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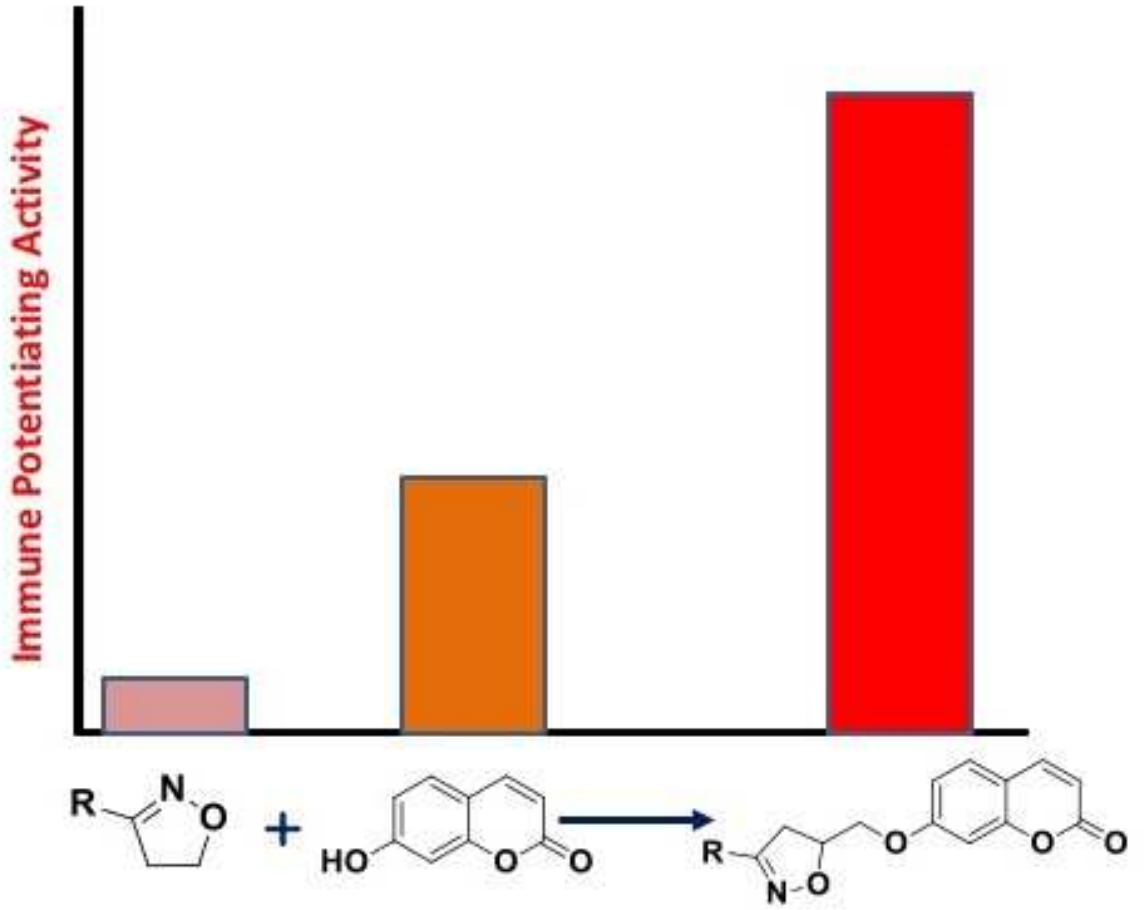

Synthesis and Immunopotentiating activity of novel Isoxazoline functionalized Coumarins

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Abstract

A novel series (13) of isoxazoline functionalized coumarins was synthesized through 1,3-dipolar cyclization of nitrile oxides with Allylatedcoumarins. Synthesis of effective and target selective immunostimulators through conjugation of diversely substituted isoxazolines and 7-hydroxycoumarins is the focus of the present article. The proposed synthetic scheme was observed to be highly regiospecific yielding attempted conjugates in good yield (>90%). Kinetic resolution of the racemates was carried out by employing lipase B from *Candida antarctica* (CALB). The synthesized compounds were screened *in vitro* and *in vivo* for their biological activities *viz.* toxicity and impact on splenocyte proliferation (T- and B-cell proliferation), antibody production (HA titer), delayed-type hypersensitivity reaction (DTH), T-cell subtypes (CD4 and CD8), cytokine production (IL-2, IFN-γ, and IL-4) and NO (macrophage) production. Our results establish that isoxazoline functionalized coumarins exhibit excellent immune potentiating activity especially compounds **2**, **4** and **8** whose activity is more than that of Levimasole as standard. The structure activity relations are explained in light of the structural/functional aspects of tested compounds. To the best of our knowledge the presented work is first of its kind and is presaged to prove very useful for the

design and synthesis of *bis*-heterocycle based novel, therapeutically selective and effective immunopotentiators.

Keywords: 7-hydroxycoumarin; 2-isoxazoline; *Bis*-heterocycles; kinetic resolution; Immunopotentiation.

Introduction

Encouraging results from immunomodulation based immunopharmacological approaches as a cure for many biological malfunctions has stimulated an intense research activity towards design of appropriate, less toxic and effective immunopotentiators. As an outcome of this intense research activity, a huge library of immunopotentiators, of natural and synthetic origin is available today [1-6]. Among the currently known immunopotentiators like, muramyl dipeptide derivatives [7], levamisole [8], niridazole [9], and those from the groups of interferons and interleukins, many have been found to express undesirable side effects and/or high toxicity during the clinical trials [10-13].The presaged potential of immunomodulation based therapies and the safety and therapeutic effectiveness concerns about currently known immunopotentiators, makes studies related to design of novel, safe to use and effective immunopotentiators indispensable. The development of natural product based low molecular weight immunopotentiators is considered as one of the promising approaches in this direction [6].

Aromatic heterocycles have served as valuable synthetic templates for the preparation of new compounds with specific biological or material properties. Biological activities of *bis*-heterocycles, especially those with nitrogen and oxygen as heteroatoms are well documented in literature [14-16]. In addition to their use in synthetic chemistry, these compounds have been found to express cytotoxic, immunostimulatory, antimicrobial, tuberculostatic, plant growth regulative properties and various other biological activities [17]. Since recent years, attention is increasingly being paid towards the synthesis of aromatic *bis*-heterocyclic compounds. The diverse functional groupings and the synergistic activity of the fused pharmacophores make *bis*-heterocyclic compounds divalent dual target interacting with improved pharmacological functions. Individually both coumarins and isoxazolines in view of their immunostimulatory effects have been employed for diverse applications and pharmacological activities [14-16, 18-25]. Coumarins have been demonstrated to exhibit a broad spectrum of pharmacological activities like antidepressant, antimicrobial, antioxidant,

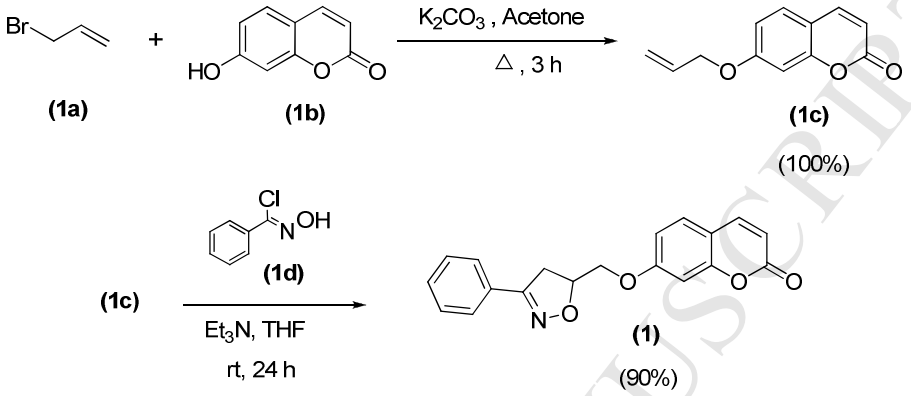
antitumor, antihistaminic, antiviral, CNS stimulant and anticoagulant [26-34]. Similarly isoxazolines; the O- and N-containing heterocycles have been extensively explored for synthetic and biological applications [35-37]. Isoxazolines have been demonstrated to exhibit interesting and diverse biological properties like antiinflamatory, antagonistic, anticancer, anti HIV, antidepressant and are currently used as pharmacophores of many clinically approved therapeutic agents [25, 38]. The compounds with two or more heterocycles play a vital role in natural and synthetic bioactive compounds [39-40]. Also it has been established that biological efficacy of coumarins can be desirably altered through incorporation of another heterocyclic moiety and the resulting compound often exhibiting promising and unprecedented properties. These above cited facts and literature documented biological expressions of coumarins and isoxazolines imply that conjugation of isoxazolines with coumarins can serve as a promising synthetic strategy for the design of effective and target selective immunostimulators; the same is demonstrated through the present work. Herein we report a simple scheme for the synthesis of novel immunopotentiators through conjugation of bioactive pharmacophoric isoxazolines with 7-hydroxycoumarin (7-OHC). A novel series of 13 isoxazoline functionalized 7-OHC (**Table 1**) was synthesized and tested *in vitro* and *in vivo* to probe the immune potentiating potential and relevant structure activity relationship (SAR) of these conjugates. The structures of new compounds were confirmed by chemical and spectroscopic methods like IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The synthesized conjugates were found to express excellent immunopotentiating activity. To the best of our knowledge the presented work is first of its kind and is presaged to prove very useful for the design and synthesis of *bis*-heterocycle based novel, therapeutically selective and effective immune potentiators.

Results and discussion

Chemistry

For the synthesis of aimed conjugates, the strategy of 1,3-dipolar cyclization between an Allylatedcoumarin and various substituted nitrile oxides (**Scheme 1**) was followed. Dipolarophile was obtained by alkylating 7-OHC with allylbromide using K_2CO_3. Nitrileoxides used as 1,3-dipoles, were synthesized according to the literature procedures [41]. The novel isoxazoline conjugates of 7-OHC synthesized in the ensuing work are presented in **Table 1**. Formation of 3,5-disubstituted isoxazolines possessing *O*-allyl derivative of 7-OHC at 5th position, was established through the characteristic chemical shift

values with multiplets at 4.00-4.26 ppm for the C5 protons and 3.00-3.65 ppm for C4 protons. Similarly, characteristic chemical shift values for CH_2 group of *O*-allyl derivative were established with double-doublets/multiplets in the range of 4.06-4.30 ppm and 5.05-5.29 ppm for the two non equivalent protons. The details of synthetic and characterization procedures are given in the experimental section.



Scheme 1: *Synthesis of bis-heterocycles encompassing 7-hydroxycoumarin and isoxazolines.*

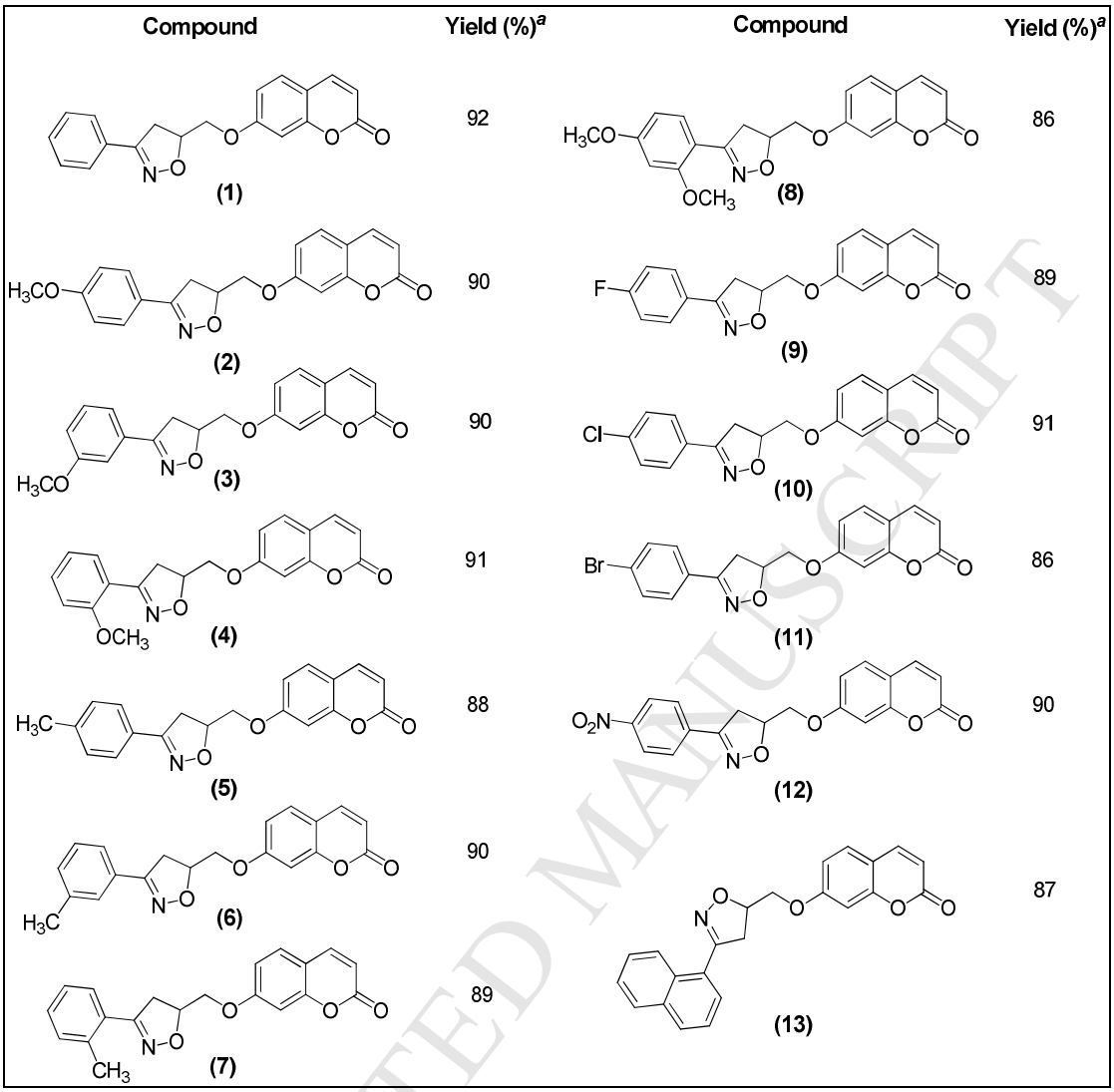


Table 1: *Synthesis of various isoxazoline derivatives of umbelliferone*
[a]*Isolated yields after chromatographic purification*

The isoxazoline derivatives as presented in **table 1**, possess one chiral centre in the isoxazoline ring thereby raising the possibility that derivatives synthesized as per **Scheme 1**, would be a mixture of two possible enantiomers. In order to investigate the impact of stereochemistry of *bis*-heterocyclic conjugates on their immune potentiating potential, kinetic resolution of the racemate by employing lipase B from *Candida Antarctica* (CALB) was carried out. The details of the kinetic resolution procedures are presented in the experimental section.

Evaluation of biological activity

The isoxazoline-coumarin conjugates synthesized for the present work (**Table 1**) were tested for their biological expressions *viz.* lymphocyte proliferation, antibody titre, delayed type

hypersensitivity response, spleenocyte proliferation, cytokine release, macrophage production and toxicity following procedures whose details given elsewhere [6] are also included in the supporting information. To begin with, all the synthesised compounds were tested for their impact on lymphocyte proliferation under *in vitro* conditions.The significant activity observed for compounds **2**, **4** and **8** in *in vitro* investigations motivated us for their further *in vivo* investigations. In the *in vivo* investigations, compounds **2**, **4** and **8** were tested for their impact on primary and secondary antibody and DTH response, spleen cell subtyping and proliferation, cytokine (IL-2, IFN-γ, and IL-4) and NO production. The efficacy of tested compounds was compared to the results observed for Levamisole (Lev), a commonly used standard for such investigations [8]. Lev was administered orally at a dose of 2.5 mg/kg body weight as a positive control. In view of the encouraging immunopotentiating activity of compounds **2**, **4** and **8** noticed during *in vivo* investigations these were also tested for their toxicity.

Effect of test compounds on *in vitro* lymphocyte proliferation by MTT assay

The results recorded during the *in vitro* screening of isoxazoline functionalized coumarins synthesised for the present study, for their impact on lymphocyte proliferation are presented as **Figure 1.** As is evident from these results, the screened compounds exhibit a dose dependent response. Compounds **2**, **3**, **4**, **5**, 7 and **8** were observed to significantly enhance the immunostimulation, especially compounds **2, 4** and **8** whose immunostimulating potential even at lower doses (0.1 µg/mL) seems significantly higher than that observed for levamisole (Lev) and 7-OHC. Immunostimulatory effect, as evident from Figure 1 was found to decrease with increase in dosage from 0.1-10 µg/mL in compounds **9-13**. These compounds did not enhance the lymphocyte proliferation significantly.

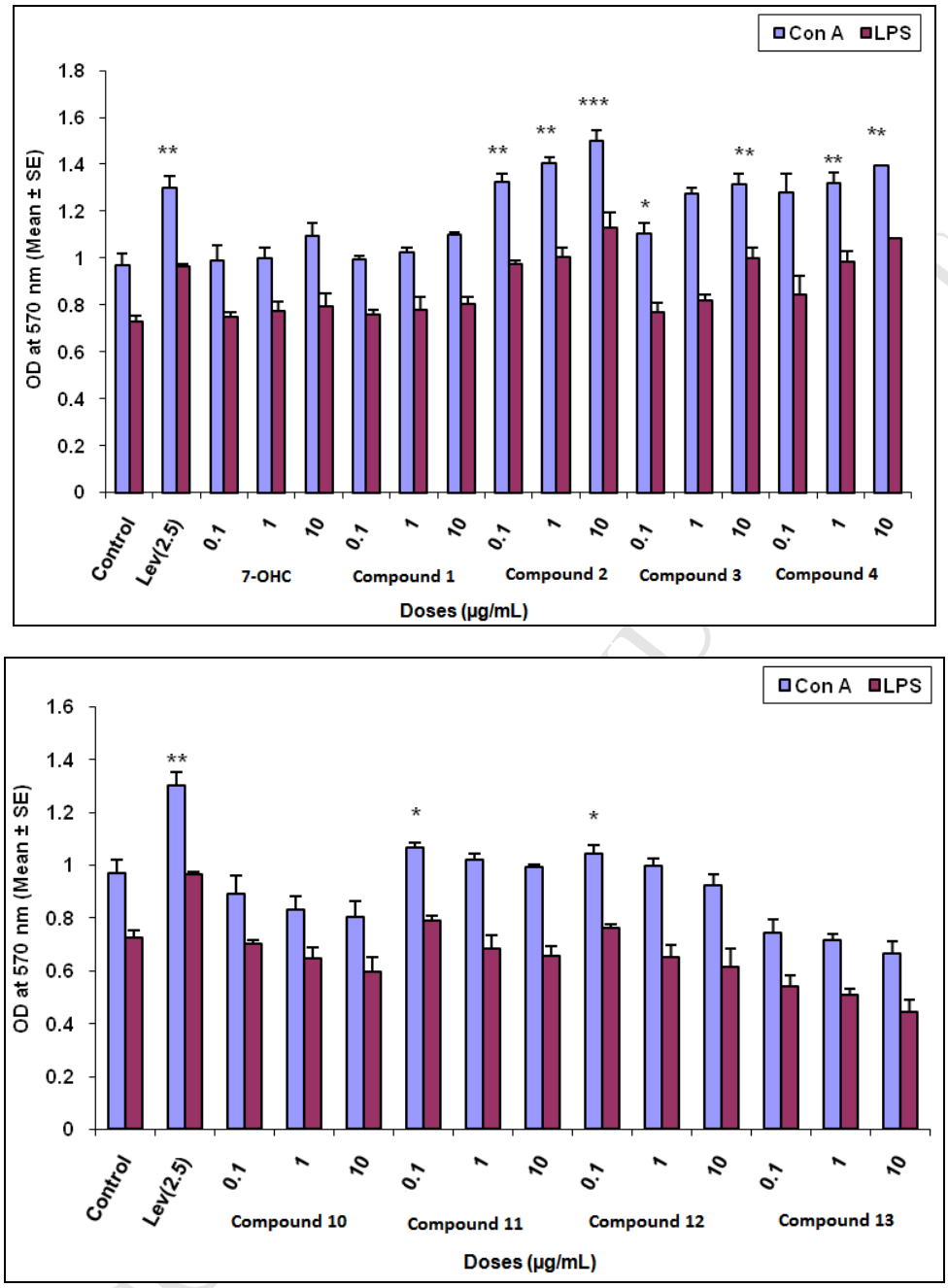


Figure 1: *Effect of test compounds on lymphocyte proliferation in vitro. The proliferation was calculated based on MTT assay. Absorbance was recorded at 570 nm. Values are expressed as mean ± S.E. of three observations.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).*

Effect on antibody titre

The compounds 2, 4 and 8 were tested for their impact on B- cell activation. For this we monitored the impact of these compounds at dose level of 0.001 mg/kg, 0.01 mg/kg and 0.1

mg/kg on primary and secondary antibody response through estimation of total antibody titre. Assuming that the response recorded on day 7 corresponds to primary antibody titre and that recorded on day 14 corresponds to secondary antibody titre; the results presented as Figure 2 suggest that in comparison to standard, compounds 2, 4 and 8 enhance the primary and secondary antibody synthesis by a significantly higher extent. In view of the above cited assumption it may be inferred that the extent of increase in antibody at 14 day (usually attributed to IgG titre) seems to be more than that of antibody response on day 7 (usually attributed to IgM titre). Comparatively, the immunostimualation activity of compound 2 at a dose of 0.1 mg/kg is highest.

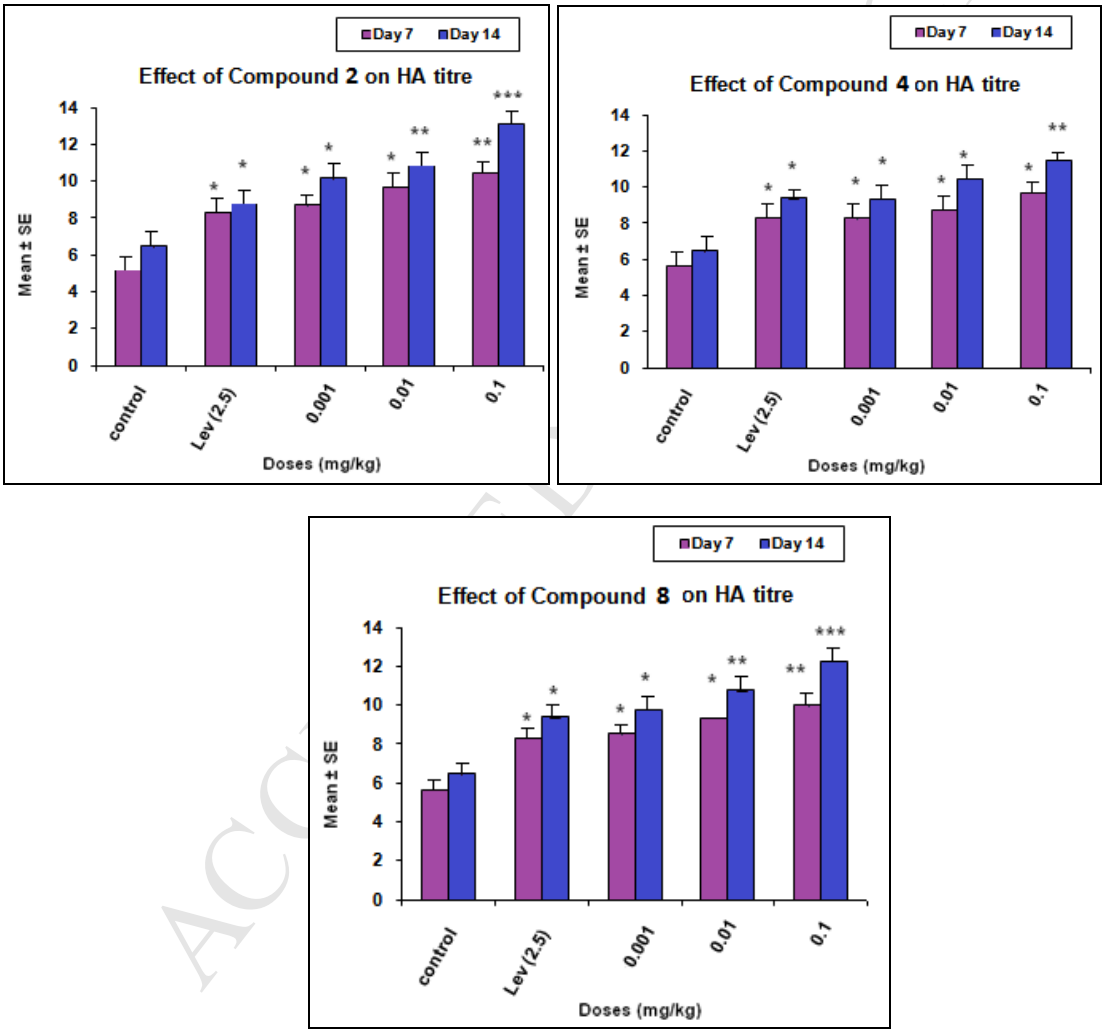


Figure 2: *Effect of compounds* **2**, **4** *and* **8***on antibody titres in mice. Data are mean ± S.E. of six animals.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

Delayed type hypersensitivity (DTH) response

Compounds **2**, **4** and **8** were administered at varying doses to mice and the impact on SRBC-induced DTH reaction was monitored. The results from these studies presented as **Figure 3** clearly indicate a significantly higher DTH response than that observed with Lev even at doses as low as 0.001 mg/kg. The DTH response was observed to decrease with time (24 hr > 48 hr > 72 hr) with compound **2** being the most effective.

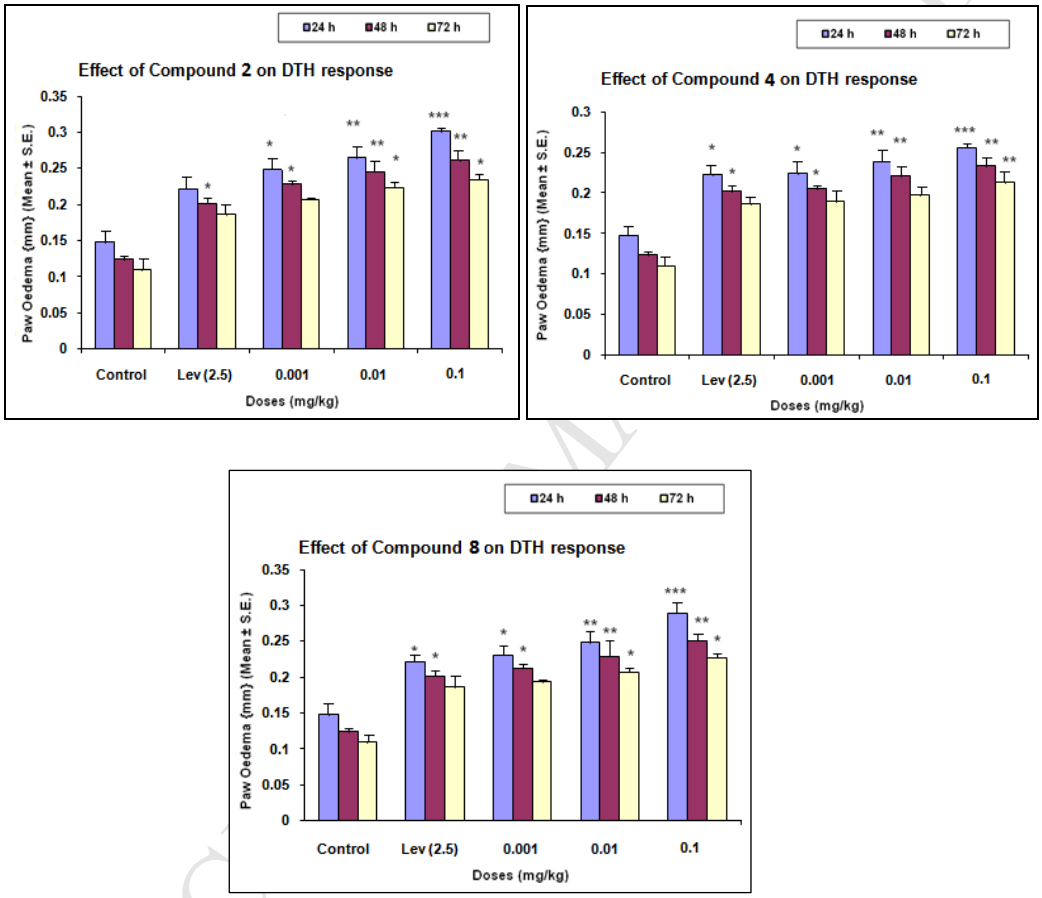


Figure 3: *Effect of compounds* **2**, **4** *and* **8** *on DTH response. Data are expressed as mean ± S.E. of five observations of left hind foot pad thickness measured at 24, 48 and 72 hr.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).*

Effect on spleenocyte proliferation *ex vivo* (T and B cell proliferation)

The results from *ex vivo* splenocyte proliferation investigations wherein compounds **2, 4** and **8** were administered at various doses to mice are depicted as **Figure 4**. Cell proliferation showed a regular increase with increase in the dose for all these compounds. As is evident

from **Figure 4**, compound **2** resulted in a significant increase in T and B cell proliferation at all doses in comparison to Lev and the other two compounds. Compound **4** showed proliferation equivalent to Lev at a dose of 0.01 mg/kg. Compound **8** also increased cell proliferation to a significant extent.

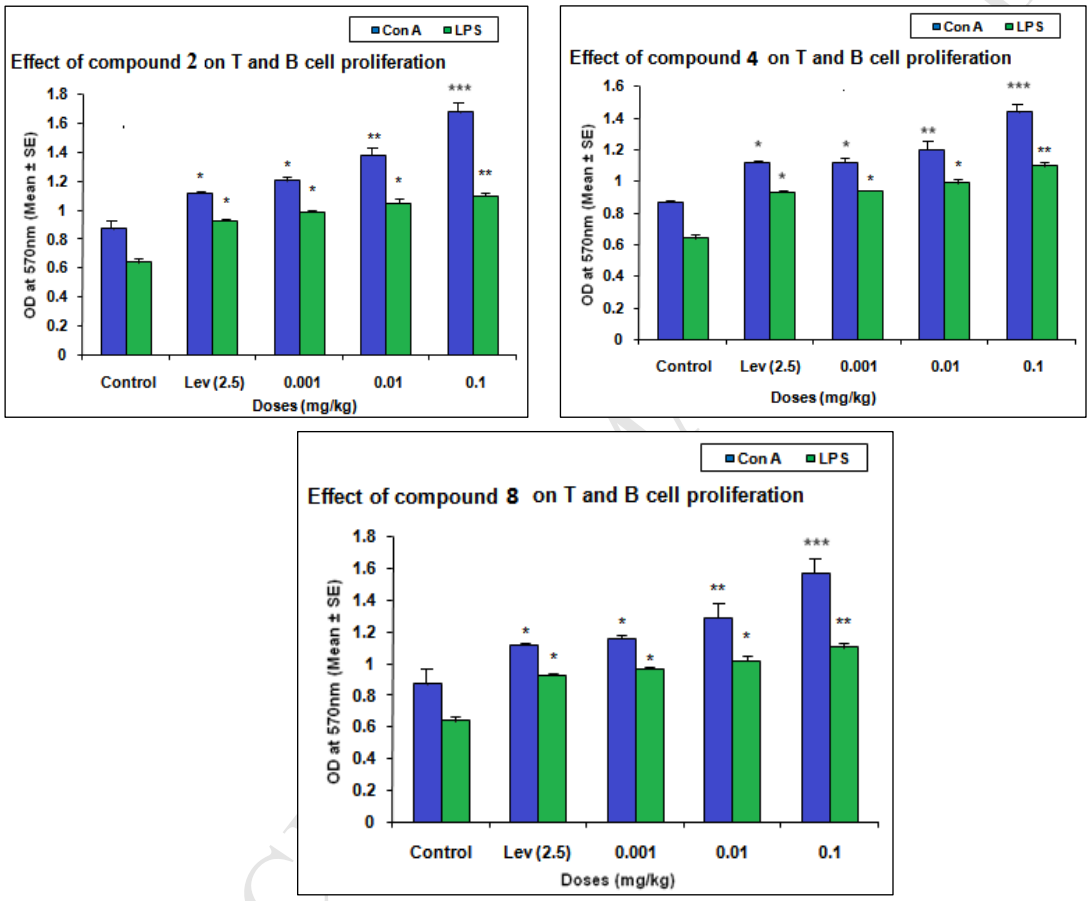


Figure 4: *Effect of compounds* **2**, **4** *and* **8** *on T and B cell proliferation.Splenocyte proliferation expressed as the absorption at 570 nm ex vivo. Data are mean ± SE of six animals.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

Effect on spleen T-cell subtyping

The impact of compounds **2**, **4** and **8** on T-cell subtyping ($CD4^{+}/CD8^{+}$) in spleen single cell suspension was monitored by flow cytometry. For these investigations fluoresceine-isothiocyanate (FITC) conjugated anti-CD4 and phycoerthyrin (PE) conjugated CD8 monoclonal antibodies were employed. The total amounts of CD4 and CD8 subtypes were

calculated by multiplying their estimated ratios with the total spleen cell contents and the results are enlisted in **Table 2**. In these studies compound **2** seems to be very effective yielding 40.2% $CD4^+$ and 21.6% $CD8^+$ T cells at a dose of 0.1 mg/kg which is much better than that observed for the control values of 20.7% of $CD4^+$ and 13.3% of $CD8^+$ T cells under similar conditions. Even with an oral dose of 2.5 mg/kg Lev stimulated $CD4^+$ and $CD8^+$ T cells only to the extent of 30.8% and 18.3% respectively.

Treatment	**Dose (mg/kg)**	**$CD4^+$ T-Cell (%)**	**$CD8^+$ T-Cell (%)**	**CD4/CD8 Ratio**	**Spleen $CD4^+$ Content ($\times10^7$)**	**Spleen $CD8^+$ Content ($\times10^7$)**
Control (vehicle)		20.7 ± 0.90	13.3 ± 0.34	1.56 ± 0.09	2.70 ± 0.12	1.47 ± 0.04
Levamisole	2.5	30.8 ± 1.30*a*	18.3 ± 0.53*a*	1.68 ± 0.06*a*	1.48 ± 0.10*a*	1.38 ± 0.10*a*
Compound **2**	0.001	30.3 ± 0.58	16.4 ± 0.37	1.32 ± 0.06	1.23 ± 0.06	1.26 ± 0.03
	0.01	34.2 ± 0.62	19.8 ± 0.39	1.72 ± 0.06	1.61 ± 0.06	1.35 ± 0.03
	0.1	40.2 ± 0.70	21.6 ± 0.46	1.86 ± 0.07	1.85 ± 0.07	1.48 ± 0.04
Compound **4**	0.001	20.3 ± 0.63	15.4 ± 0.35	1.32 ± 0.07	1.02 ± 0.07	0.88 ± 0.04
	0.01	24.2 ± 0.59	15.3 ± 0.38	1.58 ± 0.07	1.31 ± 0.06	1.18 ± 0.03
	0.1	31.1 ± 0.59	18.4 ± 0.39	1.69 ± 0.07	1.51 ± 0.06	1.37 ± 0.03
Compound **8**	0.001	20.3 ± 0.58	15.4 ± 0.34	1.32 ± 0.06	1.16 ± 0.05	1.19 ± 0.03
	0.01	27.2 ± 0.59	16.2 ± 0.38*b*	1.67 ± 0.06	1.54 ± 0.06*b*	1.29 ± 0.03*b*
	0.1	35.3 ± 0.64*b*	19.9 ± 0.42*b*	1.77 ± 0.07	1.67. ± 0.07	1.41 ± 0.04*b*

Table 2: *Effect of different doses of compounds* **2**, **4** *and* **8***on spleen T cell subtypes*
Number of observation =6
(a) P < 0. 01; (b) P < 0.05

Effect on cytokine release (IL-2, IFN-γ& IL-4)

The impact of compounds **2**, **4** and **8** on the release of cytokines IL-2, IFN-γ, and IL-4 was monitored to point out their specific effects on cytokine profiles. The results presented as **Figure 5**, indicate a dose dependent impact on the release of IL-2, IFN-γ, and IL-4 wherein

IL-4 release was always the least one. Compound **2** seems to be the most effective followed by compound **8**. At a dose of 0.01 mg/kg the observed effect of compound **4** is comparable to that of Lev while at dose of 0.1 mg/kg it proves to be more effective than the later.

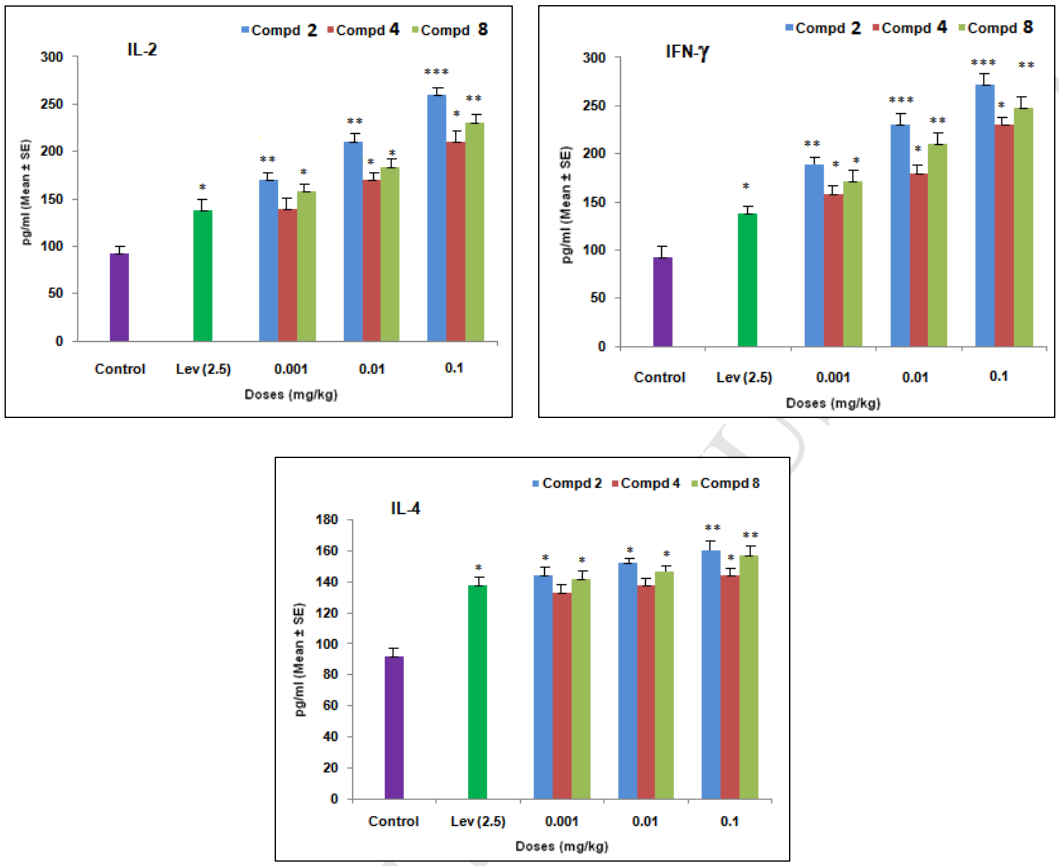


Figure 5: *The bars in the depicted graphs are a mean± SE of results from three different experiments carried under similar conditions. Mouse spleen cells (2 × 10^6 cells/mL) were stimulated for 48 h with and without (control) 2.5 µg/well Con-A in the presence of each of these compounds after which the cell supernatant was collected and tested for IL-2, IFN- γ and IL-4, by employing commercially available standard kits (Quantikine, R&D Systems).Impact of isoxazoline functionalized coumarins **2**, **4** and **8** on IL-2, IFN-γand IL-4 cytokine production at changing doses.*

Effect on NO production (macrophage production)

To test the impact of compounds **2, 4** and **8** on macrophage production, NO production in response to different doses (0.001, 0.01 and 0.1 mg/kg) of these compounds and the standard under similar conditions was monitored. The statistically analysed results from these experiments are depicted as **Figure 6.** As evident from the presented results, the NO production (an index of macrophage production) in response to administration of **2**, **4** and **8** is

significantly more than that of standard, and the production increases with the increase in dosage. Comparatively compound **2** seems to be the most active followed by **4** and **8**.

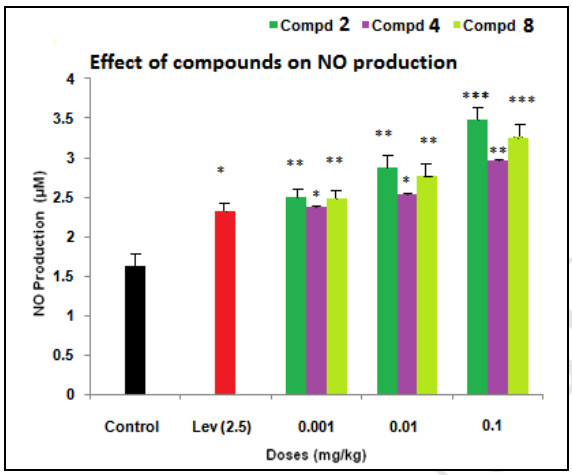


Figure 6: *Effect of compounds* **2**, **4** *and* **8** *on NO production.Results are expressed in µM. Data are mean ± SE of six animals.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

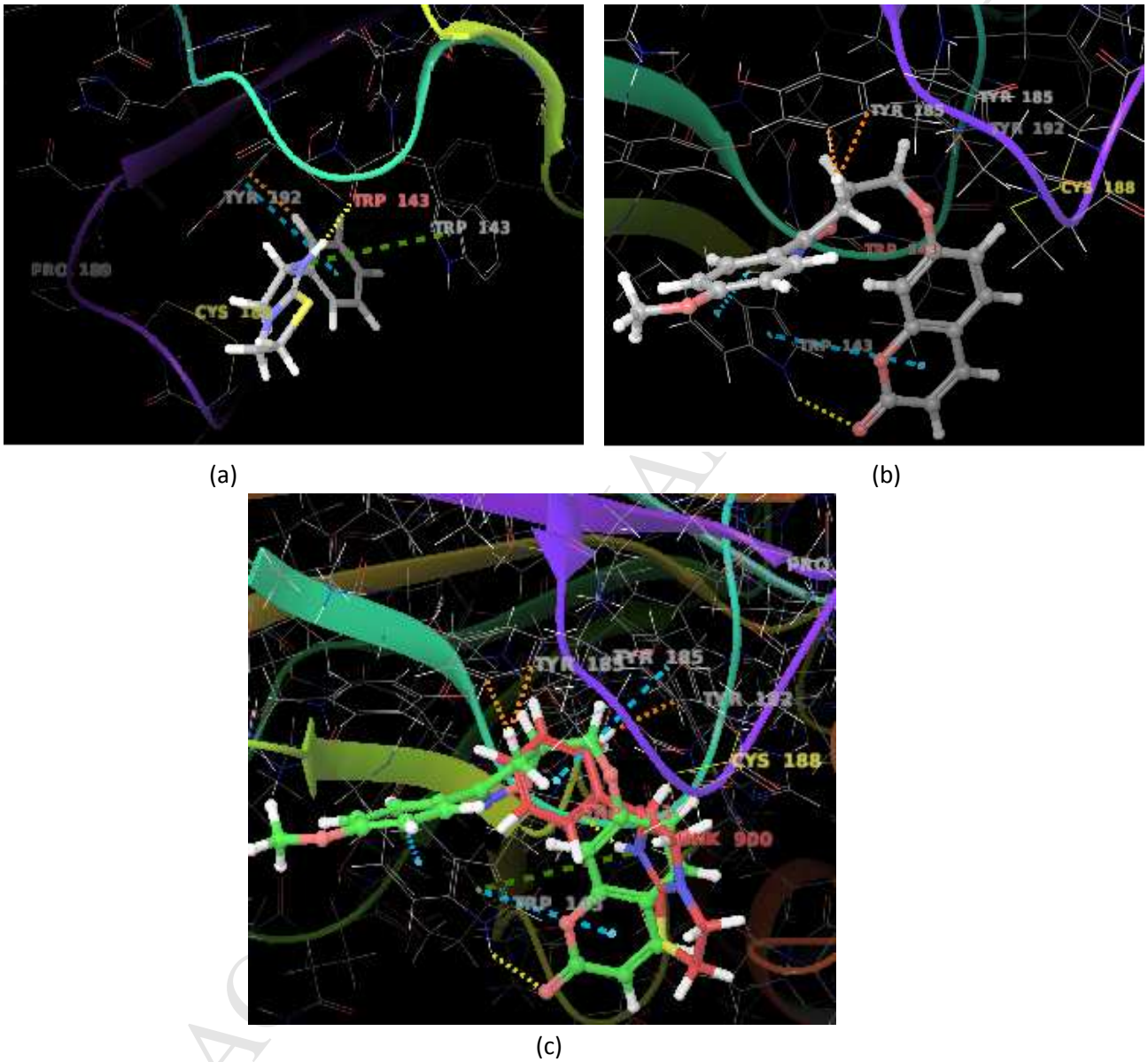
Toxicity studies

The synthesised compounds were tested for possible cellular toxic effects on spleen cells by MTT assay [42]. Our observations vis-a-vis toxicity studies suggest that even at a dose of as high 100 µg/mL the test compounds do not exhibit any toxic effect after 72 h incubation.

Docking Results

In this study we carried out the molecular docking studies, in which Levamisole, compounds **2**, **4** and **8** were docked into ATP binding site of Acetylcholine binding protein at 2.38 Å resolution (PDB code: 4MU1). The docking studies were performed with Glide (Grid-Based Ligand Docking with Energetics) program [43, 44] incorporated in the Schrödinger molecular modeling package (Schrödinger, LLC, New York, NY, 2015). The binding poses found by maestro 9.0 for Levamisole, compounds **2**, **4** and **8** are given in Figure 7 (also see figure S1). All the four compounds were successfully docked into the active site of 4MU1. On analyzing the docking poses the positioned compounds shows interactions with try 89 and ser186. In addition, we have observed a relatively consistent interaction of carbonyl group with trp 143. In order to validate the interaction results we superimposed our ligand with

standard (levamisole) ligand and observed that our ligands and the levamisole shares similar binding orientation against the 4MU1 (Figure 7 also see figure S2). Results from the docking studies indicate that binding affinity of the most active compounds follows the order 2> 8 > 4, and with almost similar binding affinity of R and S isomers for their respective enantiomers.



(a) (b)

(c)

Figure 7: Binding interactions of (a) levamisole, (b) comp-2, with against acetylcholine binding protein and (c) superimposition of compd-2 (green) with levamisole

Discussion

Our main interests for the work presented in current MS were; 1) Designing of a simple synthetic strategy for conjugation of isoxazolines and coumarins for the synthesis of novel isoxazoline derivatized coumarins as novel immune potentiators. 2) To explore the SAR of the resulting conjuagtes vis-à-vis their immunopotentiating potential. A major challenge in the synthetic approaches involving the conjugation of isoxazoline and coumarin is to ensure the stability of the chemically very sensitive functional groups present on each of these counterparts [45-47]. To ensure the same we followed a simple low cost but efficient and chemically less demanding strategy for the functionalization of hydroxyl coumarins with isoxazolines. The synthetic scheme involved 1,3-dipolar cyclization between an Allylatedcoumarin and *in situ* generated variedly substituted nitrile oxides. For all the compounds synthesized in present work we observed the formation of only one regioisomer (see **Table 1**), this suggests that nitrile oxide cycloaddition to allyliccoumarin is highly regiospecific. For exploration of SAR in isoxazoline conjugates of 7-OHC as immunopotentiators, correlation of their activity with the substitution pattern in the basic skeleton seems quite informative. To decipher such information, for present work we attempted varied type of substitutions on the aryl ring attached to isoxazoline from simple H, electron donating (OCH_3 and CH_3) and electron withdrawing groups (F, Cl, Br and NO_2) to bulky aryl (naphthyl) groups for the synthesis of coumarin-isoxazoline conjugates.

In the biological experiments compounds (**1-13**) showed no or little toxicity in comparison to the cell control and compounds **2**, **4** and **8** showed excellent dose dependent Con-A proliferative activity (**Figure. 1**). The activity observed for compounds **2**, **4** and **8** under *in vitro* conditions motivated us to test these for *in vivo.* In *in vivo* studies levamisole was used as standard, different doses of compounds **2**, **4**, **8** and the standard were administered to the animals and the blood collected at day 7 and 14 was tested for HA titre. Significant HA titre observed at day 14 (**Figure 2)** after administration of compounds **2**, **4**, **8** suggests that these compounds appreciably enhance the production of antibody against SRBC's. Similarly these compounds were also observed to enhance the secretion of cell-secreted cytokines, such as IL-2, IL-4, and IFN-γ and splenocyte proliferation significantly more than the administration of standard. The observations *vis a vis* immunophenotyping expressions like CD4 and CD8 (**Table 2)**, DTH responses and NO production (**Figure 3 and 6)** also support observations regarding the cytokine expression. The well-known synergism between the cytokines in iNOS

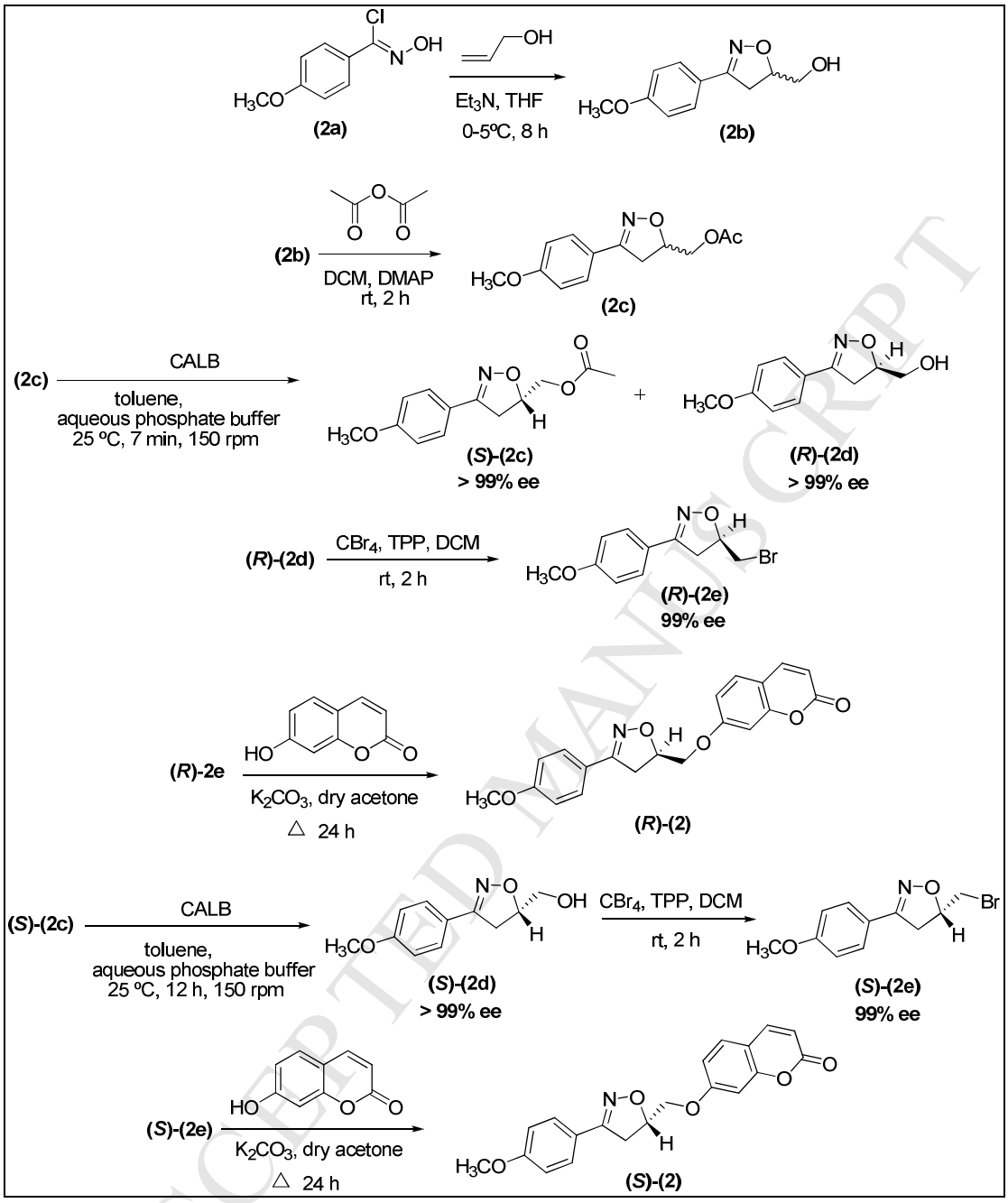
expression [42], suggests that the induction of cytokines observed after treatment with compounds **2**, **4**, **8** can be attributed to the release of NO. In the *in vivo* experiments, compound **2** exhibited highest activity at all doses in comparison to control, levamisole and compounds **4** and **8**. At lower doses compound **4** showed activities almost similar to that of standard; however at higher doses the activity was found to increase with increase in dosage per kg body weight.

Our observations *vis a vis* the production of primary and secondary antibodies (**Figure 2**, IgG > IgM) suggests that administration of compounds **2**, **4**, **8** leads to generation of a good number of memory cells. The observations about the impact of compounds **2**, **4** and **8** on spleen T-cell sub typing and the relative $CD4^+$ and $CD8^+$ counts (**Table 2**) are in conformity with their antibody response. The observed enhancement in $CD4^+$ values for these compounds clearly implies that the immunogenic response follows MHC-class II pathway.

It is important to mention that in addition to the electronic effects the substituents used for the current investigations are also expected to change the lipophilicity and hence the membrane permeation properties of the coumarins. To know the impact of the substituents on the lipophilicity of coumarins, we estimated the Log P values for the compounds 1-13. The estimated Log P values (Table S2 Supporting Information) were found to vary in the order 13>12>11>10>5>6>7>9>2>8>3>4>1. Relative Log P values of the investigated coumarins in light of their observed immune potentiating activities clearly indicate that the substituent effects of the attempted substitutions in the present study are dominated by the electronic effects of the substituents.

It is pertinent to mention that the isoxazoline-coumarin conjugates presented in **table 1**, possess one chiral centre in the isoxazoline ring thereby raising the possibility that derivatives synthesized as per **Scheme 1**, would be a mixture of two possible enantiomers. In order to investigate the impact of stereochemistry of *bis*-heterocyclic conjugates on their immune potentiating potential, kinetic resolution of the racemate of the most active of all these compounds *viz*. (**2**) was carried out following the steps depicted in **Scheme 2**. The lipase showed excellent differential enantioselectivity in catalysing the hydrolysis of racemic ester. It was observed that the enzyme in presence of *S*-isomer selectively hydrolysed the *R*-counterpart without affecting *S*-isomer, producing the corresponding enantiopure alcohol with high enantiomeric excesses (>99% *ee*) in accordance with the Kazlauskas rule. However, in the absence of *R*-counterpart, the ester with *S*-configuration at chiral carbon in the ring is

slowly hydrolysed to the corresponding enantiopure alcohol with high enantiomeric excesses (>99% *ee*). The enantiomeric excesses of acetate **(*S*)-2c**, alcohols **[(*S*)-2d**, **(*R*)-2d]** and bromo compounds **[(*S*)-2e**, **(*R*)-2e]** were determined by chiral column chromatography. The absolute configurations of compounds **(S)-2c**,**(*S*)-2d**, **(*R*)-2d**, **(*S*)-2e** and **(*R*)-2e** were determined comparing the specific rotation signs measured for the products with that reported in the literature [48-51]. The spectroscopic data (^{1}H-NMR, MS and IR) and melting point of compounds **2c**, **2d** and **2e** matches well with the data reported in the literature [49, 51-52]. The known absolute configuration of enantiomers **(*S*)-2d**, **(*R*)-2d**, **(*S*)-2e** and **(*R*)-2e** provided the way to determine the absolute configurations of the *bis*-heterocycles [**(*R*)-2** and **(*S*)-2]**.



Scheme 2: *Stereoselective synthesis of R and S enantiomers of bis-hetrocycle (2)*

Both the compounds [**(R)-2** and **(S)-2**] as well as their racemate were explored for their *in vitro* lymphocyte proliferation potential. It was observed that both the tested enantiomers as well as their racemate show excellent dose-related increase of titre (**Figure 8)** even at lower doses (0.1 µg/mL) in comparison to levamisole. It was noticed that the activity of the enantiomers in their pure form as well as their racemate form is almost similar at all doses. We attribute this stereo-independent biological activity of the tested *bis*-heterocycles to their

flexible molecular structure. On prima facie the results from the *in vitro* lymphocyte proliferation studies, the toxicity studies of the racemate and the docking studies on the R and S isomers of compound **2** indicate that these isomers can be expected to exhibit similar stereo-independent activity under *in vivo* conditions, however a thorough investigation on the toxicity of these isomers needs to be carried out to support the said claim.

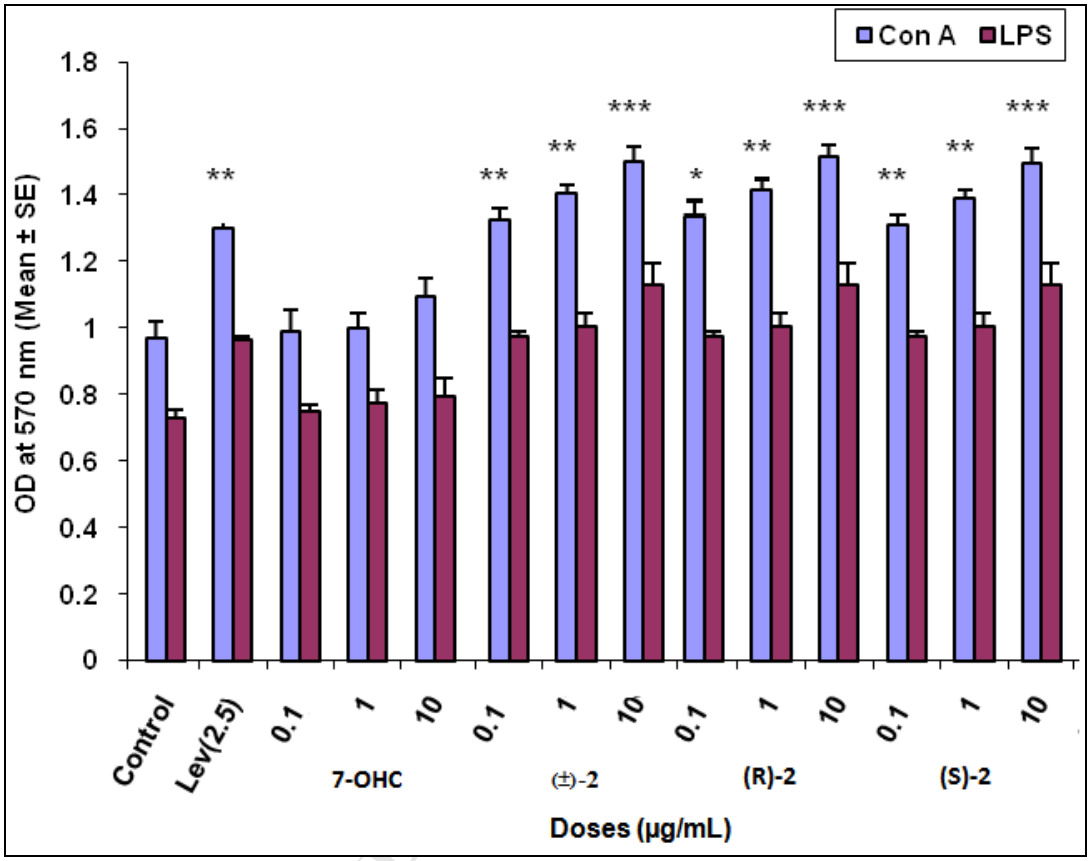


Figure 8: *Effect of enatiomers for compound **2** in [(R)-2, (S)-2] and their racemate on lymphocyte proliferation. The depicted bars present mean ± S.E. of three observations. The proliferation was calculated based on MTT assay. Absorbance was recorded at 570 nm. Values are expressed as.* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).*

Analysis of the results from biological experiments in light of the structure activity relations (SAR) points to some interesting conclusions about the immune potentiating activity of coumarin based immunopotentiators. The immune-stimulating potential of isoxazoline functionalized coumarins synthesized for the present study is evidently more than that observed for the control. The results of preliminary assays and structures of synthesized

conjugates indicate that isoxazolines possessing electron donating groups on the aryl ring (**2**-**8**) are stronger immunopotentiators (4-OCH_3> 2,4-OCH_3> 2-OCH_3 > 3-OCH_3 > 4-CH_3 > 2-CH_3> 3-CH_3) in comparison to those possessing electron withdrawing groups (**9**-**12**) (4-Cl > 4-Br > 4-F ≈ 4-NO_2). The results further indicate that the immunostimulatory activity is enhanced by increasing dosage of electron rich isoxazoline derivatives. Introducing a bulky aryl (naphthyl) group (**13**) decreases the immnostimulating potential with increase in dose. This may be attributed to high molecular mass and overcrowding of aryl rings, which probably inhibit the effective interaction of the pharmacophore with its corresponding binding receptors. The attachment of 7-OHC at 5^{th} position *via* an *O*-allyl intermediate formation on the isoxazoline ring together with appropriately functionalized aromatic ring at the 3^{rd} position of isoxazoline ring seems to impart immune-enhancing activity to the molecule (compounds **2**, **4** and **8**).

Coumarins are reported to interact with ubiquitous intracellular receptor proteins through binding of their aromatic hydrocarbons [53]. From the data observed for present study it can be inferred that electron donating groups at *ortho* or *para* positions with respect to isoxazoline ring increase the overall electron density and hence lead to their stronger interaction with the receptor binders. The possible single bond rotations between an aryl ring and isoxazoline ring in the conjugates ensure that presence of later does not impede the binding of coumarin unit to its relevant receptors. The presence of electron donating groups seems to facilitate the kinetics and thermodynamics for the binding of these conjugates with the relevant intracellular receptor proteins.This is well in agreement with the results we obtained in the current study. Compounds with OCH_3 and CH_3 groups on the phenyl rings *ortho*/*para* to isoxazoline ring (compounds **2**, **4**, **5** and **7**) displayed significantly higher activity than OCH_3 and CH_3 groups meta to isoxazoline ring (compounds **3** and **6**). Conjugate occupied with OCH_3 group at both *ortho* and *para* positions with respect to isoxazoline ring (compound **8**) also resulted in better activity. The difference observed in activity of **2** and **4** may be attributed to the fact that compound **2** being *para* substituted is more symmetrical and stable, that leads to its better interaction with the receptor binders in comparison to compound **4**. In contrast, compound **8** being slightly bulkier and less symmetrical than **2** may show lesser interaction with the receptors. This is further supported by the results from the docking studies where binding affinity and Log P value of **2** was observed to be higher than that of compound **4** and **8**.

The results from biological activity experiments suggest that compounds **2**, **4** and **8** are a good addition to the library of till date known immunostimulators that need to be tested for their prophylactic and therapeutic potential. Results from our present work suggest that isoxazoline functionalized coumarins can serve as promising lead compounds for the design of clinically safe and effective immunepotentiators. In view of the short half-life of the coumarins, it seems that multiple oral or intravenous administrations of these compounds may be required for threshold saturation of the relevant receptors for the optimal activation of lymphocytes and macrophages.

Conclusion

A simple synthetic scheme was attempted for the synthesis of novel isoxazoline functionalized coumarins. The kinetic resolution of the racemate was carried out by employing lipase B from *Candida antarctica* (CALB). The synthesized compounds when tested for their immune potentiating activity exhibited appreciable activity, especially compounds **2**, **4** and **8.**The observed immune potentiating activity for compounds **2**, **4** and **8** in our opinion makes them a promising addition to the library of till date known immunostimulators that need to be tested for their prophylactic and therapeutic potential**.** The results from our presented work suggest that isoxazoline functionalized coumarins can serve as useful immnopotentiators whose thearupatic efficacy can be tuned through appropriate substitutions in the fused heterocyclic moieties.

Experimental

Chemistry

All solvents were dried and freshly distilled prior to use. IR spectra were recorded on a Bruker Vector 22 instrument using KBr pellets and in $CHCl_3$. ^{1}H and ^{13}C NMR were recorded on a Bruker DPX 500 instrument in $CDCl_3$ using TMS as internal standard for protons. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument and MALDI TOF-TOF. Elemental analysis was carried out using Elemental Vario EL III elemental analyser. Chemicalshift values are mentioned in δ (ppm) and coupling constants (*J*) are given in Hz. Mass-spectrometric (MS) data is reported in *m/z*. Elemental analysis data is reported in % standard. The progress of all reactions was monitored by TLC on 2 cm × 5 cm pre-coated silica gel 60 F254 plates of thickness 0.25 mm (Merck). The chromatograms were

visualized under UV 254-366 nm and iodine. Melting points were determined on Buchi B-542 apparatus by an open capillary method and are uncorrected**.** Optical rotations were measured on Perkin-Elmer 241 polarimeter at 25 °C using sodium D light. Enantiomeric excess (ee%) was determined by chiral HPLC on OJH and ADH chiral columns. Chemicals were purchased from M/s Aldrich Chemicals, Mumbai. Reagents and solvents used were mostly of LR grade.

7-(Allyloxy)-2H-chromen-2-one (1c):

In a typical procedure, **1b** (0.50 g, 3.08 mmol) was dissolved in dry acetone (20 mL). K_2CO_3 (4.26 g, 30.85 mmol) was added and the reaction mixture stirred for 5 min at ambient temperature. **1a** (0.44 g, 3.70 mmol) was charged to the above reaction mixture and the reaction mixture allowed to reflux for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to attain room temperature, filtered and the filtrate concentrated under vacuum to afford **1c** in pure form (100%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.61 (d, 2H, *J* = 5.27 Hz), 5.31-5.37 (m, 1H), 5.40-5.49 (m, 1H), 5.96-6.12 (m, 1H), 6.26 (d, 1H, *J* = 9.48 Hz), 6.86 (d, 1H, *J* = 9.58 Hz), 6.89 (s, 1H), 7.38 (d, 1H, *J* = 8.36 Hz), 7.64 (d, 1H, *J* = 9.49 Hz); Mass (ESI-MS): 224.8 (M^+ + Na); C, H analysis for $C_{12}H_{10}O_3$: Calculated C, 71.28; H, 4.98. Found C, 71.24; H, 4.93.

Phenylhydroxymoyl chloride (1d):

Phenylhydroxymoyl chloride was synthesized as per the literature procedure [54]. In a typical procedure, $NH_2OH.HCl$ (0.50 g, 7.22 mmol) was dissolved in water and neutralized with NaOH. To the neutralized solution of hydroxylamine hydrochloride, benzaldehyde (1.00 g, 9.43 mmol) was added and the reaction mixture stirred for 1 h at ambient temperature. After completion of the reaction (monitored by TLC), excess of water was added to the reaction mixture and the organic compound extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford pure oxime in 99% yield. Benzaldoxime (1.00 g, 6.42 mmol) was dissolved in DMF (20 mL). *N*-chlorosuccinimide (0.95 g, 7.18 mmol) was added to the above solution and the reaction mixture stirred for 8-10 h. After completion of the reaction (monitored by TLC), excess of water was added to the reaction mixture and organic compound extracted with Et_2O (3 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford pure **1d** (74%).

^{1}H NMR (200 MHz, $CDCl_3$): δ 7.42 (m, 3H), 7.85 (m, 2H); Mass (ESI-MS): 155.58 (M^+); C, H, N analysis for C_7H_6ClNO: Calculated C, 54.04; H, 3.89; N, 9.00. Found C, 53.98; H, 4.00; N, 9.11.

7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (1):

In a typical procedure, **1d** (0.10 g, 0.04 mmol) was dissolved in THF (4 mL) and to it was added Et_3N (0.02 g, 0.02 mmol). A solution of **1c** (0.05 g, 0.02 mmol) in THF (4 mL) was added to the above solution. The reaction mixture was stirred at ambient temperature for 4 h. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 80 mL of water and extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase] to yield the pure 7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (**1**) as a white solid (90%).

White solid; mp: 132-133 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.39-3.42 (dd, 1H, J_1 = 15.54 Hz, J_2 = 6.93 Hz), 3.54-3.59 (dd, 1H, J_1 = 15.55 Hz, J_2 = 10.82 Hz), 4.16-4.22 (m, 2H), 5.04-5.09 (m, 1H), 6.15 (d, 1H, J = 8.78 Hz), 6.27 (d, 1H, J = 8.46 Hz), 6.83 (s, 1H), 6.86 (m, 3H), 7.38 (d, 1H, J = 8.56 Hz), 7.63 (d, 1H, J = 9.47 Hz), 7.71 (d, 2H, J = 9.61 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 37.50, 69.03, 78.35, 101.75, 112.80, 113.05, 113.56, 126.79, 128.81, 128.89, 129.11, 129.57, 130.39, 143.29, 155.74, 156.42, 161.06, 161.50, 176.86; IR (KBr, cm^{-1}): 693, 761, 853, 913, 1124, 1230, 1280, 1355, 1402, 1448, 1507, 1558, 1614, 1723, 2358, 2853, 2924, 3386. ESI-MS: 321.8 (M^+); C, H, N analysis for $C_{19}H_{15}NO_4$: Calculated C, 71.02; H, 4.71; N, 4.36. Found C, 71.04; H, 4.74; N, 4.30.

7-{(3-(4-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (2):

White solid; mp:140-141 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.25-3.29 (dd, 1H, J_1 = 16.45 Hz, J_2 = 6.82 Hz), 3.42-3.47 (dd, 1H, J_1 = 16.65 Hz, J_2 = 10.75 Hz), 3.88 (s, 3H), 4.06-4.09 (m, 1H), 4.12-4.15 (dd, 1H, J_1 = 9.75 Hz, J_2 = 4.98 Hz), 5.05-5.08 (dd, 1H, J_1 = 10.93 Hz, J_2 = 5.44 Hz), 6.20 (d, 1H, J = 9.47 Hz),6.75 (s, 1H), 6.79 (d, 1H, J = 8.56 Hz), 6.90 (d, 2H, J = 8.64 Hz), 7.31 (d, 1H, J = 8.52 Hz), 7.52 (d, 1H, J = 8.46 Hz), 7.56 (d, 2H, J = 9.52 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 39.69, 55.72, 69.10, 79.15, 101.77, 112.83, 113.07, 113.57, 114.98, 117.69, 124.20, 128.90, 129.22, 131.41, 143.29, 155.78, 156.56, 158.29, 161.04, 161.56; IR (KBr, cm^{-1}): 669, 810, 837, 897, 1022, 1063, 1095, 1126, 1232, 1275, 1356, 1402,

1507, 1558, 1615, 1650, 1714, 2337, 2361, 2852, 3389; MALDI-mass: 352 (M^+ + H); C, H, N analysis for $C_{20}H_{17}NO_5$: Calculated C, 68.37; H, 4.88; N, 3.99. Found C, 68.32; H, 4.85; N, 3.94.

7-{(3-(3-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (3):

White solid; mp: 96-97 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.48-3.51 (dd, 1H, J_1 = 16.50 Hz, J_2 = 6.93 Hz), 3.65-3.69 (dd, 1H, J_1 = 14.64 Hz, J_2 = 10.54 Hz), 3.82 (s, 3H), 4.10-4.14 (m, 2H), 5.13-5.16 (dd, 1H, J_1 = 11.68 Hz, J_2 = 6.72 Hz), 6.27 (d, 1H, J = 9.47 Hz), 6.85-6.95 (m, 3H), 7.20 (d, 1H, J = 8.56 Hz), 7.30-7.41 (m, 3H), 7.64 (d, 1H, J = 9.50 Hz);^{13}C NMR (500 MHz, $CDCl_3$): δ 37.81, 55.39, 69.18, 78.11, 101.85, 112.79, 113.35, 114.25, 114.83, 115.44, 124.53, 128.35, 128.89, 129.40, 143.26, 155.10, 155.97, 160.34, 161.31, 161.61; IR (KBr, cm^{-1}): 759, 835, 908, 1027, 1124, 1231, 1291, 1377, 1402, 1462, 1507, 1557, 1613, 1728, 2360, 2850, 2921, 2955, 3416; Mass (ESI-MS): 374 (M^+ + Na);C, H, N analysis for $C_{20}H_{17}NO_5$: Calculated C, 68.37; H, 4.88; N, 3.99. Found 68.39; H, 4.84; N, 3.91.

7-{(3-(2-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (4):

White solid; mp: 142-143 ^oC;^1H NMR (500 MHz, $CDCl_3$): δ 3.41-3.44 (dd, 1H, J_1 = 16.56 Hz, J_2 = 7.05 Hz), 3.62-3.67 (dd, 1H, J_1 = 16.00 Hz, J_2 = 10.80 Hz), 3.86 (s, 3H), 4.12-4.18 (m, 2H), 5.08 (m, 1H), 6.27 (d, 1H, J = 9.45 Hz), 6.83 (s, 1H), 6.86-6.89 (m, 2H), 7.26-7.39 (m, 3H), 7.64 (d, 1H, J = 9.50 Hz), 7.74 (d, 1H, J = 9.40 Hz); ^{13}C NMR (500 MHz, $CDCl_3$):δ 39.80,55.95, 69.26, 78.67, 101.75, 111.82, 112.76, 112.89, 113.53, 117.88, 121.11, 128.93, 129.09, 131.12, 143.39, 155.10, 155.97, 160.34, 161.31, 161.61; IR (KBr, cm^{-1}): 755, 808, 835, 891, 1023, 1096, 1124, 1231, 1277, 1340, 1401, 1460, 1487, 1556, 1614, 1727, 2284, 2853, 2924, 3405; Mass (ESI-MS): 374 (M^+ + Na); Anal Calcd. for $C_{20}H_{17}NO_5$: Calculated C: 68.37, H: 4.88, N: 3.99; found C: 68.33, H: 4.82, N: 3.93.

7-{(3-*p*-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (5):

White solid; mp: 158-159 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 2.39 (s, 3H), 3.38-3.39 (m, 2H), 3.49-3.54 (dd, 1H, J_1 = 15.15 Hz, J_2 = 10.86 Hz), 4.14-4.17 (dd, 1H, J_1 = 12.39 Hz, J_2 = 4.94 Hz), 5.13-5.14 (m, 1H), 6.27 (d, 1H, J = 9.50 Hz), 6.83 (s, 1H), 6.87 (d, 1H, J = 8.60 Hz), 7.23 (d, 2H, J = 7.62 Hz), 7.38 (d, 1H, J = 8.59 Hz), 7.59 (d, 2H, J = 7.08 Hz), 7.69 (d, 1H, J = 8.78 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 20.44, 36.63, 68.11, 77.17, 100.78, 111.77, 112.03, 112.52, 125.28, 125.77, 125.79, 127.85, 128.22, 128.48, 139.65, 142.35, 154.74, 155.34, 160.02, 160.54; IR (KBr, cm^{-1}): 634, 818, 837, 887, 920, 986, 1027, 1123,

1184, 1231, 1280, 1532, 1394, 1448, 1506, 1616, 1710, 2049, 2854, 2922, 3444; Mass (ESI-MS): 357.9 (M^+ + Na);C, H, N analysis for $C_{20}H_{17}NO_4$: Calculated C, 71.63; H, 5.11; N, 4.18. Found C, 71.66; H, 5.15; N, 4.14.

7-{(3-*m*-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (6):

White solid; mp: 133-134 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 2.34 (s, 3H), 3.48-3.51 (dd, 1H, J_1 = 11.56 Hz, J_2 = 6.63 Hz), 3.65-3.69 (dd, 1H, J_1 = 14.49 Hz, J_2 = 10.07 Hz), 4.20 (m, 2H), 5.13-5.18 (dd, 1H, J_1 = 12.01 Hz, J_2 = 5.27 Hz),6.27 (d, 1H, J = 9.41 Hz), 6.84 (s, 1H), 6.90-6.95 (m, 3H), 7.19-7.41 (m, 3H), 7.64 (d, 1H, J = 9.36 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ23.96, 37.36, 69.10, 78.74, 101.85, 112.85, 113.03, 113.65, 127.80, 128.06, 128.33, 129.05, 129.15, 134.65, 141.33, 143.30, 160.77, 161.38; IR (KBr, cm^{-1}): 694, 757, 787, 835, 912, 1025, 1123, 1158, 1231, 1280, 1349, 1401, 1507, 1615, 1711, 2109, 2850, 2918, 3441; Mass (ESI-MS): 358 (M^+ + Na);C, H, N analysis for $C_{20}H_{17}NO_4$: Calculated C, 71.63; H, 5.11; N, 4.18. Found C, 71.64; H, 5.19; N, 4.20.

7-{(3-*o*-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (7):

White solid; mp: 92-93 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 2.51 (s, 3H), 3.00-3.04 (dd, 1H, J_1 = 15.32 Hz, J_2 = 9.71 Hz), 3.32-3.36 (dd, 1H, J_1 = 16.42 Hz, J_2 = 7.41 Hz), 4.18-4.20 (m, 2H), 5.16-5.19 (dd, 1H, J_1 = 12.15 Hz, J_2 = 5.60 Hz), 6.26 (d, 1H, J = 9.22 Hz), 6.83 (s, 1H), 7.25-7.44 (m, 4H), 7.60-7.69 (m, 3H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 19.57, 37.50, 69.01, 78.41, 101.79, 112.78, 113.10, 113.61, 122.56, 123.03, 126.46, 128.69, 128.89, 143.24, 155.77, 161.00, 161.49; IR (KBr, cm^{-1}): 615, 718, 759, 835, 892, 989, 1031, 1123, 1230, 1279, 1347, 1377, 1400, 1456, 1493, 1507, 1556, 1613, 1726, 2110, 2853, 2869, 2924, 2956, 3384; Mass (ESI-MS): 357.9 (M^+ +Na); C, H, N analysis for $C_{20}H_{17}NO_4$: Calculated C, 71.63; H, 5.11; N, 4.18. Found C, 71.61; H, 5.17; N, 4.13.

7-{(3-(2,4-Dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (8):

White solid; mp: 141-142 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.55-3.59 (dd, 1H, J_1 = 13.34 Hz, J_2 = 6.76 Hz), 3.72-3.76 (dd, 1H, J_1 = 14.65 Hz, J_2 = 10.74 Hz), 3.90 (s, 3H), 3.91 (s, 3H), 4.17 (m, 1H), 4.18-4.21 (dd, 1H, J_1 = 12.46 Hz, J_2 = 5.20 Hz), 5.14-5.16 (m, 1H), 6.27 (d, 1H, J = 9.46 Hz),6.84 (s, 1H), 6.85- 6.90 (m, 3H), 7.27 (d, 1H, J = 8.22 Hz), 7.39 (d, 1H, J = 8.58 Hz), 7.64 (d, 1H, J = 9.48 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 39.84, 56.27, 69.16, 79.15, 101.74, 112.19, 112.89, 113.16, 113.54, 113.90, 124.20, 128.93, 129.30, 143.38,

155.78, 156.39, 156.56, 158.29,161.04, 161.56; IR (KBr, cm^{-1}): 664, 792, 841, 868, 934, 990, 1052, 1129, 1161, 1172, 1210, 1232, 1255, 1281, 1430, 1457, 1518, 1548, 1618, 1735, 2049, 2927, 3452; Mass (ESI-MS): 381 (M^+); C, H, N analysis for $C_{21}H_{19}NO_6$: Calculated C, 66.14; H, 5.02; N, 3.67. Found C, 66.19; H, 5.06; N, 3.62.

7-{(3-(4-Fluorophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (9):

White solid; mp: 114-115 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.34-3.39 (dd, 1H, J_1 = 17.21 Hz, J_2 = 7.04 Hz), 3.51- 3.56 (m, 1H), 4.15-4.18 (dd, 1H, J_1 = 9.86 Hz, J_2 = 4.84 Hz), 4.21-4.23 (dd, 1H, J_1 = 9.68 Hz, J_2 = 4.75 Hz), 5.14-5.17 (m, 1H), 6.26 (d, 1H, J = 9.44 Hz), 6.82 (s, 1H), 6.85 (d, 1H, J = 8.54 Hz), 7.12-7.15 (m, 1H), 7.37-7.46 (m, 4H), 7.63 (d, 1H, J = 9.44 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 38.71, 70.38, 80.18, 103.16, 114.26, 114.55, 115.00, 115.05, 118.71, 124.02, 130.38, 131.88, 131.94, 144.76, 157.07, 157.18, 162.51, 162.87, 165.22; IR (KBr, cm^{-1}): 615, 718, 759, 835, 892, 989, 1031, 1123, 1230, 1279, 1347, 1377, 1400, 1456, 1493, 1507, 1556, 1613, 1726, 2110, 2853, 2869, 2924, 2956, 3384; Mass (ESI-MS): 361.8 (M^+ + Na); C, H, N analysis for $C_{19}H_{14}FNO_4$: Calculated C, 67.25; H, 4.16; N, 4.13. Found C, 67.22; H, 4.19; N, 4.18.

7-{(3-(4-Chlorophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (10):

White solid; mp: 111-112^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.27-3.32 (dd, 1H, J_1 = 17.93 Hz, J_2 = 7.08 Hz), 3.44-3.50 (dd, 1H, J_1 = 19.04 Hz, J_2 = 10.87 Hz), 3.53 (m, 1H), 4.08-4.11 (dd, 1H, J_1 = 10.12 Hz, J_2 = 4.93 Hz), 5.13-5.15 (m, 1H), 6.12 (d, 1H, J = 8.95 Hz), 6.75 (s, 1H), 6.81 (d, 1H, J = 9.48 Hz), 7.30-7.34 (m, 3H),7.56-7.58 (m, 3H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 37.02, 68.74, 78.40, 101.85, 111.56, 112.85, 113.65, 127.37, 128.43, 128.63, 128.81, 128.90, 129.22, 131.41, 143.00, 155.10, 155.97, 160.34, 161.31; IR (KBr, cm^{-1}): 666, 760, 919, 1049, 1093, 1159, 1230, 1351, 1402, 1558, 1615, 1650, 1714, 2360, 2853, 2924, 3416, 3672; Mass (ESI-MS): 355.3 (M^+); C, H, N analysis for $C_{19}H_{14}ClNO_4$: Calculated C, 64.14; H, 3.97; N, 3.94. Found C, 64.12; H, 3.93; N, 3.91.

7-{(3-(4-Bromophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (11):

Brown solid; mp: 134-135 ^oC; ^1H NMR (500 MHz, $CDCl_3$):δ 3.28-3.32 (dd, 1H, J_1 = 13.95 Hz, J_2 = 5.93 Hz), 3.50-3.56 (dd, 1H, J_1 = 18.45 Hz, J_2 = 11.04 Hz), 3.64 (m, 1H), 4.26 (m, 1H), 5.26 (m, 1H), 6.29 (d, 1H, J = 9.17 Hz), 6.90 (s, 1H), 6.95 (d, 1H, J = 6.79 Hz), 7.17 (m, 2H), 7.46 (d, 2H, J = 8.43 Hz), 7.74 (d, 2H, J = 9.15 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 36.23, 67.89, 77.69, 100.67, 111.78, 112.51, 112.56, 116.22, 116.39, 121.55, 128.89,

129.39, 129.45, 142.27, 158.43, 159.64, 163.70, 164.24; IR (KBr, cm^{-1}): 683, 835, 896, 1032, 1125, 1158, 1231, 1281, 1351, 1403, 1512, 1556, 1614, 1726, 2361, 2852, 2923, 3411; Mass (ESI-MS): 401 (M^+ + H); C, H, N analysis for $C_{19}H_{14}BrNO_4$: Calculated C, 57.02; H, 3.53; N, 3.50. Found C, 57.10; H, 3.58; N, 3.52.

7-{(3-(4-Nitrophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (12):

Yellow solid; mp: 130-131 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.40-3.45 (dd, 1H, J_1 = 17.01 Hz, J_2 = 7.25 Hz), 3.55-3.61 (dd, 1H, J_1 = 19.21 Hz, J_2 = 10.71 Hz), 4.23 (m, 2H), 5.20-5.29 (m, 1H), 6.28 (d, 1H, J = 9.48 Hz), 6.83 (s, 1H), 7.39 (d, 1H, J = 8.20 Hz), 7.60-7.67 (m, 2H), 8.13 (d, 2H, J = 7.85 Hz), 8.30 (d, 2H, J = 7.40 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 37.68, 73.35, 76.92, 101.44, 102.20, 109.68, 109.75, 115.05, 122.73, 126.83, 127.17, 132.58,136.99, 137.01, 151.39, 151.44, 157.30; IR (KBr, cm^{-1}): 679, 738, 854, 983, 1097, 1191, 1236, 1298, 1345, 1404, 1490, 1523, 1533, 1557, 1623, 1730, 2058, 2927, 3450; Mass (ESI-MS): 389 (M^+ + Na); C, H, N analysis for $C_{19}H_{14}N_2O_6$: Calculated C, 62.30; H, 3.85; N, 7.65. Found C, 62.38; H, 3.82; N, 7.69.

7-{(3-(Naphthalen-1-yl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (13):

White solid; mp: 119-120 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.56-3.60 (dd, 1H, J_1 = 13.86 Hz, J_2 = 6.13 Hz), 3.75-3.80 (dd, 1H, J_1 = 15.32 Hz, J_2 = 10.92 Hz), 4.11-4.24 (m, 2H), 5.16 (m, 1H), 6.26 (d, 1H, J = 9.15 Hz), 6.84 (s, 1H), 7.36 (d, 1H, J = 8.04 Hz), 7.50 (d, 1H, J = 7.46 Hz), 7.56-7.63 (m, 4H), 7.89-7.93 (m, 3H), 8.99 (d, 1H, J = 8.01 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 37.45, 69.30, 77.31, 104.44, 115.47, 115.72, 116.19, 126.49, 126.80, 127.45, 128.90, 129.15, 129.60, 130.30, 130.42, 133.31, 133.72, 136.68, 145.95, 158.43, 159.64, 163.70, 164.24; IR (KBr, cm^{-1}): 615, 751, 775, 800, 834, 1026, 1122, 1230, 1279, 1348, 1401, 1508, 1612, 1724, 2341, 2361, 2852, 2923, 2955, 3423; Mass (ESI-MS): 394 (M^+ + Na); C, H, N analysis for $C_{23}H_{17}NO_4$: Calculated C, 74.38; H, 4.61; N, 3.77. Found C, 74.32; H, 4.67; N, 3.74.

{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol (2b):

In a typical procedure, **2a** (0.20 g, 1.07 mmol) was dissolved in THF (5 mL) and to it was added Et_3N (0.054 g, 0.534 mmol) at 0 ºC. A solution of allyl alcohol (0.062 g, 1.07 mmol) in THF (4 mL) was added to the above solution. The reaction mixture was stirred for 8 h between 0-5 ºC. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 60 mL of water and extracted with EtOAc (2 × 30 mL). The combined

organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase] to yield the pure racemic {3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol (90%).

White solid; mp: 166-167 ^oC; ^1H NMR (200 MHz, $CDCl_3$): δ 3.18-3.31 (m, 2H), 3.57-3.63 (dd, 1H, J_1 = 6.00 Hz, J_2 = 4.00 Hz), 3.74-3.80 (m, 1H), 3.87 (s, 3H), 4.76-4.82 (dd, 1H, J_1 = 10.00 Hz, J_2 = 8.00 Hz), 6.85 (d, 2H, J = 8.00 Hz), 7.47 (d, 2H, J = 8.00 Hz); IR (KBr, cm^{-1}): 626, 707, 814, 906, 955, 1018, 1063, 1178, 1258, 1274, 1300, 1343, 1415, 1459, 1508, 1560, 1604, 2849, 2925, 3391; Mass (ESI-MS): 208 (M^+ + H); C, H, N analysis for $C_{11}H_{13}NO_3$: Calculated C, 63.76; H, 6.32; N, 6.76. Found C, 63.62; H, 6.41; N, 6.69.

{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methyl acetate (2c):

2b (0.19 g, 0.917 mmol) was dissolved in DCM (5 mL). Acetic anhydride (0.093 g, 0.917 mmol) was charged to the above solution followed by the addition of DMAP (catalytic amount). The reaction mixture was then allowed to stir at ambient temperature for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was quenched with $NaHCO_3$ solution and extracted with DCM (2 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The semisolid left behind was recrystallized from hexane-ethyl acetate to yield racemic compound **2c** in pure form (100%).

White solid; mp: 82-83 ^oC; ^1H NMR (200 MHz, $CDCl_3$): δ 2.02 (s, 3H), 2.98-3.09 (dd, 1H, J_1 = 14 Hz, J_2 = 8.00 Hz), 3.39-3.38 (dd, 1H, J_1 = 8.00 Hz, J_2 = 6.00 Hz), 3.74-3.78 (m, 1H), 3.88 (s, 3H), 4.16-4.19 (m, 1H), 4.83-4.94 (dd, 1H, J_1 = 14 Hz, J_2 = 10.00 Hz), 6.87 (d, 2H, J = 8.00 Hz), 7.49 (d, 2H, J = 10 Hz); IR (KBr, cm^{-1}): 626, 706, 816, 904, 1045, 1064, 1103, 1175, 1249, 1274, 1301, 1344, 1362, 1441, 1462, 1510, 1605, 1742, 2855, 2930, 3458; Mass (ESI-MS): 288 (M^+ + K); C, H, N analysis for $C_{13}H_{15}NO_4$: Calculated C, 62.64; H, 6.07; N, 5.62. Found C, 62.59; H, 6.17; N, 5.33.

Typical lipase-catalyzed kinetic resolution of (±)-2c:

The racemic acetate (.100 g, .401 mmol), aqueous phosphate buffer (4 mL, 0.1 M, pH. 7.0), toluene (300 µL) and immobilized lipase B from *Candida antarctica* (80 mg) were shaken (150 rpm) continuously at 25 ± 1 °C for 7 min. After a certain degree of conversion (~50%) as indicated by high performance liquid chromatography (HPLC), the reaction was terminated by adding ethyl acetate and centrifuging the mixture at 10,000–15,000 g to remove the

enzyme and the suspended particles. The clear solution was decanted and the centrifuged mass extracted separately with ethyl acetate (3 × 30 mL). The organic layer was combined and washed with water. The combined organic layer was then dried and evaporated under reduced pressure to furnish a mixture of hydrolyzed alcohol **(*R*)-2d** and unhydrolyzed ester **(*S*)-2c**, which was separated by column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase]. The unhydrolyzed ester **(*S*)-2c** (.040 g, 0.16 mmol), aqueous phosphate buffer (2.5 mL, 0.1 M, pH 7.0), toluene (200 µL) and immobilized lipase B from *C. antarctica* (30 mg) were shaken (150 rpm) continuously at 25 ± 1 °C for 12 h. After complete hydrolysis (as indicated by TLC), the mixture was filtered with suction, and the filtrate concentrated in vacuo to afford pure **(*S*)-2d.**

Enantiomeric purities of **(*S*)-2c, (*R*)-2d** and **(*S*)-2d**were determined by chiral HPLC:

(*S*)-{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methyl acetate 2c:

HPLC purity >99%; HPLC ee>99%; $[\alpha]_D^{25}$ = +41.90 (c 0.5, $CHCl_3$), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 62.767 min.

(*R*)-{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol 2d:

HPLC purity >99%; HPLC ee>99%; $[\alpha]_D^{25}$ = -94.50 (c 0.5, $CHCl_3$), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time31.53 min.

(*S*)-{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol 2d:

HPLC purity >99%; HPLC ee>99%; $[\alpha]_D^{25}$ = +96.00 (c 0.5, $CHCl_3$), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 52.00 min.

(*R*)-5-{bromomethyl)-3-(4-methoxyphenyl}-4,5-dihydroisoxazole 2e:

(*R*)-2d (.04 g, 0.193 mmol) was dissolved in DCM (5 mL) and CBr_4 (.096 g, 0.289 mmol) was charged to the above solution. TPP (.075 g, 0.289 mmol) was added to the reaction mixture slowly for 10 min at 0 ºC. The reaction mixture was then allowed to stir at ambient temperature for 2 h. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 60 mL of water and extracted with DCM (2 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400

mesh as stationary phase, hexane: EtOAc; (9:1) as mobile phase] to yield the pure **(*R*)-2e** (90%).

HPLC purity >99%; HPLC ee>99%; $[\alpha]_D^{25}$ = -21.37 (c 0.5, $CHCl_3$), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 49.995 min.

White solid; mp: 73-75 ^oC; ^1H NMR (200 MHz, $CDCl_3$): δ 3.19-3.34 (m, 2H), 3.37-3.50 (dd, 1H, J_1 = 12.00 Hz, J_2 = 6.00 Hz), 3.55-3.60 (m, 1H), 3.95 (s, 3H), 4.94-5.04 (dd, 1H, J_1 = 14.00 Hz, J_2 = 8.00 Hz), 6.93 (d, 2H, J = 8.00 Hz), 7.55 (d, 2H, J = 6.00 Hz); IR (KBr, cm^{-1}): 666, 706, 755, 813, 902, 1020, 1064, 1215, 1259, 1274, 1361, 1416, 1462, 1507, 1602, 2848, 2916, 3018, 3400; Mass (ESI-MS): 269 (M^+); C, H, N analysis for $C_{11}H_{12}BrNO_2$: Calculated C, 48.91; H, 4.48; N, 5.19. Found C, 48.83; H, 4.41; N, 5.27.

(*S*)-5-{bromomethyl)-3-(4-methoxyphenyl}-4,5-dihydroisoxazole 2e:

Same procedure as described for **(R)-2e** was used.Yield of the compound was found to be 90%. HPLC purity >99%; HPLC ee>99%; $[\alpha]_D^{25}$ = +20.99 (c 0.5, $CHCl_3$), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 52.248 min.

(*R*)-7-{(3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (2):

In a typical procedure, 7-OHC (0.269 g, 1.66 mmol) was dissolved in dry acetone (5 mL). K_2CO_3 (4.26 g, 30.85 mmol) was added and the reaction mixture stirred for 5 min at ambient temperature. **(*R*)-2e** (0.30 g, 1.11 mmol) was charged to the above reaction mixture and the reaction mixture allowed to reflux for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to attain room temperature, filtered and the filtrate concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase] to yield the pure **(*R*)-2** (92%).

White solid; mp: 140-141.5 ^oC; Specific rotation $[\alpha]_D^{25}$: –32.51 (c 0.5, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 3.25-3.29 (dd, 1H, J_1 = 16.45 Hz, J_2 = 6.82 Hz), 3.41-3.48 (dd, 1H, J_1 = 16.65 Hz, J_2 = 10.75 Hz), 3.88 (s, 3H), 4.05-4.10 (m, 1H), 4.13-4.18 (dd, 1H, J_1 = 10 Hz, J_2 = 4.98 Hz), 5.04-5.10 (dd, 1H, J_1 = 10.00 Hz, J_2 = 5.00 Hz), 6.19 (d, 1H, J = 9.47 Hz), 6.76 (s, 1H), 6.80 (d, 1H, J = 8.56 Hz), 6.92 (d, 2H, J = 8.64 Hz), 7.33 (d, 1H, J = 8.52 Hz), 7.45 (d, 1H, J = 8.46 Hz), 7.56 (d, 2H, J = 8.00 Hz); IR (KBr, cm^{-1}): 669, 810, 837, 897, 1022, 1063,

1095, 1126, 1232, 1275, 1356, 1402, 1507, 1558, 1615, 1650, 1714, 2337, 2361, 2852, 3389; Mass (ESI-MS): 351 (M^+);C, H, N analysis for $C_{20}H_{17}NO_5$: Calculated C, 68.37; H, 4.88; N, 3.99. Found C, 68.32; H, 4.85; N, 3.94.

(S)-7-{(3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (2): Same procedure as described for compound **(*R*)-2** was used.Yield of the compound was found to be 92%.

White solid; mp: 140-141.5 ^oC; Specific rotation $[\alpha]_D^{25}$: +31.91 (c 0.5, $CHCl_3$).

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Supporting Information

The details of procedures employed for the biological investigations are presented in Supporting Information which can be accessed free of any charge from www.sciencedirect.com

Abbreviations:

Candida antarctica	CALB
HA titer	Hemagglutination assay
Delayed-type hypersensitivity reaction	DTH
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
IL-2	Interleukin 2
IFN-γ	Interferon-gamma
IL-4	Interleukin 4
NO production	Nitric oxide production
Levamisole	Lev
7-hydroxycoumarin	7-OHC
Con A	Concanavalin A
LPS	Lipopolysaccharides
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
Anova	Analysis of variance
SRBC	Sheep red blood cells
IgM	Immunoglobulin M
IgG	Immunoglobulin G
Fluoresceine-isothiocyanate	FITC
Phycoerthyrin	PE
MHC	Major Histocompatibility Complex

References

1. S.S. Agarwal, V.K. Singh, Immunomodulation: A review of studies on Indian medicinal plants and synthetic peptides, Proc. Indian Natl. Acad. Sci. B65 (1999) 79-204.
2. J. Sany, Prospects in the immunological treatments of rheumatoid arthritis, Scand. J. Rheumatol. Suppl. 66 (1987) 129-136.
3. S.L. Schreiber, Chemistry and biology of the immunophilins and their immunosuppressive ligands, Science 251 (1991) 283-287.
4. A. Pashine, J.B. Ulmer, N.M. Valiante,Innate immune mechanisms and the identification of immune potentiators as vaccine adjuvants,Immunopotentiators in Modern Vaccines (2006) 57-72.
5. T. Ismail, S. Shafi, A. Gupta, A. Khajuria, T. Sidiq, D.M. Reddy, M.S. Alam, G.N. Qazi, H.M.S. Kumar, Study of the adjuvanticity of lysine lipopeptides; carbamateanalogs elicit strong Th1 and Th2 response to ovalbumin in mice, Int. Immunopharmacol. 11 (2011) 1855-1863.
6. T. Ismail, S. Shafi, I. Hyder, T. Sidiq, A. Khajuria, M.S. Alam,H.M.S. Kumar, Design and synthesis of novel 1,2,3-triazole- and 2-isoxazoline-based *bis*-heterocycles as immunepotentiators, Arch. Pharm. Chem. Life Sci. 348 (2015)1-12.
7. Y.C. Yoo, K. Yoshimatsu, Y. Koike, R. Hatsuse, O. Tanishita, J. Arikawa, I. Azuma, Adjuvant activities of muramyl dipeptide derivatives to enhance immunogenicity of hantavirus-inactivated vaccine, Vaccine16 (1998) 216-224.
8. M.A. Tempero, Y. Haga, C. Sivinsk, D. Birt, L. Klassen, G. Thiele, Immunologic effects of levamisole in mice and humans: Evidence for augmented antibody response without modulation of cellular cytotoxicity, J. Immunother. 17 (1995) 47-57.
9. K. Sainis, M.B. Gadgil, G.P. Phondke. Modulation of delayed type hypersensitivity of sheep erythrocytes by Niridazole. Ind. J. Exp. Biol. 21 (1983) 304-309.
10. M.Z.X.J.C. Zhang, Synthesis of F-alkylated MDP analogs, Chin. Chem. Lett. 7 (1996) 993-94.
11. K.S.I. Kamisango, Y. Tanio, S. Kobayashi, T. Fukuda, I. Sekikawa, I. Azuma, Y. Yamamura, Chemical synthesis and adjuvant activity of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) analogs, Chem. Pharm. Bull. 29 (1981) 1644-54.

12. J. Symoens, E. Veys, M. Mielants, R. Pinals, "Adverse reactions to levamisole," Cancer Treat. Rep. 62 (1978)1721-30.
13. D.N.K. Sarma, R.L. Khosa, Immunomodulators of plant origin – A review Ancient Science of Life, Vol No. XIII Nos. 3 & 4, January-April 1994, pp. 326-331.
14. Y.H. Knysh, V.V. Parchenko, O.I. Panasenko, A.H. Kaplaushenko, Y.V. Makovyk, S.M. Kulish, A.S. Hotsulia, V.Y. Izdepskyi, B.P. Kyrychko, O.H. Mysyk, 1,2,4-triazole-3-ylthioacetic acid derivatives exhibiting the antioxidant, hepatoprotective and immunopotentiating activity. Patent No 87184, 25.06.2009.
15. T.J. Hardt, W.A. Ritschel. The effect of coumarin and 7-Hydroxycoumarin on *in vitro* macrophage phagocytosis of latex particles, Met. Exp. Clin. Pharmacol. 5 (1983) 39-43.
16. W.G. Lewis, L.G. Green, F. Grynszpan, Z. Radi, P.R. Carlier, P. Taylor, M.G. Finn, K.B. Sharpless, Click chemistry in situ: Acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks,AngewChemInt.Ed. 41 (2002) 1053-1057.
17. A.M.S. El-Shariefa, Y.A. Ammara, M.A. Zahrana, H.K. Sabeta, 1,4-Phenylenediisothiocyanate in the synthesis of *bis*-(thiourea, benzothiazole, quinazoline, 1,3-benzoxazineand imidazolidineiminothiones) derivatives.Phosphorus, Sulfur, and Silicon and the Related Elements. Copyright C Taylor & Francis Inc. 179 (2004) 267-275.
18. G. Keating, R. O'Kennedy, The chemistry and occurrence of coumarins. Coumarins: Biology, applications and mode of action. (Eds: O'Kennedy R, Thornes RD), Chichester, John Wiley & Sons, 1997, pp. 23-66.
19. O. Kayser, H. Kolodziej, A.F. Kiderlen, Immunomodulatory principles of *Pelargonium sidoides*, Phytother. Res. 15 (2001) 122-126.
20. T. Bolton, J.R. Casley-Smith, An *in vitro* demonstration of proteolysis by macrophages and its increase with coumarin, Experientia31 (1975) 271-273.
21. S. Iwaki, T. Nakamura, J. Koyama, Inhibitory effects of various synthetic substrates for aminopeptidases on phagocytosis of immune complexes by macrophages, J. Biochem. 99 (1986) 1317-1326.
22. M.E. Marshall, The coumarins. A review of the mechanisms of action and applications to clinical oncology, Dtsch. Zschr. Onkol. 24 (1992) 141-151.

23. T.H. Stefanova, N.J. Nikolova, R.A. Toshkova, H.O. Neychev, Antitumor and immunomodulatory effect of coumarin and 7-hydroxycoumarin against Sarcoma 180 in mice,J. Exp. Ther. Oncol. 6 (2007) 107-115.
24. F.A. Jimenez-Orozco, J.S. Lopez-Gonzalez, A. Nieto-Rodriguez, M.A. Velasco-Velazquez, J.A. Molina-Guarneros, N. Mendoza-Patino, M.J. Garcia-Mondragon, P. Elizalde-Galvan, F. Leon-Cedeno, J.J. Mandokki, Decrease of cyclin DI in the human adenocarcinoma cell line A-427 by 7-hydroxycoumarin, Lung Cancer 34 (2001) 185-194.
25. H.M.S. Kumar, N.A. Qazi, S.D. Sawant, P.P. Singh, S. Shafi, S.C. Taneja, K. Anamika, G. Amit, B. Sarang, G.N. Qazi, 5-Butenyl isoxazolines useful as novel immunomodulators. Patent No 0844/DEL/2006, 26.03.2007.
26. G.F. Medina, J.G. Marrero, M. Macias-Alonso, M.C. Gonzalez, I. Cordova-Guerrero, G. Ariana. T. Garcia, S. Osegueda-Roblesa, Coumarin heterocyclic derivatives: chemical synthesis and biological activity, Nat. Prod. Rep. 32 (2015) 1472-1507.
27. A. Lacy, R. O'Kennedy, Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer, Curr. Pharm. Des. 10 (2004) 3797-3811.
28. I. Kostova, S. Bhatia, P. Grigorov, S. Balkansky, V.S. Parmar, A.K. Prasad, L. Saso, Coumarins as antioxidants, Curr. Med. Chem. 18 (2011) 3929-3951.
29. Y. Bansal, P. Sethi and G. Bansal, Coumarin: a potential nucleus for anti-inflammatory molecules, Med. Chem. Res. 22 (2013) 3049-3060.
30. S.R. Trenor, A.R. Shultz, B.J. Love, T. E. Long, Coumarins in Polymers: From Light Harvesting to Photo-Cross-Linkable Tissue Scaffolds, Chem. Rev. 104 (2004) 3059-3077.
31. P.O. Patil, S.B. Bari, S.D. Firke, P.K. Deshmukh, S.T. Donda, D.A. Patil, A comprehensive review on synthesis and designing aspects of coumarin derivatives as monoamine oxidase inhibitors for depression and Alzheimer's disease,Bioorg. Med. Chem. 21 (2013) 2434-2450.
32. G.B. Bubols, D.D. Vianna, A. Medina-Remon, G.V. Poser, R.M. Lamuela-Raventos, V.L. Eifler-Lima, S.C. Garcia, The antioxidant activity of coumarins and flavonoids, Mini-Rev. Med. Chem. 13 (2013) 318-334.

33. I. Kostova, Synthetic and natural coumarins as cytotoxic agents, Curr. Med. Chem.: Anti-Cancer Agents, 5 (2005) 29-46.
34. A.A. Al-Amiery, K.Y. Al-Majedy, A.A.H. Kadhum, A.B. Mohamad,Novel macromolecules derived from coumarin: synthesis and antioxidant activity, Sci. Rep. 5 (2015) 11825.
35. S. Cicchi, M. Cordero, D. Giomi,Five-membered ring systems: With O&N atoms,Prog. Heterocycl. Chem. 15 (2003) 261-283.
36. P. Mondal, S. Jana, A. Balaji, R. Ramakrishna, L.K. Kanthal,Synthesis of some new isoxazoline derivatives of chalconisedindoline 2-one as a potential analgesic, antibacterial and anthelmimtic agents, J. Young Pharm. 4 (2012) 38-41.
37. A.P. Kozikowski, The isoxazoline route to the molecules of nature, Acc. Chem. Res. 17 (1984) 410-416.
38. K. Kaur, V. Kumar, A.K. Sharma, G.K. Gupta, Isoxazoline containing natural products as anticancer agents: a review, Eur. J. Med. Chem. 77 (2014) 121-33.
39. X. Wang, K.F. Bastow, C.M. Sun, Y.L. Lin, H.J. Yu, M.J. Don, T.S. Wu, S. Nakamura, K.H. Lee, Antitumor Agents. 239. Isolation, Structure Elucidation, Total Synthesis, and Anti-Breast Cancer Activity of Neo-tanshinlactonefrom*Salvia miltiorrhiza,* J. Med. Chem. 47 (2004) 5816-5819.
40. R. Hoerr, M. Noeldner, Ensaculin (KA-672 HCl): a multitransmitter approach to dementia treatment, CNS Drug Rev. 8 (2002)143-158.
41. K.C. Liu, R.S. Becky, K.H. Robert, A particularly convenient preparation of benzohydroximinoyl chlorides (nitrile oxide precursors), J. Org. Chem.45 (1980) 3916 - 3918.
42. S.L. Born, D. Caudill, B.J. Smith, L.D. Lehman-McKecman, *In vitro* kinetics of coumarin 3,4-epoxidation: Application tospecies differences in toxicity and carcinogcnicity, Toxicol. Sci. 58 (2000) 23-31.
43. R.A. Friesner, J.L. Banks, R.B. Murphy, T.A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, M. Shelley, J. K. Perry, D. E. Shaw, P. Francis, P. S. Shenkin, J. Med. Chem. 47 (2004) 1739.
44. T.A. Halgren, R.B. Murphy, R.A. Friesner, H. S. Beard, L. L. Frye, W. T. Pollard, J. L. Banks, J. Med. Chem. 47 (2004) 1750.

45. T.S. Jagodzinski, Thioamides as useful synthons in the synthesis of heterocycles, Chem. Rev. 103 (2003) 197-227.
46. J.Y. Lee, H.J. Lee, Y.D. Gong, Traceless solid-phase synthesis of 2,4,5-trisubstituted thiazoles, Synlett 16 (2005) 2483-2485.
47. B. Shi, A.J. Blake, W. Lewis, I.B. Campbell, B.D. Judkins, C.J. Moody,Rhodium carbene routes to oxazoles and thiazoles. catalyst effects in the synthesis of oxazole and thiazole carboxylates, phosphonates, and sulfones, J. Org. Chem. 75 (2010) 152-161.
48. V.F. Hercules, C.R. Lenilson, P.S. Richele, L.M.P. Andre, Syntheses of enantiopure aliphatic secondary alcohols andacetates by bioresolution with Lipase B from *Candida Antarctica,* Molecules 17 (2012) 8955-8967.
49. S. Masakazu, U. Yutaka, I. Katsuhiko, Preparation of novel N-sulfonylated (*S*,*S*)-2,3-diaminosuccinatetype chiral auxiliaries and application to an asymmetric 1,3-dipolar cycloaddition reaction of nitrile oxides to allyl alcohol, Tetrahedron Asym, 17 (2006) 3075-3083.
50. R.J. Kazlauskas, N.E. Weissfloch, A.T. Rappaport, L.A. Cuccia, A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia,* and lipase from *Candida rugosa*,J. Org. Chem. 56 (1991) 2656-2665.
51. D.M. Michael, L.N. Amber, A.S. Khriesto, 5-Bromomethyl-4,5-dihydroisoxazoles, Tetrahedron Lett. 50 (2009) 5647-5648.
52. S. Takashi, M. Hiroshi, K. Toshinobu, E. Tadashi, Kinetic resolution of 5-(hydroxymethyl)-3-phenyl-2-isoxazoline by using the 'low-temperature method' with porous ceramic-immobilized lipase, Tetrahedron Asym. 16 (2005) 1535-1539.
53. E.D. Chan, B.W. Winston, S.T. Uh, M.W. Wynes, D.M. Rose, D.W. Riches, Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-γ and TNF-α in mouse macrophages, J. Immunol. 162 (1999) 415-422.
54. T. Ismail, S. Shafi, P.P. Singh, N.A. Qazi, S.D. Sawant, A. Intzar, I.A. Khan, H.M.S Kumar, G.N. Qazi, M.S. Alam,Biologically active hydroxymoyl chlorides as antifungal agents, Ind. J. Chem. B.47 (2008) 740-747.

Research Highlights:

1. Synthesis of isoxazoline functionalized coumarins was attempted.
2. A series of 13 novel isoxazoline functionalized coumarins was synthesised.
3. The synthesized compounds exhibit excellent immune potentiating activity.
4. The activity of **2**,**4** and **8** is more than that of Levimasole as standard.