Determination of Anti-genotoxic Properties of Baicalin by Comet Assay

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Abstract

It has been reported that baicalin (7-glucuronic acid,5,6-dihydroxyflavone; molecular weight= 446.36) has antioxidant, antitoxin and anti-inflammatory properties, antitumor, antiviral, hepatoprotective, antibacterial, anti-inflammatory and antidiarrheal effects. The purpose of the present study is determined the anti-genotoxic and antioxidative effects of baicalin against carbon tetrachloride in the human lymphocytes cell. For the study, the anti-genotoxic potential of baicalin was investigated by comet assay. In addition, biochemical analysis including superoxide dismutase, catalase activities and malondialdehyde level, were performed. According to the obtained results; when the concentrations were evaluated in terms of reducing the level of DNA damage, it was observed that baicalin gave the best results in the applications of 100 µM concentration. In conclusion, baicalin was determined to exhibit anti-genotoxic activity by virtue of its antioxidant potential, which is the genotoxic effect caused by carbon tetrachloride. Baicalin, an important compound for clinical applications and known to have a short half-life; more successful results can be obtained in treatment with high concentrations and repeated applications.

Keywords: Baicalin, Comet assay, Genotoxicity, Carbon tetrachloride.

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1. Introduction

Scutellaria baicalensis, a plant that generally grows in China, is widely used in the treatment of cardiovascular, inflammatory diseases, bacterial-borne diseases, and cancer (Khanal et al. 2012; Lin et al. 2011; Pan et al. 2012; Shiau et al. 2014). The root of this plant is very rich in flavonoids and more than 50 different compounds have been identified (Takahashi et al. 2011). Baicalin (BA), one of these flavonoids and isolated from dried plant root, has been used for centuries as a traditional Chinese herbal medicine to treat allergic and inflammatory ailments (Tarragó et al. 2008).

Hwang et al. (2005) showed that BA inhibits tert-butyl hydroperoxide (t-BHP) induced cytotoxicity and lipid peroxidation in the mouse liver system. In the same study, it was reported that BA eliminates the glutathione (GSH) deficiency created by t-BHP and repairs DNA synthesis (Hwang et al. 2005). In addition, in a study on mice, BA was shown to protect hepatocytes from oxidative damage caused by carbon tetrachloride (CCL_4) (Park et al. 2008). BA showed strong antioxidant, anti-inflammatory and anticancer properties (Srinivas 2010). In addition, BA has been determined to increase anti-inflammatory activity in different *in vivo* and *in vitro* culture media such as human mucosal tissue (Zhang et al. 2012), old mouse kidneys (Lim et al. 2012), and cigarette smoke induced inflammatory models (Li et al. 2012). Researchers have reported that BA can reduce fever in mice and remove free oxygen radicals with its antioxidant properties (Guo et al. 2015). In the literature review conducted on the anti-genotoxic and antimutagenic properties of BA in human lymphocyte cells, it was determined that there are very few studies, and the existing studies were carried out on *Drosophila melanogaster* using prokaryotic cells and plant total extract. In this context, this study aimed to determine the anti-genotoxic and anti-oxidative properties of BA in human lymphocyte cells.

2. Materials and Methods

2.1 Procurement of Baicalin

BA (≥99.0%) purchased from Sigma - Aldrich. Stock solutions were prepared with distilled water.

2.2 Single-Cell Gel Electrophoresis Assay (Comet)

Comet test is widely used to measure DNA damage and repair in many areas such as ecotoxicology, biomonitoring studies, clinical studies, radiation biology, nutritional studies, and cancer studies (Gyori et al. 2014). Comet test is based on the principle that damaged DNA is released from the nucleus by electrophoresis (Kurtulmuş and Aydın 2007).

For the Comet test system, Singh et al. (1988) protocol has been applied. Peripheral blood from healthy men and women aged 23-25, that did not smoke and drink alcohol, have non-infectious diseases, and were not exposed to any physical agent such as X-ray was used.

Lymphocytes were extracted from whole blood using the ficoll method and gradient centrifugation (Dhawan et al. 2003). First, 1 ml of peripheral blood was diluted with the same amount of buffer solution [phosphate buffered saline (PBS)] and loaded into ficoll (Histopaque). This solution was centrifuged at 2600 rpm for 20 min. The uppermost serum layer was removed. Then the buffy coat was removed and placed in the falcon tube. After mixing with 5 mL of PBS, it was centrifuged at 1400 rpm for 10 min. Afterwards, the pellet was resuspended in 1 ml of RPMI 1640 medium and counted over a Hemocytometer.

Approximately $2x10^4$ lymphocytes per 100 µL of medium were placed in eppendorf tubes with 1 mL medium (without FBS). Different concentrations of BA (25, 50, 100 and 200 µM) were added to eppendorf tubes with CCL_4. The eppendorf was inverted to mix the cells and test material. Eppendorfs tubes were cultured for 3 h at 37 °C. After the incubation, lymphocytes were centrifuged at 3000 rpm for 5 min and their supernatants were discarded. The pellet was suspended in 100 µL PBS. Then 100 µL of low melting point agarose (LMPA) solution was added to the pellet (suspended in 100 µL PBS). The agar was mixed with lymphocytes by pipetting immediately before it solidified. Subsequently, 75 µL of the mixture was spread on a slide pre-coated with thin a layer of 1% normal melting point agarose (NMA), and immediately covered with a cover slip, and maintained for 5 min on 4 ◦C to solidify. After removing the cover slips the slides were submersed in freshly prepared cold (4 ◦C) lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% dimethyl sulfoxide and 1% Triton X, pH 10.0 adjusted with NaOH) for at least 2h. Slides were then placed in alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA-Na2, pH >13.0) prepared at 4 ° C for unwinding of the DNA (25 min) and then electrophoresis was applied (24 V / 300 mA, 30 min). All stages were performed under the least illumination possible to prevent DNA damage. After electrophoresis, the slides were removed from the buffer. Slides were covered with neutralization buffer (0.4 M Tris - HCl, pH 7.50) and left for 5 min. The neutralization process was repeated twice. The dried microscope slides were stained with 2 µg/mL ethidium bromide (5 min). Slides were visually evaluated under a fluorescence microscope (Leica® DM5500B) at x400 magnification without waiting. DNA damage in cells was divided into five groups according to the degree of DNA released from the nucleus: (a) no damage, <5%; (b) low level damage, 5–20%; (c) medium level damage, 20–40%; (d) high level damage, 40–95%; (e) total damage, >95% (Anderson et al. 1994). Cells evaluated in groups a, b, c, d, and e were calculated as 0, 1, 2, 3 and 4 points, respectively. The total comet score was calculated by counting a total of 100 cells (n = 4). As a result, the total score ranges from 0 to 400 arbitrary units (AU), depending on the level of DNA damage, as reported by Collins (1995).

2.3 Oxidative Stress Parameters

Catalase activity was determined according to the method of Aebi (1984). Superoxide dismutase (SOD) activity and malondialdehyde (MDA) level were measured as indicated in our previous studies (Ceker et al. 2015).

2.4 Statistical Analysis

All experiments were performed in three replicates and data was compared for reproducibility.

For these procedures, SPSS 18.0 version for Windows was used. The results are expressed as the mean ± standard error (SE), and one-way analysis of variance (ANOVA) test was used for statistical comparison between groups. Significance was determined by Duncan's test. The level of significance was regarded $p<0.05$ for all statistical analysis.

3. Results

CCL_4 was used as a genotoxic agent in the study. DNA damage levels and oxidative stress parameter results determined by the treatment of different concentrations of BA (25, 50, 100 and 200 µM) against CCL_4 were presented in Table 1.

According to these results, lymphocyte DNA damage parameters were higher in the group exposed only to CCL_4 compared to the control group, and DNA damage levels decreased with the addition of BA. However, in the application of 200 µM concentration, the level of DNA damage increased again compared to 100 µM concentration. When evaluating between concentrations in terms of reducing the level of DNA damage caused by CCL_4, it was observed that BA gave the best result in applications at a concentration of 100 µM ($p <0.05$) (Table 1).

On the other hand, by adding CCL_4 as a genotoxic substance to culture media; It was observed that CAT and SOD activities decreased, while the level of MDA increased. By treating different concentrations of BA (25, 50, 100 and 200 µM) together with CCL_4; It was determined that CAT and SOD enzymes increased their activities significantly, and the level of MDA decreased. Different concentrations of BA used with CCL_4; It has been determined that its effect on CAT, SOD and MDA were significant ($p <0.05$) (Table 1).

Table 1. The effects of CCL_4 and BA on Comet and enzymes activities (SOD, CAT and MDA)

Test Items	Concentrations	Visual Score (AU) ± S. E	CAT ± S. E (k/g protein)	SOD ± S. E (U/mL)	MDA ± S. E (n mol/mL)
Control		7.45 ± 0.38^{a}	1207.1 ± 27.2^{a}	1.34 ± 0.07^{a}	0.84 ± 0.21^{a}
CCL_4	5 µM	122.10 ± 4.52^{e}	986.3 ± 34.7^{d}	0.72 ± 0.02^{d}	1.20 ± 0.08^{d}
BA	50 µM	9.21 ± 0.72^{a}	1240.8 ± 26.9^{a}	1.38 ± 0.03^{a}	0.80 ± 0.07^{a}
CCL_4 + BA	5 + 25 µM	86.17 ± 3.92^{d}	1178.3 ± 22.3^{b}	1.22 ± 0.04^{c}	1.10 ± 0.12^{cd}
CCL_4 + BA	5 + 50 µM	74.43 ± 1.98^{cd}	1184.6 ± 32.7^{ab}	1.25 ± 0.02^{b}	0.92 ± 0.21c
CCL_4 + BA	5 + 100 µM	38.62 ± 2.10^{ab}	1189.2 ± 1.3^{ab}	1.29 ± 0.03^{ab}	0.86 ± 0.02^{ab}
CCL_4 + BA	5 + 200 µM	43.19 ± 1.45^{b}	1175.2 ± 1.6^{c}	1.36 ± 0.01^{a}	0.89 ± 0.08^{a}

[a-e] Means ± SE; values within each column not sharing a common superscript are significantly different ($p<0.05$) as determined by Duncan test.

4. Discussion

Phenolic compounds derived from plants can protect cellular components against oxidative damage due to their antioxidant properties, thereby reducing the risk of many degenerative diseases associated with oxidative stress. However, it has been reported that the chemical properties and activities of phenolic compounds commonly found in plants may have both antioxidant and pro-oxidant effects (Hadi et al. 2007). In this context, in this study in which we investigated the anti-genotoxic and anti-oxidative properties of BA, it was observed that the treatment of BA with CCL_4 reduced the levels of DNA damage. In addition, thanks to its anti-oxidative properties, BA increased the CAT and SOD activities and decreased the MDA level by removing the oxidative stress induced by CCL_4 (Table 1).

Liu et al. (2007) BA's; reported that it scavenges free radicals, prevents the formation of reactive oxygen species, and exhibits cytotoxic properties in inflammatory liver diseases. It has been reported that BA can scavenge free oxygen species by preventing platelet accumulation and, moreover, it can reduce endotoxin production *in vivo* studies (Zhang et al. 2007). Against the liver toxicity induced by CCL_4 administration, the concentration of 100 mg / kg provided the most effective reduction on serum alanine aminotransferase and aspartate aminotransferase activity levels in the groups given BA 30 minutes before and 120 minutes after CCL_4 administration at 25, 50, 100 and 200 mg / kg concentrations, it has been observed that it decreases the elevated MDA level and increases the GSH level (Park et al. 2008). Researchers have reported that BA protects hepatocytes against oxidative damage caused by CCL_4 and BA protects oxidants from brain and ischemia / reperfusion damage with its cleansing effect (Kim et al. 2010a). Kim et al. (2010b) reported that BA causes an increase in GSH level and a decrease in MDA level. In a study model done by giving high amounts of iron to mice, BA; It has been determined that it increases catalase activity, decreases lipid peroxidation levels and protein oxidation (Zhang et al. 2011). These data are in line with the results we have obtained from biochemical analyzes (Table 1).

In a study investigating the anti-genotoxic effect of *Scutellaria lindbergii Rech. f.* extract containing large amounts of BA, it was determined that the plant extract prevented DNA damage induced by H_2O_2, and this was explained by the phenolic compounds with antioxidant properties contained in the plant extract (Ehtesham-Gharaee et al.2015). Ding et al. (2015) attributed the antioxidant effect of BA to its ability to activate Nrf2, which regulates gene induction and is known to be an important transcription factor.

The limited number of studies conducted with the anti-genotoxic property of BA support our results. Hwang et al. (2005) determined that BA concentrations of 2, 22 and 220 M do not have a cytotoxic effect on mouse hepatocytes but reduce the lactate dehydrogenase level. Zhang et al. (2007) because of his study in mouse kidneys; It has been reported that because of the short half-life of BA, more successful results can be obtained in treatment with high concentrations and repeated applications of BA. In another study, it was reported that BA, which does not show toxic and carcinogen properties, is an important compound in clinical applications (Wang et al. 2015).

The results obtained coincide with the findings of different studies on the anti-genotoxic property of BA (Hwang et al. 2005; Zhang et al., 2007) and anti-oxidative properties (Kim et al. 2010b; Zhou et al. 2011). The protective effect of BA is due to its feature on DNA repair mechanism (Zhou et al. 2011); Its anti-oxidative effect is due to its ability to activate Nrf2, which regulates gene induction and is known to be an important transcription factor (Ding et al. 2015). In this context, BA, which is an important compound candidate for clinical applications with its non-toxic feature; it can be used as an alternative treatment for many diseases; It is thought that successful results can be obtained in the treatment of many diseases, especially cancer, by using the optimum concentration and time.

References

AEBI, H. 1984. [13] Catalase in vitro. *In Methods in enzymology*, 105:121-126.

ANDERSON, D., YU, T.W., PHILLIPS, B., SCHMEZER, P. (1994). The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay, Mutation

Research/Fundamental and Molecular *Mechanisms of Mutagenesis*, 307:261-271.

CEKER, S., ORHAN, F., KIZIL, H. E., ALPSOY, L., GULLUCE, M., ASLAN, A., AGAR, G. 2015. Genotoxic and anti-genotoxic potentials of two Usnea species. *Toxicology and industrial health*, 31(11): 990-999.

COLLINS, A.R. 2004. The comet assay for DNA damage and repair. *Molecular biotechnology*, 26(3):249.

DHAWAN, A., BAJPAYEE, M.M., PANDEY, A.K., PARMAR, D. (2003). Protocol for the single cell gel electrophoresis/comet assay for rapid genotoxicity assessment. *Sigma*, 1077(1).

DING, H., WANG, H., ZHAO, Y., SUN, D., ZHAI, X. 2015. Protective effects of baicalin on Aβ 1–42-induced learning and memory deficit, oxidative stress, and apoptosis in rat. *Cellular and molecular neurobiology*, 35(5):623-632.

EHTESHAM-GHARAEE, M., ESHAGHI, A., SHOJAEE, S., ASILI, J., EMAMI, S. A., BEHRAVAN, J., MOSAFFA, F. 2015. Protective effects of Scutellaria lindbergii root extract against oxidative-induced cell and DNA damage in mouse fibroblast-like cells. *Drug and chemical toxicology*, 38(3):293-299.

GUO, X., CHI, S., CONG, X., LI, H., JIANG, Z., CAO, R., TIAN, W. 2015. Baicalin protects sertoli cells from heat stress-induced apoptosis via activation of the Fas/FasL pathway and Hsp72 expression. *Reproductive Toxicology*, 57:196-203.

GYORI, B.M., VENKATACHALAM, G., THIAGARAJAN, P., HSU, D., CLEMENT, M.V. 2014. OpenComet: an automated tool for comet assay image analysis. *Redox biology*, 2:457-465.

HADI, S., BHAT, S.H., AZMI, A.S., HANIF, S., SHAMIM, U., ULLAH, M. 2007. *Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. Seminars in cancer biology*, 17(5): 370-376.

HWANG, J.M., WANG, C.J., CHOU, F.P., TSENG, T.H., HSIEH, Y.S., HSU, J.D., CHU, C.Y. 2005. Protective effect of baicalin on tert-butyl hydroperoxide-induced rat hepatotoxicity. *Archives of toxicology*, 79(2):102-109.

KHANAL, T., KIM, H.G., CHOI, J.H., PARK, B.H., DO, M.T., KANG, M.J., JEONG, T.C. 2012. Protective role of intestinal bacterial metabolism against baicalin-induced toxicity in HepG2 cell cultures. *The Journal of toxicological sciences*, 37(2): 363-371.

KIM, T.J., SILVA, J.L., KIM, M.K., JUNG, Y.S. 2010a. Enhanced antioxidant capacity and antimicrobial activity of tannic acid by thermal processing. *Food Chemistry*, 118:740-764.

KIM, S.J., MOON, Y.J., LEE, S.M. 2010b. Protective effects of baicalin against ischemia/reperfusion injury in rat liver. *Journal of natural products*, 73(12): 2003-2008.

KURTULMUŞ, S., AYDIN, K. 2007. Dental döküm alaşımlarının genotoksisite, mutajenisite ve karsinojenisitesi. *Selçuk Üniversitesi Dişhekimliği Fakültesi Dergisi, 16*:73-78.

LI, L., BAO, H., WU, J., DUAN, X., LIU, B., SUN, J., LUO, Q. 2012. Baicalin is anti-inflammatory in cigarette smoke-induced inflammatory models in vivo and in vitro: A possible role for HDAC2 activity. *International immunopharmacology*, 13(1):15-22.

LIM, H. A., LEE, E.K., KIM, J.M., PARK, M.H., KIM, D.H., CHOI, Y.J., YU, B.P. 2012. PPARγ activation by baicalin suppresses NF-κB-mediated inflammation in aged rat kidney. *Biogerontology*, 13(2):133-145.

LIN, Y., VERMEER, M.A., BOS, W., VAN BUREN, L., SCHUURBIERS, E., MIRET-CATALAN, S., TRAUTWEIN, E.A. 2011. Molecular structures of citrus flavonoids determine their effects on lipid metabolism in HepG2 cells by primarily suppressing apoB secretion. *Journal of agricultural and food chemistry*, 59(9):4496-4503.

LIU, L.L., GONG, L.K., WANG, H., XIAO, Y., WU, X. F., ZHANG, Y.H., REN, J. 2007. Baicalin protects mouse from Concanavalin A-induced liver injury through inhibition of cytokine production and hepatocyte apoptosis. *Liver International*, 27(4): 582-591.

PAN, T.L., WANG, P.W., LEU, Y.L., WU, T.H., WU, T.S. 2012. Inhibitory effects of Scutellaria baicalensis extract on hepatic stellate cells through inducing G2/M cell cycle arrest and activating ERK-dependent apoptosis via Bax and caspase pathway. *Journal of ethnopharmacology*, 139(3):829-837.

PARK, S.W., LEE, C.H., KIM, Y.S., KANG, S.S., JEON, S.J., SON, K.H., LEE, S.M. 2008. Protective effect of baicalin against carbon tetrachloride–induced acute hepatic injury in

mice. *Journal of pharmacological sciences*, 106(1):136-143.

SHIAU, A.L., SHEN, Y.T., HSIEH, J.L., WU, C.L., LEE, C.H. 2014. Scutellaria barbata inhibits angiogenesis through downregulation of HIF-1 α in lung tumor. *Environmental toxicology*, 29(4): 363-370.

SINGH, N.P., MCCOY, M.T., TICE, R.R., SCHNEIDER, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental cell research*, 175(1):184-191.

SRINIVAS, N. 2010. Baicalin, an emerging multi-therapeutic agent: pharmacodynamics, pharmacokinetics, and considerations from drug development perspectives. *Xenobiotica*, 40(5):357-367.

TAKAHASHI, H., CHEN, M.C., PHAM, H., ANGST, E., KING, J.C., PARK, J., REBER, H.A. 2011. Baicalein, a component of Scutellaria baicalensis, induces apoptosis by Mcl-1 down-regulation in human pancreatic cancer cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1813(8):1465-1474.

TARRAGÓ, T., KICHIK, N., CLAASEN, B., PRADES, R., TEIXIDÓ, M., GIRALT, E. 2008. Baicalin, a prodrug able to reach the CNS, is a prolyl oligopeptidase inhibitor. *Bioorganic & medicinal chemistry*, *16*(15),:7516-7524.

WANG, K., FENG, C., LI, C., YAO, J., XIE, X., GONG, L. AND REN, J., 2015. Baicalin protects mice from aristolochic acid I-induced kidney injury by induction of CYP1A through the aromatic hydrocarbon receptor. *International journal of molecular sciences*, **16**(7), 16454-16468.

ZHANG, N., VAN CROMBRUGGEN, K., HOLTAPPELS, G., BACHERT, C. 2012. A herbal composition of Scutellaria baicalensis and Eleutherococcus senticosus shows potent anti-inflammatory effects in an ex vivo human mucosal tissue model. *Evidence-based complementary and alternative medicine, 2012*.

ZHANG, Y., GAO, Z., LIU, J., XU, Z. 2011. Protective effects of baicalin and quercetin on an iron-overloaded mouse: comparison of liver, kidney and heart tissues. *Natural product research*, 25(12):1150-1160.

ZHANG, X.P., TIAN, H., LAI, Y.H., CHEN, L., ZHANG, L., CHENG, Q.H., HE, Q. 2007. Protective effects and mechanisms of Baicalin and octreotide on renal injury of rats with severe acute pancreatitis. *World Journal of Gastroenterology: WJG*, 13(38): 5079.

ZHOU, B.R., LIU, W.L., LUO, D., 2011. Protective effect of baicalin against multiple ultraviolet B exposure-mediated injuries in C57BL/6 mouse skin. *Archives of pharmacal research*, 34(2):261-268.