https://doi.org/10.31925/farmacia.2020.6.11 **ORIGINAL ARTICLE**

ANTIOXIDANT ACTIVITY AND DRUG PROFILE OF SEVERAL THIOUREA DERIVATIVES OF 2-THIOPHENE CARBOXYLIC ACID

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Abstract

Oxidative stress plays an important role in the development of several diseases (cancer, stroke, diabetes etc.). Therefore, the discovery of new compounds with antioxidant activity and a safe pharmacological profile has become an area of great interest. The aim of our study was the evaluation of antioxidant capacity and drug profile of several 2-thiophene carboxylic acid thiourea derivatives. The antioxidant activity was determined by means of scavenger activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) free radicals and ferric reducing power assay. The pharmacological profile of the analysed compounds was evaluated by *in silico* protocols (bioavailability, pharmacokinetics etc.). The electronic and steric molecular descriptors of the thiourea derivatives have been also determined. According to our results, all compounds showed antioxidant activity. For the most part, *in silico* studies revealed a safe pharmacological profile for the analysed compounds.

Rezumat

Stresul oxidativ reprezintă un factor cheie în patologia multor afecțiuni (cancer, boli cardiovasculare, metabolice etc.). În ultimii ani, s-a observat un interes crescut în descoperirea de noi compuși cu proprietăți antioxidante și cu un profil farmacologic sigur. Scopul lucrării a constat în evaluarea potențialului antioxidant și a profilului medicamentos ale unor tioureide obținute de la acidul 2-tiofencarboxilic. Activitatea antioxidantă a fost determinată pe baza capacității de chelatare a radicalilor liberi DPPH (2-difenil-1-picrilhidrazil), $ABTS^{\bullet+}$ (acidul 2,2-azino-bis-(3-etil-benzotiazolin-6-sulfonic) și pe baza capacității de reducere a ferului. Profilul farmacologic a fost evaluat prin studii *in silico* (biodisponibilitate, parametri farmacocinetici etc). Suplimentar, au fost determinați și descriptorii chimici ai acestor derivați. Conform rezultatelor obținute, toți compușii au prezentat activitate antioxidantă. Studiile *in silico* au evidențiat, în general, un profil farmacologic sigur.

Keywords: thiourea derivatives, 2-thiophene carboxylic acid, antioxidant activity, bioavailability

Introduction

The role of oxidative stress in aging and development of human diseases (cancer, cardiovascular, metabolic conditions, diabetes, autoimmune and neurodegenerative disorders) is well known [5].

Reactive oxygen species (ROS) including free radicals (hydroxyl - OH•; superoxide – O_2•; peroxyl – RO_2•; nitric oxide – NO•) and non-radicals (hypochlorous acid – HOCl; hydrogen peroxide – H_2O_2; singlet oxygen – $^1O_2$; peroxynitrite – $ONOO^-$) are constantly formed in the human body [5]. Indigenous sources of ROS result from mitochondria functions, cytochrome P450 metabolism or inflammatory cell response [5]. Among exogenous factors, exposure to UV light, X-ray irradiation, pesticides, pollution, or smoking are of great importance for ROS production [5].

Oxidative stress is considered as an imbalance between the production of ROS and the ability of the natural antioxidant defence system to fight against free radicals and prevent oxidation of lipids, proteins, or DNA [5].

It is well known that the human antioxidant defence system is represented by enzymatic (superoxide dismutase – SOD, catalase, glutathione peroxidase – GPx) and non-enzymatic antioxidants (GSH – glutathione, vitamin E, vitamin C etc.). Antioxidants are molecules that neutralize free radicals and prevent oxidation of a

substrate by means of various mechanisms: (i) keeping ROS production at a minimum level; (ii) scavenger activity; or (iii) repair of affected cells and tissues [5].

Taking into consideration the negative effect of oxidative stress upon human health, considerable research has been directed towards synthesis of new organic compounds with potential antioxidant properties.

Over the years, thiourea derivatives have become an important class of compounds due to their various biological properties: antibacterial [21], antitubercular [23], antiparasitic [24], anticonvulsant [12], analgesic [22], antiarrhythmic [6], local anaesthetic, antiproliferative, antiplatelet [19], antifungal [26], insecticidal [13], or plant-growth regulators [18].

The aim of our paper was the evaluation of antioxidant activity and drug profile of several 2-thiophene carboxylic acid thiourea derivatives.

Materials and Methods

Tested compounds (Table I) were 2-thiophene carboxylic acid thiourea derivatives encoded as follows: N-(o-iodophenyl)-N'-(2-thienyl)-thiourea (**C1**), N-(m-iodophenyl)-N'-(2-thienyl)-thiourea (**C2**), N-(p-iodophenyl)-N'-(2-thienyl)-thiourea (**C3**), N-(2,4-dichlorophenyl)-N'-(2-thienyl)-thiourea (**C4**), N-(2,5-dichlorophenyl)-N'-(2-thienyl)-thiourea (**C5**), N-(2,6-dichlorophenyl)-N'-(2-thienyl)-thiourea (**C6**) and N-(3,4-dichlorophenyl)-N'-(2-thienyl)-thiourea (**C7**). All compounds were synthesized and characterized, as previously described [3].

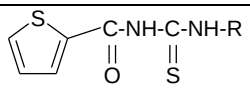
All reagents were commercially obtained (Fluka or Sigma-Aldrich, Germany), unless otherwise stated.

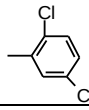
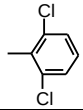
Preparation of samples for antioxidant activity evaluation: thiourea derivatives were dissolved in a mixture of dimethylsulfoxide (DMSO):96% ethanol = 1/99 (v/v). The concentration of each stock solution was 2 mM. Subsequent analysis, were performed using the same concentration range (0.025 - 2 mM).

The *antioxidant activity* was determined based on several well-known methods – the scavenger activity towards DPPH (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) free radicals and ferric reducing power assay [2, 4, 10].

Table I

Characterization of tested compounds



Compound	R	Molecular formula	Molecular weight	Melting point (°C) (isopropanol)	Yield (%)
C1		$C_{12}H_9S_2ON_2I$	388.25	168 - 172	53
C2		$C_{12}H_9S_2ON_2I$	388.25	148 - 150	61
C3		$C_{12}H_9S_2ON_2I$	388.25	171 - 173	62
C4		$C_{12}H_8S_2ON_2Cl_2$	331.24	181 - 183	70
C5		$C_{12}H_8S_2ON_2Cl_2$	331.24	197 - 199	71
C6		$C_{12}H_8S_2ON_2Cl_2$	331.24	191 - 193	69
C7		$C_{12}H_8S_2ON_2Cl_2$	331.24	159 - 162	57

DPPH radical scavenging assay was determined according to Ohnishi M *et al*. with some modifications [7, 15]. Briefly, 0.5 mL of 0.025 - 2 mM solutions were mixed with 3 mL DPPH ethanolic solution (0.1 mM). The mixture was kept in the dark, at room temperature for 30 min. The absorbance was measured at $\lambda = 515$ nm (Jasco V-530 spectrophotometer, Jasco, Japan) before ($A_{control}$) and 30 min. after adding the sample (A_{sample}). The absorbance was measured against a blank (A_{blank}) that contained 0.5 mL of each solution, except for DPPH reagent.

$$\text{DPPH free radical scavenger activity (\%)} = [A_{control} - (A_{sample} - A_{blank})]/A_{control} \times 100$$

$ABTS^{\bullet+}$ radical cation scavenging assay was determined according to Re R *et al*. [20] with slight modifications [7]. Briefly, the $ABTS^{\bullet+}$ free radical was generated by incubation of ABTS diammonium salt (7 mM) with potassium persulphate (2.45 mM) in the dark, at room temperature, for 16 h. The absorbance of the $ABTS^{\bullet+}$ solution was equilibrated to a value of 0.700 ± 0.02, at λ = 734 nm, after dilution with 96% ethanol. 0.5 mL of 0.025 - 2 mM solutions were mixed with 3 mL $ABTS^{\bullet+}$ solution and kept in the dark, at room temperature for 6 min. The absorbance was measured at 734 nm before ($A_{control}$) and after adding the samples (A_{sample}). The absorbance was measured against a blank (A_{blank}) that contained 0.5 mL of each solution, except for the free radical.

$$ABTS^{\bullet+} \text{ radical scavenger activity (\%)} = [A_{control} - (A_{sample} - A_{blank})]/A_{control} \times 100$$

Ferric reducing power assay was determined according to Oyaizu protocol [16]. Briefly, 2.5 mL of 0.025 - 0.5 mM solutions were mixed with 2.5 mL of 0.2 M phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide solution. Samples were kept in a water bath, at 50ºC, for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid solution was added and the mixture was centrifuged at 2500 rpm for 5 min (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL water and 0.5 mL of a 0.1% ferric chloride solution. The absorbance was measured at λ = 700 nm, after 10 min, against a blank that contained all reagents except for the analysed samples. The antioxidant activity was expressed as ascorbic acid equivalents (mM ascorbic acid *per* g substance) as previously described [7].

Statistical analysis. Antioxidant assays were carried out in triplicate. Results are presented as mean ± standard deviation (SD) and were statistically analysed using GraphPad Prism vers. 5 for Windows (Graph Pad, USA), using one-way ANOVA with Tukey post-test. The correlation between antioxidant assays was determined using Pearson coefficients. A value of $p < 0.05$ was considered the threshold for a statistically significant difference.

In silico studies: Computational strategy

Molecular modelling and minimum energy evaluation of thiourea derivatives. In this study, the 3D chemical structure of tested compounds was designed using molecular modelling tools from Discovery Studio software (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego, CA, USA: Dassault Systèmes, 2016). First, we have obtained the 2D chemical structure of the analysed compounds. Then, by using the Builder module, the spatial structure of each compound, with complete hydrogen atoms was obtained as a .mol2 file and also SMILES files. The electronic stability of thiourea derivatives was obtained by ruling the minimum potential energy of compounds. The minimum potential energy of compounds **C1**-**C7** was calculated using the mechanistic force field MMFF94x, at a gradient of 0.05. In the end, the Gasteiger electric charges for each molecule were downloaded.

Drug-like character and bioavailability of thiourea derivatives. With a view to evaluate the drug- and lead-likeness features, compounds **C1**-**C7** were subjected to Lipinski [14], Ghose [11], Veber [25] and Egan [9] filters using SwissADME website [28]. These filters imposed: (i) molecular weight no more than 500, log P no more than 5, hydrogen bond acceptors no more than 10, and hydrogen bond donors no more than 5 (Lipinski's filter); (ii) molecular weight between 160 and 480, log P between -0.4 and 5.6, molar refractivity between 40 and 130, number of atoms between 20 and 70 (Ghose filter); (iii) number of rotatable bonds no more than 10 and the total polar surface area no more than 140 (Veber filter) and (iv) log P no more than 5.88 and total polar surface area no more than 131.6 (Egan filter).

Predicted molecular features of thiourea derivatives. The molecular features of a certain compound (induced by its specific chemical structure) represent a crucial selection criterion during the evaluation of a drug profile. The molecular properties of compounds **C1**-**C7** were estimated using the descriptors computation facility of Discovery Studio. Due to the discrete structural differences among thiourea derivatives, we have determined the most important molecular features that may describe their therapeutic effects. Few of them were taken into account for subsequent analysis: (i) electronic features represented by orbital energies (e_HUMO, e_LUMO), dipole moment (dipole); (ii) steric features represented by accessible solvent surface area (ASA) and its derivative ASA-negative and (iii) charge distribution.

Computational ADME-Tox profile of thiourea derivatives. Predicted ADME (Absorption Distribution Metabolism Excretion) profile of tested compounds was performed using pkCSM database [29] and SwissADME website [28], based on their chemical SMILES files. By using SwissADME website the following parameters were quantified: (i) gastro- intestinal (GI) absorption; (ii) blood-brain barrier permeability (BBB); (iii) P-gp (P-glycoproyein 1) substrate and (iv) inhibitory activity upon CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 isoforms. By using pkCSM database, we estimated: (i) AMES toxicity; (ii) hepatotoxicity; (iii) hERG (potassium channels encoded by hERG genes) affinity; (iv) LD_{50} (median lethal dose); (v) skin sensitisation and (vi) the maximum tolerated dose (humans).

Results and Discussion

Antioxidant activity

Antioxidants methods are mainly based on single electron transfer (SET) or hydrogen atom transfer

(HAT). These mechanisms usually occur together, but the balance is determined by the antioxidant chemical structure and pH values [17]. A single method is not sufficient for the evaluation of a certain compound antioxidant activity, since antioxidants act by various mechanisms [1, 2, 17].

DPPH is a stable violet coloured free radical, with a high absorption value at λ = 518 nm [8]. As its odd electron becomes paired off in the presence of a free radical scavenger, the absorption diminishes and the observed degree of decolourization can be taken as a measure of the antioxidant activity [2, 8]. *DPPH assay* is mainly a single electron transfer based method [8], nevertheless some authors consider that it has a mixed mechanism [17]. For all tested compounds, the free radical inhibition (%) increased with concentration (Figure 1A). The inhibition varied among iodine thiourea derivatives from 13.12% (for **C1** – 0.025 mM) to 40.40% (for **C3** – 2 mM). Among dichloro derivatives, at the highest concentration (2 mM) compound **C4** had the best scavenger activity (38%) (Figure 1A). As shown in Table II, higher values for ascorbic acid equivalents were seen for **C1**, **C3** and **C4** compounds, similar to ferric reducing power assay.

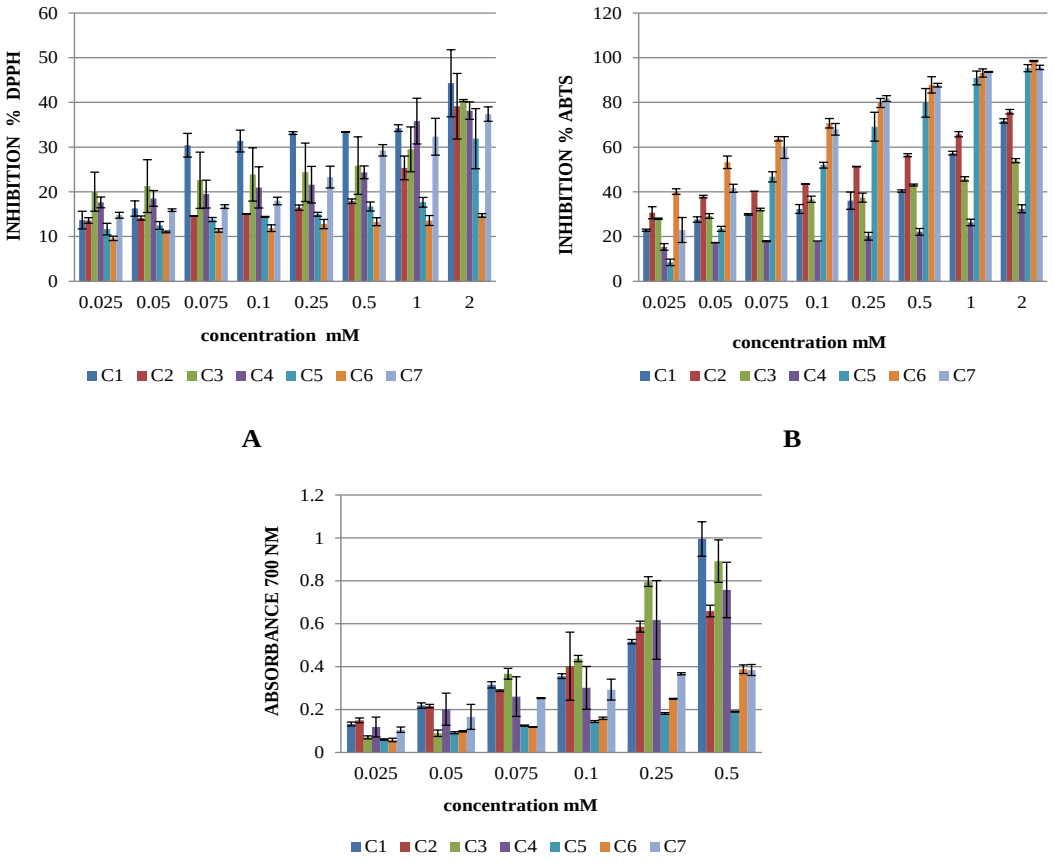


Figure 1.
Radical scavenging activity and ferric reducing power of the analysed compounds
(A – DPPH assay; B – $ABTS^{\bullet+}$ assay; C – Ferric reducing power assay)

Regarding the *$ABTS^{\bullet+}$ assay*, the bluish – green radical cation is reduced in the presence of antioxidants (both lipophilic and hydrophilic compounds) [17]. The method is based on both HAT and SET mechanisms [1, 2, 8, 17].

Among the analysed compounds, $ABTS^{\bullet+}$ scavenger activity varied between 9.94% (for **C5** – 0.025 mM) to 96.61% (for **C7** – 2 mM) (Figure 1B). According to our results, the dichloro-substituted derivatives (compounds **C5**, **C6** and **C7**) showed a higher antioxidant capacity compared to iodine derivatives (Table II). The highest antioxidant capacity was found for compound **C6**.

Ferric reducing power activity is a SET method, a colorimetric assay that measures the ability of antioxidants to reduce Fe^{+3} to ferrous (Fe^{+2}), thereby changing the absorbance [2, 8]. A higher absorbance indicates a stronger ferric reducing power capacity. According to our results (Figure 1C), for the iodine-substituted thiourea derivatives the absorbance values varied between 0.0699 (for **C3** – 0.025 mM) to 0.9947 (for **C1** – 0.5 mM), while for dichloro derivatives the absorbance values were 0.0574 (for **C6** – 0.025 mM) and 0.7570 (for **C4** – 0.5 mM). Among iodine derivatives, **C3** showed the highest antioxidant capacity, whereas among dichloro derivatives, **C4** had the best ferric

reducing power properties (Table II). Therefore, we assume that the structural differences between thiourea derivatives have a major impact upon their antioxidant potential [1, 2, 8]. Among thiourea derivatives, compound **C5** showed the weakest ferric reducing power effect, with absorbance values ranging between 0.05 (for 0.025 mM) to 0.1901 (for 0.5 mM) (Figure 1C).

Table II

Antioxidant activity of the analysed compounds

Compound	METHOD – mM ascorbic acid/g		
	DPPH	**ABTS$^{\bullet+}$**	**Ferric reducing power**
C1	1.0880 ± 0.9479^{a}	$3.4864 \pm 2.7751^{af***}$	$1.6200 \pm 0.2470^{ac*}$
C2	0.3538 ± 0.3117^{b}	$5.4634 \pm 2.6112^{bf***}$	$1.5466 \pm 0.4505^{bc*/be**}$
C3	$1.5545 \pm 0.6829^{cf*}$	$4.1647 \pm 3.8540^{cg***}$	$2.5085 \pm 0.1743^{cd*/ce***}$
C4	$1.5965 \pm 1.5927^{df*}$	$2.9954 \pm 2.9156^{dfg***}$	$1.7065 \pm 0.2982^{de**}$
C5	0.6019 ± 0.5187^{e}	$7.2755 \pm 2.9389^{ef***}$	nd
C6	0.2017 ± 0.1385^{f}	$16.5125 \pm 1.3805^{fc***}$	$0.4191 \pm 0.0682^{ea**}$
C7	0.4726 ± 0.3176^{g}	$10.8840 \pm 0.8533^{ga***/gb**}$	$0.9830 \pm 0.3430^{fc***}$

Results are mean ± SD (n = 3), nd – not determined. Two different letters for a compound (in the same column) means significant difference between antioxidant activity; $^{*}(p < 0.05)$; $^{**}(p < 0.001)$; $^{***}(p < 0.0001)$

We have found a positive correlation between the antioxidant assays (Table III). However, a negative correlation (although not significant) was observed between ABTS$^{\bullet+}$ and the ferric reducing power assays. This is probably the consequence of a different mechanism of action that is strongly influenced by thiourea derivatives chemical structure [1, 2].

Table III

Pearson correlation coefficients

	DPPH	**ABTS$^{\bullet+}$**	**Ferric reducing power**
DPPH	-	0.2857^{ns}	0.0375^{*}
ABTS$^{\bullet+}$	0.2857^{ns}	-	-0.1501^{ns}

ns – not significant ($p > 0.05$); * significant ($p < 0.05$)

In silico studies

Drug-likeness, lead-likeness and ADME-Tox features of thiourea derivatives

Our results generated by the medicinal chemistry filters (Lipinski, Ghose, Veber and Egan) (Table IV) and ADME-Tox analyses (Table V) revealed that all thiourea derivatives comply with drug-likeness rules, suggesting that these compounds may have therapeutic effects and an appropriate bioavailability (Table IV). Since medicinal chemistry filtering was respected, we had the opportunity to evaluate ADME-Tox features for all analysed thiourea derivatives.

Table IV

The bioavailability score and drug-likeness filtering of THE analysed thiourea derivatives

Compound	Lipinski	Ghose	Veber	Egan	Bioavailability score
C1	yes	yes	yes	yes	0.55
C2	yes	yes	yes	yes	0.55
C3	yes	yes	yes	yes	0.55
C4	yes	yes	yes	yes	0.55
C5	yes	yes	yes	yes	0.55
C6	yes	yes	yes	yes	0.55
C7	yes	yes	yes	yes	0.55

Table V

Predicted ADME features of the analysed thiourea derivatives

ADME	C1	C2	C3	C4	C5	C6	C7
GI absorption	high	high	high	high	high	high	high
BBB permeability	no	no	no	no	no	no	no
P-gp substrate	no	no	no	no	no	no	no
CYP1A2 inhibitor	yes	yes	yes	yes	yes	yes	yes
CYP2C19 inhibitor	yes	yes	yes	yes	yes	yes	yes
CYP2C9 inhibitor	yes	yes	yes	yes	yes	yes	yes
CYP3D6 inhibitor	no	no	no	no	no	no	no
CYP3A4 inhibitor	yes	yes	yes	yes	yes	yes	yes

GI – gastro-intestinal; BBB – blood-brain barrier; P-gp – P-glycoprotein1

According to our ADME analyses (Table V), all thiourea derivatives have an appropriate gastro-intestinal absorption. The high GI absorption together with a proper bioavailability score promotes thiourea derivatives as future candidates for oral administration. None of the analysed compounds had blood brain barrier permeability. Moreover, analysed compounds are not a substrate for P-glycoprotein 1. On addition, all thiourea derivatives inhibit CYP1A2, CYP3A4, CYP2C9 and CYP1A2 isoforms.

Interesting results were obtained regarding the toxicological profiles of the tested compounds (Table VI).

Table VI

The predicted toxicity profile of compounds **C1-C7**

Toxicity profile	Compound						
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
AMES toxicity	no	no	no	no	no	no	no
Max. tolerated dose (human) (log mg/kg/day)	0.22	0.429	0.41	0.32	0.61	0.61	0.48
hERG I/II inhibitors	no/no	no/no	no/no	no/yes	no/yes	no/no	no/no
Oral Rat Acute Toxicity(LD_{50}) (mol/kg)	2.198	2.328	2.26	2.38	2.51	2.53	2.35
Hepatotoxicity	no	no	no	no	no	no	no
Skin sensitisation	no	no	no	no	no	no	no

hERG I/II – potassium channels encoded by hERG genes; LD_{50} – median lethal dose

Our results have shown that analysed thiourea derivatives don't have a mutagenic effect; they don't induce skin sensitisation or hepatotoxicity (Table VI). Regarding cardiovascular toxicity, our results pointed out those dichloro-derivatives (mainly **C4** and **C5** compounds) are hERG I/II inhibitors (responsible for large QT syndrome or torsade de pointes arrhythmia) [27]. Regarding oral rat acute toxicity, our results marked out that all compounds have similar LD_{50} values (2.19 - 2.53 mol/kg).

Predicted molecular features of thiourea derivatives

In silico tool Discovery Studio was used for evaluation of electronic and steric molecular descriptors of analysed compounds (Table VII).

Table VII

The evaluation of electronic and steric molecular descriptors of compounds **C1-C7**

Compound	E_LUMO (eV)	E_HUMO (eV)	Dipole (D)	ASA (Å^2)	ASA-negative (Å^2)
C1	-0.87	-8.66	4.06	515.08	246.25
C2	-0.86	-8.68	4.45	522.14	250
C3	-1.12	-8.81	5.22	518.75	248.70
C4	-0.99	-8.74	5.20	526.62	273.40
C5	-0.96	-8.72	5.42	521.24	266.88
C6	-0.84	-8.62	6.35	514.65	258.20
C7	-0.95	-8.80	4.53	521.34	261.60

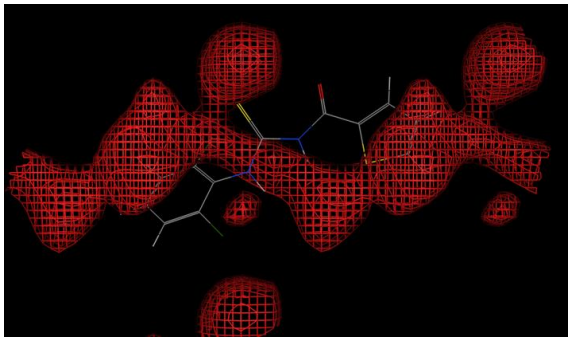


Figure 2.
Charge distribution on **C6** compound

We have noticed a significant variation of LUMO orbital energies, with values ranging from -1.12 eV (for **C3**) to -0.84 eV (for **C6**). Still, the HUMO orbital energies were similar among analysed compounds. Regarding the dipole moment, a higher value (6.35) was determined for compound **C6** (Table VII). A discrete variation was observed for the steric descriptors ASA, yet analysing the ASA negative forms a significant variation was observed (273.40 Å^2 for **C4** and only 246.25 Å^2 for **C1**). According to our results, dichloro derivatives have higher values for ASA-negative and dipole moment compared to iodine derivatives. Among dichloro derivatives, **C4** is more active than **C6** regarding orbital energy and charge distribution (Figure 2 and Figure 3).

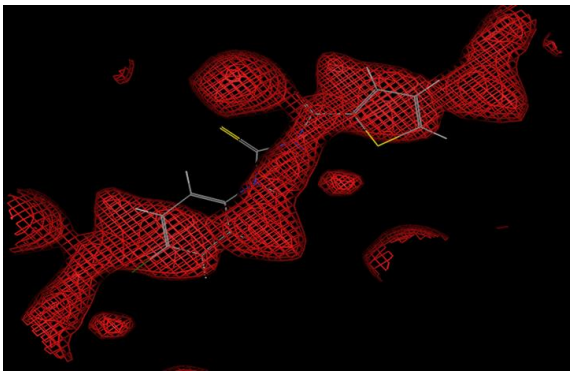


Figure 3.
Charge distribution on **C4** compound

Conclusions

All thiourea derivatives showed *in vitro* antioxidant capacity. Overall, iodine thiourea derivatives (compounds **C1**, **C2**, **C3**) act as DPPH free radical scavenger agents, whereas dichloro derivatives (compounds **C5**, **C6**, **C7**) showed better results regarding the $ABTS^{\bullet+}$ assay. In general all tested compounds had ferric reducing power properties. *In silico* studies have demonstrated the drug profile of each tested compound. On the whole, thiourea derivatives showed a safe pharmacological profile, except for dichloro derivatives (compounds **C4** and **C5**) that act as hERG I/II inhibitors. Future research is needed in order to establish the exact mechanism of action, regarding the antioxidant activity and the *in vivo* pharmacological profile.

Conflict of interest

The authors declare no conflict of interest.

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