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ANTIMICROBIAL PROPERTIES AND IMPORTANCE OF TANNINS FOR THE FOOD INDUSTRY

BY

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Abstract. The present paper is aimed to perform extended studies on the tannins extracted from the green nuts with a special attention paid to the separation methods and testing of the anti-microbial activity against some gram-positive and gram-negative micro-organisms as well as against the fungi. For finding the optimum extraction method the extractions by mechanical stirring and in ultrasonic field were carried out while the polyphenolic compounds were determined by applying the spectrophotometric method. The sensitivity of micro-organisms toward the tannin compounds was tested under the optimum standard conditions. Following the antibacterial test the inhibitory action of the tannin compounds on the microorganisms under study was estimated which affords their use in food industry by adding them in various food products (cake creams, certain sausage and cheese types).

Keywords: green nuts; separation methods; microorganisms; difusimetric method; tested activity.

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1. Introduction

As revealed by literature the tannins are natural polyphenolic compounds of a large structural heterogeneity and a rather high molecular weight showing strong anti-bacterial and anti-fungi activities against microorganisms, being thus suitable for being used in pharmaceutical and medical industries as well as in the food industry (Aires *et al*., 2016; Kim *et al*., 2012; Panagiotis, 2012; Viswanath *et al*., 2009). They show an astringent taste and may give complexes with proteins, starch, cellulose and also with some minerals (Laurichesse and Averous, 2014). The slightly acid-tart taste of tannins is due to the free carboxyl groups while the astringency comes from the low-molecular phenolic compounds (Khoddami *et al*., 2013; Bennickm, 2002). Certain phenolic hydroxyl groups on the molecule surface of tannins are responsible for some biological properties which make them suitable for treating certain human diseases (Bhattacharya *et al*., 2011; Grigore *et al*., 2013; Xie *et al.*, 2011).

The anti-microbial activity of tannins is attributable to the inactivation of microbial adherence, of transporting enzymes and proteins in the cell membrane (Cowan, 1999; Kolodziej *et al.*, 1999; Lupaşcu *et al*., 2017).

The main mechanism of the tannin action consists in their ability of binding the hydrogen in the available polyhydroxyphenol groups with some proteins especially those of a proline high content. Thus, the polyvalent intercellular junctions are troubled by the precipitation and aggregation phenomena (Bennickm, 2002), since the tannins may give stable macro-molecular complexes with proteins (Lim *et al*., 2006).

Some hypotheses have been advanced on the antimicrobial mechanisms of the tannins among which those based on the direct action of the tannins on the microbial mechanism by inhibiting the oxidative phosphorylation seem the most plausible (Girarda *et al*., 2018). This mechanism involves the binding of the tannins to the metabolic ions which results in decreasing the availability of the essential ions involved in the inhibition of the enzymatic activity by forming complexes with bacterial and fungi substrata (Girarda *et al*., 2018). The complete or partial inhibition of growth of various bacteria and yeast species by the hydrolysable tannins is due to their interference with the active principles of the tannin extracts. Thus, the tannins may act by inhibiting the extra-cell microbial enzymes interfering thus with the compounds necessary to bacterial growth (Girarda *et al*., 2018).

The aim of the present study is to find a more efficient method for extracting the phenolic derivatives from the raw material and to estimate their anti-microbial activity.

2. Material and Methods

Material

The high quality tannins coming from vegetal raw material were obtained from green nuts and the anti-microbial activity tested on the *Escherichia coli, Pseudomonas aeruginosa* (gram-negative), *Staphylococcus aureus* (gram-positive) bacteria and the fungus *Candida albicans*.

Extraction method of tannins

The extracts were obtained from the vegetal material by means of the static (maceration) and dynamic (extraction in ultrasonic field) methods. The optimum parameters of the extraction process were settled by taking the following factors into account: solvent concentration, vegetal material to solvent ratio and extraction time (Hoyos-Martínez *et al.,* 2019; Lupaşcu *et al.,* 2017).

For the extraction by mechanical stirring the vegetal raw material was prepared by extraction, then dried at room temperature and the mechanical impurities removed. The vegetal product was weighted, the solvent added (60% and 80% ethylic alcohol) in a mass/ volume ratio of 1:10 and 1:7, respectively, being then submitted to mechanical stirring for 24 h at room temperature (this procedure was accomplished into five stages). The obtained extracts were subsequently brought together and concentrated by distillation. The resulting product was dried at 50°C.

In case of extraction in ultrasonic field the vegetal product prepared for extraction was weighted and treated with the solvent (60 and 80% ethylic alcohol), in ratios of 1:10 and 1:7, then suspended in a water bath and submitted to the ultrasonic action for 35 min at room temperature (the procedure was run into five stages). The extracts were then brought together, concentrated by distillation and dried at 50°C.

Determination of total content of polyphenolic compounds

The total content of polyphenolic compounds was estimated by the spectrophotometric method by using the Folin-Ciocalteu color reagent (Pusztahelyi *et al.,* 2015; Lupaşcu *et al*., 2017). This method affords the determination of the hydroxyl groups in the sample under study in alkali conditions (adjusted with sodium carbonate in ratios of 1:10 and 1:7). At the wavelength of 765 nm the absorbance increases proportionally to the number of hydroxyl groups in the tannin. The total content of phenols was expressed as equivalents on gram.

To 0.2 mL of sample to be analyzed 0.45 mL of Folin-Ciocalteu (F-C) reagent diluted with water (1:7) were added. After 15 min 1.85 mL of 1M Na_2CO_3 were added and the material kept then in dark for 30-40 min. Subsequently the solution absorbance was noticed at the 765 nm wavelength on a Jenway UV/Vis 7310, 320-1000 nm apparatus. The sample was examined in comparison with the standard sample as 60% ethanol solution.

The antimicrobial activity

The microorganisms were cultivated on an agar-agar medium with addition of tannins of 0.1% concentration. With the young microorganism cultures microbial suspensions of 1/100, 1/1000, 1/10000 have been prepared (Jorgensen and Turnidge, 2007). The nutritional medium without tannins was taken as a standard. In the center of the Petry plates containing the solidified medium the mycelium disk of a diameter of 4 mm was sowed and the plates kept then at the temperature of 23-24ºC. The colonies were found to grow in the days 2, 5 and 7, with insignificant modifications in the other days.

3. Results and Discussions

The method of mechanical stirring and that of treating with ultrasounds are applied for settling the optimum extraction conditions for a maximum amount of phenolic compounds from the green nuts. The extraction method with ultrasounds was applied since it is much more quicker (5 cycles of 35 min each) with an extraction yield higher compared to the method of mechanical stirring (5 cycles of 24 h each). The extraction yield of the polyphenolic compounds was of 8.20% by mechanical stirring and of 8.97% by treating with ultrasounds. The total amount of the polyphenolic compounds extracted from the raw material was found not to depend mainly on the extraction method.

The optimum ratios between the vegetal product amount and the volume of the solvent used for the extraction of a maximum content of polyphenolic compounds were of 1:7 and 1:10. The solvent volume taken for the extraction was found to influence insignificantly the total content of the extracted compounds. In the following researches the 1:7 value was chosen for the vegetal product amount/solvent volume ratio. As regards the influence of the extraction solvent concentration the conclusion was drawn that a 60% solvent is suitable since it affords the obtaining of polyphenolic compound amounts almost equal to those resulting with the 80% concentration. Consequently, for extracting a maximum content of polyphenolic compounds from the green nuts the method with ultrasonic field under a controlled temperature, with 60% ethylic alcohol taken in a vegetal product/solvent ratio of 1:7 was chosen.

The sensitivity of the microorganisms under study against the tannin compounds was tested *in vitro* under the optimum standardized cultivation conditions: culture medium, inoculum, incubation time. The agar-agar (gelose) with tannins of 0.1% concentration was taken as culture medium and the agar-agar free of tannins was the standard sample. The diffusion method was applied and the growth of colonies noticed in the days 2, 5 and 7. The method was performed in Petry plates by deposing 200 μL of the samples under study on the surface of a solid medium; the diffusion through the medium followed, then a constant decrease in the concentration gradient to the periphery. When the area

diameter is larger the germ is more sensitive and the substance amount necessary to the inhibition lower and inversely.

The agar-agar culture medium with tannins of 0.1% concentration added was used for the bacteria: *Staphylococcus aureus, Escherichia coli, Pseudomonas Aeruginosa* and the yeast *Candida.* The media were put into Petry plates as an uniform layer of 4mm thickness. The optimum Ph was selected at 7.4 for bacteria and 6.5 for yeasts. From the young cultures of microorganisms microbial suspensions of 1/100, 1/1000, 1/10000 have been prepared.

The fact must be taken into account that the inoculum in the germ to be studied must be a representative one, namely to include microbial populations of every category.

Every plate was inoculated with 4 mL of the obtained suspensions and then let to stay for 3-5 min at room temperature for the inoculum absorption. After inoculum removing the plates were kept for 30 min at room temperature and applied subsequently on the medium surface. The incubation was performed at 23°C for 24 h for bacteria and 26°C for 72 h for fungi. Microorganism cultures were used for sowing both the samples and the standard sample (gelose).

Only the plates with cultures corresponding in purity and density were read. The reading was made to the naked eye by measuring 2-3 times the diameter of the inhibition area /mm in different directions by means of a rule.

The data obtained with the antifungal test revealed the tannins obtained from the green nuts to affect the growth and development of the microorganisms and fungi under study depending on the compound concentration, fungi species and its growing time on the nutritional medium (Table 1).

Table 1

The Levels of Sensitivity to the Tested Compounds

Microorganism tested	*Escherichia coli*			*Pseudomonas aeruginosa*		
Day	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
x, mm	3.9	5.5	6.2	4.1	5.2	5.5
Standard	3.7	4.9	6.0	3.9	4.5	4.9
Microorganism tested	*Staphylococcus aureus*			*Candida albicans*		
Day	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
x, mm	4.7	5.3	5.6	5.1	6.2	6.5
Standard	3.8	5.2	5.6	5.1	5.2	6.4

The strongest inhibition power of the tannins was noticed with the dilution of 1/1000. Thus, the diameter of the microorganism colonies growing in the 2-nd day compared to the standard sample. In the 5^{th} growing day the difference between the standard sample and that with tannins of 1/1000 concentration was found to decrease. The obtained data made evident the fact that the microorganisms get accustomed to the tannin presence during the growing process.

As revealed by the experimental results the tannins extracted from nuts may inhibit completely the growth of microorganisms. The data were red after 7 days and within this time the evolution of the microorganisms to be tested with tannin extract noticed. When the antibacterial activity was tested on *Escherichia coli* the antibacterial effect compared to the other samples tested was estimated. In case of activity tested with the fungi *Candida albicans* a much more higher antibacterial effect than with the other samples was also noticed.

4. Conclusions

The tannins may be introduced in the food industry in order to inhibit the antibacterial activity of certain microorganisms.

For extracting the polyphenolic compounds the method with ultrasounds was applied being much more quicker than that with mechanical stirring giving also a higher extraction yield of the compounds.

The following main factors influencing the extraction were found: the action of ultrasonic field, the controlled temperature, the vegetal product to solvent ratio.

The sensitivity of the used microorganisms to the tannin compounds was tested "*in vitro*", under the cultivation optimum and standardized conditions: culture medium, inoculum, incubation time. A comparison of the antibacterial activity of the tested compounds made evident a clear inhibition against *Escherichia coli* and *Candida* and rather lower against *Pseudomonas Aeruginosa* and *Staphylococcus aureus.*

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PROPRIETĂȚILE ANTIMICROBIENE ȘI IMPORTANȚA TANINURILOR ÎN INDUSTRIA ALIMENTARĂ

(Rezumat)

Scopul lucrării constă în extinderea studiilor asupra taninurilor izolate din nucile verzi, insistând pe metoda de izolare și testarea activități antimicrobiene față de o

serie de microorganisme gram pozitive, gram negative și asupra levurilor. Pentru determinarea metodei optime de extracție s-a realizat extracția prin agitare mecanică și în câmp ultrasonic, iar pentru determinarea compușilor polifenolici, s-a utilizat metoda spectrofotometrică. S-a testat sensibilitatea microorganismelor la acțiunea compușilor taninici în condiții optime și standardizate de cultivare. În urma testului antibacterian s-a determinat acțiunea inhibitoare a compușilor taninici, asupra microorganismelor utilizate, ceea ce face posibilă utilizarea acestora în industria alimentară ca adaosuri în diverse produse alimentare (creme pentru prăjituri, diverse tipuri de salam, brânzeturi).