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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTE SUSPENSION AFTER *IN VITRO* INCUBATION WITH LEAF EXTRACT OBTAINED FROM *RHODODENDRON MYRTIFOLIUM* SCHOTT & KOTSCHY (ERICACEAE)

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A noteworthy European member of the genus is *Rhododendron myrtifolium* Schott & Kotschy, an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1,400–2,500 m. It has been shown that *R. myrtifolium* possesses antibacterial and antiviral effects of their extracts, while the biochemical features and bioactive potentials of *R. myrtifolium* remain unexplored. In the current study crude aqueous extract from the leaves of *R. myrtifolium* was assessed for antioxidant activities, cytotoxicity, and anti-hemolytic potential. The aim of this study was to assess possible antioxidant and anti-hemolytic effects of an extract derived from *R. myrtifolium* leaves using oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity (TAC)] and HCl-induced hemolysis assay on equine erythrocytes' model. Our results demonstrated that the treatment by extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL decreased the TBARS level in the equine erythrocytes' suspension, while aldehydic and ketonic derivatives of oxidatively modified proteins and total antioxidant capacity increased. These changes were statistically non-significant. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl. The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min compared to 12 min in the control sample. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract. On the other hand, no changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were noted. The erythrocytes maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation. Therefore, extract obtained from the leaves of *R. myrtifolium* in dose 5 mg/mL Interestingly, when assayed for its cytotoxic evaluation against equine erythrocytes,

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the extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL showed mild hemolytic activity, while the oxidative stress biomarkers were non-significantly changed. Our results further suggest that *Rhododendron myrtifolium* could be further investigated for the isolation and purification of the active constituents.

Keywords: *Rhododendron myrtifolium* Schott & Kotschy, leaf extracts, 2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity, hemolysis

Introduction

The genus *Rhododendron* L. (Ericaceae) is one of the most species-rich among angiosperms, comprising over 1,000 species spreading across the northern hemisphere and with the center of diversity in southeastern Asia (Irving and Hebda, 1993). This genus is a rich source of phenolic compounds, especially flavonoids, essential oils, chromones, terpenoids, and steroids. It has many biological properties such as antioxidant, anti-inflammatory, antiviral, antibacterial, anticancer, antidiabetic, immunomodulatory, cardioprotective and hepatoprotective among others due to their polyphenolic constituents (Demir et al., 2016). *In vivo* and *in vitro* testing of plant extracts and isolated compounds determined diverse biological activities including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, insecticidal and cytotoxic activity (Popescu and Kopp, 2013).

Plants belonging to the genus *Rhododendron* generally used for traditional medicine. For example, *R. anthopogonoides* Maxim, a kind of traditional Tibetan medicine, has been used to remove body heat, body detoxification, cough, asthma, stomachic and swelling, eliminate abundant phlegm and inflammatory for a long time (Jing et al., 2015). The ethyl acetate and *n*-butanol fractions of *R. anthopogonoides* had significant antioxidant activity and could prevent PC12 cells against hypoxia-induced injury. So it might be regarded as an excellent source of antioxidants and had great potential to explore as a therapeutic agent for preventing hypoxia-related sickness in the future (Jing et al., 2015). *R. fauriei* Franch. (syn. *R. brachycarpum*) is a flowering plant generally used for traditional medicine in Korea to treat hypertension, neuralgia, and sterility. Previous studies have also shown that *R. fauriei* extract alleviates inflammation and antimicrobial activity (Yang et al., 2017). The roots of *R. mucronulatum* Turz. have been used in Oriental traditional medicine for the treatment of dysuria, fever, an increase of digestive activity and tonics in China and Korea (Choi et al., 2011).

The various studies also reveal the strong antioxidant activity of plants belonging to the *Rhododendron* genus. The biological activities of major procyanidins isolated from the leaf extract of *R. formosanum* Hemsl. were investigated by Wang et al. (2015). All compounds showed pronounced antioxidant activities and the activities are enhanced as the amount of OH groups in procyanidins increased. The pleiotropic effects of procyanidins isolated from the leaves of *R. formosanum* can be a source of promising compounds for the development of future pharmacological applications (Wang et al., 2015). On the other hand, Guo et al. (2017) have revealed that the ultrasound-assisted extraction of *R. aganniphum* Balf. f. & Kingdon-Ward presented good radical scavenging activity, superoxide and hydroxyl radicals *in vitro*

and could be used as a natural antioxidant in the food and medical industries. The methanolic extracts of *R. pseudochrysanthum* Hayata leaves have excellent antioxidant activities and great potential as a source for natural health products (Lin et al., 2014).

A noteworthy European member of the genus is *R. myrtifolium* Schott & Kotschy, an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1,400–2,500 m. The species is featured in small narrowly elliptic to obovate coriaceous leaves abaxially covered with glandular scales containing essential oils, terminal inflorescences of tubular-campanulate pinkish flowers, and long-pedunculate dry multilocular capsules containing numerous diminutive seeds (Cullen, 1980, 2005; Mircea, 2005; Boratyński et al., 2006; Voloschuk and Prokopiv, 2011). Although endangered in countries of its distribution, *R. myrtifolium* has been used in folk medicine for the preparation of herbal teas (Dihoru and Boruz, 2014; Nedelcheva and Draganov, 2014) and presents a major touristic attraction during its mass flowering period in mountains (Rivers, 2017). Its evolutionary closest relatives, *R. ferrugineum* L. and *R. hirsutum* L. (e.g., Sosnovsky et al., 2017) have been shown to possess cytotoxic, antibacterial, and antiviral effects of their extracts (Louis et al., 2010; Gescher et al., 2011; Seephonkai et al., 2011; Rezk et al., 2015b), while the biochemical features and bioactive potentials of *R. myrtifolium* remains unexplored.

In the current study crude aqueous extracts from the leaves of *R. myrtifolium* was assessed for antioxidant activities, cytotoxicity, and anti-hemolytic potential. The aim of this study was to assess possible antioxidant and anti-hemolytic effects of an extract derived from *R. myrtifolium* leaves using oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity (TAC)] and HCl-induced hemolysis assay on equine erythrocytes' model.

The erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure (Pagano and Faggio, 2015). Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with hemolysis (Pagano and Faggio, 2015; Farag and Alagawany, 2018). Red blood cells along with its membrane have always been an important medium for the study due to the important role it plays in varied physiological and metabolic processes (Jha et al., 2009; Karabulut et al., 2009; Pandey et al., 2009). The erythrocyte could be isolated and handled easily so that they could provide a good model for many assays (Alagawany et al., 2016; Farag and Alagawany, 2018). Additionally, the high concentration of polyunsaturated fatty acids in RBCs membrane, the high oxygen tension, and redox-active hemoglobin molecules [the source of reactive oxygen species (ROS) in erythrocyte] make them a good biological lipid membrane model especially for screening the oxidative stress conditions induced by various substances (Farag and Alagawany, 2018).

Material and methodology

Collection of Plant Materials

Leaves of *Rhododendron myrtifolium* were harvested on the side of the road between the Menchul valley and Rogneska valley (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 28.4“, E 24° 20‘ 05.6“, 1,485 m a.s.l.). Plant samples were thoroughly washed to remove all the attached materials and used to prepare the ethanolic extract.

Preparation of Plant Extract

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

Eighteen clinically healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 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 h, and water available *ad libitum*. Before sampling, all horses were thoroughly examined clinically by a veterinarian and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma Blood was stored into The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control, phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of per 1 L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

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

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension 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 et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Assay of Acid Resistance of Erythrocytes

The acid resistance of erythrocytes was measured spectrophotometrically with 0.1 M HCl (Terskov and Gitelson, 1957). The assay is based on the measuring of the dynamics of erythrocytes disintegration into hemolytic reagent action. The time of hemolytic reagent action serves as the measure of erythrocyte resistance. The assay mixture contained 5 mL of 1% erythrocyte suspension and 0.05 mL of 0.1 M HCl. The absorbance was read at 540 nm every 30 seconds after HCl addition till the end of hemolysis. The difference of absorbance at the beginning and at the end of hemolysis was determined as 100% (total hemolysis). The disintegration of erythrocytes (%) at every 30seconds was expressed as a curve.

Morphological alterations of erythrocytes

The smears were fixed by dipping the slides in absolute methanol, allowing them to air-dry, and then staining with May-Grunwald solution for 5 min, followed by 6% Giemsa stain for 15 min. Slides were selected on the basis of staining quality. In each group, 10,000 cells (minimum of 1.000 per slide) were examined under a 40× objective with a 10× eyepiece (Microscope Leica DM300) to identify morphologically-altered erythrocytes in separate studies.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney U-test (Zar, 1999). In addition, the relationships between oxidative stress biomarkers were

evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes' suspension after *in vitro* incubation with *R. myrtifolium* leaf extract was shown in Figure 1.

As can be seen in Figure 1, treatment by extract caused the reduced erythrocytes' TBARS level by 7.5% (p >0.05), while the ketonic aldehydic and ketonic derivatives level was increased (by 5.4 and 5.3 5, p >0.05, respectively) as compared to untreated erythrocytes. However, these changes were statistically non-significant. The TAC level was statistically non-significant changes.

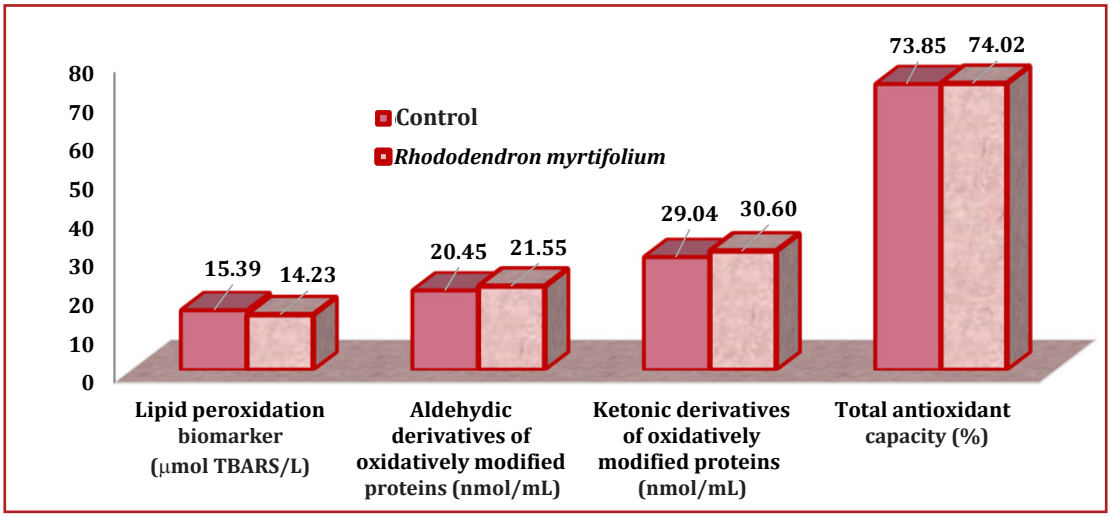

Figure 1 The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes' suspension after *in vitro* incubation with *Rhododendron myrtifolium* leaf extract (M ±m, n = 18)

The representative Figure 2 shows the observed values of % hemolysis with time at 5 mg/mL after the treatment by extract obtained from *R. myrtifolium* leaves.

In the control group (untreated erythrocytes' suspension), erythrocytes incubated with 0.1 M HCl remained stable and demonstrated slight hemolysis. The maximum level of hemolysis was (14.6 ±0.85%); the total duration of hemolysis was 12.0 min. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl (18.1 ±1.21%). The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract (Figure 2).

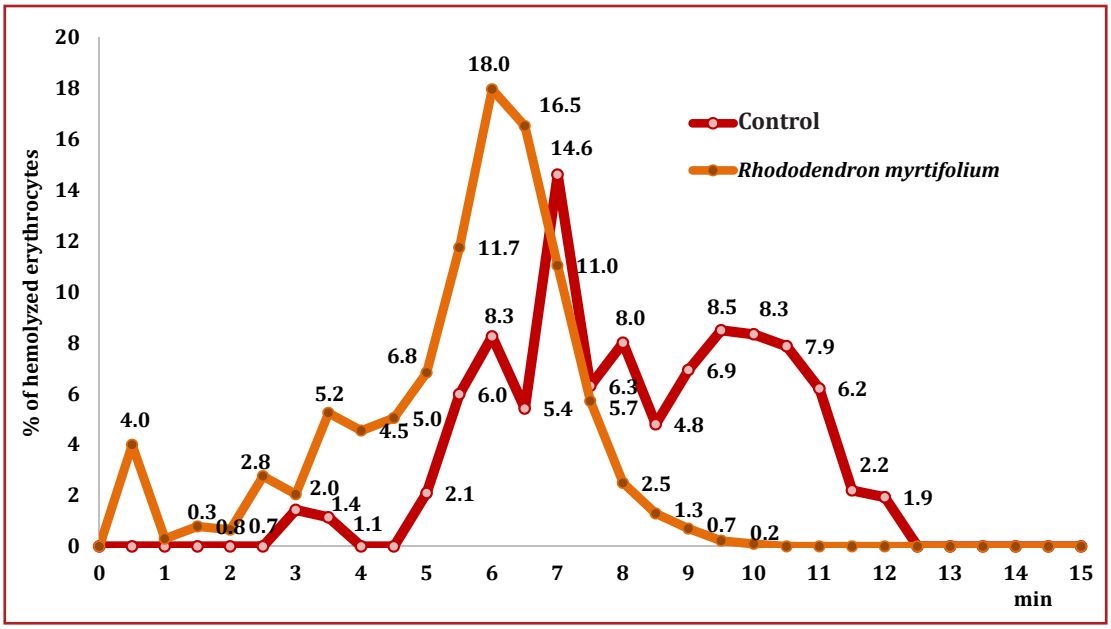

Figure 2 Effects of *Rhododendron myrtifolium* leaf extract (5 mg/mL) on HCl-induced hemolysis in equine erythrocytes (n = 18)

The morphological changes of equine erythrocytes for 1 h of incubation with *R. myrtifolium* extract is shown in Figure 3.

The normal red blood cell shape is a biconcave discocyte. Intercalation of a drug in the outer half of the membrane lipid bilayer leads to echinocytosis, intercalation in the inner half to stomatocytosis (Reinhart et al., 2014). The observation of the photomicrographs revealed that the untreated erythrocytes are a normal biconcave shape (Figure 3A). No changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were also demonstrated. The erythrocytes in the presence of *R. myrtifolium* extract maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation (Figure 3).

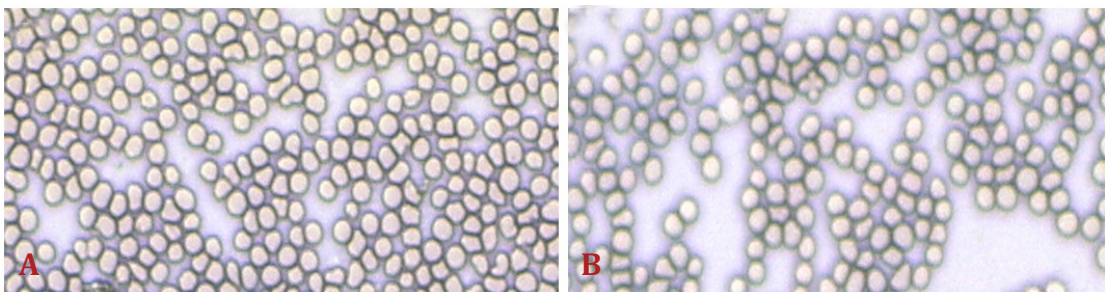

Figure 3 Effect of *Rhododendron myrtifolium* extract on morphological changes of equine erythrocytes for 1 h of incubation. Control sample (A), *R. myrtifolium* extract (5 mg/mL) (B)

The results of this study are consistent with other studies that have previously been reported that chemicals derived from various *Rhododendron* plants are promising candidates as antioxidant and anti-inflammatory agents. For example, Choi et al. (2011) have investigated the antioxidative and anti-inflammatory effects of compounds derived from *R. mucronulatum* Turcz., their 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities and the protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated HaCaT cells and their end products, nitric oxide (NO) and prostaglandin E2 (PGE2), respectively. All compounds showed potent DPPH radical scavenging compared with positive controls (L-ascorbic acid). Also, taxifolin and taxifolin 3-O-β-D-glucopyranoside dose-dependently inhibited the expressions of inflammatory mediators, NO and PGE2, suggesting they are promising candidates as anti-inflammatory agents (Choi et al., 2011).

On the other hand, *R. album* Blume may prove to be an effective therapeutic agent for the treatment of inflammatory diseases. The biological effects of *R. album* methanol extract (RAME) on inflammation was investigated in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 cells by Park et al. (2015). These researchers have investigated the effects of RAME on the production of nitric oxide (NO) and prostaglandin E2 (PGE2) in LPS-stimulated RAW264.7 cells. To explore the anti-inflammatory mechanisms of RAME, they measured the mRNA and protein expression of pro-inflammatory mediators induced by RAME in the LPS-stimulated RAW264.7 cells by RT-PCR and western blot analysis, respectively. RAME significantly inhibited the production of NO, PGE2, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α in the LPS-stimulated RAW264.7 cells. It also suppressed the mRNA and protein expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and mitogen-activated protein kinases (MAPKs) with a concomitant decrease in the nuclear translocation of nuclear factor-κB (NF-κB) in the LPS-stimulated RAW264.7 cells. Therefore, RAME inhibits LPS-induced inflammatory responses. These effects were considered to be strongly associated with the suppression of NF-κB activation (Park et al., 2015).

Medicinal plants and herbs are possessed compound that acts as antioxidants mainly due to the presence of polyphenolics and flavonoids. Antioxidant flavonoids have been isolated from the flower of *R. yedoense* var. *poukhanense* by Jung et al. (2007). One new flavonoid and three known flavonoids, quercetin-5-O-beta-D-glucopyranoside (1), quercetin (3), and quercitrin (4), were isolated from the butanol and ethyl acetate extracts of the plant. The new flavonoid was identified as myricitrin-5-methyl ether (2). The isolation of these flavonoids from this plant, for the first time, is a valuable finding. The flavonoids were evaluated for their antioxidant activities using 1.1-diphenyl-2-picrylhydrazyl free radical (DPPH), TBARS (2-thiobarbituric acid reactive substance) and superoxide anion radical ($O_{2}^{\cdot-}$) in the xanthine/xanthine oxidase assay system. The antioxidant activities of myricitrin-5-methyl ether (2) and quercetin (4) were higher than that of L-ascorbic acid when evaluated using a TBARS assay (Jung et al., 2007).

The timing of the harvest of plants impacts the plant's phenolic content and its antioxidant and anti-inflammatory activities. For instance, Black et al. (2011) have assessed seasonal variation of phenolic constituents and medicinal activities of Northern Labrador tea, *R. tomentosum* ssp. *subarcticum*. The antioxidant potency was measured in a DPPH radical scavenging

assay, and the anti-inflammatory activity was determined with a TNF-α production assay. The most abundant constituent was (+)-catechin, which made up 19% of the total weight of characterized phenolics. There was significant seasonal variation in the quantity of all constituents assessed, whereas there was no seasonal variation of their total sum. The antioxidant activity was positively correlated with phenolic content and negatively correlated with daylight hours. The anti-inflammatory activity was negatively correlated with caffeic acid derivative 1 and daylight hours (Black et al., 2011).

The antioxidant and antimutagenic effects of the hexane, chloroform, and ethyl acetate fractions of *R. arboreum* Sweet leaves and their chemical composition was carried out by Gautam et al. (2018). The different fractions inhibited lipid peroxidation, repressed the production of nitric oxide radicals, and prevented deoxyribose degradation. The antimutagenic activity of the leaf fractions was analyzed against 4-nitro-O-phenylenediamine, sodium aside and 2-aminofluorene mutagens in two test strains (TA-98 and TA-100) of Salmonella typhimurium (Gautam et al., 2018). The evaluation of the antioxidant properties and cytotoxic activity of dimethyl sulfoxide extract of flowers of *R. luteum* was done by Denim et al. (2016). *R. luteum* extract exhibited selective cytotoxicity against colon and liver cancer cells compared to normal fibroblast cells, while this selective cytotoxicity was not observed in breast cancer cells (Denim et al., 2016).

Our results suggested that extracts from these plants can be further used for the isolation and structural characterization of valuable bioactive compounds and investigated in *in vivo* experimental model of the disease.

Conclusions

In our study, treatment by extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL decreased the TBARS level in the equine erythrocytes' suspension, while aldehydic and ketonic derivatives of oxidatively modified proteins and total antioxidant capacity increased. These changes were statistically non-significant. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl. The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min compared to 12 min in the control sample. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract. On the other hand, no changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were noted. The erythrocytes maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation. Therefore, extract obtained from the leaves of *R. myrtifolium* in dose 5 mg/mL Interestingly, when assayed for its cytotoxic evaluation against equine erythrocytes, the extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL showed mild hemolytic activity, while the oxidative stress biomarkers were non-significantly changed. Our results further suggest that *Rhododendron myrtifolium* could be further investigated for the isolation and purification of the active constituents.

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