Antioxidant Effect of Caffeic Acid Derivatives on Sod and Glutathione Defective Yeasts[1]

E. R. Prudêncio[a], *, C. M. Cardoso[c], R. N. Castro[b], and C. J. Riger[a]

[a]Laboratory of Oxidative Stress in Microorganisms, Department of Biochemistry. Federal Rural University of Rio de Janeiro, Seropédica, Rio de Janeiro CEP: 23897-000 Brazil

[b]Laboratory of Medicinal Chemistry and Honey Chemistry, Department of Chemistry, Federal Rural University of Rio de Janeiro, Seropédica, Rio de Janeiro, CEP: 23897-000 Brazil

[c]Biochemistry Laboratory, Institute of Zootechnics. Federal Rural University of Rio de Janeiro, Seropédica, Rio de Janeiro, CEP: 23897-000 Brazil

*e-mail: edleneribeiro@gmail.com

Abstract—Hydroxycinnamic acids have received particular attention because they are the most abundant phenolic class in our diet and because of the increasing interest in reducing oxidative damages. These damages are related to diseases such as cancer and atherosclerosis, as well as neurodegenerative disorders. The objective of this study was to evaluate the antioxidant protection of chlorogenic and caffeic acids and caffeic acid phenethyl ester (CAPE) against oxidative stress *in vivo*. Antioxidant activity was evaluated using BY4741 strain and superoxide dismutase- and glutathione-deficient ($\Delta sod1$ and $\Delta gsh1$) mutants of *Saccharomyces cerevisiae* through cell viability assays, lipid peroxidation levels and glutathione quantification. In the cell viability tests, caffeic acid promoted higher stress tolerance, with a 106% increase in *S. cerevisiae* BY4741. However, in the $\Delta sod1$ mutant, the effect of chlorogenic acid was more prominent, showing a 3.3-fold increase and in the $\Delta gsh1$ mutant all treatments provided a similar level of protection. The phenolic acids protected cell membranes in control and mutant cells at the same level. CAPE maintained the GSH concentration at levels similar to the non-stressed control (48.6 ± 10.9 nmol/mg of cells). The maintenance of cytoplasmic levels of GSH that was promoted by CAPE, despite the induction of stress, indicates its superior antioxidant protection to its precursor, caffeic acid.

Keywords: CAPE, hydroxycinnamic acids, phenolic compounds, *Saccharomyces cerevisiae*

DOI: 10.1134/S0003683819030116

Dietary polyphenols are known to be beneficial to human health and associated with the prevention or reduction of oxidative stress in the body, that comes from a redox unbalance in cells and is connected with the etiology of a number of diseases. These include atherosclerosis, neurodegenerative diseases, cardiovascular diseases and some types of cancer [1, 2]. Hydroxycinnamic acids (HCAs) are a class of polyphenols with a high antioxidant power in biological systems [3] and the caffeic acid (CA, 3-(3,4-dihydroxyphenyl)-2-propenoic acid) is one of the main representatives of HCAs, highly abundant in foods [4, 5].

Caffeic acid phenethyl ester (CAPE) and chlorogenic acid are compounds that are derived from caffeic acid. CAPE is formed by the esterification of CA with phenethyl alcohol, while chlorogenic acid is obtained from the esterification of quinic acid with CA. The chemical characteristics of the esterified group influence the polarity of the molecule and consequently its absorption by the cells, in addition to specifically influencing its antioxidant activity.

These compounds have several biological and pharmacological effects. These include immunomodulatory [6], anti-inflammatory [7], anticarcinogenic [8] and neuroprotective [3] properties, as well as antioxidant activities [9]. However, the intracellular response and the mode of action of these substances depend intrinsically on the site of action and how these substances alter the oxidative environment within the cells. For example, phenolic compounds can mobilize radical species from endogenous or exogenous sources [10], and the induction of endogenous antioxidant defense systems involves several different steps and pathways [11], many of which are still unknown.

Some organelles and cellular structures can potentially be affected by oxidative stress, in particular the DNA, mitochondria and the plasma membranes of cells [12]. Additionally, antioxidant defense systems show variations in their activities depending on

---

[1] The article is published in the original.

changes to the redox state of the cells [7]. In this context, antioxidant assays in *Saccharomyces cerevisiae* cells are interesting because these cells are a simplified biological model. They are a unicellular eukaryotic organism, showing a high level of orthology in mammalian cells; especially regarding the macromolecules and organelles of the antioxidant defense system [13, 14]. Furthermore, knowledge of the yeast genome allows genetic manipulation, to obtain more sensitive strains to oxidative stress, such as the Δ*sod1* (deleted in the enzyme superoxide dismutase) and Δ*gsh1* (deleted in the tripeptide glutathione) mutants. These strains are deficient in protection systems linked to the cell's redox homeostasis, allowing a more precise evaluation of the compound's actions [15, 16].

In this study, we used *S. cerevisiae* as a unicellular eukaryotic model to verify the differences in the antioxidant action of CAPE, chlorogenic acid and caffeic acid on yeast strains deficient in the superoxide dismutase (SOD) or tripeptide glutathione (GSH) under oxidative stress caused by 1 mM hydrogen peroxide.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* BY4741 control strains (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and their isogenic mutants Δ*sod1* and Δ*gsh1*, which respectively harbor versions of the SOD1 and GSH1 genes that have been interrupted by the gene *KanMX4*, were acquired from Euroscarf (Frankfurt, Germany). Stocks of yeast strains were maintained on a solid YPD medium containing (g/L): yeast extract—10.0, glucose—20.0, 2% peptone—20.0 and agar—20.0. For the mutant strains, the medium also contained 0.02% geneticin. Media components were obtained from Difco (USA). Chlorogenic acid, CAPE and caffeic acid were obtained from Sigma-Aldrich (USA). A stock solution of phenolics (2 mg/mL) was prepared using dimethylsulfoxide and water (1 : 1). For all experiments, cells were cultivated on a liquid 2% YPD using an orbital shaker with 160 rpm at 28°C. The cell growth continued until the population reached the mid-exponential phase (OD_{570} of 0.6 and 1.0 mg dry weight/mL). The factor used to convert absorbance into dry weight was calculated using the centrifugation of 10 mL of the cell suspension at 2000× g for 5 min, followed by the dehydration of the pellet at 80°C until it achieved a constant weight.

Pretreatment and stress conditions. Cells (20 mg) were centrifuged at 2000× g for 5 min, resuspended in 50 mM K-phosphate buffer (pH 6.0) and treated with chlorogenic acid, CAPE or CA taken in concentration of 1, 10 or 100 µg/mL for 2 h. Cells resuspended in the same buffer were used as the control. For the toxicity test, pretreatment was terminated at this stage.

For the assays of cell viability, lipid peroxidation and GSH:GSSG quantification, cells in the exponential phase were exposed to chlorogenic acid, CAPE or caffeic acid taken in concentration of 10 µg/mL for 2 h, harvested, washed twice with 50 mM K-phosphate buffer (pH 6.0), resuspended in YPD medium and then incubated with 1.0 mM hydrogen peroxide for 1 h. Both unstressed cells and cells that were only treated with hydrogen peroxide for 1 h (stress control) were maintained for comparison purposes.

Toxicity and cell viability. Pre-treated cells were collected (40 µg), diluted (1000×) with 50 mM K-phosphate buffer (pH 6.0), plated on YPD 2% and incubated at 28°C for 72 h and the number of colonies was counted [13, 17]. For toxicity, the results were expressed as the survival percentage and for cell viability they were expressed in CFU/mL.

Lipid peroxidation. Fifty mg of pre-treated cells were collected, centrifuged at 2000× g for 5 min and washed twice with ultra-pure water. The pellets were resuspended in 0.5 mL 10% trichloroacetic acid (TCA) and 1.5 g of glass beads (40 µm, Sigma-Aldrich, USA) were added. Six cycles agitation on a vortex (Gehaka, Brazil), followed by ice (20 s each one) were performed to lyse the samples. Extracts were centrifuged at 2000× g for 4 min and the supernatants were mixed with 0.15 mL 0.1 mM EDTA and 0.60 mL 1% thiobarbituric acid. The reaction mixture was incubated at 100°C for 15 min and, after cooling, the final product of the lipid oxidation (malondialdehyde − MDA) was measured spectrophotometrically at 532 nm [18].

Glutathione determination. Twenty mg of pretreated cells were collected, centrifuged at 2000× g for 5 min and washed twice with ultra-pure water. The pellets were resuspended in 0.5 mL 10% TCA and 1.5 g of glass beads were added. For lysing the samples, they were kept 20 min in ice bath vortexing vigorously every 5 min. Extracts were centrifuged at 19 000× g for 5 min and the supernatants were neutralized with 5.0 M NaOH. Reduced glutathione (GSH) was spectrophotometrically determined in extracts following S-lactoyl-GSH production at 240 nm. Glutathione disulfide (GSSG) was determined in the same cuvette by the addition of NADPH and glutathione reductase, and reading at 340 nm [19].

Data analysis. At least three replicates for all assays were performed and the collected data was subjected to the ANOVA and the Tukey tests ($p < 0.05$).

RESULTS AND DISCUSSION

Cytotoxicity. Only innocuous concentrations of HCAs could be used to evaluate the antioxidant actions on yeast. In the *S. cerevisiae* BY4741 strain, the toxicity evaluation showed no significant decrease in survival percentage of the cells at the 1 and 10 µg/mL concentrations, when exposed to the 3 compounds (Table 1). However, 100 µg/mL CAPE was extremely toxic leading to total cell death. For mutant strains, the compound's toxicity was evaluated only at 1 and 10 µg/mL concentrations. For CAPE, chlorogenic

Table 1. Survival percentage of the *S. cerevisiae* BY4741, Δ*sod1* and Δ*gsh1* strains treated with chlorogenic acid, CA and CAPE at 1, 10 or 100 µg/mL for 2 h. Results presented are the average and standard deviation of at least three independent experiments, n.e. represent combinations that were not evaluated, equal a or b symbols show similar results

Strains	Chlorogenic acid	CA	CAPE
1 µg/mL			
BY4741	96.67 ± 4.16^a	92.33 ± 13.28^a	91.00 ± 15.99^a
Δ*sod1*	98.33 ± 2.89^a	100.00 ± 0.00^a	92.00 ± 13.86^a
Δ*gsh1*	100.00 ± 0.00^a	100.00 ± 0.00^a	100.00 ± 0.00^a
10 µg/mL			
BY4741	96.67 ± 5.77^a	96.00 ± 6.93^a	92.00 ± 13.86^a
Δ*sod1*	97.00 ± 2.65^a	100.00 ± 10.00^a	97.67 ± 2.52^a
Δ*gsh1*	100.00 ± 0.00^a	94.00 ± 6.00^a	93.33 ± 15.00^a
100 µg/mL			
BY4741	92.67 ± 12.70^a	92.33 ± 13.28^a	1.33 ± 1.53^b
Δ*sod1*	n.e.	n.e.	n.e.
Δ*gsh1*	n.e.	n.e.	n.e.

and CA, cell survival remained above 90%. Therefore, the 10 µg/mL concentration of tested compounds was chosen to be used in the subsequent assays: cell viability, lipid peroxidation and glutathione ratio.

Phenolic compounds are produced by plant's secondary metabolism, and protecting the plant against microorganisms is one of their functions. Consequently, they have antibacterial and antifungal activities [20–22], which might become a limitation in analyses using yeast cells as the biological model of investigation. However, antifungal activity is directly related to the concentration of the analyzed substances [23]. Toxicity verified for CAPE in *S. cerevisiae* BY4741 corroborates to toxicity results found in *Candida albicans*, where CAPE revealed a high fungicidal effect at 100 µg/mL [24]. Cigut et al. [25] used a chromatographic analysis to evaluate the uptake of 50 µg/mL of CA and CAPE by *S. cerevisiae* cells. Phenolic compounds were quantified in the supernatant suspension, before and after a 1 h incubation period with *S. cerevisiae*. Results showed that CAPE was absent in the supernatant after treatment time, indicating that the only compound was absorbed within 1 h. The higher toxicity observed for CAPE may be associated to its faster absorption.

Cell viability. Determination of the antioxidant activity was performed through cell viability assays. Compounds were pre-incubated with chlorogenic acid, CA and CAPE, and subsequently submitted to oxidative stress with 1.0 mM H_2O_2 [17]. All tested compounds increased yeast tolerance to hydrogen peroxide stress during the test, with different compounds highlighted in each strain (Fig. 1).

In control *S. cerevisiae* BY4741 cells, CA promoted higher stress tolerance, with a 106% mean increase compared to cells that were only stressed (S), reaching

the same level of stress tolerance as non-stressed cells (NS). Additionally, both chlorogenic acid and CAPE were effective in comparison with the control strain, obtaining increases in viability of around 76 and 91%, respectively.

In the Δ*sod1* strain, all compounds achieved protection against stress. Chlorogenic acid was more efficient than the others, producing cell survival similar to the control (NS), increased survival 3.3-fold when compared with stressed cells (S). The addition of CAPE and CA led to 2.6- and 2.2-fold survival increase, respectively. In the Δ*gsh1* strain, all com-
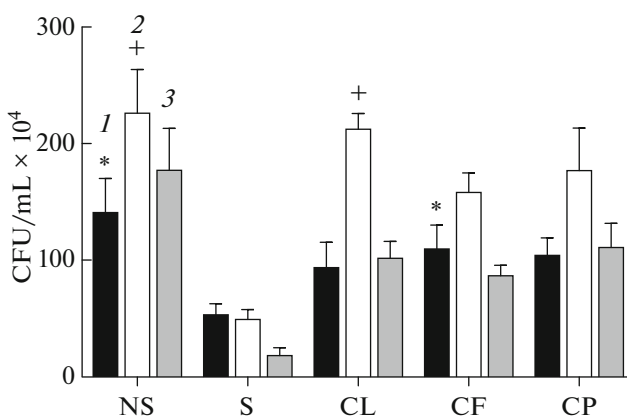
Fig. 1. Assessment of the stress tolerance of *S. cerevisiae* BY4741 (*1*), Δ*sod1* (*2*) and Δ*gsh1* (*3*) strains resulting from exposure to H_2O_2 after pretreatment with chlorogenic acid (CL), caffeic acid (CF) or CAPE (CP). NS—non-stressed cells; S—stressed cells. Results presented are the average and standard deviation from at least three independent experiments. The statistical analysis was separately performed for each strain ($p < 0.05$). Equal symbols show similar results.

pounds were similar in their promotion of stress tolerance, increasing survival about 4-fold compared to the survival of stressed cells (S). Curiously in the cell viability assays, the $\Delta sod1$ strain presented a higher resistance to hydrogen peroxide than the control strain, represented by the number of colonies (CFU). The unstressed control also presented a higher amount of CFU than the *S. cerevisiae* BY4741 strain did (140 ± 29 in BY4741 and 231 ± 37 in $\Delta sod1$), however the stressed control did not present this discrepancy (53 ± 7 in BY4741 and 51 ± 8 in $\Delta sod1$). This result contradicts the expectation that the absence of an antioxidant system would result in a more sensitive strain. However, this deviation from the expectation has already been verified in a survey performed on flavonoid hesperidin by Wilmsen et al. [26], and in a survey performed on resveratrol by Dani et al. [13]. Fernandes et al. [27] pointed out that in the absence of a defense system, a cell increases its expression of the remaining antioxidant enzymes in order to protect itself. It is possible that this mechanism is associated to the protection exerted by phenolic compounds that are absorbed by the cells, which might overcome the absence of antioxidant enzymes and produce similar viabilities to non-stressed cells.

Lipid peroxidation. Lipid peroxidation can be defined as the cascade of biochemical events resulting from the action of free radicals on the unsaturated lipids of cell membranes. This mainly generates lipid radicals (L, LO, eLOO), which initiate a primary cytotoxic events that trigger a sequence of cell lesions [28]. In three tested strains, all treatments with caffeic acid derivatives resulted in a reduction of peroxidation levels compared to cells that were only stressed (S). In addition, the peroxidation levels achieved were statistically similar to the non-stressed control (NS) (Fig. 2).

Treating the mutant strains with hydroxycinnamic compounds produced lipid peroxidation levels similar to those of the unstressed control strain (NS). However, according to the results obtained from the cells under stress (S), the compounds had a much more pronounced effect on the $\Delta gsh1$ than on the other strains, since the $\Delta gsh1$ strain showed a greater sensitivity to oxidative stress induced by hydrogen peroxide.

Lipid peroxidation results differed from the viability results, where the same treatments produced different results among the strains. Mekoue Nguela et al. [29] suggested that polyphenols are adsorbed by yeast cell walls and initially located in the space between the cell wall and plasma membrane. This initial interaction with the membrane may justify the greater protection exerted on this portion of the cell, which is verified by the similar lipid peroxidation levels produced by all treatments, as the compounds would primarily protect this location.

Reduced (GSH) and oxidized (GSSG) glutathione determination. Cell viability and lipid peroxidation results obtained for $\Delta gsh1$ demonstrate that this strain
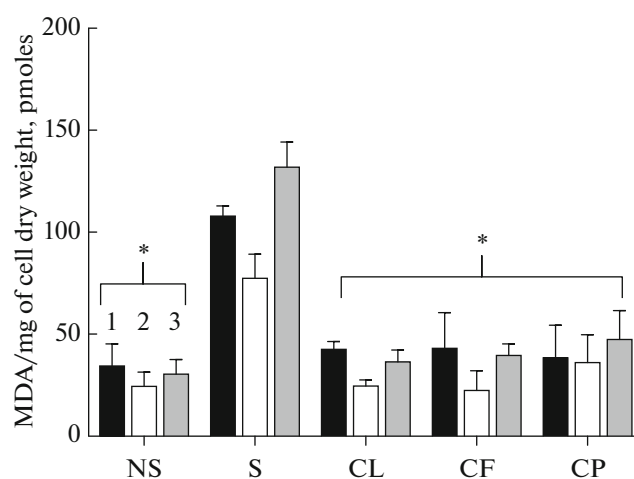

Fig. 2. Lipid peroxidation in the control *S. cerevisiae* BY7471 (*1*), and pretreated $\Delta sod1$ (*2*) and $\Delta gsh1$ (*3*) mutant strains which were exposed to chlorogenic acid (CL), caffeic acid (CF) or CAPE (CP) and subsequently stressed with H_2O_2. Extracts from non-stressed, stressed and HCAs-treated cells were used to determine MDA levels. The results presented are the average and standard deviation from at least three independent experiments ($p < 0.05$). Equal symbols show similar results.

is more sensitive than the $\Delta sod1$ strain to hydrogen peroxide oxidative stress (Figs. 1 and 2). The relationship of oxidized to reduced glutathione (GSSG : GSH) is used to evaluate the intracellular redox environment and also to verify if antioxidant substances act on cellular homeostasis, which would be reflected in the GSH and GSSG levels. In this analysis, only the control strain (*S. cerevisiae* BY4741) was used, which had a preserved endogenous antioxidant defense system.

Both chlorogenic and caffeic acids treatments increased the concentration of reduced glutathione, by 3.0 and 2.3-fold respectively, when compared to its concentration in stressed cells (S) (Fig. 3). However, treatment with CAPE was shown to be the most effective, since it obtained a reduced glutathione concentration statistically similar to that of non-stressed cells (NS), with a 4.1-fold increase compared to the stressed cells. Overall, all three compounds contributed to the maintenance of the intracellular reducing environment, contributing to the antioxidant defense of the *S. cerevisiae* BY4741 when it is under oxidative stress. Oxidized glutathione levels were not altered in any of the treatments, revealing that an increase in the GSH level does not necessarily represent a decrease in the level of GSSG.

The effects of HCAs in other biological models have already been tested by several authors, whose reports support the findings of the present study. Celik and Erdogan [30] found that CAPE reduced the levels of some oxidative stress markers induced by drugs causing diabetes. Rats treated for 60 days with CAPE (10 µg/(kg day)) showed reduced lipid peroxidation
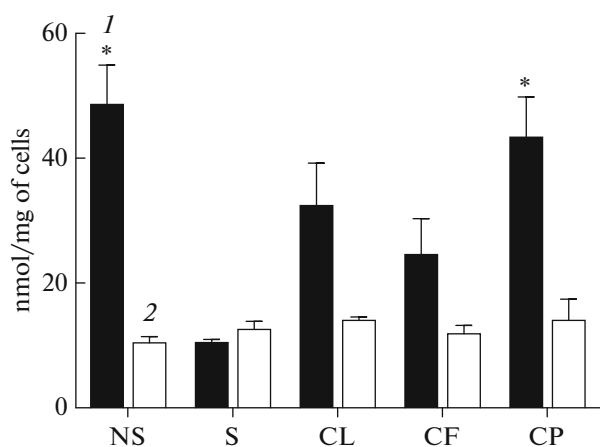
Fig. 3. GSH (*1*) and GSSG (*2*) in *S. cerevisiae* BY7471 treated chlorogenic acid (CL), caffeic acid (CF) or CAPE (CP) followed by hydrogen peroxide stress. Untreated cells (NS) and cells only treated with hydrogen peroxide (S) are represented. Mean values and standard errors form at least three independent experiments are shown. ($p < 0.05$). Equal symbols show similar results.

levels. Chen and Gong [31] revealed that treatment with 10 μM/kg CAPE might reduce the oxidative stress caused by cadmium (1 mg/kg) in rat livers and GSH concentration was also reestablished after the CAPE treatment. Coelho et al. [32] found that the CA treatment was able to reduce free radical and lipid peroxidation levels in brain tissue from guinea pigs treated with antiepileptic drugs. Sato et al. [33] reported that chlorogenic acid might reduce the oxidative damage caused by ischemia/reperfusion in the intestinal loop of rats.

In previous studies, it has been observed that GSH plays an essential role in the antioxidant response to stress caused by hydrogen peroxide [34]. Both reduced and oxidized glutathione lead to cell redox homeostasis, and these results showed that CAPE increased the amount of GSH to levels similar to those observed in non-stressed cells. It was found that CAPE is lethal to yeast at the concentration of 100 μg/mL, however treatment with 10 μg/mL CAPE provided maintenance of the GSH concentration at levels similar to those of the non-stressed cells. Although the CAPE at 10 μg/mL concentration did not cause survival decrease in the toxicity test, the compound at this concentration may have functioned as an oxidative stress-inducing agent mobilizing endogenous antioxidant defenses.

The literature predicts that phenolic compounds may have concentration-dependent pro-oxidant action, and act as modulators of antioxidant enzyme defense system with a low toxicity. Amari et al. [35] evaluated the antioxidant mechanism of quercetin and other low molecular weight phenolic compounds in yeast cells. They proposed that these compounds function as xenobiotics and stimulate the stress response

machinery of cells, making them more resistant to subsequent stress with H_2O_2. Fernandes et al. [27] reported that pretreatment with sublethal (0.2 mM) hydrogen peroxide concentrations for 1 h increases the survival of *S. cerevisiae* when exposed to lethal concentration of H_2O_2 (2.5 mM). CAPE was the compound with the highest toxic potential among those analyzed here, and it was also the compound that promoted a higher level of GSH, in addition to showing antioxidant activity in all treatments. Consequently it could be assumed that it acts similarly to quercetin, inducing an antioxidant defense system and thereby increasing tolerance to subsequent stress caused by H_2O_2.

CA is an active antioxidant. However, its derived compounds chlorogenic acid and CAPE have also been useful in increasing stress tolerance. The survival of each strain was differentiated between treatments, indicating structural changes may interfere in the antioxidant mechanism. Although all tested molecules prevented the peroxidation of membrane lipids, CAPE had a greater influence on redox homeostasis as it maintained constant GSH levels.

The effect of CAPE activity on *S. cerevisiae* BY4741 is superior to effect of its precursor, CA, indicating that esterification with phenethyl acid is relevant within its mechanism of action.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Losada-Barreiro, S. and Bravo-Díaz, C., *Eur. J. Med. Chem.*, 2017, vol. 133, pp. 379−402.
2. Salas-Salvadó, J., Becerra-Tomás, N., García-Gavilán, J.F., Bulló, M., and Barrubés, L., *Prog. Cardiovasc. Dis.*, 2018, vol. 61, no. 1, pp. 62−67.
3. Moosavi, F., Hosseini, R., Rajaian, H., Silva, T., Magalhães e Silva, D., Saso, L., et al., *Bioorg. Med. Chem.*, 2017, vol. 25, no. 12, pp. 3235−3246.
4. Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P.E., Tognolini, M., Borges, G., and Crozier, A., *Antioxid. Redox Signal.*, 2013, vol. 18, no. 14, pp. 1818−1892.
5. El-Seedi, H.R., El-Said, A.M.A., Khalifa, S.A.M., Göransson, U., Bohlin, L., Borg-Karlson, A.K., and

Verpoorte, R., *J. Agric. Food Chem.*, 2012, vol. 60, no. 44, pp. 10877−10895.

6. Búfalo, M.C. and Sforcin, J.M., *J. Pharm. Pharmacol.*, 2015, vol. 67, no. 5, pp. 740−745.

7. Zhang, H. and Tsao, R., *Curr. Opin. Food Sci.*, 2016, vol. 8, pp. 33−42.

8. Roleira, F.M.F., Tavares-da-Silva, E.J., Varela, C.L., Costa, S.C., Silva, T., Garrido, J., and Borges, F., *Food Chem.*, 2015, vol. 183, pp. 235−258.

9. Khan, F.A., Maalik, A., and Murtaza, G., *J. Food Drug Anal.*, 2016, vol. 24, no. 4, pp. 695−702.

10. Procházková, D., Boušová, I., and Wilhelmová, N., *Fitoterapia*, 2011, vol. 82, no. 4, pp. 513−523.

11. Baranowska, M., Suliborska, K., Chrzanowski, W., Kusznierewicz, B., Namieśnik, J., and Bartoszek, A., *Redox Biol.*, 2018, vol. 17, pp. 355−366.

12. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., and Telser, J., *Int. J. Biochem. Cell Biol.*, 2007, vol. 39, no. 1, pp. 44−84.

13. Dani, C., Bonatto, D., Salvador, M., Pereira, M.D., Henriques, J.A.P., and Eleutherio, E., *J. Agric. Food Chem.*, 2008, vol. 56, no. 11, pp. 4268−4272.

14. Lushchak, V.I., *Acta Biochim. Pol.*, 2006, vol. 53, no. 4, pp. 679−684.

15. Lushchak, V.I., *Biochemistry*, 2010, vol. 75, no. 3, pp. 281−296.

16. Wu, M.J., O'Doherty, P.J., Fernandez, H.R., Lyons, V., Rogers, P.J., Dawes, I.W., and Higgins, V.J., *FEMS Yeast Res.*, 2011, vol. 11, no. 4, pp. 379−387.

17. Sá, R.A., de Castro, F.A. V, Eleutherio, E.C.A., Souza, R.M., da Silva, J.F.M., and Pereira, M.D., *Brazilian J. Microbiol.*, 2013, vol. 44, no. 3, pp. 993−1000.

18. Steels, E.L., Learmonth, R.P. and Watson, K., *Microbiology*, 1994, vol. 140, no. 1994, pp. 569−576.

19. Bernt, E. and Bergmeyer, H.U., *Methods of Enzymatic Analysis*, New York: Academic Press, 1974, 2nd ed.

20. Venu Gopal, J., *Pharmacogn. J.*, 2013, vol. 5, no. 3, pp. 123−126.

21. Salgueiro, F.B., Lira, A.F., Rumjanek, V.M., and Castro, R.N., *Quim. Nova*, 2014, vol. 37, no. 5, pp. 821−826.

22. Demidchik, V., *Environ. Exp. Bot.*, 2014, vol. 109, pp. 212−228.

23. Martins, N., Barros, L., Henriques, M., Silva, S., and Ferreira, I.C.F.R., *Ind. Crops Prod.*, 2015, vol. 74, pp. 648−670.

24. Coleman, J.J., Komura, T., Munro, J., Wu, M.P., Busanelli, R.R., Koehler, A.N., et al., *Future Med. Chem.*, 2012, vol. 2, pp. 11−19.

25. Cigut, T., Polak, T., Gašperlin, L., Raspor, P., and Jamnik, P., *J. Agric. Food Chem.*, 2011, vol. 59, no. 21, pp. 11449−11455.

26. Wilmsen, P.K., Spada, D.S., and Salvador, M., *J. Agric. Food Chem.*, 2005, vol. 53, no. 12, pp. 4757−4761.

27. Fernandes, P.N., Mannarino, S.C., Silva, C.G., Pereira, M.D., Panek, A.D., and Eleutherio, E.C.A., *Redox Rep.*, 2007, vol. 12, no. 5, pp. 236−244.

28. Ayala, A., Muñoz, M.F., and Argüelles, S., *Oxid. Med. Cell. Longev.*, 2014, vol. 2014, pp. 1−31.

29. Mekoue Nguela, J., Sieczkowski, N., Roi, S., and Vernhet, A., *J. Agric. Food Chem.*, 2015, vol. 63, no. 2, pp. 660−670.

30. Celik, S. and Erdogan, S., *Mol. Cell. Biochem.*, 2008, vol. 312, no. 1−2, pp. 39−46.

31. Chen, F. and Gong, P., *Procedia Environ. Sci.*, 2011, vol. 8, pp. 633−636.

32. Coelho, V.R., Vieira, C.G., De Souza, L.P., Moysés, F., Basso, C., Papke, D.K.M., et al., *Life Sci.*, 2015, vol. 122, pp. 65−71.

33. Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., et al., *Int. J. Pharm.*, 2011, vol. 403, no. 1−2, pp.136−138.

34. Morgan, B., Ezerina, D., Amoako, T.N., Riemer, J., Seedorf, M., and Dick, T.P., *Nat. Chem. Biol.*, 2013, vol. 9, no. 2, pp. 119−125.

35. Amari, F., Fettouche, A., Samra, M.A., Kefalas, P., Kampranis, S.C., and Makris, A.M., *J. Agric. Food Chem.*, 2008, vol. 56, no. 24, pp. 11740−11751.