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Cytoprotective activity of *Sambucus ebulus* fruit extracts in conditions of oxidative *tert*-buthyl-hydroperoxyde induced cell toxicity

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Dwarf elder (*Sambucus ebulus* L.) is a popular herb in Bulgarian folk medicine known for its antiseptic, anti-inflammatory and diuretic effect. *Sambucus* sp. berries are rich in bioactive compounds, most abundant of them being polyphenols, and particularly anthocyanins. The aim of the present study was to evaluate *in vitro* proliferative and cell-protective potential of SE extract and its hydrophilic and anthocyanin enriched fraction in conditions of *t*-ButOOH induced cell death on J774A.1 macrophage cell line. Total extract (TE) and its hydrophilic (HF) and anthocyanin fraction (AF) were analyzed *in vitro* for their polyphenol, flavonoid and anthocyanin content, and antioxidant capacity. Cytoprotective activity of these preparations was assessed in a model of *t*-ButOOH induced cytotoxicity. TE, HF and AF were tested in different concentrations (0.5-64%, v/v). The lowest applied HF concentration (0.5% v/v) caused a 6% significant increase in the cell viability. All the other samples caused a gradual decrease in the cell viability. In order to measure their protective activity, extract (0.5%, 2%, 8% and 32%, (v/v)) were applied as pretreatment. Significant improvement in cell viability of *t*-ButOOH treated cells was detected for all of the extracts, however the most prominent effect was found for the TE, followed by HF and AF. TE significantly improved cell viability by 116, 230, 1165 and 1767%, respectively. Lower, but similar was the effect of the hydroxyl and anthocyanin fraction where improvement of cell viability was up to 564% and 300%. Pretreatment with *Sambucus ebulus* total extract and its hydrophilic and anthocyanin fractions protects J774A.1 cells in a model of *t*-ButOOH induced cytotoxicity.

Key words: *Sambucus ebulus*, cytoprotection, *t*-ButOOH

INTRODUCTION

Medicinal herbs are extensively studied *in vitro* and *iv vivo* in an attempt to understand their healing properties, including the molecular mechanisms. Impaired redox balance and oxidative stress are an important pathogenetic factors in pathological processes due to their potential ability to trigger cell death [1]. Tert–butyl hydroperoxide (*t*-ButOOH) is widely used in cell culture models to induce oxidative stress [2,3]. Dwarf elder, known also as elderberry (*Sambucus ebulus* L., SE) is a popular herb in Bulgarian folk medicine known for its antiseptic, anti-inflammatory and diuretic effect. SE ripe fruits are applied in the form of tea, juice, jam and dried fruits. Their popular usage is in the autumn-winter period for prevention and treatment of respiratory infectious diseases and for amelioration of gastrointestinal disorders. All of these conditions are associated with increased generation of reactive oxygen species and oxidative stress [4, 5]. Acute and chronic inflammation may lead to increased cell death [6]. *Sambucus* sp. berries are rich in bioactive compounds, most abundant of them being polyphenols, and particularly anthocyanins [7, 8, 9]. Having in mind the abovementioned, the aim of the present study was to evaluate *in vitro* proliferative and cell-protective potential of SE extract and its hydrophilic and anthocyanin enriched fraction in conditions of *t*-BuOOH induced cell death on J774A.1 macrophage cell lines. The results will be of support for SE fruits application in folk medicine as heling and complementary remedy and for prevention of their incompetent usage. Data will be of interest for application of SE as rough material for medicinal remedies, functional foods and food additives production.

MATERIALS AND METHODS

Plant material

Ripe *Sambucus ebulus* fruits were collected during maturity period (September-October 2017) from Shkorpilovtsi region, Bulgaria. After appropriate transportation in light protected containers and careful removal peduncles without disturbing their integrity, fruits were freshly frozen at -20ºC. Botanical verification of plant material was performed.

Extraction procedure

Three extracts were obtained: total acetone extract (TE) (70% acetone); hydrophilic fraction (HF) obtained from TE by chloroform fractionation, and anthocyanin fraction obtained from HF by solid phase extraction (SPE). At each step dry residues were obtained by vacuum evaporation under ≤40ºC

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(Genevac SP Scientific, United Kingdom). The resulting dry material from each step was dissolved either in dH_2O for chemical analyses or in cell culture media DMEM for cell culture experiments.

Chemical analysis of extracts

Total phenolics. The total phenolic content (TPC) of the extracts and fractions was analyzed according to the Folin-Ciocalteu method [10]. Absorbance at 765 nm was recorded spectrophotometrically (Camspec M501, United Kingdom). All measurements were performed at least in triplicate. Gallic acid was used as a standard and the TPC was expressed as mg/L gallic acid equivalents (GAE) using the standard curve equation: $y = 0.0007728x - 0.005631$, $r^2 = 0.9982$. All measurements were performed at least in triplicate.

Total flavonoids. Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric assay [11]. The absorbance was measured at 510 nm on spectrophotometer (Camspec M501, United Kingdom). All measurements were performed at least in triplicate. Rutin was used as a standard and the TFC was expressed as mg/L rutin equivalents (RE) using the standard curve equation: $y=0.001170x - 0.01354$, $r^2=0.9900$. All measurements were performed at least in triplicate.

Total monomeric anthocyanins. Total monomeric anthocyanin pigment content (TMAC) was determined by the pH-differential method [12]. Two types of reaction mixtures were prepared - one with potassium chloride buffer (pH 1.0), and another with sodium acetate buffer (pH 4.5). Absorbance was measured at 510 nm and 700 nm, respectively, on spectrophotometer (Camspec M501, United Kingdom). All measurements were performed at least in triplicate. TMAC was expressed as mg/L cyanidin-3-glucoside equivalents. Calculations were performed using the following equations:

$$A = (A_{510}–A_{700})_{pH\ 1.0} – (A_{510}–A_{700})_{pH\ 4.5}$$

TMAC (mg/L) was calculated using the following equation:

$$TMAC\ (mg/L)=(A \times MW \times DF \times 1000)/(\varepsilon \times 1),$$

A is absorption; MW is molecular mass of cyanidin-3-glucoside, equal to 449.2 g/mol; DF is dilution factor; **ε** is molar absorptivity of cyanidin-3-glucoside, equal to 26900 mol/L. All measurements were performed at least in triplicate.

Measurement of radical scavenging capacity

ABTS assay. $ABTS^{\cdot}$ radical cation scavenging activity was determined according to the method described by Re and coauthors [13] with some modifications [14]. Decolorization was recorded at 734 nm. Radical scavenging activity was calculated as uric acid equivalents (UAE) calibration curve: $y = 0.2991*x + 0.007800$, $r^2= 0.9972$. mAll mesurements were performed at least in triplicate.

DPPH assay. The hydrogen donating ability of the tested praparations was evaluated by diphenylpicrylhydrazil assay [15]. The ability of the tested samples to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - (A_{sample} - A_{sample\ blank})/ A_{control}] \times 100,$$

where, $A_{control}$ is the absorbance of the control DPPH solution without sample; A_{sample} is the absorbance of the test sample (DPPH solution plus test sample), and $A_{sample\ blank}$ is the absorbance of the sample only (sample without DPPH solution). The absorbance was measured at 517 nm against methanol as a blank. All measurements were performed at least in triplicate.

Cells culture

J774A.1 mouse macrophage cell line was obtained from American Type Culture Collection (ATCC). Cells were cultured in 75 cm^2 flasks at 37°C in a humidified chamber containing 5% CO_2 in DMEM (Sigma-Aldrich) with 4,5g/L glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to final concentration of 10% and penicillin/streptomycin mixture to a final concentration of 100U/ml of each. Cells were grown until 80% confluence was achieved.

MTT assay

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the standard procedure [16] and as described previously [17]. Absorbance of DMSO cell lysates was detected at λmax=550 nm using Synergy 2 plate reader (BioTek, USA). Viability of treated cells was presented as percentage of the viability of the non-treated cells (control group), which was considered as 100%:

$$\text{Cell viability\%}=A_{sample} \times 100/A_{\ nontreated\ control}$$

All treatments were performed in triplicate. Absorbance of each sample was detected in two individual replicated. Data were presented as mean±SD.

Experimental procedure

The cells were collected and seeded in 6 well flasks at density $2x10^5$ cells/well for different treatments. In order to determine the effect of *t*-ButOOH as oxidizing agent on cell viability, the substance in different concentrations (50-800 μM) in phenol red free DMEM (Sigma-Aldrich) without

fetal bovine serum (FBS) supplementation was applied for 24h before MTT test was performed. In separate experiments for assessment of possible protective effect of plant extracts in a model of *t*-ButOOH induced cytotoxicity a pretreatment for 24h was undertaken. Plant extracts and fractions were dissolved in cultured media (phenol red free DMEM, Sigma-Aldrich, without FBS supplementation) to achieve treatment solutions with extract content from 0.5% (v/v) to 64% (v/v) in a total volume of 2 mL/well. After the pretreatment period *t*-ButOOH in concentration of 400 μM was applied for additional 24h followed by MTT test.

Statistical analysis

Differences between the means of the groups were analyzed by Student's *t*-test (GraphPad Prism 5.0). Values of $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Chemical analysis and radical scavenging capacity

Data about the content of the total polyphenols, flavonoids and anthocyanins, and antioxidant capacity of TE, HF and AF are presented in Table 1. All the measured parameters decrease during fractionation procedure, which may be due to elimination of bioactive substances with chloroform and column fractionation and also destruction during the manipulations.

Table 1. Chemical composition and radical antioxidant capacity of extract and fractions from *S. ebulus* fruits

	TPC [mg/L] (n=3)	TFC [mg/L] (n=3)	TMAC [mg/L] (n=3)	% TMAC of TPC (n=3)	ABTS [UAE] (n=3)	DPPH [% inhibition] (n=3)
TE	522.80±4.83	630.20±7.25	161.97±0.80	30,98	18.49±0.21	58.53±2.59
HF	502.30±6.26	551.30±14.50	148.12±4.62	29.48	17.71±0.25	53.51±2.61
AF	395.60±2.90	402.30±6.04	140.02±4.93	35.39	15.64±0.36	49.03±1.19

MTT test

t-But OOH cytotoxicity

In this study we examined different concentrations of *t*-ButOOH (50-800 μM) with regards to their effects on cell viability aiming to establish a concentration contributing to a substantial cell death for the needs of subsequent experiments. Results demonstrated that the lowest applied concentration (50 μM) had a very low, but statistically significant cytotoxic effect in J744A.1 cell culture leading to about 4% cell death ($p<0.001$) (Fig. 1).

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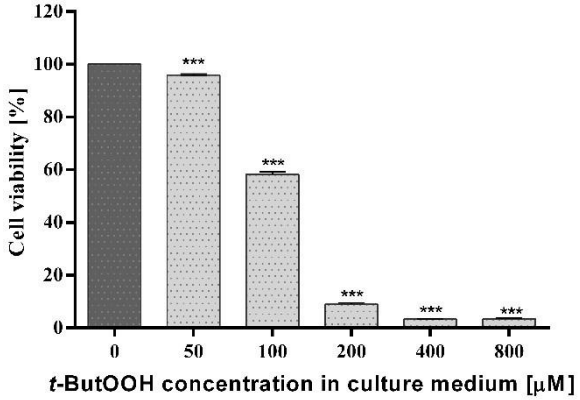

Figure 1. Cell viability of J774A.1 macrophages treated with *t*-ButOOH in a concentration range from 50 to 800 μM for 24h (n=6 for each group). Values are calculated in comparison to nontreated control (0 μM *t*-ButOOH). *** vs. nontreated control.

The next applied higher concentration (100 μM) demonstrated strong toxic effect leading to more than 40% cell death ($p<0.001$). Cell viability decreased abruptly in the other three applied concentrations achieving cell viability less than 10% (for 200 μM) and 4% (for 400 and 800 μM *t*-ButOOH). In this experiment the toxic effect of *t*-ButOOH was confirmed in agreement to other studies in different cell culture models where *t*-ButOOH has been used as effective oxidative agent in order to promote cell death. The cell survival rate under specified concentrations depends on cell type and the exposure time. In experiments with isolated peritoneal mouse macrophages Pang et al. [18] established 24% survival for 100μM *t*-ButOOH after 24h treatment. RAW 264.7 macrophages when

treated with 5 mM *t*-ButOOH for 3h revealed more than 60% cell death [19]. In HepG2 cells 300 μM *t*-ButOOH caused about 50% cell death after a 24h of exposure [20]. For the needs of the planned experiments about the establishment of the possible cytoprotective effect of SE extracts the concentation of 400 μM *t*-ButOOH was selected due to its pronounced cytotoxic effect.

Plant extracts

The establishment of the cytoprotective activity of plant extracts is important with regard to their usage as preventive and therapeutic agents in conditions associated with increased cell death, such as inflammation and oxidative stress. Plant extracts have been widely studied for their protective effects in a variety of models, including models of oxidative stress. In this study initially, the effect of fruit extracts/fractions without oxidative treatment was tested. This experiment was undertaken in order to select extract/ fractions concentrations suitable for the needs of the subsequent treatments in the model of *t*-ButOOH induced cytotoxicity. The results of the performed MTT test are presented in Figure 2.

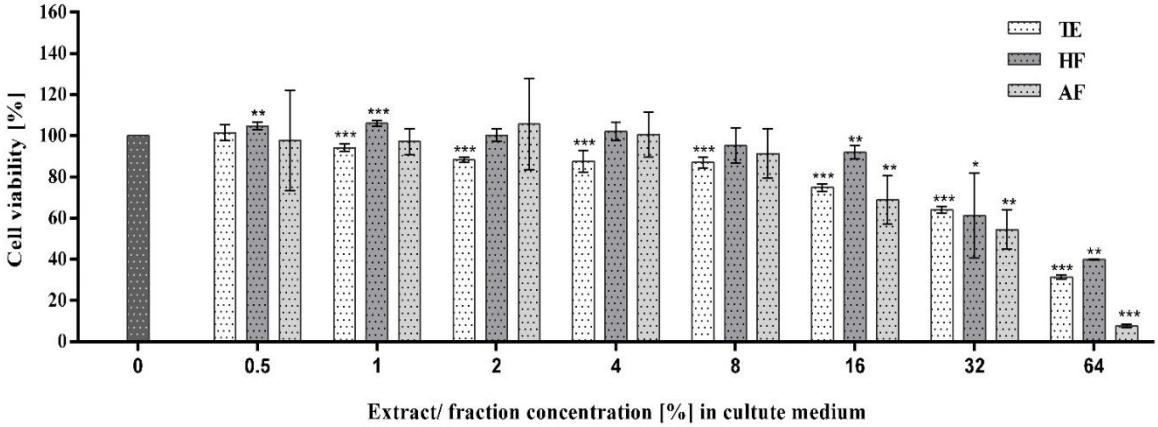

Figure 2. Cell viability of J774A.1 macrophages treated with TE, HF and AF in increasing concentrations (0.5-64%) of the extract/fractions in culture media for a period of 24h (n=6 for each group). Values are calculated in comparison to nontreated control. * vs. nontreated control.

TE, HF and AF were tested in different concentrations (0.5-64%, v/v). Slight, but significant increase in cell viability was detected for the first lowest applied HF concentrations (0.5% and 1%, v/v), which caused a 4% ($p<0.01$) and 6% ($p>0.001$) increase in the cell viability, respectively. Most likely, the observed stimulatory effect could be due to proliferative activity of constituents in the extract. Similarly, although not reported to be significant, an increased cell viability (about 20%) has been detected in mouse fibroblast L929 cells treated with anthocyanins and anthocyanidins [21]. In our previous experiments with another medicinal plant - *Agrimonia eupatoria*, a stimulation of the cell proliferation at low concentrations of the plant extract has also been established in 3T3-L1 preadipocyte cell line [22]. Except 0.5% HF, a gradual decrease in the cell viability was established for the three tested extracts in the abovementioned concentration concentration range for the experiment. The decreasing cell vitality could be explained by the manifestation of toxic effects of some of the components of the extract on the cells when administered at high concentrations. Whether a plant extract will have a cytotoxic or proliferative effect would depend on its administered concentration, time of exposure, as well as the cells type treated. For the needs of the present experiment 0.5%, 2%, 8% and 32% (v/v) extract/fractions were selected. The volumetric ratios were selected so as to use nontoxic, relatively low and high toxic concentrations in the experiments with 4 fold difference between them.

Plant extracts in a model of t-ButOOH induced cytotoxicity

In order to investigate possible protective activity of *S. ebulus* fruit preparations in *t*-ButOOH induced cell toxicity model we performed 24h pretreatment of the cells with 0.5%, 2%, 8% and 32% (v/v) extract/fraction in culture media. Significant improvement in cell viability of *t*-ButOOH treated cells was detected for all of the extracts, however the most prominent effect was found for the TE, followed by HF and AF (Figure 3).

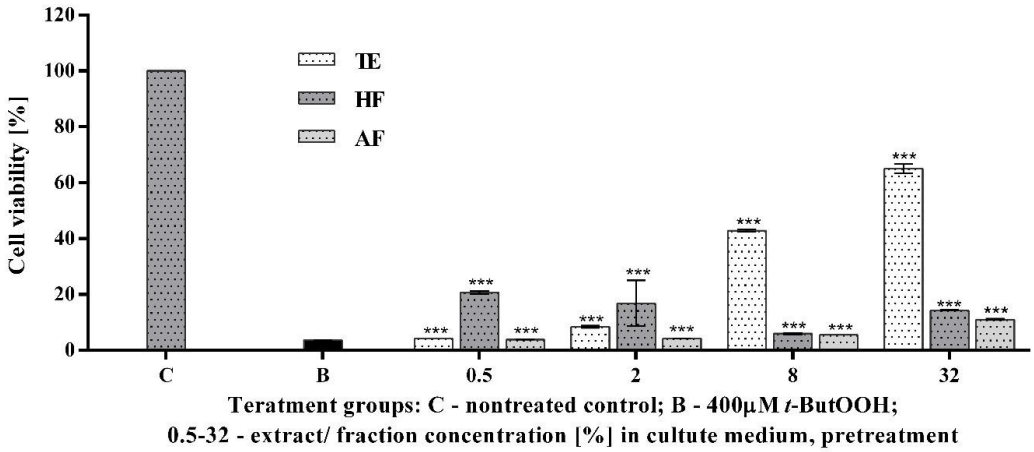

Figure 3. Cell viability of 400 mM *t*-ButOOH treated J774A.1 macrophages (24h) pretreated (24h) with TE, HF and AF in increasing concentrations (0.5-32%) in culture medium (n=6 for each group). Values are calculated in comparison to nontreated control. *** vs. nontreated control.

Table 2. Percentage of improved cell viability of pretreated J744A.1 cells as compared to *t*-ButOOH treated cells

Type of extract/fraction	TE		HF		AF	
% extract in culture medium	Cell viability [%] (n=6)	p value vs. t-ButOOH (n=6)	Cell viability [%] (n=6)	p value vs. t-ButOOH (n=6)	Cell viability [%] (n=6)	p value vs. t-ButOOH (n=6)
0.5	115.5	0.001	564.0	0.001	105.8	0.01
2	229.6	0.001	458.5	0.05	116.2	0.001
8	1165.2	0.001	162.2	0.001	150.3	0.001
32	1767.4	0.001	389.3	0.001	300.3	0.001

Cell viability%=$A_{\text{pretreated cells}}$ x 100/$A_{\text{t-ButOOH treated cells;}}$ TE: total extract; HF: hydrophilic fraction; AF: anthocyanin fraction

With the aim to assess the level of improvement of cell viability by extract/fractions, pretreatment in comparison to *t*-ButOOH treated cells, cell viability was calculated as compared to the oxidative agent treatment, where it was considered to be equal to 100% (Table 2). Total extract in applied concentrations significantly improved cell viability by 116, 230, 1165 and 1767%, respectively ($p<0.001$ for all concentrations). Lower, but similar was the effect of the hydroxyl fraction and anthocyanin fraction where up to 564% ($p<0.001$) and 300% ($p<0.001$) improvement of cell viability was detected.

Obviously, pretreatment with SE extracts significantly lowered the toxicity of the subsequently applied oxidative agent.

Total extract in the applied concentrations significantly improved cell viability by 116%, 230%, 1165% and 1767%, respectively ($p<0.001$ for all concentrations). Lower, but close to that of the TE was the effect of the hydroxyl and anthocyanin fraction. We found up to 564% ($p<0.001$) and 300% ($p<0.001$) improvement of cell viability for HF and AF, respectively. The observed decrease in the protective properties of the tested preparations depends on the type of extract/fraction and is probably due to the elimination of bioactive cytoprotective componds during the process of fraction preparation with chloroform and column fractionation. The results from chemical composition and antioxidant activity (Table 1) also confirmed the decrease in antioxidants content and activity after the fractionation procedure. Partial degradation of bioactive phytochemical complexes during fractionation could be also a possible mechanism leading to decrease in antioxidant and bioactivity properties of the SE fruit preparations.

Different antioxidant compounds have been demonstrated to attenuate cell death. For example daphnetin, a natural coumarin with antioxidant properties, attenuated *t*-ButOOH induced cell toxicity in RAW264.7 macrophages [19]. In U937 human monocytes caffeic acid (50µM) markedly induced the proliferative capacity of cells exposed to oxidative challenge by t-ButOOH [23]. In RAW 264.7 and J774A.1 murine macrophage cell line protective effect of methyleugenol was established against t-ButOOH (2mM) triggered oxidative injury

and cell death [24]. Anthocyanins have also been found to exert cytoprotective activity in culture models. Anthocyanin fraction from purple-fleshed sweet potatoes improved cell viability of HegG2 cells [20] incubated with 300 μM t-ButOOH, as detected with MTT assay and decreased malondialdehyde production. Similar effects of pure anthocyanin compounds has been established in rat smooth muscle and hepatoma cells [25].

CONCLUSIONS

Obtained data demonstrated cytoprotective activity of *Sambucus ebulus* total extract and its hydrophilic and anthocyanin fractions. Pretreatment of macrophage J774A.1 cells with studied SE preparations that had been established to be rich in polyphenols, prevent *t*-ButOOH induced cell death. In addition

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REFERENCES

1. S.W. Ryter, H. P. Kim, A. H. l Jeong, W. Park, K. Nakahira, X. Wang, A. Choi., Antioxidants & Redox Signaling, 2006, vol. 9, NO. 1.
2. Z. Liu, X. Tao, C. Zhang, Y. Lu, *D. Biomed. Pharmacoth.*, 59, 481 (2005).
3. X. Yang, Y. Zhao, Y. Lv, Y. Yang, Y. Ruan. *J. Biochem. Molec. Biol.*, **40**, 928 (2007).
4. M. Liu, F. Chen, T. Liu, F. Chen, Sh. Liu, J. Yang. *Microbes &Infection*, **19,** 80 (2017).
5. M. Nikkhah-Bodaghia, I. Malekib, Sh. Agahc, A. Hekmatdoosta. *Complem. Ther. Med.*, **43,** 1 (2019).
6. Y. Yang, G. Jiang , P. Zhang, J Fan. *Military Med. Res.*, **2**, 12 (2015).
7. Y. Kiselova, S. Marinova, D. Ivanova, D. Gerova, B. Galunska, T. Chervenkov, T. Yankova. Proceedings of the Balkan scientific conference of biology in Plovdiv, Bulgaria, 233 (2005)
8. H. Wu, M. Johnson, C. Lu, K. Fritsche, A. Thomas, Z. Cai, C. Greenlief. *Acta Hortic*. **1061**, 43 (2015).
9. G.T. Ho, H. Wangensteen, H. Barsett. *Int J Mol Sci.*, **18,** 584 (2017).
10. V. Singleton, J. Rossi. *Am J Enol Vitic*, **16**, 144 (1965)
11. J. Zhishen, T. Mengcheng, W. Jianming. *Food Chem*, **64**, 555 (1999)
12. M. Giusti, R Wrolstad, in: Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc, 2001
13. R. Re, N. Pellegrini, A. Proteggente, Y. Pannala, M. Yang, C. Rice-Evans. *Free Radic Biol Med*, **26**, 1231 (1999).
14. Y. Kiselova, D. Ivanova, T. Chervenkov, D. Gerova, B. Galunska, T. Yankova. *Phytotherapy Res.*, **20**, 961 (2006).
15. M. Akter, M. Ahmed, J.B. Eun. *Int. J. Food Sci. Technol.*, **45**, 2258 (2010).
16. T. Mosmann. *J. Immunol. Methods*., **65**, 55 (1983).
17. Y. Kiselova-Kaneva, O. Tasinov, D. Vankova, D. Ivanova. *Scripta Sci. Med.*, **44** , 31 (2012).
18. Z. Pang, M. Zhou, Y. Chen, J. Wan. *Atherosclerosis*, **147,** 33 (1999).
19. H. Lv, Q. Liua, J. Zhoua, G. Tana, X. Denga, X. Ci. *Free Radical Biol. Med.*, **106,** 38 (2017).
20. Y. Hwang, Y. Chung, J. Chou, J. Chou, Y. Chung, G.Jeong. *Food Chemical Toxicol.*, **49,** 2081, (2011).
21. E. Wang, Y. Liu , C. Xu, J. Liu. *Food Nutr Res*, **61**, 1325308 (2017).
22. D. Ivanova, O. Tasinov, D. Vankova, Y. Kiselova-Kaneva. *Bul. J. Agric. Sci.*, **19**, 171 (2013).
23. M. Nardini, P. Pisu, V. Gentili, F. Natella, M. Di Felice, E. Piccolella, C. Scaccini. *Free Radical Biology & Medicine*, **25**, 1098 (1998).
24. J. Zhou, X. Ma, Y. Cui, Y. Song, L. Yao, Y. Liu, S. Li. *J. Pharmacol. Sci.*, **135,** 55 (2017).
25. M.C. Lazzé, R.Pizzala, M.Savio, A.Stivala, E.Prosperi, L.Bianchi. *Mutation Res./Genetic Toxicol. Environ. Mutagenesis*, **535**, 103 (2003).