Summary. This paper presents the dependence of antibacterial activity on the structure of phenolic acids as biologically active substances of natural origin. The biological activity of selected compounds was assessed by means of microbiological tests on selected strains of tested bacteria Staphylococcus aureus, Proteus vulgaris and Bacillus subtilis. Microbiological assays were correlated with spectroscopic data. The dependencies between the percentage growth inhibition of microorganisms under the influence of test compounds vs. values of the wavenumbers for individual bands present in the infrared spectra (FT-IR) of these compounds, were determined. These results suggest that the biological activity of the compounds depends on the electron density distribution both in the ring and the carboxylate anion. Therefore, the dependence structure-activity may be useful for predicting the biological properties of a series of novel synthetic compounds without the need for biological tests for each compound.

Key words: antimicrobial activity, phenolic acids, microorganisms, biologically active substances

INTRODUCTION

Correlations between structure of chemical compounds and their biological activity have long been the object of a scientific interest. Recognized dependencies are often used in the synthesis of new medicines, pesticides, plastics etc. Considering the chemical
structure of a chemical compound, toxicity of other substances of similar structure can be predicted. Even a small change in the chemical structure greatly influences the physicochemical properties, which in turn often change the biological effects. For instance, introduction to chemicals the carboxyl (–COOH), sulfonic (–SO₃H), thiol (mercaptane) (–SH), methoxy (–OCH₃), or acetyl group (–COCH₃) significantly decreases their toxicity, and in some cases it completely neutralizes the adverse properties of an initial substance. Such impact of these radicals is the result of, among others, increasing the polarity of a compound, which contributes to the rapid dissolution, absorption and excretion, and also facilitates the metabolic processes. A typical example of this phenomenon is introduction of carboxyl group into the benzene ring [De Flora 1998].

Effect of hydroxyl group (–OH) on the toxicity of a compound may vary. In aliphatic compounds, the introduction of a hydroxyl group weakens the narcotic effect. Another hydroxyl radicals lead to a complete abolition of this effect. Introduction to the aliphatic compounds, 5-6 hydroxyl groups, e.g. into sugars, completely neutralizes their hazardous effects. A very different effects of the hydroxyl group were demonstrated in aromatics. The presence of the hydroxyl group significantly increases the toxicity of the compound. Benzene with its narcotic properties, after the introduction of –OH group, as a phenol, in addition to the neurotoxicity, is strongly irritant and causes denaturation of cellular proteins. The introduction of a second hydroxyl group in the ortho (catechol) or para position (hydroquinone) causes oxidation of hemoglobin, which is not observed when the hydroxyl group is in the meta position (resorcinol) [Baer-Dubowska 2003].

Among a spectrum of physicochemical and structural parameters characterizing a molecule, the quantitative research upon the relationship between structure and action of the compounds most commonly takes into account: hydrophobicity and hydrophilicity, electronic properties, and steric parameters. They all can be relatively easy to measure. The hydrophobicity and hydrophilicity refers to both the entire molecule, as well as individual substituents. Quantitative evaluation of the impact of steric and electron parameters, however, are more complicated and is only possible for selected substituents. Studies of the effect of chemical structure on the biological activity of compounds from different chemical groups are extremely rare and confined to the hydrophobicity tests. Compounds belonging to the same chemical group are more often analyzed, wherein the change in their biological activity resulting from the change of substituents, aromatic ring or functional groups, are studied. Friedman et al. studied the bactericidal activity of benzoic acid derivatives with respect to Campylobacter jejuni, Escherichia coli, Listeria monocytogenes and Salmonella enterica [Friedman et al. 2003]. In addition, the biological activity of preservatives was also assessed using microbiological tests on other bacterial strains, such as Staphylococcus aureus, Proteus vulgaris, Bacillus subtilis, Candida albicans, Pseudomonas aeruginosa [Świsłocka 2013].

The literature data indicate that a variety of phenolic acids: ferulic (4-hydroxy-3-methoxycinnamic), p-coumaric (4-hydroxycinnamic), caffeic (3,4-dihydroxycinnamic), vanillic (4-hydroxy-3-methoxybenzoic), isovanillic (3-hydroxy-4-methoxybenzoic), syringic (4-hydroxy-3,5-dimethoxybenzoic), gentisic (2,5-dihydroxybenzoic) and protocatechuic (3,4-dihydroxybenzoic) show some activity towards Gram-positive bacteria, while in the case of Gram-negative ones, these substances are inactive. Increased activity of phenolic acids in relation to Gram-positive bacteria results from different structure of
their cell membrane. The activity degree also depends on the structure of the compound, which is the theme of presented scientific achievement. Friedman et al. [2003] studied the bactericidal activity of benzoic acid derivatives with respect to *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Some authors reported that m-coumaric acid inhibits growth of such bacteria as: *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, whilst o-coumaric acid is inactive towards these bacteria [Middleton et al. 2004, Burt 2004, Salameh et al. 2008].

Caffeic, p-coumaric, ferulic, and protocatechuic acids are characterized by high fungicidal activity. Of a group tested fungi: *Verticillum* sp., *Fusarium oxysporum*, *Rhizopus* sp., *Penicillium italicum*, *Rhizoctonia solani*, *Stemphylium solani*, *Cladosporium* sp., *Mucor* sp., *Colletotrichum* sp., *Pythium* sp., *Alternaria* sp., these acids had the strongest fungicidal action to *Fusarium oxysporum* and *Verticillum* sp., while no activity was observed with respect to *Alternaria* sp. [Denyera and Stewart 1998]. The fungicidal properties are also presented by gallic acid (3,4,5-trihydroxybenzoic) and its methyl ester that appeared to be active towards *Magnaporthe grisea*, *Botritis cinerea*, *Puccinia recondita*, and gallic acid towards *Magnaporthe grisea* and *Erispinphe graminis* [Maiorella et al. 1983]. Comparing two phenolic acids: ferulic with gallic, it appears that the former has stronger antimicrobial activity. Panizzi et al. [2002] found that ferulic acid is active towards following bacteria and fungi: *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, while gallic acid towards: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Candida albicans* [Narendranath et al. 2001]. Phenolic compounds and their derivatives exhibit anti-HIV and anti-rabies features, although caffeic acid and its derivatives are the most effective. These compounds are of natural origin and are biodegradable in the environment.

The aim of this study was to investigate the relationship between the molecular structure of selected acids and their salts and their biological properties in relation to the bacteria *Staphylococcus aureus*, *Proteus vulgaris* and *Bacillus subtilis*. A logical series of metals: Li, Na, K, Rb, Cs (the atoms of these elements have the same oxidation degree but different atomic radius) meeting the following criteria were selected for the study: (1) practical applicability due to good solubility of alkali metal compounds in water and polar solvents (better than acids); (2) least harmfulness to living organisms and the environment; (3) availability, stability, and ease of preparation.

**MATERIAL AND METHODS**

In own studies, below acids and their salts with lithium, sodium, potassium, rubidium, and cesium were subject to microbial assays: 2,3-dihydroxybenzoic, 2,4-dihydroxybenzoic, 2,5-dihydroxybenzoic (gentisic), 2,6-dihydroxybenzoic, 3,4-dihydroxybenzoic (protocatechuic), 3,5-dihydroxybenzoic, o-coumaric, m-coumaric, p-coumaric, cinnamic, caffeic, vanillic, syringic, homovanillic, nicotinic, isonicotinic, picolinic, and for comparisons benzoic acid and sodium benzoate. Biological activity of the test compounds was assessed by means of microbial assays using selected test strains bacteria: *Staphylococcus aureus* (PCM 2267), *Proteus vulgaris* (PCM 2269), *Bacillus subtilis* (PCM 2021). The
microorganisms originated from the collections of Polish Collection of Microorganisms, Polish Academy of Sciences at the Institute of Immunology and Experimental Therapy in Wroclaw. The selection of bacteria was dictated by the PCM instructions for tests for antibacterial agents and literature reports in this regard.

Proteus vulgaris bacteria of the *Enterobacteriaceae* family are found in food. This family includes many microbial species important for food microbiology, because in majority, they are pathogenic bacteria, yet being a part of natural gut microflora of humans and animals. The percentage of *Enterobacteriaceae* in a digestive tract increases in the case of large amounts of animal-origin food in a diet. *Proteus vulgaris* bacilli grow well on common substrates, under aerobic or relatively anaerobic conditions, at the optimum temperature at pH 7.5. The optimum growth temperature is 34–37°C. The growth of this bacteria is accompanied by medium alkalinization. Ciliated forms give a characteristic nebular growth on a solid substrate that after 24 hours covers the whole surface area of the medium. *Proteus vulgaris* is undesirable microorganism in food. Secondary infection of food products with this microorganism can cause severe diarrhea and vomiting at humans. Diarrhea caused by *Proteus vulgaris* are prolonged and difficult to treat, especially at immunocompromised patients [Halon et al. 2000]. *Proteus vulgaris* can also be a cause of adverse organoleptic changes of food, in particular protein products.

Bacteria from *Bacillus* genus are widely distributed in the soil and nature, they occur mainly on plants, from where they enter the food. They include aerobic and facultatively anaerobic bacilli, that are often arranged in chains. These bacteria are a major problem in canning, because spores of these organisms are resistant to high temperatures. The food can contain thermophilic species with optimal growth temperature of 37–55°C as well as mesophiles. These species are distinguished by high saccharolytic, proteolytic, biochemical, and lipolytic activity. Their development is promoted by starch, and therefore they can be found in potatoes, maize flour, mashed potatoes. *Bacillus cereus* contaminates mainly cereal products and plants. In contrast, *Bacillus subtilis* genus is the cause of changes, among others, in wheat bread due to mucus [Biswas and Roymon 2008].

*Staphylococcus aureus* is a Gram-positive coccus belonging to *Micrococcaceae* family. Staphylococci form oval or round cells with a diameter of 0.7 to 1.0 µm. Cell dimensions vary for different strains, e.g. size of staphylococcus from saliva is larger amounting from 1.0 to 1.2 µm. In the environment and in the liquid medium, *Staphylococcus aureus* is present singly in the form of diplococci, quadruplets, or short chains containing 3–4 cells. All staphylococci species do not produce spores and are devoid of cilia. Only a few strains produce envelope. *Staphylococcus aureus* bacteria are concentrated in clusters forming irregular arrangements. This is particularly evident on preparations from solid substrates [Duda and Gertig 2007]. *Staphylococcus aureus* is common in nasopharyngeal cavity, as well as on human and animal skin. At humans, these bacteria colonize in the groin, perineum, nasal vestibule, navel cavity, and navel. People associated with hospital environments are particularly vulnerable to staphylococcus infection. Carrier ship of the bacteria in the nostrils of healthy individuals ranges from 36 to 50%, and at a hospital population up to 70%. It is estimated that 10 to 50% of the population are carriers of the bacteria, even though no symptoms of disease. Staphylococci are also responsible for gastric infections as a result of consuming the food products contaminated with enterotoxic strains.
The tested microorganism strains were grown in broth for 24 hours at 36°C. The broth medium for microbiological assays was inoculated with the bacterial culture (playing also the role of a control medium). The test substances were added into inoculated broth in the amounts in order to achieve 0.1% concentration in the broth solution. Aliquot of 4.75 ml broth inoculated with bacteria was used, and 0.25 ml of the test compounds solution was added. Test compounds solutions were prepared by dissolving 0.2 g in 10 ml of a solvent. Control medium was a broth inoculated with the test strain without addition of the studied formulations. All samples were incubated in an incubator at 36°C for bacteria. The increase in the number of microbial cells in the broth culture was tested at 24 and 48 hours of incubation by measuring the optical density of the solution at $\lambda = 600$ nm on a UV-VIS spectrophotometer by JASCO [Howard and Whitcombe 1995]. The amount of bacterial cells in broth medium, recalculated onto 5 ml culture, was determined by streaking method on Petrie dishes filled with agar medium. The aim was to standardize the culture, which allowed for the repeatability of measurement conditions.

The above-described method for determining the microbial activity of investigated formulations is a modification of the basic antibacterial assays performed in microbiology laboratories. It allows for selecting compounds with potential inhibitory or stimulating properties in respect to each of bacterial strains. This method is quick and easy to perform. Results of microbiological assays and spectroscopic tests (experimental and theoretical) were used to analyze the relationships between the molecular structure of compounds and their biological properties. The plots illustrating the uniform microbial data in a form of graphs, where microorganism growth (%) in relation to broth cultures with no test preparations (100%), was presented.

A Fourier transform infrared spectroscopic (FT-IR) analysis of the tested acids and salts was performed using an Alfa-Eco spectrophotometer from Bruker. The solid samples were prepared using the technique of compressing potassium bromide pellets (weight ratio of sample to KBr was 1 : 100). The spectra were recorded in the wavenumber range 4000–400 cm$^{-1}$ on the transmittance scale, using a resolution of 4 cm$^{-1}$.

Statistical calculations were performed with the use of the Statistica 13. On the basis of the spectroscopic data: the values of wavenumbers of infrared spectra of the compounds and the parameters describing their antimicrobial activity the principal component analysis (PCA) was performed.

**RESULTS AND DISCUSSION**

Among tested compounds (Fig. 1), the most effective after 24 hours of incubation in relation to *Proteus vulgaris* bacteria proved to be: o-coumaric acid and its salts with lithium, sodium and potassium, benzoic acid (as reference standard) and rubidium 2,4-dihydroxybenzoate, bacterial growth inhibition degree of which was at the level of 90%, while after 48 hours, o-coumaric acid, p-coumaric acid, benzoic acid (as reference standard), and rubidium 2,4-dihydroxybenzoate most strongly inhibited the growth of test strain – in 95%. Other preparations also in most cases inhibited bacterial growth, but to a much lesser degree [Kowczyk-Sadowy et al. 2012, Świsłocka et al. 2012, Kowczyk-Sadowy et al. 2015].
The own study upon microbiological analysis of microbiological results showed that after 24 hours of incubation, the strongest inhibitory effect on the growth of *Bacillus subtilis* bacteria (Fig. 2) was recorded in the case of *ortho*-coumaric acids (99%). Sodium *p*-coumarate (62.8%), potassium *p*-coumarate (64.1%), and lithium *m*-coumarate (71.7%) inhibited the growth of test microorganisms to a lesser, but also high degree. While benzoic acid derivatives containing hydroxyl and methoxy groups, i.e. syringic and vanillic acid and their salts showed an inhibiting growth of bacteria at the level of 20–30%, after 48 hours of incubation, majority of test compounds showed inhibitory effect on the multiplication of *Bacillus subtilis* only in 10–20%. Only benzoic acid (as reference sample) efficiently inhibited the growth of microorganisms in 85% after 24 hours and in 72% after 48 hours of incubation [Świsłocka et al. 2012, Piekut et al. 2012, Świsłocka et al. 2013].

The source of poisoning are food products such as meat and meat products, canned meats, brawn, black pudding, milk and dairy products, ice cream, cakes with cream and pudding, salads, poultry, eggs, processed fish, especially in vinegar, because staphylococci are resistant to acetic acid. The above-mentioned food products can be contaminated originally, e.g. due to milk of animals suffering from staphylococcal mastitis or meat of animals with staphylococcal purulent process. However, the main source of food products contamination (secondary) is the contact of patients with purulent skin complications or staphylococci carriers who are employed in the food industry. The source of infection may also be the equipment and production areas for food production. To prevent the staphylococcal development, the food should be cooled as soon as possible and kept within the cold chain. Staphylococcal food poisoning usually occurs as outbreaks. Staphylococci ferment sugars not producing any gas, and are resistant to salting and drying.

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Zeszyty Problemowe Postępów Nauk Rolniczych

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**Fig. 1.** Growth of *Proteus vulgaris* bacteria (PV) after 24 and 48 hours of incubation under influence of tested compounds with respect to the broth cultures with no tested preparation addition

*Rys. 1.* Wzrost bakterii *Proteus vulgaris* (PV) po 24 i 48 godzinach inkubacji pod wpływem badanych związków w odniesieniu do hodowli bulionowych bez dodatku badanego preparatu
These microorganisms are resistant to the effects of some pharmaceutical preparations, e.g. antibiotics.

Effect of the test compounds on *Staphylococcus aureus* bacteria strain is highly variable (Fig. 3), however, the most effective at inhibiting the growth of the broth culture were: *o*-coumaric acid (99%) and its salts with rubidium (92%) and cesium (89%), salts of *p*-coumaric acid with sodium and potassium (88 and 90%, respectively) after 24 hours of incubation. A similar trend was also shown after 48-hour incubation [Kowczyk-Sadowski et al. 2012, 2015].

Friedman et al. [2003] and Pennington [2002] reported lower activity of the carboxyl rather than aldehyde group and the increase in activity with increasing number of hydroxyl groups in the molecule. It was found that 2,4,6-trihydroxybenzoic acid was inactive, whereas 3,4,5-trihydroxybenzoic acid showed high activity. The 4-methoxybenzoic acid (*p*-anisic) was more toxic than 4-hydroxybenzoic acid, which might be associated with a reduction in hydrophilicity of the compound. It was also shown that the increase in the number of methoxy groups in molecules of this type of compounds has contributed to the decrease in their toxicity. In addition, it was found that toxicity of benzoic acid derivative is closely related to the number of hydroxyl groups and depends on the position of the functional group, which significantly correlates with hydrophobic properties of the compound [Pennington 2002].

The biological activity of compounds can be varied depending on their structure. This is due the type of substituent, position of substituent, type of metal substituted within the carboxylic moiety. Infrared spectroscopy applied in our study provides information about the molecular structure of compounds. Infrared spectra are specific to particular
chemical compounds. The study recorded and interpreted infrared spectra of tested acids and their salts. On the basis of spectroscopic data, values of the wavenumbers from the infrared spectra for the compounds, and parameters describing their anti-microbial activity, analysis of principal components was performed. This is the method most commonly used for analysis of multivariate experimental data. The analysis was based on the value of loads, which are linear correlation coefficients between the antimicrobial properties of the formulations and selected wavenumbers for bands in the FT-IR spectra. From the graph, two main components were selected to data analysis. To investigate which of the bands described with values of wavenumbers are strongly positively or negatively correlated with microbiological data, the weight projection on the planes defined as pairs of main factors, was made. Correlations were determined in regard to the angles formed by vectors having a common origin at the same point and ends defined by respective values of weight outside the circle.

Microbiological assays were correlated with spectroscopic data. The dependencies between the percentage growth inhibition of microorganisms such as \textit{Proteus vulgaris}, \textit{Bacillus subtilis} as well as \textit{Staphylococcus aureus} under the influence of test compounds vs. values of the wavenumbers for individual bands present in the infrared spectra (FT-IR) of these compounds, were determined. Statistically significant correlations between these values were found, especially in the case of \textit{Staphylococcus aureus}. Analysis included only the bands and parameters of the statistical evaluation, for which the highest correlation coefficient values were achieved at least for the individual microorganisms. It was found that the stretching vibration bands of carboxylic group $\gamma_{\text{as}}$COO$^-$ and deformation vibration

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**Fig. 3.** Growth of \textit{Staphylococcus aureus} bacteria (SA) after 24 and 48 hours of incubation under influence of tested compounds with respect to the broth cultures with no tested preparation addition

**Rys. 3.** Wzrost bakterii \textit{Staphylococcus aureus} (SA) po 24 i 48 godzinach inkubacji pod wpływem badanych związków w odniesieniu do hodowli bulionowych bez dodatku badanego preparatu
bands in the plane βC-H (18b) for all salts were best correlated with biological properties of the compounds. These data suggest that the biological activity of the compounds depends on the electron density distribution both in the ring and the carboxylate anion.

Statistical processing described above were in part quoted in publications Kowczyk-Sadowy et al. [2012, 2015].

Fig. 4. Analysis of individual bands in FT-IR spectrum in relation to the degree of growth inhibition of *Staphylococcus aureus* bacteria (SA) after 24 and 48 hours of incubation in the system of two principal components

Rys. 4. Analiza poszczególnych pasm w widmie FT-IR w odniesieniu do stopnia zahamowania wzrostu bakterii *Staphylococcus aureus* (SA) po 24 i 48 godzinach inkubacji, w układzie dwóch składowych głównych
Statistical analysis of results was based on an interpretation of two principal components, made on the basis of the variable loads representing the linear correlation coefficients between the antimicrobial properties of the formulations and the wavenumbers of selected bands in the spectra recorded by means of FT-IR technique (Fig. 4). The most statistically significant correlation has been shown between the degree of bacterial growth inhibition after 24 hours of incubation and the wavenumbers of bands 19a ($r = 0.956$), 8b ($r = 0.815$), $\beta_s$COO ($r = 0.868$), 19b ($r = -0.902$), $\beta_a$COO ($r = -0.901$), 8a ($r = -0.876$). No statistically significant correlation was indicated after 48 hours of incubation.

Associations between variables were presented as a graph of points configuration representing variables in the system of the first two principal components (Fig. 4). Corresponding coefficients of the variables were used as coordinates of these points, i.e. the wavenumber of selected bands in the spectra made applying FT-IR technique and the length of the directional vectors connecting the points with origin of the coordinate system from the first two principal components. The closer to the circle a variable is, the better its representation by principal components that define the coordinate system, i.e. major part of information contained within that variable is explained by the principle components.

**CONCLUSIONS**

Analysis of the test results indicates that statistically significant correlations were demonstrated between the degree of microbial growth inhibition and wavenumbers for selected bands in the spectra of acids: $\nu$C–OH, $\nu$C = O, $\beta$C = O, 18a, 19b, and in the case of salts of these acids, the biological action of the compounds was the strongest correlated with wavenumbers of bands: $\gamma_s$COO, and 18b, 8b, 19b.

Therefore, it is reasonable to search for the model of linear correlation between microbial activity and spectroscopic data, which describe the electron charge distribution within the molecule and, hence the chemical properties of the compound. Relationship structure-activity studied by our team may be useful for predicting the biological properties of a series of novel synthetic compounds without the need for biological tests for each compound, which is highly important in the case of designing the synthetic analogs of biologically active compounds, the molecular structure of which may be crucial at assessing their interaction with living organisms (e.g. bacteria).

**Acknowledgements**

The research was carried out as part of work no. WZ/WB-IIŚ/3/2020 at the Białystok University of Technology and financed from a subsidy provided by the Minister of Science and Higher Education.
Dependence between the antimicrobial activity...
Streszczenie. W pracy przedstawiono zależność pomiędzy aktywnością przeciwdrobnoustrojową a strukturą molekularną kwasów fenolowych jako substancji biologicznie czynnych pochodzenia naturalnego. Aktywność biologiczną wybranych związków oceniono na wybranych szczepach bakterii: *Staphylococcus aureus*, *Proteus vulgaris* i *Bacillus subtilis*. Badania mikrobiologiczne skorelowano z danymi spektroskopowymi. Wyznaczono zależności pomiędzy procentowym zahamowaniem wzrostu drobnoustrojów pod wpływem badanych związków, a wartościami liczb falowych dla poszczególnych pasm występujących w widmach podczerwieni (FT-IR) tych związków. Wyniki te sugerują, że aktywność biologiczna związków zależy od rozkładu gęstości elektronowej zarówno w pierścieniu, jak i anionie karboksylanowym. Dlatego te zależności te mogą być użyteczne do przewidywania właściwości biologicznych serii nowych związków syntetycznych bez konieczności wykonywania testów biologicznych dla każdego związku.

Słowa kluczowe: aktywność przeciwdrobnoustrojowa, kwasy fenolowe, mikroorganizmy, substancje biologicznie czynne