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Phenolic Acids Modulating Epigenetic Mechanisms in HepG2 Human Hepatoma Cells

Dogukan MUTLU[1], Nevin KAYA DIKICI[1], Naime Nur BOZBEYOGLU KART[2], Sevki ARSLAN[1*]

Highlights:

- The cytotoxicity of CA, FA, and OCA on hepatoma cells was investigated by MTT assay
- Phenolic compounds have reduced the expression levels of HATs, HDACs, and DNMT activity in HepG2 cells
- CA, FA, and OCA were investigated by quantitative analysis of protein and gene expression levels of epigenetic modulatory genes in hepatocarcinoma cells

ABSTRACT:

Phenolic compounds derived from plants exhibit an epigenetic modulatory impact in various cancer types by reversing DNA methylation patterns and chromatin modulation. In this study, caffeic acid (CA), ferulic acid (FA), and o-coumaric acid (OCA) mediated epigenetic alterations in hepatocarcinoma (HepG2) cells were investigated. For this purpose, changes DNMT enzyme activity and protein and mRNA levels of proteins involved in DNA methylation and histone acetylation were determined. The CA, FA, and OCA had cytotoxic activity at 48 h, with an EC50 value of 1.02, 3.1, and 5 mM in HepG2 cells, respectively. All compounds reduces the expression levels of histone acetyl transferases (HATs), histone deacetylases (HDACs) and DNA methyltransferases (DNMTs). All these results showed that phenolic acids may be used in cancer therapy as a potential epigenetic modifier.

Keywords:
- DNA methylation
- Caffeic acid
- Ferulic acid
- O-coumaric acid
- Phenolic componds

[1]Dogukan MUTLU (**Orcid ID:** 0000-0003-3259-5822), Nevin KAYA DIKICI (**Orcid ID:** 0000-0002-6483-7403), Sevki ARSLAN (**Orcid ID:** 0000-0002-4215-5006), Pamukkale University, Faculty of Science, Department of Biology, Denizli, Türkiye
[2]Naime Nur BOZBEYOGLU KART (**Orcid ID:** 0000-0002-7972-919X), Pamukkale University, Plant and Animal Production Department, Tavas Vocational High School, Denizli, Türkiye

***Corresponding Author:** Sevki ARSLAN, e-mail: sevkia@pau.edu.tr

INTRODUCTION

Epigenetics explores how cells manage gene expression without altering the DNA sequence itself. (Deans and Maggert 2015). Different epigenetic modifications have the capacity to modify regular cellular functions including cell growth and progression (Shankar et al., 2016; Feinberg et al., 2016) Regulatory mechanisms such as chromatin remodeling, histone modifications, microRNAs, and DNA methylation, coordinate the organization of these cellular processes (Huang et al., 2011; Ong et al., 2012; Ho et al., 2013; Shankar et al., 2016; Wang et al., 2021). Such epigenetic processes disrupt in cancer cells. The overexpression of DNA methyltransferases (DNMTs) was associated with the repression of tumor suppressor genes (TSGs) through DNA methylation at CpG residues in their promoters (Sen et al., 2018; Wang et al., 2021). Similarly, histone proteins undergo modifications by epigenetic enzymes like HDAC, HATs, TETs, and phosphorylases, leading to the activation or repression of gene activity. Any dysregulation may lead to the progression of cancer, as well (Kanwal et al., 2012). In addition, epigenetic alterations such as DNA methylations (hyper- or hypomethylation), disruption of histone modification patterns, changes in chromatin structure, and abnormal expression of long noncoding RNAs (lncRNAs) and micro-RNAs (miRNAs) have been linked to hepatocellular carcinoma (HCC) (Zhang 2015). For these reasons, HepG2 cell line is suitable for studying the effects of epigenetic drug action mechanisms.

Recently, there has been great interest towards epigenetic modification for cancer treatment due to its reversible nature. Several drugs were approved by FDA, such as, vorinostat and belinostat (HDAC inhibitors), and decitabine and azacytidine (DNMT inhibitors) that have shown promising results in myelodysplastic syndromes (MDS) and solid malignancies (SM) (Ho et al., 2013; Shankar et al., 2016; Hillyar et al., 2020). The combined strategy including use of HDAC and DNMT inhibitors at the same time, has proven to be more effective. However, their usage is restricted due to their lower specificity and higher systemic toxicity. Therefore, chemopreventive agents from plants are focused by researchers due to their less side effects.

Previous studies have been reported that plant-derived dietary agents such as curcumin, quarcetin, and resveratrol regulate the DNMT and HDAC activities (Yu et al., 2013; Venturelli et al. 2013; Kedhari et al., 2019). Caffeic acid (CA), ferulic acid (FA), and o-coumaric acid (OCA) is naturally found in coffee, fruits, and vegetables (El-Seedi et al., 2012). These phenolic acids exhibit antioxidant, anti-inflammatory, antiproliferative properties (Cheng et al., 2007; Kim et al., 2012). Additionally, they have been reported to inhibit the proliferation of cancer cells by inducing apoptosis, inhibiting the cell adhesion and cellular migration, and enhancing the efficacy of chemotherapy drugs (Li et al., 2012; Sen et al., 2013; Nasr Bouzaiene et al., 2015). Nevertheless, the role of CA, FA, and OCA in epigenome modulation have not been thoroughly investigated. In previous studies, CA and CA phenethyl ester have been shown to inhibit DNA methylation *in vitro* and *in vivo* (Hu et al., 2020; Wang et al., 2020; Kumar et al., 2023). Moreover, HDAC inhibitory activities of CA, FA, OCA, and its derivatives have been investigated on different cancer cells such as breast and cervical cancer cell lines (Waldecker et al., 2008; Wang et al., 2013; Saenglee et al., 2016). In all these studies, it has been reported that CA, FA, and OCA exhibits antiproliferative activity against a human cancer cells through biological mechanisms including modulation of HDAC activity, cell cycle arrest and apoptosis induction. In this regard, this study was undertaken to explore the effect of CA, FA, and OCA on epigenetic enzymes involved in DNA methylation, histone acetylation and deacetylation. Research into the relationship between the consumption of fruits and vegetables and the risk of cancer in humans indicates that dietary phenolic acids may offer protective effects against various types of human

cancers because of higher daily consumption and their diverse mode of action (Anantharaju et al. 2016). But, studies on the effects of phenolic acids on epigenetic mechanisms in Hepg2 cells are very limited in the literature. In this regard, the epigenetic modulation of CA, FA, and OCA in HepG2 cells was evaluated first time with present study.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Media (DMEM) and 2.5% trypsin-EDTA were purchased from Gibco (USA). Pen/strep antibiotic mixture and fetal bovine serum (FBS) were purchased from Capricorn (Germany). Dimethyl sulfoxide (DMSO) was purchased from a Carlo Erba (Italy). Caffeic acid (mol wt. 180.16 g/mol), o-coumaric acid (mol wt. 164.16 g/mol), and ferulic acid (mol wt. 194.18 g/mol) were purchased from Sigma-Aldrich (Germany). MTT reagent was purchased from Biovision (USA).

Cell Culture

HepG2 human hepatocarcinoma cell line was obtained from ECACC. Cells were cultured in DMEM. A complete medium containing 10% FBS and 1% pen/strep mix was used to grow cancer cells at 37 °C in a 5% CO2 incubator as described previously (Kurt-Kizildogan et al., 2022).

MTT Assay

HepG2 cells (2×10^3 cells per well) were plated in 96 well plates with DMEM and incubated overnight for attachment. After, culture medium was refreshed with DMEM containing various concentrations of CA, FA, and OCA dissolved in 100% DMSO and cells were treated for 48 h. DMEM with 0.5% DMSO was used as a control. MTT assay was used to determine the cell viability (Sahin et al., 2021). The absorbance was determined using a Biotek plate reader at 590 nm (Epoch, USA). EC_{50} values were calculated using GraphPad Prism 9 (GraphPad Software, CA, USA) as described previously (Mutlu et al., 2023)

DNMT Activity Assay

Cells ($1x10^6$) were exposed to EC50 doses of phenolic acids for 48 h. Then, they were collected by centrifugation (2000 rpm for 5 min) and EpiQuik™ Nuclear Extraction Kit (Epigentek, USA) was used for preparing nuclear extracts. This extract was used to measure Methyltransferase Activity (Biovision, USA) accoording to manufacturer's procedure. The absorbance was measured at 450 nm and standard calibration curve was used to calculate the DNMT activity. Cells treated with only DMSO was used as a control.

RNA Extraction, cDNA Synthesis and RT-qPCR

HepG2 cells ($3x10^4$ cells) were treated with the phenolic acids at EC50 concentration for 48 h and total RNA was extracted by using commercial kit (innuPREP RNA Mini Kit, Analytik Jena, Germany). 2.5 µg of total RNA was transformed into cDNA (OneScript Plus cDNA Synthesis Kit, ABM, USA). Changes in levels of DNMT1, DNMT3A, DNMT3B, TET1, HDAC1, HDAC3, and HAT1 mRNAs were determined by SYBR green RT-qPCR assay following the $2^{-\Delta\Delta Ct}$ method as a described previously (Liman et al., 2022). GAPDH was used as a housekeeping gene. The sequence of the primers were presented in Table 1.

Table 1. Primer sequences used for this study

Genes	Accession number	Primers sequences (5' – 3')
GAPDH	NM_002046	**F:** GTCTCCTCTGACTTCAACAGCG **R:** ACCACCCTGTTGCTGTAGCCAA
DNMT1	NM_001379	**F:** AGGTGGAGAGTTATGACGAGGC **R:** GGTAGAATGCCTGATGGTCTGC
DNMT3A	NM_175629	**F:** CCTCTTCGTTGGAGGAATGTGC **R:** GTTTCCGCACATGAGCACCTCA
DNMT3B	NM_006892	**F:** TAACAACGGCAAAGACCGAGGG **R:** TCCTGCCACAAGACAAACAGCC
TET1	NM_030625	**F:** CAGGACCAAGTGTTGCTGCTGT **R:** GACACCCATGAGAGCTTTTCCC
HDAC1	NM_004964	**F:** GGTCCAAATGCAGGCGATTCCT **R:** TCGGAGAACTCTTCCTCACAGG
HDAC3	NM_003883	**F:** GAGTTCTGCTCGCGTTACACAG **R:** CGTTGACATAGCAGAAGCCAGAG
HAT1	NM_003642	**F:** GGTAGCCTGTCAACAATGTTCCG **R:** CGTGTTTGTGCAAAATCCAGGTG

F: Forward, R: Reverse.

Western-blot Analysis

For western-blotting, HepG2 cells ($1x10^6$) were treated with phenolic acids (EC50 doses) for 48 h. After treatment period, cells were trypsinized and collected by centrifugation. Afterwards, pellet was dissolved in the RIPA buffer and all samples were incubated on ice for 30 min. Then, they were centrifuged at 10000 rpm for 10 min. Protein concentrations were quantified with the BCA Protein Assay Kit (BioVision, USA) by using bovine serum albumin (BSA) as a standard. Cell lysates (100 µg protein) were separated 12% SDS-PAGE as previously described (Laemmli UK 1970). Then, they were transferred to a nitrocellulose membrane (UltraCruz, USA). Following transfer, the membranes were blocked with blocking solution (non-fat dry milk) and then incubated with DNMT3B, HDAC1, HDAC3, HAT1, and TET1 antibodies overnight. After washing with TBST (3 times, 5 min), it was incubated with an ALP conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) and again washed with TBST. Finally, protein bands were visualized by using the NBT/BCIP substrate system as described previously (Arinc et al., 2005). The band densities were evaluated by densitometric analysis with Scion Image 4.0 and were reported relative to Histone H3 protein level.

Statistically Analysis

The results were expressed as the mean ± SD of three distinct experimentations using GraphPad Prism 9 software. The data were examined by using student's t-test. The accepted p-value for significance was less than 0.05.

RESULTS AND DISCUSSION

Cytotoxicity of phenolic compounds

The MTT assay was used to evaluate the cytotoxicity of phenolic acids against HepG2 cell line. As shown in Figure 1, all phenolic compounds showed a dose-dependent inhibition of cell viability on HepG2 cells after exposure ($P<0.05$). The EC50 values for 48 h of phenolic acids were found to be 1.02, 3.1, and 5 mM for CA, FA, and OCA, respectively.

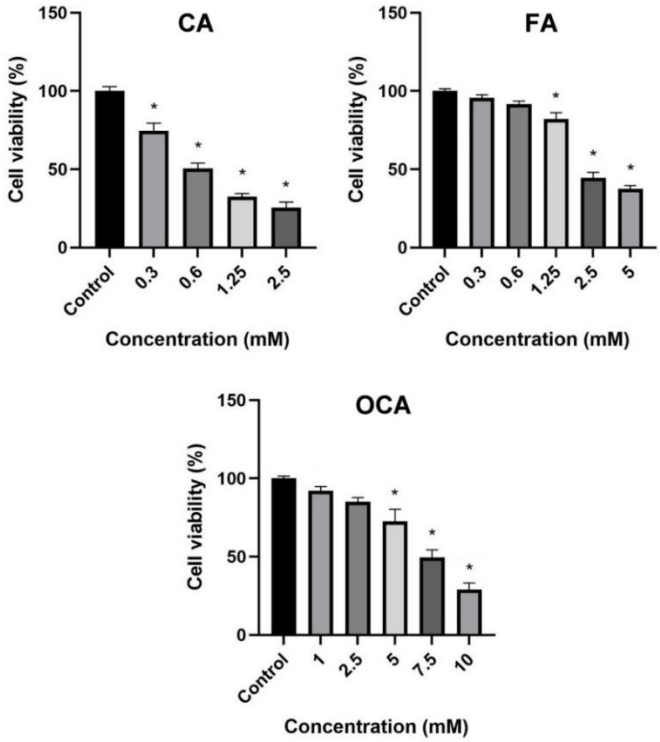

Figure 1. Cytotoxicity of phenolic compounds on HepG2 cell line after 48 h treatment. Data are expressed as the mean ± SD of each group. *P < 0.05

DNMT Enzyme Activity

Tested phenolic acids were inhibited DNMT activity in HepG2 cells (Figure 2). The nuclear extract incubated with EC50 concentration of CA, FA, and OCA resulted in the inhibition of DNMT activity by 55.03, 60.93, and 30.71% respectively (P<0.05).

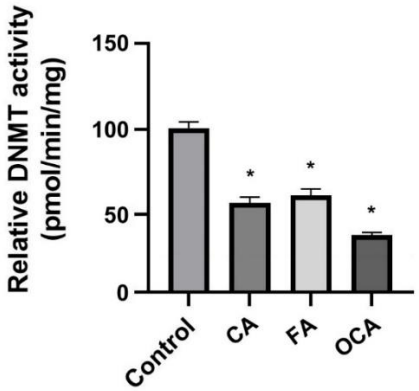

Figure 2. Relative DNMT activity in nuclear extracts of HepG2 cells after 48 h treatment of phenolic compounds. Data are expressed as the mean ± SD of each group. *P < 0.05

Gene Expression Analysis

Following the CA, FA, and OCA treatments to HepG2 cells, changes in the mRNA levels of DNMT1, DNMT3A, DNMT3B, TET1, HAT1, HDAC1 and HDAC3 genes were determined by the RT-qPCR method (Figure 3). After CA treatment, DNMT1, DNMT3A, DNMT3B, TET1, HAT1, HDAC1 and HDAC3 mRNA levels were decreased 87.6%, 76.4%, 99.3%, 98.6%, 81%, 96.4% and 87.8% in HepG2 cells (P<0.05). Similarly, 64%, 49.3%, 84.5%, 95.6%, 36.7%, 89.4% and 69.1% decreases were observed in mRNA levels of the same genes after FA treatment, respectively.

Moreover, OCA treatment was caused 64.4%, 48.6%, 91.2%, 76.1%, 36.9%, 97.2% and 80.8% decreases mRNA levels of the same genes in HepG2 cells (P<0.05).

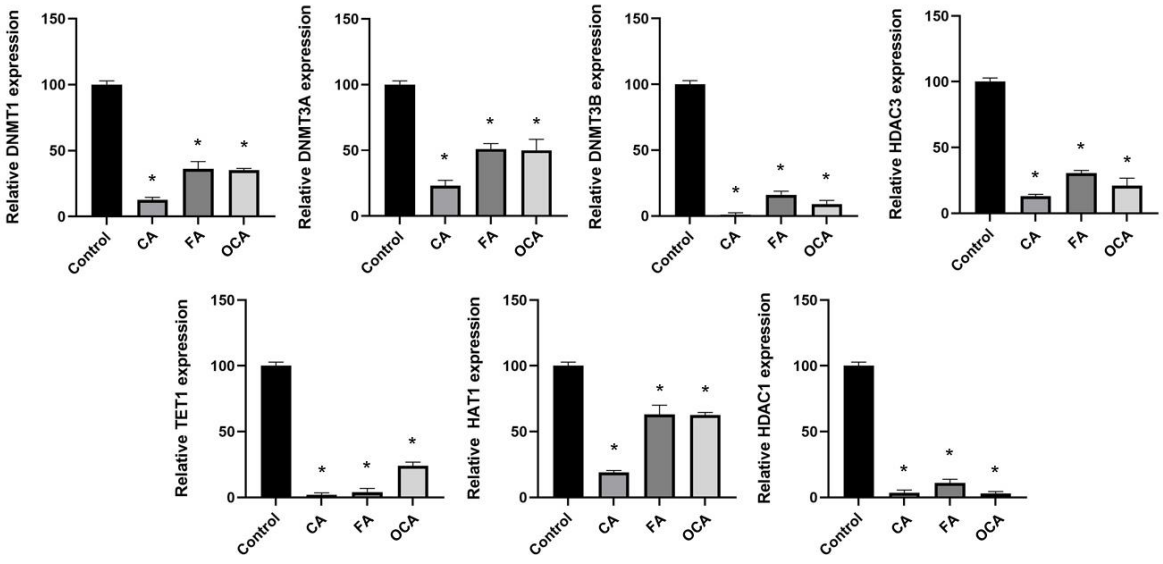

Figure 3. Relative gene expression of HepG2 cells after 48 h treatment with phenolic compounds for 48 h by RT-qPCR. GAPDH was used as an internal control. *P < 0.05

Protein Levels Expression

Western blot analysis was performed to measure the levels of DNMT3B, HDAC1, HDAC3, HAT1, and TET1 in HepG2 cell line. As shown in Figure 4, treatment with phenolic compounds at a concentration of EC50 reduced the TET1, HDAC3, and HAT1 expression in HepG2 cells (P<0.05). Similar results were obtained by FA and OCA treatment. FA, and OCA were caused a 26.4% and 26.8% decrease in HDAC1 protein level, respectively. They were caused a decrease in DNMT3B protein level by 21.9% and 70.9%, respectively. On the contrary, after CA treatment, DNMT3B protein expression was increased 76% in HepG2 cells (P<0.05). Moreover, HDAC1 protein expression was increased 9% after CA treatment, but it is not found statistically significant.

Drugs currently targeting critical epigenetic enzymes like HDACs and DNMTs are available. However, these synthetic drugs have disadvantages due to their side effects and drug resistance (Galicia-Moreno et al., 2021; Mondal et al., 2022). Therefore, it is crucial to exploring plant-based natural agents, capable of regulating various intracellular activities, including epigenetic mechanisms. These compounds have a potential to reverse epigenetic patterns associated with malignancy (Thakur et al., 2014; Shukla et al., 2014).

Moreover, plant-based natural agents taken by diet have got great interests in the field of epigenetics. A number of bioactive dietary components have been identified with potential in treating and preventing diseases (Yao et al., 2011; Busch et al., 2015; Rodríguez-García et al., 2019). In fact, various phytochemicals from natural sources have been shown to exhibit anticarcinogenic activities and may play a role in regulating cellular processes (Busch et al., 2015; Selvakumar et al., 2020; Izzo et al., 2020). Numerous studies have shown that these natural products can act as anti-cancer agents and have epigenetic targets in cancer cells. Compounds found cruciferous vegetables, grapes, teas, and mushrooms are now accepted to health-promoting role against the development of different types of tumors by acting as epigenetic modulators (Ferraz de Costa et al., 2020; Abe et al., 2021; Agagunduz et al., 2022).

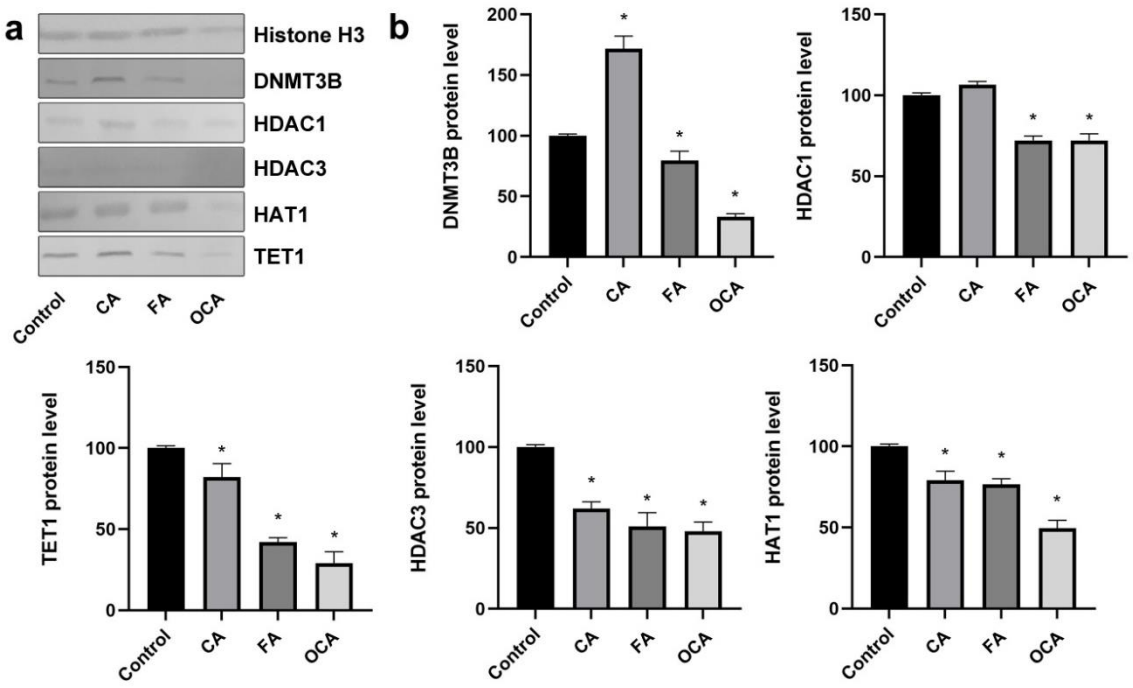

Figure 4. a. Western blot analysis of protein levels in HepG2 cells. Histone H3 was used as a loading control to normalize the data. **b.** Protein levels were analyzed with Scion Image 4.0. *P < 0.05

The chemical constituents of vegetables and edible fruits are particularly beneficial, and several reports have been published, indicating that phytochemicals may reactivate genes silenced due to abnormal methylation. In this context, it has been demonstrated that common phenolic compounds like curcumin and epigallocatechin gallate (EGCG) inhibits the DNMTs and the restore the expression of specific genes silenced in cancer cells. It was demonstrated that curcumin inhibits the methylation of the promoter region of RARβ in lung cancer cell lines (Jiang et al., 2015). It was reported that EGCG inhibits DNMT activities and DNA methylation in head and neck squamous cell carcinoma cells (Agarwal et al., 2023). In another study, it was showed that EGCG reactivated the silenced p16INK4a and Cip1/p21 genes by downregulation of DNMT activity in human skin cancer cells (Nandakumar et. al, 2011).

Western-blot, RT-qPCR and DNMT activity results obtained in this study showed that CA, FA, and OCA can modulate DNMT3B, HDAC1, TET1, HDAC3, and HAT1. As discussed previously, there were limited number of previous studies that have reported similar findings with our results (Wang et al., 2013; Du et al., 2017; Shin et al., 2019; Wang et al., 2020; Kumar et al., 2023).

Caffeic acid (3,4-dihydroxy-cinnamic acid) is an antioxidant and apoptotic polyphenol. It is nearly fully absorbed in the small intestine and is also non-toxic, even at daily doses ranging from 0.5–1 g (Olthof et al., 2001). Wang et al., (2020) reported that caffeic acid phenethyl ester (CAPE) inhibits DNA methylation *in vivo* and *in vitro*. It was reported CAPE can attenuate the HAT activity in human dermal fibroblasts (Shin et al., 2019). Additionally, computational analyses showed that CAPE binds to substrate binding site of human DNMT3A and DNMT1 (Wang et al., 2020; Kumar et al., 2023).

Ferulic acid is a common phenolic compound that has been frequently investigated. Many studies have reported the pharmacological activities of FA in addressing various diseases, such as cancers, neurodegeneration, diabetes, and cardiovascular diseases (Balasubashini et al., 2004; Sultana 2012; Zhang et al., 2016). Du et al., explored the impact of FA on the epigenetic changes in stem cells derived from human bone marrow. In this study, the treatment of FA to mesenchymal stem cells

resulted in an increased expression of β-catenin, a pivotal transcription signaling pathway crucial for stem cell differentiation (Du et al., 2017). It was reported that FA derivatives inhibits the HDAC activity *in silico* (Wang et al., 2013).

O-coumaric acid (2-hydroxycinnamic acid) is a hydroxy derivative of cinnamic acid. The presence of hydroxyl groups on the phenolic ring has been demonstrated to contribute to its antioxidant activity (Velkov et al., 2007; Hsu et al., 2009). Additionally, it exhibits antiproliferative, proapoptotic, and antitumor properties. (Sen et al., 2015; Gutiérrez Mercado et al., 2022).

Epigenetic mechanisms play a significant role in carcinogenesis. Among these mechanisms, DNA methylation stands out as a key process that influences gene expression by modifying the accessibility of transcription factors to chromatin, potentially contributing to cancer development (Song et al., 2011). DNA methylation predominantly occurs in the promoter CpG islands of the genome, facilitated by various enzymes like DNMT1 (Gailhouste et al., 2018). In HepG2 cells, there is an observed upregulation in the expression and activity of DNMTs (Wahid et al., 2017, Gailhouste et al., 2018). Ferulic acid in peanut testae can inhibit HDAC activity in colon cancer cell lines (Saenglee et al., 2016). Our results showed that treatment with CA, FA, and OCA reduced the DNMT activity and the mRNA levels of mRNA levels of DNMT1, DNMT3A, DNMT3B, TET1, HAT1, HDAC1 and HDAC3 in HepG2 cells. In addition, these phenolic acids reduced the protein expression of TET1, HDAC3, and HAT1. In accordance with our results, induced an increase in miR-124 levels by promoting DNA-demethylation, which consequently suppressed NF-κB/p65, STAT-3, and IL-6 in HepG2 cells (Wang et. al, 2015). It was also reported that CA has a strong inhibitory effect on HDAC enzymes in MCF-7 and MDA-231 breast cancer cell lines (Omene et al., 2013).

CONCLUSION

In conclusion, treatment with CA, FA, and OCA impacts in DNA methyltransferase activity and affects expression of different epigenetic enzymes like a DNMT3B, HDAC1, TET1, HDAC3, and HAT1 in HepG2 cells for the first time. These results showed that CA, FA, and OCA can reactivate the expression of specific genes silenced in HepG2 hepatocellular carcinoma cells. Therefore they may have great potential as an effective liver cancer treatment. However, further studies including animal model and clinical trials are needed to validate the efficacy in the treatment and prevention of liver cancer.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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