



Comparative evaluation of oxidatively modified proteins in the equine plasma after treatment with extracts derived from leaves of various *Camellia japonica* L. cultivars

Halyna Tkachenko^{1*}, Lyudmyla Buyun², Natalia Kurhaluk¹, Igor Kharchenko²,
Myroslava Maryniuk², Maryna Opryshko², Oleksandr Gyrenko²

¹Institute of Biology and Earth Sciences, Pomeranian University in Słupsk, Poland

²M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine, Kyiv, Ukraine



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The main goal of the current study was to determine the antioxidant activity of leaf extracts of six *Camellia japonica* L. cultivars, using the biomarkers of protein oxidation [aldehydic and ketonic derivatives content] in the *in vitro* equine erythrocyte model. The leaves of *Camellia japonica* cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (Kyiv, Ukraine). Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) for 2 min at room temperature. The extracts were then filtered and used for analysis after two weeks. A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine plasma. Phosphate buffer phosphate buffer was used for positive control. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged and plasma aliquots were used in the study. The aldehydic and ketonic derivatives content as a biomarker of protein oxidation was non-significantly altered after *in vitro* incubation with extracts obtained from the selected *Camellia japonica* cultivars. The percent of the increase had oscillated from 0.2 % (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* cv. La Pace exhibited the highest increase of the level of ketonic derivatives of oxidatively modified proteins (OMP) (by 15.3 %, $p > 0.05$). Cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, respectively. Some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. Such differences could be related to the plant's metabolic state. Screening of *Camellia* species and their cultivars for other biological activities including antioxidant and anti-inflammatory activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Camellia japonica*, cultivars, leaves, extracts, antioxidant activity, aldehydic and ketonic derivatives, protein damage, equine plasma

Introduction

Camellia genus belongs to the Theaceae family, found in southern and eastern Asia, from the Himalayas east to Japan and Indonesia. Green tea (*Camellia*) has received much attention as a beverage worldwide during the last few decades due to its various beneficial effects on human health, including different types of cancer, heart

disease, and liver disease, etc. (Chacko et al., 2010; Bashir et al., 2014). The studies reveal that green tea possesses diverse pharmacological properties, in particular, to lower the incidence of metabolic syndromes, such as obesity, type II diabetes, and cardiovascular risk factors (Chacko et al., 2010). Long-term consumption of tea catechins could be beneficial against high-fat

*Corresponding Author: Halyna Tkachenko, Institute of Biology and Earth Sciences, Pomeranian University in Słupsk, Arciszewski Str. 22b, 76-200 Słupsk, Poland
✉ halyna.tkachenko@apsl.edu.pl

diet-induced obesity and type II diabetes and could reduce the risk of coronary disease (Chacko et al., 2010). Green tea was also reported as useful against HIV strains (Fassina et al., 2002). An aqueous extract of *Camellia sinensis* L. was found to be effective against Gram-positive, Gram-negative bacteria, and fungi (Khan et al., 2019). The most important finding of this study is that its aqueous extract shows inhibitory effect against drug-resistant microorganisms e.g. MRSA, *Pseudomonas aeruginosa*, and *Candida albicans* (Khan et al., 2019). The effects of green tea include anti-oxidative, anti-inflammatory, anti-arthritic, anti-stress, hypolipidaemic, hypocholesterolemic, skin/collagen protective, hepatoprotective, anti-diabetic, anti-microbial, anti-infective, anti-parasitic, anti-cancerous, inhibition of tumorigenesis and angiogenesis, anti-mutagenic, and memory and bone health-improving activities (Alagawany et al., 2020).

Camellia japonica, native to southern Asia (China, Taiwan, Korea, and Japan), is mainly used as an ornamental plant due to its colorful flowers presenting over 32000 recognized cultivars (Savige, 1993; Vela et al., 2013; Páscoa et al., 2019). A thorough literature survey carried out on *C. japonica* revealed that this species has been used traditionally in oriental ethnomedicine for health purposes, such as the treatment of stomach disorders, blood vomiting and bleeding due to internal and external injury, as well as a tonic and anti-inflammatory agent (Yoshikawa et al., 2007; Salinero et al., 2012). The fruits of this plant are used as traditional phytomedicine for the treatment of inflammatory and immunomodulatory diseases (Akanda and Park, 2017). The extract prepared from mature leaves of *C. japonica* has been widely used as an anti-aging material in foods and cosmetics (Mizutani and Masaki, 2014).

It is well known that the *C. japonica* leaf exhibits antioxidant activity due to its high content of polyphenolic compounds (Mizutani and Masaki, 2014). Some studies have already demonstrated that this plant possesses several biological benefits due to the presence of some phenolic compounds in the flowers (Nakajima et al., 1984) as well as aglycon flavonoids (quercetin, kaempferol, and apigenin) and glycosylated flavonoids (rutin and quercetin), and a mixture of saturated fatty acids in the leaves (Azuma et al., 2011). This composition of secondary metabolites seems to be responsible for its anti-plaque, anti-inflammatory, antioxidant activity, antimicrobial, anti-tumoral, antiviral, anti-histaminic, anti-allergic properties, and skin healing activity (Azuma et al., 2011; Mizutani and Masaki, 2014; Jeong et al., 2010; Salinero et al., 2012).

Moreover, *C. japonica* possesses a protective effect against oxidative stress-induced neurotoxicity and hypoglycemic potential (Jeong et al., 2010; Páscoa et al., 2019).

The study of Lee et al. (2017) demonstrated that *C. japonica* extracts promoted antioxidative protein expression and suppressed apoptosis in human corneal epithelial (HCE) cells. Piao et al. (2011) investigating the antioxidant properties of the ethanol extract of the flower of *C. japonica* (*Camellia* extract), revealed that *Camellia* extract exhibits antioxidant properties by scavenging reactive oxygen species (ROS) and enhancing antioxidant enzymes. *Camellia* extract contained quercetin, quercetin-3-O-glucoside, quercitrin, and kaempferol, which are antioxidant compounds. It exhibited 1,1-diphenyl-2-picrylhydrazyl radical and intracellular ROS scavenging activity in human HaCaT keratinocytes. Also, *Camellia* extracts scavenged superoxide anion generated by xanthine/xanthine oxidase and hydroxyl radical generated by the Fenton reaction. Furthermore, it increased the protein expressions and activity of cellular antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Piao et al., 2011).

Nevertheless, we can conclude from the bibliography analyzed that the antioxidant properties have not been comprehensively investigated among *C. japonica* cultivars. In the study of Páscoa et al. (2019), the antioxidant profile (total phenolic and flavonoid content and total antioxidant capacity) of 31 *C. japonica* cultivars leaves was determined and further assessed by near- and mid-infrared spectroscopy.

It is believed that the accumulation of oxidatively damaged proteins is associated with an age-related decline in cellular function. Recently, mammalian animal models have been used to evaluate the levels of these damaged proteins as biomarkers of oxidative stress (Friguet and Baraibar, 2019; Kämpf et al., 2019). The susceptibility of horses to oxidant-induced erythrocyte damage is demonstrated (Walter et al., 2014). Numerous studies using erythrocytes for the evaluation of the biological effects of medicinal plant extracts in cytotoxicity and toxicity assays have been published in the recent literature (Figueirêdo Júnior et al., 2019).

In this context, we have undertaken an attempt to determine the antioxidant activity of six cultivars of *Camellia japonica* i.e. Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants using the biomarker of oxidative modification of proteins [aldehydic and ketonic derivatives] in the

in vitro equine plasma model. Investigations of this type are indicated and used for the preliminary *in vitro* toxicological evaluation of substances with the potential pharmacological application.

Material and methodology

Collection of plant materials and preparation of plant extracts

The leaves of *Camellia japonica* cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (Kyiv, Ukraine). Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) for 2 min at room temperature. The extracts were then filtered and used for analysis after two weeks. The extract was stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54° 30' 48.0" E 16° 57' 44.9"), aged 8.9 ± 1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses, and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 hr, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical, and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood samples were processed for analysis less than 12 hr after blood withdrawal. Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine plasma. For positive control, phosphate buffer was used. After incubating the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Plasma aliquots were used in the study.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract obtained from leaves of *Camellia* cultivars against free radical-induced protein damage in equine plasma, a carbonyl derivatives content of OMP assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocyte suspension and plasma was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1M DNPH (dissolved in 2M HCl) was added to 0.1 ml of the sample after denaturation of proteins by 20 % trichloroacetic acid (TCA). After the addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 hr at 37 °C. The tubes were spun in a centrifuge for 20 min at 3000 g. After centrifugation, the supernatant was decanted and 1 mL of ethanol-ethylacetate solution was added to each tube. Following the mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then spun again (20 min at 3,000 g). The supernatant was decanted and the pellet washed thrice with ethanol-ethylacetate. After the final wash, the protein was solubilized in 2.5 mL of 8M urea solution. To speed up the solubilization process, the samples were incubated in a 90 °C water bath for 10–15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22000 M⁻¹·cm⁻¹. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP₃₇₀) and 430 nm (ketonic derivatives, OMP₄₃₀).

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p > 0.05). The significance of differences between the OMP level (significance level, p < 0.05) was examined using the Kruskal-Wallis one-way analysis of variance (Zar, 1999). The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica software, version 8.0 (StatSoft, Poland) (Zar, 1999).

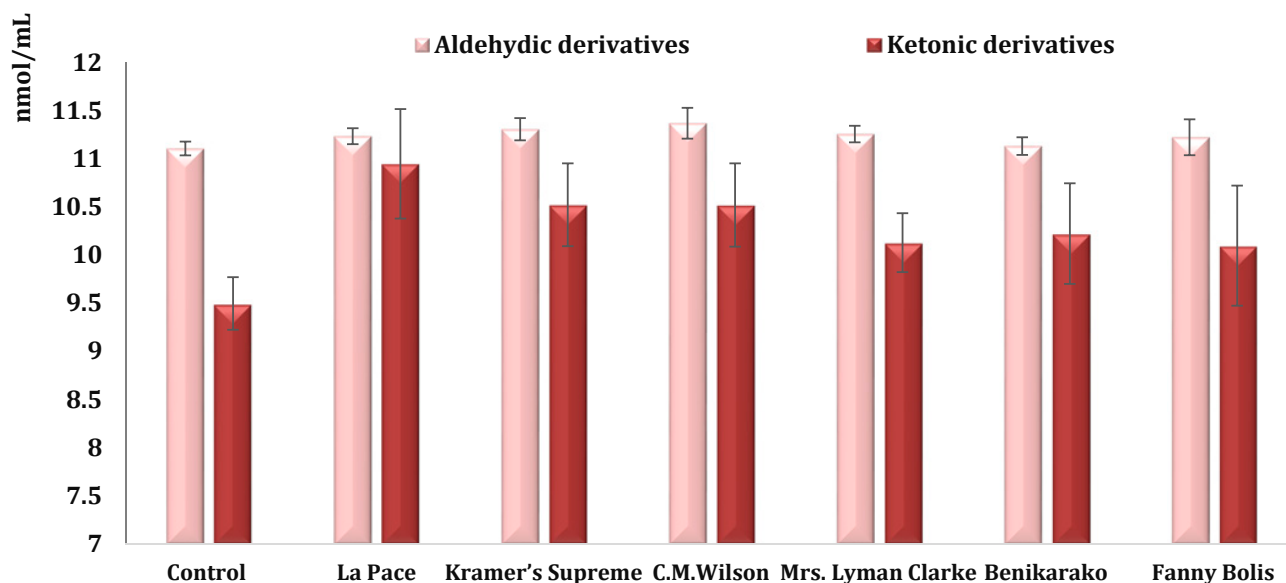


Figure 1 The content of aldehydic and ketonic derivatives as a biomarker of oxidatively modified proteins in the equine plasma after *in vitro* incubation with leaf extracts obtained *Camellia japonica* L. cultivars ($M \pm m$, $n = 18$)

Results and discussion

The present study enabled the determination of the profile of oxidatively modified proteins in equine plasma after *in vitro* incubation with leaf extracts of several *C. japonica* cultivars, namely Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis, through standard biochemical methods as previously described.

The data on the content of aldehydic and ketonic derivatives as a biomarker of oxidatively modified proteins in the equine plasma after *in vitro* incubation with leaf extracts obtained *C. japonica* cultivars was demonstrated in Figure 1.

When equine plasma was incubated with extracts obtained from *C. japonica* cultivars, the content of aldehydic derivatives of OMP was non-significantly increased. The percent of the increase oscillated from 0.2 % (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* 'La Pace' exhibited the highest increase of the level of ketonic derivatives of OMP (by 15.3 %, $p > 0.05$). *Camellia* cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level (by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, $p > 0.05$, respectively), as shown in Figure 1.

As seen from Figure 1, some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. It would be reasonable to suggest that such differences could be related to the plant's

metabolic state. Moreover, it could be explained by the various genetic background of various cultivars used in the current study. It is known, that cultivated *Camellia japonica* has been domesticated for centuries. In *C. japonica*, like most other ornamental flowers, the domestication process has resulted in several types of double flowers characterized by varying degrees and morphology of excessive petals (Sun et al., 2014). Its remarkable diversity of floral forms imparts a rich resource for understanding the genetic regulation of floral patterning and forms (Gao et al., 2005; Sun et al., 2014). Cultivated *Camellia* contains more than 5 types of double flowers with distinctive floral forms semi-double, formal-double, anemone, rose or peony doubles, mainly distinguished by number and arrangement of petals and stamens form (Li et al., 2017).

The *Camellia japonica* cultivars included in this study represent various double flowers types, i.e. "paeony" (Kramer's Supreme and Mrs. Lyman Clarke), "anemone" (C.M. Wilson and Benikarako), "formal double" (La Pace), and "semi-double" (Fanny Bolis).

Results obtained in our previous study showed that there is a possibility of using leaf extracts of various *C. japonica* cultivars as antioxidant agents in intensive aquaculture. The lipid peroxidation (2-thiobarbituric acid reactive substances (TBARS) as biomarker) level in the muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) after incubation with extracts obtained from leaves of various *C. japonica* cultivars was evaluated in our previous study (Kharchenko et

al., 2017a). All extracts (except cultivars Benikarako and Fanny Bolis) reduced the TBARS level in the extracts-treated muscle tissue, but these results were non-significant. Furthermore, the use of such plant products as antioxidants and immunostimulants in aquaculture systems may also have environmental value because of their biodegradability (Kharchenko et al., 2017a). The superoxide dismutase (SOD) activity, an antioxidant enzyme, was increased in the muscle tissue after incubation with *C. japonica* cv. Kramer's Supreme' extract (by 52 %, $p = 0.004$), cv. C.M. Wilson (by 88 %, $p = 0.001$), cv. Mrs. Lyman Clarke (by 87.2 %, $p = 0.000$), and cv. Fanny Bolis (by 40.7 %, $p = 0.044$) compared to the control group. Likewise, the SOD activity in the muscle tissue after incubation with cv. La Pace and cv. Benikarako were also increased (Kharchenko et al., 2018). The results of the investigation revealed quite a high level of total antioxidant capacity (TAC) in samples of muscle tissue incubated with leaf extracts of *C. japonica* cv. C.M. Wilson and cv. Benikarako. The levels of TAC were increased by 41.7 and 44.8 % ($p < 0.05$) as compared with the control group. Leaf extracts of cv. La Pace and cv. Kramer's Supreme being incubated with muscle tissue have not changed the level of TAC, while the effect of the leaves extracts of cv. Mrs. Lyman Clarke and cv. Fanny Bolison the decreasing TAC level was insignificant ($p > 0.05$). The results of the study suggested the high antioxidant capacity of *Camellia* cultivars screened give reason to believe that application of these plant extracts signifies a rational curative strategy to prevent and cure various fish diseases involving oxidative stress by increasing the ability of a fish organism to adapt (Kharchenko et al., 2017b).

In our other study, designed to estimate the possible antioxidant potential of the leaf extracts of *C. japonica* cultivars incubating with equine erythrocytes suspension, the TBARS content as a biomarker of lipid peroxidation was non-significantly altered (accept cv. Mrs. Lyman Clarke). Of the six plant extracts screened, *C. japonica* cv. Mrs. Lyman Clarke exhibited the highest decrease of TBARS level (by 14.4 %, $p < 0.05$). Similarly, cultivars C.M. Wilson, Kramer's Supreme, Benikarako and Fanny Bolis exhibited a non-significant decrease of TBARS level (by 8.8 %, 4.8 %, 3.6 %, and 3.1 %, $p > 0.05$ respectively). TBARS level was non-significantly increased by 3.1 % ($p > 0.05$) after incubation of erythrocyte suspension with leaf extract of 'Fanny Bolis' (Kharchenko et al., 2019). When equine erythrocytes were incubated with leaf extracts obtained from *C. japonica* cultivars, the TAC level was non-significantly altered (Tkachenko et al., 2020).

Recent investigations have associated plants belonging to the *Camellia* genus with anti-carcinogenic, immune-boosting, and antioxidative properties that may impact human health. For example, Higashi-Okai et al. (2001) have analyzed the antioxidant activity of the non-polyphenolic fraction of the residual green tea (*C. sinensis*) after hot water extraction. The non-polyphenolic fraction of residual green tea caused a significant suppression against hydroperoxide generation from oxidized linoleic acid in a dose-dependent manner. The ranks of suppressive activity against hydroperoxide generation were chlorophyll *a* > lutein > pheophytin *a* > chlorophyll *b* > β -carotene > pheophytin *b*. These results suggest that the non-polyphenolic fraction of residual green tea has potent suppressive activity against hydroperoxide generation from oxidized linoleic acid, which is derived from the antioxidant activities of chlorophylls *a* and *b*, pheophytins *a* and *b*, β -carotene, and lutein (Higashi-Okai et al., 2001).

The phenolic profiles, antioxidant and antiproliferative activities of 27 tea cultivars were determined by Zeng et al. (2017). Wide ranges of variation were found in analyzed cultivars for the contents of water-soluble phenolics (121.6–223.7 mg/g dry weight (DW)), total catechins (TC) (90.5–177.2 mg/g DW), antioxidant activities [peroxyl radical scavenging capacity (PSC) values 627.3–2332.3 μmol of vitamin C equiv./g DW, Oxygen radical absorbance capacity (ORAC) values (1865.1–3489.3 μmol of vitamin C equiv./g DW), cellular antioxidant activity (CAA) values (37.7–134.3 μmol of QE/g DW without PBS wash and 25.3–75.4 μmol of QE/g DW with PBS wash)] and antiproliferative activity (53.0–90.8 % at the concentration of 400 $\mu\text{g}/\text{mL}$ extracts). The PSC, ORAC, and CAA values were significantly correlated with phenolics, epicatechin gallate (ECG), CC, and TC (Zeng et al., 2017). Ohmori et al. (2005) have assessed the antioxidant activity of six teas, including the aqueous extracts of green tea and oolong tea (*Camellia sinensis*), tochu (*Eucommia ulmoides*), *Gymnema sylvestre*, Japanese mugwort (*Artemisia princeps*), and barley (*Hordeum vulgare*), against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and LDL oxidation, and examined the association of LDL oxidizability with the plasma catechin levels in 10 healthy volunteers with a single dose of 5 g green tea powder. Green tea, therefore, showed the strongest antioxidant activity among the six different tea, and the inhibitory effects of green tea on LDL oxidation depended on the plasma catechin levels (Ohmori et al., 2005).

Many research papers have evidenced that the anti-inflammatory and gastroprotective mechanism of *C. japonica* is mediated by the modulation of oxidative stress, inflammatory cytokines, and enzymes via suppression of MAPK/NF- κ B signaling pathways. Akanda and Park (2017) have investigated the immunopharmacological activities of *C. japonica* and have validated its pharmacological targets. These researchers found the production of NO and reactive oxygen species in RAW 246.7 cells were both suppressed by *C. japonica*. Moreover, *C. japonica* mitigated the HCl/EtOH-induced oxidative stress in gastric mucosa via the reduction of lipid peroxidation and elevation of NO production. Gastric mucosal damages were prominently improved by *C. japonica*, as confirmed by the histopathological evaluation. The gene expression of inflammatory cytokines and enzymes tumor necrosis factor α (TNF- α), interleukin 6 and 1β (IL-6, IL- 1β), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) was notably downregulated by *C. japonica*. Also, *C. japonica* markedly attenuated the mitogen-activated protein kinases (ERK1/2, JNK, and p38) phosphorylation, COX-2 expression, and activation of transcription factor NF- κ B and as well as phosphorylation and degradation of I κ B α in the gastric mucosa (Akanda and Park, 2017).

Other species of *Camellia* plants also are a promising source of natural antioxidants and further studies might be a likely source of its use in remedy of different diseases. *C. sinensis* L. is traditionally used in many polyherbal preparations for the treatment of different diseases and infections. Its action has been associated with its antioxidant activities. As tea is a very popular beverage, tea polyphenols are expected to be a potent antioxidant and chemopreventive agents that can be taken with a normal diet and can be nontoxic due to their natural origin (Bag and Bag, 2020). Catechins are powerful antioxidants, and laboratory studies have suggested that these compounds may inhibit cancer cell proliferation. Some experimental and nonexperimental epidemiological studies have suggested that green tea may have cancer-preventative effects (Filippini et al., 2020).

Conclusions

The aldehydic and ketonic derivatives content as a biomarker of protein oxidation was non-significantly altered after *in vitro* incubation with extracts obtained from selected *Camellia japonica* cultivars. The percent of the increase had oscillated from 0.2 (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* cv. La Pace exhibited the highest

increase of the level of ketonic derivatives of OMP (by 15.3 %, $p > 0.05$). Cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, $p > 0.05$ respectively. Some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. We attribute the observed differences to the use of *C. japonica* cultivars with various genetic backgrounds. Moreover, such differences could be related to the plant's metabolic state. Overall, our analysis suggests that screening of *Camellia* species for other biological activities including antioxidant and anti-inflammatory activities is essential and may be effective for searching the preventive agents to be used in the pathogenesis of some metabolic diseases.

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