



# Hydroxycinnamic Acids in Wild Blueberry and Effects of Hydroxycinnamic Acids on Apoptosis Induction in Cancer Cell Culture <sup>†</sup>

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**Abstract:** In this study, the fruits and leaves of wild blueberries grown naturally in our country will be evaluated by using different infusion and boiling methods. Blueberry teas; leaves, raw fruit, dried and shredded raw fruit, fruit beans and seedless raw fruit of different infusions were used after boiling them for 1 min, 3 min, 5 min, 7 min, 10 min. Phenolic levels were determined by LC MS/MS technique. The antioxidant and activities of all products in the vitro HCT-116 colon cancer cell line were analyzed by spectrophotometric methods. MDA and TEAC were evaluated for antioxidant activity. Cytotoxicity and viability tests were performed by adding WST-8 (Water Soluble Tetrazolium Salt-8) solution. For apoptosis, TRAIL and Apaf-1 ELISA Kit were used for the activation of caspases of intrinsic and extrinsic pathways.

**Keywords:** blueberries; apoptosis; colon cancer; Apaf-1; TRAIL

## 1. Introduction

Recently, the fruit of the wild merit is very popular in our country. Blueberry is an aromatic plant which is a promising source of antioxidant compounds that have medicinal properties due to their hydroxycinnamic acids. Blue-blackish fruits, which are firming but sweet and edible, contain flavonoids with strong antioxidant properties and Hydroxycinnamic acids (Caffeic, p-Coumaric and Ferulic acid). Cancer is a pathological state occurs due to disruption of balance between excessive cell proliferation and decreased apoptosis. Improved or decreased apoptosis has been reported to play an important role in the process of cancer formation [1]. According to data from the American Cancer Society, approximately 2% and 3% of annual deaths in the world are caused by cancer and approximately 3.5 million people die every year from cancer [2]. It is estimated that more than 4700 new cancers have been diagnosed daily in 2018 [3]. It has been known that certain plants are used in the fight against human and animal diseases for centuries. Today, approximately 50% of modern drugs used to suppress the proliferation of cancer cells have been obtained from natural products [4]. According to the World Health Organization estimates, approximately 80% of the people living in developed countries are applying traditional treatment to eliminate primary health problems [5]. Therefore, they are regarded as a potential pharmaceutical raw material for natural products [1]. The cytotoxic and apoptotic effects of the highest ferulic, Caffeic, P-coumaric acid-containing blueberry

extracts to investigate the possible beneficial effect in Hct-116 colon cancer cell will be determined. The aim of this study is to determine the content of hydroxycinnamic acids (Ferulic, Caffeic, P-coumaric acid) found in the structure of teas obtained different products of blueberries and their antioxidant activities. The possible beneficial cytotoxic and apoptotic effects of the highest ferulic, Caffeic, P-coumaric acid-containing blueberry extracts on Hct-116 colon cancer cells were investigated.

## 2. Material and Methods

### 2.1. Blueberry Tea Preparation

Dry leaf, Dry raw fruit, Dry crushed fruit, Frozen raw fruit, Seedless fruit, Blueberries kernels and their fresh states from Turkey's Aegean region were collected and some of them were dried. Each sample of 1 g, weighed with distilled water of 10 mL, was prepared as brewed and boiled at different infusion temperatures of 80 °C and 100 °C.

### 2.2. Determination of the Amount of Phenol Compounds LCMS/MS Analysis

Previously prepared samples were used. Quantitative analysis of the components was performed using the external standard method. The analysis was performed on Waters Xevo TQD system containing automatic sample injection and UHPLC.

### 2.3. Analysis in Cell Culture

Control-only medium, Ferulic acid, Caffeic acid, p-Coumaric acid, Ferulic acid + caffeic acid + p-coumaric acid, blueberry (in p-coumaric, caffeic and ferulic acids) combinations were administered in the Hct-116 colon cancer cell line.

### 2.4. Cytotoxicity and Cell Viability Analysis

In our study, cells were seeded to 96-well cell culture plates in each well of  $2 \times 10^4$  cells in 50  $\mu$ L medium. After 24 h of incubation, the active ingredients were added in 50  $\mu$ L medium at different concentrations of  $\mu$ g/mL. The experiment was established on the 24th, 48th and 72nd hours of 3 days. After incubation of every three days, 10  $\mu$ L of WST-8 (Water Soluble Tetrazolium Salt-8) solution was added and the optical absorbance at 450 nm was read in the 620 nm reference range at the micro plate reader after the end of the 4th hours. 3 wells were used for each concentration and experiments were repeated 3 times.

### 2.5. Application for Ferulic, Caffeic Acid and P-Coumaric Acid Binary Combination

For the combination of Caffeic acid + Ferulic acid + p-Coumaric acid triple, combination in combination with synergism is considered as a single active agent. 2nd time dual combination application was made. For the combination of Caffeic acid + Ferulic acid + p-Coumaric acid triple, firstly dual combination state was applied, the chosen combination in combination with emergent synergism was considered as a single active agent and the 2nd time dual combination application was made.

### 2.6. Determination of the Effect on Apoptosis in Cell Culture

After the HCT-116 colon cancer cells were plated in 6-well plates at 1 million/mL, the active ingredients were applied and the cells were collected after 72 h, centrifuged for 20 min at 2000–3000 rpm. Later, they were centrifuged at 2000–3000 rpm for 20 min again making icing-deicing application three times. Human (TRAIL) ELISA Kit and Human Apoptosis protease activating factor-1 (Apaf-1) ELISA Kit were used to determine the effect.

### 3. Results

In our study, the infusion method was applied using the highest hydroxycinnamic acid content and boiled the seedless wild blueberries for 5 min. After boiling these seedless fruits for 5 min they were injected into the cell culture so as to have the mass of 1 g/10 mL. Concentrations of the blueberries in cell culture were prepared based on the IC<sub>50</sub> values of the applied hydroxycinnamic acid's LC/SMS results. In the cell culture *in vivo* studies, when we individually evaluated the hydroxycinnamic acids, IC<sub>50</sub> concentration was found to be 39.9 µg/mL at the 48th hours. Ferulic and p-coumaric acid are considered to be the most effective hydroxycinnamic acids. The doses were applied utilizing the results of the table, shown above, for combination applications. All dose applications were made by selecting IC<sub>50</sub> values of Caffeic acid and Ferulic acid in the 72th hour, respectively, 58.3 µg/mL and 478.8 µg/mL. It was found IC value to show between 0.3 and 0.7 (+++) synergism. The combination of caffeic acid (29.15 g/mL) and ferulic acid (239 µg/mL) was accepted as one active substance and the IC<sub>50</sub> (39.9 µg/mL) value of p-coumaric acid was added to them, so that the triple combination was obtained. It was seen in the concentrations of double and triple hydroxycinnamic acid that cytotoxicity, made by unification of half IC<sub>50</sub> dose of the caffeic and the ferulic acid in the ratio of 1:1, was found to be 2.6-fold greater than that of made by the half of IC<sub>50</sub> dose of every acid. In the triple combination, cytotoxicity, made by unification of half IC<sub>50</sub> dose of each the hydroxycinnamic acid in the ratio of 1:1, was found to be 4.5-fold greater than that of made by the half of IC<sub>50</sub> dose of every acid. The LC/MSMS results of the blueberry tea extract containing hydroxycinnamic acids, prepared using the 5 min boiling infusion method, when the hydroxycinnamic acids in the wild teas were calculated by using the 5 min boiling method, it was found that the blueberry tea is more effective than the single and combined hydroxycinnamic acids. According to the results of TRAIL, the highest value was found to be 644.6 ng/L for the blueberry tea extract. In our control with no active substance, the lowest value was calculated as 590.4 ng/L. According to the results of Apaf-1, the highest value was found to be 169.6 pg/mL for the blueberry tea extract. In our control with no active substance, the lowest value was calculated as 150.4 pg/mL.

### 4. Discussion

The best way to use plant extracts is to eliminate solvent damages and take such plants through water extraction. In our study, boiling with water and brewing methods were used that is the most reliable way in terms of health. Studies have shown that the duration of brewing and boiling is effective on the separation of phenolic compounds from plants. In the studies related to brewing time, it was shown that the brewing time was effective in increasing the solubility and the solubility of the diffusion coefficient. The fact that water extracts are safe and usable according to other extraction methods distinguishes the present study from the other studies.

In this study, the extracts were prepared with both brewing and boiling methods, and the amount of ferulic, caffeic and P-coumaric acid in the extracts were determined at different time intervals between 1 and 10 min. In this way, the optimum conditions are determined to obtain the highest active molecules.

In addition, the hydroxycinnamic acid contents of the blueberry dried and its raw fruit and leaves were determined by selecting the highest phenolic containing species.

In the study on traditionally used medical plants, the effect of the total phenolic composition and the antioxidant content on different extraction times (15 min and 5 min) were investigated. It was determined that the total phenolic contents and the antioxidant properties of the plants, which had been kept for 15 min and extracted, to be higher and to be better, respectively, according to the extracted plants [6]. In the light of this information, we investigated the ferulic, caffeic and P-coumaric acid content of the blueberry tea, brewing and boiling in different times of 1, 3, 5, 10 min in different fractions of the blueberry. When the results were compared to other methods, it was found the highest p-coumaric acid to be (6.11 ng/mL), caffeic acid (3.34 ng/mL) and ferulic acid (77.56 mg/mL) in comparison to the hydroxycinnamic acids.

In our study, depending on the excitations of the extrinsic pathway of the HCT-116 colon cancer cell line, when they are together, TRAIL level of the caffeic acid and ferulic acid at related IC<sub>25</sub>

concentrations were found to be greater than that of every one. In another study, phenolic acids, human lungs (A549) and colon (HT29-D4) showed that cancer cell lines improve anti-cancer activities through proliferation, adhesion and migration reduction [7]. In light of the study, we can indicate caffeic, coumaric and ferulic acids, as potential anti-metastasis agents in vitro, seem to be very promising. Exploring the molecular mechanisms associated with cellular signaling pathways will support our study. Hydroxycinnamic acids in the structure of this effect can be indicated to have a role.

**Author Contributions:** E.Y.S. designed the study and prepared the study protocol. B.D. obtained literature data. H.K.Y. prepared the tea samples B.D. and H.M. performed LC MS/MS analysis. B.D., N.S.G., L.O. designed the experiments and performed cell culture studies. E.S., B.D. analysed the data.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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