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Research Article

ANTIOXIDANT AND ANTI TUMORAL ACTIVITIES OF HYDRAZYLPYRROLIDINE 2, 5 DIONE SUBSTITUTED AND 2-THIOXO IMIDAZOLIDINE 4-ONE

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ABSTRACT

In continuation of our research efforts to evaluate biological properties of synthesized compounds, we have investigated the *in vitro* antioxidant activity of hydrazylpyrrolidine 2, 5 dione substituted **2**, **3**and**4**or 2-thioxo imidazolidine 4-one**5**, **6**and**7** by spectrophotometric DPPH and lipid peroxide inhibition. The IC_{50} of both compounds was higher excepted compounds **6**b, **7**a and **4**c witch have a low value (4.9, 6.18, 6.70 µg/mL) respectively.Thesecompounds exhibited strong antioxidant activity. Their IC_{50} are approximatively equal to standard quercetin for **4**c and **7**a or to ascorbic acid standard for **6**b. *In vitro* lipid peroxydation was determined by antioxidant activity using the β-Carotene/ linoleic acid system. The synthesis compounds have revealed a low or moderate antioxidant activity. The maximum relative antioxidant activity (AAR) was observed for derivatives **2**a and **7**c respectively (53.31, 48.72 µg/mL). *In vitro* antitumoral activities of compounds **2**a and **6**b at 370 µg/mLhave exhibited significant antitumoral activities against HEp2 (Human Laryngeal CarcinomaCells).

Keywords: Pyrrolidine 2, 5 one,Thiohydantoin, antioxidant and antitumoral activities.

INTRODUCTION

In recent years, researches about the natural or synthesis antioxidants have considerably increased. So free radicals or reactive oxygen species (ROS) are formed in vivo from different biochemical reactions and also from the respiratory chain as a result of occasional leakage from metabolic circuits. These free radicals are the main sources of lipid peroxidation[1]. Free radicals induced oxidative stresses have been implied in the pathogenesis of wide variety of clinical disorders, emanating usually from deficiency of natural antioxidative defense[2].Antioxidants scavenger free radicals are associated with reduced risk of cancer and cardiovascular diseases[3].

The most known antioxidants are ß-carotene (provitamin A), the ascorbic acid (vitamin C), the tocopherol (vitamin E) as well as phenolics compounds and flavonoids[4-5].

Indeed, most of synthesis or natural origin antioxidants possess hydroxyl phenolics groups in their structures and the antioxidizing properties are partially awarded, in the capacity of these compound natures to trap the free radicals such as the radical hydroxyl ($OH^{\cdot}$) and superoxidize ($O2^{-}$). Free radical scavenging by polyphenols has been widely studied[6]. Other heterocyclic compounds like pyrrolidine 2, 5

dione substituted or 2-thioxo imidazolidine 4-one incorporating amide, amine or others functions could have antioxidizing activities. The presence of heterocyclic moieties or functions into 2-thioxo imidazolidine 4-one or thiohydantoin skeleton is a widely used procedure in the preparation of biologically active compounds[7-9].

In this study, and in the search of new biological properties of pyrrolidine 2, 5 dione nuclei and 2-thioxo imidazolidine 4-one, we have exploited the structural diversity of substituted hydrazylpyrrolidine 2, 5 dione **2**, **3** and **4** or 2-thioxo imidazolidine 4-one **5**, **6** and **7** previously reported by us, to evaluate their in vitro antioxidant activities using standard procedures of DPPH based free radical scavenging activity or lipid peroxidation inhibition. We investigated also the possible effects of compounds on proliferation (Human Laryngeal Carcinomacells) HEp2 cells, the malondialdehyde (MDA) were measured after cells treatment as indicator of lipid peroxidation.

MATERIALS AND METHODS

1. Evaluation of antioxidant capacity

1. 1.DPPH radical scavenging activity.

In the DPPH radical scavenging test, the scavenging of DPPH is followed by monitoring the decrease in absorbance at 517 nm that occurs due to the reduction by the antioxidant or reaction with a radical species. DPPH radical scavenging effect was determined according to the method[10]. DPPH is widely used to test the ability of compounds to act as hydrogen donors or free radical scavengers and to evaluate antioxidant activity. A solution of DPPH was prepared by dissolving 4 mg in 100 mL of methanol, and the solution was kept in the dark at 4 °C. A stock solution of the compounds was prepared at 1 mg/mL in methanol. The stock solution was diluted to varying concentration in tube assay. Different levels of methanol solution (5, 20, 50, 100, 200, 300, 1000 µg/mL) were added to 2 mL of DPPH solution (0.004%w/v). After incubation for 30 min in the dark at room temperature, absorbance (A) was read at 517 nm, against the blank (methanol). The blank was used to remove the influence of the samples color. The radical-scavenging activity was expressed as percentage of inhibition according to the following formula[11].

Inhibition (%) = (A control−A sample) x100/ A control

1.2. Antioxidant Activity Using the β-Carotene/ linoleic Acid System

Antioxidant activity of all solutions was estimated spectrophotometrically based on the β carotene discoloring induced by the oxidative degradation of linoleic acid described by[12] and modified by[13]. The decrease in the absorbance of level solution was measured at 30 min intervals for a total of 120 min.

β-carotene (2 mg) was dissolved in 10 mL of chloroform and blended with 20 mg of linoleic acid and 200 mg of Tween 40 followed by removal of chloroform with subsequent addition of 50 mL of distilled water with vigorous shacking to prepare β-carotene linoleate emulsion. An aliquot of solution (50 µL) was mixed with 1mL of the emulsion, vortexed and absorbance was determined at 470 nm immediately against the BHT control (blank).The β-Carotene bleaching inhibition was estimated by the Antioxidant Activity Relative[14] (AAR) following the equation: Bleaching inhibition (%)= (At/ ABHT) x 100

At: the absorbance value for the test solution

ABHT: the absorbance value for the test sample control

2. Cell Proliferation Assay

The cells were trypsinised (0.1% of trypsin Gibco, USA) and suspended. After incubation during 48 h, cells were exposed to compounds **2**a or **6**b during 48 h, the cells were trypsinized and the evaluation of proliferation rate was performed on 100 µL cell suspension by counting on Mallasez cell.

3. Morphological and morphometic study

The suspended cells hasbeenincubated during 48h and exposed during 48 h at 370 µg/mL to compounds **2**a and **6**b. After that, the mediums were eliminated and the cells were washed with a phosphate-buffered salin (PBS,1x) (Gibco) and fixed in the aqueous Bouin and colored with May Grumwald–Giemsa (MGG) ((V/V, 1/1) and 100 mg/mL orange acridine. The observation was done with and an inverted microscope.

4. Measurement of lipid peroxidation using MDA assay

MDA level of cells was measured spectrophotometrically. MDA reacts with TBA as a thiobarbituricacide reactive substance (TBARS) to produce a red colored complex that has a peak absorbance at 532 nm[15].

The MDA was determinate in the intracellular compartments of control cells during 48 h of incubation and submitted at 370 µg/mL to compounds **2**a and **6**b.

After reaction with thiobarbituric acid TBA[16] cells were homogenized in buffered (Na_2 HPO4 /Na H_2PO4) 0.2 M, pH 6.5 and centrifuged for 20 mn at 4°C. The MDA contained supernatant in presence of 10% TCA reacts with TBA and

causes the formation of a complex. The absorbance at 532 nm against a blank (TBA, 1mL) that contained all reagents except the sample. The amount of MDA equivalents formed was calculated using MDA standard graph prepared under similar reaction conditions.

RESULTS AND DISCUSSION

The preparation of hydrazylpyrrolidine 2, 5 dione**2**, **3** and **4** or 2-thioxo imidazolidine 4-one **5**, **6** and **7** was described in Scheme 1. Thiohydantoine structures **5**, **6** and **7** were synthesized from appropriate maleimide and binucleophilic reagents according to the reported procedures[17-19].

1. Evaluation of antioxidant capacity DPPH scavenging

Antioxidant capacity is widely used as a parameter for medicinal bioactive components. The antioxidant most made known for its potential benefits to cancer patients is vitamin C (ascorbic acid). Antioxidant supplements may reduce the frequency and severity of toxicity associated with anticancer therapy.

The antioxidant activity of diverse synthesis molecules was expressed as IC_{50} (half inhibitory concentration). The calculated IC_{50} of **2**, **3** and **4** derivatives was consigned in Table 1, and the vitamin C or the quercetin were used as standard references.

According to the data in Table 1, the tested compounds proved generally to reduce the DPPH. Derivatives **3**a-c, substituted with a methyl, an ethyl or a phenyl group in position 1 of the pyrrolidine 2, 5 dionering, were characterised by IC_{50} values ranging between 90 and 905.87 µg/mL and have a low or moderate free radical scavenging activity. With the exception of **3**c, pyrrolidine 2, 5 dionewith an aryl group showsa very low DPPH-scavenging activity (905.87 µg/mL) than those with alkyl substituent's (methyl or ethyl) as compared with quercetin (6.6 µg/mL) or ascorbic acid (3.67 µg/mL). In the case of compounds **2**a-d, we have observed the different results comparatively to derivatives **3**a-c. Products **2**a-d displaying an even higher DPPHscavenging activity, and the IC_{50} values ranging were including 83- 101.21µg/mL.A phenyl group shows a good effect scavenging than the alkyl radical. The best DPPH-scavengers was observedhydrazylpyrrolidine 2, 5 dione **4**a-d, substituted with a cyanic group. The IC_{50} values were ranging between 6.70-11.93µg/mL. The comparison with quercetin and ascorbic acid as antioxidants standardsshowed that derivative **4**c (6.70µg/mL) with alkyl (ethyl) has a similar antioxidant scavenging activity as quercetin.

The anti-oxidant data of series **5**, **6** and **7** provided from substituted hydrazylpyrrolidine 2, 5 dione nuclei **2**, **3** and **4** revealed that IC_{50} values of all compounds were modified by the introduction of a new function. The presence of the amide function on the position 4 or sulfurin the C=S group in position 2 of thiohydantoin nuclei did not shown any significant influence on antioxidant activity accepted **6**b (4.9 µg/mL) and **7**a (6.18 µg/mL). Antioxidant activity of compound **7**a could be explained and assigned to the presence of the attracted cyanic group (CH_2CN) inserted on the 2-thioxoimidazolidin 4-one in position 3. This group (CH_2CN) has a great impact on the activity. Moreover, the presence of two amid groups in position 3 or 4 on the thiohydantoine ring increases in compound **6**b the scavenger activity (4.9µg/mL), similar to vitamin C.

In all cases 2-thioxo imidazolidine 4-one structures showed a better DPPH scavenging activity than hydrazylpyrrolidine 2, 5 dione with the same substituent. The assumption is that 2-thioxo imidazolidine 4-one, when donating an electron to a free radical, can form a structure stabilized by delocalization of electrons throughout the molecule.

2. Peroxydation of lipid using the β-Carotene/ linoleic Acid System

The antioxidant activity was also evaluated by the ß Carotene/ linoleic assay, and the results, expressed as the percent inhibition of β-carotene oxidation, is shown in Table 2. Maximum anti oxidant activity relative was observed for compound **2**a and lowest for compound **3**c with AAR values 48.72 and 26.27 % respectively.

The percentage inhibition induced of the second molecules series (**5**, **6** and **7**)was approximately ranged from 53.31 to 13.29 %. The compound **5**f possessed the high AAR. This result could be assigned to the presence of ester, methyl and phenyl groups.

3. Evaluation of anticancer activity in vitro

We investigate also potential anti-tumoralactivity; compounds **2**a and **6**b are selected for their difference in antioxidant effect (low and moderate IC_{50}).We performed a preliminary cytotoxicity study with cells HEp2 (Human Laryngeal Carcinomacells) exposed to two concentrations (5 µg/mL and 370 µg/mL) for compounds **2**aand **6**b cells treated with DMSO were used as controls. Treatment with a low dose (corresponding to IC_{50}) did not revealed any alteration in morphology of HEp2.

When cells were exposed to compounds **2**a or **6**b at 370 µg/mL for 48 h as shown in Figure 1

cells proliferations was not reduced after treatment, although we observed cytotoxicity effects of these products. Microscope image analysis revealed the presence of hypertrophic cell and oncosis this is can be indicate morphological signs of apoptosis. According to Figure 1, we noted that the derivative **6**b presented a large number of variable sizes of oncosis compared to compound **2**a. This result has shown that **6**b was the most active compound.

Treatment with compound **2**aand **6**b at dose of 370 µg/mL significantly increased MDA levels by respectively 322% and 361% compared to HEp2 cells control, we observed that both compounds exhibita similar increase in MDA represented in Figure 2.

Reactive Oxygen Species (ROS) accumulation in cell is responsible of the peroxidation lipid. This result confirmed the cytotoxic effect. Accumulation of ROS generates oxidative stress, which mostly results in cell apoptosis[20].As know free radicals are important for apoptosis signals.

CONCLUSION

In this study antioxidant and anticancer properties of 2-thioxo imidazolidine 4-one or hydrazylpyrrolidine 2, 5 dione were examined. The 2-thioxo imidazolidine 4-one structures showed a good antioxidant activity than hydrazylpyrrolidine 2, 5 dione nuclei. Compounds **6**b, **4**c and **7**a were revealed as a potent antioxidant, derivative **6**b possessed a low IC_{50} compared to standard quercetin. We have also observed that compound 5f have the high Antioxidante Activity Relative (AAR).

The cytotoxic activities of **2**a or **6**b were evaluated against human cancer HEp2 (Human Laryngeal Carcinomacells) by morphological study. Cells undergoing derivatives **2**a or **6**b treatment showed signs of apoptosis marked by the hypertrophy and oncosis. These compounds could have potent anti-tumor effects against malignant HEp2 cells.

Table 1: IC_{50}(half inhibitory concentration) of all synthesized compounds

Antioxidant activity was determinate by DPPH radical scavenging test.
Standard references:IC_{50}quercetin : 6.9 µg/mL, ascorbic acid IC_{50} 3.6µg/mL.

Compounds	IC50 (µg/mL)	Compounds	IC50 (µg/mL)	Compounds	IC50 (µg/mL)
2a	101.21	4c	6.70	5g	99
2b	89.92	4d	11.23	6b	4.9
2c	83.33	5a	103.3	6d	25.27
2d	83	5b	309.91	6e	52.06
3a	120.97	5c	107	6f	307.12
3b	90.86	5d	87	7a	6.18
3c	905.87	5e	105.04	7b	10.97
4a	11.93	5f	90	7c	32.93
4b	9.86	5h	104	7d	21.81

Table 2: Inhibition percentage of β-carotene oxidation of all compounds

The β-Carotene bleaching inhibition was estimated by the Antioxidant Activity Relative (AAR)

Compounds	2a	2b	2c	2d	3a	3b	3c	4a	4b
AAR	48.72	43.11	32.39	41.32	31.12	26.78	26.27	27	26.27
Compounds	**4**c	**4**d	**5**a	**5**b	**5**c	**5**d	**5**e	**5**f	**5**g
AAR	29.33	27.55	35.20	33.67	33.92	34.43	46.93	53.31	34.94
Compounds	**5**h	**6**b	**6**d	**6**e	**6**f	**7**a	**7**b	**7**c	**7**d
AAR	41.32	13.29	15.02	22.25	17.91	33.92	35.45	31.37	29.33

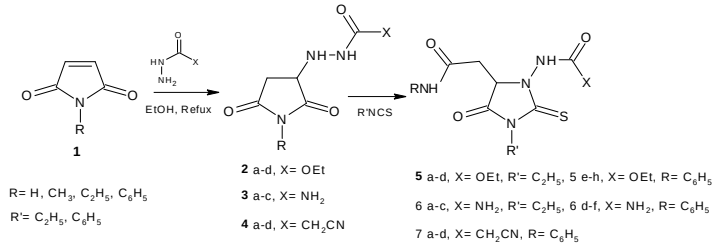

Fig. 1: Synthesis of all compounds

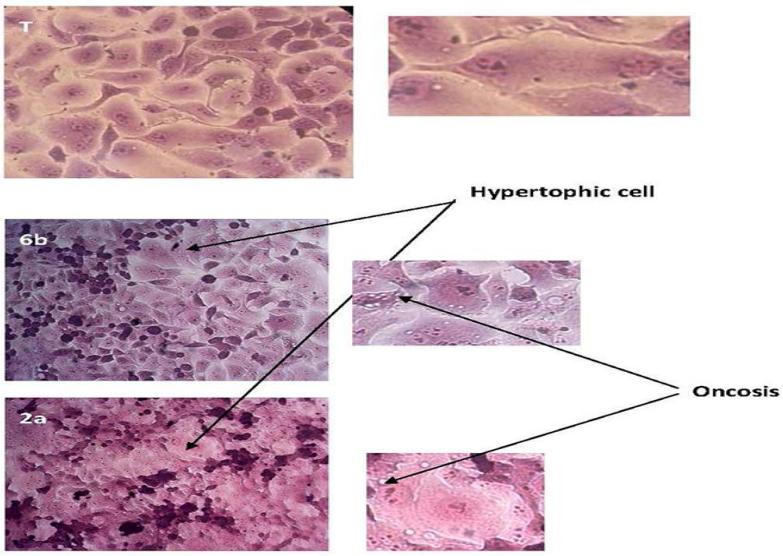

Fig. 2: Effects of compounds 2a or 6b at 370 µg/ mL on the morphology of Human Laryngeal Carcinoma cells (HEp2) after 48 h of treatment. The cells were colored withMayGrumwald –Giemsa (M G G)(G x 86,95 µm)

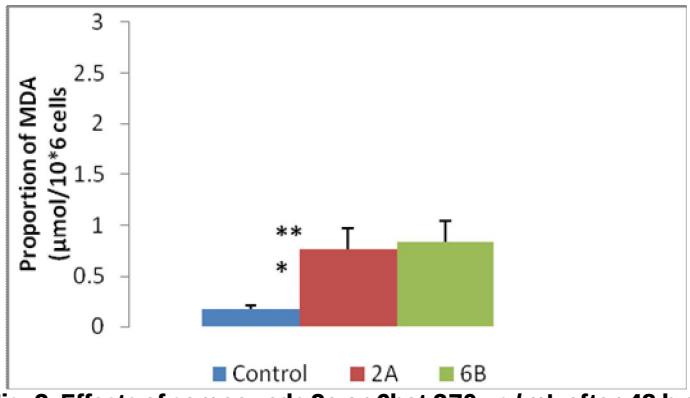

Fig. 3: Effects of compounds 2a or 6bat 370 µg/mL after 48 h of treatment on the level of malondialdehyde (MDA µmol/ l 10^6 cells) in cells HEp 2 type *** P≤ 0.001

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