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Original Research

Investigation of biological effects of some Mannich Bases containing Bis-1,2,4- Triazole

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Abstract: In this study, the effects of Mannich bases containing bis-1,2,4-triazole on the levels of *in vivo* malondialdehyde (MDA) and antioxidant vitamins (A, E, C) were examined in serum, livers and kidneys of rats. DA and vitamin (A, E, C) levels were determined by high performance liquid chromatography (HPLC). Antioxidant effect was investigated by determining the MDA levels in *Saccharomyces cerevisiae* cells as *in vitro*. Furthermore, the antitumor effects of compounds were investigated against MCF-7 human breast cancer cells. Interrelations of results among control and compound groups were evaluated using SPSS statistical software package. As a result, some of the compounds showed effective biological activity when compared to control conditions. The test compounds used in this study may be effective for utilization in the selection and design of model compounds for further studies.

Key words: Mannich bases, 1,2,4-triazole, Biological activity.

Introduction

Having potential in biology, Mannich bases have found quite a large application area in medicine in recent years. Nowadays, there is an increasing interest in heterocyclic molecules containing nitrogen because of their intense range of biological activity. Intensive studies have been carried out on these compounds by many research groups because of quite remarkable biological characters of triazole compounds (1, 2). Various biological activities like antineoplastic (3, 4), antimalarial (5) and antiviral (6) effects of Mannich bases have been reported.

One of the methods that have become commonly used in the study of drug designs is a set of Mannich reactions that lead to the synthesis of organic molecules in a single step containing different functional groups in a single molecular structure. Mannich bases, with high water solubility, are chemical structures preferred in preparing prodrugs (7, 8). Pharmaceutical chemistry is the most important usage area of Mannich bases, and 35% of the publications in this area are about Mannich bases. In pharmaceutical chemistry; they are used in analgesics, antibiotics and anti-cancer drugs (9). Triazoles were introduced to the scientific world by Bladin (10), and studies of Andreocci in the 1880s (11) on this subject have been taken intensively further until today. Triazoles and thiadiazole constitute a practical set of subjects with their chemical activities suitable to be placed on the structure of various substituents, and especially their tautomeric properties (12, 13). In the studies performed with triazole derivatives in the literature, effective compounds of antimicrobial (14), virostatic (15), cytostatic (16), anti-inflammatory (17), analgesic (18), anticonvulsant (19), central nervous system depressant (20), antihistaminic (21), hypotensive (22), diuretic (23), anthelmintic (24), antifungal (25), pesticide (26) and insecticide (27) properties have been found.

The fact that both triazoles and Mannich bases are biologically active compounds has led to the idea of synthesizing Mannich bases over 1, 2, 4 - triazoles, and caused these compounds to gain a wide range of applications in pharmaceutical chemistry. This study aimed to contribute to other works on this issue by searching the bioactivity of some Mannich bases containing bis-1, 2, 4 - triazoles. Towards this aim in this study, antioxidant properties were investigated with MDA assay through synthesizing some Mannich bases containing bis-1, 2, 4 – triazoles and by using an in vitro method in Saccharomyces cerevisiae cells. Saccharomyces cerevisiae, which belongs to the kingdom of fungi, is a unicellular microorganism. Saccharomyces cerevisiae is an ascomycete yeast. This type of yeast has dominantly a unicellular talus that produces ascospores in a free ascus that is composed of a zygote or a single somatic cell as a parthenogenetic, and it reproduces asexually with methods like budding and transverse division. Saccharomyces cerevisiae ferments carbohydrates like other yeasts. It is used in beer production and bakery. High vitamin content of yeasts increases their value as food ingredients. Many types of Saccharomyces cerevi-

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Table 1. UIPAC Nomenclature of compounds.

L1	*5,5'-Butane-1,4-diylbis(4-ethyl-2-({4-(3-(trifluoromethyl)phenyl)piperazin-1-yl} methyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione)*
L2	*5,5'-Butane-1,4-diylbis(4-ethyl-2-(pyrrolidin-1-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione)*
L3	*5,5'-Butane-1,4-diylbis{2-((dipropylamino)methyl)-4-ethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione}*
L4	*5,5'-Butane-1,4-diylbis{4-ethyl-2-((4-methylpiperidin-1-yl)methyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione}*

siae particularly synthesize vitamin B as well as other vitamins. Moreover, in this study, antitumor properties of bis-1,2,4 - triazole compounds were investigated by also using MCF-7 human breast cancer cells. Additionally, liver and kidney tissues and blood samples were taken from rats with in vivo method, and then the effects of these compounds on antioxidant vitamins A, E, C, and MDA were examined. When Mannich compounds with triazole ring show high levels of biological activity, they may prove important for further research on the pharmaceutics industry. On the other hand, introducing this subject to the literature is also important.

Materials and Methods

Experimental section

The following experiments were approved by the Ethics Committee for the care and use of laboratory animals in Firat University.

The age of male rats from Long-Evans species used in this study ranged between 12 and 14 weeks, while they had an average weight of 250 g. Rats were obtained from Firat University Experimental Research Center (FÜDAM), and experiments were carried out in the same center. Rats were fed in specially prepared cages in a ventilated environment and the bottoms of the cages were cleaned every day. Feeds were given in special steel pots and water was given in feeding bottles with stainless steel balls.

Used animals in the experiments were fed with rat feeds in the form of pellets specially prepared in Elazig feed factory. Care of the rats was continued in this manner through the duration of the experiments. None of the experiment subjects died for any reason in the course of the study.

Creating groups and implementations

In the study, used rats were classified into 1 control group and 4 experiment groups. After the relevant literature review (28), it was decided to make the implementations with an interval of 3 days on day 0, day 3, day 6, day 9 and so on for the control and experiment groups, during a course of 30 days. Subcutaneous injections were carried out with 3-day intervals for 30 days in defined doses in a suitable research environment. At the end of this period, rats were decapitated, and their kidney, liver tissue and blood samples were taken. Following the clotting, blood samples taken from the rats were centrifuged, and their serums were separated. The separated serums were analyzed within three days at the least. Serum samples were kept at -20 °C till the analysis.

The tissue samples were cut into a defied size in preparation. Doses of the implementation substances were decided upon based on the literature (29, 30). The doses were firstly dissolved in DMSO (Dimethyl sulfoxide) to achieve a body weight of 25 mg kg^{-1} according to the conditions. After being diluted with corn oil to reach a DMSO proportion of less than 10%, 0.5 mL of the solution was injected subcutaneously into the animals. In summary, in the experiment groups, the subcutaneously injected solutions were prepared to ensure that ligand concentration in the solution of 0.5 mL would provide a 25 mg kg^{-1} body weight dose.

Chemical compounds used in implementations

In implementations, Mannich bases containing bis-1,2,4-triazole were used. Inspired by the ligand term, coding was applied on the compounds in the form of L1-4. These compounds were synthesized and characterized by the researchers of Firat University Faculty of Science, Department of Organic Chemistry (31). The synthesized compounds bearing bis-1,2,4-triazole ring were characterized by elemental analysis, IR, ^{1}H and ^{13}C NMR spectral data. The IR spectra were measured with Perkin-Emler spectrum one FT–IR spectrophotometer. Electronic spectral studies were conducted on a Shimadzu model UV-1700 spectrophotometer in the wavelength 1100-200 nm. The ^{1}H and ^{13}C spectra were taken on Bruker AC-300 and Bruker AC-400 NMR spectrometer operating at 400 MHz for ^{1}H, 100 MHz for ^{13}C NMR. Structures of the compounds are shown in Table 1 and Figure 1.

Determination of vitamins A and E in tissue samples

Proteins were precipitated on fragmented 0.3 g tissue sample by adding 4 mL of ethyl alcohol containing 1% H_2SO_4. After mixing thoroughly by vortex, the mixture was centrifuged at 4500 rpm for 25 minutes, and then 0.3 mL of n-hexane was added to the samples (32). Vitamins dissolved in fat medium were extracted with hexane and mixed by vortex once again, and the tubes were centrifuged. At the end of centrifuging, the hexane phase was taken to a glass tube by separating carefully. The sample was centrifuged again by adding 250 µL of n-hexane over it and mixed. The n-hexane phase was combined with the other hexane phase in the glass tube. The extracted hexane was removed carefully under dry nitrogen. The residue was dissolved in methanol 100 µL. 20 µL was taken from this solution and injected to the HPLC.

Determination of vitamin C and MDA in tissue samples

1.5 mL of 0.5 M $HClO_4$ was added to approximately 0.3 g of the fragmented tissue sample. After the precipitation of proteins, total volume was completed to 3 mL by adding 1.5 mL of distilled water. After centrifuging at 4500 rpm for 25 minutes, 20 µL of the clear part was taken to vials. The clear solution was analyzed by HPLC

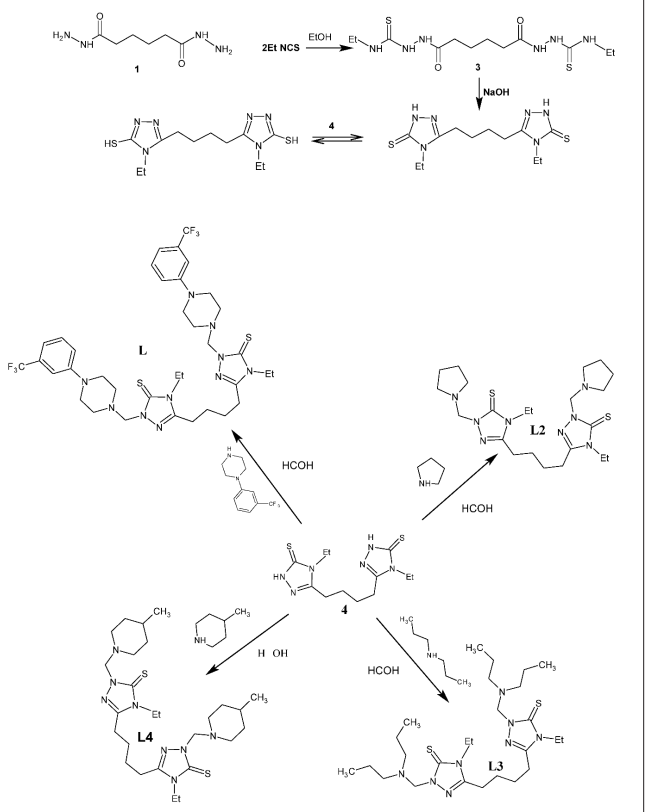

Figure 1. Synthesis and the structures of the compounds.

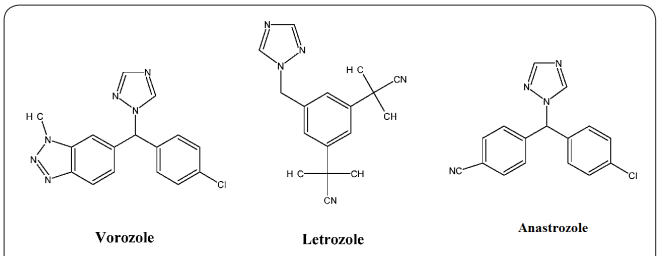

Figure 2. Some of triazole derivatives used in the treatment of breast cancer.

(33). For the mobile phase used in the analysis, flow rate have been made ml min^{-1} by using the column of inertsil 5µ C-18 (15 cm x 4.6 mm) at 250 nm in the mixture of 30 mM KH_2PO_4 - methanol (% 82,5 – 17,5; pH:4).

Determination of vitamin C and MDA in blood samples

Proteins were precipitated by taking 0.3 mL of the serum sample and adding 0.3 mL 0.5 M $HClO_4$ to it. Then, total volume was completed to 1 mL by adding clear water to it. After centrifuging for 15 minutes (2500 rpm), the mixture was analyzed by HPLC by taking 20 µL carefully from the clear part of the samples. For mobile phase used in the assay, flow rate was measured in units of mL min^{-1} by using the column of inertsil 5µm C-18 (15 cm x 4.6 mm) at 250 nm in the mixture of 30 mM KH_2PO_4 - methanol (82.5-17.5%; pH: 4) (33).

Determination of vitamins A and E in blood samples

After serum samples taken from the deepfreeze were dissolved, proteins were precipitated by adding 0.3 mL of ethyl alcohol containing 1% H_2SO_4 to 0.3 mL of the serum sample. After being put into vertices, the mixture was centrifuged at 2.500 rpm for 5 minutes. 250 µL of n-hexane was then added to samples. Vitamins dissolved in fat in the medium with hexane addition were extracted into the hexane phase. After the addition of the hexane and being mixed in the vortex again, the tubes were centrifuged. At the end of centrifuging, the hexane phase was taken into the glass tube by careful separation. After adding 250 µL of n-hexane to the sample and mixing, the mixture was centrifuged and the n-hexane phase was combined with the hexane phase in the glass tube. The extracted hexane was removed carefully under dry nitrogen. The residue was dissolved in 100 µL of methanol. It was analyzed by HPLC (HPLC column: 5µm C-18 (15 cm x 4.6 mm)). In the samples, vitamin E was analyzed at a wavelength of 296 nm and vitamin A was analyzed at a wavelength of 326 nm.

Measurements in the samples of *Saccharomyces cerevisiae* as *in vitro* antioxidant method

S. cerevisiae yeast cells were used to determine antioxidant activity. *Saccharomyces cerevisiae* was inoculated in malt extract broth and was incubated at 25 °C in an oven, and experiments were conducted by taking 10^6 cells mL^{-1} at the exponential phase. These cells are often used as a model in studies of metabolism response to oxidative stress on the molecular level (34). Each solution containing a 2 mL cell was placed into a test tube for MDA measurements. Substances were dissolved in DMSO, their solutions were prepared at precise concentrations, and final mixtures were added to the tube in samples of 50 µM and 100 µM. Adjustments were made for all experiments to make the amount of mixtures equal to the amount of DMSO in the tubes. Tubes containing the same amounts of DMSO were used as the control group. After the addition of the substances, certain durations were estimated. Considering the relevant literature, attention was paid for cell number to be 10^6 cell mL^{-1}, and doses were determined taking solubility of the substances into account. The cells treated with chemical substances for MDA analysis were mixed by adding 250 µL of 15% of trichloroacetic acid (TCA) and 750 µL of 0.5 M $HClO_4$. Cells were divided into smaller samples, and after centrifuging at 4500 rpm for 5 minutes, supernatant were analyzed by HPLC.

Investigation of *in vitro* antitumor properties

Antitumor activity evaluation cell cultures

Human breast cancer cell lines (MCF-7) were maintained in Dulbecco's Modified Eagle Medium culture supplemented by 4 mM L-glutamine, 4500 mg L^{-1} glucose (10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin - streptomycin), and with addition of 10 mM of nonessential amino acids for culture of breast cancer cells. The cells were preserved at 37 °C in 5% CO_2 humidified incubator.

MTT assay

MCF-7 cell lines were treated with concentrations of 0.1 µM, 1 µM, 10 µM and 100 µM of the test compounds L1, L2, L3 and L4. The cytotoxic properties of the compounds L1-L4 against MCF-7 cell lines were determined using MTT assay (3-(4,5-dimethylthiazol)-2-yl]-2,5-diphenyl-2H-tetrazolium bromide). MTT was transformed by active mitochondria to form a dark blue

formazan which was detected by a microplate reader.

The MTT method provides a simple way to detect living and growing cells without using radioactivity. Briefly, 15 x 10^3 breast cancer cells were plated in triplicate in 96-well flat bottom tissue culture plates, and treated with DMSO (for dissolvent control group) and different concentrations of test compounds in DMSO. The cells were then incubated for 24 and 48 hours at 37 °C in 5% CO_2 humidified incubator. After 24 and 48 hours, MTT (0.005 g mL^{-1} in phosphate buffer saline) was added to the cell culture and incubated for 3 hours. The formazan crystals formed during the reaction of active mitochondria with MTT were dissolved in 0.04 N (100 mL) of isopropanol and measurements were taken in a microplate reader (Biotek Synergy) using a 550 nm filter (35). Relative cell viability (%) was expressed as a percentage of the untreated control cells. Each data represented an average of 10 measurements. Test compounds (L1-L4) were dissolved in DMSO. There was no effect of 0.2% DMSO on any of the measured parameters. All cellular results were compared with control cells line.

Statistical analysis

All of the tests and analyses were performed in triplicate and then their average was calculated. SPSS 15.0 statistical software package was used for statistical analysis. Comparison among experimental groups and control groups was made using analysis of variance (ANOVA) and LSD (Least significant Difference) tests. Results were expressed as mean ± SD. $P < 0.05$, $P < 0.01$ and $P < 0.001$ values were used to measure the significance of the differences between the control and the other groups.

Results and Discussion

Measurements in samples of *Saccharomyces cerevisiae* as *in vitro* antioxidant method

S. cerevisiae is very useful in investigation of cell cycle, because it is easy to culture, and has complex internal structures of animals and plants, because it is an eukaryote. Eukaryotes are the first organisms whose genome sequence was determined (36). Due to its favorable properties, *S. cerevisiae* was preferred in this study as a material. Results related to MDA levels of *S. cerevisiae* yeast cells treated with test substances are given in Table 2 and Figure 3.

According to the results, 50 µM and 100 µM concentrations of the substances L1 and L4 significantly decreased MDA levels in comparison to the control group, and the amount of change was close to the level of significance in the L3 substance. The substance L2 increased the MDA levels significantly in comparison to the control group and showed significant differences in comparison to other test substances.

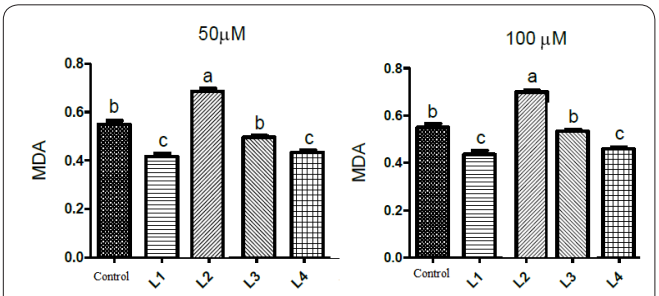

Figure 3. MDA leves of *Saccharomyces cerevisiae* yeast cells treated with compounds L1-L4.

Statistically significant differences emerged in all other test compounds of 50 µM and 100µM concentrations compared to control, except for the L3 compound. The compounds L1 and L4 significantly decreased the level of MDA. The compound L2 significantly increased the level of MDA when compared to the control group. For the compound of L3, although the difference was not found highly significant, a decrease in MDA levels was observed in comparison to the control. Thus, the compound L2 showed pro-oxidant effect by increasing the MDA level relative to other compounds. Previous studies determined that the compound L2 has low activity in other antioxidant methods. Additionally, the L2 compound's amplifying effect on levels of MDA, which is the indicator of lipid peroxidation, may imply that L2 has no effect as an antioxidant and it may even have a negative effect. L1 and L4 compounds showed good activity in other antioxidant methods. A connection may be established through the reduction of MDA levels in this method by the compounds of L1 and L4. The findings support the idea that these compounds have antioxidant potential. In the literature, there are a limited number of studies on MDA analysis of 1,2,4 - triazole in S. cerevisiae cell culture. Hence, this study may contribute to the elimination of shortcomings in this aspect.

Investigation of in vitro antitumor properties MCF-7

The results related to the viability rate of the MCF-7 (human breast cancer) cells treated with test substances are given in Table 3 and Figure 4.

Based on the results of the analysis, the L4 substance in 0.1 µM concentration, the L2 substance in 1 µM concentration, the L1 substance in 10 µM concentration and the L4 substance in 100 µM concentration were substances which destroyed the cancer cells in the most significant amounts ($P < 0.001$). Besides, as it can be seen in Table 3, when compounds were compared

Table 2. Average values by doses of MDA leves of *Saccharomyces cerevisiae* yeast cells treated with compounds.

Parameters (mg/2.10^6cell)	Groups					
	Control	**L1**	**L2**	**L3**	**L4**	*P*
MDA 50 µM	0.55±0.02^b	0.42±0.02^c	0.69±0.01^a	0.50±0.01^b	0.43±0.01^c	***
MDA 100 µM	0.55±0.02^b	0.44±0.02^c	0.70±0.01^a	0.54±0.01^b	0.46±0.01^c	***

a-c Difference among groups with different letters in the same row are statistically significant.
*P < 0.05;**P < 0.01;***P < 0.001

Table 3. Viability status of MCF-7 cells treated with compounds according to the dose rate in %.

% Cell Viability (MCF-7)	Groups						
	Control	Dissolvent Control	L1	L2	L3	L4	*P*
0,1 µM	100±0,00^{a}	94,05±0,83^{ab}	84,33±1,21^{c}	88,72±2,17^{bc}	93,36±2,21^{ab}	75,67±3,56^{d}	***
1 µM	100±0,00^{a}	94,05±0,83^{ab}	88,08±1,72^{bc}	73,49±2,45^{e}	83,90±1,53^{cd}	77,34±3,9^{de}	***
10 µM	100±0,00^{a}	94,05±0,83^{a}	67,23±1,76^{c}	76,45±3,41^{bc}	79,78±3,00^{b}	76,11±1,8^{bc}	***
100 µM	100±0,00^{a}	94,05±0,83^{a}	34,25±1,58^{b}	37,77±1,56^{b}	25,54±2,58^{c}	22,87±1,47^{c}	***

a-e Difference among groups with different letters in the same row are statistically significant.
*P<0.05; **P<0.01; ***P<0.001.

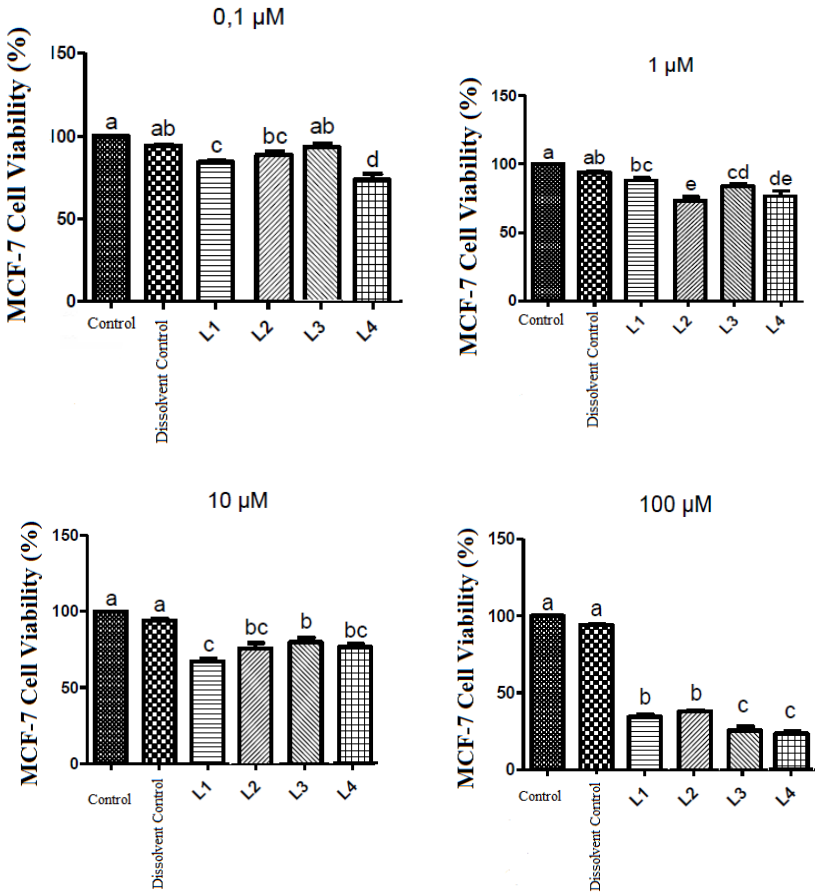

Figure 4. The cell viability results of MCF-7 cells with test compounds L1-L4.

among themselves and to the controls, the L3 substance showed significant differences in some concentrations.

Results show that all of our test substances show statistically significant differences in comparison to control and solvent groups. Additonally, significant differences were observed in comparison of the substances among themselves. Based on the analysis, the compound L4 mostly decreased the viability of MCF-7 cancer cell lines in 0.1 µM concentration. Moreover, the compound L4 in 0.1 µM concentration had highly significant effects in comparison to the control group and the groups where other compounds were used. The other compound that drew attention in this concentration and decreased the viability of the cell significantly was the compound L1. In concentration of 1 µM, the compound that significantly decreased MCF-7 cancer cell viability was L2. The L2 and L4 compounds showed similar magnitudes of effect. The compound L1 in 10 µM concentration significantly decreased the viability of MCF-7 cancer cell lines. In 100 µM, which was the highest concentration used, the compound L4 decreased the viability of cancerous cell lines and showed the highest effect. Considering all these results, particularly the substance L4 showed the most effective antitumor activity. Additionally, the substances L1 and L2 showed the most effective anticancer activity. L3 particularly showed good activity in 100 µM. Thus, almost all compounds had antitumor effects at certain doses.

The most noteworthy compounds may be L2 and L4 in the light of these data, because, while the compound L2 was ineffective in antioxidant methods, it increased MDA in Saccharomyces cerevisiae cell culture. It had toxic effects on MCF-7 cancerous cells as in vitro. The data led to a deduction that the compound L2 has a cytotoxic effect. The compound L4 was the compound that showed the best antitumor activity in the lowest concentration of 0.1 µM and 100 µM. Furthermore, the compound L4, unlike the compound L2, had antioxidant

activity and was effective as antitumor. Therefore, the compound L4, showing antioxidant effect, decreased the oxidative stress load in the cells, and had a different mechanism that was effective in triggering antitumor effects in comparison to the compound L2.

In order to support this study, various studies in the literature on the anti-tumor activity of 1, 2, 4 - triazole ring derivatives were utilized. Regarding the structure of some drugs used as anticancer, there are various studies that explain the presence of 1, 2, 4 - triazole derivatives and thiazole derivatives considered as the drugs' iso-esters (37). Mannich base derivatives of 1,2,4-triazole compounds are known to be used as anti-tubercular, antimalarial, anticancer and analgesic drugs. Today, various methods have been developed and compounds with different structures are being used in this context for treatment of cancer. However, cancer is still one of the diseases with higher probability of death. In one of the recent studies, arilidenhidrazids containing 1, 2, 4 - triazole ring were synthesized as compounds effective against breast cancer (38). For utilizing the antitumor properties of compounds containing 1, 2, 4 -triazole, different molecules' derivatives have been synthesized and they continue to be synthesized. A base derivative of 1, 2, 4 - triazole ring compounds has been synthesized, and the base derivate of acetic acid hydrazides was an effective antitumor agent solely in breast cancer, while the compounds 2-phenyl-ethyl amino and 2-phenyl-ethylamine were effective antitumor agents in non-small cell lung carcinoma and breast cancer. Some compounds like therapeutic agents Vorozole, Letrozole and Anastrozole containing azole rings such as 1,2,4 - triazole rings are also being used in the treatment of breast cancer (Figure 2) (39).

There are 1,2,4 - triazole derivatives which have been developed for usage in treatments of cancer as antitumor drugs. 4-amino-3-phenyl-5-oxo-4,5-dihydro (1,2,4) triazole-1-il-acetic acid 2,4-dichlorbenzylhidrazit has anticancer effects against breast cancer (40). These studies support the data and the antitumor effects found in this study. Thus this study may contribute to further research by scientists who are working on these issues.

Vitamin A, E, C and MDA levels in liver tissue as in vivo measurement of antioxidant activity

The effects of test compounds on vitamins A, E and C in liver tissue and MDA are given in Table 4. Considering the results of control and compounds among themselves, it is seen that the compound L2 significantly decreased the concentration of vitamin C in comparison to both the control group and other compounds.

When data were evaluated, it was seen that the compound L2 also had a significantly different effect in the liver tissue in which it was implemented in terms of vitamins A, E and C, relative to other compounds and the control group ($P < 0.05$). After the implementation of all compounds except for the substance L2, a significant difference was observed compared to control group.

In the groups where the compounds L3 and L4 were implemented, a lower vitamin C concentration was observed in comparison to the control group, although the difference was not significant. In the groups where the compound L1 was implemented, it was seen that vitamin C concentration appears to be close to the level of the control group. When we analyze the effects of compounds on MDA levels, there was not any statistically significant difference in experimental groups in comparison to the control group. Nevertheless, it is noteworthy that the compound L2 increased MDA levels more than the control group. The fact that the compound L2 increased MDA levels in the method of S. cerevisiae proves that this compound has an activity that increases MDA in both in vivo and in vitro. It is possible to say that the compound L1 decreased MDA levels compared to the control group, although the difference did not come out statistically significant. The levels for the substances L3 and L4 were close to the levels of the control group.

When we investigated the vitamin A levels; the compound L2 significantly decreased the concentration of vitamin A in comparison to control and other compounds. On the other hand, it was detected that the compound L2 significantly decreased the level of vitamin E relative to the control group. Based on the differences, it was detected that other compounds were effective on the level of vitamin E at a degree close to the level in the control group, which was not statistically significant ($P > 0.05$).

Vitamin A, E, C and MDA levels in kidney tissue as in vivo measurement of antioxidant activity

When we look at the control group values and the comparison of the compounds among themselves, it is seen that the compound L2 increased MDA, decreased the vitamin levels, while other compounds decreased MDA at a degree close to the control, holding the vitamins at the same level (Table 5).

In this case, it is seen that the compound L2 increased MDA levels in kidney tissue as well as in liver tissue.

Table 4. Vitamin A, E and C and MDA levels in liver tissue that have been treated with compounds.

Parameters (µg/g)	Groups					
	Control	L1	L2	L3	L4	*P*
C Vit	20,72±1,47[a]	25,18±1,14[a]	7,16±0,95[b]	16,10±0,55[a]	16,28±1,27[a]	***
MDA	4,96±1,08	2,24±0,23	6,01±1,61	5,20±0,95	4,96±0,68	ND
A Vit	0,24±0,01[a]	0,26±0,02[a]	0,11±0,02[b]	0,21±0,02[a]	0,21±0,01[a]	***
E Vit	0,55±0,06[ab]	0,66±0,06[a]	0,31±0,10[b]	0,55±0,04[a]	0,62±0,01[a]	**

a-c Difference among groups with different letters in the same row are statistically significant.
*P<0.05; **P<0.01; ***P<0.001;ND: Not Determined, P > 0.05.

Table 5. Vitamin A, E and C and MDA levels in kidney tissue that have been treated with compounds.

Parameters (µg/g)	Groups					
	Control	L1	L2	L3	L4	*P*
C Vit	9,26±1,09	7,40±1,60	8,40±1,33	8,24±0,27	8,42±0,42	ND
MDA	2,90±0,05^{ab}	2,28±0,22^{b}	3,84±0,33^{a}	2,58±0,17^{b}	2,54±0,45^{b}	**
A Vit	0,24±0,02^{a}	0,24±0,01^{a}	0,11±0,01^{b}	0,24±0,02^{a}	0,26±0,02^{a}	**
E Vit	0,74±0,06^{b}	1,03±0,01^{a}	0,31±0,05^{c}	0,75±0,01^{b}	0,71±0,08^{b}	***

a-c Difference among groups with different letters in the same row are statistically significant.
*P<0.05; **P<0.01; ***P<0.001; ND: Not Determined, P>0.05.

It is seen that other test compounds values are at the level close to each other, and it is detected that they are not statistically significant; they have decreased MDA level compared to control group. On the other hand, it is seen that all other compounds do not have statistically significant effect on vitamin C level both in control and the comparisons among themselves. When we investigate the levels of vitamin A, it has been detected that the compound of L2 has significantly decreased the level of vitamin A compared to both control and other compounds. Also, it has been detected that the compound of L2 significantly decreased the level of vitamin E.

Vitamin A, E, C, and MDA levels in blood samples as in vivo measurement of antioxidant Activity

As shown in Table 6, according to control and comparison of the compounds among themselves, it was detected that the compound L2 had a more significant effect compared to other compounds. It was seen that other compounds were not effective on MDA and vitamin levels, and the present results were very close to the results in the control group (Table 6).

Looking at the control group values and the results of the compounds among themselves, it was detected that the compound L2 decreased the level of vitamins A, E, C significantly compared to the control group values and other compounds' values. In the groups where other test compounds were implemented, the values obtained were close to the control group values; there was no statistically significant difference. On the other hand, considering the effects on MDA, statistically significant differences were found in the test groups of L1, L3, and L4, where MDA levels were determined as close to the control. The compound L2 was determined to increase MDA levels significantly.

When we evaluate the results in tissue and blood samples, it is seen that MDA levels increased significantly relative to the control in the group where the test compound L2 was implemented. The L2 compound also decreased the levels of the vitamins. Generally, in other test groups, while there was an activity that increased MDA levels compared to the control group, levels were close to the control group in terms of vitamins.

We have not found a study that would be comparable to our in vivo methods with 1,2,4-triazoles in the literature. According to the results of other groups and derivatives, the increase in free radical formation causes an increase in MDA levels, and a decrease in the levels of vitamin E (41-43). In another study where researchers (44) gave antioxidant substances (naringin and probucol) to rabbits with diet, they observed that these substances did not affect plasma MDA and the level of vitamin A, while increasing the level of vitamin E by decreasing its use. Accordingly, in our study, it can be put forward that the compound L2 increases MDA by showing the radical effect on the tissue. Therefore, vitamins decrease by consumption. On the contrary, it can be suggested that the other test compounds also decreased MDA by showing fairly antioxidant effects, and therefore, their use decreased as the vitamins were not consumed. Hence, this resulted in values that were close to the control or slightly higher.

Conclusions

Considering the measured in vitro and in vivo antioxidant parameters and antitumor effects in this study, the results may be seen encouraging. Significant antitumor properties of the substances of L1, L3 and L4 which may be demonstrated with good antioxidant activity, can be the indicators that these compounds showed significant pharmacological bioactivity. Although the antioxidant effect of the compound L2 is low, it was seen that it is effective against breast cancer.

As a result, we think, the chemical compound classes that have been tested within this study and the parameters we investigated in addition to many of the studied pharmacological properties will contribute to the literature. In fact, we believe that our test compounds are

Table 6. Vitamin A, E and C and MDA levels in Blood samples that have been treated with compounds.

Parameters (mg/lt)	Groups					
	Control	L1	L2	L3	L4	*P*
C Vit	4,00±0,19^{ab}	4,52±0,24^{a}	3,00±0,71^{c}	3,88±0,23^{b}	4,036±0,36^{ab}	***
MDA	0,57±0,02^{b}	0,48±0,03^{b}	0,68±0,03^{a}	0,56±0,01^{b}	0,56±0,02^{b}	***
A Vit	0,57±0,02^{a}	0,62±0,03^{a}	0,30±0,09^{b}	0,53±0,06^{a}	0,55±0,02^{a}	**
E Vit	3,34±0,20^{bc}	3,93±0,05^{a}	2,07±0,04^{d}	2,91±0,10^{c}	3,20±0,06^{c}	***

a-d Difference among groups with different letters in the same row are statistically significant.
*P<0.05; **P<0.01; ***P<0.001.

effective to be utilized in the selection and design of model compounds for further studies.

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