Antioxidant activity of novel selena-diazole derivative against oxidative stress induced by dipyrone in female rats

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Abstract
Introduction: Selena-diazole has antioxidant, and antitumor activities. Also selena-diazol exhibited promising antifungal, antibacterial, viral infection and neurodegenerative disease. The aim of the study is to evaluate the antioxidant activity of a novel 4,4’-(4,5,6,7-tetrahydro-[1,2,3]-selenadiazolo-[4,5-e] pyridine-4,6-diyl) bis(benzene-1,3-diol) (T) against dipyrone (Di) induced oxidative stress.

Methods: In vitro antioxidant using DPPH, concentrations of T and ascorbic acid (AA) at 10, 20, 30, 40 and 50µg was measured. In vivo study conducted using four groups, received 50mg/kg of T or/and Di and DW for 30 days. Antioxidant estimated in vivo by serum superoxide dismutase activity (SOD); Glutathione Peroxidase enzyme GPx measured by using Rat SOD1 kit and Rat GPX1 ELISA Kit respectively. Furthermore, Malondialdehyde (MDA) is reliable biomarkers to predict oxidative stress.

Results: The results indicate IC50 rate using DPPH of T compound 48.888µg/ml. GPx of T and T&Di groups were significantly increased. SOD of T was significantly increased than other groups. MDA results presented essential reduction in T group value than Di group.

Conclusion: The study concluded that synthesized novel selena-diazole derivative T has a good effect as an anti-oxidant.

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Keywords: Antioxidant; Selena-diazole; DPPH; MDA; GPx; SOD

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Introduction
Selena-diazole has antioxidant, and antitumor activities. Also selena-diazol exhibited promising antifungal, antibacterial, viral infection and neurodegenerative disease. In general, selenium containing compounds restores antioxidant enzyme activity like glutathione peroxidase (GPx). Selenium is an oligo nutritional constituent with important biological roles. It acts as a prosthetic group in thioredoxin reductase enzyme and glutathione...
peroxidase enzyme thus selenium considered as an effective free radicals scavenger (Ghorbel et al., 2017). Also, selenium has ability to oxidize thiol groups in both non-protein and protein as a consequence superoxide particle thus it can act as pro-oxidant. Selenium insufficiency is detected in numerous metabolic diseases, such as hyperlipidemia, hyper-glycemia and hyper-phenylalaninemia. Addition of selenium may recover hyper-cholesterolemia, type1 diabetes mellitus, atherosclerosis and phenylketonuria. Furthermore, selenium has detoxification activity to heavy metal intoxication, when selenium added to diet and its deficiency related with several diseases such as chronic degenerative disease (Ansar et al., 2017). Imbalance in homeostasis in pro-oxidant results in creation of highly reactive oxygen species (ROS)/reactive nitrogen species (RNS) which can be an essential mediator of destruction to cell components such as DNA and lipids. Oxidative impairment acts as a vital role in health. ROS/RNS creation in low to moderate concentration induce molecular signals that mange a series of physiological actions specially regulates ventilation, preserves vascular tone and maintain rodex homeostasis supporting (Kurutas, 2016). Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury and assists as a reliable biomarker of oxidation; therefore MDA is mutagenic and carcinogenic. Organic selenium has larger bioavailability, with lower toxicity in comparison with inorganic compound. Firstly unstable compounds were synthesized like selenols. Various studies concentrated on development of more stables and easily purified organo-selenium compounds. Novel compounds have potential therapeutic effects towards several diseases, for example: cancer, microbial infections and neurodegenerative diseases. Selena diazole

Fig.1. Schematic illustration of the synthesis path way for preparation of novel selena-diazole derivative (Neamah et al., 2020)
Antioxidant activity of selena-diazole derivative

**Materials and methods**

**Animal preparation**

Forty female rats obtained from College of Veterinary Medicine/Basrah University. All procedures of this study were approved by Veterinary Medicine College/University of Basrah (Approved No:7/18/2420 At Nov 2nd 2019), the animals kept in cages from polypropylene lined with sawdust (3-4 rats/cage) and separated as groups. Rats were providing usual pellet diet and tap water. First, they were adapted to laboratory circumstance, natural day and night (12h day and 12h night). Room temperature was 21±4°C (Kumar et al., 2017). Body weight of all rats was measured. Each animal group consist 10 female rats, the rats were orally received through oral gavage 50mg/kg of body weight (BW) of T or/and Di, dissolved in 2ml distilled water (DW) for 30 days. Control group received only 2ml of DW. The rats after 30 days were sacrificed using chloroform anesthetic drug, the blood pull directly from the heart, blood sample kept in serum-separating tubes on room temperature for 20-30 minutes. Serum collected using centrifuges for 10min at 4000 RPM.

**Preparation of DPPH**

DPPH solution was prepared by adding 7.89mg of DPPH to 100ml of DMSO, the resultant 0.2mM DPPH solution was kept for 2h in a dark by casing the test tubes and flask with aluminum foil, at room temperature (23-25°C). The next step is adding 1ml of prepared DPPH to 3ml of DMSO, the absorbance was measured using 517nm, and recorded as control data (Mostafa et al., 2017).

**Preparation of tested sample (T) and ascorbic acid (AA) concentrations**

Five milligram of T and AA were weighted 3 times and then each dissolved in 10ml of DMSO. From this solution different concentrations of T and AA were prepared. First 1mg of each T and AA dissolved in 2ml of DMSO, each ml contain 500µg of T or AA. Only 400µl (50 µg/100µl) were added to 1600µl of DMSO, the resultant solution contain 200µg/2ml. T and AA concentrations at 10, 20, 30, 40 and 50µg was prepared by sequent dilution as mention in Table 1 (Sherikar and Mahanthesh, 2015).
Measurement assay

The spectrophotometric technique for measuring the antioxidant ability of T and AA are based on the reduction of the absorbance of the DPPH radical. From each concentration of T and AA, 3ml was added into separated test tube. One ml of 0.2mM DPPH was added to each T and AA test tube. The mixture was shaking and stands for 10min at room temperature. Then the absorbance measured via spectrophotometer at 517nm. AA first measured as reference standard material, inhibition of absorbance or DPPH scavenging activity was calculated by the equation:

\[ \text{Percent inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]

Where A0 refer to the absorbance of control reaction and A1 the absorbance in presence of (T), or standard sample AA. The IC50 rate of the test sample was measured from concentration and inhibiting percentage curve. The IC50 of each diagnostic sample was measured according to the following formula:

\[ \text{IC50 for T: } y = 13.161x + 14.635; \text{ IC50 for AA: } y = 5.8935x + 54.502 \]

Y are inhibition ratios, plotted against concentrations of the sample X, the regression line for each (T) and AA (Y=aX+b) is drawn. Basically all point that measured is on the regression line and at least 2 points around 50% do not have deviation from the line (Shimamura et al., 2014). The lower IC50 value indicates the greater overall effectiveness of the antioxidant. The IC50 of the compound was measured by spectrophotometric method (Hangun-Balkir and McKenney, 2012).

Serum MDA levels assay

Determination of MDA in different samples such as plasma, serum or tissue homogenates is one of the reliable biomarker to predict the oxidative stress levels (Singh et al., 2014). In the present study MDA measurement is carried out as reported by Yagi method (Yagi, 1998). The reaction achieved in acidic situations and at 95°C. The resultant substance was TBARS “thiobarbituric acid reactive substances”. TBARS can be measured by either fluorimetry (excitation at 532nm and emission at 553nm) or colorimetry (532-535nm), (Atiba et al., 2016). The results estimated by the following equation:

\[ \text{MDA (µmol/l) = } (A1-A0 / 1.56) \times 10 \]

Where A1 is test sample absorbance and A0 is control absorbance (Antolovich et al., 2002).

SOD activity

The serum SOD is measured by Rat SOD1 kit (Superoxide Dismutase 1, Soluble) from Elabascience Biotechnology Inc. China. Detection rate 0.16—10 ng/ml. Serum, plasma and other biological fluids can be used as a sample for SOD measurement.

Measurements of GP

Serum GPx enzyme (pg/ml) measured by Rat GPX1 (Glutathione Peroxidase 1) ELISA Kit, Elabascience Biotechnology Inc. China. Serum, plasma and other biological fluids can be used for GPx measurement.

Results

DPPH radical scavenging assay (antioxidant action)

The results of significant antiradical activity were showed in Table 2. Both of tested samples (T and AA), in different concentration were evaluated for antioxidant effects. High scavenging effects observed up to (80.027 µg/ml ±7.30) for (T) compound and (82.74 µg/ml ±4.19) for standard antiradical AA. At low dose 10µg/ml there were significant differences between (T) 28.4362±7.28132 and AA 57.1962 µg/ml ±10.18565. The antioxidant effects increased with increased concentration. The regression line for each (T) and AA (Y = aX + b), and IC50 was calculated. Median antiradical inhibition rate IC50 of AA is 40.75 and 48.88 of T compound (Fig 2).

Serum SOD activity

Serum SOD concentrations numerically elevated in all treated groups. When compared with DW group, the mean SOD of T group (60.01pg/ml ±9.08), was significantly greater than in DW group level (37.15pg/ml ±2.18) and T&Di group. While in Di and T&Di groups, SOD values were (39.97±3.28) and (43.58±1.99) respectively, the concentration increments were statistically non-significant. The results are shown in Table3.

Serum MDA levels assay

Serum MDA of rats that received Di quantity was
Antioxidant activity of selena-diazole derivative

Physiol Pharmacol 24 (2020) 202-210  | 206

DPPH antiradical measurents  \( IC_{50} \)  \( N = 10 \)

\[
y = 5.8935x + 54.502 \\
AA IC_{50}=40.7518
\]

\[
y = 13.161x + 14.635 \\
(T) IC_{50}=48.888
\]

\( y = 13.161x + 14.635 \)  
\( (T) IC_{50}=48.888 \)

% of antiradicle

-----AA

-----T

concentration of T & AA

Fig. 2. From the histogram median concentration inhibition (\( IC_{50} \)) calculated for both test selendiazole derivative (T) and ascorbic acid (AA). \( N \)=times of examinations of each AA and each of T concentrations. The differences between T groups, \( P \leq 0.05 \) vs. AA at concentrations of 10, 20, 30 and 40 \( \mu \text{g/ml} \).

Table 1: preparations of different concentrations of Test selendiazole derivative (T) and ascorbic acid (AA)

| Concentration of T, and AA(1) | 500 \( \mu \text{g/ml} \) of T, and AA(1) |
|-----------------------------|----------------------------------|
| 400\( \mu \text{l} \) added to 1600 \( \mu \text{l} \) DMSO | T, AA conc. are 100\( \mu \text{g/ml}(2) \) |
| 500 \( \mu \text{l} \) added to 500 \( \mu \text{l} \) | T, AA conc. are 50\( \mu \text{g/ml} \) |
| 400 \( \mu \text{l} \) added to 600 \( \mu \text{l} \) | T, AA conc. are 40\( \mu \text{g/ml} \) |
| 300 \( \mu \text{l} \) added to 600 \( \mu \text{l} \) | T, AA conc. are 30\( \mu \text{g/ml} \) |
| 200 \( \mu \text{l} \) added to 800 \( \mu \text{l} \) | T, AA conc. are 20\( \mu \text{g/ml} \) |
| 100 \( \mu \text{l} \) added to 900 \( \mu \text{l} \) | T, AA conc. are 10\( \mu \text{g/ml} \) |

Table 2: Antiradical effects of (T) compound enhanced with increase its concentration compare with standard antiradical AA.

| Treatment groups | Mean of Concentrations (\( \mu \text{g/ml} \)) \( \pm \) SEM |
|-----------------|----------------------------------|
|                 | 10\( \mu \text{g/ml} \) | 20\( \mu \text{g/ml} \) | 30\( \mu \text{g/ml} \) | 40\( \mu \text{g/ml} \) | 50\( \mu \text{g/ml} \) |
| (T) Compound    | 28.43\( \pm \) | 38.63\( \pm \) | 56.44\( \pm \) | 67.05\( \pm \) | 80.03\( \pm \) |
| Ascorbic acid (AA) | 7.8\( ^\dagger \) | 14.63\( ^\dagger \) | 6.4\( ^\dagger \) | 7.8\( ^\dagger \) | 7.31 |
| Ascorbic acid (AA) | 57.20\( \pm \) | 69.94\( \pm \) | 73.26\( \pm \) | 77.77\( \pm \) | 82.75\( \pm \) |
| Ascorbic acid (AA) | 10.19 | 10.16 |

*Significant differences at \( P<0.05 \). Results are expressed as mean\( \pm \) SEM of the mean of the ten replicates.

statistically non-significant elevated to (3.35 \( \mu \text{mol/l} \pm 1.87 \)) than in DW group MDA (1.96 \( \mu \text{mol/l} \pm 1.5 \)). Also in rats received T&Di group, the concentration reduced to 1.69\( \mu \text{mol/l} \pm 0.41 \), but not statically significant. Only T group (0.61\( \mu \text{mol/l} \pm 0.40 \)) showed significant reduction than Di group, the results are shown in Table 3.

GPx levels
Administration of T, Di and T&Di compounds to female rats for 30days, then serum GPx was measured, after rats were sacrificed. It can be seen from the data in Table 3 that the results indicate significant enhancement in serum GPx concentration T group (4532.67\( \mu \text{g/ml} \pm 444.94 \)) and T&Di (3599.67\( \mu \text{mol/l} \pm 1058.63 \)) GPx level compare to DW group.
Discussion
Numerous studies have been revealed that ROS generated the epithelial cells damage, to reduce or prevent cellular damage. There are two endogenously antioxidant systems; the first system is preventive the antioxidant system and the second is cleaning antioxidant enzyme system, e.g. vitamin E, C and GPx that eliminate produced ROS, so plasma membrane lipid. GPx (selenium-dependent glutathione peroxidase) is one of the chief antioxidative enzyme in the cells and it has been shown that Se is a structural component of GPx (Hasanvand et al., 2017). Antioxidant activity is not determined based on a single test model. Several in vitro and in vivo test techniques carried out for assessing antioxidant activities of the studied samples. Another characteristic is that models of antioxidant test differ in different respects. Consequently, it is difficult to compare completely between any methods with the others (Alam et al., 2013). As, it is difficult to compare among different methods, therefore the present study attempts to involve a wide diversity of antiradical procedures including in vitro DPPH scavenging test and in vivo GPx, SOD and MDA tests.

DPPH radical scavenging assay
DPPH in vitro technique considered to be the simplest methods in comparison with other antioxidant in vitro technique characterized with fewer steps and reagents and practically low-cost, requires only a UV-V is spectrophotometer; therefore frequently used for in vitro antioxidant evaluation (Karadag et al., 2009). The reduction ability of DPPH radical is measured by the decline in its absorbance at 517nm induced by antioxidants. The reduction in its absorbance caused by antiradical, due to the progresses of antioxidant molecules and radicle reaction; results in the radicle scavenging by hydrogen donation (Shekhar and Anju, 2014). This study indicates that synthesized (T) has a DPPH radicals scavenging in "dose dependent manner" due to its antioxidant capacity, compared with ascorbic acid (S.D.Sanja et al., 2009). The present findings by (Ruberte et al., 2018), seem to be consistent with other research which found selena-diazole derivatives" twenty-seven novel benzo [c][1,2,5]seleno-diazole-5-carboxylic acid (BSCA) derivatives" have potent in vitro radical scavenging activity using DPPH antioxidant method. This series of compounds can serve as an excellent scaffold to achieve novel and potent antioxidant complexes beneficial for several diseases. Several studies have confirmed that a lot of novels selena-diazole derivatives have DPPH radicle scavenging effects (Zhang et al., 2010).

Glutathione peroxidase
GPx and selenoproteins, which are generated by selenium consumption, have been involved to enhance the ability of antioxidant activity in the animal tissues and cells, which breakdown H2O2 to water. In mitochondria, GPx converts lipid peroxidation to conforming alcohols. GPx enzyme has a vital inhibitory role of lipid peroxidation and protects cells from oxidative stress (Ighodaro and

| Table 3: illustrate the antioxidant activity of the experimental groups. |
|-----------------------------|-------------------------|-------------------------|-------------------------|
| Groups                      | Parameters (mean±SEM)   |                         |                         |
| Distilled water             | GPx (pg/ml) N=10         | SOD (ng/ml) N=10         | MDA (µmol/l) N=10       |
|                            | 1761.47±1055.02 A       | 37.15±2.18 A            | 1.96±1.57 A             |
| Dipyrone                    | 1526.01±627.18 A        | 39.97±3.28 A            | 3.35±1.87 A             |
| Test                        | 4532.66±444.94 B        | 60.01±9.08 B            | 0.61±0.40 AB            |
| Test and Dipyrone           | 3599.66±1058.63 B       | 43.58±1.99 AC           | 1.69±0.41 A             |
| LSD (Lesser Significant Differences) | 1838.19 | 16.43       | 2.74       |

Means bearing different capital letters vertically differs significantly at 0.05 level (P<0.050). N refers to number of rats.
Antioxidant activity of selena-diazole derivative

Physiol Pharmacol 24 (2020) 202-210 | 208

Akinloye, 2019), also reduced ROS and RNS and protects lipids of DNA and protein. Furthermore, anti-inflammatory properties of selenium have involved in cyclooxygenase and lipooxygenase sequences (Karaveloglou et al., 2015). GPx enzyme low activity is one of the early consequences of a disorder of the prooxidant/antioxidant equilibrium (Alam et al., 2013). In recent years, a number of studies focused on antioxidant effects of selenium compounds. It was documented that a decrease in oxidation with higher activity of GPx by addition of selenium into the diets (Amirkolaie et al., 2014). Also, the antiradical activity of selenoneine was measured, the results that reported similar to those obtained by authors in the present study (Yamashita et al., 2010). The result of present study is also supported by a study which revealed that important elevation in both GPx actions after administration of many organic-selenium compounds (Stefanello et al., 2015). Many authors recently found comparable results of antioxidant action of selenium (Ansar et al., 2017); (Hasanvand et al., 2017). The Di anti-oxidant effects in the current study was consistent with other study showed that Di decreases endogenous glutathione levels and inhibits GPx action in dose dependent manner (Sanchez et al., 2002); however, the results were contradicted by the experiments of peroxyl Radical ROO, that dipyrone has antioxidant activity (Costa et al., 2006).

Superoxide dismutase

SOD is the initial detoxification enzyme and more potent antioxidant enzyme that acts as a primary defense system in the cell. It activates the transformation of 2 molecules of superoxide anion (O−2) first to H2O2 and then molecular oxygen (O2), changing the possibly destructive superoxide anion less (Ighodaro and Akinloye, 2019).

The results show that SOD of T group exert significant elevation with SOD level of DW group, as well as with T&Di group. These results might be due to hepato-protective special properties were evaluated by the capability to prevent lipid peroxidation, protein oxidation and to modify in the SOD (Schaffer et al., 2016). The results of current research support the idea that selenium containing compounds are more potent SOD inducer. Data from several sources have identified the increased SOD activity. The GPX and SOD plasma levels increased significantly in Se-supplemented-diet to diabetic rats for one month (Ghaffari et al., 2011). Another study demonstrates that diphenyl diselendite (PhSe) active in contradiction of increased oxidative stress in kidney and liver (Sartori et al., 2017). Pre-treatment with diphenylmethyl selenocyanate found to be the most powerful SOD and CAT inducer (Jayanta Kumar Das et al., 2013). Also, it was reported that 1, 4-Anhydro-4-seleno-d-talitol (SeTal) has powerful antioxidant action in vitro and it's likely to stimulate wound healing in diabetic mice due to elevate SOD production and reduced NO formation (Ng et al., 2017). Similar results revealed that Se effectively elevated significantly the activities of SOD, GPx and CAT compared to the Ag nano-particles treated group (Ansar et al., 2017).

Serum MDA levels assay

Once lipid peroxides are unstable compounds, they tend to degrade rapidly in a variety of sub products. MDA is one of the most known secondary products of lipid peroxidation and it can be used as a marker of cell membrane injury (Grotto et al., 2009). Enhance level of MDA is considered as an indicator of change of cell function and tissue damage. This event appears in the liver or kidneys due to ROS formation or free radicals. It is common that natural antiradicals can diminish intracellular oxidative stress by prevent free radicals generation (Rjibi et al., 2017).

In the present study MDA concentrations of all testing groups are measured and the results indicate the only significant difference was between T and Di groups. The result of the present study agrees comparatively with a study of the effects of selenium on oxidative stress of cadmium in bone tissue in rats, the results reported decreased in MDA when Se added to rats previously exposed to cadmium compare to control group (Ibrahim et al., 2014). The results also supported a study showed that Se decreased high-sensitivity C-reactive protein in serum and elevated GSH level, resulting in reduction of plasma MDA (Wang et al., 2017) which reveals that the marker of lipid peroxidation MDA had a negative association with serum level of selenium in both normal, and hyper cholesterolemic (specifically negative associated with low-density lipoprotein) patients. Furthermore (Hasanvand et al., 2017) showed that administration of Se remarkably decreased MDA. Recent study (Amraoui et al., 2018) has reported that rats treated concomitantly with
biphenol A (BPA) and (Se + Vit E) displayed a significant decrease in MDA concentration as compared to BPA-treated group. Selenium could reduce the elevated MDA level by about 5-fold compared to the diabetic rats (Ghaffari et al., 2011). A study by (Orhan and Sahin, 2001) observed that Di at the high concentration exerted an inhibition which is statistically significant on H2O2 forced erythrocyte membrane lipid peroxidation. The results agree with our data and explain the elevation in MDA. In contrast, (Costa et al., 2006) concluded that none of pyrazolones that studied including Di was able of scavenging O2− or H2O2. Pervious study by Batu and Erol (2007) found that elevated lipid peroxidation after Di administration, which confirms the results of current study. The study concluded that synthesized novel selena-diazole derivative T has a good effect as an anti-oxidant.

Conclusion
which reveals that the marker of lipid peroxidation MDA had a negative association with serum level of selenium in both normal, and hyper cholesterolemic (specifically negatively associated with low-density lipoprotein) patients.

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Conflict of interest
The authors declare that there is no conflict of interest.

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Antioxidant activity of selena-diazole derivative

Physiol Pharmacol 24 (2020) 202-210 | 210

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