3.1.0. Introduction to isoxazoles:-

Isoxazoles (Gilchrist 1985) are unsaturated aromatic heterocyclic compounds containing a ring with three carbon atoms, one oxygen atom and one nitrogen atom. Isoxazole being an azole with an oxygen atom next to the nitrogen, exhibits broad spectrum of biological activity and also forms a part of various biodynamic agents. The substituted isoxazoles are also considered to be important synthons due to their versatility towards chemical transformations to useful synthetic intermediates such as 1,3-dicarbonyl, 1,3-iminocarbonyl (McMurry 1973) and γ-amino alcohols (Stork and McMurry 1967). The significance of this class of molecules gets further impetus due to their involvement as intermediates in the synthesis of various natural products.

3.1.1. Biological importance of isoxazole containing molecules:-

Isoxazoles constitute an important family of five membered heterocycles in view of their use in many natural products synthesis and occurrence in pharmaceutical agents viz., COX-2 inhibitor (Talley et al., 2000), fungicides (Tomita et al., 1973), dopamine D4 receptors antagonist (Rowley et al., 1996), GABAγ antagonist (Frolund et al., 2002), analgesic (Daidone et al., 1999), antiinflammatory (Daidone et al., 1999), ulcerogenic (Daidone et al., 1999), antinociceptive (Giovannoni et al., 2003) and anticancer activity (Li et al., 2003) etc. In the last few decades, isoxazole containing natural and non natural compounds gained importance due to their immunomodulatory properties. (S,R)-3-Phenyl-4,5-dihydro-5-isoaxasole acetic acid VGX-1027 (A, Fig 1) exerts anti-diabetogenic effects by limiting cytokine-mediated immuno-inflammatory events which leads to inflammation and destruction of pancreatic islets (Grujicia et al., 2007). N-(4’-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide B (Leflunomide, Fig 1) is an isoxazole immunomodulatory agents which inhibits dihydroorotate dehydrogenase (Wozel and Pfeiffer 2002; Osiri et al., 2003). 5-amino-3-methyl-4-isoxazolecarboxylic acid semicarbazides C and thiosemicarbazides D (Fig 1), a new class of isoxazole derivatives shown immunotropic activity (Maczynski et al., 2008). Isoxazole derivatives mostly possess potential immunosuppressive property, but in certain cases within the family of isoxazoles, both immunostimulatory and immunosuppressive activity have been reported. 5-Amino-3-methylisoxazole-4-carboxylic acid amide E (Fig 1) exhibits promising immunomodulatory activity (Ryng et al., 1999). 3-Methylisoxazole[5,4-d]-
1,2,3-triazin-4-ones \( F \) (Fig 1) exhibits immunostimulatory activity (Maczynski et al., 2003; Jezierska et al., 2004). RM-11, an isoxazole derivative \( G \) is a potent stimulator of the humoral and cellular immune responses in mice (Ryng et al., 2000; Zimecki et al., 2008).

Fig 1: Structures of biological important isoxazole derivatives

3.1.2. Literature background:

Many synthetic methods have been employed in the preparation of isoxazoles (Wakefield 2001; Jaeger and Colinas 2002). Despite the numerous methods to construct these pharmacologically important heterocycles, there is still a strong need to further explore synthetic methods to efficiently synthesize novel heterocyclic structures from readily available reagents. Following are the literature known methods utilized for the synthesis of isoxazoles and their derivatives.

**General methods for the synthesis of isoxazoles**

The reaction of asymmetrically substituted \( \beta \)-diketones with hydroxylamine to give 3,5-diarylisoazoles in high yields (Bandiera et al., 2009).
5-Silylisoxazoles have been prepared by condensation of silylalkynones with hydroxylamine hydrochloride (Cuadrado et al., 2002).

Regiospecific synthesis of isoxazoles has been reported in excellent yield by acylation of syn-1,4-dilithio oximes with amides (DMF) followed by a mineral acid induced cyclization-dehydration (Barber and Olofson 1978).

A [3 + 2] cycloaddition reaction between alkynyldimethylsilyl ethers and aryl/alkyl nitrile oxides to produce isoxazolylsilanols has been developed. The cross-coupling reactions of these heterocyclic silanols with a variety of aryl iodides afford 3,4,5-trisubstituted isoxazoles (Denmark and Kallemeyn 2005).

A variety of 3,5-disubstituted 4-halo(seleno)isoxazoles are readily prepared in good to excellent yields under mild reaction conditions by the reaction of 2-alkyn-1-one with O-methyl oximes with ICl, I₂, Br₂ or PhSeBr (Waldo and Larock 2005).
A 1,3-dipolar cycloaddition of phenyl vinylic selenide to nitrile oxides and subsequent oxidation-elimination furnished 3-substituted isoxazoles with good yields in a one-pot, two-step transformation (Sheng et al., 2003).

The reaction of activated nitro compounds such as phenyl nitro methane with terminal acetylenes affords isoxazoles derivatives in higher yields compared with those of other methods. However, the reaction is not compatible with nitroalkanes (Cecchi et al., 2006).

3,5-Disubstituted isoxazoles are regioselectively obtained in good yields by a mild and convenient one-pot, three-step procedure utilizing a copper(I)-catalyzed cycloaddition reaction between in situ generated nitrile oxides and terminal acetylenes (Hansen et al., 2005).
A series of 4-alkyl-5-aminoisoxazoles have been synthesized in high yields by nucleophilic addition of lithiated alkyl nitriles to chlorooximes (Bourbeau and Rider 2006). However, these methods often require strong bases, strong mineral acids, or high temperatures and provide poor regioselectivity.

Various synthetic procedures have so far been proposed for the synthesis of 3-aryl-5-substituted isoxazoles themselves. (Grunanger and Vita-Finzi 1999). Following are the few methods present in the literature utilized for the synthesis of 5-alkyl isoxazole derivatives.

**Methods for the synthesis of 3-aryl-5-substituted isoxazoles**

3-Aryl-5-substituted were prepared by the 1,3-dipolar addition of arylnitrile oxides to stable enolate derivatives (silyl enol ethers or enol acetates) followed by final aromatization of the formed isoxazolines (Micetich 1970).

3-Aryl-5-alkylisoxazoles were prepared by the addition of benzonitrile oxide to the enolate ions (regio-selectively generated from methyl ketones) instead of the corresponding silyl enol ethers and enol acetates (Nunno et al., 2002).
A series of novel 3-(substituted phenyl)isoxazole derivatives were prepared from phenyl butan-1,3-dione. (Zhou et al., 2003)

Eventhough a plethora of methods available for isoxazole synthesis, there are only few methods described in the literature for the preparation of 3-aryl-5-substituted isoxazoles. Thus exploring new route to 3-aryl-5-substituted isoxazoles is a very important synthetic exercise.

3.1.3. Present work:- Synthesis of 3-aryl-5-substituted isoxazoles:

As part of our continued interest in exploring organometallic addition reactions to various C≡N compounds which lead to the development of novel regio-selective route for the synthesis of 5-butenylisoxazolines (Qazi et al., 2005) and 5-vinyl isoxazolines (Qazi et al., 2007). Here, our particular interest would be the addition of nitrile oxides to acetylenic compounds to generate synthetically and pharmacologically valuable novel substituted isoxazoles. This section present the nucleophilic addition of allenylmagnesium bromide to nitrile oxides, the resulting intermediate undergoes C–O heterocyclization followed by the addition to another molecule of allenylmagnesium bromide to generate 5-butynylisoxazoles in good yields. Several benzonitrile oxides 1 (Grundmann and Dean 1964) generated \textit{in-situ}, were reacted with excess (>2 mol equiv) propargylmagnesium bromide 2 in THF together with a catalytic quantity (3% w/w) of mercuric (II) chloride (Hopf 1990) under an inert
atmosphere (mercuric chloride on interaction with propargylmagnesium bromide generates allenylmagnesium bromide in situ). In most cases, 3-aryl-5-butynilisoxazoles were isolated in good yields (67–84%, Table 1) after 5–6 h reaction at an ambient temperature with only a trace amount of the corresponding 3-aryl-5-methylisoxazoles (5–8%) shown in Scheme 14.

![Scheme 14: Synthesis of 3-aryl-5-methylisoxazoles](image)

The reaction was found to be general with regard to various substituted nitrile oxides bearing electron-donating or electron withdrawing groups on the aromatic ring (Table 1). However, hindered nitrile oxides such as 2,6-dichlorobenzonitrile oxide gave trace amounts of 5-methylisoxazole without any butynylated product. When this reaction was attempted in the absence of mercuric chloride, no product was observed even after a prolonged reaction. The formation of 3-aryl-5-butynilisoxazoles could occur in a domino fashion, nucleophilic addition of allenylmagnesium bromide to the nitrile oxide followed by C–O-heterocyclization to generate an organometallic isoxazole intermediate 3a (Scheme 15), which undergoes reaction with an additional mol of allenylmagnesium bromide 2 to generate 4. A plausible mechanism for the generation of 3-aryl-5-butynilisoxazole 4 from intermediate 3a can be visualized either through, (i) Wurtz-type of coupling of organometallic intermediate 3a with an additional mol of free propargyl bromide if present in the medium or (ii) SN$_1$ type reaction via intermediate 3c generated through Schlenk equilibrium.

Since the propargyl bromide was treated with an excess of metal to completely convert it into allenylmagnesium bromide, the possibility of a Wurtz-type coupling between intermediate 3a and propargyl bromide can be ruled out. This was further confirmed by the fact that no trace of the cycloaddition product arising from the dipolar addition of nitrile oxide to propargyl bromide (unreacted, if any) could be detected in the crude product mixture. It is pertinent to mention here that propargyl bromide readily undergoes dipolar cycloaddition with nitrile oxides to generate 5-bromomethylisoxazole under the given experimental conditions.
Scheme 15: Plausible mechanism for the synthesis of 3-aryl-5-substituted isoxazole

Hence, product formation can be attributed to a SN1 reaction as shown in Scheme 15 involving Schlenk equilibrium (similar coupling between two Grignard species has already been explained mechanistically by Schlenk (Schlenk 1929)). 3-Aryl-5-methylisoxazole 5 is likely to be formed through proton capture by intermediate 3a during quenching. The possibility of a 1,3-dipolar cycloaddition of nitrile oxide to allenylmagnesium bromide to generate the isoxazole nucleus can be ruled out since allenylmagnesium halides do not form Diels Alder adducts with any dienes under the given experimental conditions, which shows the poor dipolarophilic nature of these resonance stabilized species. To sum up, the present work involving the reaction of nitrile oxides with resonance stabilized organometallics provides the invention of another class of anionic domino reactions for the high yield synthesis of 3-aryl-5-substituted isoxazole.
<table>
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<tr>
<th>Entry</th>
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\(^a\) All products are characterized by IR, 1H/13C NMR, Mass spectral analysis.
\(^b\) Isolated yields after column chromatography.
\(^c\) Corresponding 5-Methyl isoxazoles were isolated in 5-8% yields.

**Table 1: Synthesis of 3-aryl-5-butynyl isoxazoles**
3.1.4. Biological activity: Evaluation of immunomodulatory activity of 3-aryl-5-substituted isoxazoles:

The isoxazoles possess both immuosuppressive and immunostimulatory activity. The immunological response of isoxazoles derivatives depends upon the nature and position of substituents on the isoxazole rings. Most of the isoxazole derivatives possess immunosuppressive activity such as leflunomide (isoxazole derivative), used for the treatment of autoimmune disorders. However, many 5-amino-3-methylisoxazole-4-carboxylic acid phenylamides possess immunostimulatory activity. For example, 3-phenyl-5-phospho-dihydroisoxazole also possess immunostimulatory activity. Literature survey revealed that isoxazole having substituent at third and fifth position shown immunostimulatory activity. Furthermore, there is no report on the evaluation of 3-aryl-5-alkyl substituted isoxazoles for immunomodulatory activity.

Keeping in view the importance of the isoxazole moieties as immunomodulators and to further explore the nature and position of substituents on isoxazoles to decide whether the molecules show immunosuppressive or immunomodulatory activity, a novel series of 3-aryl-5-butynyl isoxazoles was synthesized through the nucleophilic addition of allenylmagnesium bromide and to various nitrile oxides.

All the derivatives, thus generated screened for their effects on different aspects of immune response i.e., immunostimulant and immunosuppressive activities. In order to evaluate the immunomodulatory activity, splenocyte proliferation assay (T & B Cell proliferation) was employed followed by a number of assays in a variety of immune responsive cells for active derivatives such as antibody titre (IgM and IgG), DTH reaction, cytokine analysis (IFN-γ, IL-4 and IL-2) and cell surface marker population (CD4/CD8). Levamisole and cyclophosphamide, a known immunostimulant and immunosuppressive agents respectively drug was used as a standard in this study. In all 13 different derivatives of 5-butynyl isoxazoles for their possible immunomodulator activity were tested. Out of these, 3 isoxazoles derivative viz., isoxazole 4a, isoxazole 4b and isoxazole 4l exhibited varying degrees of immunostimulatory activities, whereas rest of the compounds have shown either moderate or no activity. Therefore, these compounds were used for detailed evaluation and the results are summarized below.
3.1.5. Results and Discussion:

Results

**Effect of 3-aryl-5-butynylisoxazole on *in vitro* T & B cell proliferation**

To confirm the effect of the 3-aryl-5-butynylisoxazole on the immune response, the proliferation of splenocytes in response to Con A (5 µg/mL) and LPS (10 µg/mL) was evaluated. The results shown in Table 2 indicate that the proliferation in 3-aryl-5-butynylisoxazole treated groups at 0.001, 0.01, 0.1 and 1 µg/ml was stimulated in a dose-dependent manner compared with the control group. Cellular proliferation was significantly increased in Con-A and LPS treated cells with isoxazole 4a, isoxazole 4b and isoxazole 4l at a dose of 1 µg/ml compared to the control. These 3-aryl-5-butynyl isoxazole derivatives showed significant rise in splenocytes proliferation, therefore, these derivatives have been chosen for further detailed investigation.

**Effect of 3-aryl-5-butynyl isoxazole derivatives on SRBC induced antibody in mice**

Group of five mice (Balb/c) was immunized intraperitoneally with SRBC followed by concomitant treatment of isoxazoles derivatives *viz.*, isoxazole 4a, isoxazole 4b and isoxazole 4l (0.001, 0.01 and 0.1 mg/kg p.o) on day 0 & day 7. Effect of these compounds on SRBC induced antibody titre (both IgM and IgG) in Balb/c mice clearly showed that 3-aryl-5-butynyl isoxazoles exhibited dose dependent immunomodulatory effect ([Fig 2](#)). The isoxazole 4a shown maximum potentiating effect at 0.001 mg/kg whereas, isoxazole 4b and isoxazole 4l showed effect at dose 0.01 and 0.1 mg/kg respectively. These 3-aryl-5-butynyl isoxazole derivatives showed significant rise in antibody titre therefore, these derivatives were chosen for further detailed investigation.

**Effect of 3-aryl-5-butynyl isoxazole derivatives on SRBC induced delayed type hypersensitivity reaction (DTH) in mice**

The effect of isoxazole 4a, isoxazole 4b and isoxazole 4l on DTH reaction in mice was given in [Fig 3](#) in which data is expressed in terms of the swelling of the footpad. Administration of the isoxazole 4a, isoxazole 4b and isoxazole 4l (0.001, 0.01 and 0.1 mg/kg, p.o.), a significant dose related change in footpad thickness was observed at 24 h as compared to control group as well as groups treated with reference drug *viz.*, levamisole and cyclophosphamide. Isoxazole 4a had shown a significant enhancement in DTH response at a lower dose of 0.001 mg/kg, as compared to isoxazole 4b and isoxazole 4l which showed
enhancement at a dose of 0.01 and 0.1 mg/kg respectively. Among three derivatives, the effect of isoxazole 4a was the most promising.

**Effect of 3-aryl-5-butynyl isoxazole derivatives on Th1 (IFN-\(\gamma\) and IL-2) and Th2 (IL-4) cytokines release**

The effect of isoxazole 4a, isoxazole 4b and isoxazole 4l on cytokine release in serum is shown in Fig 4 A-C. At doses of 0.001 mg/kg isoxazole 4a significantly enhanced the Th1 (IFN-\(\gamma\) and IL-2) and Th2 (IL-4) cytokines as compared to control group whereas, isoxazole 4b and isoxazole 4l significantly enhanced the Th1 (IFN-\(\gamma\) and IL-2) and Th2 (IL-4) cytokines at a dose of 0.01 and 0.1 mg/kg respectively. Out of three molecules, the results were more promising in case of isoxazole 4a, which enhanced significantly both Th1 and Th2 type of immune response.

**Effect of 3-aryl-5-butynyl isoxazole derivatives on spleen T cells subtypes CD4 and CD8**

The effect of isoxazole 4a, isoxazole 4b and isoxazole 4l (0.001, 0.01 and 0.1 mg/kg) on CD4 and CD8 population in spleen determined by flow cytometry is shown in Fig 5. Administration of isoxazole 4a at a dose of 0.001 mg/kg increased significantly CD4 and CD8 population in comparison with control where as levamisole and cyclophosphamide showed a significant increase and decrease in CD4/CD8 production in mice. Similarly, isoxazole 4b and isoxazole 4l also showed moderate enhancement in CD4/CD8 production at a dose of 0.1 and 0.01 mg/kg respectively as compared to control group.

**Discussion:** A number of assays such as HA titre, DTH reaction, cytokines response and CD4/CD8 were used to investigate the immunomodulating effect of 3-aryl-5-butynyl isoxazole derivatives on different immune responses (Table 1 and Fig 2-5). The findings outlined in result have demonstrated that 3-aryl-5-butynyl isoxazole derivatives possess a potent immunostimulant activity. In literature, most of the isoxazole derivatives possess potential immunosuppressive activity such as leflunomide (Wozel and Pfeiffer 2002; Osiri et al. 2003) and there are few reports where isoxazole derivatives also possess immunostimulatory activity (Ryng et al. 2000; Zimecki et al. 2008). 3-Aryl-5-butynyl isoxazole derivatives possesses potential immunostimulatory activity indicated by increase in the level of HA titre, DTH reaction, cytokines response and CD4/CD8 population in various *in vivo* and *ex vivo* experiments.
The immune response of the body is mainly composed of specific and non-specific immunity. The specific immune response includes humoral and cellular immunity. Humoral immunity, via the antibody response is regulated by B cells and other immune cells involved in antibody production. The stimulation of the humoral response against SRBCs by 3-aryl-5-butynyl isoxazole derivatives was evidenced by the increase in HA titer. As shown in Fig 2, isoxazole 4a, isoxazole 4b and isoxazole 4l enhanced significantly HA titre at a dose of 0.001, 0.01 and 0.1 mg/kg as compared to control group. The results were most promising in case of isoxazole 4a.

A DTH reaction is an expression of cell-mediated immunity and plays a role in many inflammatory disorders. Such reactions are characterized by large influxes of non-specific inflammatory cells, of which the macrophage is a major example. Several lines of evidence suggest that DTH reactions are important in host defense against parasites and bacteria that can live and proliferate intracellularly. Administration with 3-aryl-5-butynyl isoxazole derivatives viz. isoxazole 4a, isoxazole 4b and isoxazole 4l enhanced the DTH reaction at a dose of 0.001, 0.01 and 0.1 mg/kg, as reflected by the increased footpad thickness compared to the control group, and also suggesting heightened infiltration of macrophages to the inflammatory site. Among three derivatives, isoxazole 4a gave most significant response. These results suggested that presence of para substituted aryl group at 3rd position along with butynyl group at fifth position on isoxazoles is important for immune enhancing activity. Moreover, electron donating aryl group further enhances the activity as evidenced by the results. The results were more promising with isoxazole 4a having electron donating methoxy group at lower dose (0.001 mg) as compared to isoxazole 4b and isoxazole 4l having electron withdrawing fluoro and cyano group respectively.

We also determined the possible effect of 3-aryl-5-butynyl derivatives viz. isoxazole 4a, isoxazole 4b and isoxazole 4l on soluble mediators of Th1 and Th2 response. 3-Aryl-5-butynyl isoxazole derivatives also enhanced the Th1 and Th2 immune responses as shown in Fig 4A-C by significantly increasing the production of Th2 (IL-4) and Th1 cytokines (IL-2 and IFN-γ) as compared with control. Out of the above three analogues, isoxazole 4a significantly increased the production of Th1 (IFN-γ and IL-2) and Th2 (IL-4) at a dose of 0.001 mg/kg. Here again the observed CD4/CD8 values shown in Fig 5 for isoxazole 4a, isoxazole 4b and isoxazole 4l were in conformity with the antibody response, which qualifies
3-aryl-5-butynyl isoxazole as immunostimulator and among three, isoxazole 4a as the highest active molecules, studied here. The proliferation of HA titre, DTH reaction, and cytokine production Th2 (IL-4) and Th1 cytokines (IL-2 and IFN-γ) suggested that 3-aryl-5-butynyl isoxazoles may enhance both humoral and cellular immunity in a mouse model.

The results of preliminary assays and structural investigation of 3-aryl-5-butynyl isoxazole derivative revealed that presence of butynyl chain at fifth position and suitably substituted aryl group at third position seems to impart immune-enhancing activity to the molecule. Furthermore, the presence of substitution at para position of aryl group is responsible for activity and moreover, the presence of electron donating at para position of aromatic further enhances the immunomodulatority activity. The varying degree of activity among the 5-butynyl isoxazole derivatives may be attributed to the functionalization of aromatic ring and the delicate balance between the optimum substitution pattern on both fifth position of isoxazole ring and the substitution on the aromatic ring at the third position of the isoxazole ring, which decides the final activity of the molecule and substitution patterns may facilitate or restrict the molecule to interact with cells. In summary, para substituted aryl at third position and butynyl group at fifth position on isoxazoles are responsible for imparting immunostimulatory activity.
Table 2: Effect on *in-vitro* T & B cell proliferation

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<th>Treatment</th>
<th>Dose µg/ml</th>
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<th>Stimulated cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Con-A 2.5 µg/ml</td>
<td>LPS 2.5 µg/ml</td>
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<tr>
<td>Control</td>
<td></td>
<td>1.034 ± 0.01</td>
<td>0.998 ± 0.03</td>
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<tr>
<td>Levamisole (0.25 µg/ml)</td>
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<tr>
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<tr>
<td>(1 µg)</td>
<td>1.657 ± 0.10</td>
<td>1.60</td>
<td>1.185 ± 0.02</td>
</tr>
<tr>
<td>Isoxazole 4f</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Isoxazole 4g</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Isoxazole 4h</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Isoxazole 4i (0.001 µg)</td>
<td>1.211 ± 0.11</td>
<td>1.17</td>
<td>1.027 ± 0.05</td>
</tr>
<tr>
<td>(0.01 µg)</td>
<td>1.130 ± 0.05</td>
<td>1.09</td>
<td>1.058 ± 0.10</td>
</tr>
<tr>
<td>(0.1 µg)</td>
<td>1.161 ± 0.05</td>
<td>1.12</td>
<td>1.063 ± 0.12</td>
</tr>
<tr>
<td>(1 µg)</td>
<td>1.885 ± 0.13</td>
<td>1.18</td>
<td>1.092 ± 0.02</td>
</tr>
<tr>
<td>Isoxazole 4j</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Isoxazole 4k (0.001 µg)</td>
<td>1.200 ± 0.13</td>
<td>1.16</td>
<td>1.019 ± 0.06</td>
</tr>
<tr>
<td>(0.01 µg)</td>
<td>1.129 ± 0.04</td>
<td>1.09</td>
<td>1.037 ± 0.15</td>
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<table>
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<th>Dose (µg)</th>
<th>SI (Mean ± S.E.)</th>
<th>Values ± S.E.</th>
<th>SI (Mean ± S.E.)</th>
<th>Values ± S.E.</th>
</tr>
</thead>
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<tr>
<td>(0.1 µg)</td>
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<td>1.11</td>
<td>1.045 ± 0.16</td>
<td>1.04</td>
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<tr>
<td>(1 µg)</td>
<td>1.347± 0.10</td>
<td>1.30</td>
<td>1.125 ± 0.02</td>
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<tr>
<td>Isoxazole 4l (0.001 µg)</td>
<td>1.171± 0.0 7</td>
<td>1.13</td>
<td>1.009± 0.03</td>
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<tr>
<td>(0.01 µg)</td>
<td>1.168 ± 0.05</td>
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<td>1.007 ± 0.14</td>
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<td>(0.1 µg)</td>
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<td>0.89</td>
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<tr>
<td>(1 µg)</td>
<td>2.103 ±0.04</td>
<td>2.03</td>
<td>1.193 ±0.02</td>
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</tr>
<tr>
<td>Isoxazole 4m (0.001 µg)</td>
<td>1.170± 0.01</td>
<td>1.13</td>
<td>1.013 ± 0.06</td>
<td>1.01</td>
</tr>
<tr>
<td>(0.01 µg)</td>
<td>1.158 ± 0.01</td>
<td>1.11</td>
<td>1.023 ± 0.15</td>
<td>1.02</td>
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<tr>
<td>(0.1 µg)</td>
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<td>1.14</td>
<td>1.082 ± 0.16</td>
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<td>(1 µg)</td>
<td>1.986 ± 0.01</td>
<td>1.92</td>
<td>1.185 ± 0.02</td>
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The proliferation was calculated based on MTT assay. Absorbance was recorded at 570 nm. Values are expressed as Mean ± S.E. of three observations. SI is the stimulation Index which is calculated as T/C (Mean of Test Drug/Mean of Control). N.T. = Not Tested

Fig 2: Effect on SRBC induced antibody in mice
Effect of isoxazole 4a, isoxazole 4b and isoxazole 4l (0.1, 0.01 and 0.001 mg/kg) on humoral immunity by haemagglutination antibody titre. Data are means ± S.E. of five animals. Levamisole and cyclophosphamide was taken as standard.*P < 0.05, **P < 0.01 and ***P < 0.001 when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).
Fig 3: Effect on SRBC induced DTH reaction in Balb/C mice
Effect of isoxazole 4a, isoxazole 4b and isoxazole 4l (0.1, 0.01 and 0.001 mg/kg) on cell mediated immunity as assessed by delayed type hypersensitivity reaction in mice. Data are means ± S.E. of five animals. Levamisole and cyclophosphamide was taken as standard. *P < 0.05, **P < 0.01 and ***P < 0.001 when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

A)

B)
Fig 4A-C: Effect on Th1 (IFN-γ and IL-2) and Th2 (IL-4) cytokines release
Effect of isoxazole 4a, isoxazole 4b and isoxazole 4l (0.1, 0.01 and 0.001 mg/kg) on cytokine release in serum. Levamisole and cyclophosphamide was taken as standard. Data are means ± S.E. of five animals. *P < 0.05, **P < 0.01 and ***P < 0.001 when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

Fig 4A-C: Effect on spleen T-cells subtypes (CD4 and CD8)
Effect of isoxazole 4a, isoxazole 4b and isoxazole 4l (0.1, 0.01 and 0.001 mg/kg) on CD4/CD8 population in spleen cells by flow cytometry. Levamisole and cyclophosphamide was taken as standard. Data are means ± S.E. of five animals. *P < 0.05, **P < 0.01 and ***P < 0.001 when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).
3.1.6. Conclusion:-

In conclusion, we presented the synthesis of 3-aryl-5-butynyl isoxazoles in high yields with high product selectivity and method described in this work may find utility as an alternative to existing protocols for the synthesis of 3-aryl-5-butynylisoxazoles. Furthermore, isoxazoles with butynyl group at fifth position acts as potential immune-stimulators when properly tuned with the substitution pattern on the aromatic ring at the third position. Furthermore, the present study has shown the immunostimulatory activity of 5-butynyl-3-aryl isoxazole particularly isoxazole 4a suggests its possible therapeutic usefulness. However, further derivatization and mechanism-based and safety studies would lead to a better understanding of the mode of action of isoxazoles on immune system for immunestimulatory activity.
3.1.7. Experimental section:

**Synthesis of 3-(4'-Methoxyphenyl)-5-butynyl isoxazole (Typical procedure)**

In a typical procedure, to a suspension of magnesium turnings (0.12 g, 5 mmol, 5 equiv.) in specially dried tetrahydrofuran with mercury (II) chloride (5 mg, 1 % w/w of propargyl bromide) was added propargyl bromide (80 wt.% solution in toluene, 4 mmol, 4 equiv) in small portions while stirring the reaction mixture at room temperature (Note: A small grain of iodine is generally required to promote formation of the Grignard reagent.). The mixture was stirred at room temperature for 2 h to give a cloudy light green solution. The allenylmagnesium bromide generated as above was cooled to 0-5 °C and added drop wise to a solution of various phenyl substituted benzonitrile oxide (1 mmol, generated *in situ* by the treatment of triethylamine with the corresponding chlorooxime, 1 mmol) in THF (15 ml) over a period of 10 minutes while maintaining the temperature between 0-5 °C. The reaction mass was allowed to attain rt. and stirring was continued at ambient temperature for 6 h followed by quenching with aqueous ammonium chloride solution (10 ml) and diluting with dichloromethane (50 ml). The organic layer was separated and the aqueous layer extracted with dichloromethane (2x20 ml). The combined organic layers were dried (anhydrous Na₂SO₄) and evaporated under reduced pressure to afford crude product which was subjected to chromatography (silica gel, 200-400 mesh, elution; *n*-hexane/EtOAc gradient) to afford pure 3-*substituted aryl*-5-butynylisoxazole with good yields and characterized by ¹H, ¹³C NMR, Mass and IR spectroscopy.

1. 3-(4'-Methoxyphenyl)-5-butynyl isoxazole (4a):

![Image of 3-(4'-Methoxyphenyl)-5-butynyl isoxazole](image)

**¹H NMR** (CDCl₃, 200 MHz): δ 2.02 (t, 1H, *J* = 2.5 Hz), 2.63 (m, 2H), 3.02 (t, 2H, *J* = 7.2 Hz), 3.87 (s, 3H), 6.37 (s, 1H), 6.98 (dd, 2H, *J* = 11.7 and 2.8 Hz), 7.72 (dd, 2H, *J* = 11.4 and 2.7 Hz).

**¹³C NMR** (CDCl₃, 50 MHz): δ 17.08, 26.13, 55.35, 69.76, 82.11, 99.39, 114.26, 121.72, 128.16, 160.92, 162.05, 171.38.

**IR (KBr, cm⁻¹)**: 3281, 2966, 2937, 1608, 1527, 1459, 1431, 1256, 1176, 1064, 840, 790, 659, 533.
MS (EI, 70eV): m/z 227 (M⁺) and 249 (M⁺ + Na).

Elemental anal. for C₁₄H₁₃NO₂: C = 73.99, H = 5.77, N = 6.16: Found C = 73.97, H = 5.79, N = 6.16.

2. 3-(4′-Fluorophenyl)-5-butynyl isoxazole (4b):

\[
\text{\textsuperscript{1}H NMR (CDCl₃, 200 MHz): } \delta 2.02 (t, 1H, J = 2.5 Hz), 2.59 (m, 2H), 3.02 (t, 2H, J = 7.2 Hz), 6.52 (s, 1H), 7.01 (dd, 2H, J = 10.9 and 2.1 Hz), 7.57 (dd, 2H, J = 11.0 and 2.3 Hz).
\]

\[
\text{\textsuperscript{13}C NMR (CDCl₃, 50 MHz): } \delta 19.06, 28.03, 70.66, 83.11, 101.39, 115.26, 123.72, 129.16, 161.72, 164.15, 172.56.
\]

IR (KBr, cm⁻¹): 3282, 2980, 1620, 1560, 1459, 1016, 840, 755, 659, 543.

MS (EI, 70eV): m/z 215.27.


3. 3-(4′-Chlorophenyl)-5-butynyl isoxazole (4c):

\[
\text{\textsuperscript{1}H NMR (CDCl₃, 200 MHz): } \delta 2.02 (t, 1H, J = 2.6 Hz), 2.66 (m, 2H), 3.23 (t, 2H, J = 7.2 Hz), 6.65 (s, 1H), 7.12 (d, J = 8.9 Hz, 2H), 7.53 (d, J = 8.7 Hz, 2H).
\]

\[
\text{\textsuperscript{13}C NMR (CDCl₃, 50 MHz): } \delta 18.06, 29.03, 69.66, 82.11, 100.39, 125.06, 128.72, 130.16, 135.72, 164.15, 171.56.
\]

IR (KBr, cm⁻¹): 3281, 2982, 1620, 1562, 1460, 1015, 1064, 841, 756, 660, 545.

MS (EI, 70eV): m/z 232.8 (M⁺ + 1).

4. 3-(3′-Bromo-4′-methoxyphenyl)-5-butylnyl isoxazole (4d):

\[ \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta 2.12 \text{ (t, } 1H, J = 2.6 \text{ Hz), 2.70 (m, } 2H, 3.04 \text{ (t, } 2H, J = 7.2 \text{ Hz), 3.82 (s, } 3H), 6.45 \text{ (s, } 1H), 7.23 \text{ (s, } 1H), 7.71 \text{ (d, } 1H, J = 2.1 \text{ Hz), 7.97 (d, } 1H, J = 2.1 \text{ Hz).} \]

\[ \text{C NMR (CDCl}_3, 50 \text{ MHz): } \delta 16.24, 25.32, 56.04, 79.44, 82.45, 98.73, 112.76, 117.67, 127.21, 129.37, 134.44, 161.55, 166.25, 171.87. \]

\[ \text{IR (KBr, cm}^{-1}) \text{: } 3091, 2906, 1618, 1507, 1479, 1401, 1243, 1096, 1042, 798, 636. \]

\[ \text{MS (EI, 70eV): } m/z 307.8 \text{ (M}^+ +1). \]

Elemental anal. for C_{14}H_{12}BrNO_{2}: C = 54.92, H = 3.95, N = 4.58: Found C = 54.90, H = 3.98, N = 4.57.

5. 3-(4′-Methylphenyl)-5-butylnyl isoxazole (4e):

\[ \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta 2.01 \text{ (t, } J = 2.6 \text{ Hz, } 1H), 2.37 \text{ (s, } 3H), 2.63 \text{ (m, } 2H), 3.00 \text{ (t, } J = 7.2 \text{ Hz, } 2H), 6.39 \text{ (s, } 1H), 7.24 \text{ (d, } J = 7.9 \text{ Hz, } 2H), 7.68 \text{ (d, } J = 8.1 \text{ Hz, } 2H). \]

\[ \text{C NMR (CDCl}_3, 50 \text{ MHz): } \delta 17.06, 21.39, 26.11, 69.78, 82.09, 99.53, 126.34, 126.65, 129.55, 139.99, 162.37, 171.45. \]

\[ \text{IR (KBr, cm}^{-1}) \text{: } 3300, 3123, 2922, 2854, 1604, 1526, 1463, 1428, 1385, 1254, 1158, 1116, 1043, 1019, 990, 949, 908, 831, 803, 651, 514. \]

\[ \text{MS (EI, 70eV): } m/z 211.9 \text{ (M}^+). \]

6. 3-(3′-Methylphenyl)-5-butynyl isoxazole (4f):

\[ \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta 2.02 (t, 1H, \text{J} = 2.6 \text{ Hz}), 2.52 (s, 3H), 2.64 (m, 2H), 3.01 (t, 2H, \text{J} = 7.1 \text{ Hz}), 6.32 (s, 1H), 7.22 (m, 3H), 7.57 (d, 1H, \text{J} = 6.7 \text{ Hz}). \]

\[ \text{C NMR (CDCl}_3, 50 \text{ MHz): } \delta 17.11, 21.06, 26.07, 69.77, 82.08, 102.31, 125.94, 128.95, 129.37, 129.41, 131.02, 136.86, 163.07, 170.69. \]

\[ \text{IR (KBr, cm}^{-1}): 3297, 2925, 2853, 1599, 1503, 1433, 1402, 1350, 1271, 1153, 1117, 1017, 950, 901, 800, 765, 726, 643. \]

\[ \text{MS (EI, 70eV): m/z 211.7 (M^+).} \]

\[ \text{Elemental anal. for C}_{14}\text{H}_{13}\text{NO: C = 79.59, H = 6.20, N = 6.63: Found C = 79.57, H = 6.23, N = 6.63.} \]

7. 3-(2′-Methylphenyl)-5-butynyl isoxazole (4g):

\[ \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta 2.04 (t, 1H, \text{J} = 2.6 \text{ Hz}), 2.50 (s, 3H), 2.71 (m, 2H), 3.22 (t, 2H, \text{J} = 7.1 \text{ Hz}), 6.31 (s, 1H), 7.45 (m, 3H), 7.54 (m, 1H). \]

\[ \text{C NMR (CDCl}_3, 50 \text{ MHz): } \delta 17.19, 21.06, 26.07, 69.77, 82.08, 102.31, 125.94, 128.95, 129.37, 131.41, 133.02, 137.86, 162.07, 168.69. \]

\[ \text{IR (KBr, cm}^{-1}): 3233, 2925, 2853, 1599, 1503, 1433, 1402, 1350, 1271, 1153, 1017, 949, 902, 7990, 765, 726, 645. \]

\[ \text{MS (EI, 70eV): m/z 211.0 (M^+).} \]

\[ \text{Elemental anal. for C}_{14}\text{H}_{13}\text{NO: C = 79.59, H = 6.20, N = 6.63: Found C = 79.59, H = 6.22, N = 6.60.} \]
8. 3-(3’-Hydroxy-4’-methoxyphenyl)-5-butynyl isoxazole (4h):

\[
\text{\begin{figure}[h]
\centering
\includegraphics[width=0.45\textwidth]{image}
\caption{Structure of 3-(3’-Hydroxy-4’-methoxyphenyl)-5-butynyl isoxazole (4h)}
\end{figure}}
\]

\[\begin{align*}
^1H\text{ NMR} (\text{CDCl}_3, 200\text{ MHz}): & \quad \delta 2.21 (t, 1H, J = 2.5\text{ Hz}), 2.71 (m, 2H), 3.02 (t, 2H, J = 7.2\text{ Hz}), 3.94 (s, 3H), 6.30 (s, 1H), 7.22 (m, 2H), 7.53 (d, 1H, J = 6.8\text{ Hz}). \\
^13C\text{ NMR} (\text{CDCl}_3, 50\text{ MHz}): & \quad \delta 17.18, 26.07, 55.66, 69.77, 82.08, 102.31, 112.01, 116.89, 122.45, 126.94, 148.95, 159.86, 163.07, 170.69. \\
\text{IR (KBr, cm}^{-1}) & \quad 3300, 3092, 2908, 1619, 1511, 1477, 1401, 1243, 1096, 1045, 796, 646; \\
\text{MS (EI, 70eV)} & \quad m/z 266 (M^{+} + \text{Na}). \\
\text{Elemental anal.} & \quad \text{for C}_{14}\text{H}_{13}\text{NO}_3: \text{C} = 69.12, \text{H} = 5.39, \text{N} = 5.76; \text{Found C} = 69.16, \text{H} = 5.37, \text{N} = 5.75.
\end{align*}\]

9. 3-(3’-Nitrophenyl)-5-butynyl isoxazole (4i):

\[
\text{\begin{figure}[h]
\centering
\includegraphics[width=0.45\textwidth]{image}
\caption{Structure of 3-(3’-Nitrophenyl)-5-butynyl isoxazole (4i)}
\end{figure}}
\]

\[\begin{align*}
^1H\text{ NMR} (\text{CDCl}_3, 200\text{ MHz}): & \quad \delta 2.12 (t, 1H, J = 2.6\text{ Hz}), 2.66 (m, 2H), 3.12 (t, 2H, J = 7.1\text{ Hz}), 6.54 (s, 1H), 7.50 (m, 2H), 8.0 (m, 1H), 8.93 (s, 1H). \\
^13C\text{ NMR} (\text{CDCl}_3, 50\text{ MHz}): & \quad \delta 17.07, 26.11, 70.11, 84.98, 101.53, 122.76, 124.72, 128.16, 134.45, 135.67, 150.50, 168.25, 172.87. \\
\text{IR (KBr, cm}^{-1}) & \quad 3382, 3365, 3283, 2982, 1620, 1561, 1459, 1014, 1063, 842, 751, 658, 543; \\
\text{MS (EI, 70eV)} & \quad m/z 243.2 (M+1). \\
\text{Elemental anal.} & \quad \text{for C}_{13}\text{H}_{10}\text{N}_2\text{O}_3: \text{C} = 64.46, \text{H} = 4.16, \text{N} = 11.56; \text{Found C} = 64.48, \text{H} = 4.14, \text{N} = 11.57.
\end{align*}\]
10. 3-(2'-Nitrophenyl)-5-butynyl isoxazole (4j):

![Chemical Structure](image)

^{1}H NMR (CDCl$_3$, 200 MHz): δ 2.22 (t, 1H, $J = 2.6$ Hz), 2.60 (m, 2H), 3.22 (t, 2H, $J = 7.1$ Hz), 6.53 (s, 1H), 7.5 (m, 2H), 8.31 (m, 2H).

^{13}C NMR (CDCl$_3$, 50 MHz): δ 17.07, 26.11, 70.12, 83.98, 99.53, 121.76, 123.72, 128.23, 131.16, 134.55, 150.11, 166.25, 171.87.

IR (KBr, cm$^{-1}$): 3380, 3283, 2981, 1620, 1560, 1459, 1016, 1064, 842, 754, 658, 543.

MS (EI, 70eV): m/z 243.23 (M$^+$ + 1).

Elemental anal. for C$_{13}$H$_{10}$N$_2$O$_3$: C = 64.46, H = 4.16, N = 11.56: Found C = 64.54, H = 4.10, N = 11.60.

11. 3-Phenyl-5-butynyl isoxazole (4k):

![Chemical Structure](image)

^{1}H NMR (CDCl$_3$, 200 MHz): δ 2.01 (t, 1H, $J = 2.6$ Hz), 2.62 (m, 2H), 3.03 (t, 2H, $J = 7.1$ Hz), 6.49 (s, 1H), 7.19 (m, 4H), 7.32 (m, 1H).

^{13}C NMR (CDCl$_3$, 50 MHz): δ 19.17, 26.11, 69.86, 81.98, 99.53, 126.45, 128.78, 129.22, 129.72, 131.22, 135.75, 164.25, 170.77.

IR (KBr, cm$^{-1}$): 3283, 2980, 1622, 1560, 1459, 1018, 1063, 842, 758, 660, 543.

MS (EI, 70eV): m/z 198.4 (M$^+$ + 1).

Elemental anal. for C$_{13}$H$_{11}$NO; C, 79.16; H, 5.62; N, 7.10: Found C, 79.19; H, 5.64; N, 7.13.

12. 3-(4'-Cyanophenyl)-5-butynyl isoxazole (4l):

![Chemical Structure](image)
**Chapter-III, Section-A**

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 2.12 (t, 1H, $J = 2.7$ Hz), 2.62 (m, 2H), 3.01 (t, 2H, $J = 7.1$ Hz), 6.42 (s, 1H), 7.81 (d, 2H, $J = 8.8$ Hz), 8.42 (d, 2H, $J = 8.9$ Hz).

$^{13}$C NMR (CDCl$_3$, 50 MHz): $\delta$ 17.07, 26.11, 69.86, 81.98, 99.53, 112.33, 115.76, 128.16, 134.55, 161.55, 166.25, 171.87.

IR (KBr, cm$^{-1}$): 3206, 2943, 2224, 1638, 1569, 1462, 1016, 824, 779, 549.

MS (EI, 70eV): m/z 246.4 (M$^+$ +1 + Na).

Elemental anal. for C$_{14}$H$_{10}$N$_2$O: C = 75.66, H = 4.54, N = 12.60: Found C = 75.68, H = 4.52, N = 12.58.

13. 3-(4′-Hydroxyphenyl)-5-butynyl isoxazole (4m):

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 2.13 (t, 1H, $J = 2.4$ Hz), 2.73 (m, 2H), 3.15 (t, 2H, $J = 7.2$ Hz), 6.50 (s, 1H), 7.02 (d, 2H, $J = 8.7$ Hz), 7.52 (d, 2H, $J = 8.7$ Hz).

$^{13}$C NMR (CDCl$_3$, 50 MHz): $\delta$ 18.18, 26.13, 69.76, 83.11, 101.39, 129.26, 130.72, 132.16, 148.92, 163.05, 172.48.

IR (KBr, cm$^{-1}$): 3300, 3206, 2943, 1638, 1547, 1462, 1249, 1146, 1036, 824, 779, 652;

MS (EI, 70eV): m/z 214.7 (M$^+$ + 1).

Elemental anal. for C$_{13}$H$_{11}$NO$_2$: C = 73.23, H = 5.20, N = 6.57: Found C = 73.20, H = 5.25, N = 6.56.

3.1.8. Biological Methodologies:-

A). *In-vitro* study: Splenocyte proliferation assay

Spleen collected under aseptic conditions in HBSS, was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × g at 4 °C for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FCS]. The cell
number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. To evaluate the effect of the test samples on the proliferation of splenic lymphocytes, the spleen cell suspension (1×10^7 cell/mL) was pipetted into 96-well plates (200 µL/well) and cultured at 37 °C for 72 h in a humid saturated atmosphere containing 5% CO₂ in the presence of Con-A (5 µg/mL) and LPS (10 µg/mL). After 72 h, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 µL of a DMSO working solution (192 µL DMSO with 8 µL 1 M HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

B). In-vivo study: Treatment and Immunization

SRBC collected in Alsever’s solution, were washed three times in large volumes of pyrogen-free 0.9% normal saline and adjusted to a concentration of 5 × 10^9 cells/mL for immunization and challenge. The animals were divided into groups of five animals each. The test samples were dissolved in 1% gum acacia and were administered orally for 14 days. The dose volume was 0.2 mL.

Group of five mice was immunized intraperitoneally with SRBC (5 × 10^9 cells/ml) followed by treatment of different doses of test samples and challenge on day 7 in the footpad in a final volume of 20 µl (5 × 10^9 cells/ml). On day 7 and 14, the primary (IgM) and secondary (IgG) antibody titre was observed. After challenge injection on day 7, delay type hypersensitivity in mice was expressed in terms of the swelling of the foot pad after different 24 h.

HA titre

Blood was collected on days 7 and 15 from the retro-orbital plexus of each mouse for serum preparation. Serial two-fold dilutions of serum samples were made in 50 µL of PBS (pH 7.2) in 96-well microtitre plates and mixed with 50 µL of 1% SRBC suspension in PBS. After mixing, the plates were kept at room temperature for 2 h. The value of the antibody titre was assigned to the highest serum dilution showing visible haemagglutination.

DTH reaction

The test samples were administered 2 h after SRBC injection and once daily on consecutive days. Six days later, the thickness of the left hind footpad was measured with a spheromicrometer (pitch, 0.01 mm) and was considered as the control. The mice were then
challenged by injecting 20 μL of $5 \times 10^9$ SRBC/mL intradermally into the left hind footpad. The foot thickness was measured again after 24 h.

**Determination of IFN-γ, IL-2 and IL-4 by ELISA method**

Serum was collected 4 h after the final oral administration of test samples (0.001, 0.01 and 0.1 mg/kg). The interleukin-2 (IL-2), interferon-gamma (IFN-γ) and interleukin (IL-4) concentration were measured with an enzyme-linked immunosorbent assay (ELISA kit, R & D Systems) according to the instructions of the manufacturer.

**Lymphocyte immunophenotyping (spleen)**

The spleen (one-third of the organ) was placed in PBS buffer (without Mg$^{2+}$ and Ca$^{2+}$) and stored on ice prior to preparation of single cell suspensions. Splenic erythrocytes were lysed with red blood cell lysing buffer (BD Pharmingen). Cell suspensions were refrigerated (ca. 4 °C) pending staining with antibodies. All reagents *viz.*, anti-CD4 FITC and anti-CD8a PE antibodies were purchased from BD Pharmingen. For each sample, $2 \times 10^6$ cells were stained with conjugated anti-CD4 FITC and anti-CD8a PE antibodies. After staining with antibodies, the cells were washed and resuspended in PBS for flow cytometric analysis, which was performed on a FACS Calibur flow cytometer equipped with Cell Quest software (Becton Dickinson).
References:


