Four *in vitro* activities of apigenin to human colorectal carcinoma cells susceptible to air-oxidative and heating treatments

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**ABSTRACT**

Flavonoids as the important natural phytochemicals have been widely assessed for their healthcare functions in recent years. Apigenin, one member of the flavonoid family, was dissolved in aqueous solution, and subjected to respective air-oxidative and heating treatments in this study to result in partial apigenin degradation. Impacts of the two treatments on apigenin's four *in vitro* activities to a human colorectal carcinoma line (HCT-116 cells) were assessed, in terms of growth inhibition, apoptosis induction, mitochondrial membrane potential (MMP) loss, and generation of intracellular reactive oxygen species (ROS). The results indicated that apigenin in dose-time-dependent manner inhibited the growth of the cells, and showed stronger anti-proliferation at 160 mmol/L than the air-oxidized or heated apigenin (67.4% *versus* 65.1% or 50.1%) at same dose level. Apigenin was also more powerful than the air-oxidized or heated apigenin to cause the cells with death morphology and MMP loss. In comparison with the air-oxidized or heated apigenin, apigenin at 160 mmol/L conferred the treated cells with more apoptotic cells (29.3% *versus* 28.3% or 8.3%) and much enhancement of the intracellular ROS (300.7% *versus* 247.3% or 201.7%), demonstrating that the treated apigenin had weaker apoptosis induction and pro-oxidation than the untreated apigenin. It is thus concluded that the four anti-cancer properties of apigenin in terms of anti-proliferation, MMP loss, death or apoptosis induction, and pro-oxidation were all susceptible to (i.e. weakened by) the air-oxidative and especially heating treatments.

**Keywords:** Apigenin; Heating treatment; Air-oxidation; Anti-cancer activity; Human colorectal carcinoma cells

**INTRODUCTION**

Colorectal carcinomas following lung cancer are the second leading causes of cancer related deaths in the whole word (Ahmed et al., 2013). Modern medical treatment of advanced colon cancer is still limited. At the same time, chemotherapeutics often lead to unpleasant side effects (Siddique et al., 2008). Consumption of fruits and vegetables has been demonstrated to have chemopreventive potential to prevent cancer development in the body, as some small molecules (e.g. polyphenols) from these plant foods have anti-oxidant, anti-inflammatory, and anti-carcinogenic properties (Marchand, 2002). Many studies have assessed anti-cancer activities of these small molecules. Citrus flavonoids have growth inhibition on the HepG2, HL-60 and MDA-MB-231 cancer cells (Zhang et al., 2014). Total flavonoids isolated from Korean citrus can arrest the A549 cells at G2/M-phase, and induce apoptosis via regulating the expression levels of caspase, cleaved PARP, and Bax/Bcl-xl (Park et al., 2012). Flavones and flavonols show *in vitro* anti-cancer activities to both human oesophageal adenocarcinoma and squamous cell carcinoma cells by inducting G2/M arrest and apoptosis (Zhang et al., 2008; Zhang et al., 2009). Artocarpesin, cycloartocarpesin, and isobavachalcone all have cytotoxic effects on the hepatocarcinoma HepG2, colon carcinoma HCT-116, leukemia CCRF-CEM, and other six cancer cells (Kuete et al., 2015). Following other four flavonoids quercetin, kaempferol, myricetin, and luteolin, apigenin (4’,5,7-trihydroxy flavone) is one of the most common flavonoids in plant foods, and estimated to be consumed by Hungarian children and adults at 0.57-0.87 mg/day (Meyer et al., 2006). Apigenin is considered as non-mutagenic and non-toxic to normal cells (van Dross et al., 2003), but has anti-cancer activities to several cancer cells in terms of anti-proliferation, cell cycle alteration, and apoptosis induction.
(Shukla et al., 2014). Apigenin in a dose-time-dependent manner can inhibit cell growth and cause G2/M cell cycle arrest in three cancer cells (SW480, HT-29, and Caco-2) (Wang et al., 2000). Apigenin also has anti-cancer effect on the breast cancer MDA-MB-468 cells via inhibiting cell growth and reducing phosphorylation of Akt (protein kinase B) (Harrison et al., 2014), as Akt phosphorylation is a fetal in the phosphatidylinositide 3-kinase pathway to promote tumor growth and progression. A recent study has shown that apigenin is a potent promoter to activate caspase pathway in the human leukemia cells (U937, THP-1 and HL60) (Jayasooriya et al., 2012).

However, human beings consume raw or fresh plant foods as well as the processed plant foods. The quality, quantity, and bio-activities of flavonoids in these processed plant foods can be affected by processing conditions (Igual et al., 2013; Escriche et al., 2014; Rodríguez-Roque et al., 2015). Processing methods (e.g. heating, mechanical, and domestic processes) and the storage period can directly affect flavonoid contents and consequently anti-oxidant properties of these processed plant foods (Yao and Ren, 2011; Marszalek et al., 2015). Several studies prove that food processing can enhance anti-oxidant properties of flavonoids, whereas others studies give opposite conclusions (Trumbo et al., 2003; Ryan et al., 2010; Velázquez-Estrada et al., 2013). In general, most of the studies are focused on changed anti-oxidation of flavonoids (Yang et al., 2001; Brusselmann et al., 2005). Anti-cancer activities of the flavonoids after heating or oxidative treatments, however, are less assessed so far, and should be paid for special attention.

Heat treatment is a common or necessary step during food cooking or processing, whilst air-oxidation of food components often occurs during food storage. Flavonoid compounds in these processed foods might undergo some chemical changes and therefore have altered anti-cancer activities. In this study, a flavonoid compound apigenin before and after air-oxidative and heating treatments was assessed and compared for its anti-cancer activities to a human colon carcinoma cell line (HCT-116 cells), in terms of anti-proliferation, mitochondrial membrane potential (MMP) loss, death or apoptosis induction, and pro-oxidation. The aim of this study was to reveal possible impacts of the two treatments on anti-cancer activities of apigenin or those apigenin-containing plant foods.

**MATERIALS AND METHODS**

**Materials and chemicals**

Apigenin (>99% of purity) was purchased from Shanghai Yousi Biotechnology Co. Ltd. (Shanghai, China). McCoy’s 5A medium and trypsin-EDTA solution (containing 2.5 g/L trypsin and 0.2 g/L EDTA) both were bought from Sigma-Aldrich, Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Penicillin, streptomycin, Cell Counting Kit-8, Cell Cycle and Apoptosis Analysis Kit, Annexin V-FITC Apoptosis Detection Kit, Hoechst 33258 and Mitochondrial Membrane Potential Assay kit with JC-1 were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Solarbio Science and Technology Co. Ltd. (Beijing, China). Other chemicals used were of analytical grade. The water used was prepared from Milli-Q Plus system (Millipore, New York, NY, USA).

**Apigenin treatments**

Two apigenin solutions (30 and 160 mmol/L) were prepared, heated in glass tubes in a thermostatic water bath operated at a normal temperature of food cooking (100°C) for 30 min in the dark, and then immediately cooled in ice-water till the solutions reached a temperature of 4°C. This treatment generated the heated apigenin (i.e. H-apigenin). At the same time, air at 0.2 MPa was introduced continuously from a compressed gas cylinder into the two apigenin solutions via a stainless steel pipe, to conduct air-oxidation of apigenin at room temperature in the dark for 6 h. This treatment obtained the air-oxidized apigenin (i.e. O-apigenin).

The heated and air-oxidized apigenin solutions of 160 mmol/L were filtrated with 0.22 mm micro-pore membranes, and then stored at 4°C before being used in cell experiments. The treated apigenin solutions of 30 mmol/L were detected immediately for their absorbencies to generate absorption spectra (wavelength range of 340-400 nm), using an UV-visible spectrophotometer (UV-2401 PC, Shimadzu Co., Kyoto, Japan).

**Cell line and culture conditions**

A human colorectal cancer cell line (HCT-116 cells) was obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were recommended to be cultured in the McCoy’s 5A medium, supplemented with 10% FBS and maintained at 37°C in a humidified incubator with 5% CO₂. The cells were sub-cultured every 2 d, and used for the experiments after reaching the exponential growth phase.

**Hoechst 33258 staining**

Hoechst 33258, a bis-benzimidazole derivative compound capable of binding the minor groove of DNA, was used for DNA staining to show cell death. Hoechst stains completely dissolve in aqueous solutions and emit blue light under UV excitation. The cells (1×10⁴ per chamber) were seeded into
The effects of two treatments on degradation and anti-proliferation of apigenin

The untreated apigenin solution and the two treated apigenin solutions were all scanned at the same concentration (30 mmol/L) to obtain their UV spectra. The spectra were recorded and compared to reflect possible apigenin degradation upon the heating and air-oxidative treatments. The obtained results (Fig. 1) demonstrated that the conducted heating and air-oxidative treatments both resulted in partial apigenin degradation, as the heated and air-oxidized apigenin solutions both had lower absorbencies than the untreated apigenin solution. This fact implied that
The chemical structure of apigenin during the two treatments was damaged (e.g., ring-cleavage), which led to decreased absorbency. The applied heating treatment was observed to degrade apigenin severely than the air-oxidative treatment, because the heated apigenin solution showed much lower absorbencies at 340-400 nm than the air-oxidized solution.

Apigenin, O-apigenin, and H-apigenin were then used in cell culture to assess their anti-proliferation on the HCT-116 cells. The results (Fig. 2) showed that apigenin at five dose levels of 20-160 mmol/L inhibited the growth of the cells in a dose-time-dependent manner, verifying apigenin’s anti-cancer activities to the cells. The maximum growth inhibition of apigenin was detected to be 78.5% if treatment time of the cells was 72 h. When the cells were treated by apigenin for 24, 48, and 72 h, the respective half maximal inhibitory concentration (IC$_{50}$) values of apigenin were calculated to be 91.9, 88.4, and 77.1 mmol/L. Based on these results, apigenin of 160 mmol/L and a treatment time of 24 h were applied on the cells in the future evaluation. With these two selections, O-apigenin and H-apigenin were detected to have respective growth inhibition of 65.1% and 50.1% on the cells. The two values were lower than that of apigenin (67.4%), indicating that the two treatments (especially heating treatment) resulted in apigenin decreased growth inhibition.

Flavonoids as natural anti-oxidants are well-known to have instability in aqueous solutions, which depends on chemical structures of flavonoids and medium conditions. Chemical instability of flavonoids therefore brings about concentration loss (i.e., degradation), and has been observed in several researches. Heating of fisetin and quercetin solutions leads to continuously decreased absorption intensities as heating time progresses, as the results of fisetin and quercetin degradation (Wang et al., 2016). During storage, ripe pistachios show decreased total phenolics and flavonoids contents (Tsantili et al., 2011). When apple juice is heated at several temperatures, 39 phenolic compounds show degradation especially at higher temperature (De Paepe et al., 2014). In consistent with these reported results, the heated and air-oxidized apigenin solutions both were observed in this study to have decreased absorbencies at 340-400 nm, which also was the result of apigenin degradation. Flavonoid degradation contains a serial reaction such as oxidation, hydroxylation, and especially ring-cleavage. For example, the chalcone-trione structure of quercetin can be opened by a nucleophilic attack at C$_3$ (Barnes et al. 2013). Surely, degraded products of apigenin had chemical structure different from apigenin. However, the treated apigenin had impaired growth inhibition on the cells than the untreated apigenin. It is thus suggested that intact chemical structure of apigenin is very important to apigenin’s anti-proliferative activity.

**Fig 1.** UV spectra of apigenin and the two treated apigenin solutions of 30 mmol/L scanned at 340-400 nm.

**Fig 2.** Growth inhibition of apigenin at different dose levels on the HCT-116 cells. All the measurements were conducted three times (n = 3), while 5-fluorouracil (5-FU) was used as positive control. Different lowercase letters above the columns indicate that one-way ANOVA of the mean values at the same incubation time was significantly different (p < 0.05).

Anti-oxidant and anti-inflammatory properties of apigenin have been well-studied (Nielse et al., 1999). Anti-cancer, anti-mutagenic, anti-viral, and purgative potentials of apigenin are also studied in the recent years (Nichenametla et al., 2006). The effects of food processing and storage on healthcare functions of apigenin (and more important flavonoids) are thus needed detailed investigation. Flavonoids are sensitive to both heating and oxidative treatments. Heating treatment can cause flavonoid loss in plants and fruits (Ranilla et al., 2009; Zielinski et al., 2009; Igual et al., 2011). However, flavonoids after heating treatment show decreased but relatively high anti-oxidation, as their degraded products also possess anti-oxidant activities (Buchner et al., 2006). Oxygen has been found to promote flavonoids’ degradation. Higher CO$_2$ (i.e. lower O$_2$) level applied in the storage of pomegranate...
fruit results in high maintenance of physiological and biochemical properties (Selcuk et al., 2015). This result indicates an adverse effect of O₂ on the bio-activities of flavonoids. In this study, as the result of apigenin degradation, both heating and air-oxidative treatments were proved to have adverse impacts on anti-cancer activities (i.e., anti-proliferation) of apigenin to the cells.

Effects of the two treatments on apigenin’s ability to cause MMP loss
If apigenin, O-apigenin, and H-apigenin were used to treat the cells, the treated cells were detected to have integrity loss of mitochondrial transmembrane depolarization potential (Fig. 3). The results indicated that the cells treated with apigenin severely lost their MMP (shown as much enhanced green/red ratio). However, O-apigenin and especially H-apigenin were detected less powerful than the untreated apigenin to cause MMP loss, as the measured green/red ratios were decreased significantly ($p < 0.05$) in the two cases (especially using H-apigenin). These results indicated that the air-oxidative and especially heating treatments decreased apigenin’s ability to cause MMP loss of the cells.

Depolarization of MMP causes the releases of cytochrome c and other apoptosis-inducing factors from the mitochondria into the cytoplasm. Thus, the initiator (i.e., caspase-9) and executor (i.e., caspase-3) are activated, which afterward leads to the so-called apoptosis. Many natural compounds have been evidenced able to cause MMP loss. Cinnamon can inhibit the growth and break down MMP of the SiHa cells (Koppikar et al., 2010). Physcion can induce mitochondrial apoptosis in the human gastric cancer SGC-7901 cells by reducing MMP and releasing cytochrome c (Xiong et al., 2015). In the human gastric cancer AGS cells, tangeretin induces mitochondrial/caspase-dependent apoptosis by causing MMP loss and up-regulating pro-apoptotic proteins (Dong et al., 2014). This study also proved that apigenin or the treated apigenin could cause MMP loss in the HCT-116 cells. Unfortunately, air-oxidative and especially heating treatments of apigenin led to structure changes and consequentially resulted in weaker ability to cause MMP loss.

Effects of the two treatments on cell death induction of apigenin
Potential cell death induction of apigenin and the treated apigenin to the HCT-116 cells was firstly reflected by the Hoechst 33258 staining. Compared with the control cells treated by DMSO (Fig. 4a), the cells treated with apigenin showed typical morphology in terms of condensation and fragmentation of nuclei shrinkage, and formation of apoptotic bodies (Fig. 4b). It is thus briefly proved that apigenin could induce cell death in the cells. O-apigenin and H-apigenin both were also able to induce cell death; however, they were observed with weaker induction than the untreated apigenin, as fewer cells were observed with typical death morphology in the two cases (Fig. 4b versus Fig. 4c and d).

Apoptosis induction of apigenin or the treated apigenin to the cells was then assessed by flow cytometry using
the Annexin V-FITC/PI double staining. As seen from these obtained results (Fig. 5), the cells treated with apigenin had total apoptotic cells (Q2 + Q4) about 29.3% (Fig. 5b). However, the control cells only contained total apoptotic cells about 7.4% (Fig. 5a). Apigenin treatment of the cells led to an increase in the numbers of apoptotic cells, demonstrating apoptosis induction of apigenin. If O-apigenin and H-apigenin were used to treat the cells, respective total apoptotic cells were detected to be 28.3% and 8.3% (Fig. 5c and d). These results verified that the air-oxidative and especially heating treatments of apigenin led to impaired apoptosis induction. Clearly, these results were consistent with these Hoechst 33258 staining results shown in Fig. 4.

Apigenin does not induce apoptosis in normal cells (Way et al., 2004). Several studies have assessed apoptosis induction of apigenin to some mammalian cancer cells. Apigenin induces the androgen-refractory human prostate cancer PC-3 and DU145 cells apoptosis, associated with release of cytochrome C and deregulation of Bax/Bcl2 pathway (Shukla et al., 2014). Apigenin causes DNA fragmentation in the human prostate carcinoma LNCaP cells, and induces apoptosis by cleaving PARP, up-regulating Bax/Bcl-2 ratio and down-regulating NF-kB/p65 expression (Gupta et al., 2002). Apigenin is capable of inducing caspase-dependent apoptosis in the human bladder cancer cell line (T24 cells), via activating caspase-3, cleaving PARP, and down-regulating Bcl-2 (Zhu et al., 2013). The present results were consistent with these mentioned results, proving apigenin and the treated apigenin had apoptosis induction to the HCT-116 cells. However, it was interesting to see that the prior air-oxidative and especially heating treatments of apigenin led to structure damage of some apigenin molecules and therefore, resulted in impaired apoptosis induction.

Effects of the two treatments on pro-oxidation of apigenin

After being treated with apigenin or the treated apigenin, the cells were assessed for their intracellular ROS generation, to reflect the pro-oxidation of apigenin or the treated apigenin. Apigenin and the treated apigenin were able to enhance ROS generation in the cells (Fig. 6).
In comparison with the control cells, the cells treated with apigenin had much increase in ROS level (300.7%), whilst those treated with O-apigenin and H-apigenin had respective 247.3% and 201.7% increases in ROS levels. That is, apigenin had stronger pro-oxidation than the treated apigenin \((p < 0.05)\). These results indicated that air-oxidative and especially heating treatments were adverse to anti-cancer activities of apigenin in terms of pro-oxidation.

ROS are a group of highly reactive chemicals in the body. In normal conditions, ROS level in the body is controlled by several anti-oxidants. Once the oxidation/anti-oxidation balance in the body is disturbed, the subsequence oxidative stress will occur (Droge, 2002; Sequeda-Castañeda et al., 2016). ROS levels in cancer cells are usually higher than that in normal counterparts. If cancer cells are treated with chemotherapeutic agents and natural anti-cancer compounds, ROS can reach to very high levels, which therefore cause irreversible oxidative damages and induce cell death (Ozben, 2007). Flavonoids can act like pro-oxidants other than anti-oxidants in cancer cells to increase intracellular ROS levels (Zhang et al., 2015). Apigenin can increase ROS level in the monocytic and HL-60 leukaemic cells (Morrissey et al., 2005). Apigenin, luteolin, kaempferol, and quercetin can induce mitochondrial apoptosis via triggering intracellular ROS production in the human hepatoma HepG2 cells (Vargo et al., 2006). These mentioned studies all agree that apigenin and the treated apigenin could increase ROS generation in the HCT-116 cells. However, the prior air-oxidative and especially heating treatments resulted in apigenin degradation, which damaged apigenin’s structure and therefore resulted in weaker pro-oxidation.

**CONCLUSIONS**

Apigenin had *in vitro* anti-cancer effects on the human colorectal cancer HCT-116 cells, reflecting by four activities in terms of inhibiting cell growth, inducing death and apoptosis, causing MMP loss, and enhancing intracellular ROS generation. When being heated or air-oxidized, apigenin was partially degraded with changed chemical structure; subsequently, the air-oxidized and especially heated apigenin had weakened anti-cancer activities to the cells than the untreated apigenin. We thus demonstrated for the first time that the four anti-cancer activities of apigenin were susceptible to (and could be weakened by) the air-oxidative and especially heating treatments. Impacts of other food processing on anti-cancer properties of flavonoids or the flavonoid-containing foods are thus suggested for future investigation.

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**Author contributions**

B. Wang and X. H. Zhao jointly carried out this study, compiled data, conducted the statistical analysis, and wrote the manuscript.

**Conflicts of interest**

The authors declare no conflict of interest.

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