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Potassium Salts of Terpene Acids of Siberian Pine Resin as Effective Drug in the Cultivation of Potatoes in Organic Farming

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Siberian pine is a source of a wide range of biologically active substances for use as biopesticides and growth stimulators in organic farming. Ten components, among which the share of abietic acid reached 54.8%, have been determined with the help of the gas chromatography/mass spectrometry method. Water-soluble potassium salts of terpene acids were obtained from Siberian pine resin during the work. Potassium salts of terpene acids from Siberian pine resin has bacteriostatic, fungistatic, bactericidal, and fungicidal properties against phytopathogens. Relatively high indicators of antioxidant activity of aqueous solutions of potassium salts from resin at 1 mg/mL were revealed. Pre-planting treatment of tubers with an aqueous solution of terpene acids salts of Siberian pine resin in a concentration of 10 g/L contributed to an increase in the yield of potato. Also, an increase in the mass fraction of dry matter and starch was also observed.

Keywords: Potassium salts, Terpene acids, Siberian pine, Antimicrobial activity, Antioxidant activity, Potato.

INTRODUCTION

According to Tripathi *et al*. [1], the world's population is increasing by 1.13% every year and will reach 8.1 billion by 2025 and 9.6 billion by 2050. In this regard, the demand for affordable, high-quality, and safe food is constantly increasing. In world agricultural production, potato occupies a special place as the most important food, technical and fodder crop, which is of great importance in improving the live sustenance of the population.

In the context of organic farming development, special requirements are imposed on the range of used fertilizers and plant protection products, such as efficiency, selective ability, biodegradability, absence and/or low toxicity [2]. In recent years, the interest in natural substances extracted from such plants has been increased due to their significant environmental impact, as well as an effective alternative to industrially synthesized chemicals [3].

The Pinus (Pinaceae) genus includes 250 species and is the largest coniferous genus naturally occurring in the Northern hemisphere [4-6]. By having various biological activities *viz*. fungicidal, antibacterial, antioxidant and insecticidal [7-10] coniferous metabolites are of interest as a source of obtaining plant extracts that cause induced resistance of agricultural plants to pathogens and adverse environmental conditions.

Some pine species (for example, *P. pinaster*, *P. halepensis*, *P. brutia*, *P. radiata*, *P. pinea*, *P. nigra*, etc.) have been studied thoroughly. There are relatively few studies of the biological activity and phytochemical composition of other species [11]. In recent years, plant growth regulators based on coniferous extracts such as Novosil, Lariksin, Verva and others have been created and patented in the Russian Federation. Their positive effect on improving the quality and quantity of agricultural products has been found [12-14].

The potential for deep chemical processing of coniferous biomass has not been exhausted and capable of producing innovative products [15]. In this regard, the work objective was to develop a new drug based on Siberian pine resin for use in organic farming for potato growing. Moreover, the phytochemical composition of Siberian pine *Pinus sibirica* Du Tour resin was investigated before obtaining water-soluble salts of terpene acids from Siberian pine resin and the biological activity of water-soluble salts of terpene acids of Siberian pine resin by a set of indicators was also conducted.

EXPERIMENTAL

Plant material: Siberian pine *Pinus sibirica* Du Tour resin was obtained by trees tapping in the Zmeinogorsky District of the Altai territory of Russian Federation.

Potassium salts of terpene acids from Siberian pine resin: Potassium salts of terpene acids was obtained as follows: The resin was dissolved in ethanol (96%) and then added aqueous solution of KOH in an amount of 8% of resin mass and refluxed the mixture for 1.5 h at 85 ºC. Transferred the solution to the separating funnel after cooling to room temperature and settled the solution for 3 h. The lower layer containing terpene acid salts was separated by vacuum stripping of distillation solvent (10 mm Hg) at 45 ºC. As a result, light yellow, odourless crystals were obtained.

The choice of potassium salts was due to the soft conditions of synthesis and the speed of production in comparison with other salts. The increased efficiency of potassium salts in the range of alkali metals has been proved. The obtained salts are hydrolytically stable.

GC/MS analysis: Mass spectra (EI, 70 eV, *m/z*=30-550; CI, 30 eV, *m/z* 100-550) of resin chloroformic solution were recorded on a GC/MS device Agilent 6890N with a mass selective detector 5973 N (Agilent Technologies, USA) using a silica capillary column Restek-5 MS (5% biphenyl, 95% dimethylpolysiloxane, 30 m × 0.25 mm, film thickness 0.25 µm (Restek, Germany).

The sample was examined in a native form without dilution. The volume of the injected sample was 1 µL. GC separation conditions: evaporation temperature: 280 ºC; interface temperature: 290 ºC; initial temperature of thermostat: 60 ºC (holding time: 1 min); rate of rising of the column temperature: 5 ºC/min; final temperature of column: 280 ºC, isothermal analysis: 32 min. Column carrier gas flow rate (helium, 99.999%): 0.9 mL/min; separate injection: 40:1; sample volume: 1 mL/min. Analysis time: 705 min.

The analysis of mass spectral data was performed with the help of the software Turbomass Ver. 6.0 (Perkin-Elmer, USA), MS Interpreter. Ver. 2.0 (NIST, USA), AIPS IN (BelHard Grupp, Belarus).

Strains of microorganisms and culture media: Standard bacterial strain of human pathogen, *Staphylococcus aureus* 209P was obtained from the state collection of pathogenic microorganisms of the National Institute for Standardization and Control of Biomedical Preparations, and other phytopathogenic strains *viz*., *Agrobacterium tumefaciens* A-47, *Erwinia amylovora* S59/5, *Erwinia carotovora* spp. *carotovora* SCC3193, *Xanthomonas arboricola* S3, *Pantoea agglomerans* SK-1, *Pseudomonas syringae* pv. *atrofacience* AP-2, *Alternaria solani* St108, *Fusarium graminearum* PH-1, *Fusarium culmorum* 3288, *Phytophthora* sp.

Microorganisms were incubated in standard sterile nutrient broths. The concentration of bacteria was determined using a DEN-1B densitometer (Biosan, Latvia) according to standard protocols. Norfloxacin (Sigma-Aldrich, USA), chloramphenicol (Kazan Pharmaceutical Plant, Russia) and difenoconazole (Score250 EC, Syngenta, USA) were used in the experiments as reference compounds.

***in vitro* Antimicrobial analysis:** In the experiments, the minimum inhibitory concentration of an aqueous solution of potassium salts of terpene acids of Siberian pine resin was determined by the method of double sequential dilution [16,17]. The fungistatic activity was determined by the method of serial dilution in a liquid medium [18]. Liquid broth with microbial spores was prepared on standard nutrient media: Hottinger broth for bacterial pathogens, Sabouraud′s medium for fungi pathogens and broth of potato-glucose extract for phytopathogenic microorganisms from 24 h bacterial cultures and for fungal spores 7-14 days cultures, respectively. The final size of inoculates was 10^5 CFU (colony forming units)/mL in the case of bacteria analysis and 1.1-1.5 × 10^2 CFU/mL in case of fungi analysis. As a control, tubes containing only nutrient media were used.

To identify the minimum bactericidal and fungicidal concentrations (MBC and MFC, respectively), 10 µL of inoculating (or a piece of mycelium of fungi) taken from test tubes without visible growth was added to petri dishes with agarized nutrient medium using a bacteriological loop.

For *Staphylococcus aureus* 209P, the results were recorded every day for 5 days at 37 ºC, for *Agrobacterium tumefaciens* A-47, *Erwinia amylovora* S59/5, *Erwinia carotovora* spp. *carotovora* SCC3193, for *Pantoea agglomerans* SK-1, *Pseaudomonas syringae* pv. *atrofaciens* AP-2 at 30 ºC and for *Xanthomonas arboricola* S3 at 25 ºC, respectively. The time of fungi incubation in a thermostat at 26 ºC with the appropriate substance made up 14 days. The growth of microorganisms was determined visually [2]. All analyses were carried out in triplicate.

Antioxidant activity: The antiradical properties of an aqueous solution of potassium salts of Siberian pine resin were evaluated using chemiluminescent (CL) analysis [19] and adapted for the device Lum-100 (DISoft, Russia) [20] using luminol (Alfa Aesar, UK) as a luminophore, as well as 2,2′-azo-*bis*(2-amidinpropane) (AAPH) (Acros Organics, USA) to activate the luminescence. The reaction took place at 30 ºC and the main CL level was measured for 20 min. Then the test compound was added to the reaction mixture. Working concentrations of the studied aqueous solution of resin potassium salts were diluted in distilled water to a dry matter concentration of 10, 1, 0.1 and 0.01 mg/mL.

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma-Aldrich Co., USA) and quercetin were used as standard antioxidants. To estimate the CL value of the studied samples, total antioxidant activity (TAR) and total reactive antioxidant potential (TRAP) were calculated [21]. The relative inhibitory activity of each sample was estimated based on CL curve area measurement. The inhibition coefficient was calculated according to eqn. 1:

$$\text{Inhibition (\%)} = \frac{100 \times \text{AUC}_1}{\text{AUC}_0} \quad (1)$$

where AUC_0 and AUC_1 are the area under the curve observed for control and in the presence of the test solution, respectively.

The results were processed with the help of the programs PowerGraph (http://www.powergraph.ru) and OriginLab.

Experimental site description: Field experiments were conducted on the basis of experimental fields of the Federal Research Center "Kazan Scientific Center of the Russian Academy of Sciences" in the Republic of Tatarstan (Russian Federation). The experimental field is located in a broad-leaved forest natural and agricultural zone 55º38′51″N 49º18′29″E, no more than 200 m above sea level and the average annual precipitation is 440 mm. The climate is moderately continental and the average vegetation period is 160 days and the snow blanket thickness is around 39-44 cm. The soil of the experimental field is grey forest medium loam. Table-1 shows the agrochemical indicators of soil of the experimental site.

TABLE-1
AGROCHEMICAL PROPERTIES OF THE SOIL LAYER 0-20 cm

Indicator	Value
Humus (%)	5.35%
pH	5.85
Alkaline-hydrolyzable nitrogen (mg/kg)	157
Mobile phosphorus (mg/kg)	250
Exchange potassium (mg/kg)	210
Mobile aluminum (mg/kg)	257
Mobile iron (mg/kg)	210
Mobile manganese (mg/kg)	57
Mobile copper (mg/kg)	2.4
Mobile zinc (mg/kg)	2.3

Experimental design: The experiments were carried out during three consecutive growing seasons in 2017-2019 according to the different arrangement: (i) $N_{16}P_{16}K_{16}$ - background (control); (ii) pre-planting treatment of tubers with saponified Siberian pine resin. Fertilization in the form of nitrogen-phosphorus-potassium fertilizer was annual.

The object of present study was to release the potato variety *Zhukovskiy rannii*. For planting, certified vernalize tubers were used, the planting rate was 55 thousand pcs/ha. To study the biological activity, aqueous solutions of potassium salts of terpene acids were prepared at a concentration of 10 g/L. Processing was carried out immediately before planting on a special drum-type installation. The total area of the plot was 300 m^2, the accounting area was 200 m^2. Repeatability in experiments was three-fold, the placement of options was systematic. The predecessor was winter whea and the planting was carried out mechanically. The distance between plants in a row - 30 cm, between rows - 70 cm.

As means of plants protection from insects, Tanrek® and Provotox® (by August) were used. The treatment of crops against weeds was carried out manually.

RESULTS AND DISCUSSION

Phytochemical composition: At the first stage, the phytochemical composition of Siberian pine resin was analyzed using the GC/MS method. Table-2 shows the main detected compounds, which contain 88.39% of the total amount. At the same time, the share of abietic acid accounts for 54.76%.

TABLE-2
MAIN COMPONENTS OF SIBERIAN PINE RESIN (CHLOROFORMIC EXTRACT)

Component	RR_t	Total (%)
α-Pinene	5.356	17.948
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	6.387	2.303
Cyclofenchene	7.105	2.410
D-Limonene	7.668	1.612
β-Phellandrene	7.763	1.576
Sandaracopimaral	33.974	3.017
9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-	34.212	1.221
Isopimaral	34.745	2.012
Cholest-14-ene, (5α)-	35.125	1.527
Abietic acid	38.054	54.764

Coniferous resin is synthesized by many plant organs and consists of a volatile mixture of monoterpenes, monoterpenoids, sesquiterpenes, sesquiterpenoids, and less volatile diterpenes and diterpenic acids [22,23]. According to the literature, the essential oil, gum and other components of coniferous trees contain a wide range of biologically active substances, including α-pinene (from 20 to 30%), β-pinene (from 5 to 10%), β-3-carene (from 55 to 65%), longifolene, longicycline, α-humulene, α-terpineol, limonene, caryophyllene, camphene and other terpenes (from 2 to 10%) [6,24-28].

Antimicrobial activity: The minimum inhibiting concentrations of potassium salts of terpene acids of Siberian pine resin that inhibit the growth of pathogenic bacteria ranged from 0.0156 to 0.25% (Table-3). The minimum bactericidal concentrations varied in the range from 0.031 to 0.25%. The most sensitive to the drug were *Erwinia amylovora* (Gram -ve) and *Staphylococcus aureus* (Gram +ve). The phytopathogen *Pantoea agglomerans* was the most stable among the studied strains.

TABLE-3
ANTIMICROBIAL ACTIVITY OF POTASSIUM SALTS OF SIBERIAN PINE RESIN AGAINST PATHOGENIC BACTERIA

Strains of microorganisms	MIC	MBC	MIC	MBC
Human pathogen	Siberian pine (%)		Norfloxacin (%)	
Staphylococcus aureus	0.0310	0.0310	0.00024	0.00024
Phytopathogenic bacteria	Siberian pine (%)		Chloramphenicol (%)	
Agrobacterium tumefaciens	0.1250	0.2500	0.0250	0.0500
Erwinia carotovora	0.0625	0.2500	0.0125	0.0125
Xanthomonas arboricola	0.1250	0.1250	0.0250	0.0500
Erwinia amylovora	0.0156	0.0313	0.0250	0.0500
Pantoea agglomerans	0.2500	0.2500	0.0250	0.0500
Pseaudomonas syringae pv. atrofaciens	0.0313	0.1250	0.0250	0.0500

The minimum inhibiting concentrations of potassium salts of terpene acids of Siberian pine resin that inhibit the growth of phytopathogenic fungi ranged from 0.0625 to 0.25% (Table-4). The minimum fungicide concentrations were 0.125-0.25%. The most sensitive to aqueous solution of potassium salts of resin terpene acids was *Alternaria solani* strain.

TABLE-4
ANTIMICROBIAL ACTIVITY OF POTASSIUM SALTS OF SIBERIAN PINE RESIN AGAINST PATHOGENIC FUNGI

Phytopathogenic strains of fungi	Siberian pine (%)		Difenocanozole (%)	
	MIC	MFC	MIC	MFC
Alternaria solani	0.0625	0.125	0.00019	0.00313
Fusarium graminearum	0.125	0.125	0.00039	0.00625
Fusarium culmorum	0.125	0.25	0.00039	0.0125
Phytophthora sp.	0.125	0.125	0.00078	0.00078

Plants contain a large number of organic compounds with a huge chemical diversity and many of them have antimicrobial properties [2,29]. According to Zafar *et al*. [25] and Salem *et al*. [27], α-pinene in the composition of pine needle oil and wood of *Pinus roxburghii* was active against *Staphylococcus aureus* and *Bacillus subtilis*, but inactive against *Escherichia coli*, *Salmonella typhi* and *Enterobacter aerogenes*.

There are reports about the antifungal activity of pine (*Pinus sylvestris* L.) and spruce (*Picea abies* L.) extracts and essential oils against a wide range of fungi: *Fusarium culmorum*, *F. poae*, *F. solani* [30], *Heterobasidion parviporum*, *H. annosum*, *Fomitopsis pinicola*, *Ophiostoma piceae*, *Ceratocystis polonica*, *Phacidium coniferarum* [31], *Penicillium funiculosum* and *Ulocladium oudemansii* [32].

There are two possible ways to implement antimicrobial activity: initiation of the protective mechanism in plants, leading to system-induced resistance [33] or the ability of extract compounds to violate the integrity of the fungal cell wall and membrane [34].

Antonenko and Smirnov [12] reported that there is no unambiguous effect of preparations based on coniferous Novosil (active substances are triterpenoid acids), Lariksin (active substance is dihydroquercetin), Terpenol (active substances are triterpenoid acids) on the aggressiveness of oomycete of *Phytophthora infestans* (Mont.) de Bary *in vitro*. Similar results were also found when analyzing the formation of zoosporangia and zoospores of *P. infestans* [13].

Antioxidant activity: The results of the study of antioxidant activity of potassium salts of Siberian pine resin are presented in Table-5. Relatively high values were found for the studied aqueous solutions of resin potassium salts only at 1 mg/mL (highest analyzed concentration). TAR values was 99.13% and the The TRAP values were more than 3,000 s.

Fig. 1 shows the chemiluminescent curves of an aqueous extract of potassium salts of Siberian pine and control antioxidants. A long latent period in concentrations of 1 mg/mL or more was detected for Siberian pine. At the same time, the TRAP indicators were high.

The interaction rate of potassium salts of Siberian pine resin is maximal compared to the studied compounds, which means that the system has an imbalance between the number of unbound radicals and the amount of antioxidant, with a preponderance in the first case. There is no latent period in the chemiluminescence kinetics (except for the concentration of Siberian pine dry matter 1 mg/mL), which may be an indicator of a small amount/absence of an antioxidant in the system. The direct correlation between the concentration of components in the resin potassium salt and the degree of TAR is observed, *i.e.*, with increasing concentration, the degree of quenching of CHL intensity increases. It can be concluded that the kinetics

TABLE-5
TAR AND TRAP VALUES OF AQUEOUS SOLUTIONS OF POTASSIUM SALTS OF SIBERIAN PINE RESIN

Option	TAR (%)				TRAP (s)			
	1 mg/mL	0.1 mg/mL	0.01 mg/mL	0.001 mg/mL	1 mg/mL	0.1 mg/mL	0.01 mg/mL	0.001 mg/mL
Siberian pine	99.13	81.6	11.7	-133.49	3,000+	2.26	1.58	1.3
Trolox	10.83	4.9	11.07	-0.27	555.3	8.4	0.8	0
Quercetin	98.07	98.02	-36.68	-1.76	3,000+	3,000+	136.4	19.9

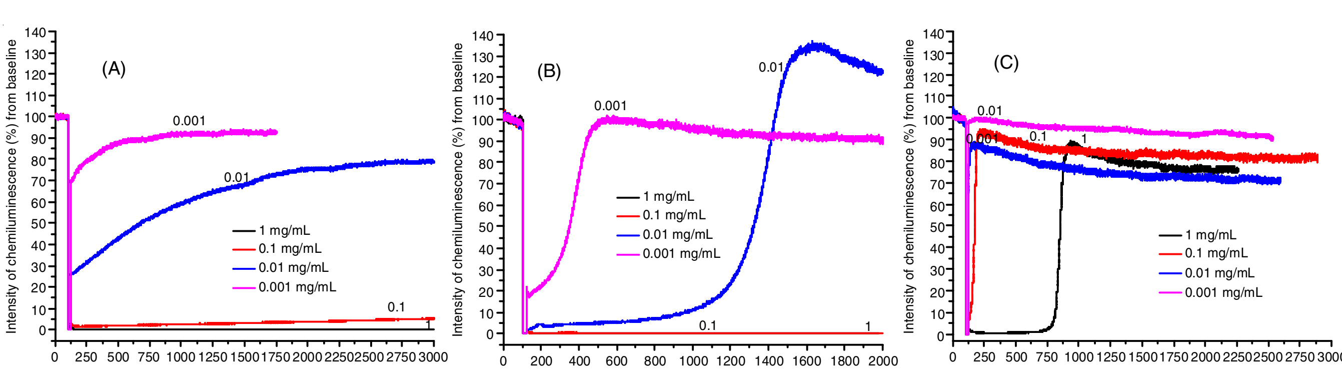

Fig. 1. Influence of potassium salts of Siberian pine resin (A), quercetin (B), Trolox (C) on chemiluminescence kinetics. Concentrations of the system components: Tris-buffer – 0.1 M, luminol – 250 μm, AAPH – 40 μm. The numbers above the curves are the initial concentration of the antioxidant (mg/mL)

TABLE-6
INFLUENCE OF PRE-PLANTING TREATMENT OF POTATO TUBERS WITH A SOLUTION OF POTASSIUM SALTS OF TERPENE RESIN OF SIBERIAN PINE ON THE QUALITATIVE AND QUANTITATIVE INDICATORS OF THE YIELD (AVERAGED DATA FOR 2017-2019)

Experience option	Yield (c/ha)	Mass fraction of dry matter (%)	Starch content (%)
Background (control) $N_{16}P_{16}K_{16}$	33.1 ± 1.9	20.3 ± 0.9	11.4 ± 0.9
Treatment with an aqueous solution of potassium salts of Siberian pine resin	40.0 ± 1.8	22.3 ± 0.9	13.8 ± 0.6

TABLE-7
INFLUENCE OF PRE-PLANTING TREATMENT OF POTATO TUBERS WITH A SOLUTION OF POTASSIUM SALTS OF TERPENE RESIN OF SIBERIAN PINE ON THE ELEMENTAL COMPOSITION OF THE POTATO (AVERAGED DATA FOR 2017-2019)

Experience option	Element content (mg/100 g)						
	P	Cl	S	K	Na	Ca	Mg
Background (control) $N_{16}P_{16}K_{16}$	98	35	19	450	–	9	57
Treatment with an aqueous solution of potassium salts of Siberian pine resin	117	48	27	603	5	6	67

of this resin potassium salt is characterized by a short-term significant pro-oxidant response and potassium salt can extinguish the intensity of luminescence, which correlates with the amount of active component in the solution.

Yield and product quality: Pre-planting treatment of tubers with a solution of saponified resin had a stimulating effect on potato yield (Table-6). The average yield increase was 6.9 t/ha (20.8%) compared to the control. The main indicator of the quality and value of potato is its chemical composition. The dry matter content is an important indicator for both fresh potato sales and processing. The mass fraction of dry substances in the experiment options was 20.3-22.3% with the highest indicators in the case of pre-planting treatment (Table-6). The most valuable and industrially significant among the dry substances of potato is starch. The starch content in the option with treatment with saponified Siberian pine resin was 13.8%.

The content of the main macro and microelements was also highest during pre-planting treatment of tubers with saponified salts of terpene acids of Siberian pine resin, especially potassium and increase compared to the control was 1.34 times.

Conclusion

As a result of present study, the water-soluble salts of terpene acids of Siberian pine resin were obtained and the effectiveness of their use in growing potato was found. The obtained salts showed inhibitory, bactericidal and fungicidal properties against phytopathogenic microorganisms. Among bacteria, *Erwinia amylovora* (MIC 0.0156%) was the most sensitive to the action of an aqueous solution of potassium salts of terpene acids, and among fungi *Alternaria solani* (MIC 0.0625%). In case of pre-planting treatment of tubers with an aqueous solution of terpene acid salts of Siberian pine resin in a concentration of 10 g/L, the yield of potato increased by an average of 21% compared to the control. The values of mass fraction of dry substances and starch content in the products exceeded the control indicators by 2-3.5%. The biological activity of resin salts was due to the presence of such compounds as α-pinene, D-Limonene and abietic acid, which consists a total amount of 74.3%.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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