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Evaluation of the *in vitro* antioxidant effect of novel 3-methoxysalicylaldehyde derived hydrazones

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In an effort to continue the exploration of hydrazone analogues with highly diverse structural features based on variation in the skeleton we present a comparative evaluation of the antioxidants properties of novel 3-methoxy hydrazones derived from salicylaldehyde benzoylhydrazone. The determination of the antioxidant properties was performed *in vitro* in biologically relevant cell-free model systems with different mechanism of generation of ROS using chemiluminescent (potassium superoxide (KO_2)-produced superoxide and luminol-dependent CL in a system of iron dependent hydroxyl radical formation) and spectrophotometric (ABTS, DPPH) methods to monitor and compare the investigated properties. We demonstrated that the initial compound salicylaldehydebenzoylhydrazone and its 3-methoxy derivatives exhibited antioxidant activity with variable efficiencies in relation to the experimental model system and the used method for registration. In both chemiluminescent model systems the incorporation of the electron donating methoxy group had increased considerably the studied properties, while in the ABTS assay we observed a slight but significant decrease. According tothe obtained by the ABTS method results of TEAC the four hydrazones have demonstrated better scavenging properties compared to the reference Trolox. All the studied compounds have demonstrated high antioxidant activity in terms of oxidation inhibition and free radical scavenging activity, thus indicating their potential direct or indirect beneficial effect in case of oxidative stress processes.

Key words:Hydrazones, antioxidant activity, ABTS, DPPH,chemiluminescence

INTRODUCTION

In medical chemistry hydrazones derivatives have been very well known for their vast spectrum of biological activities, coordinative capability and applications in analytical chemistry.The scientific interest in this group of compounds, as an alternative for the development and design of new therapeutic agents with improved toxicity profile and increased efficiency is determined by the combination of the relatively easy and cheap technology of synthesis, their physicochemical properties and their pharmacological activities.Scientific evidences obtained during the extensive investigations of these compounds have proved their capability for versatility in coordination, tendency to yield stereochemistry of higher coordination number, ability to behave as deprotonated or neutral ligands and capacity to generate flexible molecular architecture in assuming different conformations[1, 2].It has been reported that derivatives of this class of organic compounds possessing different functional moieties in the aldehyde or hydrazide part of the molecule exhibit antimicrobial, antimalarial, analgesic, anti-inflammatory, antitumoral and antioxidant properties[3]. All those effects are connected with opportunity of hydrazones to influence directly or indirectly free radicals processes.

In an effort to continue the exploration ofhydrazone analogues with highly diverse structural features based on variation in the skeleton we present a comparative evaluation of the antioxidants properties of novel hydrazones derived from salicylaldehyde benzoylhydrazone.We determined their antioxidant activity in *in vitro* biologically relevant cell-free model systems with different mechanism of generation of ROS using various methods to monitor and compare the investigated properties. This offersthe unique advantages to delineate the chemical and molecular mechanisms of action of these compounds and to investigate the correlation of their reactivity in radical reactions with their composition and molecular structure.The studied molecules have more drug-like structures compared to the initial compound and the current investigation was carried out in order to establish their value as prospective multifunctional antioxidant candidates.

MATERIALS AND METHODS

Materials

All the reagents used in the antioxidant model systems were analytical grade. 1,1-Diphenyl-2-picryhydrazyl (DPPH), 2,2’-azinobis-(3-ethyl-

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benzothiazoline-6-sulfononic acid), and Trolox(6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxyl acid) were obtained from Sigma-Aldrich. Ethylenediamine-tetraacetic acid(EDTA) and $FeCl_3.6H_2O$ were purchased from Genaxxon.

The derivatives of SBH used in the present work - 3-methoxysalicylaldehydebenzoylhydrazone (3mSBH), 3-methoxysalicylaldehyde-4-hydroxybenzoylhydrazone (3mShBH) and 3-methoxysalicylaldehydeisonicotinoylhydrazone (3mSIH)were synthesized and characterized in the Faculty of Pharmacy at the Medical University of Sofia by assistant professor B. Nikolova-Mladenova. The hydrazones were obtained by Schiff base condensation. Their structure was confirmed by elemental and thermo-gravimetric analysis, IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy[4].

Methods

Evaluation of the antioxidant properties in chemiluminescent model systems

Luminol-dependent CL was used for registrationof ROS [5-7].The fundamental chemistry of CL assay is based on the two-electron oxidation of the luminol molecule and formation of endoperoxide. This compound is unstable and upon decomposition yields an electronically excited 3-aminophthalate, emitting photons on its return to the ground sate whichare amplified by the phototube of the luminometer. The chemiluminescent response is determined by calculating the area under the obtained chemiluminescent light curves. We assumed that the CL ratio in the presence and in the absence of the tested compound should indicate the scavenging properties of the derivatives. Therefore, this ratio was termed chemiluminescence scavenging index (CL-SI).

Two types of CL model systems were chosen - luminol-dependent CL in a system of potassium superoxide (KO_2)-produced superoxide and luminol-dependent CL in a system of irondependent hydroxyl radical formation. For the purpose of these methods an LKB 1251 luminometer (BioOrbit, Turku, Finland) set at 37°C was used. It was connected with an AT-type computer via serial interface and MultiUse program ver. 1.08 (Bioorbit, Turku, Finland) which was used for collection of the experimental data.

For both assaysthe chemiluminescent reagent was prepared by dissolving luminol in a small amount of 0.01 M NaOH. Then the solution was diluted to luminol concentration of 1 mMwith a 50mMphosphate buffer solution (PBS) and the pH was adjusted to 7.4 with 0.01 M HCl. Drugs were dissolved in 10mMNaOH. Their final concentrations in the investigated samples are shown in the figure legends.

Luminol-dependent CL in a system of potassium superoxide (KO_2)-produced superoxide.The assay was carried out using 1 ml samples of phosphate buffer solution, pH 7.4, containing 0.1 mMluminol, and the studied hydrazone derivative. In the control sample hydrazones were omitted. The release of the superoxide is a fast process. Therefore, the CL response was measured immediately after addition of 20 µl KO_2 solution using the "flash assay" option of the MultiUse program, every 50 milliseconds.

Luminol-dependent CL in a system of irondependent hydroxyl radical formation.One ml samples of PBS, pH 7.4, contains 0.1 mMluminol, 0.1 mM Fe^{3+} ($FeCl_3$), 0.1 mM EDTA, 0.1 mM ascorbate, 1mM H_2O_2 and any of the tested hydrazones at concentrations between 0.1 and 100 µM, or buffer for the controls. The CL was measured using the "flash assay" option of the MultiUse program, every 50 milliseconds.

Evaluation of the radical scavenging activity against stable free radicals

The total antioxidant capacity of the tested hydrazones was measured spectrophotometricallyby using the stable 1,1-diphenyl-2-picrul-hydrazilor 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfononic acid)free radicals in vitro assays. $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ have different mechanism of neutralizing their free radical character.$DPPH^{\bullet}$ absorbance decreases in presence of hydrogen-donating antioxidants due to the formation of the stable DPPH-H compound and ABTS is involved in an electron transfer processes.Unicam UV 500 Spectrophotometer (ThermoSpectronic, UK) and 1 cm disposable cuvettes (Brandt, Germany) were used for all the absorption measurements.

The antioxidant activity was calculated by the formula:

$$AOA\% = \frac{Abs_{control} - Abs_{hydrazone\ probe}}{Abs_{control}}.100$$

The procedure followed the method as described by Re et al., [8]. The $ABTS^{\bullet+}$working solution was produced by the mixing of 14 mM ABTS stock solutions with potassium persulfate 2.45 mM (final concentration), allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The suspension was then diluted by mixing 1 ml $ABTS^{\bullet+}$with PBS to obtain final solution with absorbance 0.70 ± 0.01 units at 734 nm. The reduction in absorbance of 2 ml of $ABTS^{\bullet+}$ after

adding the hydrazones derivatives was recorded at 734 nm exactly 60 min after the mixing. Fresh $ABTS^{\bullet+}$solution was prepared for each assay.

Trolox was used as a reference compound. The concentration that provides 50% AOA (C-50) for different hydrazone derivatives andTrolox equivalent antioxidant capacity (TEAC) were calculated to quantify the antioxidant capacity.

The DPPH freeradical-scavengingactivityofthehydrazoneswasdetermineda ccordingtothemethoddescribedbyGoupy*etal*.[9].For this experiment all studied compounds were dissolved in ethanol to a concentration of 1 mM/L. $DPPH^{\bullet}$ solution in ethanol was prepared with initial optical absorbance of 1 at 518 nm.2 mL of $DPPH^{\bullet}$ solution were allowed toreact with 200 µl sample solution. The probes were left to stay at room temperature in the dark. After 1 hour of incubation, the absorbanceof the solution was measured at 518 nm. The scavenging activity was expressed as a percentage of the absorbance of the control $DPPH^{\bullet}$ solution containing ethanol instead of hydrazones derivatives solution.

The concentration that corresponds to 50% decreasing of radicals was termed C-50. Its value was calculated, using the following equation:

$$AOA = 100/[1 + 10^{B(logC - \log(C-50))}]$$

The data processing included fitting of the experimental results to sigmoid dose-response curves, whereby B is the coefficient (hill slope) and C is the substrate concentration. The formula was used to calculate C-50 for the data obtained by all experimental model systems.

All statistical analyses were performed using IBM SPSS Statistics v.19.0. for Windows (Chicago, Illinois, USA, 2010). The assays were carried out in triplicate and the quantitative variables are presented as mean ± standard deviation. P-values lower than 0.05 were considered statistically significant.

RESULTS

The effects of the studied 3-methoxy derivatives on the luminol-dependent CL in a system of KO_2 generated $O_2^{-\bullet}$ (assay 1) are shown on figure 1. The chemiluminescent luminosity is dependent on the quantity of the generated $O_2^{-\bullet}$ in the reaction system. Therefore this CL reaction system can be used to evaluate the scavenging activity of the tested derivatives on $O_2^{-\bullet}$.SBH had the lowest inhibitory effect among the studied hydrazones. It didn't demonstrate any antioxidant properties at concentrations above 1µmol/L and with increasing its concentration to 100 µmol/L the inhibition effect exhibited slight but significant escalation to 74%.Due to the strong inhibitory effect of the rest of the tested compounds under investigation their antioxidantpropertieswerefollowedover a wide

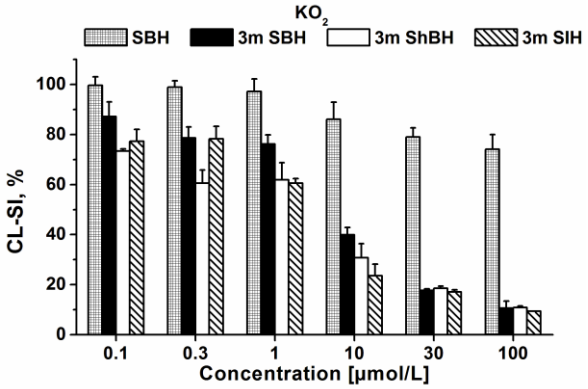

Fig. 1. Effect of SBH and its 3-methoxy derivatives on the luminol-dependent chemiluminescence scavenging index in the presence of KO_2 generated $O_2^{\bullet-}$. The solution in the sample cuvette comprises 1 ml phosphate buffer solution pH 7.4, containing 0.1 mMluminol and the hydrazone derivatives at concentration as indicated. In the control sample the studied hydrazones were omitted.

concentration range from 0.1 to 100 µmol/L. A small and not statistically significant difference was observed between the obtained values for their chemiluminescent scavenging index at concentrations upper than 30 µmol/L, suggesting no influence and involvement of the incorporation of the electron-donating atoms or groups in their molecular structureon the radical scavenging activity.At the maximal studied concentration of 100 µmol/L the chemiluminescent response in percentage for SBH is equal to 74,09 %, for 3mSBH = 10.62 %, for 3mShBH = 10.94 % and for 3mSIH = 9.39%. The diminished SBHs' effectiveness is due to the lack of methoxy substitution in its structure which is required for the high antioxidant activity in the experimental chemiluminescent model system.

We investigated the ability of the selected drugs to interact with $OH^{\bullet}$ in a system containing the Fe^{2+}-EDTA complex (Figure 2). In this system the $OH^{\bullet}$ are formed in the water phase in which are located the rest of the participants of the system– luminol and the studied hydrazones.The tested compounds exhibit different antioxidant activities in a concentration-dependent manner. At the lowest tested concentration 3µmol/L the obtained results for the studied parameter for all compounds except 3mSIH were almost identical to those of the untreated control sample. Further increasing of the concentrations to 10 µmol/L decreased moderately the CL-SI index. In the whole studied concentration range the initial compound SBH again

demonstrated the lowest antioxidant properties. At the highest tested concentration the 3mSBH, 3mShBH and 3mSIH values of the CL-SI were respectively 78,4%, 58,3% and 25,7% from the one obtained from the initial SBH compound. In this experimental chemiluminescent model system we can observe again the effect of diminished activity of the initial compounds due to the absence of methoxy group in its molecular structure.

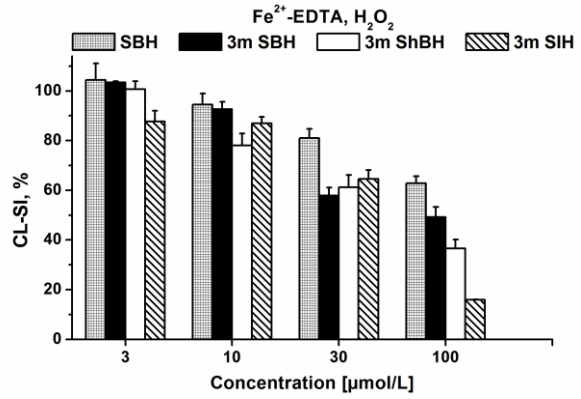

Fig. 2. Effect of SBH and its 3-methoxy derivatives on the luminol-dependent chemiluminescence scavenging index in a system of iron-dependent hydroxyl radical formation. The solution in the sample cuvette comprises 1 ml phosphate buffer solution pH 7.4, containing – 0.1 mMluminol, 0.1 mM Fe^{3+}, 0.1 mM EDTA, 0.1 mM ascorbate, 1mM H_2O_2 and the hydrazonesat concentrations as indicated. The control sample contained solvent in place of the studied hydrazones.

The C-50 values for all the studied 3-methoxy derivatives in both chemiluminescent model systems were determined and reported in Table 1.

Table 1. C-50 inhibition values of the chemiluminescence scavenging index for the investigated SBH derivatives in the studied model systems. SBH C-50 values are not presented due its weak effect on the tested systems they were out of investigated concentration range.

	C-50 [µmol/L] CL system of KO_2-produced superoxide	*C-50 [µmol/L] CL system of iron dependent hydroxyl radical formation*
SBH	*	*
3mSBH	3.58 ± 0.13	79.86 ± 5.61
3mShBH	1.43 ± 0.04	50.12 ± 0.001
3mSIH	1.89 ± 0.07	54.00 ± 0.43

SBH values are not reported because of its relatively weak inhibition activity in order to avoid extrapolation error. Comparing the structures and the demonstrated activities of the studied compounds it is inferred that the presence of a methoxy group in the molecular structure of the studied compounds is a crucial factor affecting the antioxidant behavior of these compounds in the tested chemiluminescent systems.The values of C-50 suggested that the strength of the radical-scavenging effect decrease in the order $O_2^{-\bullet}$>$OH^{\bullet}$.In both assays the 3-methoxy derivatives containing hetero atom or hydroxyl group (3mShBH and 3mSIH) exhibited lower C-50 values, compared to 3mSBH. The results obtained for these two compounds in each of the tested systems lead to the assumption that these types of molecular modifications seems to have slight but significant influence on the studied properties.

Scavenging potential

The free radical scavenging potential of the studied hydrazones derivatives and the initial compound were tested by the ABTS and DPPHdecolorization assays – operationally simple, reproducible, sensitiveand relatively cheap methods. The most important advantages of these model systems are their flexibility allowing to use the preformed stable radicals in aqueous and nonpolar organic solvents and the possibility to determinesimultaneouslythe antioxidants capacity of both hydrophilic and lipophilic constituents of the tested sample[10]. Both assays are considered as representative methods for preliminary screening of broad spectrum of complex samples (plant and food extracts, dietary supplements, biologic fluids, natural and synthetic compounds).

ABTS method

In the investigated concentration range (from 0.9 to 9 µmol/L) a significant decrease in the concentration of ABTS radical due to the demonstrated excellent scavenging effect of the tested compounds was observed. From the analysis represented at Figure 3 it can be concluded that the scavenging activity of all derivatives compounds on ABTS rises with increase in the concentration.

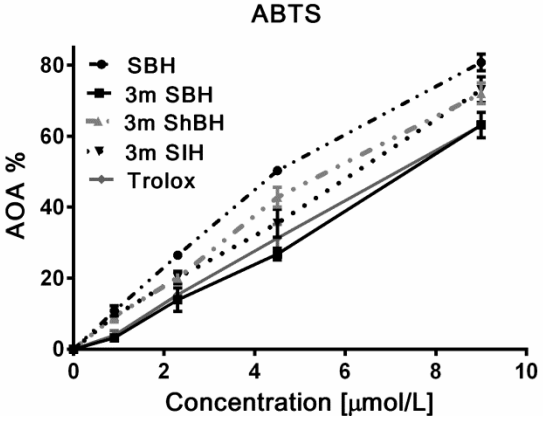

Fig. 3. Antioxidant activity of salicylaldehydebenzoylhydrazone and its 3-methoxy derivatives measured by the ABTS assay.

The initial compound SBH demonstrated higher effect compared to the 3-methoxy derivatives and the reference Troloxfor all of the applied concentrations. According to the results presented at figure 1 and the performed calculation the C-50 values decrease in the following order – trolox (C-50 = 8.53 µmol/L) < 3mSBH (C-50 = 7.44µmol/L) < 3mSIH (C-50 = 6.16µmol/L) < 3mShBH (C-50 = 5.98 µmol/L) < SBH (C-50 = 5.17 µmol/L).

The C-50 values from the antioxidant activity in the ABTS spectrophotometric model system were evaluated by measuring the Trolox equivalent antioxidant capacity (TEAC). The method allow to determine the relative of antioxidant substances to scavenge the radical action 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) compared with a standard amount of the synthetic antioxidant Trolox. In this experiment TEAC values are defined as concentrations of standard Trolox with the same antioxidant capacity as 1 mMconcentration of the hydrazone under investigation.

Table 2 summarized the obtained results in terms of Trolox equivalent capacity. Because the initial compound has demonstrated strongest antioxidant effect in the studied model system its TEAC value is the smallest one. From the presented in table 2 results we can conclude that the electron donating properties of the studied hydrazones and the reference trolox increase in the following order:Trolox< 3mSBH < 3mSIH < 3mShBH < SBH.

Table 2. Determination of antioxidant activity as TEAC of the studied hydrazones by ABTS.

Compound	TEAC
SBH	1.65 ± 0.028
3mSBH	1.16 ± 0.007
3mShBH	1.44 ± 0.013
3mSIH	1.40 ± 0.002

Contradictory to the chemiluminescent model system the obtained by the ABTS assay results demonstrate only slight but significant variation in the studied properties due to the studied structural modifications. The scavenging activity of the studied compounds diminished with the incorporation of the electron donating methoxy group in the molecular structure of the initial structure of SBH. The subsequent alterations in the structure of 3mSBH by the addiction of hydroxyl group or hetero atom have increased equally the AOA of 3mShBH and 3mSIH but the obtained values were still smaller compared to SBH.

DPPH method

The results of the antioxidant activity using the DPPH radical scavenging assay indicate that the studied hydrazone derivatives demonstrate very weak (3mSBH, 3mShBH and 3mSIH) or absence (SBH) of antioxidant properties compared to the reference Trolox (). The tested hydrazones were used at final concentration 90 µmol/L. The scavenging effect of the compounds and the reference antioxidant on DPPH decreases in the following order: Trolox> 3mSIH >3mSBH > 3mShBH>SBH. The 3-methoxy derivatives have demonstrated close but not similar antioxidant activity compared with the initial compound SBH. There was no statistically significant difference between the obtained values for these structural analogues - 3mSIH = 88.4%, 3mShBH = 94.7% and 3mSBH = 92.7%.

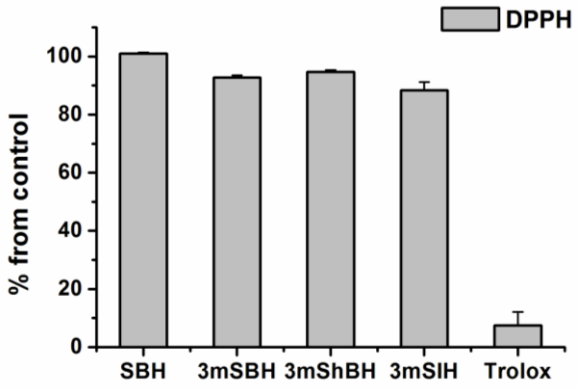

Fig. 4. Antioxidant activity of salicylaldehydebenzoylhydrazone and its 3-methoxy derivatives measured by the DPPH assay. The concentration of the studied hydrazones in the sample cuvette is 90 µmol/L. Trolox at concentration 46 µmol/L was used as reference.

The obtained results could be explained by the quite difficult possibility of abstraction of hydrogen atom from the phenolic ring of the studied compounds under the examined conditions. This is due to the strong intermolecular bonds with the adjacent methoxy group. According to the recent work of Dolens et al. regarding the autoxidation processes ofhydrazone derivatives and the results obtained by Belkheiri et al., as a consequence of the interaction with the DPPH radical may be produced a nitrogen radical of the investigated compound[11]. After studying the influence of the incorporation of different functional moieties in the structure of the initial hydrazone compound these authors proposed the formation of N• centered hydrazonyl radical which is stabilized through formation of delocalisated C• centered radicals.

The modest increase of the scavenging properties of the studied derivatives compared to the initial compound could be due to the increased ability of N-H hydrogen atom extraction due to the incorporation of the methoxy group in the molecular structure.

CONCLUSION

Using four experimental methods, we demonstrated that the studied 3-methoxy derivatives of salicylaldehyde benzoylhydrazones exhibited antioxidant activity with variable efficiencies in relation to the experimental model system and the used method for registration of the studied properties. The compounds have demonstrated high antioxidant activity in terms of oxidation inhibition and free radical scavenging activity, thus indicating their potential direct or indirect beneficial effect in case of oxidative stress processes.

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АНТИОКСИДАНТИ СВОЙСТВА НА НОВОСИНТЕЗИРАНИ 3-МЕТОКСИ САЛИЦИЛ-АЛДЕХИДБЕНЗОИЛХИДРАЗОНИ В *in vitro* ТЕСТ СИСТЕМИ

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(Резюме)

В настоящата статия са представени резултатите от сравнително проучване на антиоксидантните свойства на три новосинтезирани 3-метокси структурни аналози на активния хелаторсалицилалдехидбензоилхидразон. Антиоксидантната и радикал-улавящата активности на съединенията са изследвани в биологично релевантни тест системи на генериране на АФК. За регистриране на радикалите са използвани спектрофотометрични и хемилуминесцентни методики. Получените резултати показват, че новосинтезираните аналози проявяват различна от базисното съединение антиоксидантна активност. В системата с хидроксилни радикали и в тази със супероксидни радикали се наблюдава значително нарастване на инхибиращият ефект върху луминол-зависимата хемилуминесценция при наличие на електрон донорен заместител в алдехидното ядро на молекулата. Съединенията проявяват силни редукционни свойства спрямо ABTS радикала. Отчетеният антиоксидантен ефект надвишава този на използваният референт Trolox, като въвеждането на метокси група е съпроводено със слабо статистически значимо потискане на изследваните свойства.

Въз основа на получените експериментални данни може да заключим, че изследваните съединения проявяват мощен антиоксидантен ефект по отношение на $HO^{\bullet}$ и $O_2^{-\bullet}$ и спрямо стабилният ABTS радикал. Получените резултати ще са от значение за рационалния лекарствен дизайн на фармакологично активни агенти притежаващи антиоксидантни свойства на основата на хидразоните.