In Vitro Antioxidant Evaluation of Some Mannich Bases which Contain Bis-1,2,4 -Triazole Derivative

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Summary: This study aims to examine the antioxidant effects of some Mannich bases containing bis-1, 2, 4 triazole, which are synthesized afresh. The antioxidant activities of the derivatives were measured using different methods in this study, including reducing power capacity, metal chelating activity, superoxide anion radicals scavenging activity, H_2O_2 scavenging activity and hydroxyl radical scavenging. As a result, derivatives had efficient antioxidant and free radical scavenging activity when compared to ascorbic acid, BHT and α-tocopherol as associated antioxidants.

Keywords: 1,2,4 – Triazole; Mannich bases; Antioxidant; Radical scavenging; Biological activities

Introduction

Mannich bases have strong biological activities such as antibacterial, antifungal, anti-inflammatory, antimalarial and pesticide features [1].

It was proclaimed that 1,2,4-triazole and its derivatives show numerous pharmacological activities. The 1, 2, 4-triazole is a heterocyclic compound with five members and contains two carbons and three nitrogen with a molecular formula of $C_2H_3N_3$. It has two tautomeric forms. 1*H* and 4*H*-1, 2, 4-triazole is regarded as a pharmacologically crucial nucleus. The literature review demonstrates that 1, 2, 4-triazole have a wide range of biological activities. Compounds with 1, 2, 4-triazole nucleus are especially renowned with their distinguished antibacterial, antifungal, anti-tubercular, antioxidant, anticancer, anti-inflammatory, analgesic, anticonvulsant, anxiolytic activities [2-4].

As a result of their beneficial biological and pharmacological features, heterocyclic compounds and their equivalents and derivatives have drawn a great deal of attention recently.

It was aimed to examine the antioxidant effects of some Mannich bases containing bis-1, 2, 4 triazole, which are synthesized afresh, on certain antioxidant processes such as reducing power capacity, metal chelating activity, superoxide anion radicals scavenging activity, H_2O_2 scavenging activity and hydroxyl radical scavenging.

Experimental

Materials and Methods

All of the solvents were made up of analytical-grade reagents. Mannich bases containing bis-1,2,4 - triazole derivative, which were used in the applications, were synthesized afresh, and they were described as proclaimed before [5]. Derivatives structure is shown below (Sch. 1).

In Vitro Antioxidant Methods

Reducing Power

The method of Oyaizu was used in order to establish the reducing power of the synthesized compounds [6]. Different sample concentrations (50-100-250 μg/mL) in DMSO (1 mL) were blended with phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for duration of 20 minutes, then a portion (2.5 mL) of trichloroacetic acid (10% w/v) was added to the mixture, and the mixture was centrifuged for 10 min at 2000 rpm. The upper layer of solution (2.5 mL) was blended with distilled water (2.5 mL) and $FeCI_3$ (0.5 mL, 0.1% w/v), after which the absorbance at 700 nm was measured using a spectrophometer. As standard antioxidant compounds, α-tocopherol, ascorbic acid and Butylated hydroxytoluene (BHT) were used. Higher absorbance of the reaction mixture meant that there was greater reducing power.

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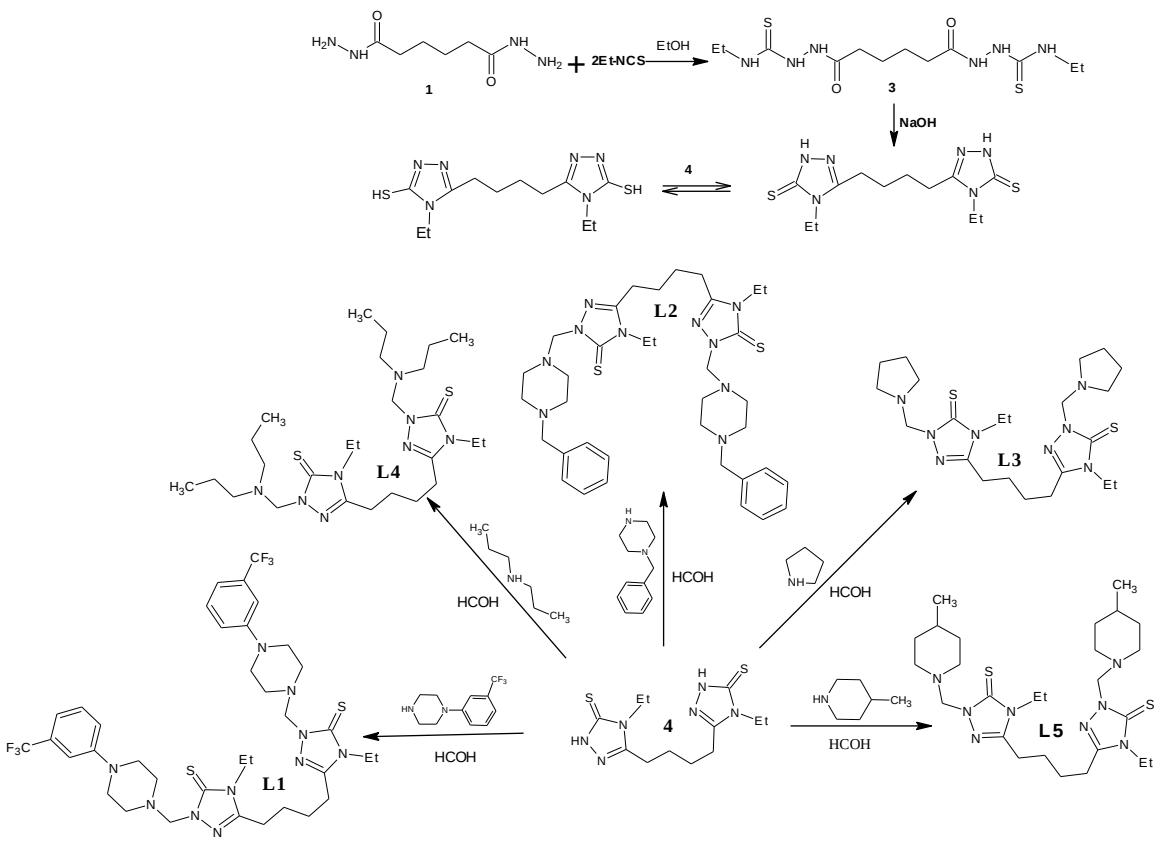

Scheme-1: Chemical structure of derivatives.

Metal Chelating Activity

The method of Dinis *et al*. was used in order predicts the chelation of ferrous ions by the synthesized compounds and standards [7]. Shorty, the synthesized compounds (50-100-250 µg/mL) were added to a 2 mM solution of $FeCI_2$ (0.05 mL). The reaction was started by adding 5 mM of ferrozine (0.2 mL) and the mixture was shaken robustly, and then left to mature at room temperature for duration of 10 minutes.

The absorbance of the solution was measured at 562 nm using a spectrophotometer after the mixture reached stability. All of the test and analyses were performed in triplicate and then their average was calculated. Decreased absorbance of the reaction mixture meant that the metal chelating activity increased. The following formula gave the percentage of inhibition of ferrozine-Fe^{2+} complex formation: % Inhibition = $(A_0 – A_1/A_0) \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the samples or standards. There was no compound or standard in the control. α-Tocopherol and Butylated hydroxytoluene (BHT) were chosen as standard antioxidant compounds.

Hydrogen Peroxide Scavenging Activity

The procedure of Ruch et al. was followed while the hydrogen peroxide scavenging assay was carried out [8]. This method is based on the principle that there is a decrease in the absorbance of H_2O_2 when H_2O_2 is oxidized. The solution of 43 mM H_2O_2 was prepared in 0.1 M phosphate buffer (pH 7.4). Different compound concentrations (50-100-250 µg/mL) in 3.4 mL phosphate buffer were added to 0.6 mL H_2O_2 solution (43 mM), and then the absorbance of the reaction mixture was recorded at 230 nm. The sodium phosphate buffer without H_2O_2 was included in a blank solution. All of the test and analyses were performed in triplicate and then their average was calculated. Decreased absorbance of the reaction mixture meant that the Hydrogen peroxide scavenging activity increased. The following formula was used in order to calculate the percentage of H_2O_2 scavenging by compounds and standards = (A_0 –

A_1/A_0) x 100, where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the samples or standards. As standard antioxidant compounds, ascorbic acid and butylated hydroxytoluene (BHT) were used.

Superoxide Radical Scavenging Activity

The method of Liu et al. was used with slight changes in order to create the superoxide radicals [9]. Approximately 1 mL of nitro bluetetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of solution of compounds at different concentrations (50-100-20 µg/mL) DMSO were blended. 100 µL of phenazinemethosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) was added to the mixture in order to initialize the reaction. The reaction mixture was incubated at 25 ^{0}C for duration of 5 minutes. Then the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture meant that superoxide anion scavenging activity increased. All of the data were obtained by calculating the average of the triplicate analyses. The following formula was used in order to calculate the percentage of the inhibition of superoxide anion scavenging formation: % Inhibition = ($A_0 - A_1/A_0$) x 100, where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the samples or standards. As standard antioxidant compounds, ascorbic acid and butylated hydroxytoluene (BHT) were used.

Hydroxyl Radical (˙OH) Scavenging Activity

2-deoxy-D-ribose oxidative degradation through hydroxyl radicals as described by Halliwell et al. [10] was used with slight changes in order to establish the compounds' hydroxyl radical-scavenging ability. The reaction mixture, which included various compound dilutions (50-100-20 µg/mL) in DMSO (1 mL), 150 µL of 2.8 mM 2-deoxy-D-ribose (200mM 5ml), 60 µL of $FeSO_4.7H_2O$ (20mM 50ml), 30 µL of EDTA (10mM 50ml), 150 µL of H_2O_2(1 M 50ml) and 60 µL of ascorbic acid (500mM 5ml) was vortexed. Then, 2.5 ml KH_2PO_4 buffer (pH: 7.4) was added and the mixture was incubated at 37^{0}C for a duration of 60 minutes. Thenceforth, 1 mL of %2 thiobarbituric acid in 10% trichloroacetic acid was added. Then the mixtures were vortexes and heated in a water bath at 100^{0}C for 15 minutes. The reaction was halted by a 5 min. ice water bath. The mixtures were then centrifuged at room temperature at 12,000x*g* for 5 min. The absorbance resilient was measured at 532 nm. The following formula was used in order to calculate the scavenging effect of hydroxyl radicals (%): Hydroxyl radical scavenging activity (%) = ($A_0 - A_1/A_0$) x 100, where A_0 the control reaction (containing all reagents except the test compound) at 536 nm and A_1 is the absorbance in the presence of the samples or standards. As standard antioxidant compounds, Ascorbic acid and butylated hydroxytoluene (BHT) were used.

Radical scavenging activities were calculated with inhibition percentage equation. Data obtained was then analyzed using linier regretion equation to determine IC_{50}. Percent of inhibitions were plotted against concentrations, and the equation was the line used to obtain IC_{50} value. The IC_{50} value denotes the concentration of a sample that is required to scavenge 50% of free radicals. A lower IC_{50} value indicates greater antioxidant activity.

Results and Discussion

Mannich reactions that lead to the synthesis of organic molecules in a single step containing different functional groups in a single molecular structure have become one of the commonly used methods in the study of drug designs. Mannich bases, with high water solubility, are preferred chemical structures in preparing pro drugs [11, 12].

Pharmaceutical chemistry is the most important usage area of Mannich base, and %35 of the publications in this area are about Mannich base. In Pharmaceutical Chemistry; it is used in analgesics, antibiotics and anti-cancer drugs [13].

Triazoles and thiadiazole constitute a practical subject with chemical activities suitable to be placed on the structure of the various substituents, especially their tautomeric properties [14, 15]. In the studies done with triazole derivatives in literature, effective compounds of antimicrobial [16], antiviral [17], cytotoxic [18], anti-inflammatory [19], analgesic [20], anticonvulsant [21], antidepressant [22], diuretic [23], anticancer [24], antifungal [25], anti-parasitic [26] and antinociceptive [27] have been found.

The fact that both triazoles and Mannich bases are biological active compounds has suggested the idea of synthesizing of Mannich bases

over 1,2,4-triazoles, and has caused these compounds to acquire a wide application area in pharmaceutical chemistry. In our study, we think that we can contribute to the works on this issue by searching the antioxidant activity of some Mannich bases containing bis-1,2,4-triazoles. Antioxidants are closely strictly associated with their bio functionalities, such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacteria growth. These bio functionalities are usually related to the cessation of free radical reproduction in biological systems. As a result, antioxidant capacity is extensively used as a medical bioactive component parameter.

A wide variety of in vitro methods have been set up to assess radical scavenging ability and antioxidant activity. The antioxidant activity of the target compounds was compared with BHT, α-tocopherol and ascorbic acid in this study. In vitro tests (reducing power, metal chelating activity, hydrogen peroxide scavenging activity, superoxide radical scavenging activity, and hydroxyl radical scavenging activity) were used in order to evaluate the antioxidant activity of the test compounds.

In the literature, there are studies investigating potential antioxidant effects of 1,2,4-triazole derivatives and mannich bases simultaneously using all or some of these methods we planned in our study. Furthermore, there are many studies revealing the antioxidant effects of 1,2,4-triazoles with methods other than the methods we study on. [28 - 39]. For example, in one of the studies, it was observed that the phenyl achyl derivative in 1,2,4-triazole ring possesses antioxidant activity for oxygen and nitric oxide free radicals [40]. In another study, the antioxidant properties of 8-chloro- [1,2,4] triazole derivatives were revealed [41]. Compounds with good antioxidant effect among 1,2,4-triazole derivatives were determined using hydrogen peroxide scavenging activity as the standard ascorbic acid method in another study [42]. In another study [43] potential antioxidant 1,2,4 triazole compounds were reported.

We are unable to compare the results of similar studies in the literature with the results of our test compounds due to antioxidant method differences and the differences of groups linked to the triazole ring. However, there are studies in the literature containing groups close to our test compounds, which show the antioxidant effect of 1,2,4 triazole derivatives by using one or more of the methods we use and support our results [35, 44]. When we assess these studies and our results, we can assert the idea that especially piperazine secondary amine bis 1,2,4 triazole derivative L1 and L2 compounds may have potential antioxidant effects in the light of the data we obtained.

As for L4 and L5 compounds, we can say that the antioxidant effects of these compounds are at a level that can be considered well, having regard to the fact that they are able to yield results close to standard antioxidants. We determined that L3 compound remain at very low levels in all methods other than the hydroxyl trapping activity, when compared to the activities of standard antioxidants. Thus, we can say that the potential antioxidant activity of L3 compound is low when compared to other compounds.

Determination of reducing power

The reducing capacity of a compound may strongly indicate its potential antioxidant activity [45]. Fe^{3+}/ferricyanide complex is reduced to the ferrous form as a result of the reductants such as antioxidant substances content of the antioxidant samples. Thus, Fe^{2+} can be examined by measuring the formation of Perl's Prussian blue at 700 nm [46]. The reducing powers of the compounds were monitored in different concentrations. Then, the results obtained were compared with BHA, BHT and α-tocopherol. The Fe^{3+}– Fe^{2+} transformation was examined using Oyaizu's method in the presence of target compounds in order to measure the reductive ability [6].

The antioxidant activity of a putative antioxidant has been linked to numerous mechanisms, including the prevention chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [13]. In this method, absorbance of 50, 100 and 250 μg/ml of compound at 700 nM, values are expressed. IC_{50}-values can not be determined.

In Table 1 and Figure 1, the reducing power of the target compounds and standards (ascorbic acid, BHT and α-tocopherol) using the potassium ferricyanide reduction method is shown. With the increase in the concentration, the reducing power of the test compounds also increases. The L1 sample

showed a strong reducing capability when compared to all other compounds at 50 µg/ml L1. The compound L1 also showed a significant reducing power (Fig. 1) when compared to the standards (BHT and α-tocopherol) in all concentrations. The compound L3 showed a lower absorption at all concentrations (50-100-250 µg/ml) than the standarts and other compounds, while the compounds L2, L4 and L5 were determined to have higher absorption than α-tocopherol but a lower one than the other standards at maximum concentration (250 µg/ml).

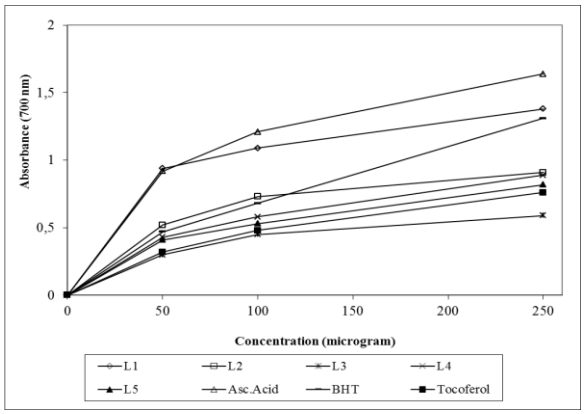

Fig. 1: Comparison of reducing power capability of test compounds and standard antioxidant compounds such as BHT, α-tocopherol, ascorbic acid at the concentrations (50–100-250 µg/mL).

The reduction capacity of a compound is associated with its capability of transferring electrons and of is consider as an important indicator of its potential antioxidant activity. The following order shows the reducing power of the compounds and standards at maximum concentration (250 µg/ml): Ascorbic acid > L1 > BHT > L2 > L4 > L5 > α-tocopherol > L3. It was particularly observed that L1 compound exhibits the best activity. We can therefore think that among the compounds, the L1 compound can be the best potential antioxidant. In the literature, there are studies suggesting that 1,2,4-triazole and Mannich bases complying with the results we obtained have antioxidant effects depending on the reduction force. For example, in a study [28] it was shown that 1,2,4-triazole derivatives show a good reductant activity. In another study [47] it was demonstrated that certain mannich bases have antioxidant effects by showing a much better reduction power than BHT and Trolox that are used as a standard. 1,2,4-triazole derivatives were examined in another study [29]. In another study, and it was determined that they exhibit a reduction activity that can be considered well, although it is lower than the standards. A different study [48] showed that triazole derivatives are good reductants.

Compound L1 containing trifluoromethylphenyl-piperazine moiety showed a better reducing capability than the other compounds. The existence of trifluoromethylphenyl-piperazine moiety may have a significant role as a better electron donor and thus may help the stabilization of the free radical form after donating electron. This may then lead to a maximum reducing capability. Compound L3 containing pyrrolidin moiety showed a feeble reducing capability. The other compounds L2, L4 and L5 exhibited a relatively better absorbance than α-tocopherol and L3, and thus good reductive activities. Metal ions complexes may be reduced to their lower oxidation states or to take part in electron transfer reaction by L2, L4 and L5 compounds. That is, these compounds exhibit the capability electron donor to scavenge free radicals.

Determination of Hydrogen peroxide scavenging activity

Hydrogen peroxide exhibits powerful oxidizing effects. It can be generated in vivo by various oxidizing enzymes, including superoxide dismutase. Hydrogen peroxide can cut across the membranes and slowly oxidize several compounds [49].

The hydrogen peroxide scavenging capability of the test compounds were given in Figure 2 and Table 2, and they were compared to those of the reference compounds BHT and ascorbic acid. The results obtained from Figure 2 and Table 2 showed that the superoxide radical scavenging activity of the samples was not dependent on the concentration. Hydrogen peroxide scavenging activity of L1 at maximum concentration (250 µg/Ml) was determined as 51.5%; while BHT and ascorbic acid showed 41.3% and 52% hydrogen peroxide scavenging activity at the same concentration, respectively. The hydrogen peroxide scavenging effect of compounds and standard compounds at this concentration decreased in the following order: Ascorbic acid > L1 > L2 > L4 > BHT > L5 > L3. Compound L1scavenged hydrogen peroxide much more strongly than the other compounds. It was considered that such a strong scavenging power resulted from the existence of trifluoromethylphenyl - piperazine moiety.

Table-1: Reducing power of test compounds.

Test Compounds	Reducing power[a] 50 µg/ml	Reducing power[a] 100 µg/ml	Reducing power[a] 250 µg/ml
L1	**0,94±0.04**	**1,09±0.04**	**1,38±0.04**
L2	**0,52±0.07**	**0,73±0.05**	**0,91±0.02**
L3	**0,3±0.06**	**0,45±0.03**	**0,59±0.03**
L4	**0,43±0.02**	**0,58±0.04**	**0,89±0.06**
L5	**0,41±0.03**	**0,53±0.03**	**0,82±0.05**
Ascorbic acid[b]	**0,92±0.05**	**1,21±0.04**	**1,64±0.04**
BHT[b]	**0,47±0.03**	**0,68±0.04**	**1,31±0.09**
α-tocoferol[b]	**0,32±0.06**	**0,48±0.02**	**0,76±0.05**

[a]Absorbance of 50, 100 and 250 µg/ml of compound at 700 nM, values are expressed as mean ± SD [b] Ascorbic acid, BHT and α-tocoferol were used as controls.

Adding hydrogen peroxide to cells in culture may cause the transition metal ion dependent OH radicals mediated oxidative DNA damage. It appears that the levels of hydrogen peroxide at or below around 20–50 mg have restricted cytotoxicity to many cell types. So, the elimination of hydrogen peroxide is critical for the protection of pharmaceutical and food systems [49]. Except for compounds L3 and L5, all of the new compounds showed greater hydrogen peroxide scavenging activity than that of BHT. L1 and L2 exhibited a comparable or similar H_2O_2 scavenging activity to that of standard reference ascorbic acid under in vitro conditions. The L5 test compound exhibited good reducing capability when compared to L4 and BHT at 100 µg/ml. Compound L3 exhibited the lowest activity among the standards and other groups in all concentrations, and thus no H_2O_2 scavenging activity.

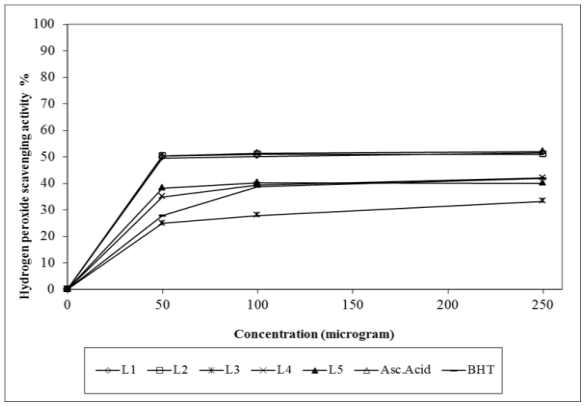

Fig. 2: Comparison of H_2O_2 scavenging activities of test compounds and standard antioxidant compounds such as BHT and ascorbic acid at the concentrations (50–100-250 µg/mL).

According to the extrapolated IC50-values, L2 compound exhibits the highest inhibitory activity. These results show that L1 and L2 among all of the test compounds proved to be the best compounds for H_2O_2 scavenging activity. In the literature there are several studies showing that 1,2,4 triazole compounds showing compliance with our results have the effect of H_2O_2 scavenging. In a study [189], the intermediate level of H_2O_2 scavenging activity of certain mannich bases were revealed. Also in another study [190], hydrogen peroxide activities were examined in order to evaluate antioxidant effects and triazoles showing a good level of activity were determined.

Determination of Superoxide Scavenging Activity

Superoxide anions are indicators of active free radicals with the potential of reacting with biological macromolecules and thus causing tissue damage [10]. Superoxide has also been blamed in several pathophysiological processes for its transformation into more reactive species such as hydroxyl radical that lead to lipid peroxidation. It has also been observed that superoxide directly starts lipid peroxidation [50]. Again, it has been indicated that antioxidant features of certain flavonoids are effective principally by means of the scavenging of superoxide anion radical [51]. Superoxide anion has an important role in the generation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, that induce oxidative damage in lipids, proteins, and DNA [52].

Furthermore, superoxide anion is an oxygen-centred radical, which has selective reactivity. Several enzyme systems in autoxidation reactions and enzymatic electron transfers univalently reducing molecular oxygen generate these species. Some iron complex, including cytochrome *c*. can also be reduced by it [53].

NBT in this system is reduced by superoxide anion that is derived from dissolved oxygen by PMS–NADH coupling reaction. Superoxide anion reduces the yellow dye (NBT^{2+}) in order to produce the blue formazan measured spectrophotometrically at 560 nm in this method. Antioxidants can prevent the formation of blue NBT [54]. The decrease of absorbance at 560 nm with antioxidants shows the consumption of superoxide anion in the reaction mixture.

Table-2: Hydrogen peroxide scavenging activity of test compounds.

Test Compounds	Hydrogen Peroxide Scavenging[a] (%Inhibition) 50 (µg/ml)	Hydrogen Peroxide Scavenging[a] (%Inhibition) 100 (µg/ml)	Hydrogen Peroxide Scavenging[a] (%Inhibition) 250 (µg/ml)	IC_{50}[a] (µg/ml)
L1	**49,58±2.61**	**50,23±2.84**	**51,55±3.35**	**86,25±3.70**
L2	**50,35±3.33**	**51,05±1.40**	**51,03±3.43**	**47,60±2.81**
L3	**24,88±1.82**	**27,91±1.67**	**33,23±1.23**	**nt**
L4	**34,89±2.15**	**39,31±2.89**	**42,03±1.66**	**nt**
L5	**38,23±2.43**	**40,25±1.12**	**40,12±1.45**	**nt**
Ascorbic acid[b]	**50,43±3.14**	**51,45±2.42**	**52,01±4.43**	**48,12±3.35**
BHT[b]	**27,82±1.80**	**38,71±2.98**	**41,83±1.38**	**nt**

[a]Values are expressed as mean ± SD, nt: not tested
[b] Ascorbic acid and BHT were used as controls.

Table-3: Superoxide scavenging activity of test compounds.

Test Compounds	Superoxide radical scavenging activitya (%Inhibition) 50 (µg/ml)	Superoxide radical scavenging activitya (%Inhibition) 100 (µg/ml)	Superoxide radical scavenging activitya (%Inhibition) 250 (µg/ml)	IC50a (µg/ml)
L1	**62,88±4.27**	**64,84±4.10**	**66,18±4.02**	**nt**
L2	**62,11±4.12**	**63,98±3.60**	**67,01±5.40**	**nt**
L3	**53,21±3.35**	**51,01±3.35**	**48,98±2.65**	**75,30±2.42**
L4	**60,33±3.65**	**60,77±4.67**	**62,19±3.22**	**nt**
L5	**59,48±2.41**	**61,01±4.26**	**61,66±2.43**	**nt**
Ascorbic acid[b]	**64,11±4.32**	**68,33±5.01**	**69,45±5.76**	**nt**
BHT[b]	**69,3±3.24**	**75,05±5.51**	**77,29±4.86**	**nt**
α-tocoferol[b]	**57,44±2.35**	**63,12±2.33**	**65,73±4.16**	**nt**

[a]Values are expressed as mean ± SD, nt: not tested
[b] Ascorbic acid, BHT and α-tocoferol were used as controls

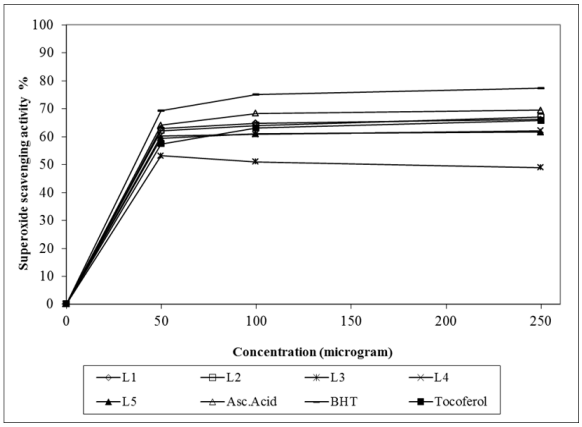

Fig. 3: Comparison of Superoxide scavenging activity of test compounds and standard antioxidant compounds such as BHT, Ascorbic acid and α –tocopherol at the concentrations (50–100-250 µg/mL).

In Figure 3, the superoxide radical scavenging activity of the test compounds at 50–100-250 µg/mL when compared to the same amount of BHT, α-tocopherol and ascorbic acid is shown. The results obtained from Figure 3 showed that the superoxide radical scavenging activity of the samples was not dependent on the concentration.

As is seen in Table 3, IC_{50}-values for the samples tested were not determined except L3; the percentage inhibition of superoxide anion radical generation at 250 µg/mL concentration of L2 is 67,01 %; while BHT, ascorbic acid and α –tocopherol showed 77.2%, 69.4% and 65.7% superoxide anion radical scavenging activity at the same concentration, respectively. L2 exhibited the highest superoxide radical scavenging activity among α-tocopherol and the other test compounds at a concentration of 250 µg/mL. The superoxide radical scavenging activity of test compounds were in the following order at this concentration, respectively: BHT >Ascorbic acid > L2 > L1> α –tocopherol > L4 > L5 > L3. The compounds L2 and L1 exhibited a similar antioxidant potential to that of the standard. Compounds L4 and L5 exhibited limited superoxide anion scavenging activity, while L3 compound exhibited the lowest activity among the standards and other compounds in all concentrations.

The results showed that compound L1 containing trifluoromethylphenyl-piperazine moiety and L2 containing benzylpiperazine moiety exhibited the best superoxide anion scavenging activity among all other compounds from all synthesized derivatives. Thus, the existence of trifluoromethylphenyl-piperazine and benzylpiperazine moiety was determined to be crucial for their superoxide anion scavenging activity.

According to these results, it can be said that L1 and L2 compounds have the best superoxide radical scavenging activity among the compounds. In the literature, superoxide radical scavenging activities of certain derivatives in the structure of 1,2,4, triazole. For example, certain derivatives that may

have a good level of superoxide radical scavenging activity were examined determined when examining 1,2,4, triazole derivatives [55].

Determination of Chelating Capacity

Ferrous chelating capacity was important as it reduced the catalyzing transition metal concentration in lipid peroxidation. It was indicated that chelating agents are useful as secondary antioxidants as they reduce the redox potential and thus stabilize the metal ion's oxidized form. Ferrozine can form complexes with Fe^{2+} quantitatively. The complex formation is obstructed when chelating agents are present, and this decreases the red colour of the complex. Thus, measuring the reduction of colour enables the estimation of the chelating activity of the coexisting chelator [56]. Transition metals play an important role in the formation of oxygen free radicals in living organisms. The ferric iron (Fe^{3+}) is the comparatively biologically inactive form of iron. Yet, it can be reduced to the active Fe^{2+} depending on the circumstances, and especially pH [57]. It can also be oxidized back by means of Fentontype reactions with the production of hydroxyl radical or Haber-Weiss reactions with superoxide anions.The production of these radicals may cause lipid peroxidation, protein modification, and DNA damage. Chelating agents may not trigger metal ions and possibly prevent the metal dependent processes [58]. The production of highly active ROS, including $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$ is also catalyzed by free iron by means of Haber-Weiss reactions:

$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$ [59].

Iron is known to be the most significant lipid oxidation pro-oxidant among all transition metals as a result of its high reactivity. The ferrous state of iron decomposes the hydrogen and lipid peroxides into reactive free radicals by means of the Fenton reactions, thereby speeding up the lipid oxidation:

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$. [60]

Fe^{3+} ion also generates radicals from peroxides. However, the rate is ten times less than the rate of Fe^{2+} ion, the most powerful pro-oxidant among various metal ion types [61].

In Figure 4, the ferrous ion chelating activities of the compounds and standards are demonstrated. Figure 4 shows that the chelating capacity of the samples is not concentration dependent. In addition, only L1 demonstrated a good activity when compared to the other compound. However, it was not a good a activity when it was compared to the standards. On the contrary, other compounds showed exhibited quite low ion chelating activities. The chelating capacity of the test compounds were in the following order at maximum concentration (250 µg/mL): BHT > α –tocopherol> L1 > L2 > L5 > L4 > L3 respectively.

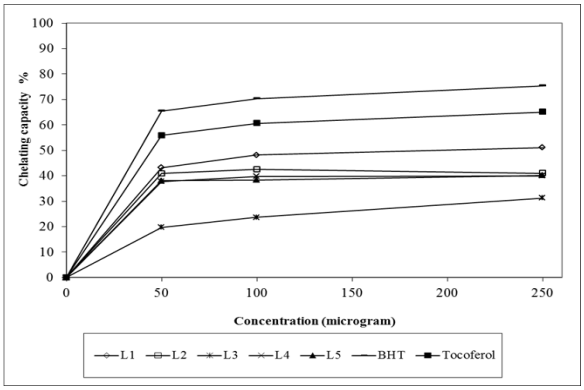

Fig.4: Comparison of iron chelating capacity of test compounds and standard antioxidant compounds such as BHT and α –tocopherol at the concentrations (50–100-250 µg/mL).

As is seen in Table 4, IC_{50}-values for the samples tested were not determined except L1. For Table 3, it can be said that the test compound possesses a good level of metal chelating activity among the test compounds as the L1 compound exhibits chelating at a level close to standard antioxidants. In the literature, there are studies on the metal chelating activity in order to put forth the antioxidant effects of triazoles. In one of these studies [62], it was shown that triazole derivatives are good chelators and possess antioxidant potential. In another study [48], triazole derivatives that come out to be good chelators were determined. As for another study [63], antioxidant effects of (1,2,4)-Triazole derivatives were put forth with regard to their metal chelating. Data obtained as a result of our study also support these studies in the literature and revealed the chelating activities of bis 1,2,4- triazole derivatives.

Determination of Hydroxyl radical scavenging activity

Hydroxyl radicals generated by the reduction of hydrogen peroxide and radiation, contribute significantly to the molecular and cellular damage within biological systems. Thus, scavenging and preventing the formation of hydroxyl radicals is of upmost importance.

Table-4: Metal chelating activity of test compounds.

	Metal chelating activity[a] (%Inhibition) 50 (µg/ml)	Metal chelating activity[a] (%Inhibition) 100 (µg/ml)	Metal chelating activity[a] (%Inhibition) 250 (µg/ml)	IC_{50} (µg/ml) [a]
L1	**43,25±1.42**	**48,22±2.71**	**51,09±3.23**	**125,42±9.28**
L2	**40,91±1.40**	**42,5±2.23**	**41,03±2.43**	**nt**
L3	**19,8±1.90**	**23,71±1.96**	**31,33±1.13**	**nt**
L4	**37,77±1.32**	**39,74±1.48**	**40,03±1.55**	**nt**
L5	**38,08±1.38**	**38,37±1.43**	**40,22±1.64**	**nt**
BHT[b]	**65,42±2.52**	**70,33±5.76**	**75,42±5.40**	**nt**
α-tocoferol[b]	**55,88±3.14**	**60,58±4.16**	**65,02±4.23**	**nt**

[a] Values are expressed as mean ± SD, nt: not tested
[b] BHT and α-tocoferol were used as controls

Table-5: Hydroxyl radical scavenging activity of test compounds.

Test Compounds	Hydroxyl radical scavenging activity[a] (%Inhibition) 50 (µg/ml)	Hydroxyl radical scavenging activity[a] (%Inhibition) 100 (µg/ml)	Hydroxyl radical scavenging activity[a] (%Inhibition) 250 (µg/ml)	IC_{50}[a] (µg/ml)
L1	**43,01±2.23**	**75,55±5.15**	**89,23±6.38**	**60,35±2.44**
L2	**42,11±2.12**	**74,52±4.77**	**90,25±6.85**	**58,49±3.14**
L3	**40,13±1.89**	**71,03±3.76**	**89,21±6.18**	**64,04±4.17**
L4	**44,33±2.40**	**73,58±4.74**	**87,77±3.70**	**65,33±4.23**
L5	**37,85±1.40**	**72,54±3.42**	**89,84±1.66**	**59,14±3.16**
Ascorbic acid[b]	**45,21±2.54**	**78,07±3.22**	**92,54±7.72**	**56,12±3.68**
BHT[b]	**38,52±5.05**	**72,82±4.04**	**90,02±7.28**	**64,82±3.40**

[a]Values are expressed as mean ± SD,
[b] Ascorbic acid and BHT were used as controls.

According to the results ın Table 5, hydroxyl trapping activity of our compounds was found to be in a good level. In a study, it has been reported that there are derivatives containing 1,2,4 triazole and exhibit good hydroxyl trapping activity [48].

As is seen in Table 5, of all the samples tested, compound L2, which has the lowest IC_{50}-value, exhibits the strongest antioxidant activity against hydroxyl radical. Hydroxyl radical scavenging activities of the positive controls increased ascorbic acid and BHT respectively.

Figure 5 shows the radical inhibition percentages of the compounds and standards. It can be inferred from the results that Hydroxyl radical scavenging activity of the test compounds and standards (Ascorbic acid and BHT) increases with the increase of the concentration. In addition, all of the compounds exhibited similar or equal hydroxyl radical scavenging activity with one another. Nonetheless, compound L2 exhibited the highest Hydroxyl radical scavenging activity among BHT and the other test compounds at a concentration of 250 µg/mL. The superoxide radical scavenging activity of test compounds were in the following order at this concentration: Ascorbic acid> L2 > BHT >L5 > L1 > L3 > L4 respectively.

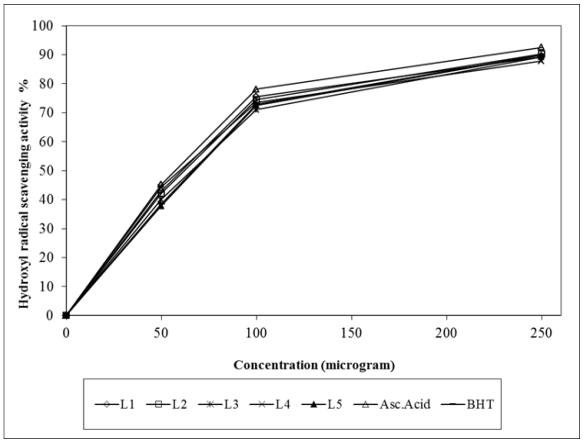

Fig. 5: Comparison of Hydroxyl radical scavenging activity of test compounds and standard antioxidant compounds such as BHT and Ascorbic acid at the concentrations (50–100-250 µg/mL).

Conclusion

To conclude, the successful synthesis and biological activity of new Mannich bases which contain bis-1,2,4 – triazole groups were shown in the study. The results obtained from this study shows that the test compounds (especially L1 and L2) are effective antioxidants when compared to standard antioxidant compounds such as BHT, α- tocopherol and ascorbic acid in different in vitro tests: reducing power, superoxide anion radical scavenging, hydrogen

peroxide scavenging, metal chelating activities and Hydroxyl radical scavenging activity.

The antioxidant activity showed that all of the compounds that were tested have good antioxidant activity under in vitro conditions. This may result from the existence of piperazine, dipropylalmino, methyl piperidine moiety in addition to phenyl, methyl, benzyl and ethyl group. The results of the present study demonstrated that these new bases containing bis-1,2,4 – triazole groups can be useful as a reactive oxygen scavenger. Hence, test compounds may emerge as a new type of reactive oxygen species scavenger. According to the data above, test compound can be used for delaying or preventing the oxidation of the compounds and extending the shelf life of pharmaceuticals. Synthesizing new derivatives can be used as a protection tool in biological systems and in modern chemistry. Thus, it is concluded that further study should be carried out in such a large scope of investigation.

References

1. G. Roman, Mannich Bases in Medicinal Chemistry and Drug Design, *Eur. J. Med. Chem*, **89** (2015).
2. S. Kucukguzel and P. Cıkla-Suzgun, Recent Advances Bioactive 1,2,4-triazole-3-thiones, *Eur. J. Med. Chem*, **1** (2014).
3. D. Kumudha, R. R. Reddy and T. Kalavathi, 1, 2, 4-Trıazoles: As Bıologıcally Important Agents, *IJPSR*, **3** 12 (2012).
4. A. K. Wahi, Arti Singh, Triazole: Recent Development and Biological Activities, *AJBPR*., **1**, 193 (2011).
5. M. Koparır, Synthesis and Biological Activities of Some New Mannich Bases of 5,5'-Butane-1,4-diylbis[4-ethyl-2,4-dihydro-3H-1,2,4-triazole-3-thiones, *ChemSci Trans*., **2**, 701 (2013).
6. M. Oyaizu, Studies on Products of Browning Reaction Prepared from Glucosamine, *Japan. Nutri*., **44**, 307 (1986).
7. T. C. P. Dinis, V. M. C. Madeira, L. M Almeida, Action of Phenolic Derivatives (Acetaminophen, Salicylate, and 5-aminosalicylate) as İnhibitors of Membrane Lipid Peroxidation and as Peroxyl Radical Scavengers, *Arch. Biochem. Biophys*., **315**, (1994).
8. R. J. Ruch, S. J. Cheng and J. F. Klaunig, Prevention of Cytotoxicityandinhibition of İntercellular Communication by Antioxidant Catechins İsolated from Chinese Greentea, *Carcinogenesis*., **10**, 1003 (1989).
9. F. Liu, V. E. C. Ooi and S. T. Chang, Free Radical Scavenging Activities of Mushroom Polysaccharide Extracts, *Life Sci*., **60**, 763 (1997).
10. B. Halliwell, J. M. C. Gutteridge and O. I. Aruoma, The Deoxyribose Method: a Simple ‘‘Test-Tube” Assay for Determination of Rate Constants for Reactions of Hydroxyl Radicals, *Analytical Biochemistry*, **165**, 215 (1987).
11. D. D. Reynolds and B. C. Cossar, *J Heteroc. Chem*. (1971) **8**, 597, 605 and 611, and Def Publ. U.S. Put. Ofi, 900,001; (1972) *Chem. Abs*.,78,708-47.
12. M. Arend, B. Westermann and N. Rısch, Modern Variants of the Mannich Reaction, Angew, Chem. Int. Ed, **37** 1044 (1998).
13. M. Tramontini and L. Angiolini, Mannich Bases Chemistry and Uses. 5. Londra: CRC,**34**,254 Pir, (1994).
14. O. F. Ozturk, A. Cansiz and M. Koparir, Preparation and Characterization of Co(II), Ni(II) and Cu(II) Complexes of a Ligand Containing 1,3,4-thiadiazole groups, *Transit. Metal Chem*. **32** 224 (2007).
15. S. Ozturk, M. Akkurt, A. Cansiz, M. Koparir, M. Sekerci, F. W. Heinemann, 2-Amino-5-phenyl-1,3,4-thiadiazole at 110 K, *Acta Crystallogr*. **E 60** o820 (2004).
16. N. Ulusoy, N. Ergenç, G. Otük and M. Kiraz, Synthesis of some 4-(alkylidene/ arylidene)amino-2,4-dihydro-5-(2-thienyl)-3H-1,2,4-triazole-3-thiones Tested for Antimicrobial Activity, *Boll. Chim. Farm* **140** (2001).
17. R. Kharb, M. Shahar Yar, P. C. Sharma, Recent Advances and Future Perspectives of Triazole Analogs as Promising Antiviral Agents, Mini Rev. *Med. Chem.* **11** (2011).
18. A. T. Mavrova, D. Wesselinova, Y. A. Tsenov and P. Denkova, Synthesis, Cytotoxicity and Effects of Some 1,2,4-triazole and 1,3,4-thiadiazole Derivatives on İmmunocompetent Cells, *Eur. J. Med. Chem*. **44** (2009).
19. A. K. Wahi, A. K. Sing and A. Singh. Design and Synthesis of Novel Schiff’s bases Having N-(4*H*-1, 2, 4-triazole-4-yl)benzamido Moiety as Antimicrobial and Anti-İnflammatory Agents. *Der Pharma Chemica*; **3,** 146 (2011).
20. U. Salgin-Goksen, N. Gokhan-Kelekçi, O. Goktas¸ Y. Koysal, E. Kiliç, S. Isik, G. Aktay

and M. Ozalp, 1-Acylthiosemicarbazides, 1,2,4-triazole-5(4H)-thiones, 1,3,4-thiadiazoles and hydrazones containing 5-methyl-2-benzoxazolinones: Synthesis, Analgesic-Anti-İnflammatory and Antimicrobial Activities, *Bioorg. Med. Chem*. **15**, 5738 (2007).

21. T. Plech, B. Kapro_n, J. J. Luszczki, M. Wujec, A. Paneth, A. Siwek, M. Koaczkowski, M. Zonierek and G. Nowak, Studies on the Anticonvulsant Activity and İnfluence on GABA-ergic Neurotransmission of 1,2,4-triazole-3- thione- Based Compounds, *Molecules* **19** 11279 (2014).
22. J. M. Kane, M. W. Dudley, S. M. Sorensen, F. P. Miller, 2,4-Dihydro-3H-1,2,4- triazole-3-thiones as Potential Antidepressant Agents, *J. Med. Chem*. **31** 1253 (1988).
23. Jag Mohan. Heterocyclic Systems Containing Bridgehead Nitrogen Atom: Synthesis and Bioactivity of 3-(2-thienyl)-s-triazolo[3,4-b][1,3,4]thiadiazole, 2(2-thienyl) thiazolo[3,2-b]-s-triazole and isomeric 3-(2-thienyl)thiazolo[2,3-c]-s-triazole. *Ind. J. Chem*; **42B**: 401 (2003).
24. S. Holla, B. Veerendra, M. K. Shivananda, B. Poojary, Synthesis Characterization and Anticancer Activity Studies on Some Mannich bases Derived from 1,2,4-triazoles, *Eur. J. Med. Chem*. **38** (2003).
25. Anees A. Siddiqui, Amit Arora, N. Siddiqui and Amit Misra. Synthesis of some 1,2,4-triazoles as Potential Antifungal Agents. *Ind. J. Chem* **44B**: 838 (2005).
26. F. H. Havaldar, A. R. Patil, Synthesis of 1,2,4 Triazole Derivatives and their Biological Activity, *E. J. Chem*. **5** (2008).
27. N. Upmanyu, J. K. Gupta, K. Shah and P. Mishra, Anti-İnflammatory and Antinociceptive Evaluation of Newly Synthesized 4-(substituted ethanoyl) amino-3-mercapto-5-(4-methoxy) phenyl-1,2,4-triazoles, *J. Pharm. Bioallied Sci*. **3** (2011).
28. M. Alkan, H. Yüksek,, Ö. G. Kol and M. Çalapoğlu, Synthesis, Acidity and Antioxidant Properties of Some Novel 3,4-disubstituted-4,5-dihydro-1H-1,2,4-triazol-5-one Derivatives, *Molecules*, **13**, 107 (2008).
29. O. G Kol and E. Ayazoglu, Antioxidant Activities and Acidic Properties of Some Novel 4-[3,4-di-(4-nitrobenzoxy)-benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one derivatives, *Arabian J. Chem. (2014).*
30. C. Kuş, G.Ayhan-Kilcigil, B Can Eke and M Iscan, Synthesis and Antioxidant Properties of Some Novel Benzimidazole Derivatives on Lipid Peroxidation in the Rat Liver, *Arch. Pharm. Res*. **27**, 156 (2004).
31. G. Ayhan-Kilcigil, C. Kuş, T. Çoban, B. Can-Eke, S. Ozbey and M. Iscan, Synthesis,Antioxidant and Radical Scavenging Activities of Novel Benzimidazoles, *J. Enzyme Inhib. Med. Chem*. **20**, 503 (2005).
32. C. Kuş, G. Ayhan-Kilcigil, S. Ozbey, F.B. Kaynak, M. Kaya,T. Çoban and B. Can-Eke, Synthesis and Antioxidant Properties of Novel N-methyl-1,3,4-thiadiazol-2-amine and 4-methyl-2H-1,2,4-triazol-3(4H)-Thione Derivatives of Benzimidazoleclass, *Bioorg. Med. Chem*. **16**, 4294 (2008).
33. I. Khan, S. Ali, S. Hameed, N. H. Rama, M. T. Hussain, A. Wadood, R. Uddin, Z. Ul-Haq, A. Khan, S. Ali, M. I. Choudhary, Synthesis, Antioxidant Activities and Urease İnhibition of Some New 1,2,4-triazol and 1,3,4-thiadiazole Derivatives, *Eur. J. Med. Chem.* **45**, 5200 (2010).
34. M. Hanif, M. Saleem, M. T. Hussain, N. H, Rama, S. Zaib, M. A. M. Aslam, P. G. Jones, J. Iqbal, ‘‘Synthesis, Urease İnhibition, Antioxidant and Antibacterialstudies of some 4-amino-5-aryl-3H-1,2,4-triazol-3-thiones and their 3,6-disubstituted 1,2,4-triazolo[3,4-b]1,3,4-thiadiazole Derivatives, *J. Braz.Chem. Soc.* **23**, 854 (2012).
35. C. Aswathanarayanappa, E. Bheemappa, Y. D. Bodke, P. S. Krishnegowda, S. P. Venkata, R. Ningegowda, Synthesis and Evaluation of Antioxidant Propertiesof novel 1,2,4-Triazol-Based Schiff Base Heterocycles, *Arch. Pharm.(Weinheim.,)* **346** 922 (2013).
36. H. Nadeem, M. Mohsin, H. Afzaal, S. Riaz, A. Zahid and S. A. Muhammad, Synthesis and in vitro Biological Activities of 4,5-disubstituted 1,2,4-triazol-3-thiols, *Adv. Microbiol.* **3** 366 (2013).
37. M. Koparir, C. Orek, A.E. Parlak, A. Soylemez, P. Koparir, M. Karatepe and S. D. Dastan, Synthesis and Biological Activities of Some Novel Aminomethylderivatives of 4-substituted-5-(2-thienyl)-2,4-dihydro-3H-1,2,4-triazol-3-thiones, *Eur. J. Med. Chem*. **63**, 340 (2013).
38. M. Koparır and C. Orek, Synthesis and Biological Activities of Some Novel Aminomethylderivatives of 5,50-butane-1,4-

diyl-bis[4-allyl-2,4-dihydro-3H-1,2,4-triazol-3-thiones, *Chem. Sci. Trans*. **2** 181 (2013).

39. E. Dügdü, Y. Unver, D. Unlüer and K. Sancak, Synthesis and Biological Properties of Novel Triazol-Thiol and thiadiazole Derivatives of the 1,2,4-triazol-3(5)-one class, *Molecules* **19** 2199 (2014).
40. V. Sorrenti, L. Salerno, C. Di Giacomo, R. Acquaviva, M. A. Siracusa, A. Vanella, Imidazole Derivatives as Antioxidants and Selective Inhibitors of nNOS, *Nitric Oxide*, **14**, 45 (2006).
41. M. Suresh, P. Lavanya, D. Sudhakar, K. Vasu and C. Venkata Rao, Synthesis and Biological Activity of 8-chloro-[1,2,4]triazolo [4,3-a]quinoxalines, *J. Chem. Pharm. Res*., **2,** 497 (2010).
42. P. Valentina, K. Ilango, M. Deepthi, P.Harusha, G. Pavani, K. L. Sindhura and C. G. Keerthanan, *J. Pharm. Sci. And Res***, 2**, 74(2009).
43. R. Singh,A. Chouhan, Important Methods Of Synthesıs And Bıologıcal Sıgnıfıcance Of 1, 2, 4-Triazol Derıvatıves, *W. J. P. P. S*, **3**, 8, 874 (?).
44. M. M. El Sadek, N. S. Abd E-Dayem, S. Y. Hassan, M. A. Mostafa, G. A. Yacout, Antioxidant and Antitumor Activities of New Synthesized Aromatic C-Nucleosidederivatives, *Molecules* **19**, 5163 (2014).
45. S. Meir, J. Kanner, B. Akiri, S. P. Hadas, Determination and İnvolvement of Aqueous Reducing Compounds in Oxidative Defense Systems of Various Senescing Leaves, *J. Agri. Food. Chem*.,**43**, 1813 (1995).
46. Y. C.Chung, C.T.Chang, W.W.Chao, C.F.Lin, S.T.Chou, Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by Bacillus subtilis IMR-NK1,*J. Agric. Food Chem*., **50**, 2454 (2002).
47. L. Ma, , L. Xiao, L. Xie, M. Wang, H.L. Zhu, M.H. Wang, Y.H. Ye, Synthesis and antioxidant activity of novel Mannich base of 1,3,4-oxadiazole derivatives possessing 1,4-benzodioxan,'' *Bioorg. Med. Chem*. **21**, 6763–6770 (2013).
48. S.Y. Çiftci, N.G.Kelekçi, U.S. Gökşen, G. Uçar, Free-Radical Scavenging Activities of 2-Benzoxazolinone Derivatives Containing Thiosemicarbazide, Triazole, Thiadiazole and Hydrazone Units, *Hacettepe University Journal of the Faculty of Pharmacy*, **31**,1, 27-50(2011).
49. I. Kilic , Y. Yeşiloglu , Y. Bayrak, Spectroscopic studies on the antioxidant activity of ellagic acid, *SpectrochimicaActa Part A:Molecular and Biomolecular Spectroscopy.*,**130**, 447 (2014).
50. A.P.Wickens, Aging and the free radical theory. *Resp. Physio*l.,**128**, 379 (2001).
51. G.C.Yen, P.D.Duh, Scavenging effect of methanolic extract of peanut hulls on free radical and active oxygen species, *J. Agric.Food Chem.*,**42**,629(1994).
52. Pietta. P.G, Flavonoids as antioxidants. *J. Nat. Prod*.,**63**,1035–1042(2000).
53. I. Gulcin, Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid), *Toxicology*.,**217**, 213 (2006).
54. I.Parejo, F.Viladomat, J.Bastida, A.Rosas-Romero, N.Flerlage, J.Burillo,C.Codına, Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants. *J. Agric. Food Chem*., **50**, 6882 (2002).
55. Y. Kotaiah ,K. Nagaraju , N. Harikrishna , C. Venkata Rao , L. Yamini ,M. Vijjulatha, Synthesis, docking and evaluation of antioxidant and antimicrobial,activities of novel 1,2,4-triazolo[3,4 b][1,3,4]thiadiazol-6 yl)selenopheno[2,3-d]pyrimidines, *European J. Med. Chem.* **75** 195-202 (2014).
56. F. Yamaguchi, T. Ariga, Y. Yoshimira, H. Nakazawa, Antioxidative and anti-glycation activity of garcinol from Garciniaindica Fruit Rind, *J. Agric. Food Chem*., **48**, 180 (2000).
57. M. Strlič, T. Radovič, J. Kolar, B. Pihlar, Anti- and Prooxidative Properties of Gallic Acid in Fenton-Type Systems, *J. Agric. Food Chem.*,**50**, 6313 (2002).
58. A. E. Finefrock, A. I. Bush, P. M. Doraiswamy, Current Status of Metals as Therapeutic Targets in Alzheimer's Disease, *J Am Geriatr Soc..*, **51**, 1143 (2003).
59. S. Papakonstantinou-Garoufalias, N. Pouli, P. Marakos and A. Chytyroglou-Ladas, Synthesis Antimicrobial and Antifungal Activity of some new 3-substituted derivatives of 4-(2,4-dichlorophenyl)-5-adamantyl-1*H*-1,2,4-triazole, *Farmaco*, **57**, 973 (2002).
60. A. A. Ikizler, F. Ucar, H. Yuskek, A. Aytin, I. Yasa and T. Gezer, Synthesis and Antifungal Activity of Some New Arylidenamino Compounds, *ActaPoloniaePharmaceutica*, **54**,135 (1997).

61. I. Çaliş, M. Hosny, T. Khalifa and S. Nishibe, Secoiridoids from Fraxinusangustifolia, *Phytochemistry*., **33**, 1453 (1993).
62. H. Yuksek, O. Akyildirim, and O. G. Kol, Synthesis and In Vitro Antioxidant Evaluation of New 1,3,5-Tri-{2-methoxy-4-[(4,5-dihydro-1*HH*-1,2,4-triazol-5-on-4-yl) -azomethine]-phenoxycarbonyl}-Benzene Derivatives, *Hindawi Publishing Corporation J. Chem..*, **8** (2013).
63. G. K. Nagaraja, K. Reshma, B. Manjunath, S. K. Peethambar, T. Arulmoli, Antioxidant and Metal Chelating Activities of some Novel Imidazoquinolie Incorporated [1,2,4]-Triazolo Heterocycles, *J. Pharm. Res*., **3**, 23 (2014).