Novel hydrazones – antioxidant potential and stabilization via polysaccharide particles

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Abstract. In this study, we aimed to: i) determine the impact of three new isonicotinoyl hydrazones derivatives in in vitro systems used to investigate free radical processes - radical scavenging approach (ABTS and DPPH) and iron induced peroxidation in lipid containing model systems and ii) evaluate the potential of polysaccharide-based particles to act as protective carriers preserving the antioxidant activity (AOA) of the tested compounds. The tested compounds revealed excellent antioxidant effectiveness in the ABTS system. In the DPPH radical scavenging assay the compounds exhibited very weak or absence of AOA. The data from the iron induced peroxidation methods disclosed better antioxidant properties of the derivatives in the system containing egg yolk homogenate which is more plausible compared to the lecithin containing one.

The incorporation of a bromine atom on 5th position in salicylaldehyde moiety is associated with diminishment of the radical scavenging activity in the systems containing stable free radicals but its AOA reduction after encapsulation during the storage was only 9.17%. The obtained data indicate that compounds have proven themselves as promising candidates for further evaluation as antioxidant agents. Their encapsulation in chitosan-alginate particles could be a useful approach for improving the stability of their antioxidant properties.

1. Introduction

During the past decades there has been an abundant amount of evidence in the scientific literature proving the connection between oxidants, free radical reactions and the pathogenesis of several incurable progressive illnesses which are among the main causes of death worldwide [1]. This fact has led to the increasing interest in the synthesis and development of novel antioxidants suppressing and preventing free radical cellular damage. The process of discovery of novel more-powerful and low toxic antioxidants comprises several steps, including rational design strategies, theoretical methods for investigation of structure activity relationship, evaluation of the kinetic and the stability of the observed properties for prolonged periods of time and the effects of the environment condition on the radical scavenging activity [2,3]. The two most commonly used strategies adopted from the research groups focused on the design of novel antioxidants are to structurally modify existing antioxidants
(ascorbic acid, α-tocopherol) in order to surpass their properties or to design an entirely new molecule [4].

The present investigation includes the evaluation of the antioxidant activity (AOA) of three new designed isonicotinoyl hydrazones – salicylaldehyde isonicotinoylhydrazone (SIH) derivatives. Hydrazones are widely studied biologically important compounds famous for a broad spectrum of biological activities - i.e. antibacterial, anticancer, anti-inflammatory, antioxidant etc [5,6]. The wide variety in the observed pharmacological properties in combination with their relatively easy and cheap methodology of synthesis has made them considerably interesting for medicinal chemists in their attempt to design concepts and synthesize novel multifunctional molecules meeting the requirements of chemical and pharmaceutical industries. The initial compounds SIH is a lipophilic tridentate iron chelator possessing noticeable antioxidant activity and modest cytotoxicity against different type of neoplastic cell lines [7]. However, the performed investigations concerning its stability in aqueous milieu have revealed rapid hydrolysis of its hydrazone bond [7,8].

For our investigation we have chosen three structurally similar isonicotinoyl hydrazones with proved cytotoxicity – two methoxy derivatives and one bromo-substituent. The previous investigations of the research group which designed the compounds studied by us - 5-bromosalicylaldehyde isonicotinoylhydrazone (5BrSIH), 3-methoxysalicylaldehyde isonicotinoylhydrazone (3mSIH) and 4-methoxysalicylaldehyde isonicotinoylhydrazone (4mSIH) includes confirmation of their structure by elemental and thermo-gravimetric analysis, IR, ¹H-NMR, ¹³C-NMR spectroscopy and evaluation of the compounds with the Lipinski's rule of five [9,10].

The study has two primary interrelated aims. The first one is to determine the impact of three new hydrazone derivatives in \textit{in vitro} systems used to investigate free radical processes. For this purpose, we have performed radical scavenging approach in systems containing stable free radicals (ABTS and DPPH) and measured the degree of oxidation of biologically important molecules via iron induced peroxidation in lipid containing model systems. The second goal is associated with the preservation of the established activity – we aimed to evaluate the potential of polysaccharide – based particles (attractive drug delivery systems possessing various advantages, i.e. nontoxicity and biodegradability) to act as protective carriers preserving the revealed antioxidant properties of the tested compounds.

2. Material and methods
2.1. UV-Vis spectra – the UV-Vis absorption spectra of the investigated compounds were taken with Shimadzu computerized spectrophotometer. The investigated compounds were diluted in 50 mM K₂HPO₄/KH₂PO₄ buffer, \textit{pH} 7.4 [0.1 mmol/L], placed in quartz cuvettes and scanned over the wavelength range from 200 nm to 700 nm.

2.2. Determination of the total antioxidant capacity
To determine the total antioxidant capacity of the studied hydrazone derivatives we have chosen two of the most popular and commonly used spectrophotometric methods used for evaluation of the antioxidative role of a wide range of biological samples and new designed compounds e.g. ABTS and DPPH assays. They are rapid, simple and inexpensive methods based on the discoloration of the preformed stable free radicals – the antioxidant efficiency of the tested compounds is inversely proportional to the absorbance of the sample solution. The experimental evaluation of the studied properties has been performed using UV-Vis Shimadzu spectrophotometer and 2 ml disposable cuvettes. Fresh solutions of the radicals were prepared for each assay. Samples were analyzed in triplets.

2.2.1. \textit{ABTS} method – The total antioxidant capacity (TAC) in the ABTS model system was determined by the method proposed by Re et al. [11]. The ABTS assay utilizes the free mono-cation radical of 2′2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), generated by the oxidation of ABTS with potassium persulfate. The blue-green colored solution was dark stored for 1 day. The working
solution was obtained by diluting the stock solution of the ABTS radical cation with buffer solution K2HPO4/KH2PO4, pH 7.4 to produce a final solution with absorbance 0.7 ± 0.005 at 734.

Hydrazones samples were reacted with 2 cm3 of ABTS+● solution for 1 hour at room temperature. The discoloration of the pre-generated ABTS+● radical was measured at 734 nm and the chemical response was compared with the one obtained under identical experimental conditions with the water soluble vitamin E analogue – Trolox.

2.2.2. Evaluation of the stability of the antioxidant properties – To evaluate the stability of the antioxidant properties of the studied hydrazones we have used the stable ABTS radical. We prepared stock solutions of the investigated hydrazones and hydrazones encapsulated in chitosan alginate particles. Chitosan and sodium alginate were selected as carriers due to their favorable biological properties [12,13]. The isonicotinoyl hydrazones were incorporated in the polysaccharide carriers by isotropic gelation. In particular, sodium alginate solution (3 mg/ml) was incubated with the respective hydrazone derivative for 30 min (ratio 1:10 with respect to polymer concentration), after which solution of calcium chloride (3.35 mg/ml) was slowly added to the mixture under gentle stirring (700 rpm). After that a solution of chitosan (0.75 mg/ml) was added drop wise in order to accomplish gelation between both polymers. The stock solutions were stored at 4 °C for thirty-day investigation period and every 5 days a comparative evaluation of the encapsulated and non-encapsulated hydrazones was performed.

2.2.3. DPPH method – the analytical procedure was performed according to Goupy et al [14]. For this experimental procedure a working purple colored solution of the DPPH radical in ethanol with absorbance of 1 at 517 nm was prepared. 2 cm3 of the working solution of the radical were reacted with the studied hydrazones for 60 minutes at room temperature. After the incubation time the decrease in the absorbance reading of the probes was measured at 517 nm. Trolox was used again as referent compound. Results were presented as percentage from the control sample.

2.3. Determination of the protection effect of the studied derivatives during iron induced peroxidation in lipid containing model systems

The thiobarbituric acid assay was used to assess lipid peroxidation according to the method of Asakawa and Matsushita [15]. The peroxidation was induced in two alternative model systems one containing egg yolk lipoproteins and the other lecithin. Hen egg yolk homogenate was used as lipoproteins rich medium. According to the literature data the total solids content of egg yolk is generally around 50% and its major constituents are proteins 16% and lipids 32% [16]. All samples were prepared in PBS, pH 7.4, containing 1 mg (lecithin or egg yolk homogenate lipids) per mL and the studied hydrazones. In the control sample hydrazones were omitted. The peroxidation was induced by the addition of FeCl3 with final concentration of 0.1 mmol/L. The samples were incubated at 37 °C for 30 min. Then 0.5 ml of 2.8% trichloracetic acid and 0.5 ml of thiobarbituric acid were added. The mixture was vortexed vigorously, heated in 100 °C water bath for 20 min and centrifuged at 3000 rpm for 20 min. The absorbance of the studied probes was measured at 532 nm. The degree damage of the lipid molecules was presented as percentage from the entreated control.

3. Results

The electronic spectra of the studied isonicotinoyl hydrazones in 50 mM K2HPO4/KH2PO4 at room temperature were recorded. The obtained data from the spectral study showed maxima in the middle and near UV region of their UV-Vis absorption spectra. We didn’t observe absorption bands over the wavelength range where DPPH and ABTS radicals are quantified. The lack of overlapping absorbance bands makes both methods suitable for the determination of the antioxidant properties of the investigated compounds.
Figure 1. Absorption spectra of the studied isonicotinoyl hydrazone derivatives diluted in 50 mM K$_2$HPO$_4$/KH$_2$PO$_4$ buffer pH 7.4, over the wavelength range from 200 nm to 700 nm.

The newly designed compounds were screened for their antioxidant potential employing the *in vitro* DPPH and ABTS free radical scavenging assays. Using these methods, we have determined their capacity to quench both preformed stable free radicals utilizing Trolox as a standard. A close survey of the obtained results indicates a different magnitude of the revealed antioxidant properties against both radicals during this approach. This could be explained by the different mechanisms of reduction.

The radical scavenging activity of the SBH derivatives against ABTS has been assessed in the concentration range from 0 to 9 µmol/L (figure 2). Results are presented as AOA and the reducing capacity in terms of C-50 [µmol/L] was calculated in order to compare the antioxidant properties. C-50 is the concentration of the hydrazone that provides 50% value of AOA.

![Absorption spectra of isonicotinoyl hydrazone derivatives](image)

Figure 2. Antioxidant activity of the isonicotinoyl hydrazones in the ABTS model system.

All the derivatives revealed themselves as excellent antioxidants. The AOA effect rises with the increase of the concentrations of the tested hydrazone ($R^2 > 0.97$). In the whole studied concentration range the compounds possessing methoxy group as substitutes in the salicylaldehyde moiety demonstrated better radical scavenging activity correlating with bigger AOA values than the bromo-substituent.

Table 1. Results for C-50 values calculated on the basis of the experimental results obtained in the ABTS model system.

| Compound | C-50 (µmol/L) |
|----------|---------------|
| 5BrSIH   | 8.16 ±0.005   |
| 3mSIH    | 6.16 ±0.015   |
| 4mSIH    | 5.93 ±0.128   |
| Trolox   | 8.53 ± 0.18   |
The comparison of the calculated C-50 values revealed better antioxidant properties of isonicotinoyl hydrazones compared to the reference Trolox (table 1). Varying the position of the methoxy group in the salicylaldehyde has led to slight but statistically significant increase of the antioxidant properties corresponding to lower C-50 value.

From the evaluation of the stability of the disclosed antioxidant properties in the ABTS model system we can see that the three compounds exhibit variable stability of their AOA which doesn’t depend on the type of the substitutes in the salicylaldehyde moiety of the molecule (figure 3). From the studied derivatives 3mSIH present itself as the more stable antioxidant. During the 30 days investigation period the AOA of its stock solution diminished only by 13.08%. The AOA activity of the other methoxy bearing isonicotinoyl hydrazones (4mSIH) and the bromo - substituent (5BrSIH) diminished respectively by 75.12% and 56.44%. During the whole investigated period 3mSIH stock solution is the most potent antioxidant from the studied compounds.

![Figure 3](image)

**Figure 3.** Changes of the AOA of non-encapsulated and encapsulated into polysaccharide particles hydrazones against ABTS radical during 30 days storage period.

The incorporation of the derivatives into the chitosan-alginate particles has had a beneficial preserving effect on the antioxidant activity of both compounds which have demonstrated unstable antioxidant properties. This effect is more prominent in the case of the bromo-substituent. At the end of the storage period the encapsulated 5BrSIH has an antioxidant activity approximately two times higher compared to the initial solution of the non-encapsulated hydrazone. During the studied period the reduction of the AOA was only by 9.17%.

From the studied derivatives 4mSIH stock solution was the less stable. The reduction in its AOA for the investigated period was 75.13%. We also observed significant diminishment of the AOA of the loaded in polysaccharide particles 4mSIH – 33.51%. At the end of the investigated period the encapsulated 4mSIH exhibit two and a half times higher activity compared to the non-encapsulated compound solution.

The comparison of the reduction of the activity of the encapsulated and non-encapsulated 3mSIH has disclosed an almost equal stability of both solutions and diminishment of the AOA by less than 20%.

The obtained results from the model system containing the stable DPPH radical are shown in figure 4. Due to the poor reactivity of the studied hydrazones in this model system only data concerning the highest investigated concentration [90 µmol/L] are presented.
The sample containing the bromo-substituent (5BrSIH) has the same absorbance as the control which indicates impossibility for hydrogen atom abstraction from its molecule and formation of hydrazonyl radical. The two methoxy bearing isonicotinoyl hydrazones displayed a slight but statistically significant increase of the investigated in the DPPH system properties. This effect is better expressed in the sample containing 3mSIH which has demonstrated the highest DPPH scavenging activity among the synthesized compounds.

Even in this case the degree of decolonization and the exhibited DPPH radical scavenging properties are very weak compared with the one of the reference Trolox. At a concentration of 46 µmol/L Trolox induced almost complete quenching efficacy and full decolonization of the DPPH solution.

In the model system of iron induced lipid peroxidation the isonicotinoyl hydrazones demonstrated different extent of protection of the molecules depending on the used substrate (figure 5).

In the lecithin containing system the bromo-substituent (5BrSIH) had an identical effect on both tested concentrations. The methoxy derivatives exhibited a similar but statistically different concentration dependent activity. The variation of the position of the methoxy group from 3rd to 4-th position had a slight beneficial effect on the evaluated in the system effect.
The data from the iron induced peroxidation methods in the system containing egg yolk homogenate which is more plausible compared to the lecithin containing one uncover significant decrease of the level of lipid peroxidation. This effect is most pronounced in the samples containing the bromo-substituent (5BrSIH) were at the highest investigated concentration of 160 µmol/L we established lack of evidences for lipid oxidative damage.

5. Conclusion
The obtained results made evident that the investigated isonicotinoyl hydrazones have proven themselves as promising candidates for further evaluation as antioxidant agents. They have demonstrated excellent ABTS radical scavenging effect and provided significant protection in the model systems containing egg yolk homogenate. The performed comparative evaluation of the stability of non-encapsulated and encapsulated in polysaccharide particles hydrazones revealed valuable information about the possible use of chitosan-alginate particles as protective carriers preserving the antioxidant properties of the compounds. The stability of the AOA of the encapsulated bromo-substituent (5BrSIH) along with the data from the iron induced lipid peroxidation denote that despite its lower reactivity against stable free radicals the structural modification associated with incorporation of bromine atom in the salicylaldehyde moiety of the isonicotinoyl hydrazones deserve more detailed analysis.

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References
[1] Florence TM 1995 Aust N Z J Ophthalmol. 23 3
[2] Thomas CE 1997 Handbook of synthetic antioxidants. New York: Marcel Dekker; 1
[3] Espinoza-Fonseca L.M 2006 Bioorg. Med. Chem. 14 896
[4] Zhang HY 2005 Curr. Comput.-Aided Drug Des 1 257
[5] Rollas S, Küçükgüzeli SG 2007 Molecules 12 1910
[6] Narang R, Narasimhan B, Sharma S 2012 Curr Med Chem. 19 569
[7] Potůčková E, Hrušková K, Bureš J, Kovaříková P, Špirková IA, Pravdíková K, Kolabová L, Hergeselová T, Hašková P, Jirkovská A, Richardson V, Lane DJ, Kalinowski DS, Richardon DR, Vávrová K, Šimůnek T 2014 PLoS One. 13 9
[8] Richardson D, Vitolo LW, Baker E, Webb J 1989 Biol Met 2 69
[9] Nikolova-Mladenova B, Halachev N, Iankova R, Momkoves G, Ivanov D, 2011 Arzneimittelforschung/Drug research, 61 714
[10] Nikolova-Mladenova B, Bakalova A, Momkoves G, Ivanov D International Scientific Journal of Medical and Biological Sciences http://bioscience.scientific-journal.com
[11] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C 1999 Free Radic Biol Med. 26 1231
[12] Kumar MN, Muzzarelli RA, Muzzarelli C, Sashiwa H, Domb AJ 2004 Chem Rev. 104 6017-84
[13] Baldrick P 2010 Regul Toxicol Pharmacol. 56 290
[14] Goup Y, Dufour C, Loonis M, Danges O 2003 J Agric Food Chem. 51 615
[15] Asakawa T, Matsushita. S 1980 Lipids 1 137
[16] Ahn D, Kim SM, Shu H 1997 Poult Sci. 76 14