Synthesis and biological evaluation of heterocyclic 1,2,4-Triazole scaffolds as promising pharmacological agents

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Abstract

**Background:** Triazole is an important heterocyclic moiety that occupied a unique position in heterocyclic chemistry, due to its large number of biological activities. It exists in two isomeric forms i.e. 1,2,4-triazole and 1,2,3-triazole and used as core molecule for the design and synthesis of many medicinal compounds. 1,2,4-Triazole possess broad spectrum of therapeutically interesting drug candidates such as analgesic, antiseptic, antimicrobial, antioxidant, antiurease, anti-inflammatory, diuretics, anticancer, anticonvulsant, antidiabetic, antimigrain agents.

**Methods:** The structure of all synthesized compounds were characterized by physicochemical properties and spectral means (IR and NMR). The synthesized compounds were evaluated for their *in vitro* antimicrobial activity against Gram-positive (*B. subtilis*), Gram-negative (*P. aeruginosa* and *E. coli*) bacterial and fungal (*C. albicans* and *A. niger*) strains by tube dilution method using ciprofloxacin, amoxicillin and fluconazole as standards. *In-vitro* antioxidant and anti-urease screening was done by DPPH assay and indophenol method, respectively. The *in-vitro* anticancer evaluation was carried out against MCF-7 and HCT116 cancer cell lines using 5-FU and cisplatin as standards.

**Results, discussion and conclusion:** The biological screening results reveal that the compounds T5 (MIC_{BS, EC} = 24.7 μM, MIC_{PA, CA} = 12.3 μM) and T17 (MIC_{AN} = 27.1 μM) exhibited potent antimicrobial activity as comparable to standards ciprofloxacin, amoxicillin (MIC_{Cipro} = 18.1 μM, MIC_{Amo} = 17.1 μM) and fluconazole (MIC_{Flu} = 20.4 μM), respectively. The antioxidant evaluation showed that compounds T2 (IC_{50} = 34.83 μg/ml) and T3 (IC_{50} = 34.38 μg/ml) showed significant antioxidant activity and comparable to ascorbic acid (IC_{50} = 35.44 μg/ml). Compounds T3 (IC_{50} = 54.01 μg/ml) was the most potent *urease* inhibitor amongst the synthesized compounds and compared to standard thiourea (IC_{50} = 54.25 μg/ml). The most potent anticancer activity showed by compounds T2 (IC_{50} = 3.84 μM) and T7 (IC_{50} = 3.25 μM) against HCT 116 cell lines as compared to standard 5-FU (IC_{50} = 25.36 μM).

**Keywords:** 1,2,4-Triazole, Antimicrobial, Antioxidant, Anti-*urease*, Anticancer, SAR
Background:

Triazole is a N-bridged aromatic heterocyclic compound that received a considerable attention in recent years due to their biological activities [1]. The name “triazole” was first use by Bladin in 1855 for describing the carbon-nitrogen ring system C₂H₃N₃[2]. It is a white to pale yellow crystalline solid with a weak, characteristic odour, soluble in water and alcohol, melts at 120 °C and boils at 260 °C [3]. Triazole exists in two isomeric forms such as 1,2,4-triazole and 1,2,3-triazole [4]. The SAR studies of triazole derivative reveals that substitution on positions 3, 4 and 5 of triazole ring can be varied but the greatest changed in physicochemical properties and biological profile is exerted by the groups attached to the nitrogen atom at the 4th position [3]. It favours the hydrogen bonding and also stable for metabolic degradation, which could be favorable in increasing solubility as well as in binding bimolecular targets [5]. The bioisosteric replacement between triazole moiety and its bioisoster triazole (imidazole, pyrazole, thiazole) has received special attention in medicinal chemistry, which represented an efficient concept for the discovery and development of novel triazole drugs, significantly extending the chemical space of triazole scaffolds possessing potent activities or enhancing biological activities [6]. Numerous medicines containing triazole moiety available in market (Fig. 1) are: Antifungal [7, 8, 9, 10]- myclobutanil, tebuconazole, posaconazole, itraconazole fluconazole, paclobutrazole Anticancer [9, 11]- anastrazole, litrozole, vorozole, Antimigrain [9, 12]- rizatriptan and Antiviral [9, 13]- ribavirin.

In spite of these achievements, many clinically important microbial isolates still present a real challenge for physicians because of their high resistance to current antimicrobial agents and also associated with serious and life-threatening infectious disease. Therefore, the need for new class of compounds possessing a broad spectrum of antimicrobial activity that are highly effective against those highly resistant Gram positive, Gram negative bacterial and fungal strain is increasing and becomes the top priority of most government heath institutions as well the world health organization [14].

Human cells face threats everyday because the attack of various viruses, infections and free radicals damage the body cells and DNA. Scientists observed that the free radicals contribute to the ageing process and also contribute in diseases, like cancer, diabetes and heart disease. Antioxidants are the chemicals that stop or limit the damage caused by the free radicals and also boost our immunity [15].
Urea amidohydrolases (*ureases*) have been known as a class of large heteropolymeric enzymes with active site containing two nickel (II) atoms to accelerate hydrolysis of urea into ammonia gas with reaction rate at least $10^4$ over the spontaneous reaction. *Ureases* are widely distributed in nature and are found in a number of plants, algae, fungi and bacteria. Medically, bacterial *ureases* have been reported as important virulence factor implicated in the pathogenesis of many clinical conditions such as pyelonephritis, hepatic coma, peptic ulceration and the formation of injection induced urinary stones and stomach cancer. The catalytic mechanism of action of all *urease* inhibitors have been believed that the active sites of the native enzymes binds three water molecules and a hydroxide ion bridged between two nickel ions. Urea replaces these three water molecules and bridges the two metal ions. In surrounding, the hydrogen-bonding network strongly activates the inert urea molecule and subsequently attacked by the hydroxide ion, forming a tetrahedral transition state. As a result, ammonia is released from the active site followed by the negatively charged carbamate and after that decomposes rapidly and spontaneously, yielding a second molecule of ammonia [16].

Colorectal cancer is the third most lethal cancer worldwide in both males and females with drug resistance and metastasis being the major challenge to effective treatments. Maximum deaths due to colon cancer are related with metastatic ailment. The growth of colorectal cancer is promoted by epigenetic factors, such as abnormal DNA methylation. Targeted therapy is a kind of chemotherapy that specifically targets the proteins that resist the development of some cancers [17].

Palmitic (hexadecanoic acid) acid is the most common saturated fatty acid found in animals, plants and microorganisms of chemical formula $\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$. As its name indicates, it is a major component of the oil from the fruit of oil palms (palm oil). Palmitic acid also found in meats, cheeses, butter and dairy products [18]. The palmitate anion is the perceived form of palmitic acid at physiologic pH (7.4). Aluminium salts of palmitic acid and naphthenic acid were combined during World War II to produce napalm. The word "napalm" is derived from the words naphthenic acid and palmitic acid [19].

1,2,4-Triazole attracts the attention of researchers due to its broad spectrum of biological activities (Fig. 2) such as antimigrain [9, 12], antioxidant [15], anti-*urease* [16], antimicrobial [20, 21], anti-inflammatory [21, 22], anticonvulsant [23], anticancer [24, 25], antiviral [26] and antiparasitic [26].
Results and discussion

Chemistry

The multistep synthetic process of 1,2,4-triazole derivatives (T1 – T20) was depicted in Scheme 1. Initially, ethylpalmitate (Int-i) was synthesized by the reaction of palmitic acid, ethanol and sulphuric acid. Palmitohydrazide (Int-ii) was synthesized from ethanolic solution of ethylpalmitate (Int-i) followed by addition of hydrazine hydrate. 5-Pentadecyl-1,3,4-oxadiazole-2(3H)-thione (Int-iii) was synthesized using Int-ii in alc. potassium hydroxide solution followed by the addition of carbon disulfide and then followed by addition of hydrazine hydrate to Int-iii yielded 4-amino-5-pentadecyl-4H-1,2,4-triazole-3-thiol (Int-iv). Finally, the Int-iv on reaction with different substituted aromatic aldehydes in ethanol yielded the title compounds (T1-T20). The physicochemical properties of the synthesized compounds are depicted in Table 1. The synthesized derivatives of 1,2,4-triazole were confirmed by Infrared (IR) and Nuclear Magnetic Resonance (1H/13CNMR). The spectro-analytical data has been depicted in Table 2. The presence of aliphatic -CH- stretch in all compounds was confirmed at 2990-2879 cm⁻¹. The intermediates (Int-ii, iii and iv) exhibited the -NH stretch in range of 3424-3319 cm⁻¹. The presence of -CONH- group in Int-ii was indicated by appearance of -CONH- stretch at 1630 cm⁻¹. The peak range 1677-1589 cm⁻¹ in Int-iii, iv and compounds T1-T20 indicated the presence of –C=N stretch. The presence of –SH stretching vibrations in Int-iv and compounds T1-T20 were indicated in a scale of 2593-2505 cm⁻¹. The compounds T4, T5 and T6 showed the -OCH₃ stretching vibrations in the range of 2860-2848 cm⁻¹. The presence of phenolic group in compounds T6, T7, T8 and T18 was indicated by peaks in the range of 3483-3400 cm⁻¹. The peak range 701-699 cm⁻¹ of compounds T13 and T14 was indicated the presence of Ar-Br group. The compounds T15, T16 and T17 showed the Ar-NO₂ stretching vibrations in the range of 1545-1424 cm⁻¹. The presence of Ar-Cl group in compounds T10, T11 and T12 was confirmed by the appearance of peaks in the range of 767-750 cm⁻¹. The presence tertiary amine in compound T9 confirmed by the appearance of peak at 3431 cm⁻¹. The presence of aromatic ring in compounds T1-T20 was indicated by the appearance of peak in the range of 1796-1719 cm⁻¹. DMSO was used as solvent for the analysis of compounds by ¹H NMR spectra. The presence of singlet signal at 1.22-2.47 δ ppm and 0.82-0.84 δ ppm indicated the presence of protons of –CH₂ and –CH₃ groups in Int-ii, iii and iv, respectively. Singlet at 2.25 δ ppm and 8.87 δ ppm showed the presence of protons of NH₂ and NH groups in Int-ii, iii and iv, respectively. The presence of proton of SH group was indicated by appearance of
singlet at 3.30 in Int-iv. The findings of elemental analysis of synthesized derivatives were recorded within theoretical results of ± 0.4%. Mass spectra of the synthesized derivatives reflected the characteristic molecular ion peaks.

**SAR (structure activity relationship) studies**

In the synthesized compounds, the substitution on m- and p-position of the aromatic ring with methoxy group improved the antimicrobial activity (compound T5, MIC\textsubscript{BS, EC} = 24.7µM, MIC\textsubscript{PA, CA} = 12.3 µM) against Gram positive (\textit{B. subtilis, P. aeruginosa}), Gram negative (\textit{E. coli}) bacterial and fungal (\textit{C. albicans}) strains, respectively. The p-substitution of nitro (compound T17, MIC\textsubscript{AN} = 27.1µM) group improved the antifungal activity against \textit{A. niger}. The substituent methyl at p-position of ring (compound T3, IC\textsubscript{50} = 54.01 µg/ml) enhanced the anti-\textit{urease} activity. The antioxidant activity has been improved by p-substituents i.e. aldehyde (compound T2, IC\textsubscript{50} = 34.83 µg/ml) and methyl groups (compound T3, IC\textsubscript{50} = 34.38 µg/ml). The most potent anticancer activity showed by compounds T2 (IC\textsubscript{50} = 3.84 µM) and T7 (IC\textsubscript{50} = 3.25 µM) against HCT 116 cell lines as compared to standard 5-FU (IC\textsubscript{50} = 25.36 µM). From the analysis of antimicrobial activity, it may be concluded that the substitution of methoxy group increase the antibacterial activity whereas introduction of nitro as electron withdrawing groups at p-position may enhance the antifungal activity of synthesized compounds. The introduction of methyl substituent as electron donating groups at p-position of aromatic ring may increase the anti-\textit{urease} as well as antioxidant activity. The substitution of of p-aldehyde and o-hydroxy group on the aromatic ring may enhance the anticancer activity against HCT 116 cells.

**Experimental Section**

The initial material, reagents and solvents were purchased from Loba Chemie. The glasswares were obtained from Borosil. The raw material was weighed on calibrated weighing balance. The synthetic scheme was drawn via Chem Draw 8.03. The confirmation of reaction at every step was done by TLC (thin layer chromatography). Melting point of the synthesized compounds was depicted by labtech melting point equipment. For spectral characterizations of the compounds, Bruker 12060280, Software: OPUS 7.2.139.1294 spectrometer using ATR for IR spectra (cm\textsuperscript{-1}) and Bruker Avance III at 600 NMR and 150 MHz for \textsuperscript{1}H and \textsuperscript{13}CNMR (DMSO-\textit{d6}, δ ppm) were used. The tested microbial strains like Gram positive, Gram negative bacteria and fungi were obtained from the Institute of Microbial Technology and Gene bank, Chandigarh for the \textit{in vitro} antimicrobial activity. Waters Micromass Q-ToF Micro instrument was used for mass
spectra. Elemental analysis was performed on Perkin-Elmer 2400 C, H and N analyzer and all synthesized compounds gave C, H and N analysis within ± 0.4% of the theoretical results.

**Procedure for synthesized 1,2,4-triazole derivatives (T₁-T₂₀)**

**Step A: Synthesis of Int-i**

A mixture of palmitic acid (2.6 g, 0.01 mol), absolute ethanol (50 ml) and few drops of conc. H₂SO₄ (0.5 ml) was refluxed for 10 h in a round bottom flask and then cooled to 5 °C. The liquid product was separated from reaction mixture by using ether on the basis of relative density and then purified [27].

**Step B: Synthesis of Int-ii**

To a solution of ethyl palmitate (Int-i, 2.8 g, 0.01 mol) in absolute ethanol (30 ml), hydrazine hydrate (0.64 g, 0.02 mol) was added and refluxed for 6 h and then left to cool. The solid product was collected by filtration and recrystallization from ethanol [27].

**Step C: Synthesis of Int-iii**

Palmitohydrazide (Int-ii, 3.12 g, 0.01 mol) dissolved in the solution of potassium hydroxide (1.12 g, 0.02 mol) in ethanol (30 ml) and then (0.76 g, 0.01 mol) carbon disulfide was added slowly in the reaction mixture. The reaction mixture was refluxed for 10-12 h and then cooled at room temperature followed by addition of hydrochloric acid for neutralization of product. The precipitated solid was filtered, washed with ethanol, dried and recrystallized from ethanol [28].

**Step D: Synthesis of Int-iv**

An ethanolic (30 ml) solution of 5-pentadecyl-1,3,4-oxadiazole-2(3H)-thione (Int-iii, 3.26 g, 0.01 mol) and hydrazine hydrate (0.38 g, 0.01 mol) was heated under reflux for 3 h and then solution was poured in ice. The resulting product was filtered, washed and recrystallized from ethanol [27, 28].

**Step E: Synthesis of 1,2,4-triazole derivatives (T₁-T₂₀)**

The reaction mixture of 4-amino-5-pentadecyl-4H-1,2,4-triazole-3-thiol (Int-iv, 3.26 g, 0.01 mol) and different substituted aldehydes (0.01 mol) in ethanol followed by addition of few drops of sulphuric acid was refluxed for an appropriate time. The reaction was monitored by thin layer chromatography. After completion of reaction, the product was poured in ice and filtered, then wash and finally solid products were collected and recrystallized from ethanol [28].
**Biological studies**

**Antimicrobial evaluation**

The *in vitro* antimicrobial screening of the synthesized 1,2,4-triazole derivatives (T1-T20) in μM was determined against Gram-positive *Bacillus subtilis*, *Pseudomonas aeruginosa*, Gram-negative *Escherichia coli* bacterium and fungal strains *Candida albicans* and *Aspergillus niger* by tube dilution method using ciprofloxacin, amoxycillin (antibacterial) and fluconazole (antifungal) as reference drugs. DMSO was used to dissolve the reference and sample derivatives (T1-T20). Dilutions were prepared in nutrient broth (I.P.) for bacterial (incubated at 37 ± 1 °C for 24 h) and Sabouraud dextrose broth (I.P.) for fungal species (37 ± 1 °C for 48 h for *C. albicans*) and (25 ± 1 °C for 7 days for *A. niger*) (Table 3, Figs. 4 and 5) [17].

*In vitro Antioxidant evaluation*

In the DPPH Free radical scavenging activity, compounds (T1-T20) were evaluated for their free radical scavenging activity with ascorbic acid as standard compound. The IC50 was calculated for each compound as well as ascorbic acid as standard and summarized in Table 4 and shown in Figs. 6-8. The scavenging effect increased with the increasing concentrations of sample compounds. DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichiometrically depending on the number of electrons taken up. Fifty millilitres of various concentrations (25, 50, 70 and 100 µg/ml) of the compounds dissolved in methanol was added to 5 ml of a 0.004 % methanolic solution of DPPH. The sample solutions were incubated for 30 minutes at room temperature in dark place and after then absorbance was recorded against the blank solution at 517 nm. The relative percent of DPPH scavenging activity was calculated according to the following equation:

\[
I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100
\]

Where,

- \(A_{control}\) = absorbance of the control
- \(A_{sample}\) = absorbance of the test compound
Urease inhibition evaluation

Urease inhibitory potential for each synthesized compound (T1-T20) was evaluated using Jack Bean Urease by Indophenol method (Table 5, Figs 9-11). 250 µL of jack bean urease (4U) was mixed with 250 µL of different synthesized test compounds and standard of different concentrations (dissolved in DMSO/H2O mixture (1:1 v/v). The mixture was pre-incubated for one hour at 37 °C in test tubes. 2 ml of 100 mM phosphate buffer (pH 6.8) containing 500 mM urea and 0.002% phenol red as an indicator were added in sample test tubes after pre incubation and again incubated at room temperature. Absorbance of reaction mixture was recorded by ELISA at 570 nm. Ammonium carbonate increased the pH of phosphate buffer from 6.8 to 7.7 which was produced from urea by urease enzyme and the end peak was measured by the colour of phenol red indicator [16].

The percentage inhibition of urease enzyme was calculated by using following formula:

\[ I\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \]

Where, \( A_{\text{control}} = \) absorbance of the control
\( A_{\text{sample}} = \) absorbance of the test compound

Anticancer evaluation

HCT 116 (human colon cancer cells) were seeded at 2,500 cells/ well (96 well plate), allowed to attach overnight, exposed to the respective compounds for 72 h and subjected to SRB assay (570nm). Data represent mean IC50 of at least triplicates. The compounds were all dissolved in DMSO as stock of 100mg/ml. DMSO of < 1.5% did not result in cell kill. The highest concentration of each compound tested (100µg/ml) contained only 0.1% DMSO. Compounds T2 (IC50 = 3.84 µM) and T7 (IC50 = 3.25 µM) exhibited the most potent anticancer activity against HCT 116 cell lines as compared to standard 5-FU (IC50 = 25.36 µM) given in Table 6 and Figs. 12-15.

Conclusion

All the compounds were synthesized according to synthetic scheme under appropriate experimental conditions and analysed by IR and 1H/13CNMR. The pharmacological potential was carried out to study the effect of different substituents on antimicrobial, antioxidant and anti-urease activities. From the outcomes of the pharmacological studies it is concluded that the substitution of tri-methoxy (T5) group increases the antibacterial activity whereas introduction of nitro (T17) group at p-position enhances the antifungal activity. The introduction of aldehyde (T2) and methyl
(T₃) at p-position of aromatic ring may increase the anti-urease as well as antioxidant activities. The substitution of p-aldehyde (T₂) and o-hydroxy (T₇) groups on the aromatic ring may enhance the anticancer activity against HCT 116 cell line.

**Author’s declaration:**

**Conflict of interest:**

The authors have no conflict of interest.

**Author's Contributions:**

Authors MK, ST, BN and SK have designed synthesized and carried out the antimicrobial, antioxidant, anti-urease activities and KR, SML, SAAS and VM have carried out the spectral analysis, interpretation and anticancer evaluation of synthesized compounds.

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