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# DPPH radical-scavenging activity of Galantamine hydrobromide and Pymadine alone and in combination

D. D. Tsvetkova*, St. A. Ivanova

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University-Sofia, Bulgaria*

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The overproduction of reactive oxygen species and the weakness of the antioxidant defense mechanisms in the human body are the main reasons for the oxidative stress, which underlies the development of neurodegenerative Alzheimer's disease. Alkaloid Galantamine is nonselective acetylcholinesterase inhibitor with antioxidant activity. Pymadine is non-depolarizing potassium channel blocker having a synergistic effect with Galantamine on the symptomatic treatment of Alzheimer's disease. The aim of the current study was the evaluation of the radical-scavenging activity (RSA) of Galantamine hydrobromide, Pymadine and the combination Galantamine hydrobromide/Pymadine towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The decrease in the absorbance of 0.05 mM methanol solution of DPPH at $\lambda = 516$ nm in presence of methanol solution of: 1 mM Butylhydroxytoluene (BHT) (standard); 1 mM ÷ 5 mM Galantamine hydrobromide; 1 mM ÷ 5 mM Pymadine and 5 mM Galantamine hydrobromide/5 mM Pymadine was monitored by spectrophotometry in equal intervals of 5 s for a total period of 30 min. The regression equations were used for calculation of the $RSC_{50}$: RSA (Galantamine hydrobromide) = $3.419.e^{0.293}.c$, $RSC_{50}$ (Galantamine hydrobromide) = 9.16 mM; RSA(Pymadine) = $0.460.e^{0.411}.c$, $RSC_{50}$ (Pymadine) = 11.41 mM. RSA of the investigated compounds was compared with the effect of standard BHT and the relative radical-scavenging activity (RRSA) and relative decrease of radical-scavenging activity (RDRSA) were calculated. The experimental results showed that the combination of 5 mM Galantamine hydrobromide/5 mM Pymadine has a higher RSA (20.19 %), compared to 5 mM Galantamine hydrobromide (15.44 %) and 5 mM Pymadine (2.48 %) itself.

**Keywords:** Galantamine hydrobromide, Pymadine, Combination, DPPH

## INTRODUCTION

The brain is particularly sensitive to the influence of free radicals due to high oxygen consumption, unsaturated fatty acids and decreased activity of oxidative sensitive endogenous antioxidant systems. In Alzheimer's disease, oxidative stress arises as a result of disturbing the balance between endogenous or exogenous overproduction of reactive free radicals and the reduction of antioxidant protective mechanisms. Oxidative disorders are one of the initial pathological changes in Alzheimer's disease and occur selectively in brain areas responsible for the regulation of memory functions [1]. Oxidative stress plays an important role in the pathogenesis of neuronal degeneration [2], because the formation of free radicals leads to inflammatory processes [3], cell membrane dysfunction [1], activation of programmed nerve cell death (apoptosis) by oxidation of proteins, lipids and nucleic acids (DNA, RNA) [4] and impaired glucose metabolism [5].

Reactive oxygen species include charged and neutral species such as: superoxide anion ($O_2^{-}$•), peroxide radical ($O_2^{2-}$•), hydrogen peroxide ($H_2O_2$), hydroxyl radical (HO•), singlet oxygen ($^1O_2$), alkoxyl radicals (RO•) and peroxyl radicals (ROO•) [6]. One of the therapeutic approaches of Alzheimer's disease is related to decreasing neuronal degeneration by antioxidants [7-9].

The widely used in clinical practice alkaloid Galantamine acts as a nonselective acetylcholinesterase inhibitor [10], but it was recently found that it also displays a considerable antioxidant activity. On the other hand, Pymadine, as a non-depolarizing muscle-relaxant antagonist (potassium channel blocker) [11], was discovered also to have a synergistic effect with the Galantamine on the symptomatic treatment of this widespread disease. The ability to pass through the blood-brain barrier and the different routes of administration (oral, intravenous, intramuscular, subcutaneous, intraocular, electrophoretic) determine the following Galantamine applications: Alzheimer's disease, Alzheimer's with cerebrovascular syndrome, vascular dementia [12-14].

The antioxidant activity of Galantamine hydrobromide was investigated *in vitro* by a luminol-dependent chemiluminescent method. The antioxidant action is bound to the enol group and disappears upon conversion of the enol group (Galantamine and Galantamine hydrobromide) to a

* To whom all correspondence should be sent:
E-mail: dobrinka30@mail.bg

carbonyl group (Narwedine, Narwedine hydrobromide). Changing Galantamine in Galantamine hydrobromide is accompanied by an increase in antioxidant activity due to the quaternary nitrogen atom [15]. Galantamine has a neuroprotective effect by reduction of oxidative neuronal damage by binding free radicals: superoxide, peroxide, hydroxyl and alkoxy [16]. The optimal dose for the activation of α7-subtype nicotinic acetylcholine receptors, whereby Galantamine protects neurons from the influence of superoxide radicals, is 1.5 mg/kg - 5.0 mg/kg [17].

Methods for examination of radical-scavenging activity are [18]: A) electron transfer based methods, in which the increase in the radical-scavenging activity of the test compounds is directly proportional to: I) the decrease of the absorbance of a solution of: (i) 1,1-diphenyl-2-(picrylhydrazyl) (λ=516 nm): DPPH method; (ii) 2,2-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid (λ=734 nm): ABTS method; (iii) hydrogen peroxide (λ=230 nm): peroxidic radical-binding method; (iv) nitroblue tetrazolium: superoxide-radical-scavenging method; II) increasing of the absorption of a solution of: (i) $Cu^{+}$-(neocuproine) (λ=450 nm), obtained by reduction of $Cu^{2+}$-(neocuproine): CUPRAC method; (ii) N,N-dimethyl-p-phenylenediamine (λ=505 nm): DMPD peroxide-radical method; (iii) $Fe^{2+}$-(2,4,6-tripyridyltriazine) (λ=595 nm), obtained by reduction of $Fe^{2+}$-(2,4,6-tripyridyltriazine): FRAP method; (iv) Prussian blue (λ = 700 nm), obtained by reaction of potassium ferrocyanide with ferric chloride: Prussian blue method; (v) $Mo^{5+}$ (λ = 765 nm), obtained by reduction of $Mo^{6+}$: Folin-Ciocalteu method; B) proton based methods, where the increase in the radical-scvenging activity of the test compounds is directly proportional to the lower rate of reduction of: I) the absorption of a solution of β-carotene (λ = 450 nm); II) the fluorescence of: (i) 4,4-difluoro-5-(4-phenyl-1,3-butadienyl) 4-bora-2-amidinopropane) hydrochloride: a method of inhibiting lipid peroxidation; (ii) 2.7-dichlorofluorescein ($\lambda_{excitation}$ = 350 nm, $\lambda_{emission}$ = 540 nm), fluorescein ($\lambda_{excitation}$ = 493 nm, $\lambda_{emission}$ = 518 nm) under the influence of AAPH [2,2'-azobis(2-amidinopropane) – ORAC method, HORAC method); (iii) the product (λ=425 nm) of the reaction between hydrogen peroxide and luminol.

The DPPH method is a rapid, simple, accurate and inexpensive assay for measuring the radical scavenging activity of flavonoids [19, 20], coumarins [21, 22] and their synthetic analogues [22] from some medical plants, to determine antioxidant capacity of wines [23, 24], beer [25], tea infusions [26] and antioxidant activity of plant foods, oils, beverages [27] and extracts from *Citrus* [28], *Crataegus oxyacantha L.* [29], *Ocimum basilicum L.* [30].

The aim of the current study was the evaluation of the radical-scavenging activity (RSA) of Galantamine hydrobromide, Pymadine and the combination Galantamine hydrobromide/ Pymadine towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by measuring the decrease in the absorbance of 0.05 mM methanol solution of DPPH radical at λ = 516 nm by using standard 1 mM methanol solution of butylhydroxytoluene (BHT).

### *Materials*

Test compounds: Galantamine hydrobromide, 4-aminopyridine (Pymadine). Reagents with pharmacopoeia purity: 1.1′-diphenyl-2-picrylhydrazyl (DPPH) (99 %), (Sigma Aldrich, N: STBD 4145 V), butylhydroxytoluene (BHT) (99%) (Sigma-Aldrich, N: BCBL 8166 V), methanol (99.9 %) (Sigma-Aldrich, N: SZBD 063 AV UN 1230).

### *Methods*

*DPPH-method for in vitro study of the radical-binding activity.* Accurately measured quantities: 0.0441 g of BHT (M = 220.35) and 0.0039 g of DPPH (M = 394.32) were dissolved in methanol to 100.0 ml to obtain solutions with concentrations of 2 mM BHT and 0.1 mM DPPH.

Precisely measured amounts of Galantamine hydrobromide (M = 368.27): 0.0737 g, 0.1473 g, 0.2210 g, 0.2946 g, 0.3683 g and 4-aminopyridine (Pymadine) (M = 94.12): 0.0188 g, 0.0376 g, 0.0565 g, 0.0753 g, 0.0941 g were dissolved separately in methanol to 100.0 ml for obtaining solutions with concentrations of 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM.

## RESULTS AND DISCUSSION

The solution of DPPH (1,1'-diphenyl-2-picrylhydrazyl) radical has an absorption maximum at λ = 516 nm. The mechanism of the DPPH method is based on the reduction of the DPPH-radical (violet solution) to the yellow colored 1,1'-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine by free radicals capture compounds.

The experimental results for the change of absorption (A) of DPPH-radical solution and for the radical scavenging activity RSA [%] for 30 min of 1 mM BHT (standard), 1 mM÷5 mM Galantamine hydrobromide and 1 mM÷5 mM Pymadine are summarised in Table 1.

The decrease of the absorbance (A) of 0.05 mM solution of DPPH-radical at λ = 516 nm under the

effect of the test compounds in every 5 s for 30 min is shown on Figure 1 (1 mM BHT, 1 mM÷5 mM Galantamine hydrobromide, 5 mM Galantamine hydrobromide/5 mM Pymadine.

**Table 1**. DPPH-radical scavenging activity of 1 mM BHT (standard), 1 mM ÷ 5 mM Galantamine hydrobromide and 1 mM ÷ 5 mM Pymadine for 30 min.

| | 1 mM BHT | | | | | |
|---|---|---|---|---|---|---|
| t [min] | A | RSA [%] | t [min] | A | RSA [%] | |
| 0 | 0.4862 | 0.00 | 20 | 0.3168 | 34.84 | |
| 5 | 0.4373 | 10.06 | 25 | 0.2847 | 41.44 | |
| 10 | 0.3925 | 19.27 | 30 | 0.2559 | 47.37 | |
| 15 | 0.3526 | 27.48 | | | | |

| 1 mM ÷ 5 mM Galantamine hydrobromide | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| t [min] | 1 mM | | 2 mM | | 3 mM | | 4 mM | | 5 mM | |
| | A | RSA [%] | A | RSA [%] | A | RSA [%] | A | RSA [%] | A | RSA [%] |
| 0 | 0.4880 | 0.00 | 0.5059 | 0.00 | 0.4845 | 0.00 | 0.4933 | 0.00 | 0.5037 | 0.00 |
| 5 | 0.4834 | 0.94 | 0.4967 | 1.82 | 0.4720 | 2.58 | 0.4782 | 3.06 | 0.4799 | 4.73 |
| 10 | 0.4794 | 1.76 | 0.4900 | 3.4 | 0.4632 | 4.40 | 0.4681 | 5.11 | 0.4654 | 7.60 |
| 15 | 0.4760 | 2.46 | 0.4843 | 4.27 | 0.4569 | 5.70 | 0.4601 | 6.73 | 0.4537 | 9.93 |
| 20 | 0.4730 | 3.07 | 0.4792 | 5.28 | 0.4522 | 6.67 | 0.4535 | 8.07 | 0.4438 | 11.89 |
| 25 | 0.4700 | 3.69 | 0.4745 | 6.21 | 04484 | 7.45 | 0.4479 | 9.20 | 0.4349 | 13.66 |
| 30 | 0.4673 | 4.24 | 0.4702 | 7.06 | 0.4451 | 8.13 | 0.4430 | 10.20 | 0.4268 | 15.27 |

| 1 mM ÷ 5 mM Pymadine | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| t[min] | A | RSA [%] | A | RSA [%] | A | RSA [%] | A | RSA [%] | A | RSA [%] |
| 0 | 0.5615 | 0.00 | 0.5671 | 0.00 | 0.5828 | 0.00 | 0.5812 | 0.00 | 0.5856 | 0.00 |
| 5 | 0.5564 | 0.91 | 0.5623 | 0.85 | 0.5775 | 0.91 | 0.5770 | 0.72 | 0.5822 | 0.58 |
| 10 | 0.5550 | 1.16 | 0.5600 | 1.25 | 0.5743 | 1.46 | 0.5753 | 1.02 | 0.5786 | 1.20 |
| 15 | 0.5554 | 1.09 | 0.5591 | 1.41 | 0.5717 | 1.90 | 0.5735 | 1.32 | 0.5760 | 1.64 |
| 20 | 0.5565 | 0.89 | 0.5588 | 1.46 | 0.5706 | 2.09 | 0.5720 | 1.58 | 0.5740 | 1.98 |
| 25 | 0.5575 | 0.71 | 0.5596 | 1.32 | 0.5715 | 1.94 | 0.5711 | 1.74 | 0.5723 | 2.27 |
| 30 | 0.5578 | 0.66 | 0.5610 | 1.08 | 0.5726 | 1.75 | 0.5707 | 1.81 | 0.5709 | 2.51 |

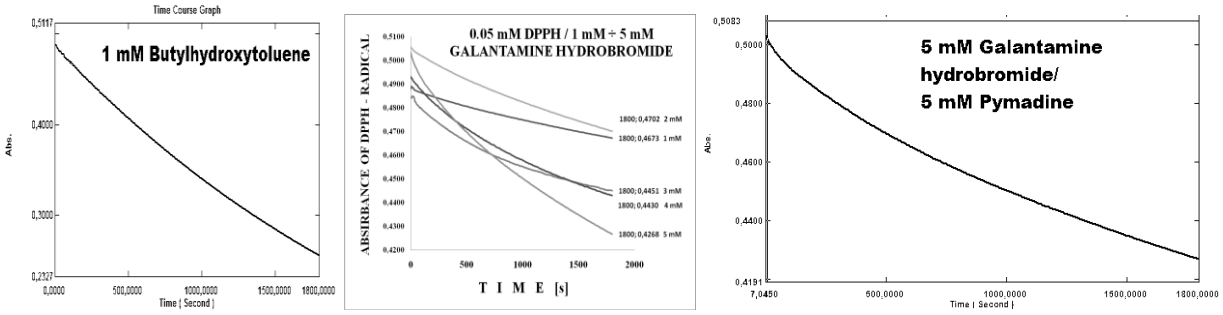



**Figure 1.** Effect of 1 mM Butylhydroxytoluene, 1 mM ÷ 5 mM Galantamine hydrobromide, 5 mM Galantamine hydrobromide/5 mM Pymadine on the absorbance of 0.05 mM solution of DPPH-radical for a period of 30 min.

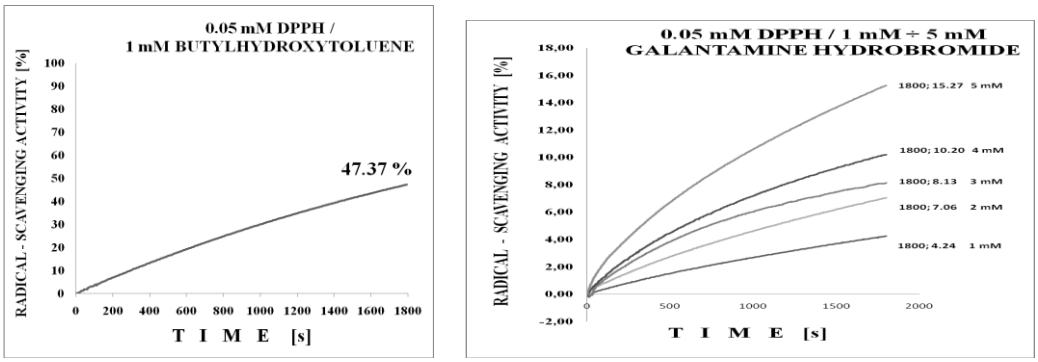



**Figure 2.** Kinetics of scavenging of 0.05 mM DPPH-radical from 1 mM BHT and 1 mM ÷ 5 mM Galantamine hydrobromide for a period of 30 min.

The experimental kinetic curves for scavenging of DPPH-radical for 30 min by 1 mM BHT and 1 mM÷5 mM Galantamine hydrobromide are illustrated in Figure 2.

The increase of the radical binding effect with an increase of the concentration of Galantamine hydrobromide and Pymadine is illustrated on Figure 3.

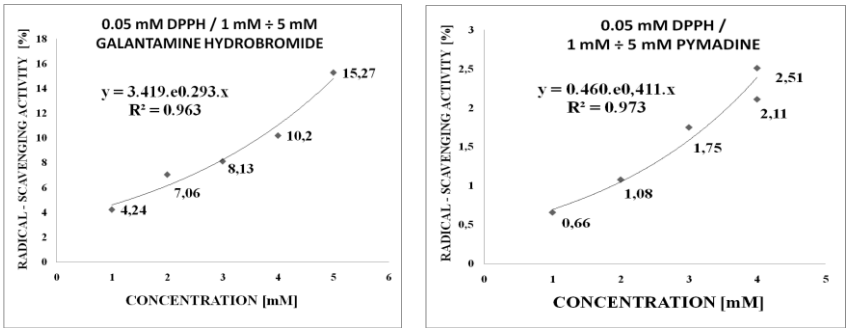
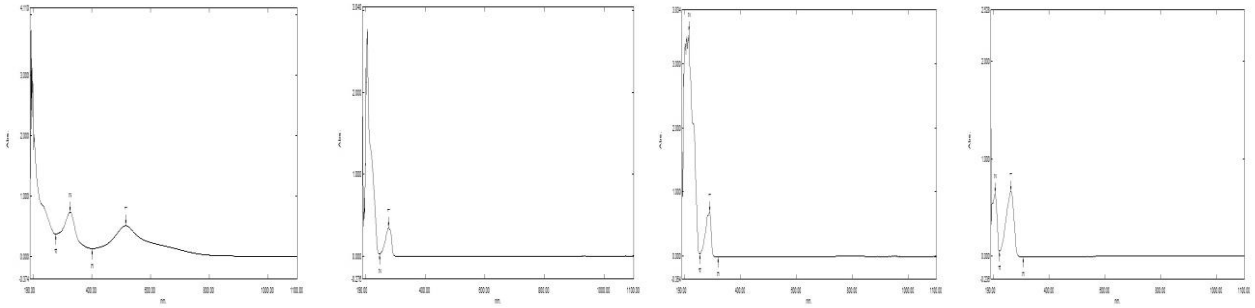



**Figure 3.** Relationship between the radical binding activity and concentration of Galantamine hydrobromide and Pymadine.



DPPH BHT Galantamine hydrdogromide Pymadine

**Figure 4.** Spectra of DPPH, BHT, Galantamine hydrobromide and Pymadine

The method is applicable for the study of radical scavenging activity of the test compounds, because they do not possess a measurable absorbance at the absorption maximum λ = 516 nm, where the DPPH-test is carried out. Spectra in methanol of DPPH, BHT, Galantamine hydrobromide and Pymadine are illustrated on Figure 4.

**Table 2.** Radical-binding activity of 1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine after 30 min reaction with 0.05 mM solution of DPPH-radical.

| N: | 1 mM BHT | | | 5 mM Galantamine hydrobromide/5 mM Pymadine | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | RSA[%] | UA | A | RSA[%] | UA | RRSA [%] | RDRSA [%] |
| 1. | 0.36263 | 47.47 | 0.84 | 0.54881 | 20.50 | 0.71 | 43.19 | 56.81 |
| 2. | 0.36066 | 47.76 | 0.15 | 0.54971 | 20.37 | 0.41 | 42.65 | 57.35 |
| 3. | 0.35957 | 47.92 | 0.69 | 0.55431 | 19.71 | 1.12 | 41.13 | 58.87 |
| $\overline{X}$ | 0.36095 | 47.72 | | 0.55094 | 20.19 | | 42.32 | 57.68 |
| SD | 0.002 | 0.23 | | 0.003 | 0.42 | | 1.07 | 2.07 |
| RSD [%] | 0.55 | 0.48 | | 0.54 | 2.08 | | 2.53 | 1.86 |

**Table 3.** Radical-binding activity of 5 mM Galantamine hydrobromide and 5 mM Pymadine after 30 min reaction with a 0.05 mM solution of DPPH-radical.

| N: | 5 mM Galantamine hydrobromide | | | | | 5 mM Pymadine | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | RSA [%] | UA | RRSA [%] | RDRSA [%] | A | RSA [%] | UA | RRSA [%] | RDRSA [%] |
| 1. | 0.58649 | 15.05 | 1.36 | 31.70 | 68.30 | 0.67228 | 2.53 | 1.0 | 5.33 | 94.67 |
| 2. | 0.58273 | 15.59 | 0.52 | 32.64 | 67.36 | 0.67342 | 2.46 | 0.35 | 5.15 | 94.85 |
| 3. | 0.58208 | 15.69 | 0.85 | 32.74 | 67.26 | 0.67354 | 2.44 | 0.65 | 5.09 | 94.91 |
| $\overline{X}$ | 0.58377 | 15.44 | | 32.36 | 67.64 | 0.67328 | 2.48 | | 5.19 | 94.81 |
| SD | 0.002 | 0.34 | | 0.57 | 0.57 | 0.0004 | 0.05 | | 0.12 | 0.12 |
| RSD [%] | 0.34 | 2.20 | | 1.76 | 0.84 | 0.06 | 2.02 | | 2.31 | 0.13 |

The results of DPPH-radical binding activity for a period of 30 min for 1 mM BHT, 1÷5 mM Galantamine hydrobromide and 1 ÷ 5 mM Pymadine (Table 1), were calculated by the equation:

$$\text{RSA [\%]} = \frac{Acontrol - Asample}{Acontrol}.100$$

$A_{control}$ - absorption of the solution of DPPH-radical before interaction with the investigated compound; $A_{sample}$ - absorption of the solution of DPPH-radical after reacting with the investigated compound.

Experimental results show that with increasing of the concentration from 1 mM to 5 mM for both compounds was observed an increase in radical-scavenging effect (Figure 3.). The equations:

$y = 3.419.e^{0.293.x}$ (Galantamine hydrobromide), $y = 0.460.e^{0.411.x}$ (Pymadine)

were used for calculatation of $[RSC_{50}]$ – the concentration which achieves 50 % binding of DPPH-radical. For Galantamine hydrobromide: $[RSC_{50}]$ = 9.16 mM and for Pymadine: [RSC50] = 11.41 mM.

For the calculation of radical binding activity under identical conditions, 2 ml of 0.1 mM of DPPH ($A_{0\ min.}$ = 0.69037) (Figure 5.), were added to 2 ml of 2 mM BHT, 2 ml of the model mixture containing 10 mM Galantamine hydrobromide/10 mM Pymadine. The spectra of the solutions after 30 min. reaction with DPPH-radical are presented in Figure 5. (1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine) and Figure 6. (5 mM Galantamine hydrobromide and 5 mM Pymadine).

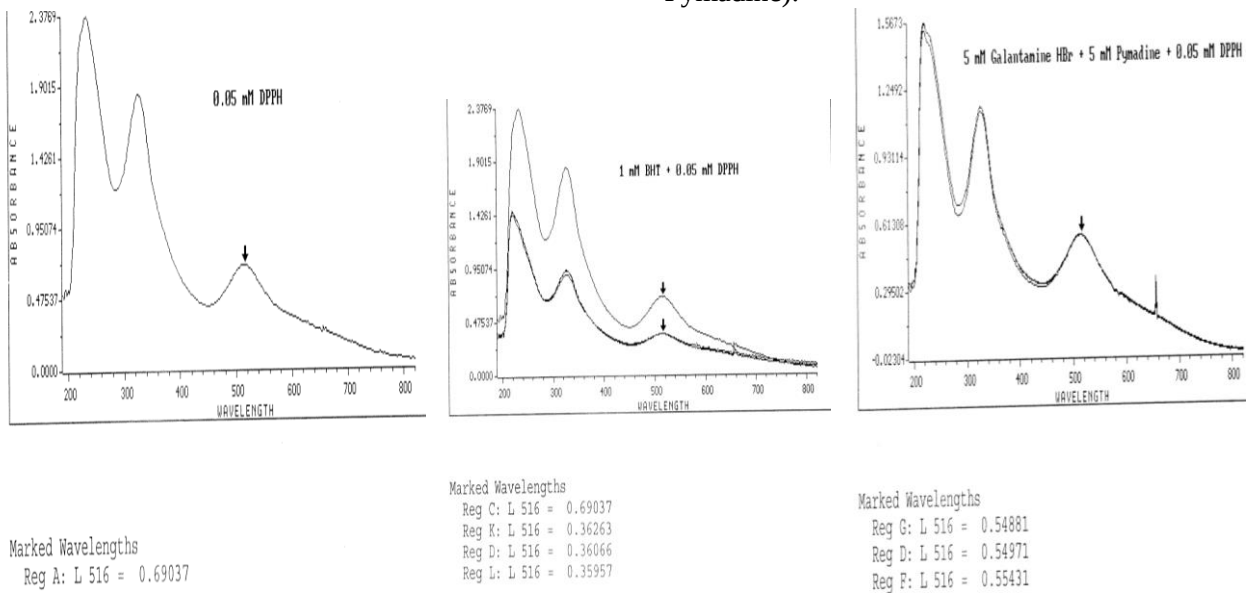



**Figure 5.** Spectra of 0.05 mM solution of DPPH-radical: A 0 min. = 0.69037 and after 30 min reaction with 1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine.

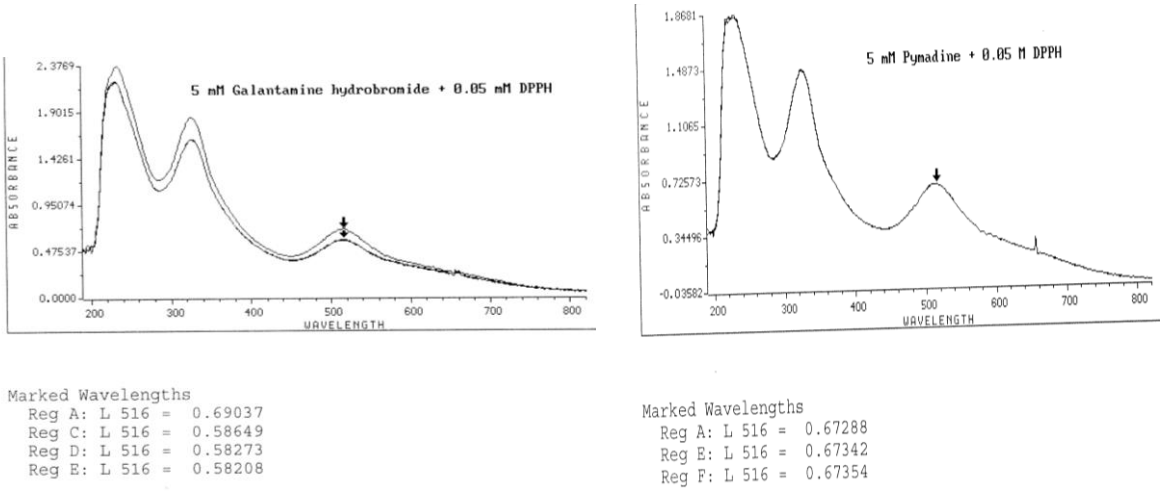



**Figure 6.** Spectra of 0.05 mM solution of DPPH-radical after 30 min. interaction with 5 mM Galantamine hydrobromide and 5 mM Pymadine**.**

The radical-scavenging activity of the test compounds was compared to the activity of the standard butylhydroxytoluene. The relative radical scavenging activity (RRSA, [%]) and the relative decrease in radical scavenging activity (RDRSA, [%]) were calculated and were compared to the standard butylhydroxytoluene.

$$\text{RRSA [\%]} = \frac{RSA\,sample}{RSA\,BHT}.100$$

$$\text{RDRSA [\%]} = \frac{RSA\,BHT - RSA\,sample}{RSA\,BHT}.100$$

$RSA_{sample}$ - radical binding activity of the test compounds; $RSA_{BHT}$ - radical binding activity of the standard butylhidroxytoluene.

The results for the antiradical activity of the test compound after 30 min interraction with DPPH-radical are shown in Table 2 (1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine), Table 3 (5 mM Galantamine hydrobromide, 5 mM Pymadine).

## CONCLUSIONS

The following results for the radical scavenging activity were obtained: 1 mM÷5 mM Galantamine hydrobromide (4.24 %÷15.27 %) and 1 mM÷5 mM Pymadine (0.66 %÷2.51 %). The results show that the combination 5 mM Galantamine hydrobromide/5 mM Pymadine (20.19 %) has a stronger radical scavenging activity than 5 mM Galantamine hydrobromide (15.44 %).

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# DPPH РАДИКАЛ-СВЪРЗВАЩА АКТИВНОСТ НА ГАЛАНТАМИН ХИДРОБРОМИД И ПИМАДИН САМОСТОЯТЕЛНО И В КОМБИНАЦИЯ

Д. Д. Цветкова*, Ст. А. Иванова

[1]*Катедра "Фармацевтична химия", Фармацевтичен Факултет, Медицински Университет-София*

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(Резюме)

Свръхпроизводството на реактивни кислородни видове и отслабването на антиоксидантните защитни механизми в човешкото тяло са основната причина за оксидативния стрес, който е в основата на развитието на невродегенеративната болест на Алцхаймер. Алкалоидът галантамин е неселективен инхибитор на ацетилхолинестеразата с антиоксидантна активност. Пимадин е недеполяризиращ блокер на калиевите канали и оказва синергичен ефект с галантамин върху симптоматичното лечение на болестта на Алцхаймер.

Целта на настоящото изследване е оценката на радикал-свързващата активност (RSA) на галантамин хидробромид, пимадин и комбинацията галантамин хидробромид/пимадин по отношение на 2,2-дифенил-1-пикрилхидразил (DPPH) радикала. Намалението на абсорбцията на 0.05 mM метанолен разтвор на DPPH при $\lambda$ = 516 nm в присъствието на метанолни разтвори на: 1 mM бутилхидрокситолуен (стандарт), 1 mM ÷ 5 mM галантамин хидробромид, 1 mM ÷ 5 mM пимадин и 5 mM галантамин хидробромид/5 mM пимадин се наблюдава през равни интервали от 5 s за общ период от 30 min чрез спектрофотометричен метод. Регресионни уравнения се използват за изчисляване на стойностите на $RSC_{50}$: RSA (галантамин хидробромид) = $3.419.e^{0.293}.c$, $RSC_{50}$ (галантамин хидробромид) = 9.16 mM; RSA (пимадин) = $0.460.e^{0.411}.c$, $RSC_{50}$ (пимадин) = 11.41 mM. RSA на изследваните съединения се сравнява с ефекта на стандартния BHT и се изчисляват относителната радикал-свързваща активност (RRSA) и относителното намаляване на радикал-свързващата активност (RDRSA). Експерименталните резултати показват, че комбинацията от 5 mM галантамин хидробромид/5 mM пимадин има по-висока RSA (20.19 %) в сравнение с 5 mM галантамин хидробромид (15.44 %) и 5 mM пимадин (2.48 %).