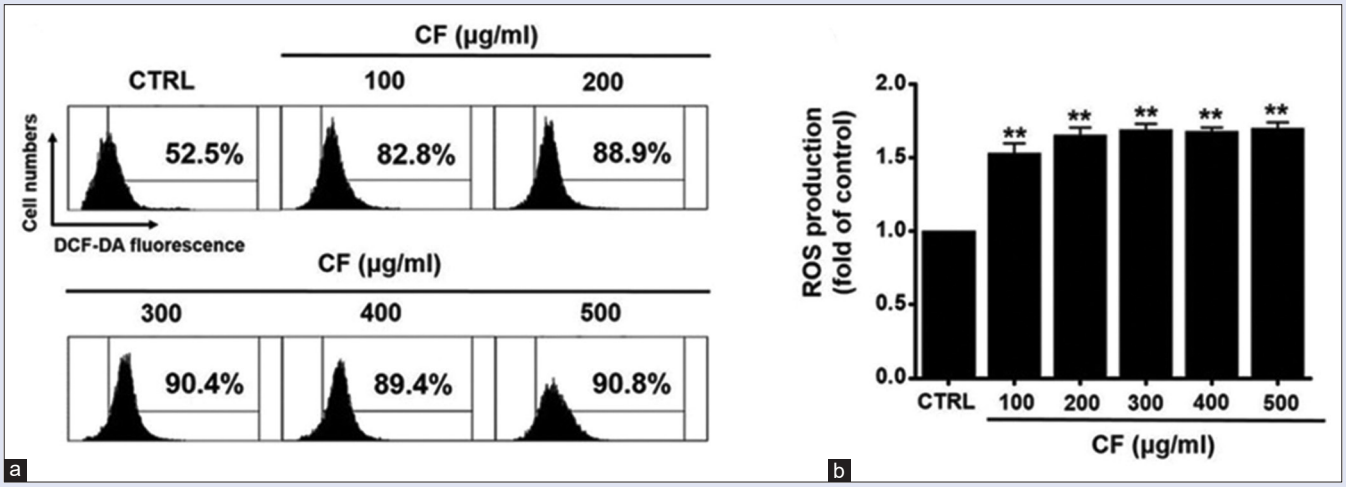

Figure 8: CF increased reactive oxygen species levels in AGS cells. (a) Intracellular reactive oxygen species levels were estimated by DCF-DA staining. (b) Intracellular reactive oxygen species levels were detected following CF treatment. reactive oxygen species levels are expressed as percentages compared to those in untreated cells (controls). Results are presented as the means ± standard error means. $^{**}P < 0.001$ versus untreated cells. CF: *Carthami flos*; CTRL: Control

MAPKs regulate various cellular processes, including apoptosis.[42,43] In the present study, CF treatment activated ERK, p38 and JNK, which correlated with the inhibition of AGS cell proliferation. In addition, MAPK signaling inhibition by PD98059, SP600125, or SB203580 prevented CF-induced apoptosis, suggesting that the activation of MAPK signaling inhibits AGS cell proliferation. In addition, ROS are important for normal physiological function control and is involved in activating various cellular signaling pathways.[44] Excess ROS can cause damage to proteins, membranes and intracellular organs, which can develop into apoptosis.[45] In Figure 8, CF increased ROS levels in AGS cells and therefore, we think that ROS may be one of the important keys in CF-induced AGS apoptosis.

Apoptosis is always related with cell death, whereas autophagy is associated with both cell survival and cell death. However, in some cases, apoptosis and autophagy are linked.[46,47] Consequently, studies on the relevance of autophagy and the molecular mechanism underlying CF-induced AGS cell death are required.

CONCLUSION

We provide evidence that CF treatment induces the apoptosis of AGS cells. Furthermore, CFinduced apoptosis is associated with decreased Bcl-2 and increased Bax levels. In addition, CF activates caspase-3 and-9 and MAPK proteins, which may play a role in CF induced cell death and increases the intracellular ROS levels. We hope that our results provide clues regarding the development of CF-based treatments for gastric cancer.

Financial support and sponsorship

The present study was supported by a Korean National Research Foundation Grant funded by the Korean Government (MSIP) (Grant no. 2017R1A2B2003764) and a grant from the Korea Food Research Institute (E0164502-05).

Conflicts of interest

There are no conflicts of interest.

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