Synthesis and Evaluation of Biological Activities of Triazoles

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ABSTRACT

A series of novel substituted triazole derivatives of 5,5'-diphenyl-4H-4',4'-'diamine 2a, 2,2'-(4,4'-'diamino-4H-4',4'-diamino-4H,4'H-3,3'-bi-1,2,4-triazole-4',4'-diamine 2c and 4,4'-(4,4'-'diamino-4H,4'H-3,3'-bi-1,2,4-triazole-5,5'-diyl)diphenol2b, 5,5'-bis(4-methoxyphenyl)-4H,4'H-3,3'-bi-1,2,4-triazole-4',4'-diamine 2c and 4,4'-(4,4'-'diamino-4H,4'H-3,3'-bi-1,2,4-triazole-5,5'-diyl)bis(2-methoxyphenol) 2d were synthesized. The structure of newly synthesized compounds was established on the basis of FT-IR and ¹H NMR. The in-vitro analysis of these compounds for their Insecticidal, antibacterial, antifungal, anti-inflammatory activities were performed. The compounds 2b, 2c and 2d showed a moderate to high insecticidal and antibacterial activity. Compound 2a showed a maximum antifungal and anti-inflammatory activity. To understand the mode of action of anti-inflammatory activity, molecular docking studies were performed using AutoDock4.2. Compound 2b showed a competitive inhibitory activity and can act as lead molecule towards the drug designing.

Keyword: Triazoles, Insecticidal, Antibacterial, Anti-inflammatory, Anti-fungal, Molecular docking

INTRODUCTION

Triazole and its derivatives embrace a vital session of biologically and pharmacologically active heterocyclic compounds such as antibacterial [1], antifungal, [2, 3] anti-inflammatory [4] and anticonvulsant activities. The various compounds derived from 1, 2, 4-triazoles have an extensive range activities including antihypertensive, and hypoglycemic properties [5, 6]. It has been reported that triazole and hydrazones poses potential insecticidal property [7] and has been used as pesticides on crops both extensively and intensively for pigeon pea (Cajanus cajan, family Fabaceae) [8]. It is estimated that within Karnataka state, a gross cultivation area under pigeon pea is about 3.82 million hectares with a total production of 2.88 million tonnes with productivity of 753 kg per hectar [9, 10]. The crop protection chemicals widely used in agriculture to control various pests are classified
as insecticides, fungicides, rodenticides, herbicides and fumigants, depending upon their mode of activity. Due to spreading resistance of plant pathogens towards fungicides of the strobilurin class [11], control of fungi such as Septoria tritici or Gibber ellazeae [12] relies heavily on triazoles. The store bought potatoes contain retardants such as triazole or tetcyclacis, which play an important role in agriculture and chemical synthesis. Triazoles are relatively stable functional groups and linkages can be used in replacing the phosphate backbone of DNA [13].

In the recent decades insects and pests are getting more resistant due to the wide spread use of insecticides and it is challenging to develop more powerful compounds with the less side effect and bio products, keeping in view of this importance of triazoles, we hereby report the synthesis, characterization and insecticidal activities of new substituted derivatives of triazoles. The synthesized compounds were screened for their insecticidal, antibacterial, antifungal, anti-inflammatory activities. The molecular docking studies of compounds were also carried out.

MATERIALS & METHODS
The homogeneity of all the newly synthesized compounds was routinely checked by thin layer chromatography (TLC) on silica gel G plates (silica gel 60F254) and spots were located by using iodine chamber. The IR spectra (in KBr disks) were recorded on Perkin Elmer and the characteristic bands obtained at the wave numbers are specific to the functional group of the molecular structure [14]. ¹H-NMR spectra were recorded by an instrument using DMSO-d₆ as solvent and tetramethylsilane (TMS) as internal reference standard. All chemical shift values were recorded at δ (ppm). Commercial grade solvents and reagents were used without further, purification. The chemicals used in the synthesis of new compounds are diethyl oxalate, hydrazine hydrate, Ethanol, AR grade, Hydrochloric acid, etc.

The determined structures were further extrapolated to molecular docking studies for their anti-inflammatory activity.

The reaction scheme employed for the synthesis of compounds 5,5'-diphenyl-4H- 4'H-3,3'-bi-1,2,4-triazole-4,4'-diamine 2a, 2,2'-(4,4'-diamino-4H, 4'H-3,3'-bi-1,2,4-triazole-5,5'-diyl)diphenol2b, 5,5'-bis(4-methoxyphenyl)-4H,4'H-3,3'-bi-1,2,4-triazole-4,4'-diamine 2c and 4,4'-(4,4'-diamino-4H,4'H-3,3'-bi-1,2,4-triazole-5,5'-diyl)bis(2-methoxyphenol) 2d are given in Scheme-1. The oxadiazoles when refluxed with hydrazine hydrate in ethanol media yielded triazoles (2a-d). The structures of the newly synthesized compounds are established by IR and NMR.

General procedure for the synthesis of Bis-(5-aryl substituted-1, 2, 4- triazole) alkanes
A mixture of oxadiazoles (0.0005 mol), and hydrazine hydrate (99%) (0.001 mol) in absolute ethanol (10-20 mL) were refluxed for 3-4 hours, cooled to room temperature and the contents were poured into ice cold water. Upon acidification, with acetic acid, a solid mass was separated and collected by filtration followed by washing with cold water, then dried and re-crystallized from Dimethylformamide (DMF). The purification of the compound has been done by using chromatography.
Fig. 1: Synthetic route for the compounds 2a-d

**Compound 2a:** 5, 5'-diphenyl-4H, 4'H-3, 3'-bi-1, 2, 4-triazole-4, 4'-diamine.
IR (KBr) (cm\(^{-1}\)) : 3247.9 (N-H Stretch aromatic), 3047.3 (C-H Stretch aromatic), 1442.7 (C-C Stretch in ring-aromatic) and 1311.5 (C-N Stretch in aromatic amines), \(^{1}\)H-NMR (DMSO-d\(_6\)) \(\delta\) (ppm): 2.0 (bs, 4H, NH\(_2\)), 7.22 - 7.48 (m, 10H, ArH), Molecular Formula; C\(_{16}\)H\(_{14}\)N\(_8\), Formula Weight; 318.33596, Anal. Cald for C\(_{16}\)H\(_{14}\)N\(_8\): C, 60.37; H, 4.43; N, 35.20. Density (g/cm\(^3\)); 1.51 ± 0.1 and Polarizability (cm\(^3\)); 35.69 ± 0.5 \(10^{-24}\).

**Compound 2b:** 2'-(4,4'-diamino-4H, 4'H-3, 3'-bi-1, 2, 4-triazole-5, 5'-diyl) diphenol.
IR (KBr) (cm\(^{-1}\)) : 2638.4 (H-C=O, aldehyde), 1481.2 (C-C Stretch in ring-aromatic), 1326.9 (C-N Stretch in aromatic amines), 1203.5 (C-O alcohols) and 3600-3000(O-H), \(^{1}\)H NMR (DMSO-
About 25mg of synthesized drugs were also screened under similar activity of the solvent. The above said standard control (DMSO) was also used to know the percentage of mortality recorded in 24, 48 and 72 hours. For contact poison, the larvae of Heliothisarmigera were placed one in each compartment of the tray. The sprayed pods were then fed and observation of the percentage of mortality was recorded after 24, 48 and 72 hours. For stomach poison, the prepared solution was sprayed on the red gram by using a micro sprayer (i.e. atomizer). On the other hand, the larvae of Heliothisarmigera were taken. These (24 in number) were placed one in each compartment of the tray. The sprayed pods were then observed and verified against untreated check by placing the larvae in each compartment of the tray. The red gram pods are fed without any treatment and were kept for observation. The mortality was observed after 24, 48 and 72 hours were as same as the test compound observed.

**Anti-bacterial activity**

The Cup-Plate Method given by Chuinckshank et al [19]. Nutrient agar was poured onto the sterilized petri dishes (20-25 mL each petri dish). The poured material was allowed to set for 2h and thereafter the "CUPS" (6 mm diameter) were made by punching into the agar surface with a sterile cork borer and scooping out the punched part of the agar. Into these cups, the test compound solution was added with the help of a sterile syringe. The plates were incubated at 37°C for overnight and the results were noted. The zone of inhibition developed. A 1% solution of Gentamycin was used as standard and solvent control (DMSO) was also used to know the activity of the solvent. The above said standard drugs were also screened under similar conditions for comparison.

**BIOLOGICAL ACTIVITIES**

**Insecticidal activity**

The activity was studied into three general classes as a stomach poison, contact poison, and untreated check [7]. About 25mg of synthesized compounds were dissolved in a minimum amount of acetone in a beaker and 4-5 drops of Tween-80 was added as an emulsifying agent and diluted to 50 mL into a volumetric flask separately. For stomach poison, the prepared solutions were sprayed on the red gram by using a micro sprayer (i.e. atomizer). On the other hand, the larvae of Heliothisarmigera were taken. These (24 in number) were placed one in each compartment of the tray. The sprayed pods were then fed and observation of the percentage of mortality was recorded after 24, 48 and 72 hours. For contact poison, the larvae of Heliothisarmigera were placed one in each compartment of the tray which was half filled by diet. The solutions above prepared were sprayed directly on the larval body by using an atomizer and the percentage of mortality was recorded after 24, 48 and 72 hours as shown in Table no.1. These stomach and contact poisons are observed and verified against untreated check by placing the larvae in each compartment of the tray.

**Antifungal activity**

The spore suspension of each test organism was...
added to sterilize the sabouraud dextrose agar medium at 40-50°C by thorough shaking. The petri dishes were seeded with the mixture and the paper discs of the methanolic solution of compounds and the reference antibiotic as the control was placed in the same manner as in antibacterial activity determination [2, 3]. These petri dishes were incubated for 24h at room temperature. The zone of inhibition developed of any was then accurately measured and recorded in the Table no.2.

Anti-inflammatory activity

Albino rats of either sex weighing between 120-150 g were selected; the animals fasted for 24 hours with water ad libitum. Animals were marked on their hind paws (right and left) just beyond the tibio-tarsal to ensure constant dipping in the mercury column up to the fixed mark. The test doses (25 mg/kg) were prepared in Tween-80 and suspended in water. After 30 mins, 0.1 mL of 1% v/v formalin solution was injected into the plantar region of the left paw of the all experimental animals. The right non-inflamed paw served as reference for comparison. At the end of 5 hours after formalin injection, the paw volume of both legs of all the experimental animals was measured with the help of plethysmograph [4]. In the same way, test group was used for evaluating the anti-inflammatory activity of the respective test compounds. The percentage inhibition of the inflammation by the drug-treated animals were recorded using the formula. Percentage inhibition= 100(1-T/C), Where T and C are volumes of edema of drugs treated and control group, respectively.

The synthesis compounds were tested for anti-inflammatory activity in-vitro against albino rats and compared with that of standard drug Diclofenac sodium.

Protein and ligand structure preparation

The X-Ray Crystallographic structures of the 1.6 Å model of Crystal Structure of the Complex formed between Russell’s Viper Phospholipase A2 and an Antiinflammatory agent DICLOFENAC (PDB: 2B17) was obtained from the protein databank (www.pdb.org). Topology file and other force field parameters were generated for all triazole derivatives using the PRODRG program (Aalten et al. 1996) [15]. Flexible torsions were defined using AUTOTORS. The docking site for triazole derivatives on 2B17 was defined at the position of the co-crystallized ligand by using PyRX 0.8 interface (Wolf 2009) [16] with grid box size of 52 x 49 x 58, a spacing of 0.375, grid center 48.098, 32.710 and 6.880 and assigning 3 Degrees of Freedom. The Lamarckian Genetic Algorithm (LGA) (Morris et al. 1998) was employed with the population size of 150 individuals, maximum number of generations and energy evaluations of 27,000 and 2.5 million respectively. From the estimated free energy of ligand binding (∆G), the inhibition constant (K) for each ligand was calculated. Only the best pose (the one with the lowest binding energy) was considered for the ligand. The best conformation was analyzed for protein and triazole derivatives interaction using Ligplot+ (Wallace et al. 1995) [17]. PyMOL (DeLano 2002) [18] was used for docking conformation representation.

RESULTS AND DISCUSSION

The synthesized compounds were characterized by using IR and NMR Spectrum, the powdered potassium bromide was used for background correction for IR spectrum. The IR spectrum of 5, 5'-diphenyl-4H- 4'H-3, 3'-bi-1,2,4-triazole-4,4'-diamine 2a, theN-H stretch aromatic band was observed at 3247.9 cm⁻¹. Further, in the ¹H NMR spectrum of 2a, the NH₂ group attached to the triazole came into resonance as a bs, at δ 2.0 integrating for four protons. A multiplet at δ 7.22 -7.48 is due to ten protons these support in assign the structure of synthesized compounds. The detailed spectrum is described in the experimental section. The substituted derivatives of triazoles were synthesized and
Screened for their insecticidal, anti-bacterial, anti-fungal and anti-inflammatory. Results of synthesized compounds (2a-d) are detailed in Tables.

On screening of stomach poison of 2,2’-(4,4’-diamino-4H,4’H-3,3’-bi-1,2,4-triazole-5,5’-diyl)diphenol 2b showed 25% of mortality while other compounds 5,5’-diphenyl-4H,4’H-3,3’-bi-1,2,4-triazole-4,4’-diamine 2a, 5,5’-bis(4-methoxyphenyl)-4H,4’H-3,3’-bi-1,2,4-triazole-4,4’-diamine 2c showed moderate mortality rate of 12.50, 20.83 and 4,4’-(4,4’-diamino-4H,4’H-3,3’-bi-1,2,4-triazole-5,5’-diyl)bis(2-methoxyphenyl) 2d exhibited low mortality of 4.17%. In case of contact poison toxicity, all the compounds showed good activity, especially the compounds 2b, 2c and 2d showed a high percentage of mortality, i.e., 37.50, 79.17 and 50.00% respectively when compared with standard and untreated control. The results are summarized in Table no.1.

The results of anti-bacterial activity study indicated that among the tested compounds 2c, 2b and 2d showed high activity against 

\textit{Kallipso calla, Escherichia coli, and Porlous vulgaris}, 2a compound exhibits moderate antibacterial activity against all the tested microbial stains as compared with the standard drug. The antibacterial activity results are summarized in Table no. 2.

Among the synthesis compounds the anti-fungal activity of the compound 2b showed high activity against 

\textit{A. niger}, 2a, 2c and 2d showed moderate results. In a case of \textit{A. flavous}, 2a and 2d compound showed maximum and average inhibition, the remaining compounds are inactive. All the compounds were moderately active against \textit{C. albicans}. The antifungal activity results are summarized in Table no.2.

Among all the compounds the 5,5’-diphenyl-4H,4’H-3,3’-bi-1,2,4-triazole-4,4’-diamine 2a has shown good anti-inflammatory activity where 2,2’-(4,4’-diamino-4H,4’H-3,3’-bi-1,2,4-triazole-5,5’-diyl)diphenol 2b has shown moderate activity, while 2c and 2d compounds did not possess any activity. The results of anti-inflammatory activity and phenyl butazone and samples are tabulated in Table no.3.

Table 1: Bio-efficacy testing of compounds 2a-d (Stomach and contact poison)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dosage (in ppm)</th>
<th>Stomach poison</th>
<th>Contact poison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mortality out of 24 larvae</td>
<td>% Mortality after (Stomach)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>2a</td>
<td>500</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2b</td>
<td>500</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2c</td>
<td>500</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2d</td>
<td>500</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Acetone (Standard)</td>
<td>-</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>U.T.C.</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2: Anti-bacterial and antifungal activity of compounds 2a-d

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Kallipsi calla ‘k’ (in mm)</th>
<th>Escherichia coli ‘EC’ (in mm)</th>
<th>Portious vulgaris ‘PV’ (in mm)</th>
<th>A. niger ‘AN’ (in mm)</th>
<th>A. flavus ‘AF’ (in mm)</th>
<th>C. albicans ‘CA’ (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>12</td>
<td>10</td>
<td>08</td>
<td>07</td>
<td>12</td>
<td>08</td>
</tr>
<tr>
<td>2b</td>
<td>14</td>
<td>09</td>
<td>09</td>
<td>16</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>2c</td>
<td>15</td>
<td>10</td>
<td>08</td>
<td>07</td>
<td>-</td>
<td>08</td>
</tr>
<tr>
<td>2d</td>
<td>18</td>
<td>10</td>
<td>09</td>
<td>08</td>
<td>08</td>
<td>06</td>
</tr>
<tr>
<td>Standard</td>
<td>29</td>
<td>28</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control (DMSO)</td>
<td>06</td>
<td>06</td>
<td>06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3: Anti-inflammatory activity of compounds 2a-d

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Mean value of hind paw oedema at different time of intervals in hours (mean 24 ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At 0</td>
</tr>
<tr>
<td>Control*</td>
<td>1%</td>
<td>0.575 ± 0.048</td>
</tr>
<tr>
<td>Standard</td>
<td>25</td>
<td>0.55 ± 0.028</td>
</tr>
<tr>
<td>Compound 2a</td>
<td>25</td>
<td>0.65 ± 0.064</td>
</tr>
<tr>
<td>2b</td>
<td>25</td>
<td>0.95 ± 0.028</td>
</tr>
<tr>
<td>2c</td>
<td>25</td>
<td>0.95 ± 0.615</td>
</tr>
<tr>
<td>2d</td>
<td>25</td>
<td>0.8 ± 0.048</td>
</tr>
</tbody>
</table>

*Molecular Docking Studies

Molecular docking simulation of 2a, 2b, 2c and 2d to Crystal Structure of Russell's viper Phospholipase A2 was performed to gain functional and structural insight into the mechanism of inhibition. AutoDock 4.2 (Morris et al. 1998)[20] suites were used as a molecular-docking tool. For the in silico docking studies, triazole derivatives were docked against Phospholipase A2, using the co-crystallised ligand structure of Diclofenac as a reference. Diclofenac was rocked to its active site to calculate the binding energy, inhibition constant value using AutoDock. The active site comprises of Leu2, Phe 5, Ile 19, Cys 29, Gly 30, Cys 45, His 48, Lys 69, and Asp 99. The triazole derivatives were allowed to dock at the active site with the complete flexibility. The best pose having least binding energy revealed the electrostatic interaction and hydrogen bonding which facilitate the binding to the active site. The experimental results of interaction of Diclofenac (PDB: 2B17) with Phospholipase A2 show that the $K_i = 6.9\mu M$ by forming hydrogen bonds with His 48 and Asp 99. The interaction of 2b with PAP2 (PDB: 2B17) shows seven hydrogen bonds with active site residues Tyr28, Gly30, Trp31, His48, Asp49, Lys69, and Asp99. The $K_i = 4.15\mu M$ was observed which is low as compare to standard drug Diclofenac. $IC_{50}$ value of diclofenac and triazole derivatives was predicted from AutoDock. Based on the very low binding energy of -7.34 kcal/mol and low $IC_{50}$ of 4.15µM, the 2b proves to be a competitive inhibitor as
compared to Diclofenac and as a potential anti-inflammatory lead molecule since the ligand interact with a relatively higher affinity and lower inhibition constant than the standard diclofenac. PLA2 catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. The cofactor Calcium uses the metal binding residues Tyr27, Gly29, Gly31, Asp48 (residue numbering according to PDB) via carbonyl oxygen for its activity. Compound 2b formed a hydrogen bond with these metal-binding residues and blocked the interaction of calcium and further disturbs the hydrolysis and inactivates the enzyme. Compound 2b formed Binding energy and an inhibition constant of all the docked compounds including both the standard drugs were presented in Table no. 4 and supported by enclosing Fig. 2.

Table 4: Molecular Docking Studies results.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Ligand structure</th>
<th>Binding energy (kcal/mol)</th>
<th>Amino acids involved in Hydrogen Bonding</th>
<th>Predicted IC50 (micromolar) from AutoDock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipase A2</td>
<td>2a</td>
<td>-7.11</td>
<td>Asp49</td>
<td>6.14</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>-7.69</td>
<td>Tyr28, Gly30, Trp31, Asp49</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>-7.03</td>
<td>Leu2, Gly30, Trp31, Asp49</td>
<td>7.04</td>
</tr>
<tr>
<td></td>
<td>2d</td>
<td>-6.01</td>
<td>Leu2, Gly30, Asp49</td>
<td>39.2</td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>Diclofenac(DIF)</td>
<td>-7.04</td>
<td>His 48(A), Asp 99(A)</td>
<td>6.9</td>
</tr>
</tbody>
</table>

Fig. 2: Molecular interaction of DIF and 2b with Phospholipase A2 from Ligplot+. (A) Molecular interaction of DIF with Phospholipase A2 as represented in PDBsum (PDB: 2B17) (B) Molecular docking interaction of 2b with Phospholipase A2.
CONCLUSION
The new series of substituted 1, 2, 4 triazoles were synthesized in reasonably good yields. The synthesized compounds were evaluated for insecticidal activities, anti-bacterial, anti-fungal and anti-inflammatory property. The compound 2b has shown high percentage of mortality compared to the standard acetone. The increased percentage of mortality of compound 2b may be due to the very low binding energy and presence of substituted phenol to 1,2,4-triazole. The compound 2a and 2c shows moderate mortality other compound shows less mortality because of moderately high binding energy. Compounds 2c, 2b, and 2d showed high activity against the tested microbial stains Kallipsi calla, Escherichia coli and portious vulgaris compared to standard. The molecular docking studies of the synthesized compounds 2a-d were performed. In silico studies revealed that all the synthesized compounds have relatively lesser binding energy as compare to the standard drug and may be considered as a good inhibitor. Compound 2b showed the least binding energy which is in agreement with the in vitro results. Hence, this study has extended the scope of developing these 1, 2, 4-triazoles derivatives as promising antimicrobial agents.

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interests.

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