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Antibacterial Activity of Polyphenols Derived Mechanochemically from Natural Raw Materials

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Abstract. This study focuses on bactericidal activity of a number of biomass-derived polyphenols (humic substances and melanins) obtained by mechanochemical activation of the brown coal and husk of buckwheat mixed with a solid oxidizing or reducing agents against opportunistic pathogens relevant to the poultry industry: Salmonella typhimurium, Shigella sonnei, Escherichia coli, Pasteurella multocida, Yersinia pseudotuberculosis, Streptococcus pyogenes, Staphylococcus aureus, and Staphylococcus epidermidis. The studied polyphenols are active against bacteria belonging to the family Enterobacteriaceae (Salmonella typhimurium, Shigella sonnei, Escherichia coli, and Yersinia pseudotuberculosis) after being subjected to mechanochemical activation in the presence of sodium percarbonate. Almost all the studied samples, except for those exposed to treatment with zinc, were active against bacteria Pasteurella multocida (the family Pasteurellaceae) and Staphylococcus epidermidis (the family Staphylococcaceae). The samples active against bacteria Streptococcus pyogenes (the family Streptococcaceae) and Staphylococcus aureus (the family Staphylococcaceae) can be obtained by mechanochemical interaction with sodium percarbonate under certain conditions.

Keywords: Fagopyrum esculentum, mechanochemical activation, polyphenols, melanin, humic substances, biological activity.

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Антибактериальная активность полифенолов, полученных механохимическим путем из природного сырья

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Аннотация. Проведено исследование бактерицидного действия ряда полифенолов (гуминовых веществ и меланинов) из природного сырья, полученных в результате механохимической обработки смесей сырья с твердым окислителем – перкарбонатом натрия и восстановителем – цинком в отношении условно-патогенных бактерий, важных для птицеводства: Salmonella typhimurium, Shigella sonnei, Esherichia coli, Pasteurella multocida, Yersinia pseudotuberculosis, Streptococcus pyogenes, Staphylococcus aureus, Staphylococcus epidermidis. Исследованные гуминовые полифенолы проявляют активность в отношении бактерий семейства Enterobacteriaceae (Salmonella typhimurium, Shigella sonnei, Esherichia coli, Yersinia pseudotuberculosis) после механохимического взаимодействия с перкарбонатом натрия. Образцы после взаимодействия с цинком активности не проявляют.

В отношении бактерий Pasteurella multocida (семейство Pasteurellaceae) Staphylococcus epidermidis (семейство Staphylococcaceae) активны практически все исследованные гуминовые и меланиновые препараты за исключением препаратов после взаимодействия с цинком. Активные в отношении бактерий Streptococcus pyogenes (семейство Streptococcaceae), Staphylococcus aureus (семейство Staphylococcaceae) могут быть получены механохимическим взаимодействием с перкарбонатом натрия в определенных условиях.

Ключевые слова: Fagopyrum esculentum, механохимическая обработка, полифенолы, меланин, гуминовые вещества, биологическая активность.

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Introduction

The interest in obtaining natural polyphenols and using them in various fields of medicine, cattle breeding, and food industry is stipulated by the fact that the humanity is becoming aware that constant supply of antibiotics into the body should be avoided as much as possible. Natural polyphenols derived from plant raw materials, peat, and brown coal exhibit a broad range of effects on the human and animal bodies [1, 2]. The antimicrobial [3–5], antiviral [6, 7], antifungal, immunostimulatory, and anti-inflammatory properties of polyphenols are reported most commonly [8, 9]. The use of certain polyphenols increases animals' productivity and improves quality of animal-based foods [10].

Meanwhile, the modern mechanochemical technologies allow one to use an environmentally friendly and economically efficient method to produce polyphenol preparations [11], which are characterized by an elevated content of soluble biologically active substances and are promising for food industry [12, 13] and fodder production [14, 15]. The preparations manufactured mechanochemically from biomass (phytobiotics) can inhibit growth of some pathogenic organisms [5, 16]. The biological activity of polyphenol preparations is usually attributed to the polymeric structure of polyphenol macromolecules and the number of functional groups in polymer molecules (mostly the oxygen-bearing ones) [17–19]. The methods for chemical modification of polyphenols (in particular humic ones) are involve performing solid-phase mechanochemical reactions in mixtures of humic substances or humic feedstock with solid oxidizing [20, 21] and reducing [22] agents. This treatment significantly changes the number of functional groups and alters properties of polyphenols (e.g., their complexation ability). Furthermore, mechanochemical activation increases the amount of low-molecular-weight polyphenols (and fulvic substances in particular) formed [23].

Appreciably simple, accurate, and rapid experimental methods for assessing the effect of biologically active substances and preparations on microorganisms have been developed due to the significant advances in microbiological analysis of bacterial invasion of human and animals' bodies and compiling thematic collections of microorganisms (including pathogenic ones) typical of animals, especially birds. This study aimed to determine the profile of biological (antagonistic and antibacterial) activity of some modern polyphenol preparations (humic and melanin ones) obtained mechanochemically against a number of opportunistic microorganisms depending on the method used for modifying natural preparations.

Materials and Methods

The preparation of raw materials (brown coal for humic acids, buckwheat husk for melanins) for the isolation of polyphenol preparations consisted in mechanochemical treatment without additives, with the addition of an oxidizing agent (solid sodium percarbonate in an amount of 5 wt.%) or a reducing agent (metal zinc in an amount of 5 wt.%). Moisture content in the raw material was 8–10 wt.%. An AGO-2 planetary activator mill designed at the Institute of Solid State Chemistry and Mechanochemistry (Russian Academy of Sciences) was used as a mechanochemical reactor (the treatment mode: calculated acceleration of grinding bodies was 200 m/s^2; treatment duration was 2 or 10 min; steel balls 5 mm in diameter were used as the grinding bodies; the ball to powder ratio was 1: 20). In all the cases, mechanochemical activation was performed in the presence of atmospheric oxygen.

Brown coal from the Itatskoye deposit of the Kansk–Achinsk coal basin (Western Siberia, Russia; 56°05'24.43"N, 89°04′23.64″E) was used. Humic acids were extracted according to the State Standard GOST 9517–94 (ISO 5073:2013). The content of humic substances in the mechanochemically manufactured powder products was up to 70 % [24].

Buckwheat (*Fagopyrum esculentum Moench)* husk has been drawing researchers' attention as an affordable source of melanins, active polyphenolic substances [25]. Their content in the mechanochemical extracts obtained in the presence of sodium percarbonate is up to 8 % [26]. Husk (hulls) of buckwheat of Dikul variety (State Standard GOST 56105–2014) cultivated in the Cherepanovo district (Novosibirsk region, Russia) was used. Melanins were extracted and analyzed using the procedure [27] elaborated and fine-tuned for melanins contained in tree mushrooms. Buckwheat husk was extracted in an apparatus equipped with a reflux condenser using a 0.1 M NaOH solution at 90 °C for 30 min. After cooling down, the solution was separated by filtering using a paper filter and a water-circulating pump. 1 M HCl solution was added to the filtrate until pH became 1–2; after 30 min, the resulting precipitate was filtered off and subjected to re-precipitation that involved dissolution in 10 % NH_3 solution and vaporization at 90 °C.

The following samples were obtained and analyzed (Table 1). A mixture of polyphenol-containing raw materials: coal as well as husk of buckwheat (*Fagopyrum esculentum Moench)* with sodium percarbonate or zinc was subjected to solid-phase mechanochemical activation. The use of both oxidizing

Table 1. The analyzed samples of preparations containing humic acid and melanin

No.	Short code for the sample	Description
1	Pristine HA	Humic acids (HAs) from untreated coal
2	HA activated for 2 min	HAs from coal subjected to mechanical activation for 2 minutes
3	HA activated for 10 min	HAs from coal subjected to mechanical activation for 10 minutes
4	HA activated with SP, 2 min	HAs from a mixture of coal and sodium percarbonate (SP) subjected to activation for 2 minutes
5	HA activated with SP, min	HAs from a mixture of coal and sodium percarbonate subjected to activation for 10 minutes
6	Pristine melanin	Melanin from untreated buckwheat husk
7	Melanin activated for 2 min	Melanin from buckwheat husk subjected to activation for 2 minutes
8	Melanin activated for 10 min	Melanin from buckwheat husk subjected to activation for 10 minutes
9	Melanin activated with SP, 2 min	Melanin from a mixture of buckwheat husk and sodium percarbonate subjected to mechanical activation for 2 minutes
10	Melanin activated with SP, 10 min	Melanin from a mixture of buckwheat husk and sodium percarbonate subjected to mechanical activation for 10 minutes
11	Melanin activated with Zn, 2 min	Melanin from a mixture of buckwheat husk and zinc subjected to mechanical activation for 2 minutes
12	Melanin activated with Zn, 10 min	Melanin from a mixture of buckwheat husk and zinc subjected to mechanical activation for 10 minutes
13	Reference samples	Biosib® Combi

and reducing reagents and various processing conditions was aimed at obtaining a range of polyphenols characterized by a wide range of oxygen-containing groups responsible for their biological activity. Next, polyphenol samples of different degrees of oxidation were isolated from the resulting powder materials. The reference sample was a Biosib® Combi, a microbial product (a combination of specially selected and freeze-dried homofermentative lactic and propionic acid bacteria and enzymes: xylanase (≥ 50,000 U/g), pectin lyase (≥ 30,000 U/g)) manufactured by SIBBIOPHARM (Berdsk, Novosibirsk region, Russia) [28]. Elemental analysis was carried out using an EA3000 automatic elemental CHNS analyzer (Eurovector Instruments, Italy). The carbon, hydrogen, nitrogen, and oxygen contents were corrected to the ash-free basis.

Spectrophotometric analysis of humic acid and melanin solutions was carried out on an SF-2000 UV/VIS spectrophotometer (LOMO Ltd., St. Petersburg, Russia) using polystyrene cells relative to 0.1 M NaOH solution. To prepare a solution, an accurately weighed sample of humic acids or melanin (0.022 g) was dissolved in 50 mL of 0.1 NaOH solution, subjected to treatment in an ultrasonic bath (Vilitek, Russia) at a frequency of 40 kHz for 5 min and centrifugation at a frequency of 6000 rpm for 5 min. After sampling a 1 mL aliquot from the supernatant, 0.1 M NaOH solution was added: 3 mL for humic acids and 1 mL for melanin. The spectra were recorded in the range of 400–700 nm (scanning pitch, 1 nm; precision mode; three accumulation cycles).

The disk diffusion method was used for studying the antibacterial activity against test microorganisms [29, 30]. This method is based on direct determination of the key quantitative parameter: the minimal concentration inhibiting visible growth of a microorganism under study on dense medium. The preset concentrations of an antibacterial preparation are added to the growth medium, into which the culture of analyzed microorganism is subsequently seeded. After incubation, it is assessed whether there is or there is no visible growth. Archival strains and field isolates of the following microorganisms were used as test cultures: *Salmonella typhimurium, Shigella sonnei, Escherichia coli, Pasteurella multocida, Yersinia pseudotuberculosis, Streptococcus pyogenes, Staphylococcus aureus,* and *Staphylococcus epidermidis.* Meat infusion agar manufactured at the State Research Center for Applied Microbiology and Biotechnology (Obolensk, Russia) was used for working with microorganisms.

Results and discussion

Characterizing polyphenol preparations

A mixture of polyphenol-containing raw materials: coal as well as husk of buckwheat (*Fagopyrum esculentum Moench)* with sodium percarbonate or zinc was subjected to solid-phase mechanochemical activation. Next, polyphenol samples of different degrees of oxidation were isolated from the resulting powder materials.

Table 2 shows the yields of brown coal humic acids before and after mechanochemical activation under different conditions, as well as the results of spectrophotometric and elemental analysis. The absorption spectra of the solutions of humic acids extracted from brown coal before and after mechanochemical activation have a falling form typical for humic acids. The samples were characterized using the E_4/E_6 ratio, which is equal to the D_{465}/D_{600} ratio and is a parameter used most commonly for describing the degree of oxidation of humic acids [31, 32].

In order to balance out the effect of molecule size and environment acidity on this ratio [33], we recorded all the spectra in a strongly alkaline medium at identical pH values. The spectrophotometric analysis data suggest that the degree of oxidation decreases after mechanochemical activation for all the samples, which correlates with elemental analysis data: the H/C ratio increases, while the O/C and H/C ratios decrease, thus demonstrating that the "average degree of oxidation" of the product declines after the humin-containing raw material is subjected to treatment in the presence of sodium percarbonate. Comparison of the triads "the pristine sample – the sample activated for 2 min – the sample activated

Table 2. Characteristics of humic acids

	Sample	Yield of humic acids, %	E_4/E_6	C, $\%_{mass}$	H, $\%_{mass}$	N, $\%_{mass}$	H/C	O/C	O/H
1	Pristine HA	17.0 ± 2.5	3.23	62.37 ± 0.05	4.55 ± 0.11	1.08 ± 0.05	0.87	0.38	0.44
2	HA activated for 2 min	38.3 ± 2.0	3.20	63.07 ± 0.09	4.60 ± 0.03	0.95 ± 0.04	0.88	0.37	0.43
3	HA activated for 10 min	72.6 ± 3.8	3.03	63.10 ± 0.05	4.85 ± 0.16	0.96 ± 0.05	0.92	0.37	0.40
4	HA activated with SP, 2 min	42.1 ± 6.1	3.20	62.91 ± 0.16	4.76 ± 0.18	0.99 ± 0.05	0.91	0.37	0.41
5	HA activated with SP, 10 min	64.6 ± 7.4	3.18	61.74 ± 0.23	4.70 ± 0.07	0.98 ± 0.05	0.91	0.40	0.43

for 10 min" demonstrates that longer treatment duration increases the degree of oxidation of coal more significantly than adding an oxidizing agent does.

This can be explained by the fact that activation in the presence of sodium percarbonate significantly raises the total yield of humic substances. The previously non-extractable portion of humic acids having lower degree of oxidation compared to that of the pristine sample starts to pass into the solution. This effect is enhanced by the fact that the most oxidized fragments of humic acids remain in the soluble, non-deposited fraction (i.e., are converted to fulvic acids) during purification, when humic acids are precipitated from the solution under acidic conditions at pH = 2 [34].

The values of light absorption of melanin solutions at 465 nm of (Table 3) demonstrate that mechanochemical activation of buckwheat husk reduces absorption in the visible region for the extracted melanin regardless of additives used during mechanochemical activation. This effect is enhanced when an oxidizing agent (sodium percarbonate) is added during mechanochemical activation, as well as when longer treatment durations are used. A 5 % sodium percarbonate additive is sufficient for, within 2 minutes, reaching an effect identical to that achieved by 10-minute activation in the absence of reagents.

For all melanin samples extracted from buckwheat husk processed under oxidizing conditions (samples **7–8** contained no additives; samples **9–10** were activated in the presence of a sodium percarbonate additive), the H/C ratio was increased (Table 3). The sample extracted from buckwheat husk subjected to activation in the presence of sodium percarbonate for 10 min was characterized by the lowest light absorption at 465 nm, which, combined with the high oxygen content and low H/C ratio, is due to the low solubility of this sample. Melanin extracted from buckwheat husk activated for 2 min in the presence of zinc is characterized by low O/H and O/C ratios (the low oxygen content). As one can see below, this very sample exhibits the lowest antibacterial activity.

The results of biological tests

The antibacterial activity of polyphenols was studied via the diffusion method by adding solutions of the samples into wells on the surface of solid growth media. The presence of growth inhibition

Table 3. Characteristics of melanin samples

	Sample	D_{465}	C, $\%_{mass}$	H, $\%_{mass}$	N, $\%_{mass}$	O, $\%_{mass}$	H/C	O/C
6	Pristine melanin	0.610	49.6 ± 0.2	5.69 ± 0.09	2.69 ± 0.05	42.4 ± 0.2	1.38	0.64
7	Melanin activated for 2 min	0.478	47.9 ± 0.1	5.71 ± 0.06	4.58 ± 0.02	41.9 ± 0.2	1.43	0.66
8	Melanin activated for 10 min	0.323	50.6 ± 0.2	6.04 ± 0.11	3.66 ± 0.04	39.7 ± 0.3	1.43	0.59
9	Melanin activated with SP, 2 min	0.310	47.3 ± 0.4	5.97 ± 0.15	4.36 ± 0.02	42.4 ± 0.5	1.52	0.67
10	Melanin activated with SP, 10 min	0.273	50.0 ± 0.3	5.67 ± 0.02	2.10 ± 0.02	42.2 ± 0.4	1.36	0.63
11	Melanin activated with Zn, 2 min	0.382	53.7 ± 0.2	6.27 ± 0.08	3.30 ± 0.11	36.7 ± 0.4	1.40	0.51
12	Melanin activated with Zn, 10 min	0.349	59.3 ± 0.1	6.84 ± 0.03	3.59 ± 0.13	30.26 ± 0.2	1.38	0.38

zones for test microorganisms around the wells containing polyphenol samples was a criterion for assessing the antibacterial activity. The results are summarized in Table 4. It has, therefore, been demonstrated that preliminary mechanochemical activation of raw material significantly changes the chemical composition of the extracted polyphenolic fraction; in turn, this alters the biological activity of the samples.

Mechanochemical activation of humin-containing preparations significantly broadens the range of antibacterial activity. Comparison of the samples obtained at identical durations of mechanochemical activation but differing in reagent added (pairs of samples **2/4** and **3/5**; Table 4) demonstrates that the effectiveness of preparations obtained by mechanochemical activation of plant raw material in the presence of a special reagent is higher than the effectiveness of plant raw material ground under the same conditions. This effect seems to be caused by the fact that soluble forms of biologically active substances are formed during the treatment and their extractability is altered as the number of oxygen--bearing groups in macromolecules is varied.

Buckwheat husk-derived preparations have a high initial activity. Mechanical and mechanochemical activation makes the spectrum of antibacterial activity of preparations (samples **6–10**) narrower, up to complete absence of activity with respect to the tested pathogenic bacterial species (sample **11**). Some polyphenolic preparations (samples **4, 5, 6, 9, 10**, and **12**) differ in terms of the number of microorganisms they can inhibit that are comparable to a modern commercial microbial product (sample **13**). The increase in biological activity of a melanin sample mechanically activated with zinc for 10 minutes requires further study, since this effect is unpredictable in terms of correlations between the degree of oxidation of the polyphenol and its biological activity.

The revealed effect can be subsequently used in the substantiated approach to designing combination drugs, which needs to be used for specific cases of the treatment and prophylaxis of humans and animals. As more data on selectivity of preparations and composition of pathogenic microorganisms that are typical of each specific case of disease become available, it can be possible to select mixtures of preparations for each disease in a tailored manner.

Some regularities related to differences in behavior of microorganism families have been detected. The studied polyphenols exhibit antibacterial activity against bacteria belonging to the family *Enterobacteriaceae* (*Salmonella typhimurium, Shigella sonnei, Escherichia coli,* and *Yersinia pseudotuberculosis*) after mechanochemical activation; after activation in the presence of zinc, the sample are inactive. Almost all humic and melanin preparations (except for those obtained via interaction with zinc) are active against bacteria *Pasteurella multocida* (the family *Pasteurellaceae*) and *Staphylococcus epidermidis* (the family *Staphylococcaceae*). The preparations exhibiting activity against bacteria *Streptococcus pyogenes* (*the family Streptococcaceae*) and *Staphylococcus aureus* (the family *Staphylococcaceae*) can be manufactured mechanochemically under certain conditions (using a sodium percarbonate additive and specific treatment duration).

In summary, the following conclusions can be highlighted:

(1) Mechanochemical activation significantly alters the chemical composition of the water-soluble polyphenolic fraction. Activation in the presence of zinc increases the yield of the more reduced polyphenol fraction, while decreasing the “total degree of oxidation” of the preparations.

(2) The preparations produced by mechanochemical activation of mixtures of natural raw materials (coal or buckwheat husk) with solid sodium percarbonate inhibit growth of opportunistic

Table 4. Antibacterial activity of the polyphenol samples

No.	Treatment method / Strain	*Salmonella typhimurium*	*Shigella sonnei*	*Escherichia coli*	*Pasteurella multocida*	*Yersinia pseudotuberculosis*	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Staphylococcus epidermidis*
1	Pristine HA	-	-	-	+	-	+	-	+
2	HA activated for 2 min	-	-	-	+	+	-	-	+
3	HA activated for 10 min	-	-	-	+	+	-	+	+
4	HA activated with SP, 2 min	+	+	+	+	+	-	+	+
5	HA activated with SP, 10 min	+	-	-	+	+	+	+	+
6	Pristine melanin	+	+	+	+	-	-	+	+
7	Melanin activated for 2 min	+	-	-	+	+	-	-	+
8	Melanin activated for 10 min	+	-	-	+	+	-	-	+
9	Melanin activated with SP, 2 min	+	+	+	+	+	-	-	+
10	Melanin activated with SP, 10 min	+	+	+	+	+	-	+	+
11	Melanin activated with Zn, 2 min	-	-	-	-	-	-	-	-
12	Melanin activated with Zn, 10 min	-	+	+	+	+	-	+	+
13	Reference sample	-	+	+	+	+	+	+	+

Note: (+) – antibacterial activity is present; (–) – no antibacterial activity was detected.

bacteria *Salmonella typhimurium, Shigella sonnei, Escherichia coli, Pasteurella multocida, Yersinia pseudotuberculosis, Streptococcus pyogenes, Staphylococcus aureus,* and *Staphylococcus epidermidis.*

(3) The studied humic polyphenols exhibit an antibacterial activity against bacteria belonging to the family *Enterobacteriaceae (Salmonella typhimurium, Shigella sonnei, Escherichia coli,* and *Yersinia pseudotuberculosis)* after mechanochemical interaction with sodium percarbonate; the samples produced after treatment in the presence of zinc are inactive.

(4) Almost all the studied humic and melanin preparations, except for those produced via treatment in the presence of zinc, are active against bacteria *Pasteurella multocida* (the family *Pasteurellaceae*) and *Staphylococcus epidermidis* (the family *Staphylococcaceae*).

(5) Preparations active against bacteria *Streptococcus pyogenes* (the family *Streptococcaceae*) and *Staphylococcus aureus (*the family *Staphylococcaceae*) can be obtained via mechanochemical interaction under certain conditions, which are characterized by the amount of sodium percarbonate added and treatment duration.

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