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**Research Article**

# SYNTHESIS AND TOXICOLOGICAL EVALUATION USING BRINE SHRIMP LETHALITY ASSAY OF NOVEL 1,2,4-TRIAZOLE DERIVATIVES WITH ANTICANCER ACTIVITY

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**A R T I C L E I N F O**

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**A B S T R A C T**

1,2,4-triazole derivatives are recognized to possess various biological activities as antiparasitic, antifungal, antimicrobial and antiproliferative. The present work reports the synthesis of six new derivatives under microwave irradiation with the purpose of developing new drugs that present high specificity for tumor cells and low toxicity to the organism. The synthesized compounds were characterized by IR, 1H-NMR, and mass spectral data. The present studies widen the scope of the brine shrimp model that may prove quite helpful as a preliminary screen in the anticancer drug designing and synthesis expeditions. In Brine shrimp lethality bioassay, compounds produced dose dependent cytotoxicity effect to brine shrimp nauplii. All the newly synthesized compounds 2a-f were further evaluated for anticancer activity against MCF-7 cell lines using MTT assay and compound 2d could be considered as possible hit as therapeutic agents. The study draws a very good relationship between a simple, inexpensive, and bench-top BSL assay and the antitumor potential of the cytotoxic compounds.



## INTRODUCTION

Triazole based compounds have a wide range of applications in analytical, industrial and medicinal chemistry. Their important biological activities includes antifungal, antibacterial, anticancer and antiviral, H1/H2 histamine receptor blockers, cholinesterase active agents, central nervous system (CNS) stimulants, antianxiety and sedatives[1]. To overcome cancer disease novel molecules are urgently needed, because the pharmacological fight against this disease has made significant progress in the last twenty years[2]. Due to the biological importance, efforts are focused on 1,2,4,-triazole derivatives for the treatment of MCF-7 breast cancer[3-4]. Nitrogen-containing five-membered heterocycles play a vital role in drug discovery to identify novel chemical entities of immense therapeutic potential[5]. The application of anastrozole and letrozole as aromatase inhibitors for the treatment of estrogen-dependent cancer as well as the anticancer properties of ribavirin led to the investigation of many 1,2,4-triazole derivatives in laboratorial conditions for their antitumor activity[6]. Additionally 1,2,4-triazole derivatives have been reported to inhibit several enzymes which play an important role in the expression of tumors such as Protein Kinase CK2, methionine aminopeptidase type II, Janus kinase 2, Tankyrases. Recently 1,2,4-triazoles have been identified as a new class of tubulin polymerization inhibitors[7-9]. Microwave heating is the best method due to the microwave couple directly with the molecule that are present in the mixture, leading to fast rise in temperature, faster reaction and cleaner chemistry. The microwave is also called as green chemistry because it does not produce any hazardous material like gas fumes or heating using external energy source. Microwave uses electromagnetic radiation that passes through material and causes oscillation of molecule which produces heat. Microwave heating produces heat in entire material in the same rate and the same time at a high speed and at a high rate of reaction. Microwave assisted synthesis has become an important tool to the medicinal chemist for rapid organic synthesis. Application of microwave technology in organic synthesis has some of the major advantages like spectacular decrease in reaction in reaction time, improved conversions, clean product formation and wide scope for the development of the new reaction conditions[10-14]. Due to its ability to couple directly with the reaction molecule and by passing thermal conductivity leading to a rapid rise in the temperature, microwave irradiation has been used to improve many organic synthesis[15-18]. Recrystallization can affect physical and chemical stability, apparent solubility, dissolution, bioavailability and bioequivalence and drug product manufacturability, which require special attention during product development as it affects drug product quality,

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protection and effectiveness. It describes to exsist in two or more crystalline phases which have different arrangement of molecules in solid state with different arrangements or conformations of constituents in crystal lattices[19]. Brine shrimp lethality assay is the most convenient system to monitor the biological activity of different species of plants. This approach is very useful for the toxicity evaluation in advance. This assay has many advantages: simplicity and low requirements[20]. However, several requirements need to be fulfilled, especially with standardized experimental conditions (temperature, medium pH, salinity, aeration, and light)[21]. Subsequently animal model for establishment is recommended. Other top assays at the bench are inhibition of crown gall tumors on potato tuber disks, frond proliferation inhibition in duckweed and yellow fever larvae lethality test. Between them, the lethality test for brine shrimps is the shortest, low cost and effective one. The larvae (nauplii; singular nauplius), about 22 mm long, are large enough to observe in a laboratory without high magnification and small enough to hatch in vast amounts without extensive workspace[22-23]. This in vivo test has been employed successively since its introduction for the bioassay-guide fractionation of active cytotoxic and antitumor agents. Furthermore, several studies have shown that there is a strong link between the results for the lethal concentration that kills 50% of the exposed population obtained using artemia salina with the brine shrimp lethality assay[24]. Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the toxicant/cell interaction. This interaction can differ depending on the toxic chemical properties and the cell membrane, as it can occur on the cell surface, in the cell body, or in the tissues below, as well as in the extracellular matrix[25]. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys. Evaluation of a substance's toxic properties is also important when thinking for the safety of public health, since exposure to chemicals can be dangerous and can result in adverse human effects[26]. Breast cancer is the world's leading cause of cancer death and the most common cancer among women. In the family of diseases cancer is known as large family. The development of the cancer cell is abnormal with possible of over run or spread to another section of the body. Neoplasm is formed as subset by them. Cancer cells are formed when normal cell lose the normal regulatory mechanism controlling development and multiplication. If the cancer is present, it is said that it is benign and if the cancer cell invade another section of the body and set up secondary tumor's known as metastasis. From milk duct lining and the lobules supplying the ducts milk, breast cancer most frequently grows in cells. Before the period mostly women observed that there breast become lumpy and tender. Breast cancer mostly observed in above 40 age women. Hence the treatment of breast cancer is depends on the identification breast cancer. We report herein the new series of 1,2,4-triazole derivatives and evaluation of the anticancer activity[27-28].

## MATERIAL AND METHODS

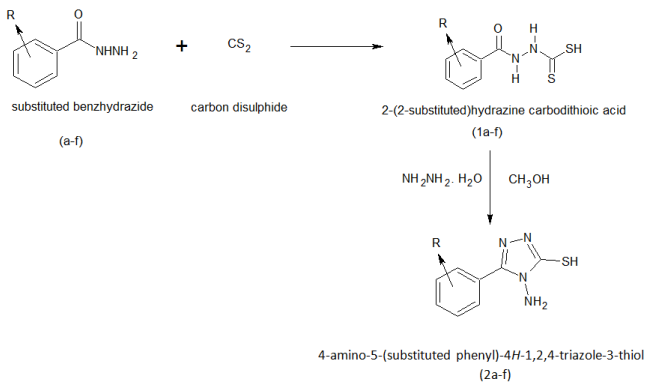
All chemicals and solvents were procured from commercial sources, purified and dried using standard procedures from literature whenever required the regents were purchased from S.D fine, Research laboratory, mumbai and marck laboratory, mumbai. The melting points of synthesized compound were determined by open capillary tube method and are uncorrected. Thin layer chromatography was used confirmation of reaction and the purity of the intermediate and the final compounds by applying a single spot on TLC plate (silica gel G) using various solvents such as butanol, chloroform, water system. TLC plates were visualized under iodine chamber. IR spectra were recorded on FTIR, 1H NMR spectra were performed in DMSO solution using Bruker 300 MHz and their chemical shift are reported in δ unit with respect to TMS as internal standard. Mass spectra were recorded on Pe sciex (model no. API 2000) software analyst 1.4.2 mode: Q1MS Q1/AUTO INJECTION from diya lab, airoli, mumbai.

### *General method for the Synthesis of 2-(2-substituted) hydrazine carbodithioic acid*

Mixture of substituted benzhydrazide (2g) and carbon disulphide (1.5ml) was irradiated for 15 min at 340 watt under microwave. The reaction was monitored by TLC using chloroform: methanol (9:1) as mobile phase.

### *General method for the Synthesis of 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol*

A product of 2-(2-substituted)hydrazine carbodithioic acid was added in hydrazine hydrate (2ml) and methanol (10ml) and mixture was irradiated for 20 min at 340 watt under microwave. The reaction was monitored by TLC using butane: chloroform: water (7:2:1) as mobile phase. The solid product was washed with water and recrystallized with methanol.




**Scheme 1** Synthetic route for the preparation of the title compound (2a-f)

### *Analytical Data of Novel 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol*

**2a. 4-amino-5-(4-chlorophenyl)-4*H*-1,2,4-triazole-3-thiol**

Yield 84%; m.p 152-154$^0$C; IR (KBr, cm-$^1$) 674.20 (C-Cl), 1635.18 (C=C), 2245.38 (C-C), 2478.29 (S-H), 1H NMR (DMSO, 300 MHz, ppm): δ 2.18 (s, 1H), 5.41-5.48 (2H, d, ArH), 7.58-7.64 (q, 2H, d, *J*=6.1 Hz ArH); mass m/z (M+) 226.7.

**2b. 4-amino-5-(4-methoxyphenyl)-4*H*-1,2,4-triazole-3-thiol**

Yield 65%; m.p 145-147$^0$C; IR (KBr, cm-$^1$) 662.34 (C-Cl), 1635.71 (C=C), 2234.82 (C-C), 2484.10 (S-H), 1H NMR (DMSO, 300 MHz, ppm): δ 2.45 (s, 1H), 5.48-5.57 (2H, d, ArH), 7.32-7.39 (q, 2H, d, *J*=6.0 Hz ArH); mass m/z (M+) 222.3.

**2c. 4-amino-5-(4-fluorophenyl)-4*H*-1,2,4-triazole-3-thiol**

Yield 78%; m.p 158-160$^0$C; IR (KBr, cm-$^1$) 698.32 (C-Cl), 1677.89 (C=C), 2242.48 (C-C), 2460.19 (S-H), 1H NMR (DMSO, 300 MHz, ppm): δ 2.91 (s, 1H), 5.78-5.85 (2H, d, ArH), 7.65-7.72 (q, 2H, d, *J*=6.1 Hz ArH); mass m/z (M+) 210.3.

**2d. 4-amino-5-(2-chlorophenyl)-4*H*-1,2,4-triazole-3-thiol**

Yield 89%; m.p 142-145$^0$C; IR (KBr, cm-$^1$) 652.18 (C-Cl), 1642.12 (C=C), 2251.24 (C-C), 2480.10 (S-H), 1H NMR (DMSO, 300MHz, ppm): δ 2.14 (s, 1H), 5.29-5.38 (2H, d, ArH), 7.32-7.45 (q, 2H, d, *J*=6.2 Hz ArH); mass m/z (M+) 226.7.

**2e. 4-amino-5-(2,4-dichlorophenyl)-4*H*-1,2,4-triazole-3-thiol**

Yield 72%; m.p 149-151$^0$C; IR (KBr, cm-$^1$) 692.11 (C-Cl), 1640.42 (C=C), 2232.18 (C-C), 2489.27 (S-H), 1H NMR (DMSO, 300MHz, ppm): δ 2.71 (s, 1H), 5.28-5.32 (2H, d, ArH), 7.32-7.45 (q, 2H, d, *J*=6.0 Hz ArH), 8.92 (s, 1NH); mass m/z (M+) 261.14.

**2f. 4-amino-5-phenyl-4*H*-1,2,4-triazole-3-thiol**

Yield 81%; m.p 142-141$^0$C; IR (KBr, cm-$^1$) 652.18 (C-Cl), 1642.12 (C=C), 2251.24 (C-C), 2480.10 (S-H), 1H NMR (DMSO, 300MHz, ppm): δ 2.14 (s, 1H), 5.59-5.64 (2H, d, ArH), 7.45-7.52 (q, 2H, d, *J*=6.2 Hz ArH); mass m/z (M+) 192.3.

**Biological Evaluation**

**Brine Shrimp Lethality Assay**

Brine shrimp lethality test has been used as a bioassay for a variety of toxic substances. A general bioassay that appears capable of detecting a broad spectrum of bioactivity, present in synthetic compounds, rather than more tedious and expensive *in-vitro* and *in-vivo* antitumor assays. Furthermore, it does not require animal serum as is needed for cytotoxicities.

***Procedure***

***Preparation of seawater***

38 gm sea salt (without iodine) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

***Hatching of Brine Shrimp***

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank, and shrimp eggs were moved to one side of the tank, and sealed on this side. The shrimp was allowed to hatch for two days and be matured like nauplii. Constant supply of oxygen was rendered during the process of hatching. The hatched shrimps are drawn to the light (phototaxis) and so egg shell-free nauplii from the illuminated portion of the tank was collected. The nauplii was taken by a pipette from the fish tank and filtered to improve visibility in fresh clear sea water, and 10 nauplii was taken carefully by micropipette.

***Preparation of test samples***

In each experiment, 0.5mL of test compound of different concentration i.e (50, 100 and 150µg/mL) was added to brine solution and maintained at room temperature for 24h under the light and surviving larvae were counted. Vehicle treated used as control for the test. Test solutions were used in sets of three tubes per dose. Replicas should be maintained to get accurate results. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a ($ED_{50}$)[29-30].

***Anticancer Evaluation***

***MTT assay and Anti proliferative activity***

The in-vitro anti-proliferative activity was carried out on human carcinoma cell lines namely MCF-7. All the cell lines were grown in DMEM-HG supplemented with 10% heat-inactivated FBS, 2% Penicillin-Streptomycin and 2.5 µg/mL Amphotericin-B solutions. Cell lines were incubated at 37°C in a humidified atmosphere of 95% air, 5% $CO_2$. Following 24-48 hrs. of incubation period, the adherent cells were detached using Trypsin-EDTA solution. Cell count was determined using the Luna automated cell counter based on trypan blue dye exclusion method. Cytotoxicity of the novel 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol derivatives have been determined using MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

***Cell Viability Assay (MTT Assay)***

200µL cell suspension was seeded in 96-well microplates at a density of 25,000 cells/well and incubated for 24hrs, all cells were seeded in duplicates with novel compounds 2a-f having range of concentrations from 50µM-500µM, incubated in a $CO_2$ incubator at 37°C. Treated cells were thereafter incubated with 10% MTT (5mg/ml) for 3 hrs. The culture medium was then aspirated and 200µL dimethyl sulfoxide (DMSO; Sigma-Aldrich, India) was added. 5-Fluorouracil (5-FU) was used as standard. Cell viability was determined by measuring the absorbance on a microplate reader at 570nm. Cell viability was calculated as a percentage of viable cells at different test concentrations relative to the control (5-FU) cells [% cell viability = (A570 of treated cells /A570 of control cells) ×100%] [31].

## RESULTS AND DISCUSSION

***Chemistry***

In first step mixture of substituted benzhydrazide and carbon disulphide was irradiated for 15 min at 340 watt under microwave. The reaction was monitored by TLC using chloroform: methanol (9:1) as mobile phase. A product of 2-(2-substituted)hydrazine carbodithioic acid was added in hydrazine hydrate and methanol and mixture was irradiated for 20 min at 340 watt under microwave. The reaction was monitored by TLC using butane: chloroform: water (7:2:1) as mobile phase. The solid product was washed with water and recrystallized with methanol. The reaction sequence is shown in Scheme 1. Microwave assisted synthesis is faster, better and safer green chemistry approach for the traditional reactions. The time taken for the synthesis of 1,2,4-triazole is drastically

reduced by the microwave assisted synthesis. This technique offers clean, simple, efficient, fast and economic for the synthesis of a number of organic molecules such reaction has new tool in the organic synthesis and highly accelerated rate of the reaction time with an improvement in yield and quality of product. The IR, 1H NMR and mass spectra are fully consistent with the structure.

### *Brine shrimp lethality assay*

All the synthesized compounds (2a-h) were tested for cytotoxic activity by the brine shrimp lethality assay. Among them compounds 2a, 2d and 2e showed a dose dependent cytotoxic activity at concentrations of (2a) 37.05µg/ml, (2d) 25.27µg/ml and (2e) 32.15µg/ml. The remaining compounds exhibited less activity when compared to the other compounds at various concentration levels. The degree of lethality is directly proportional to the concentration of the synthesized compounds.

**Table 1:** Brine shrimp lethality assay data novel of 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol derivatives (2a-f)

| Sr. no | Compound code | $ED_{50}$ (µg/ml) |
|---|---|---|
| 1 | 2a | 37.05 |
| 2 | 2b | 45.21 |
| 3 | 2c | 48.18 |
| 4 | 2d | 25.27 |
| 5 | 2e | 32.15 |
| 6 | 2f | 49.23 |

### *Anticancer activity*

The synthesized 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol (2a-f) were evaluated their anticancer activity on MCF-7 cell lines (50µM-500µM) in order to obtain the effective concentration at 50% of the inhibited cells. The results are expressed as 50% of the total available cells inhibited after 72hr. of incubation. The compound 2d showed good cytotoxicity having $IC_{50}$ of 3.8 µM on MCF-7 cell lines respectively. The results of the MTT assay of these compounds were compared with the results of the standard 5-fluorouracil.

**Table 2** $IC_{50}$ values of the novel of 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol derivatives for anticancer activity.

| Sr. no | Compound Code | $IC_{50}$ values of 1,2,4-triazole in µM |
|---|---|---|
| | | MCF-7 |
| 1 | 2a | 52.4 |
| 2 | 2b | 39.1 |
| 3 | 2c | 58.9 |
| 4 | 2d | 3.8 |
| 5 | 2e | 109.2 |
| 6 | 2f | 35.6 |
| 7 | 5-fluorouracil | 8.2 |

## CONCLUSION

All the synthesized derivatives of novel series were synthesized by microwave method. Synthesis of compounds by the microwave method gives more yield and requires less time to complete the reaction. So, the microwave synthesis better method. The brine shrimp lethality bioassay is considered as a useful tool for the preliminary assessment of toxicity. All the synthesized subjected for anticancer activity among all, the compound code 2d had significant anticancer activity. Electron with drawing groups seemed to be necessary factors in providing higher anticancer activity.

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