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MECHANOCHEMICAL PREPARATION OF WATER-SOLUBLE FORMS OF TRITERPENE ACIDS

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A waste-free technology for mechanochemical processing of fir needles into a biologically active preparation that regulates plant growth was developed. A preparation exhibiting cytokinin activity due to the presence in it of water-soluble forms of triterpene acids can be prepared using the proposed method. The effectiveness of the proposed method was confirmed by instrumental methods and biological tests.

Key words: mechanochemical processing of plant material, triterpene acids, plant-growth regulators.

Plant material is a promising source of ecologically pure plant-growth regulators [1]. Extraction by organic solvents is a necessary step in the preparation of biologically active substances from plant material. The target components are fully isolated by repeated treatment of the raw material with solvents of different polarity. The drawbacks of traditional extraction technology for isolating biologically active compounds include the use of toxic and flammable organic solvents, the low extraction in a single treatment step, and, as a result, repeated extraction, increased production losses, and environmental contamination.

The rate of extraction and the yield of target products are determined by the diffusion of solvent into the particles of plant material. Preliminary mechanical activation of the plant material can achieve the maximum effectiveness in the subsequent extraction step. Impact-shear action on the particles of the processed material during mechanical activation is accompanied by not only grinding but also destruction of the cell covering and wall. This facilitates significantly the isolation of the components in the plant material. For example, mechanical treatment of a mixture of powdered bark and alkali produces a composite with a distinct interface. The solid alkali microparticles are distributed throughout the volume of the plant material, the cellular structure of which is destroyed. Adding water to such a composite isolated in one step the water-soluble substances in limiting quantities without preliminary defatting. Direct aqueous extraction of bark containing insoluble lipids and resinous components is ineffective [2, 3].

Increasing the dispersion of the raw material increases the amount of ballast upon extraction. This problem can be solved by combining the dispersion with modification of the target or ballast substances in the plant material. Such an approach is called "solid-state mechanochemical extraction" and consists of reactive grinding of raw material [2]. The technology includes mechanochemical processing of powered mixtures of plant material and a specially selected solid phase. The solid phase may be "collectors" (adsorbents of various chemical nature) or "reagents" (compounds that can react with the target or ballast substances). Grinding is accompanied by an increase of the total contact surface area. Therefore, the substances in the plant material may bind to the collector or react with the reagent.

The role of mechanicochemical treatment is not only to increase the effective surface area of the mixture components and to eliminate or decrease hindrances to diffusion but also to transform chemically the target substances into forms that are more soluble in water or the solvent.

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Target compounds, as a rule, can be selectively extracted from the resulting composite in the limiting yield, which is achieved in the usual technology by prolonged continuous extraction [4]. Thus, use of the mechanochemical method can simplify greatly the isolation of diterpene alkaloids, sesquiterpene lactones, cardiac glycosides, etc. [2, 5-7].

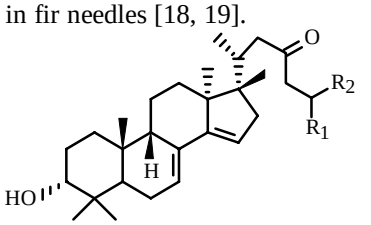
In some instances, pure compounds cannot be isolated from mechanical composites if the biological activity of the target substances is so great that the preparation must be diluted. The single mandatory condition is the presence in the mechanically treated product of biologically available forms of the target substances. These forms should be water-soluble.

We investigated the mechanochemical production from Siberian fir needles (*Abies sibirica* Ledb. [8]) of a biologically active preparation, the active principles of which are water-soluble forms of triterpene acids. The mixture of triterpene acids isolated from the ether extract of the needles acts as a plant-growth regulator. It exhibits cytokinin activity in tissue culture in vitro and facilitates cell division and shoot regeneration [9].

The process of extracting needles with organic solvents is well studied. The ether extract of fir needles contains 51% acids, a large part of which are triterpenes. The acids of the ether extract are arbitrarily divided into "weak" and "strong" ones. The strong fraction consists primarily of triterpene acids; the weak fraction, of resinous and fatty acids [10].

The chemical nature of the triterpene acids occurring in large quantities in Siberian fir gum and needles was not investigated for a long time [10, 11]. Strong abiesonic and weak abiesolidic acids were first isolated as the methyl esters from a mixture of acids from fir gum [11-13]. Then, abiesonic acid and its 24-*Z*-isomer were detected in Siberian fir needles [10].

The 9β-lanostane triterpenoids 3-hydroxy-23-oxolanostanic acids **1** and **2** and their 3-keto analogs **3** and **4** and 3,23-dioxoacids **5** and **6** were isolated as the methyl esters from a mixture of strong acids in the ether extract of needles [10, 14, 15]. Compounds **1** and **2** are 23-oxo derivatives of mariesiic A acid and its 24-*Z*-isomer, respectively [16]. Firmanic acid **5** was first described by Hasegawa et al. [17]. Its 24-*Z*-isomer **6** and acids **3** and **4** are the main components of the acidic part of the needle extract. Their total fraction of the strong acids is >55% [10]. Isofirmanic acid **7**, the Δ^8-isomer of firmanic acid **8**, the 3-methoxy derivative of acid **2**, and *cis*-sibiric and anhydrosibiric acids were added as a result of the variety of strong triterpene acids in fir needles [18, 19].



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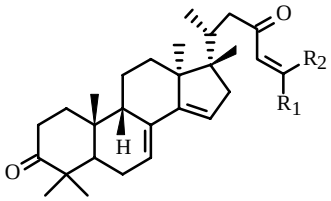
2: $R_1 = H$, $R_2 = CO_2H$



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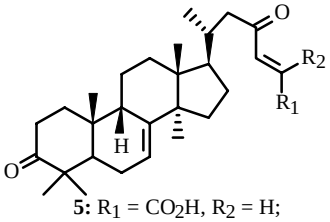

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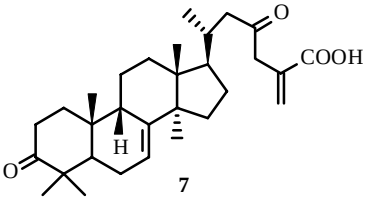

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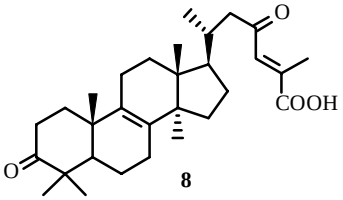

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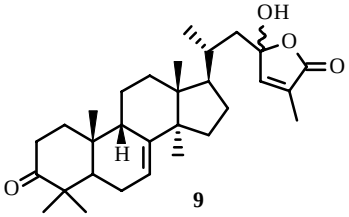

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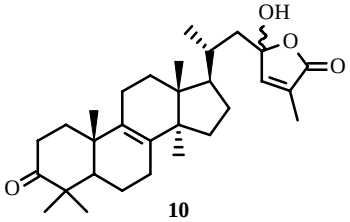

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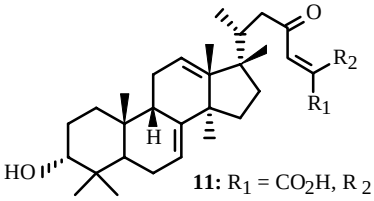

11: $R_1 = CO_2H$, $R_2 = H$;
12: $R_1 = H$, $R_2 = CO_2H$

A detailed study of the acidic part of fir needles showed that every triterpene acid has two geometric isomers at the Δ^{24}-double bond [20]. The *cis*-isomers occur as the cyclic γ-lactol form that converts in alkaline solutions to the corresponding tautomer with a β-acetylacrylic fragment. For example, tautomer **9** of acid **6** was isolated pure [21]. The 24-*Z*-isomer of acid **8** was also found in the γ-lactol form **10** [22]. The similar tautomer, which is well known for penicillinic acid, was investigated separately [23].

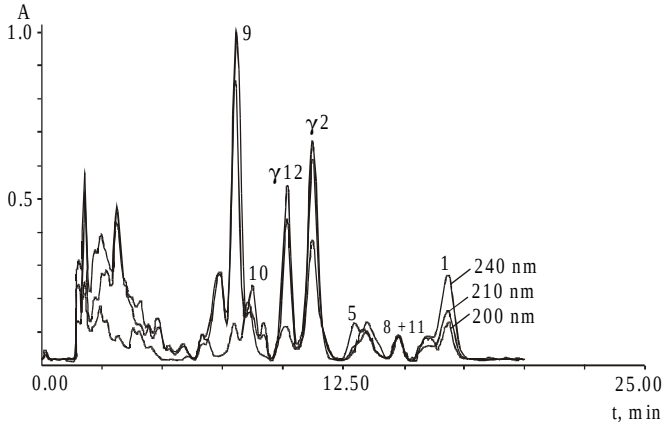

Fig.1

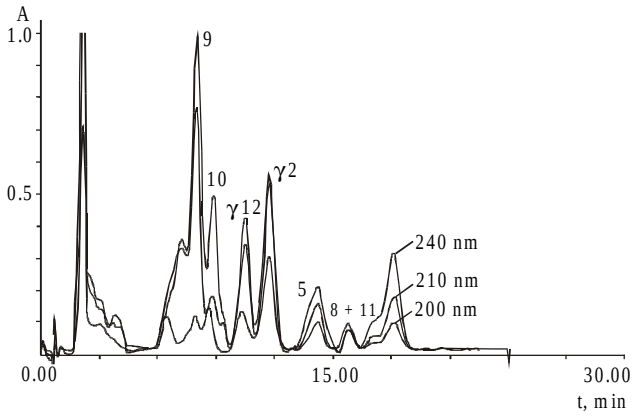

Fig.2

Fig. 1. Separation of components of total triterpene acids. The corresponding hydroxylactones are designated by γ.
Fig. 2. HPLC analysis of triterpene acids isolated from the aqueous extract of the mechanical composite.

It should be noted that only 23-oxofirmanic acid **5** and γ-hydroxylactones **9** and **10** were isolated pure. The remaining compounds were isolated as methyl esters.

Several methods for isolating the total triterpene acids are known [24-26]. The primary extract is produced by treatment of the plant material with an organic solvent or alkaline solution. Then, this is separated by extraction with various systems. The main task of these procedures is to separate strong triterpene acids from phenolic compounds and low-molecular-weight components of the acidic part. Contamination of the final product by phenolic compounds reduces markedly its activity. Many phenolic compounds of plant origin are known to be growth inhibitors. However, not being hormones themselves, they can actively affect plant growth by influencing auxin exchange [27].

We propose a method for producing a preparation containing triterpene acids in a biologically available form that does not include steps in which organic or aqueous extractants would be used. The new method is based on mechanochemical treatment, the main purpose of which is to convert triterpene acids to a water-soluble form.

The effectiveness of the proposed method was confirmed by analyzing the solutions obtained after treatment of the mechanicochemical preparations with water. Their biological activity was confirmed by a series of biological tests on wheat sprouts (*Triticum aestivum* L.) and rape tissue culture (*Brassica napus* L.) *in vitro*.

An AGO-2 high-intensity planetary activator was used in model experiments. The strength of the impact-shear action on the ground material in the selected operating mode of the AGO-2 was equivalent to the strength for BTsM and TsEM semi-industrial flow-through grinders. The model experiments could be performed in a regime with conditions similar to those during operation of VTsM and TsEM flow-through activators if the AGO-2 activator was used [28].

Mechanical activation of the plant material is accompanied by destruction of the cell covering and wall. This ensures that complete chemical transformations will occur involving additives and substances in the plant cell and that the yield of extracted compounds will increase substantially.

Extensive mechanical activation may be accompanied by destruction of organic substances. According to model experiments, triterpene acids in plant material are not degraded. Substances were extracted by diethylether from composite No. 1, a mixture containing abrasive and plant material. Then, they were purified and analyzed by HPLC. The mixture of substances isolated from composite No. 1 was identical to the total triterpene acids obtained by the usual method. The yield of triterpene acids could be increased substantially from 2.9 to 4.7% by using preliminary mechanochemical treatment.

Composite No. 2, which contained plant material, abrasive, and Na_2CO_3 and a test batch identical to it, was produced under specially selected conditions that ensured neutralization of the triterpene acids and binding of the polyphenolic compounds. Flow-through technology (VTsM-10) is about as effective for producing salts of triterpene acids as the experimental process (AGO-2). The composition of the triterpene acids converted to the water-soluble form by the flow-through regime (Fig. 1) was identical to the sum of triterpene acids isolated by the extraction method (Fig. 2).

The complex composition of the natural mixture of triterpene acids in fir needles stimulated the development of analytical methods for total triterpene acids. The most promising method was based on HPLC separation of the acids. Researchers [29] were able to identify with certainty **1**, **5**, **8**, 23-oxomariesiic acid B (**11**), the γ-lactol forms of acids **2** and **12**, and the γ-hydroxylactones **9** and **10**.

We first used HPLC—MS, a combination of HPLC and mass-selective detection, to analyze total triterpene acids from fir needles. Detection of the negative ions from total triterpene acids obtained by extraction showed that the strongest peaks in the UV chromatogram were the strongest peaks in the chromatogram of the total ion current. The main ion for most of these peaks was the ion with m/z = 467.3, which corresponds to the pseudomolecular ion $[M - H]^+$, which under these analytical conditions should form triterpene acids and their lactones with M = 468.3. Judging from the extracted-ion chromatogram for m/z = 467, there are more than 10 such substances in the analyzed mixture. Their sum is the main part of the mixture. In addition to them, there is a significant quantity of substances with M = 466.3 and a large set of components with M = 482.3. Substances with M = 500.3 make up most of the ion current in the beginning part of the chromatogram.

The HPLC—MS analytical results of triterpene acids isolated from composite No. 2 differed slightly from the analytical results for the total triterpene acids isolated by the usual method (without mechanical treatment). The principal components are also substances with M = 468.3 and 466.3 whereas the contributions from substances with M = 482.3 and 484.3 increased. The main difference is that the fraction of peaks eluting in the first 5 min increased for the sample produced by the mechanochemical method. A variety of ions, the strongest of which were peaks of high-molecular-weight compounds with M = 498, 500, 514, and 516, was observed. These compounds were assumed to be more polar (than those described earlier) triterpene acids.

We found that the ratio of component concentrations of the mechanochemically treated mixture and the parameters of the mechanochemical treatment (time in the active zone of the grinding bodies, amount and geometric characteristics of the grinding bodies loaded in the mechanochemical activator) must be selected depending on the properties of the starting plant material, i.e., moisture and content of triterpene acids and ballast substances.

The minimal content of alkali depends on the kinetics of the mechanochemical process (rate of component transfer during mechanochemical treatment) and the content of triterpene acids in the plant material. For example, the Na_2CO_3 content should be at least 3%. Otherwise the time of mechanochemical treatment needed to neutralize the acids increases to values unacceptable for the process.

The effectiveness of neutralizing the acid part decreases sharply if the water content in the plant material is <1%. Apparently the residual water makes the alkali mobile.

The mechanism making the reagents mobile is still under discussion. One issue is the presence of water in the plant material. Air-dried material with a moisture content up to 10% is usually used. According to one of the possible models, strong mechanical treatment can generate high-pressure zones and local heating. The water contained in pores can approach a supercritical state, increase its dissolving power, and transport dissolved substances [30].

The presence of an abrasive in the treated mixtures helps to destroy cell walls and grinds finely the material. The fraction of the principal fraction (5-25 μm) increases from 20 to 80%. In addition, fine particles of abrasive are included in the plant-material particles during mechanical activation. As a result, the bulk density of the product increases. This aids the settling and centrifugation of the aqueous extracts.

Thus, mechanochemical treatment of fir needles with solid bases forms mechanical composite powders that contain triterpene acids in a water-soluble form. The presence of an abrasive in the treated mixturees improves the grinding.

Comparative vegetative experiments showed that the mixture of triterpene acids produced by the standard extraction method affects significantly the sprouting of wheat seeds. The aqueous extract produced from composite No. 2 also stimulates the growth of roots (30%) and shoots (20%).

We determined previously that the mixture of triterpene acids isolated from fir needles has a biological activity that also stimulates the formation of callus tissue on rape explants and plant regeneration [9].

Tests with rape explants also showed that the aqueous extract of composite No. 2 exhibits the cytokinin activity of the total triterpene acids. Adding the aqueous extract of composite No. 2 to the culture medium increased the volume of callus tissue by 23-26% regardless of whether or not cytokinin (6-benzyladenine) was present in the medium and intensified root formation.

Soaking the mechanical composites in aqueous solution extracts the triterpene acids and most of the water-soluble substances in the starting plant material. Therefore, we performed additional biological tests in order to determine if the biological activity of the aqueous extract of composite No. 2 was due to the presence in it of water-soluble forms of triterpene

acids. We isolated from the aqueous extract the total triterpene acids and studied its effect on the sprouting of wheat seeds and the formation of callus in rape explants. The total triterpene acids obtained from the aqueous extract of composite No. 2, like the aqueous tincture, stimulated the formation of roots and wheat shoots. Triterpene acids isolated from the aqueous tincture of composite No. 2 also stimulated callus formation in rape explants.

Thus, we conclude from the results of the instrumental analysis that the biological activity of the preparation produced by the mechanicochemical method is due to the presence in it of water-soluble forms of triterpene acids.

EXPERIMENTAL

HPLC analysis was performed on a Millichrom Ob-4 chromatograph. The analytical conditions were: Nucleosil-C18 column, 2.0 × 75 mm, 5.0 μm particle size, mobile phase CH_3OH (85%) with H_3PO_4 (0.05 M), flow rate 100.0 μL/min, column temperature 25.0°C, sample volume 2.0-5.0 μL. A three-wave detector (200, 210, 240 nm) was used. Chromatograms were recorded using the Chrom V 1.02 program. Peak areas of chromatograms were calculated using the 200-nm channel of the UV detector.

HPLC—MS analysis [31, 32] was carried out on a liquid chromatograph with a diode array and mass-selective detector (Agilent 1100 Series LC/MSD). The chromatography conditions were: Zorbax XDB-C8 4.6 × 150 mm, particle size 5 μm, mobile phase H_2O:CH_3OH, linear gradient 75-95% CH_3OH from 5 to 10 min, flow rate 1.00 mL/min. UV chromatograms were recorded at 240.16 nm (control wavelength 350.10 nm) with storage in memory of the UV spectra for all chromatographic peaks. Negative ions were scanned by atomspheric-pressure chemical ionization (APCI) using a mass-selective detector with a quadrupole analyzer (model G1946C) over the range *m*/*z* 200-550. The eluent was removed in the spray chamber by drying gas (N_2, 7 mL/min) at 340°C and 4.42 atm. The vaporizer temperature was 350°C.

Chemically pure or high-purity reagents were used. Solvents were purified by standard methods [33]. Murasighe-Skoog nutrient medium, 2,4-dichlorophenoxyacetic acid, and 6-benzyladenine (Serva) were used for the biological experiments.

Plant material: Fur needles (Maslyanin Region, Novosibirsk District, collected in November 2002) were dried and ground after removal of branches. The drying was carried out in a stream of warm air (40-45°C) on special wood trays. The initial moisture of the green material was 46%; final, 5%. The dried needles were ground in a blade-type disintegrator (8255, Nossen VEB Maschinen) to particle size 2-3 mm.

Mechanical treatment was performed in an AGO-2 planetary centrifugal activator (acceleration of grinding bodies 200 m/s^2) with water cooling using steel reactors and grinding bodies (170 g, diameter 5 mm). The mass ratio of grinding bodies and treated material was 170:5; treatment time, 3 min.

Composites Nos. 1 and 2 were prepared by mechanical activation in the AGO-2. Composite No. 2 consisted of plant material, abrasive material, and Na_2CO_3. Composite No. 1 did not contain Na_2CO_3. An experimental batch of the product was prepared in a VTsM-10 flow-through activator. A mixture with a composition analogous to that of No. 2 was treated in the VTsM-10 (acceleration of grinding bodies 170-200 m/s^2, 1.5 min treatment time). The product was a finely ground light-brown powder with particle-size distribution 5-25 μm (80%), 25-40 μm (15%).

Isolation of Total Strong Triterpenes. Fir needles (10 g) were defatted by hexane and extracted with Et_2O (3 × 50 mL). The ether extracts were combined and extracted with saturated $NaHCO_3$ solution (3 × 55 mL). The aqueous extracts were combined and washed with hexane (3 × 50 mL), acidified to pH 2 with HCl, and extracted with Et_2O (3 × 50 mL). The ether extracts were combined, washed with saturated NaCl solution (3 × 30 mL), and dried over Na_2SO_4. Solvent was removed in vacuo in a rotary evaporator. Yield 2.9%. The resulting acids were analyzed by HPLC and HPLC—MS.

Composite No. 1 was extracted with Et_2O (3 × 30 mL). The ether extracts were combined and purified by the procedure used to isolate triterpene acids to afford an amorphous powder (yield 4.7% of plant material).

Composite No. 2 (2.5 g) was extracted with H_2O (25 mL, 25°C) with stirring for 10 min. The solid was separated by centrifugation. The aqueous extract was filtered through a nylon membrane filter (Osmonics, Micron Separations Inc., pore size 0.45 μm), saturated with $NaHCO_3$, washed with hexane, acidified to pH 2, and extracted with Et_2O (3 × 10 mL). Solvent was removed to afford water-soluble acids (36.6 mg, 2.32% of plant material). Repetition of the procedure using the solid obtained by centrifugation extracted 25.7 mg of triterpene acids (1.62% of plant material).

Biological Tests. Laboratory experiments studied the biological activity of the products. The studied mixture of compounds was added in various concentrations to nutrient medium for in vitro tests. Mixtures of compounds produced from the mechanical composites and needles were dissolved in water. We used the Murasighe—Skoog agar mixture as nutrient medium [34]. Callus tissue was prepared and rape plants were regenerated by the previous methods [35].

Formation of callus on explants of rape (isolated leaf fragments) was induced by adding 2,4-dichlorophenoxyacetic acid to the nutrient medium; shoot regeneration, 6-benzyladenine.

Explants were cultivated for 4-6 weeks at 18-24°C with a 16-h light period, and additional illumination of 3,000 lux. The rates of formation of callus (undifferentiated) tissue, roots, and shoots; callus volume; shoot height; quantity of leaves and roots; and root length were determined.

Vegetative tests were performed on Novosibirsk-89 wheat seeds placed in rolls of filter paper. They were sprouted in solutions of the studied compounds for 7-10 days. The shoot height, root length, and fresh mass of sprouts were determined.

The experimental results were examined by statistical analysis [36].

Biological tests showed a significant stimulating effect for accelerated plant growth in vitro and *in vivo*.

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