https://doi.org/10.31925/farmacia.2019.6.16 **ORIGINAL ARTICLE**

ENHANCED BIOMEDICAL PROPERTIES OF CHITOSAN-ENOXIL FILMS

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Abstract

Polyphenolic compounds show antioxidant activities by inhibiting the formation of free radicals. Using eco-friendly reactions, which involve oxidation and depolymerization of grape seed tannins, new products for biomedical applications are obtained. Especially, it is of high interest the valorisation of by-products such as grape seeds, which are in considerable quantities in the Republic of Moldova. However, these products are highly labile to environment risk factors. Therefore, the use of polymeric materials, especially by-products such as chitin and chitosan, is a perspective way to design dressing materials such as biomedical films. This paper studies the change of antioxidant activity and bacteria growth inhibition of the synthetized chitosan-Enoxil film. It was demonstrated that the addition of Enoxil to chitosan film in the concentration of 5% improves antibacterial properties against *Bacillus subtilis, Pseudomonas fluorescens* and *Candida utilis*. Moreover, these biofilms have proved an efficient inhibition of the cation ABTS radical (0.42 mg-echiv/mL Tox) and DPPH radical (23.66 % remaining DPPHc). Incorporation of polyphenolic acids to chitosan films could improve the overall lifetime of the food products storage.

Rezumat

Compușii polifenolici manifestă proprietăți antioxidante prin inhibarea formării radicalilor liberi. Folosind reacții *eco-friendly*, care implică oxidarea și depolimerizarea taninurilor din semințe de struguri, pot fi obținute produse noi pentru aplicații bio-medicale. În special, prezintă interes valorificarea produselor secundare, precum semințele de struguri, care sunt în cantități considerabile în Republica Moldova. Totodată, aceste produse sunt extrem de labile la factorii de risc a mediului ambiant. Prin urmare, utilizarea materialelor polimerice cum ar fi produșii secundari chitina și chitosan, reprezintă o perspectivă de a fabrica biomateriale cum sunt filmele biomedicale. În cadrul acestei lucrări a fost studiată modificarea activității antioxidante și antibacteriene a bio-materialelor sintetizate chitosan-Enoxil. S-a stabilit că la adăugarea de Enoxil în filme de chitosan în concentrație de 5% se îmbunătățesc proprietățile antibacteriene împotriva *Bacillus subtilis, Pseudomonas fluorescens* și *Candida utilis*. De asemenea, aceste biofilme sunt eficiente în inhibarea cationului radical ABTS (0,42 mg-echiv/mL Tox) și radicalului DPPH (23,66%, conc. DPPH remanent). Încorporarea acizilor polifenolici în filme de chitosan ar putea îmbunătăți durata de viață a produselor alimentare la stocare.

Keywords: tannin, chitosan, biomedical, antioxidant, antibacterial

Introduction

Winemaking industry is a significant economical branch in the Republic of Moldova, due to the favourable conditions for grapes growing in our country. Climate, water and soil play a key role in the development of this industrial area. Climate conditions and soil fertility are favourable and allow the growing of a vast variety of grapes of high quality. As a result, Moldova has a real potential for exporting the wine products to the European market, but this branch of agro-food industry is accompanied by obtaining large quantities of both liquid and solid waste.

The wine industry in the Republic of Moldova has the potential to process grapes up to 1 million tons/ year by 125 companies, but in recent years only about 550 thousand tons of grapes were purchased and processed. After grapes processing, a significant quantity of wine by-product waste is produced and can be further used. All these products are a source of environmental pollution and create major environmental problems. Therefore, it is important to processes raw material (grapes) using technological lines, which lead to no waste or minimal waste, as well as further processing of secondary wine products in order to obtain a wide range of useful products [1, 2]. Secondary winemaking products are an important source for obtaining special natural products with specific properties that cannot be obtained synthetically, and through their processing allowing a good environmental protection. Waste from the wine industry, consisting mainly from solid and liquid by-products, includes pulp, seeds, yeasts, and may account for an average about 30% (w/w) of the grapes used for

wine production. This waste mostly contains biodegradable organic materials, which can be used to produce various highly demanded products for different industries such as pharmaceutical, cosmetic and food ones. Secondary winemaking products can be processed and the quantities of waste can be significantly reduced by new or modified processing methods, obtaining a variety of products [3].

Some of these wine products contain considerable amounts of polyphenolic components, depending on the type of grapes (white or red), the part of the tissues (hides, seeds, etc.) and processing conditions (e.g. seeds). In recent years, not only secondary by-products but also a number of other agricultural wastes of vegetable origin have attracted considerable attention as potential sources of bioactive polyphenols, which can be used in the pharmaceutical, cosmetic and food industries. However, in many cases, the adequate feasibility studies on the operation of this agricultural waste are missing.

In recent years, stability and prolonged activity issues of food products have become a growing concern for the food industry. Polymer films using as primary material chitosan are known to be biocompatible, biodegradable, antibacterial, possessing antioxidant activities [4]. Biodegradable films containing active ingredients can effectively maintain food quality and safety, in addition to acting as barriers to moisture and gas [5]. In particular, polyphenolic acids, including gallic acid, grape seed and pomace extract, tea catechins and several hydroxybenzoic acids have been covalently grafted to chitosan or physically incorporated in antioxidant active chitosan films. These modifications benefited the acknowledged protective effects of foods against oxidation reactions [6-11].

The purpose of this paper is to investigate the change of antioxidant activity and bacteria growth inhibition of the synthesized chitosan-Enoxil film in order to improve its antibacterial properties.

Materials and Methods

Materials

Chitosan medium molecular weight (mol wt 190,000 - 300,000 Da (based on viscosity)), ABTS cation radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, DPPH 2,2-diphenyl-1-picrylhydrazyl, luminol, ammonium persulphate, EDTA thylenediamine-tetra-acetic acid, Trolox 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid were purchased and used from Sigma-Aldrich. NaOH, HCl, acetic acid, H_2O_2 and ethanol were purchased from a local chemicals supplier. Enoxil product was synthesized in our Ecological Chemistry laboratory. Microbiological investigations were performed in collaboration with Moldova's National Public Health Centre (NPHC).

Analysis of antioxidant scavenging properties

Antioxidant scavenging ABTS•+ assay

ABTS cation radical assay is quite often used for the determination of polyphenolic compounds antioxidant activity of polyphenolic compounds, or their complex material with different natural polymers [12]. To control the antioxidant activities of flavonoids, radical ABTS+ was used in this assay, according to the protocol of Re *et al*. [13]. For the analysis, a stock solution of cation ABTS (7 mM) was mixed with ammonium persulphate solution (2.45 mM, final volume concentration), and left to stay in dark for 12 - 16 h at room temperature. Afterwards, the mixture was transferred to a 70% ethanol solution in order to obtain a final absorbance of 0.700 (± 0.02), measured at 734 nm. The sample solution was prepared by dissolving 10 mg film into 3 mL ultra-pure water. Then, adding 40 μL sample to 4 mL of ABTS•+ solution produces an inhibition of free radicals of 20 - 80%. The measurement was performed on a Jenway Spectrophotometer 6505, exactly 1 min after initial mixing. Calibration curve for different Trolox concentrations was drawn, and the result was expressed as mg-echiv/mL Trolox (Figure 1).

Chemilumenescence measurement

In alkaline solution, H2O2 is a source of free radicals, as hydroxyl, singlet oxygen and superoxide radicals [14]. These free radicals catalyse the oxidative cleavage of luminol, followed by N_2 removal and transition to excited 3-aminophthalate anion form which then de-excite with luminesce as result [15, 16]. It is known that antioxidant is a powerful radical scavenger that could inhibit the triggering of free radical formation, decreasing the emission of photon light [17]. Therefore, inhibition percentage of free radicals produced in the luminol/Tris-EDTA chemiluminescence generation system after addition of 50 μL of aqueous hydrogen peroxide solution (50 μM), 200 μL of Luminol solution (0.1 mM), 650 μL Tris-EDTA solution (pH 8.0) and 100 μL aqueous or alcohol solution of sample. The final mixture volume of 1 mL was vortexed for 10 seconds prior to insertion in the reactor tube. Emitted light was recorded on Promega Glomax Luminometer instrument over 15 sec, after initial mixing with peroxide solution. Measurements were performed in triplicates, the fixed value being the arithmetic mean of the parallel analyses.

The antioxidant activity, AA (%) was calculated according to the following equation:

$$AA(\%) = (I_0-I_s)/I_0*100\%,$$

where I_0 and I_s are relative light emissions of blank and sample solution after 15 sec, respectively.

DPPH radical inhibition assay

The free radical DPPH$^{\bullet}$ is considered one of the most stable radicals and used for determination of the antioxidant activity [18-20]. In this study, the method described by Wootton-Beard *et al*. with some minor

modifications was used. 60 µM DPPH in a solution of ethanol in water (70%) was prepared before analysis. Its measured absorbance was 0.700 ± 0.02 at 517 nm. Prior to analysis, 10 mg of film were immersed in 3 mL ultra-pure water. In order to measure the radical scavenging activity (%, remaining DPPH) of biomedical films, 60 µL of film forming solution were transferred to 3 mL of DPPH ethanol solution. After interaction of DPPH free radicals with the antioxidant, the quantity of remaining DPPH was measured. Measurements were performed in triplicates, the fixed value being the arithmetic mean of the parallel analyses.

$$\% \text{ remaining DPPH} = \frac{Abs\ sample}{Abs\ blank} * 100,$$

where *Abs sample* is the sample absorbance measured over 240 min and *Abs blank* (DPPH ethanol solution) is the absorbance measured over 240 min.

Microbiological investigation

Frequently, the microbiological assay for the analysis of the biomedical films is similar to disk applications on inoculated agar plates and incubation at specific conditions with measurement of the inhibition zone surrounding of the disk [21, 22]. Briefly, Petri dishes were sterilized by heating and then, nutrient and Sabouraud Dextrose Agar were poured in aseptic conditions. Moreover, strains of *Pseudomonas fluorescens* CNMN-PFB-01, *Bacillus subtilis* CNMN BB-01 and *Candida utilis* were prepared accordingly to the laboratory procedure, with initial inoculum of 3 x 10^8 CFU, measured by turbidity method. The microorganisms were diluted in sterilized distilled water to the concentrations of 10^6 CFU. Further, 0.1 mL of inoculum were taken, representing the seeded dose, put into the centre of the prepared dishes and spread uniformly on the agar plate surface. Subsequently, samples of chitosan and chitosan impregnated with Enoxil (1%, 2.5%, and 5%) films with 20 mm diameter were immersed in the gelatinous agar. Afterwards, the seeded Petri dishes were placed in the thermostat at 37°C for 24 hours. The next day the results of the antimicrobial and antifungal activity were assessed by calculating the diameters of inhibitions around the impregnated discs. All measurements were doubled, the fixed value being the arithmetic mean of the parallel analyses.

Synthesis of chitosan-Enoxil films

The preparation method consists of preparing Enoxil solutions with different concentrations (1%, 2.5%, 5 %, w/1 g of chitosan) with 2% (g/100 g of Enoxil solution) chitosan left for 6 hours swelling. Afterwards, concentrated acetic acid was added to obtain a final concentration of 2.5% in film forming solution. The resulted film forming solution was pipetted on Petri dishes accurately, avoiding the air bubbles, and left to dry at 45°C for 24 hours.

Results and Discussion

Antioxidant activity of the biomedical films

ABTS free cation radical, being one of the most stable radicals is considered as a first-choice method analysis for the determination of scavenging properties of natural antioxidants. ABTS cation radical method is a universal assay for determination of both hydrophilic and lipophilic compounds [23, 24].

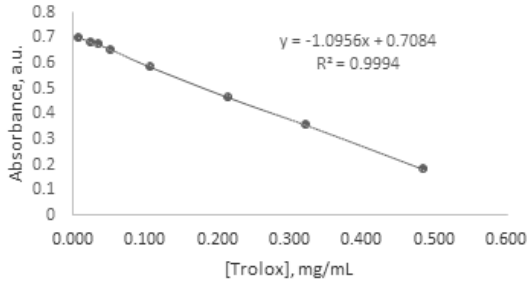

Figure 1.
Trolox calibration curve obtained after inhibition of cation ABTS radical

In this study, chitosan and chitosan-Enoxil films were investigated for antioxidant capacity in free radical scavenging. In order to measure the antioxidant activity of natural inhibitors, a calibration curve for Trolox was plotted (Figure 1). A significant increase in cation ABTS radical scavenging ability was greater for chitosan-film/Enoxil (1%, 2.5% and 5%), as compared to chitosan-film (*control*). Moreover, chitosan-Enoxil (5.00%) showed maximum inhibition power (0.42 mg-echiv/mL Trolox, Figure 2), which further illustrated that impregnation of Enoxil antioxidant to chitosan-film increased the antioxidant capacity, by a concentration dependent manner, mainly attributed to the hydrophilic polyphenolic acid components of Enoxil.

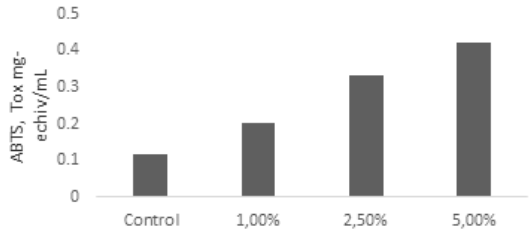

Figure 2.
Radical scavenging ability of chitosan and chitosan-Enoxil film with different concentrations expressed as Eox mg-echiv/mL

Bao *et al*. [25] Ferreira *et al*. [26] and others reported similar antioxidant activity. One should mention that chitosan film without Enoxil, possess low inhibition properties of free radicals. Such antioxidant activity of chitosan film has been reported elsewhere [27].

Chemiluminescence assay

The increased or decreased intensity of chemiluminescence signal could be attributed to an oxido-reducing

process. According to Meghea *et al*. [28], the increase of the quantum of photons emitted upon molecule transition to a lower energy state means that molecule possess pro-oxidating properties, while the decrease of the chemiluminescence signal corresponds to reducing the properties of the same molecule. As reported by Rolewski *et al*. [30], quercetin as low as 2 μmol/L exerts pro-oxidant activity. Following these, it is important to identify the efficient concentrations of Enoxil in chitosan film, which determine an anti-oxidant activity. Therefore, in Table I the results of antioxidant activity, measured by chemiluminescence assay, for chitosan and chitosan-Enoxil films, which exert enhanced antioxidant activity, are presented.

Table I

Determination of antioxidant activity by chemiluminescence assay

% of Enoxil in chitosan film	Antioxidant Activity, AA%
Control	16.2
1% Enoxil	28.9
2.5% Enoxil	45.5
5% Enoxil	79.2

DPPH assay

In order to determine the antioxidant activity of chitosan and chitosan-Enoxil films by using DPPH radical method, kinetic curves plotting absorbance (a.u.) *vs* time (min) were performed (Figure 3).

For better understanding the efficient inhibition of DPPH radical, a total time interval of 240 min was chosen to measure the absorbance at the steady state. Such an approach was reported by Kedare *et al*. [31] and Chat *et al*. [29]. These results, presented in Table II, emphasized that Enoxil showed a fast decrease time evolution followed by a slow antiradical kinetics reaching endpoint at 240 min.

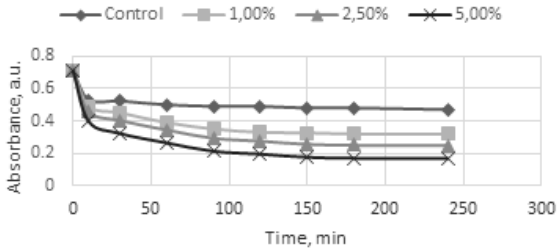

Figure 3.

Inhibition of free DPPH radical by chitosan and chitosan-Enoxil solutions

Table II

Determination of DPPH radical inhibition by antioxidant biomedical films

% of Enoxil in chitosan film	% remaining DPPH
Control	66.83
1% Enoxil	44.71
2.5% Enoxil	34.92
5% Enoxil	23.66

The results (Table II) demonstrate an increase of anti-oxidant activity by 76% for Chitosan-Enoxil (5%). According to the time reactions, the curve denotes the mechanism of fast H-abstraction, followed by a slow and more complex mechanism due to interaction between chitosan and Enoxil [19].

Microbiological investigations

Antimicrobial activity was improved with the addition of Enoxil into chitosan film. Enoxil-chitosan film increases significantly the size diameter around the film inoculated with Pseudomonas fluorescens CNMN-PFB-01, Bacillus subtilis CNMN BB-01, compared to control chitosan film after 24 h at 37°C (Figure 4).

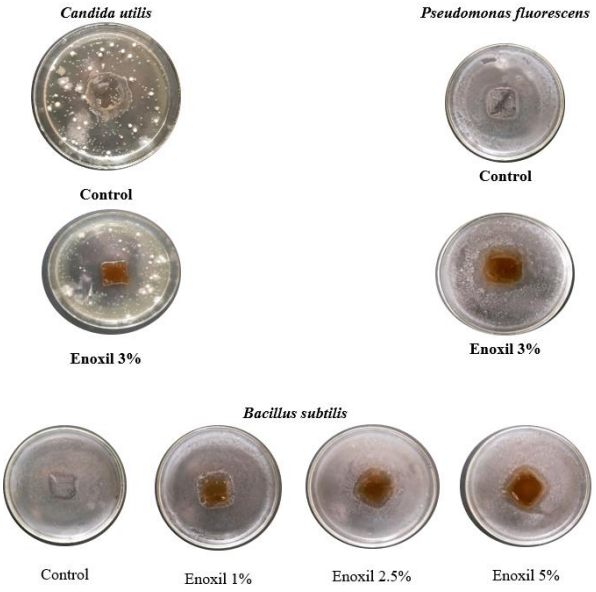

Figure 4.

Determination of the inhibition zones diameter for *Pseudomonas fluorescens* CNMN-PFB-01, *Bacillus subtilis* CNMN BB-01 and *Candida utilis* after interaction with control and chitosan-Enoxil films

Table III

Inhibition zones for *Pseudomonas fluorescens* CNMN-PFB-01, *Bacillus subtilis* CNMN BB-01 and *Candida utilis* after interaction with chitosan and chitosan-Enoxil films

% of Enoxil in chitosan film	Diameter inhibition zones, mm		
	Pseudomonas fluorescens	*Bacillus subtilis*	*Candida utilis*
Control, initial zone	20	20	20
1% Enoxil	29	26	28
2.5% Enoxil	34	30	32
5% Enoxil	40	36	42

The antibacterial properties of chitosan edible films incorporated with Enoxil is explained mainly by the presence of polyphenolic acids in the product Enoxil, which interacts with bacteria cell protein by hydrogen bonding though inhibiting bacteria further growth. Moreover, phenolic compounds can damage the bacterial cell membrane, though causing leakage of intracellular constituents. However, chitosan is also known to inhibit positive bacteria growth, where the epithelial cell wall consists of teichoic acid, by ionic interaction between cation form chitosan and anionic nature of teichoic acid. The inhibition zones for *Pseudomonas fluorescens* CNMN-PFB-01, *Bacillus subtilis* CNMN BB-01 and *Candida utilis* after interaction with chitosan and chitosan-Enoxil films are shown in Table III. The best result for inhibiting both gram-positive, gram-negative bacteria and fungus was shown by chitosan-Enoxil 5% composition film, with the diameter of inhibition zones for *Pseudomonas fluorescens* (40 mm), *Bacillus subtilis* (36 mm) and *Candida utilis* (42 mm).

Conclusions

Addition of antioxidant components to chitosan films increases the inhibition of free radicals, thus contributing to new biomedical applications. The order of antioxidant activity measured by DPPH, ABTS and chemiluminescence assay is the following: Cts-Enoxil (5%) > Cts-Enoxil (2.5%) > Cts-Enoxil (1%), which confirms that by increasing the concentration of Enoxil in chitosan film, the scavenging ability of radical sources is improved.
Bacteria growth inhibition is growing linearly, by increasing the concentration of Enoxil in the chitosan film. Chitosan film impregnated with 5% Enoxil showed promising results against both gram-positive and gram-negative bacteria. Films with enhanced antibacterial properties are very important for application in food packaging industry to delay or prevent the growth of microorganisms on the product's surface.

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