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EFFECT OF *RHODODENDRON MYRTIFOLIUM* SCHOTT & KOTSCHY LEAF EXTRACTS ON HCL-INDUCED HEMOLYSIS IN HUMAN ERYTHROCYTES

Prokopiv Andriy[1, 3], Tkachenko Halyna*[2], Honcharenko Vitaliy[1], Kurhaluk Natalia[2], Nachychko Viktor[1, 3], Sosnovsky Yevhen[3], Osadowski Zbigniew[2]

[1]Department of Botany, Faculty of Biology, Ivan Franko National University in Lviv, Lviv, Ukraine
[2]Institute of Biology and Earth Sciences, Pomeranian University in Słupsk, Poland
[3]Botanic Garden of Ivan Franko National University in Lviv, Lviv, Ukraine

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Many species of the genus *Rhododendron* L. contain a large number of chemical compounds that possessed the antioxidant properties. *Rhododendron myrtifolium* Schott & Kotschy possesses cytotoxic, antibacterial, and antiviral effects, while its biochemical features and bioactive potentials remain unexplored. In the current study, the *in vitro* hemolytic activity of *R. myrtifolium* Schott & Kotschy extract in three different doses on human erythrocytes was evaluated. The total hemolysis (%) was obtained using 0.1 M HCl compared to the phosphate saline buffer as a control sample. Therefore, the aim of the current study was to assess possible antioxidant and anti-hemolytic effects of extract derived from *R. myrtifolium* leaves using oxidative stress biomarker [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation] and HCl-induced hemolysis assay on human erythrocytes' model after incubation with plant extracts in three doses (5.0, 2.5, and 1.25 mg/mL). Our results revealed that the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level. The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted. On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest percent of hemolyzed erythrocytes and decrease hemolysis duration. This study demonstrated, that among the leaf extract of *R. myrtifolium*, the dose of 5 mg/mL exhibited the highest level of lipid peroxidation biomarker and morphological changes in the human erythrocytes. In addition, the study also demonstrated that *R. myrtifolium* leaf extract at dose 1.25 mg/mL possessed hemolytic effect *in vitro* compared to other doses studied. Therefore, further investigation of it's *in vitro* and *in vivo* activity is warranted.

Keywords: *Rhododendron*, leaf extracts, 2-thiobarbituric acid reactive substances (TBARS), hemolysis, erythrocyte suspension

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

Introduction

As one of the largest genera of vascular plants, *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). The phytochemistry and bioactivity of this genus have been investigated, and many plants have been demonstrated to have significant biological activities, including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, antioxidant, insecticidal and cytotoxic activity (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). Some members of the genus *Rhododendron* were used in traditional medicine for arthritis, acute and chronic bronchitis, asthma, pain, inflammation, rheumatism, hypertension, and metabolic diseases (Popescu and Kopp, 2013).

Recent studies have shown that many species of the genus *Rhododendron* contain a large number of phenolic compounds possessed the antioxidant properties that could be developed into pharmaceutical products (Qiang et al., 2011; Lin et al., 2014, 2016). Phenolic compounds decrease the risk of serious health concerns due to their antioxidants properties to the oxidative damage by reactive oxygen species (ROS). Plant phenolic compounds can act as antioxidants, structural polymers (lignin), attractants (flavonoids and carotenoids), UV screens (flavonoids), signal compounds (salicylic acid, flavonoids) and defense response chemicals (tannins, phytoalexins) (Lin et al., 2016). Therefore, the using of antioxidant components from natural resources such as plants is getting significant attention since the last few decades (Yasin et al., 2017).

Determination of cellular toxicity of a drug towards human erythrocytes is the basic step of drug development against any particular disease. Hemolytic assays for the plant extracts were carried out due to the fact that compounds possessing potent biological activity may not be appropriate in pharmacological preparations if they have hemolytic effects. This assay is usually used to check the biocompatibility of potential drug against erythrocytes in which the interaction of molecules with the cells (RBCs) results in the formation of pores in the membrane that ultimately leads to the release of hemoglobin (Mishra et al., 1981; Lieber et al., 1984; Katsu et al., 1986; Baumann et al., 2000). In the current study, the *in vitro* hemolytic activity of *Rhododendron myrtifolium* Schott & Kotschy extract in three different doses on human erythrocytes was evaluated. The total hemolysis (%) was obtained using 0.1 M HCl compared to the phosphate saline buffer as a control sample.

R. myrtifolium is 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 1400–2500 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., 2015a, b), while the biochemical features and bioactive potentials of *R. myrtifolium* remain unexplored.

We continue to assess the dose-dependent antioxidant potential of crude aqueous extracts from the leaves of *R. myrtifolium* sampled in the western part of Ukraine using a human erythrocytes' model. The aim of the current study was to assess possible antioxidant and anti-hemolytic effects of extract derived from *R. myrtifolium* leaves using oxidative stress biomarker and HCl-induced hemolysis assay on human erythrocytes' model after incubation with plant extracts.

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.

Human blood samples

Blood (10–20 ml) was obtained from normal volunteers via venipuncture (4 males and 5 females aged 28–53-years old). The Research Ethics Committee of Regional Medical Chamber in Gdańsk (Poland) approved the study (KB-31/18). All patients provided written informed consent before the start of the study procedures. Human erythrocytes from citrated blood were isolated by centrifugation at 3,000 rpm for 10 min and washed two times with 4 mM phosphate buffer (pH 7.4) and then re-suspended using the same buffer to the desired hematocrit level. Cells stored at 4 °C were used within 6 h of sample preparation. An erythrocytes' suspension at 1% hematocrit was incubated with 4 mM phosphate buffer (pH 7.4) (control) and pre-incubated with the extracts (5.0, 2.5, and 1.25 mg/mL, respectively) at 37 °C for 60 min. This reaction mixture was shaken gently while being incubated for a fixed interval at 37 °C. For positive control (phosphate buffer) was used. Erythrocytes' aliquots were used in the study.

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 TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of MDA per L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

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 in the human erythrocytes' suspension after *in vitro* incubation with *R. myrtifolium* leaf extract was shown in Figure 1.

Our results revealed that treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level by 47.1% (p <0.05), 31.2% (p <0.05), and 105.6% (p <0.05), respectively (Figure 1). The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL.

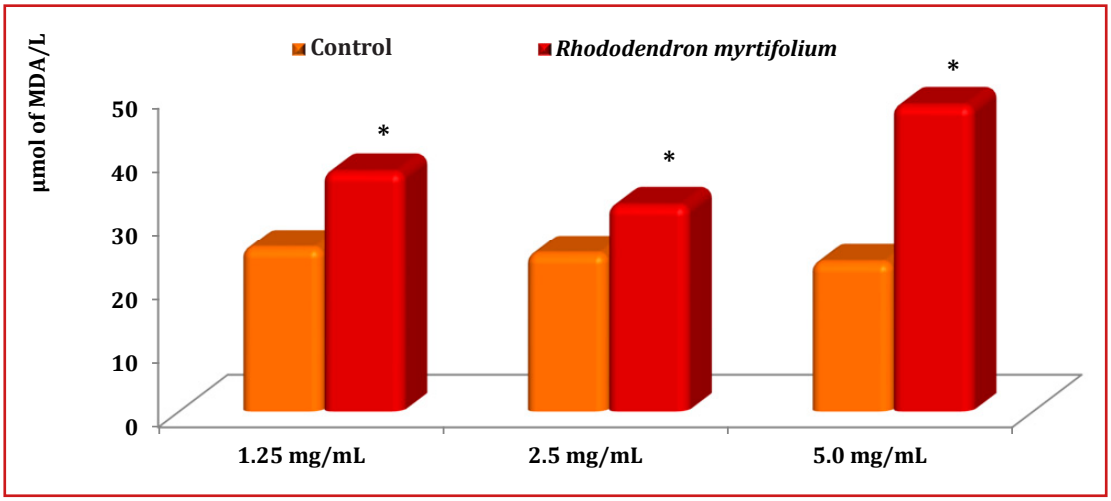

Figure 1 The TBARS content as a biomarker of lipid peroxidation in the human erythrocytes' suspension after *in vitro* incubation with *Rhododendron myrtifolium* leaf extract in different doses (1.25, 2.5, 5.0 mg/mL) (n = 9)

The representative Figure 2 shows the observed values of % hemolysis with time at 1.25, 2.5, 5.0 mg/mL after the treatment by extract obtained from *R. myrtifolium* leaves.

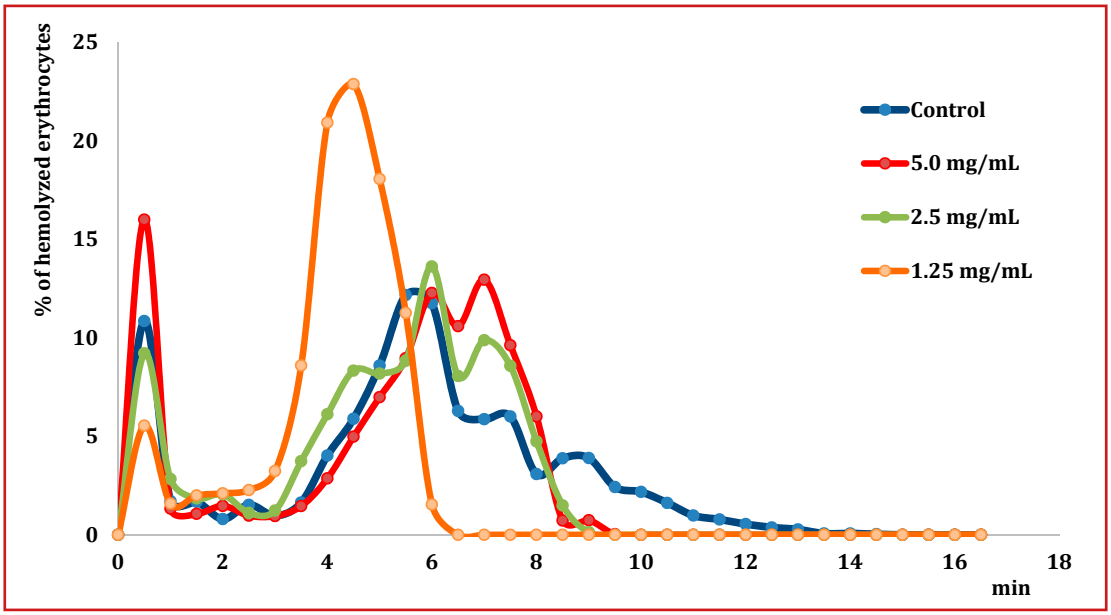

Figure 2 Effects of *R. myrtifolium* extract in different doses (1.25, 2.5, 5.0 mg/mL) on HCl-induced hemolysis *in vitro* in human erythrocytes (n = 9)

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 (12.17 ±1.02%); the total duration of hemolysis was 15.0 min. When *R. myrtifolium* extract

(5 mg/mL) was added to the erythrocytes' suspension, the maximum level of hemolysis has occurred after 0.5 min of incubation with 0.1 M HCl (16.0 ±1.11%). The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.0 min. At the concentration of *R. myrtifolium* extract of 2.5 mg/mL, it decreased the hemolysis (the maximum % of hemolysis was 13.6 ±0.98% at 6.0 min, the total duration of hemolysis was 9.5 min). Interestingly, *R. myrtifolium* extract at 1.25 mg/mL caused the highest % of hemolyzed erythrocytes (the maximum % of hemolysis was 22.9 ±2.32% at 4.5 min, the total duration of hemolysis was 6.5 min). Therefore, we selected *R. myrtifolium* extract at a concentration of 2.5 mg/mL for the next study. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract at different doses (Figure 2).

Effect of *R. myrtifolium* extract at 1.25, 2.5, 5.0 mg/mL on morphological changes of human erythrocytes for 1 h of incubation is shown in Figure 3.

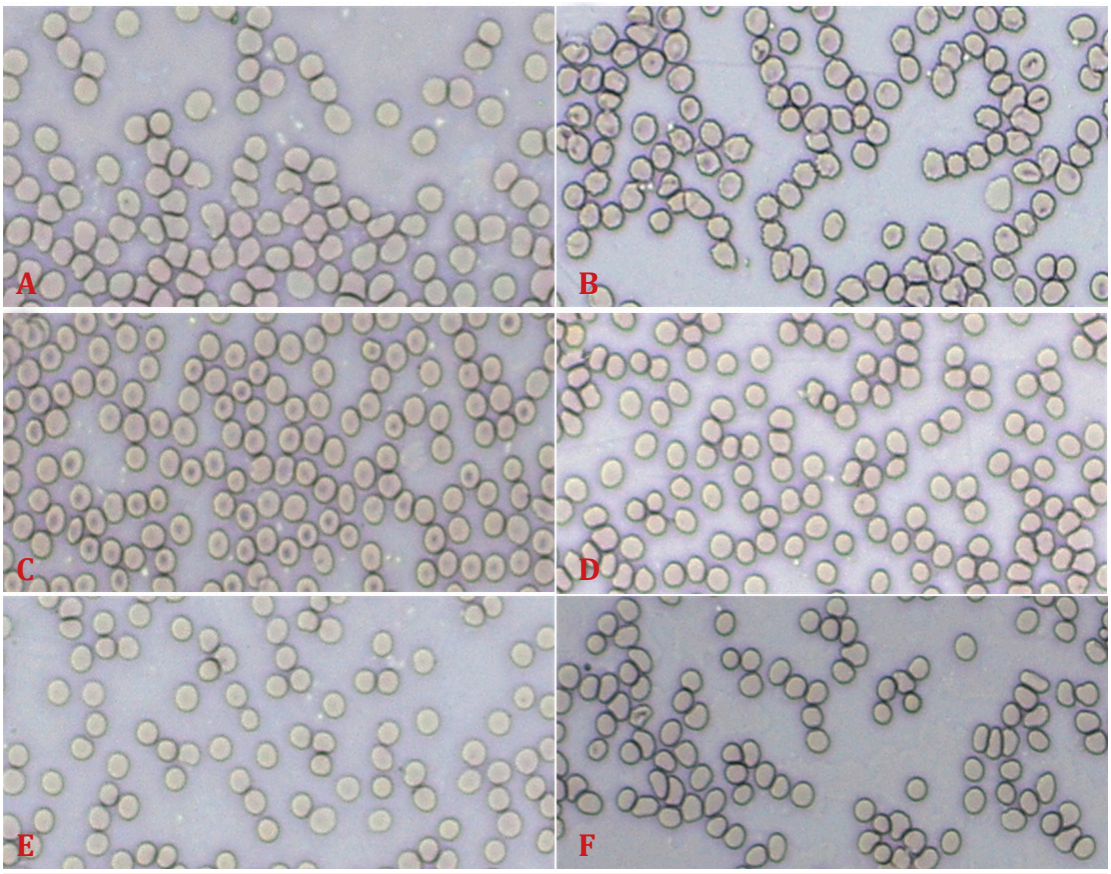

Figure 3 Effect of *R. myrtifolium* extract in different doses on morphological changes of human erythrocytes for 1 h of incubation. (A), (C), and (E) Control samples, (B) *R. myrtifolium* extract (5.0 mg/mL), (D) *R. myrtifolium* extract (2.5 mg/mL), (F) *R. myrtifolium* extract (1.25 mg/mL)

As shown in Figure 3 (A, C, E), the untreated erythrocytes are a normal biconcave shape. After exposure to *R. myrtifolium* extract in dose 5 mg/mL, protuberances and ruffled edges

on erythrocytes were noted (Figure 3B). No changes in the size and shape of cells, as well as protuberances on their surfaces, were demonstrated after exposure to *R. myrtifolium* extract in both doses 2.5 mg/mL and 1.25 mg/mL (Figure 3D, F).

Our results demonstrated that the extract deriving from the leaves of *R. myrtifolium* showed large variations in TBARS levels (Figure 1). The highest TBARS level was determined for a dose of 5 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted (Figure 3D). On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest % of hemolyzed erythrocytes and decrease hemolysis duration (Figure 2).

In vivo and *in vitro* testing of *Rhododendron* plants and isolated compounds determined diverse biological activities including cytotoxic activity (Popescu and Kopp, 2013). In several studies demonstrated that *Rhododendron* spp. can cause intoxications in humans (Popescu and Kopp, 2013; Denim et al., 2016). The toxicity is due to grayanotoxins, diterpenes which activate voltage-gated sodium channels and lead to gastrointestinal, cardiac and central nervous system symptoms (Popescu and Kopp, 2013). For example, diterpenes, such as rhodojaponin III, were considered as the toxic agents associated with the toxicities of *R. molle* G. Don. In China, the *R. molle* is traditionally and commonly recognized as a toxic plant as other plants in rhododendrons (Cai et al., 2018). *Rhododendron honey*, locally known as „mad honey", contains grayanotoxin (GTX) and thus induces toxic effects when consumed in large amounts. But, it is still popularly used for treating medical conditions such as high blood pressure or gastrointestinal disorders (Sibel et al., 2014). The effect of grayanotoxin from *R. honey* on antioxidant parameters measured from rats fed with *R. honey* was evaluated by Sibel et al. (2014). It has been observed that both grayanotoxin and high dose *R. honey* treatments showed an oxidant effect on blood plasma and organ tissues of Sprague-Dawley female rats. The biochemical analysis of the various parameters (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, creatine kinase, and creatine kinase muscle and brain) showed a significant elevation with increasing concentration of honey (Sahin et al., 2016). On the other hand, a low dose (1 g/kg/bw) of *R. honey* produces no acute adverse effects on the renal functions of rats (Silici et al., 2016).

Traditionally, *Rhododendron* species is a very important medicinal plant having oxytocic, estrogenic, anti-inflammatory, analgesic, and hepatoprotective activities; it also inhibits the prostaglandin synthetase. For example, *R. luteum* Sweet may be a great source of antioxidant and antitumor natural agents due to their capability of decreasing cancer cell proliferation (Denim et al., 2016). The essential oil of *R. tomentosum* Harmaja with the rich polyphenolic fraction possesses analgesic, anti-inflammatory, antimicrobial, antiviral, antifungal and insecticidal potential, demonstrated by *in vivo* and *in vitro* studies. In addition, recent scientific research reported promising antidiabetic, antioxidant and anticancer properties (Dampc and Luczkiewicz, 2013). 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. Therefore, 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).

The ethyl acetate fraction of *Rhododendron fauriei* Franch. (syn. *R. brachycarpum*) leaves extract has strong antioxidant, α-glucosidase, and prevention of DNA damage activities, and furthermore, ethyl acetate fraction significantly protected the cells from high glucose-induced cell death. In the study of Yang et al. (2017), the effects of *R. fauriei* and its different fractions (*n*-hexane, ethyl-acetate, and *n*-butanol) on antioxidant activity, DNA damage prevention and the activity of α-glucosidase were studied. Pretreatment of pancreatic β-cells from the Syrian golden hamster (HIT-TI5) with the ethyl acetate fraction at concentrations of 300 μg/ml significantly protected the cells from high glucose-induced cell death (Yang et al., 2017).

The total phenols and total flavonoid contents, as well as antioxidative properties of the crude extract and solvent fractions of *R. anthopogonoides* Maxim., were determined using seven antioxidant assays in the study of Jing et al. (2015). Additionally, the protective effect of the extracts on hypoxia-induced injury in PC12 cells was also investigated. The gathered data demonstrated that ethyl acetate and *n*-butanol fractions were able to protect PC12 cells against hypoxia-induced injury through direct free radical scavenging and modulation of endogenous antioxidant enzymes (Jing et al., 2015).

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

Our results revealed that the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level. The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted. On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest percent of hemolyzed erythrocytes and decrease hemolysis duration. This study demonstrated, that among the leaf extract of *R. myrtifolium*, the dose of 5 mg/mL exhibited the highest level of lipid peroxidation biomarker and morphological changes in the human erythrocytes. In addition, the study also demonstrated that *R. myrtifolium* leaf extract at dose 1.25 mg/mL possessed hemolytic effect *in vitro* compared to other doses studied. Therefore, further investigation of it's *in vitro* and *in vivo* activity is warranted.

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