

Original Article

In vitro Biological Evaluation of 1,2,4-triazole Mannich Base

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Abstract

Biological evaluation of a 1,2,4-triazole mannich base that has been synthesized for interesting behavior in medicinal chemistry, so it's interesting to synthesize triazole derivatives and investigate their biological properties to evaluate their capacity in medicine. The aim of this study evaluate the antimicrobial, anticancer, and antioxidant activities of a 1,2,4-triazole Mannich base derivative and its power in the treatment of diseases. For antimicrobial activity four bacterial strains and one fungal strain based on the agar disc diffusion. While used two cancer cell lines for anticancer activity which are known as Hep-G2 and MCF-7; the antioxidant activity of the compound used three different radical species include OH[•], ABTS^{•+}, and DPPH[•], and also determine the antioxidant activity of the compound after extracting vitamins A, C, E, and MDA in the *S. cerevisiae* yeast cell by HPLC. The results of this study show the positive role of a derivative 1,2,4-triazole mannich base as antimicrobial and antiradical assays, particularly when treated with *C. albicans* and OH[•] radical that significantly reduced growth of the fungal and the radical compared to others. In addition, it didn't exhibit any cytotoxicity to reduce both cancer cell lines. In conclusion, the compound's ability is different from one assay to another, increasing its concentration efficiency affects the radical scavenging, and inhibits microorganism growth.

Keywords: Antioxidant, Anticancer, Microorganism, Cancer cell line, 1,2,4-triazole.

INTRODUCTION

Heterocyclic compounds have cyclic structures that contain carbon atom(s) as a major atom as well as other different group atoms like nitrogen, sulfur, and oxygen which are known as heteroatom. However, they have several membered forms but the most common is five heterocyclic compounds particularly, those compounds that contain nitrogen in the structure which is low toxic and has a higher ability to be used in the medical area as a drug for any field¹⁻³. Between the heterocyclic compounds, triazole derivative compounds are significant compounds that are composed of two carbon and three nitrogen atoms, due to their structure can react with multiple receptors and proteins by making various bonds⁴. Novel heterocycle compounds that have been used in many fields, particularly chemotherapy applications, also a synthesis of heterocyclic compounds that have high nitrogen in

the structure displayed higher interest in the biological area⁵. Fluconazole is a common drug in the treatment of those pathogens that relate to fungal-like *Candida albicans* which is present triazole in its structure. 1,2,4-triazole is one of the triazole isomers that are present in the composition of several drug structures like antifungal, and others, generally its derivatives have low side effects and are used as a significant compound in medicinal chemistry⁶⁻¹⁰.

Another significant compound that has been used in several pharmacological and biological is Mannich bases, in the chemical structure and composition of drugs are affected by the polar function that rising bioactivities, hydrophilic, and bioavailability which consequently enhance the drug's pharmacodynamic and pharmacokinetic characteristics. Biological activity is the evaluation

role of compounds in the biological process especially antimicrobial and other beneficial activities that will be found in the Mannich bases¹¹⁻¹⁶. Mannich bases in medicinal chemistry have multiple advantages with heterocycles like anticancer, antiviral, anti-inflammatory, and antioxidant^{17,18}. Since these compounds are target structures for many researchers, they have been synthesized, their biological activity assessed, and several are presently undergoing clinical evaluation¹⁹.

Several studies show that a common compound that is carried out in the numerous fields of biology and medicine is a five-membered heterocyclic compound, and its nitrogen-containing structure with high activities against several pathogens like antifungal, antibacterial, antiviral, and others could be found in 1,2,4-triazoles Mannich bases²⁰.

The main radical forms are called reactive oxygen species (ROS) which contain oxygen in the structure and nitrogen in the structure of reactive nitrogen species (RNS). Both species can damage the biological system and cause oxidative damage²¹. About the outer orbital of the radical forms exist unpaired electrons, however, it has extreme energy to damage the cells, proteins, and so on²². Due to ROS containing oxygen in the structure having more influence on the aerobic organisms, also its ability is related to the concentration of radical²³. In addition, radicals count as a factor that has a role in the starting because they can make a defect in the Deoxyribonucleic acid (DNA) action carcinogenesis and other disorders²⁴. However, antioxidants like β -carotene, polyphenols, and vitamins C, E are the main compounds in the human body that maintain the body from lots of diseases by decreasing the concentration of radicals. Antioxidants destroy radicals in various ways like the reduction of superoxide anion ($O_2^{\cdot-}$) into Hydrogen peroxide (H_2O_2) by the enzymatic antioxidants such as catalase and then to those molecules that do not cause damage to the health^{25,26}. Antioxidants' ability to reduce or remove radicals passed in two ways, first by transferring hydrogen to the radical to become

stable or by transferring an electron from the antioxidant^{27,28}.

Modern cancer research places a great deal of focus on the discovery and development of new, selective, and effective anticancer medications because cancer is remained as a serious worldwide problem¹. A significant class of active pharmacological scaffolds are heterocyclic molecules having nitrogen atoms, particularly those rings containing three nitrogen atoms, such as the 1,2,4-triazole ring. Because of these scaffolds' capacity to establish hydrogen bonds with various targets, drugs' pharmacological, pharmacokinetic, and toxicological characteristics are improved. For the treatment of estrogen-dependent breast cancer, 1,2,4-triazole-based medications such as Anastrozole, Letrozole, and Vorozole are currently frequently used²⁹.

This study aims to evaluate a synthesized triazole Mannich base biological activity to know its ability against microorganisms, radical species, and tumors by several assays according to both procedure fundamentals and investigation by instrumental analysis to achieve primary information about its application in the medicine area as an agent.

MATERIALS and METHODS

The 2,6-Bis(4-phenyl-5-thioxo-4,5-dihydro-1,2,4-triazol-3-yl)-pyridine synthesized compound was taken by the Chemistry department at Firat University (as shown in Figure 1)³⁰.

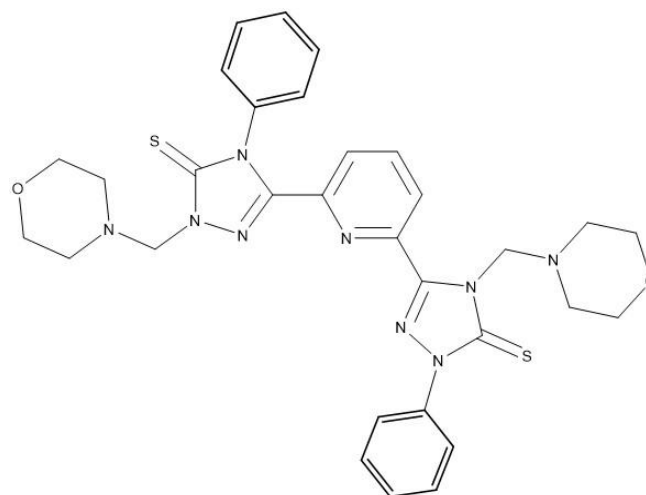


Figure 1. The structure of the synthesized triazole Mannich base derivative.

Antimicrobial assay

The study used the strains of *Bacillus megaterium* DSM32, *Escherichia coli* ATCC25322, *Klebsiella pneumoniae* ATCC700603, *Staphylococcus aureus* ATCC25923, and *Candida albicans* FMC17 to determine the antibacterial activity of the derivate. The microorganisms were received from the culture collection of Firat University's microbiology lab. Prepared fungus and bacteria in broth culture (10^6 bacteria/mL, 10^4 fungus/mL). In addition, 100 μ l of the compound was impregnated with 6 mm diameter discs, and each of them was placed into Petri dishes. After that, the yeast-infected plates were incubated for 72 hours at 25 ± 0.1 °C; the bacteria-inoculated plates were incubated for 24 hours at 37 ± 0.1 °C. Nystatin 30 mg/disk and streptomycin sulfate 10 mg/disk were used as standard controls. The inhibition zones on the medium were measured as mm after the incubation period³¹.

Antiradical assays

Dimethyl sulfoxide (DMSO) was used as a solvent to dissolve the synthetic compound and standard antioxidant at a concentration of 5000 M. Using the Brand-Williams et al. (1995) approach, the 2,2-diphenyl-1-picrylhydrazyl method was used to determine a compound's ability to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH[•]) radical. In a spectrophotometer, the mixture's absorbance was measured at 517 nm³².

The method of Re et al. (1999) was used to determine the spectrophotometric analysis of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{•+}) radical cation scavenging capacity, and product absorbance was measured at 734 nm³³.

According to Halliwell et al. (1987), the novel derivative's inhibitory capacities against hydroxyl radical (OH[•])-mediated peroxidation were tested. The solution's absorbance at 532 nm wavelength was used to measure the degree of oxidation that 2-deoxyribose³⁴.

HPLC quantification of MDA and vitamins C, A, E

Antioxidant activity was assessed using *Saccharomyces cerevisiae* yeast cells. A single-

celled microbe called *S. cerevisiae* belongs to the fungi kingdom. The yeasts have significant vitamin content, which raises their nutritional worth. The molecular responses to oxidative stress metabolism are frequently studied using these cells as a model³⁵. *S. cerevisiae* was found in dry yeast samples that were kept at +4 °C for the duration of the investigation. In the microbiology laboratory, preparation, culture of microorganisms, the addition of chemicals, and incubation of those chemicals were done to examine the impact on microorganisms. Malt Extract Broth (Difco) was inoculated and incubated for 48 hours at 25 ± 1 °C to prepare *S. cerevisiae* reproduction and growth for use in the experiment. The produced yeast broth culture is injected into YEDP (1 g yeast extract for 100 mL, 2 g glucose, 2 g Bacto Peptone, and 2 g agar) into the medium at a rate of 1% (10^4 yeast/mL) at 25 ± 1 °C for 48 hours.

The levels of malondialdehyde (MDA) and vitamin C were measured using Karatepe's method with a few minor adjustments. The cells were quickly collected and delicately washed twice in 2 mL of ice-cold water. After the incubation period, use Krebs-Ringer-Hepes buffer (128 mM NaCl, 20 mM Hepes, 1.4 mM MgSO₄, 1.4 mM CaCl₂, 1 mM NaH₂PO₄ and 5.2 mM KCl, pH=7.4). Following that, 0.1 mL of 0.5 M perchloric acid and 0.1 mL of water were added to the mediums. After removing the cells from the tubes, the lysates underwent a 5-minute centrifugation at room temperature. The supernatant was collected, and 17.5% methanol (v/v) was separated in a mobile phase of 30 M monobasic potassium phosphate buffer (pH=3.6).

Cellular lipid-soluble vitamins were measured based on Catignani's technique. For the purpose of precipitating proteins, 200 μ L of ethanol: sulfuric acid (99: 1) and 100 μ L of water were added after the compounds were incubated in 100 μ L of cell suspensions. It was thoroughly mixed with a vortex before being centrifuged for five minutes at 4500 rpm. The centrifuged materials were then mixed with 100 μ L of n-hexane (0.05% butylated hydroxytoluene). Hexane was added, and this extracted the lipid-soluble vitamins from the medium into the hexane phase. Before a second centrifugation, the tubes were combined on a

vortex. The hexane phase was carefully removed from the centrifuge at the end and placed in the glass tube. The material was once more combined and centrifuged after receiving 100 μ L of n-hexane. Nitrogen gas was used to gently evaporate the extracted hexane phase. In 100 μ L of mobile phase (methanol/ acetonitrile/ chloroform, 47:42:11, v/v), the hexane residue was dissolved. We took 20 μ L of this solution and injected it into the High-Performance Liquid Chromatography (HPLC).

Anticancer and cytotoxicity assay

This study used two cell lines which included Hep-G2 and MCF-7 abbreviations for Liver Hepatocellular Carcinoma and Michigan Cancer Foundation-7 respectively, from the Biotechnology Institute at Ankara University. To evaluate cell proliferation and cytotoxicity of the compound, the assay based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method was done. In the beginning, cell lines were cultured in 25 cm² flasks that contained DMEM (1% of both L-Glutamine and Penicillin-Streptomycin, 10% FBS) at 37°C under 5% CO₂ atmosphere, and covered the flask. Moreover, the flask was washed with 5 mL of sterile Phosphate buffered saline, to separate from the medium, increased 1 mL of Trypsin-EDTA, and again incubated in an oven with 5% CO₂. After that, to isolate the supernatant from the cells was centrifuged for 5 min. The assay was done at Firat University by the Medical School in Anatomy department. The cells were placed in 96-well plates (2 \times 10⁴/well) for 24 h at 5% CO₂. Then prepared our compound with various concentrations (5000 μ M, 2500 μ M, 1250 μ M, 650 μ M), after pouring 100 μ L of each concentration (12 times) into the 96-well plates and incubated for 3 days at 37°C with 5% CO₂ atmospheric. In the last step, removing the media from the cells, 20 μ L of stock MTT (5 mg/mL) which was prepared in neutral pH (7.2) solution was added and then incubated for 4 hours at 37 °C in a dark area under 5% CO₂. After incubation of the plates, a crystal form was produced that is called formazan, and it was dissolved by 100 μ L of DMSO and stayed in a place that was dark for 10 min. Also, standard Doxorubicin (2.5 ng/mL) as a positive control,

about negative control was used as the medium. Then, measured the compound activity against cancer cell lines at 450 nm by an enzyme-linked immunosorbent assay (ELISA) instrument.

RESULTS

Antimicrobial activity

According to the disk diffusion method, Figure 2 illustrates the result after observing the inhibition around the disks and measuring the values by millimeter (ruler). Based on the result the inhibition zone of the two bacterial groups was the same, *S. aureus* was more inhibited by the compound if compared with the standard, and our compound was powerful against this bacteria group. The *C. albicans* is fungal and was destroyed higher by the compound.

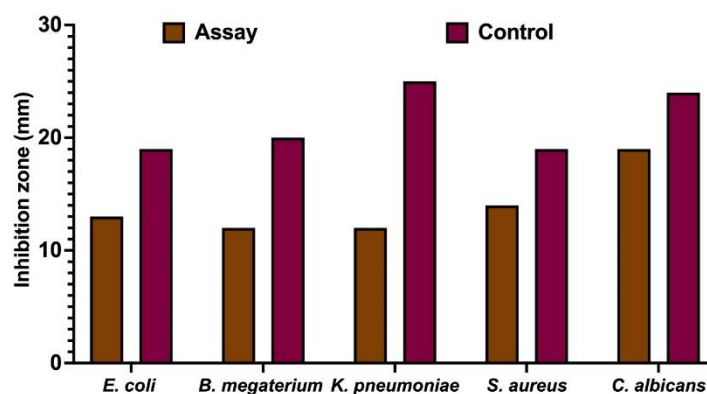


Figure 2. The inhibition zone area of the triazole derivative compound against the growth of various microorganisms.

Antiradical activity

In scavenging radical species by the compound, we observed the results (shown in Figure 3) were the importance of our compound to remove radicals when added the compound to those solutions that contained radicals. If compared between the standard used known as tert-butyl hydroxyl toluene (BHT) and the compound, it had excellent activity in reducing radical concentration in all radical species after being measured by the device. However, the results changed based on the radical types and compound values. If observed the result of scavenging DPPH[•] radical was very low at 100 μ L due to the radical (unpaired electron) located in the center of DPPH[•] and the antioxidants that have huge structure difficulty reacting with it. At 500 μ L of OH radicals, the radical ratio was decreased in the solution and more than the standard, so the

compound has more energy to interact with it and may convert it to a harmless form *in vitro* also *in vivo*, due to present OH[•] radicals inside the body, while two other radical species don't present.

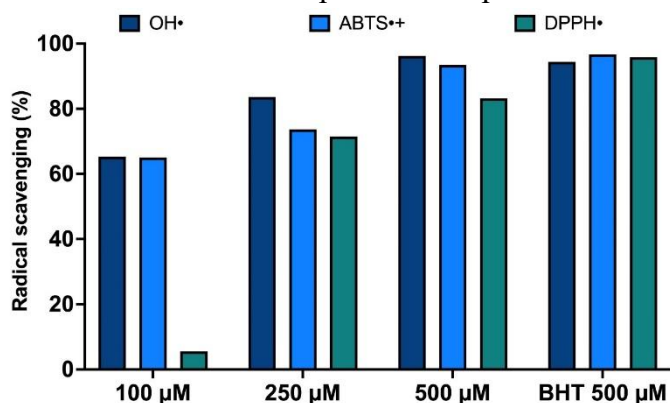


Figure 3. *In vitro* radical scavenging activity of triazole derivative against three radicals at different concentrations.

Table 1. Vitamins and MDA values in the yeast cell

Group	Vitamin A/(ppm)	Vitamin E/(ppm)	Vitamin C/(ppm)	MDA/(ppm)
Sample 50 μL	0.07±0.015	0.81±0.07	0.86±0.06	23.67±0.92
Sample 100 μL	0.09±0.002	0.89±0.04*	0.91±0.05	18.17±0.65*
Control	0.08±0.009	0.79±0.06	0.82±0.02	23.12±0.26

*p < 0.05 means statistically was significant

D. Anticancer activity

Figure 4 indicates the result of treating the compound with both cell lines based on the MTT method didn't show any interesting activity and cytotoxicity to reduce cell lines, the MCF-7 cell line was reduced but not high if compared to the cytotoxicity of doxorubicin as a reference, and the activity changed based on the compound concentration. While cancer cell lines Hep-G2 was not affected by the compound.

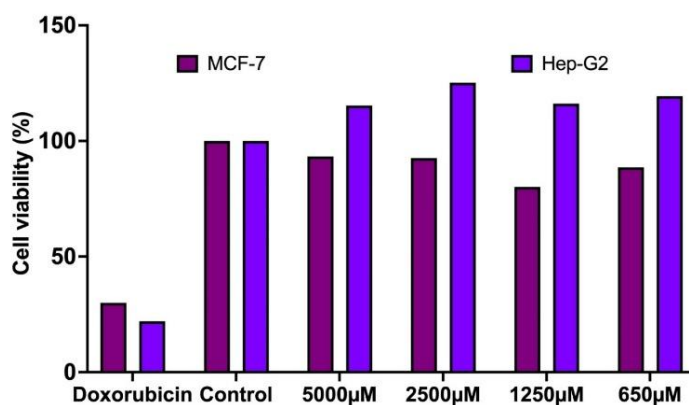


Figure 4. Cytotoxicity of triazole derivative against MCF-7 and Hep-G2 cancer cell lines

HPLC quantification of MDA and vitamins C, A, E

To determine the antioxidant power of the compound in the value of the increasing vitamins and decreasing MDA value during the increasing amount of compound when treated with the yeast that contained targets. The results displayed concentration of vitamins A, C, and E raised with the compound but when treated with 50 μL of compound lower than when combined with DMSO, the most common factor may during extraction a portion of vitamin A remained in the solution. In addition, it has a significant role in decreasing MDA concentration which was inversely proportional to the compound concentration (Table 1. shows the results).

DISCUSSION

Agar-well diffusion was used by Bayrak et al. to synthesis a few novel 1,2,4-triazoles and assess their antibacterial activities. 4-Amino-2 [(4-methylpiperazin-1-yl)methyl] is one of them has antibacterial efficacy against *S. aureus*, *P. aeruginosa*, *B. cereus*, and *E. coli*³⁶.

The compound was evaluated for its antimicrobial properties. Based on the results, the compound showed poor activity compared with Streptomycin sulfate and Nystatin. While the *C. albicans* is and was destroyed higher by the compound if compared to other organisms, lots of studies confirmed that the triazole drugs are more known as an antifungal agent³⁷⁻³⁹. Triazole forms can arrest the enzyme that is responsible for ergosterol biosynthesis, due to it being a significant agent to maintain the fungal wall.

Koparir et al. had carried out the synthesis and assessment of antioxidant activity of new derivatives of 4-substituted-5-(2-thienyl)-2,4-dihydro-3H-1,2,4-triazole-3-thiones⁴⁰. It was

discovered that every chemical has a significant antioxidant activity. Compared to ascorbic acid as a reference, the three triazole compounds exhibited the strongest DPPH radical scavenging activity at all concentrations. However, the derivatives of 4,5-disubstituted-1,2,4-triazole-3-thione compounds synthesized by Nadeem et al. were examined for their antioxidant activity using DPPH radical. The results showed a considerable reduce in the concentration of DPPH radical because of these compounds' ability to scavenge radicals⁴¹.

Utilizing diverse *in vitro* systems, such as the interaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH), scavenging of superoxide radicals, and microsomal NADPH-dependent suppression of lipid peroxidation (LP), researchers have examined the antioxidant capabilities of 1,2,4-triazole derivatives⁴²⁻⁴⁵.

The compounds described here were examined for their capacity to decrease LP and increase vitamin levels in *S. cerevisiae* as well as their ability to scavenge DPPH[•], ABTS^{•+}, and OH[•] radicals. To find out how well the chemical scavenges radicals, preliminary screening was done at various concentrations. The compound was discovered to be effective at removing OH[•] radicals. The reference antioxidant BHT displayed superior DPPH[•] and ABTS^{•+} scavenging abilities under these circumstances.

Enzymatic and non-enzymatic systems are the two main categories of cellular antioxidants⁴⁶. The non-enzymatic low molecular weight antioxidant molecules vitamins C and E are used up by the metabolism and may drop below normal levels. Analyses of the levels of antioxidants and MDA are crucial for assessing oxidative stress in biological systems.

The cells that are actively metabolizing create ROS continually. But like all living things, *Saccharomyces cerevisiae* has powerful antioxidant defense systems that detoxify ROS as they are produced and keep the intracellular redox environment in reduced form. When ROS exceeds these defenses, oxidative stress happens, causing physiological malfunction, genetic deterioration, and ultimately cell death. In

comparison to people and other animals, *S. cerevisiae*, a typical eukaryotic model microbe, has several benefits due to its straightforward structure, well-known genealogy, and simplicity in manipulation. Additionally, the *S. cerevisiae* genome exhibits an astonishingly high degree of functional conservation with the human genome and the genomes of other higher eukaryotes. Due to these benefits, *S. cerevisiae* has gained popularity as a model for assessing some cell damage to swiftly offer functional clues⁴⁷.

Numerous studies have been conducted to clarify the oxidation/antioxidation mechanisms, provide solutions, and reduce the negative effects of oxidative stress. These mechanisms are crucial to all biological systems, but particularly to humans. Mammalian cells and yeast cells are similar. Therefore, apoptosis, aging, and different illnesses brought on by oxidative stress are crucial in recent studies. The impairment of either the cellular transduction signaling or cellular regulatory system, intracellular macromolecule production could be used to explain the cytotoxicity of xenobiotics. We select antioxidant vitamins and MDA to determine the test compound's pro-oxidant and antioxidant activity⁴⁸.

Membranes undergo structural and functional changes as a result of LP and increased MDA generation, leading to cell death⁴⁹. One of the most significant indications of LP is MDA, which may be evaluated in a variety of techniques^{50,51}. The findings demonstrate that the chemical significantly increased vitamin E levels and statistically decreased MDA concentrations in *S. cerevisiae* yeast cells, both of which have not before been reported. This suggests that by reducing LP in the cells, the chemical supplements were able to preserve the vitamin E concentrations.

Because the antioxidant function of vitamin E uses all processes, including destroying free radicals, chain breaking, suppression, and rebuilding damaged structures, it has a very high and large antioxidant capacity⁴⁶. The thiol, thiosulfonic acid, and phosphorothioate and their derivatives possess antioxidant activity, according to investigations on the antioxidant activity of

1,2,4-triazoles. This action can clarify the relationship between aromatic rings containing thiols and biological function⁵².

Sunil et al.⁵³ created a number of 5-substituted-4-amino-3-mercapto-1,2,4-triazoles and their Mannich and Schiff base derivatives. The MTT assay was used to test a few of the compounds for their ability to cause cytotoxicity against the HepG2 human hepatocellular liver cancer cell line. One chemical in this investigation had an IC50 value of 0.018 g/l, which is comparable IC50 value of doxorubicin was 0.017 g/l. while, synthesis 2-[4-Phenyl-1H-1,2,4-triazole-5-thione-3-yl(methylene)] by Al Soud et al., it was assessed for its anticancer activity in vitro against a panel of 60 human tumor cell lines that were generated from nine different cancer types by using the NCI technique. The nine cancer types—non-small cell lung, ovarian, breast, prostate, colon, CNS, melanoma, leukemia, and renal cancers—were not responsive to this drug⁵⁴.

For the anticancer assay, the triazole compounds were considered to destroy cancer cell lines like standard ($P < 0.05$), while it tested and observed to kill both MCF-7 and Hep-G2 human cancer cells. Triazole compound did not have any anticancer effects when used at the given doses.

CONCLUSIONS

This study concluded that the compound's ability against diseases or factors responsible for health abnormalities changes from one to another. While it has strong antimicrobial properties, it may not be classified as an antibiotic. However, it exhibits an exceptional ability to neutralize reactive species and enhance the production of valuable antioxidant molecules. However, the substance did not exhibit a reduction in cancer cell lines, therefore considering it an ineffective agent against both cancer cell lines.

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Conflict of Interest

The authors declare they have no conflicting interests.

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