RESEARCH ARTICLE

In-vitro Preliminary Evaluation of Antioxidant and Anticoagulant Activity of Novel N-Phenyl Hydrazine 1-Carbothioamide Derivatives of (2-Methyl-3-(Substituted Thio)Propanoyl) Proline

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Received: 10th June, 2022; Revised: 20th July, 2022; Accepted: 20th August, 2022; Available Online: 25th September, 2022

ABSTRACT

A series of (2-methyl-3-(substituted thio)propanoyl)proline (2-7) were evaluated *in-vitro* for antioxidant activity and anticoagulant activity. The antioxidant were determined using the most common models, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), H_2O_2, and reducing power method. The better radical scavenging antioxidant activity was observed with compound (4) with % inhibition (93.04 ± 0.7 µg\mL and IC50 69.56 µg\mL), using the DPPH method, while with H_2O_2 model, the observed % inhibition for compound (4) was (92.18 ± 1.52). For reducing power assay, the absorbance of the formed complex at 700 nm was (1.245 ± 0.213). The anticoagulant activity of the compound (3) shows significant prolongation in clotting time compare with (3-mercapto-2-methylpropanoyl) proline (captopril) with Prothrombin time (PT) and Activated partial thromboplastin time (aPTT) values (17.1 ± 0.1 s and 75.13 ± 0.15 s), respectively. The results of this study refer to an improvement of the biological activity of novel captopril derivatives with the introduction of electron donated group as (NH) and the presence of free thiol group.

Keywords: Anticoagulant, Antioxidant, Captopril, DPPH, H_2O_2.

International Journal of Drug Delivery Technology (2022); DOI: 10.25258/ijddt.12.3.38

How to cite this article: Najeh, AH, Mahmood, KAAR. *In-vitro* Preliminary Evaluation of Antioxidant and Anticoagulant Activity of Novel N-Phenyl Hydrazine 1-Carbothioamide Derivatives of (2-Methyl-3-(Substituted Thio)Propanoyl) Proline. International Journal of Drug Delivery Technology. 2022;12(3):1156-1161.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Captopril ((3-mercapto-2-methylpropanoyl)proline) was the earliest orally active angiotensin converted enzyme inhibitor (ACEI) and was marketed in 1975 to treat congestive heart failure and hypertension.[1] Captopril is a chiral medication that consists of two chiral centers, and four stereoisomers.[2] Only (S, S) stereoisomer is effective as angiotensin-converting enzyme (ACEI)[3] (Figure 1). Captopril raises plasma renin activity while decreasing the effective vasoconstrictor angiotensin II (Ag ll) production. This rise is most likely the result of a lack of feedback inhibition mediated by Ang II on renin releasing and/or activation of reflex mechanisms via baroreceptors.[4]

Captopril clinically proved to have anti-inflammatory,[5] antibacterial[6], antiplatelet,[7] antioxidant,[8] and anticancer activity.[9] In addition, Captopril has an active role in treating essential hypertension and several related cardiovascular diseases.[10]

Thrombosis is a pathological clot involved in various diseases, including deep venous "thrombosis," "myocardial infarction," and "stroke".[11,12] Haematosis is the preservation of blood fluidity and preventing its loss following vascular injury.[13]

Platelet and coagulation variables play an essential role in hemostatic and thrombotic mechanisms engaged in "thrombus" formation and limit hemorrhage by stimulating and stabilizing thrombin.[14,15] Utilizing anticoagulant and/or antiplatelet medications is one logical strategy to avoiding and treating cardiovascular disease linked with thrombosis.

The coagulation system comprises intrinsic and extrinsic routes, with thrombin serving as the usual final mediator, triggering the formation of the V, VIII, and IX factors. Furthermore, thrombin causes platelet stimulation and the cleavage of "fibrinogen" to "fibrin," in which it stays active once binding to "fibrin,"[16] Many techniques for thromboembolic incident prevention or therapy rely on inhibiting thrombin production or blocking its action.

Oxidative stress is an imbalance among Reacting oxygen species (ROS) that may be generated either by exogenous factors such as air pollutants, cigarette smoke and/or metabolic processes and intrinsic defense mechanism.[17] ROS are classified into two types: "free radicals" and "non-radicals"[18] Free radicals are molecules possessing one or more unpaired

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electrons as hydrogen peroxide H_2O_2, superoxide radical O_2•–, and hydroxyl radical.[19] At the same time, the non-radical form is generated when two radicals share their unpaired electrons.[20] Although ROS can contribute to signal transduction, hormone synthesis, cell defense, and gene expression under normal physiological conditions,[21] the damage of cellular molecules as protein, lipid, and DNA can result from excessive exposure to free radicals due to initiated chain of reactions. Therefore, ROS plays a crucial role in developing degenerative and chronic diseases like atherosclerosis, ischemic heart disease,[22] diabetes mellitus, cancer, arthritis, immunosuppressant, and neurodegenerative disease.[23]

Antioxidants are chemicals that could decrease or protect biomolecules at minimal concentrations against oxidative damage caused by free radicals.[24] Antioxidants could either directly or indirectly reduce oxidative damage. The direct damage is caused by free radicals interacting with proton donated potential such as the OH, SH, and NH groups.[25] Indirect damage is caused by hindering the activity or expression of free radical generating enzymes, such as xanthine oxidase inhibitors[26] or by boosting the activity or expression of intracellular antioxidant enzymes, such as boosting the activity of superoxide dismutase through the anticancer drug 5-fluorouracil.[27] As a result, identifying and discovering novel natural or synthetic substances with antioxidant activity to avoid or influence oxidative stress on cells is essential.

According to our previous work, a series of novel N- phenyl hydrazine 1-carbothioamide derived from 3-mercapto-2-methylpropanoyl)proline were synthesized.[28] Scheme 1 illustrates the synthesis of N- phenyl hydrazine 1-carbothioamide. Antioxidant and anticoagulant activities were evaluated using different antioxidant methodologies such as DPPH, scavenging of H_2O_2, and reduce power by $FeCl_3$. At the same time, anticoagulant activity was calculated via measuring "prothrombin time (PT)" and "activated partial thromboplastin time (aPTT)".

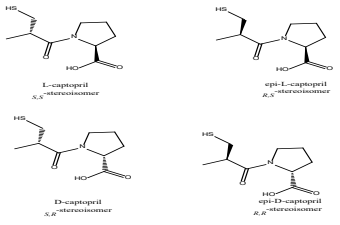
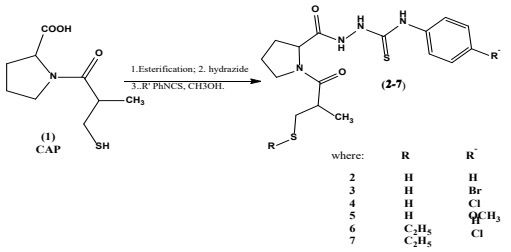


Figure 1: Captopril stereoisomers.



Scheme 1: Synthesis of N- phenyl hydrazine 1-carbothioamide derived from captopril (2-7)

EXPERIMENTAL

Materials

All reagents and anhydrous solvents used in this study were purchased from commercial vendors (Sigma-Aldrich / Munich, Germany, Fluka, Newport News/USA, and BDH, Pool Dorset/England). Sigma-Aldrich provided the L-Captopril (S, Sisomer) (Shanghai, China). UV absorption was evaluated in 10 mm quartz cells between 200 and 700 nm utilizing a UV-1100 spectrophotometer at college of pharmacy Basrah University.

Methods

In-vitro Antioxidant Activity

Free Radical Scavenging Activity

The free radical scavenging ability of produced compounds was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) utilizing the Blois technique with certain modifications.[29] A 0.1 mM DPPH solution in ethanol was produced, and 1-mL of this solution was mixed into 3 mL sample solutions with concentrations of 50 µg/mL to 250 µg/mL. The mixture was briskly stirred and left to stand in the dark for 30 minutes at room temperature. The absorbance was then measured at 517 nm with a spectrophotometer. The reaction mixture's lower absorbance showed more free radical scavenging ability. The sample's radical scavenging activity was represented as a percent of free radical inhibition and was determined utilizing the given equation:

Equation 1[30]:

$$\% \text{ inhibition} = \left(\frac{Ac - At}{Ac} \right) * 100$$

Where (A_c) is the absorbance of the control (solvent + DPPH without sample) and (A_t) is the absorbance of the test sample. All experiments were carried out in triplicate, and the findings were represented as an average, as shown in Table 1. IC_{50} values were calculated from the first-order linear equation.

Scavenging of Hydrogen Peroxide Assay

The potential of the generated derivatives (2–7) to scavenge hydrogen peroxide was evaluated using a modified technique reported by Keser *et al.*[31] In phosphate buffer, a 40 mM solution of hydrogen peroxide was produced (pH 7.4). Derivatives (2–7) were produced at concentrations ranging from 50 µg/mL to 250 µg/mL, and 0.6 mL of 40 M hydrogen peroxide solution was added. The absorbance of hydrogen peroxide at 230 nm was measured after 10 minutes using a spectrophotometer against a blank solution that having phosphate buffer but no "hydrogen peroxide". Equation 1 was used to compute the percent of hydrogen peroxide scavenging by the synthesized derivatives and the reference chemical.

Reducing Power Assay

The total reduction capability of synthesized compounds (2-7) was estimated using the method of Oyaizu, with some modification. A 50 µg/mL to 250 µg/mL of concentrations of derivatives were prepared in methanol. 1-mL of the solution

was added to 2.5 mL of 0.2M, pH 6.6 phosphate buffer, and 2.5 mL of "potassium ferricyanide ([K3Fe(CN)6], 1%)". The mixture was maintained at 50°C for 20 minutes. 2.5 mL of 10% trichloroacetic acid was introduced then the mixture was centrifuged at 3000 rpm for 10 minutes. The 2.5 mL of a supernet layer was mixed with an equal volume of distillated water and ferric chloride ("0.5 mL, 0.1%). The absorbance was characterized at 700 nm by a spectrophotometer. The higher absorbance of the reaction mixture revealed greater reducing power.[32]

In-vitro Anticoagulant Activity

Blood samples were collected from healthy adult volunteers who did not take medication for at least two weeks. The blood samples were stored in anticoagulant tubes. The plasma was prepared by centrifugation of collected blood for 5 minutes at 2500 g. Later, the PT and the aPPT tests were measured for the samples.

Prothrombin Time (PT) Test

The PT test was evaluated for action in the extrinsic pathway of coagulation. A mixture of 90 µL of plasma and 10 µL of derivatives (2-7) (50 µg/mL-250 µg/mL) was incubated for 2 min at 37°C. Heparin (1-U/mL) was utilized as a positive control, while plasma was used as a control (without anticoagulant activity).

PT 200 µL assay reagent was pre-incubated at 37°C and added to the samples, and recorded the clotting time by a coagulometer.

Activated Partial Thromboplastin Time (aPTT) Test

The aPTT measure was evaluated for the action in the intrinsic pathway of coagulation. A mixture of 90 µL of plasma and 10 µL of derivatives (2–7) (50 µg /mL-250 µg /mL) and 100 µL of aPTT reagent was incubated exactly for 3 minutes at 37°C. The $CaCI_2$ (100 µL, 0.025 M) was added to the samples, and the clotting time was recorded by coagulometer.

Plasma and Heparin (1-U/mL) were used as a negative and positive control, respectively.

Statistics

Statistical package for the social sciences (SPSS) was used to analyze the current study's experimental data (version 24). The one-way ANOVA test was performed to compare the antioxidant activities and anticoagulant properties of ascorbic acid, heparin, captopril, and innovative derivatives. *p*-values less than 0.05 were deemed meaningful.

RESULTS AND DISCUSSION

Antioxidant Activity

Free Radical Scavenging Activity

The antioxidant activity might not be conducted upon a single antioxidant measure model. *In-vitro*, there are several methods for testing that variable concerning evaluating the antioxidant activity of interesting compounds. The "free radical scavenging" is one of the best mechanisms by which antioxidant inhibits target protein oxidation. The DPPH model

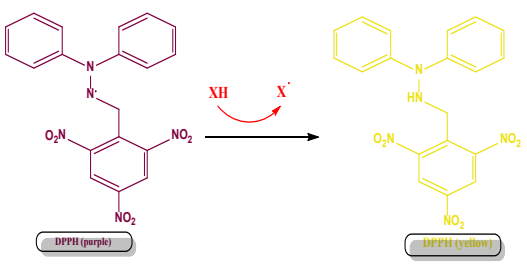


Figure 2: Mechanism of DPPH reduction by proton donated group.

Table 1: Antioxidant activity of synthesized derivatives as % inhibition and IC_{50} by DPPH.

Compound	*INH% a (250µg\mL)±SD*	*IC_{50} µg\mL*
2	90 ± 1.4*	89.36
3	91.034 ± 1.4*	88.98
4	93.04 ± 0.7*	69.56
5	84.88 ± 0.7*	130.28
6	81.49 ± 1.4*	97.93
7	89.02 ± 0.7*	103.84
Captopril	62.98 ± 0.7	147.56
Ascorbic acid	90.51724 ± 0.7	38.82

a:% inhibition was calculated as an average of 3 absorbencies for each concentration; SD: standard derivation. *: significant improvement in antioxidant activity of new derivative compare with captopril.

is simple (fewer reagents are needed) and more rapid than other methods.[33] DPPH is a stable free radical, characterized by deep visible purple color and absorbance at 517 nm attributed to the presence of odd electrons, the stability of this radical results from the virtue of the delocalization of the electron over the whole molecule so that the molecules don't dimerize.[34] The deep violet color changes into yellow upon reduction process either by proton or electron donated group; therefore, any compound with such characteristics can be considered an antioxidant (Figure 2). The % inhibition and IC_{50} values of the ethanolic solution of interested derivatives and ascorbic acid, which were considered a standard, were mentioned in Table 1 and Figure 3. It was found that compound (4) which contains electron withdrawal group (Cl) showed better antioxidant activity as compared with captopril with IC_{50} (69.56 µg/mL), while compound (5) displayed mild antioxidant activity with IC_{50} (130.28µg/mL), using the DPPH method

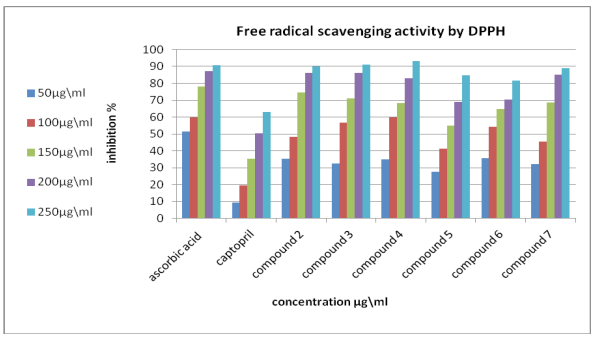


Figure 3: Free radical scavenger activity of captopril and its derivatives by DPPH.

Table 2: The antioxidant activity of synthesized derivatives by H_2O_2

Compound	*INH % a ± SD (250 µg\mL)*
2	86.26 ± 1.52*
3	90.58 ± 0.57*
4	92.18 ± 1.52*
5	87.03± 0.57*
6	81.98 ± 0.57*
7	90.12± 1*
Captopril	58.48 ± 1.52
Ascorbic acid	84.36 ± 1.15

(a % inhibition was calculated as an average of 3 absorbencies for each concentration, $p < 0.05$)

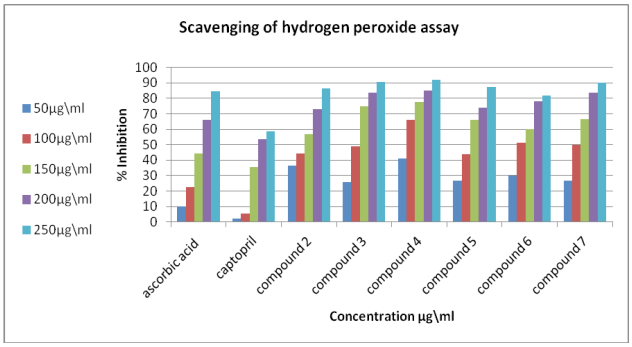


Figure 4: H_2O_2 scavenger results of captopril and its derivatives as % inhibition.

Scavenging of Hydrogen Peroxide Assay

The presence of an electron donated group can neutralize H_2O_2 into H_2O, Equation 2. Synthetic derivatives' capability to scavenge hydrogen peroxide is determined according to the method mentioned in,[35] where ascorbic acid is considered standard. The % inhibition was followed concentration-dependent manner, compound (4) was the best scavenging one, while compound (6) was the lowest,% inhibition were (92.18 ± 1.52 and 81.98 ± 0.57), respectively, as shown in Table 2 and Figure 4.

Equation 2.

$$H_2O_2 + 2H + 2e \longrightarrow 2H_2O$$

Reducing Power Assay

Where ascorbic acid is considered standard. The % inhibition was followed a concentration-dependent manner, compound (4) was the best scavenging one, while compound (6) was the lowest,% inhibition were (92.18 ± 1.52 and 81.98 ± 0.57), respectively, as shown in Table 2 and Figure 4.[36] As the measured absorbance is increased, the antioxidant activity of the tested compound increase. The presence of reductants is usually linked with the reducing characteristic. The antioxidant effect of reductants is dependent on the donation of a hydrogen atom, which breaks the free radical chain. Reductants can react with peroxide precursors, inhibiting the production of peroxide. The data illustrated in Table 3

Table 3: Absorbance of synthesized derivatives in ferric reducing power at 250 µg\mL

Compound	*Absorption ± SD*
2	0.933 ± 0.02*
3	1.14 ± 0.121*
4	1.245 ± 0.213*
5	0.926 ± 0.02*
6	0.87 ± 0.017*
7	0.973 ± 0.015*
Captopril	0.56 ± 0.01
Ascorbic acid	1.133 ± 0.057

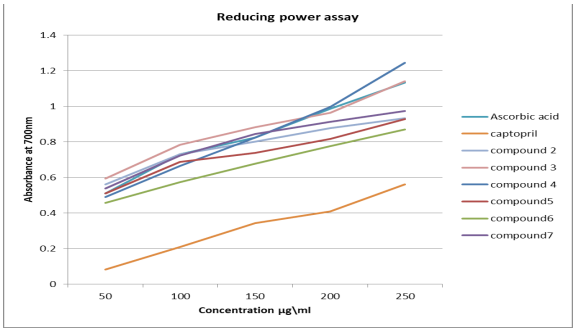


Figure 5: Reducing power activity of captopril and its derivatives as absorbance at 700 nm.

Table 4: Anticoagulant activity of synthesis derivatives (2-7) at 250 µg\mL

Compound	PT(s)	aPTT (s)
2	15.26 ± 0.05	57.16 ± 0.15
3	17.1±0.1	75.13 ± 0.15
4	17.2 ± 0.15	70.16± 0.15
5	16.5 ±0.05	67.1 ± 0.1
6	14.1 ±0.1	49.13 ± 0.15
7	16.05 ± 0.01	50.1 ± 0.2
Captopril	13.03±0.05	43.33 ± 0.416
Heparin	27	106

and Figure 5 indicated that synthetic compounds' antioxidant activity is markedly improved compared with captopril due to the presence of proton donated group in thiosemicarbazide moiety. Compounds 3 and 4 have higher antioxidant activity and absorption (1.14 ± 0.121 and 1.245 ± 0.213). This heightened activity is due to the presence of electron withdrawal group as a substitution at para- position of the aromatic ring and free thiol at a terminal aliphatic chain, when compare them with other derivatives without substation at para- position of an aromatic ring or that with ethyl protected thiol group (compound 6 and 7).The values are mean of triplicate determination ± SD

In- vitro Anticoagulant Activity

The anticoagulant effect of captopril and its derivatives were screened in PT and aPTT assays using different concentrations. The results show all the synthesized derivatives possess proper PT and aPTT values compared with captopril, as

shown in Table 4, where compound 4 has a higher PT value (17.2 ± 0.15). Compound 6 has a lower PT value (14.1 ±0.1). This improvement in biological activity is greater related to the presence of thiosemicarbazide (-NHNHCSNH-) moiety in synthesis compounds that provide suitable H-donated groups to form H-bond with a specific target protein. Besides, the present electron withdrawal group (Cl) in compound 4 showed a better effect on anticoagulant activity than the electron donated group (OCH_3) in compound 5. On the other hand, aPTT values of the synthesized derivates with a free terminal thiol group (SH), show better improvement in anticoagulant activity than that with the protected group (compound 6 and 7). Compound 3 had a higher aPTT value (75.13 ± 0.15), and compound 6 had a lower aPTT value (49.13 ± 0.15). This is also referred to the importance of electron withdrawal and proton donating groups in the anticoagulant activity.

CONCLUSION

In vitro, antioxidant activity studies showed that introducing an electron-rich group into N- phenyl hydrazine 1-carbothioamide derivative of captopril would improve its free radical scavenging activity. In addition, introducing an electron-rich group enhancing the anticoagulant activity that refers to the importance of thiosemicarbazide moiety in improving biological activity. Compound 4 can be utilized as a lead molecule in designing new drugs with antioxidant and anticoagulant activity to treat various cardiovascular diseases.

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