Evaluation of Seasonal Differences in the Antioxidant Activity of Needle Juices of *Picea abies* L. and *Pinus sylvestris* L. with Luminol-enhanced Chemiluminescence

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**ABSTRACT**

**Background:** Coniferous tree species typical of the central part of Russia can be evaluated not only as sources of wood but also as a raw material for obtaining biologically active substances.

**Methods:** A comparative evaluation of needle juices of *Pinus sylvestris* and *Picea abies* in the summer and winter periods was the objective of this study and carried out in this study by chemiluminescence analysis and testing for membrane-protective activity. Juices were evaluated for the content of flavonoids and ascorbic acid, which some authors suggest are markers of stress in plants. An increase in the content of flavonoids and ascorbic acid in *Pinus sylvestris* L. in the summer period is a sign of an adaptive reaction of plants to the high intensity of ultraviolet radiation, which is typical for the growing conditions of pine as a photophilous plant. An increase in these indicators in *Picea abies* L. in winter is a sign of the plant stress response to low temperatures.

**Result:** The membrane-protective activity of needle juices concerning peroxidation and osmotic hemolysis was revealed, which is more prominent in *Pinus sylvestris* L. needle juice. The analysis revealed more prominent antioxidant properties in the juice of *Pinus sylvestris* L. needles compared to that of *Picea abies* L. both in summer and in winter.

**Key words:** Antioxidant activity, Luminol-enhanced chemiluminescence, Membrane-protective activity, *Picea abies*, *Pinus sylvestris*, Seasonal differences.

**INTRODUCTION**

The scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L.) are the most common coniferous tree species in the Western sector of the forest and forest-steppe zone of Russia (Sacchetti *et al.*, 2005; Rehfeldt *et al.*, 2002) which means that they are exposed to a wide range of climatic conditions. Most environmental stress factors, such as low temperatures, high radiation intensity, air pollutants and drought, cause photo-oxidative stress in plants (Blokhina, 2003). Plants have evolved protective mechanisms - antioxidant protection systems and photoprotective pigments - to control reactive oxygen intermediates (ROI) (Gill and Tuteja, 2010; Singh *et al.*, 2020).

Some coniferous species that are acclimated to cold environments show resistance to photoinhibition which may be the result of increased metabolism of active oxygen scavengers (Hallgren, 1990; Singh *et al.*, 2017; Korotaeva *et al.*, 2018). The content of an effective system of antioxidants consisting of a wide range of compounds, such as ascorbic acid, flavonoids, etc. is directly related to the growth conditions and the physiological state of the plant (Lee *et al.*, 2018; Garg *et al.*, 2020; Gupta *et al.*, 2020).

A special place among the biologically active substances in needles is occupied by phenolic compounds (Mannila, 1993; Kochetova *et al.*, 2007). They are involved in processes that counteract oxidative stress and their concentration is higher in diseased trees compared to healthy ones. Phenolic compounds and other substances in the needles of plants can affect oxidative processes (Singh *et al.*, 2013). Similarly, the variability of flavonoids in plants of the same species when collected in different climatic conditions has also been proven. Plaksina *et al.* noted (2009) that the metabolism of flavonoids is largely determined by endogenous factors but mainly depends on exogenous factors.

Various studies have demonstrated the biological activity of pine and spruce juices (Kilic *et al.*, 2011; Bushmeleva *et al.*, 2019; Kormut'ak *et al.*, 2019). Significant differences in the content of ascorbic acid were found between forest species (Bianchi *et al.*, 2016), it is explained...
by the fact that the content of ascorbic acid is species-specific and also differs in the same species in different vegetation periods (Gilson et al., 2014; Haberer et al., 2006). Since important biological components that affect the antioxidant potential of plants have distinctive features of qualitative composition, the object of this study was the juices obtained from the needles of these plants (Pukacka and Pukacki, 2000).

It is important to note that the definition of antioxidants as chemical compounds does not give a complete picture of the protective properties of the object under study: they are not only determined by the amount of a particular antioxidant but also by the activity of each of them. Antioxidant activity (AOA) is the rate constant of an antioxidant reaction with a free radical. Chemiluminescence (CL) analysis allows for determining to determine the total number of radicals that bind antioxidants in a sample (total antioxidant capacity), as well as the rate of formation and reaction of radicals with antioxidants, i.e. AOA. Thus, for the study of the dynamic AOA of coniferous juices CL analysis was chosen. Determining the content of compounds involved in ROI binding and excretion will help to reveal the still-missing clear relationship between the AOA of the juices and their overall composition, including some potential interactions between components.

The work objective was to compare the AOA of two types of needles (pine and spruce) collected in summer and winter, growing in a moderate climate of the middle zone, by the number of radicals that bind antioxidants in a sample (total antioxidant capacity), as well as to study the resistance of juice antioxidants to oxidation.

MATERIALS AND METHODS

The study subjects included needle juices of Norway spruce and Scots pine collected in August 2018 and December 2019. The place of the collection was the Chuvash Republic, Russia. The choice of the collection place was due to its remoteness from industrial centers, i.e. an environmentally friendly area. The average temperature in December ranges from -9.1°C to -12.9°C, in August – from +12.5°C to +23.1°C. The study was conducted from December to April 2019 in Federal Research Center «Kazan Scientific Center of Russian Academy of Sciences», Kazan, Russia.

The juices were obtained by the pre-grinding of needles at a temperature of 20-22°C, followed by pressing on a press. The obtained juices were stored at +4°C in the absence of sunlight. The content of dry substances in the juices was determined by the refractometric method described in the work (de Silva et al., 2000). Dihydroquercetin and Trolox were used as comparison substances.

The total content of flavonoids was determined spectrophotometrically by the method described in the work of Sorokina et al. (2013). The percentage of ascorbic acid in the juices was determined by high-performance liquid chromatography (Zuo et al., 2015).

The CL analysis using luminol as a luminophore and 2,2′-azo-bis(2-amidinopropane) (ABAP) to activate the luminescence was used to determine the level of ROI generation and integral state evaluation of the antioxidant system. The CL intensity is a measure of the number of radicals. The CL analysis is described in the work (Krasowska et al., 2000) and adapted for the device chemiluminimeter Lum-100 (LLC Disoft, Russia) (Sharonova et al., 2019). The results were processed on a personal computer using the PowerGraph and OriginLab software.

In the work of Lissi et al. (1995), two approaches to measuring the total antioxidant capacity, taking into account this feature of the curves, are described – the TRAP (total reactive antioxidant potential) method and the TAR (total antioxidant reactivity) method. TRAP method has the advantage in that reflects the amount of antioxidant in a complex system and TAR – its activity, i.e. the rate of the antioxidant interaction with radicals (Desmarchelier et al., 1997). The TRAP method is based on the measurement of the CL latency period. The TAR method was used to determine the value of CL intensity quenching.

The membrane-protective activity of juices was evaluated using methods that initiate peroxidation or osmotic damage to red blood cell membranes (Sowemimo-Coker et al., 2002). Peroxide hemolysis was caused by Fenton’s reagent, the components of which were used in minimal concentrations causing 100% lysis of red blood cells: 0.01 mg/ml Fe$_2$SO$_4$·7H$_2$O (in terms of 100% hydrogen peroxide solution). To study osmotic hemolysis, the erythrocyte suspension was placed in a 0.3% NaCl solution that causes erythrocyte lysis. For the study, rat erythrocytes obtained from EDTA-treated whole blood of animals and washed with normal saline by repeated centrifugation were used. The membrane-protective effect of needle juices was evaluated as a percentage of the control parameters (without adding the test substance to the incubation medium).

RESULTS AND DISCUSSION

Evaluation of the content of biologically active compounds in juices

Usually, ROIs are produced in plant cells as a result of biological processes occurring in them (Zolfaghari et al., 2010), but it is believed that their increased production is a nonspecific response of plants to environmental stress (Taibi et al., 2015). It is known that phenolic compounds can act as low-molecular antioxidants that prevent or reduce the effects of oxidative stress (Rice-Evans et al., 1997; Blokhina et al., 2003; Plaksina et al., 2009). The content of ascorbic acid and flavonoids per dry substance of needle juice in summer was three times higher than the similar indicators of spruce needle juice (Table 1). It can be explained both by the specific characteristics of the studied plants and by the peculiarities of their growing conditions. Spruce and pine are plants of the first tier, but pine is a sun-loving plant and spruce can tolerate shade and often grows in mixed forests under the cover of other plants. Therefore, pine needles,
unlike spruce, are more exposed to photo-oxidative stress associated with the high intensity of ultraviolet radiation. In winter, the content of ascorbic acid and flavonoids in dry pine needles decreased by 1.4 and 3.3 times, respectively (Table 1), which can be explained by a decrease in radiation exposure and the pine resistance to low-temperature stress (Plaksina et al., 2009). It is known that lighting stimulates the formation of phenolic compounds by increasing the activity of some phenolic metabolism enzymes and activating phytochrome, which is involved in the non-photosynthetic formation of phenolic compounds (Agrawal et al., 2012). Therefore, a decrease in the content of phenols in pine juice in winter may be associated with a lack of illumination due to the shortening of the photoperiod (Blokhina et al., 2003).

An increase in the content of ascorbic acid by 2.7 times and flavonoids by 1.3 times was observed in the dry substance of spruce needles in winter (Table 1). In other words, according to the literature data (Treutter, 2005; Garg, 2020), one of the responses to low-temperature stress is quantitative changes in the metabolism of flavonoids, namely, an increase in their concentration (Olenichenko et al., 2006; Fuksman et al., 2015). An increase in the content of ascorbic acid, according to data outlined by Pukacka and Pukacki (2000), can also be evaluated as an indicator of the adaptive response of plants to adverse environmental conditions. The picture of seasonal changes in the content of flavonoids and ascorbic acid presented in this paper (Table 1) may indicate that these compounds play a significant role in protecting pine needles from adverse factors, including low-temperature stress and ultraviolet radiation (Fischbach et al., 1999).

### Evaluation of the chemiluminescent activity of juices

The latency period – the area where the signal intensity is minimal or close to zero – was found in all the studied juices, similar to dihydroquercetin (Fig 1, f). In the kinetics of the CL curve of spruce and pine needle juices collected in winter (Fig 1, b, d), at dry substance concentrations of 1 and 10 mg/ml, CL intensity was observed at the level of zero values for 2000 s, but at concentrations of 0.1 mg/ml and less, the latency period (TRAP) of pine needle juice obtained in winter was longer. The value of CL quenching is better observed in the pine needle juice collected in winter (Fig 1, b) – at concentrations of dry substance of 0.1 mg/ml, TAR reaches 74% of the initial level. In comparison, Trolox reaches only up to 4.5% (Fig 1, e).

The analysis of the CL kinetics of spruce needle juices collected in different periods reveals some distinctive features. Thus, the juice of spruce needles obtained in winter reveals a longer ROI-scavenging mechanism than the juice of spruce needles collected in summer, as well as a lower rate of reaction kinetics, which is explained by the high content of antioxidants in the dry substance of pine needles in winter (Table 1).

When the diluted juice of spruce needles collected in summer with a dry substance concentration of 0.1 mg/ml is added to the chemiluminescent system, an increase in the CL intensity is observed after a latent period of 95 s, exceeding the initial level by 67% (Fig. 1, c). At a similar concentration of spruce needle juice collected in winter, a latent period of 641 s was observed and subsequently a 22% decrease in the level of CL intensity by compared with the baseline. When the diluted juice of spruce needles, collected in summer with a dry matter content of 1 mg/ml was added to the system, an increase in the CL intensity was observed with an excess of the baseline after 3250 s, reaching 45% in 750 s. A similar concentration of spruce needle juice collected in winter quenched the intensity of CL up to 97%. An increase in the CL intensity above the initial level is an indicator of the increased formation of free radicals in the system and demonstrates the manifestation of pro-oxidant properties. Increased formation of free radicals may be associated with the appearance of antioxidant transformation products that have pro-oxidant properties. This effect in the CL system is described for the well-known antioxidant ascorbic acid (Vyshtakalyuk et al., 2018; Zolfaghari et al., 2010). In contrast to the summer harvest of spruce needles, similar concentrations of spruce needle juice from the winter harvest did not show pro-oxidant properties. Thus, the juice of spruce needles collected in winter in contrast to the juice of spruce needles collected in summer shows more prominent antioxidant properties and can be characterized as containing more stable antioxidants. The juice of pine needles collected in summer caused an increase in the CL intensity by 30-35% in the system when it was added at a dry substance concentration of 0.1 mg/ml (Fig 1, a). In other words, the pine needle juice from the summer period is also characterized by the manifestation of pro-oxidant properties, but less prominent in comparison with the spruce needle juice.

The analysis of the TRAP value revealed that winter pine needle juice had a higher amount of compounds capable of protecting against ROIs in the system, a lower rate of the increase in CL kinetics. All this indicates that, like winter spruce needle juice, winter pine needle juice acts

### Table 1: Characteristics of Scots pine and Norway spruce needles by the content of flavonoids, ascorbic acid per dry substance.

| Compound                                | Scots pine | Norway spruce |
|-----------------------------------------|------------|---------------|
|                                        | Pinus sylvestris L. | Picea abies L.|
| Ascorbic acid, mg/100g dry matter       | August     | February      | August     | February      |
|                                        | 3.33       | 2.41          | 1.04       | 2.77          |
| Flavonoids (in quercetin equivalents), mg/100g dry matter | 27.30      | 8.30          | 10.0       | 13.08         |
| Dry matter content, %                   | 6.3        | 11.2          | 12.5       | 6.5           |
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Fig 1: Chemiluminescence intensity (% from baseline) of Scots pine needle juice collected in August (a), Scots pine needle juice collected in February (b), Norway spruce needle juice collected in August (c), Norway spruce needle juice collected in February (d), Trolox (e), dihydroquercetin (f). Concentrations of system components: Tris-buffer solution – 0.1 M, luminol – 250 μM, ABAP – 40 μM.

The numbers above the curves are the initial concentration of antioxidant added to the chemiluminescent system (mg/ml).

The results of determining the area under the curve (Fig 1 Suppl.) showed that the rate of the curve reaction kinetics of a sample of summer spruce needles increased most rapidly and reached a maximum concentration of 0.1 mg/ml, as well as a sample of winter spruce needles that reached a maximum concentration of 0.01 mg/ml, which is confirmed by the data on the TAR capacity (Table 2).

**Evaluation of the components stability in juices**

The results of determining the CL activity after a 10-month storage of pine needle juices in the absence of sunlight at a temperature of +4°C (Table 3, Fig 2) confirm the hypothesis about the stability of the antioxidant compounds that are part of the juice. It is interesting to note that in pine needle juices after a 10-month storage, the ability to increase the formation of free radicals (pro-oxidant properties) in the system is reduced, which can serve as an indicator of the degradation of ascorbic acid in the needle juices.

**Evaluation of the membrane-protective activity of juices**

As a result of the studies conducted to evaluate the effect of juices on the membranes of erythrocytes, it was found that the juice of pine needles contributes to the stabilization of erythrocytes during peroxidation and osmotic hemolysis *in vitro* (Fig 3, 4). The pine and spruce needle juices under investigation in the growing period show the most prominent membrane-protective effect on peroxide hemolysis in the concentration of the dry substance of 0.1-1.0 mg/ml, which shows the most effective decrease in
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**Table 2:** TRAP (total reactive antioxidant potential) & TAR (total antioxidant reactivity) values obtained from the quenching of luminol-enhanced chemiluminescence.

| Method | Concentration (mg/mL) | Scots pine *Pinus sylvestris* L. | Norway spruce *Picea abies* L. | Trolox | Dihydroquercetin |
|--------|-----------------------|---------------------------------|--------------------------------|--------|------------------|
|        | August                | February                        | August                        | February | April            | February |
| TAR, % | 10                    | 95.88                           | 98.72                          | 85.18   | 96.20            | 99.96    |
|        | 1                     | 1.06                            | 97.99                          | -184.32 | 97.11            | 99.77    |
|        | 0.1                   | -35.15                          | 74.58                          | -67.59  | 21.72            | 4.50     |
|        | 0.01                  | -4.18                           | 12.38                          | -9.08   | 7.63             | 1.68     |
| TRAP, s| 10                    | 3000.0                          | 3000.0                         | 1343.2  | 3000.0           | 15000.0  |
|        | 1                     | 1180.1                          | 3000.0                         | 822.5   | 3000.0           | 15000.0  |
|        | 0.1                   | 127.0                           | 954.7                          | 93.1    | 640.9            | 2697.0   |
|        | 0.01                  | 18.9                            | 57.9                           | 12.6    | 44.2             | 443.0    |

**Table 3:** TRAP (total reactive antioxidant potential) & TAR (total antioxidant reactivity) values obtained from the quenching of luminol-enhanced chemiluminescence after a 10-month storage in the absence of sunlight at a temperature of +4°C.

| Method | Concentration (mg/mL) | Scots pine *Pinus sylvestris* L. | Norway spruce *Picea abies* L. |
|--------|-----------------------|---------------------------------|--------------------------------|
|        | August                | February                        | August                        | February |
| TAR, % | 10                    | 99.56                           | 99.19                          | 99.44    | 99.92            |
|        | 1                     | 95.65                           | 54.67                          | 69.19    | 82.97            |
|        | 0.1                   | 37.65                           | 21.96                          | -5.83    | 24.90            |
|        | 0.01                  | 23.94                           | 20.01                          | 0.57     | 14.83            |
| TRAP, s| 10                    | 12000.0                         | 12000.0                        | 12000.0  | 12000.0          |
|        | 1                     | 4145.0                          | 0                              | 0        | 383.0            |
|        | 0.1                   | 14.0                            | 0                              | 0        | 0                |
|        | 0.01                  | 0                               | 0                              | 0        | 0                |

**Fig 2:** Chemiluminescence intensity (% from baseline) of Scots pine needle juice collected in August (a), Scots pine needle juice collected in February (b), Norway spruce needle juice collected in August (c), Norway spruce needle juice collected in February (d) after a 10-month storage in the absence of sunlight at a temperature of +4 °C. Concentrations of system components: Tris-buffer solution – 0.1 M, luminol – 250 μM, ABAP – 40 μM. The numbers above the curves are the initial concentration of antioxidant added to the chemiluminescent system (mg/ml).
hemolysis by 46.8 and 64.8% relative to the control, respectively, as well as in concentrations of $10^{-6}$ and 1 mg/ml, leading to a decrease in hemolysis by 65.2 and 50.7% for spruce and pine juices in the winter period, respectively.

According to the effect on osmotic hemolysis, pine needle juices obtained in summer and winter also had a more significant effect than spruce juices. Under the influence of the studied concentrations of pine needle juice, a decrease in the intensity of osmotic hemolysis by 30-40% was also observed (Fig 3).

**Fig 3:** The effect of Scots pine needle juice collected in August (a), Scots pine needle juice collected in February (b), Norway spruce needle juice collected in August (c), Norway spruce needle juice collected in February (d) on the degree of red blood cell hemolysis during peroxidation.

**Fig 4:** The effect of Scots pine needle juice collected in August (a), Scots pine needle juice collected in February (b), Norway spruce needle juice collected in August (c), Norway spruce needle juice collected in February (d) on the degree of osmotic hemolysis of red blood cells.
The established membrane-protective activity of the studied juices is believed to be determined by the presence of triterpenoid saponins and flavonoids (quercetin and kaempferol) contained in Pinus sylvestris L. and Picea abies L. (Formazyuk, 2003).

CONCLUSION

It can be concluded that among the studied juices of conifer species, the greatest antioxidant properties, manifested in the ability to scavenge ROI, are typical for the juice of pine needles obtained in winter and Pinus sylvestris L. has stronger antioxidant capacity compared to Picea abies L. both in winter and in summer. The AOA level of the studied juices in terms of TRAP values is comparable to the activity of the medium-strength antioxidant Trolox and even exceeds it in terms of TAR values.

The membrane-protective effect of pine needle juices was revealed, which is manifested to a greater extent on the model of peroxide hemolysis and is more prominent in Pinus sylvestris L. needle juices both in winter and in summer collection periods.

High values of flavonoid content and ascorbic acid in the juice of Pinus sylvestris L. needles in the summer are an indicator of the high adaptive potential of pine needles as a photophilous plant that is subjected to strong photo-oxidative effects. An increase in the content of the above indicators in the juice of Picea abies L. needles in the winter period relative to the summer period characterizes the manifestation of an adaptive response to low-temperature stress.

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