7-Methoxyflavone and 7-Hydroxy-4'-nitroisoflavone confer ameliorative effects to human erythrocytes exposed to oxidative damage

Mucip GENISEL [1,*] and Sedat AYDIN [2]

[1] *Department of Pharmaceutical Botany, Faculty of Pharmacy, Agri Ibrahim Cecen University, 04100 Agri, Turkey*
mgenisel@agri.edu.tr
[2] *Faculty of Pharmacy, Agri Ibrahim Cecen University, 04100 Agri, Turkey*
evrimsedo@gmail.com

Abstract

Human erythrocytes are in constant motion within the blood, therefore, they contact inevitably with factors such as different toxic substances, drugs, reactive oxygen species. This situation may lead to a decline in the life span of erythrocytes by disrupting their metabolism. In the present study, the effect of 7-Methoxyflavone (MP) and 7-Hydroxy-4'-nitroisoflavone (HNF) on erythrocytes exposed to oxidative damage with hydrogen peroxide (H_2O_2) application has been investigated. The possible mitigating effect on the oxidative damage of these flavonoid derives have been tried to be revealed with biomarkers such as total superoxide dismutase (SOD), manganese SOD (MnSOD) and cupper-zinc SOD (CuZnSOD), catalase (CAT) and lipid peroxidation. H_2O_2 application caused serious decreases in TSOD and CAT enzyme activities as well as the band intensities of MnSOD and CuZnSOD isoenzymes in erythrocytes. In addition, this H_2O_2 increased critically the oxidative products of lipid peroxidation. MP and HNF treatment significantly reduced the level of lipid peroxidation by increasing the antioxidant enzymes activities of erythrocytes in oxidative stress. As a result, it has been revealed that MP and HNF contributes to the attenuation of oxidative damage in human erythrocytes with its own antioxidant effect and/or by promoting antioxidant enzymes.

Keywords: Human erythrocyte; oxidative damage; 7-Hydroxy-4'-nitroisoflavone;7-Methoxyflavone; antioxidants

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Corresponding author: Mucip GENISEL
orcid.org/0000-0002-9339-9334
E-mail: mgenisel@agri.edu.tr
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1. Introduction

Organisms are constantly exposed to reactive oxygen species (ROS) result from exogenous factors such as infectious agents, pesticides, air pollution, stress, smoking and antineoplastic drugs as well as redox reactions in cells (Yokoyama *et.al., 2017*; Boukhenouna *et. al., 2018*). While low amounts of ROS play a role as signal molecules for metabolic events, excessive ROS could lead to disruption in the functions of cell and tissues by causing oxidative damage (Murphy *et. al., 2011*).

Reactive oxygen species are tried to be eliminated by antioxidant defence systems consisting of antioxidant matters and antioxidant enzymes such as superoxide dismutase and catalase (Klaunig and Kamendulis, 2004). However, the antioxidant defence systems might be insufficient to eliminate them when ROS reach high concentrations. In this case, these radicals damage to all cell components such as DNA, proteins and lipids (Marengo *et. al.*, 2016). The cell and organelle membranes are very sensitive to ROS due to their excessive lipid contents. As a result of the reaction of reactive oxygen species with lipids in the membranes, lipid peroxidation event occurs irreversibly in the biologic membranes, this situation could lead to the loss of membrane function by breaking down the membrane integrity (Esterbauer *et. al.*, 1991).

Hydrogen peroxide does not actually show the radical property but it easily diffuses into the cytoplasm and could be the precursor molecule of ROS. It could cause oxidative damage through Fenton and similar reactions and thus is also used as a prooxidant in the in vivo and in vitro experiments aimed at investigating oxidative damage in cells (Sánchez-Gallego *et. al.*, 2010).

The human body consists of many special tissues and cells. Erythrocytes consistently carry oxygen to these tissues and cells for the continuation

in a healthy way of their special functions (Mohanty *et. al.*, 2014). However, erythrocytes have more contact than other cells with oxidant agents due to their constantly mobile in the blood tissue and this situation make them more vulnerable to oxidative damage (Ross *et al.*, 1999). They could be haemolysis due to oxidative damage in case of the inability of their antioxidant defence systems for elimination of these oxidant agents. This situation might lead to the various diseases resulting from anaemia manifesting itself with insufficient oxygen symptoms in all tissues. In recent studies, it has been emphasized that exogenous or dietary antioxidants should be taken to prevent or reduce the oxidative damage in erythrocytes. (Sánchez-Gallego *et. al.*, 2010; Tulipani *et. al.*, 2011).

Flavonoids are plant secondary metabolites that have the ability to scavenge reactive oxygen species. Recently, interest in flavonoids and their derivatives has increased significantly due to their protective effects against oxidative damage and they have been used to as antioxidant in many studies (Sánchez-Gallego *et. al.*, 2010; Rodríguez-García *et. al.*, 2019). Gopinath et al. (2011) reported that flavonoid derivatives exhibited neuroprotective effects. It has also been demonstrated that some flavonoid derives have cardioprotective and hepatoprotective effects (Luo *et. al.*,2017; Testai *et. al.*, 2015; Kim *et. al.*, 2017). Moreover, they could play a role as anti-inflammatory and immunomodulatory (García-Lafuente *et. al.*, 2009; Mesaik *et. al.*, 2009). In addition, flavonoid derivate luteolin and orientin were used to eliminate oxidative damage in erythrocytes (An *et. al.*, 2016).

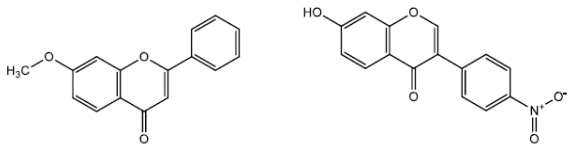


Figure 1. Chemical structure of 7-Methoxyflavone **(a)** and 7-Hydroxy-4'-nitroisoflavone **(b)**

However, to our knowledge, there is not any study explaining the effects on oxidative damage of flavonoid derivate 7-Methoxyflavone and 7-Hydroxy-4'-nitroisoflavone Figure 1. In this study, it has been tried to be revealed with biomarkers such as antioxidant enzyme activities, their isoenzymes, and lipid peroxidation whether these flavonoid derives are effective on alleviation of the oxidative damage in erythrocytes.

2. Material and methods

2.1 Preparation of erythrocyte samples and oxidative damage model

For this study, erythrocyte samples of healthy, non-smokers aged between 20-30 years were procured from the Agri State Hospital Blood Center. To separate the plasma, the samples were centrifuged at 4500 rpm for 10 minutes and then erythrocytes obtained were gently washed with physiological saline three times. RPMI 1640 medium, developed by Roswell Park Memorial Institute to be used as a medium for eukaryotic cells, was added to the erythrocytes at ten times the volume of the cells (Moore *et. al.*, 1967). This study was duly approved by Scientific Research Ethics Committee of Agri Ibrahim Cecen University. The H_2O_2 concentration to be used in the study was determined by preliminary studies and it was decided to be 100 µM by measuring the lipid peroxidation levels one day after the H_2O_2 applications. All chemicals except for MP and HNF were obtained from Sigma Aldrich. MP and HNF were purchased from Fluorochem UK. To dissolve MP and HNF, it was used to dimethyl sulfoxide (DMSO). This study was conducted with control, H_2O_2, MP and HNF (10, 50, and 100 µM) groups, respectively. The experiment groups were incubated at 37 ° C for 24 hours. Afterwards, it was determined antioxidant enzyme activities and oxidative damage levels with these samples.

2.2 Determination of antioxidant enzyme activities and the superoxide dismutase isoenzymes

The total SOD activity was determined with the method based on the measurement at 560 nm of the amount of blue complex-formazan, which is formed with a reaction of nitro blue tetrazolium chloride and superoxide anions (Beauchamp and Fridovich, 1971). This reaction takes place in a lighted environment. In this reaction, riboflavin is used to produce superoxide anion in presence of methionine which is an electron donor. One-unit of total SOD is expressed as the SOD

amount reducing in the rate of fifty percent the formazan formation.

Catalase activity was measured by the method of (Aebi, 1984). After the erythrocytes were lyzed and suspended in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C for 30 min, the lysates were mixed 30 mM hydrogen peroxide. The alteration of hydrogen peroxide was measured at 240 nm for 3 min and the enzyme activity was determined with an extinction coefficient of 0.0436 mmole−1 cm−1 for H_2O_2.

To detected the isoenzymes of SOD, it was used 10 % polyacrylamide gel electrophoresis (Native-PAGE) defined by Laemmli (1970) method. SOD isoenzymes were detected at 120 V by the gel staining method described by Beuchamp and Fridovich (1971) Isoenzymes band intensities were measured by comparing to control values in Gelanalyzer program.

2.3 Determination of lipid peroxidation

The method described by Chang *et al.* (2013) was used to measure the lipid peroxidation levels. After 24-hour from treatments, the cells were lysed and centrifuged at 3500 g for 5 minutes. HCl solution containing TBA and TCA was mixed with the supernatant and then the mixture was incubated in boiling water for 30 minutes. After incubation, the reaction was stopped by transferring the mixture into an ice bath. The mixture was centrifuged again for 5 minutes at 5000 g. Afterwards, the absorbance of the supernatant was measured at 532 nm. The amount of MDA, a lipid peroxidation product, was calculated and then it was expressed as μM. g^{-1} haemoglobin.

2.4 Statistical analysis

In this study, the results are the average of the values obtained from 3 repetitions of samples. To comparison of the results at $p<0.05$ significance level, one-way analysis of variance (ANOVA) and Duncan's Multiple Comparison Test in SPSS 20 package program were used.

3. Results

As seen from the Figure 2, H_2O_2 reduced seriously total SOD activity compared to the control. MP and HNF supplementations reverted to important degrees the H_2O_2-based reduction in total SOD activity.

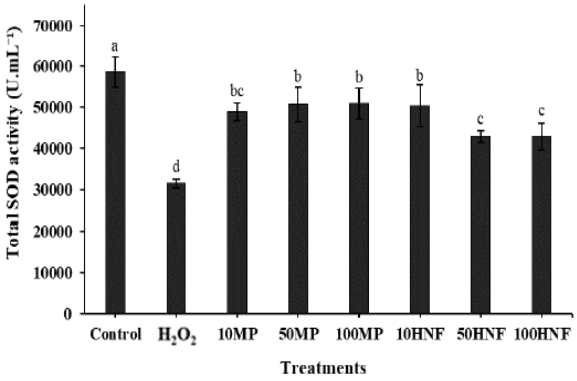
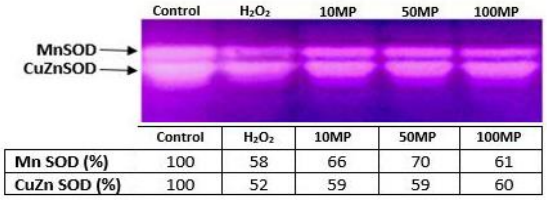
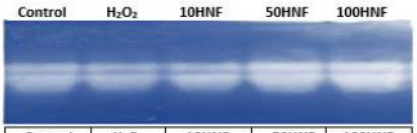


Figure 2. Effects of H_2O_2, MP and HNF (10, 50 and 100 μM) treatments on superoxide dismutase activities in erythrocytes. Significant differences are indicated by different letters ($p< 0.05$).

Figure 3 clearly shows the change in the isoenzymes of SOD in NATİVE Gel. The band densities of SOD isoenzymes were also determined with a Gel Analyzer program and expressed with numerical values under Figure 3. To calculate the density of bands obtained from treatments, the control values of Mn SOD and CuZn SOD band densities was accepted as 100 %. H_2O_2 declined critically the band intensities of Mn SOD and CuZn SOD isoenzymes. However, MP and HNF treatments significantly increased the band intensities of these isoenzymes as compared to alone H_2O_2 groups.



	Control	H_2O_2	10MP	50MP	100MP
Mn SOD (%)	100	58	66	70	61
CuZn SOD (%)	100	52	59	59	60



	Control	H_2O_2	10HNF	50HNF	100HNF
Mn SOD (%)	100	63	76	106	112
CuZn SOD (%)	100	72	86	95	109

Figure 3. Effects of H_2O_2, MP and HNF (10, 50 and 100 μM) treatments on relative band intensity of Mn SOD and CuZn SOD isoenzymes in human erythrocytes.

Figure 4 clearly shows that under oxidative stress, catalase activity exhibited an important decline. MP and HNF supplementations significantly reversed the H_2O_2-based inhibition in catalase activities ($p<0.05$).

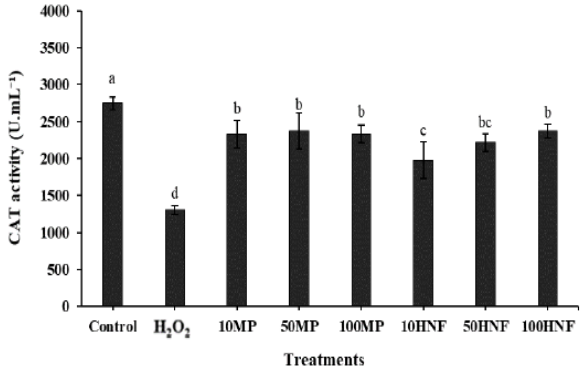


Figure 4. Effects of H_2O_2, MP and HNF (10, 50 and 100 μM) treatments on catalase activity in erythrocytes. Significant differences are indicated by different letters ($p< 0.05$).

Compared to the control, the MDA content was sharply increased under oxidative stress. However, MP and HNF treatments was reduced MDA contents under the stressed conditions (Figure 5).

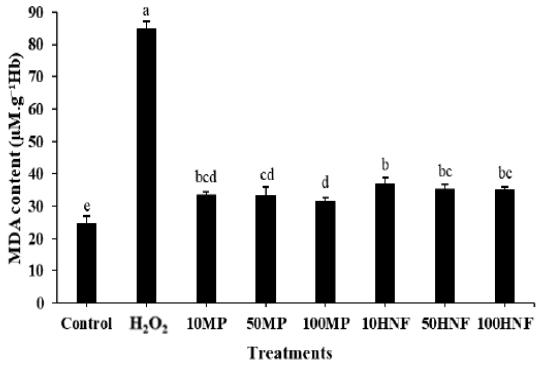


Figure 5. Effects of H_2O_2, MP and HNF (10, 50 and 100 μM) treatments on on lipid peroxidation level in erythrocytes. Significant differences are indicated by different letters ($p< 0.05$).

4. Discussion

Erythrocytes with a very short life span are the most number cells compared to other cells in the human body. The fact that erythrocytes are the only factor carrying oxygen to all tissues exert that they are very important for the continuation of metabolic functions and survival of cells, tissues, organs. However, their life span could shorten even more due to their constant movement and exposure to oxidants. Erythrocyte deficiency in blood tissue manifests as anaemia, and other all tissues and cells are seriously affected by oxygen deficiency (Libregts *et. al*., 2011).

Many factors such as smoking, stress, antineoplastic drugs could cause various damages on erythrocytes by disrupting their metabolism and structural integrity. This could lead to a decline in their oxygen-carrying capacity and end their lives. The most important factors causing oxidative damage in erythrocytes are endogenous or exogenous origin reactive oxygen species (Gonzales *et. al.*, 1984). Hydrogen peroxide is an oxidant agent commonly used for the design of in vivo and in vitro experiments. Actually, hydrogen peroxide is a non-radical molecule but it could easily pass from biological membranes and become the precursor of reactive oxygen molecules through Fenton reactions (Sánchez-Gallego *et. al.*, 2010).

The reactive oxygen species are tried to be eliminated by the defence system consisting of antioxidant enzymes and antioxidant matters in cells. In case of insufficient of the antioxidant defence system, these radicals show destructive effects by reacting with cell components such as DNA, protein, and lipid molecules (Marengo *et. al.*, 2016). It is also well known that flavonoid derivatives consisting of biologically active polyphenolic compounds provide support to the antioxidant systems in organisms (Nabavi *et. al.*, 2020). Superoxide dismutase is the first enzyme of the antioxidant defence process, and converts the superoxide radical to hydrogen peroxide and molecular oxygen. In the present experiment, the SOD activity in erythrocytes exposed to H_2O_2 showed a serious decline as consistent with the results of a previous study (An *et. al.*, 2016). On the other hand, MP and HNF applied together H_2O_2 significantly increased the SOD activity. There are studies reporting that different flavonoid applications increase SOD activity in erythrocytes exposed to oxidative damage however, the effects of MP and HNF application on SOD enzyme activity was demonstrated for the first time in the present study. Also, the changes in the SOD isoenzymes of the erythrocytes under oxidative stress were detected and

demonstrated electrophoretically for the first time in this study. H_2O_2 application caused serious decreases in the band intensities of MnSOD, CuSOD and ZnSOD isoenzymes. As seen in the Figure, while MnSOD (88 kDa) appears as a separate band in native electrophoresis, CuSOD and ZnSOD seem as a single band (CuZnSOD) together due to their same molecular weight (36 kDa) (Weydert *et. al.*, 2010). It can be clearly seen from the Figure that MP and HNF applications significantly increased the band densities of SOD isoenzymes compared to alone H_2O_2 application. This might be due to the stimulant effect of this flavonoid derivative on SOD enzymes and isoenzymes. The catalase is capable of converting the hydrogen peroxides formed by SOD or exogenous source into water and oxygen. H_2O_2 applied to the erythrocytes significantly reduced catalase activity as compatible with results by reported (An *et. al.*, 2016). MP and HNF applications caused a significant increase in catalase activity compared to only H_2O_2 application. it is highly probable that the increase in both SOD and CAT activities result from the stimulant effect on these antioxidant enzymes of MP and HNF. In addition, MP and HNF might have contributed to the stabilization of these enzymes by scavenging the reactive oxygen species with their own antioxidant effects.

ROS cause lipid peroxidation event by reacting with the biologic membrane lipids when the power of antioxidant defence systems is insufficient to scavenge radical oxygen derivatives. Lipid peroxidation continues in a chain by causing irreversible damage to membranes. Malondialdehyde formed as a product of lipid peroxidation is accepted as a good indicator in determining the level of lipid peroxidation. Lipid content of the human erythrocyte membranes is much higher than other cells and thus, they are more susceptible to the destructive effect of ROS. In the present work, H_2O_2 applied to the human erythrocytes caused a serious increase in lipid peroxidation level. It is already known that H_2O_2 damages through lipid peroxidation in many cells including erythrocytes (An *et. al.*, 2016; Hong and Liu, 2004; Ajila and Rao, 2008). This devastation effect of H_2O_2 on membranes of the human erythrocytes can be explained with its reactive precursor property although it is non-radical. On the other hand, MP and HNF treatments significantly reduced the lipid peroxidation triggered by H_2O_2. It is very likely that MP and HNF achieved this by removing the H_2O_2 directly and/or by providing a stimulant effect in antioxidant enzyme activities.

5. Conclusion

Flavonoid derivates MP and HNF significantly reduced the H_2O_2-induced oxidative damage in human erythrocytes by scavenging reactive oxygen species and/or by stimulating antioxidant defence system. It is recommended to investigate the drug active substance potentials of MP and HNF with in vivo studies.

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References

AEBI H. Catalase in vitro in method of enzymology. (1984). Meth Enzymol., 121-126.

AJILA C, RAO UP. (2008). Protection against hydrogen peroxide induced oxidative damage in rat erythrocytes by Mangifera indica L. peel extract. Food Chem Toxicol., 46(1):303-309.

AN F, WANG S, YUAN D, GONG Y, WANG S. (2016). Attenuation of oxidative stress of erythrocytes by plant-derived flavonoids, orientin and luteolin. Evid Based Complementary Altern Med., 1:1-8.

BEAUCHAMP C, FRIDOVICH I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem., 44(1):276-287.

BOUKHENOUNA S, WILSON MA, BAHMED K, KOSMIDER B. (2018). Reactive oxygen species in chronic obstructive pulmonary disease. Oxid Med Cel Longev., 2018.

CHANG D, ZHANG X, RONG S, SHA Q, LIU P, HAN T, PAN H. (2013). Serum antioxidative enzymes levels and oxidative stress products in age-related cataract patients. Oxid Med Cel Longev., 1-7.

ESTERBAUER H, SCHAUR RJ, ZOLLNER H. (1991). Chemistry and biochemistry of 4-

hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med., (1):81-128.

GARCÍA-LAFUENTE A, GUILLAMÓN E, VILLARES A, ROSTAGNO MA, MARTÍNEZ JA. (2009). Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflamm Res., 58(9):537-552.

GONZALES R, AUCLAIR C, VOISIN E, GAUTERO H, DHERMY D, BOIVIN, P. (1984). Superoxide dismutase, catalase, and glutathione peroxidase in red blood cells from patients with malignant diseases. Cancer Res., 44:4137-4139.

GOPINATH K, PRAKASH D, SUDHANDIRAN G. (2011). Neuroprotective effect of naringin, a dietary flavonoid against 3-nitropropionic acid-induced neuronal apoptosis. Neurochem Int., 59(7):1066-1073.

HONG H, LIU G-Q. (2004). Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin. Life Sci., 74(24):2959-2973.

KIM SM, KANG K, JHO EH, JUNG YJ, NHO CW, UM BH, PAN CH. (2011). Hepatoprotective effect of flavonoid glycosides from Lespedeza cuneata against oxidative stress induced by tert-butyl hyperoxide. Phytother Res., 25(7):1011-1017.

KLAUNIG JE, KAMENDULIS LM. (2004). The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol., 44:239-267.

LAEMELLI D. (1970). Cleavege of structural ptoteins during in assembly of thehead of bacteriophage. Nature, 227-680.

LIBREGTS SF, GUTIÉRREZ L, DE BRUIN AM, WENSVEEN FM, PAPADOPOULOS P, VAN IJCKEN W, NOLTE MA. (2011). Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU. 1 axis. Am J Hematol., 118(9):2578-2588.

LUO Y, SHANG P, LI D. (2017). Luteolin: A flavonoid that has multiple cardio-protective effects and its molecular mechanisms. Front Pharmacol., 8:692.

MARENGO B, NITTI M, FURFARO AL, COLLA R, CIUCIS CD, MARINARI UM, et al. (2016). Redox homeostasis and cellular antioxidant systems: crucial players in cancer growth and therapy. Oxid Med Cel Longev., 1-16.

MESAIK MA, MURAD S, KHAN KM, TAREEN RB, AHMED A, CHOUDHARY MI. (2009). Isolation and immunomodulatory properties of a flavonoid, casticin from Vitex agnus-castus. Phytother Res., 23(11):1516-1520.

MOHANTY J, NAGABABU E, RIFKIND JM. (2014). Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. Front Physiol., 5 84:1-6.

MOORE GE, GERNER RE, FRANKLIN HA. (1967). Culture of normal human leukocytes. Jama., 199(8):519-524.

MURPHY MP, HOLMGREN A, LARSSON N-G, HALLIWELL B, CHANG CJ, KALYANARAMAN B, et al. (2011). Unraveling the biological roles of reactive oxygen species. Cell Metab., 13(4):361-366.

NABAVI SM, ŠAMEC D, TOMCZYK M, MILELLA L, RUSSO D, HABTEMARIAM S, et al. (2020). Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. Biotechnol Adv., 38:107316.

RODRÍGUEZ-GARCÍA C, SÁNCHEZ-QUESADA C, GAFORIO JJ. (2019). Dietary flavonoids as cancer chemopreventive agents: An updated review of human studies. Antioxidants 8(5):137.

ROSS D, MENDIRATTA S, QU Z-C, COBB CE, MAY JM. (1999). Ascorbate 6-palmitate protects human erythrocytes from oxidative damage. Free Radic Biol Med., 26(1-2):81-89.

SÁNCHEZ-GALLEGO JI, LÓPEZ-REVUELTA A, SARDINA JL, HERNÁNDEZ-HERNÁNDEZ Á, SÁNCHEZ-YAGÜE J, LLANILLO M. (2010). Membrane cholesterol contents modify the protective effects of quercetin and rutin on integrity and cellular viability in oxidized erythrocytes. Free Radic Biol Med., 48(10):1444-1454.

TESTAI L, MARTELLI A, CRISTOFARO M, BRESCHI MC, CALDERONE V. (2013). Cardioprotective effects of different flavonoids against myocardial ischaemia/reperfusion injury in L angendorff-perfused rat hearts. J Pharm Pharmacol., 65(5):750-756.

TULIPANI S, ALVAREZ-SUAREZ JM, BUSCO F, BOMPADRE S, QUILES JL, MEZZETTI B, et al. (2011). Strawberry consumption improves plasma antioxidant status and erythrocyte resistance to oxidative haemolysis in humans. Food Chem., 128(1):180-186.

WEYDERT CJ, CULLEN JJ. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc., 5(1):51-66.

YOKOYAMA C, SUEYOSHI Y, EMA M, MORI Y, TAKAISHI K, HISATOMI H. (2017). Induction of oxidative stress by anticancer drugs in the presence and absence of cells. Oncol Lett., 14(5):6066-6070.