2.1.0. Introduction to iridoid glycosides:-

Despite the important role of adjuvants for vaccine development, relatively few adjuvants have been successfully incorporated into vaccines intended for human administration. This is in part due to the high toxicity associated with many experimental adjuvants. This lack of choice effectively hinders the ability to produce vaccines against many diseases or to improve current vaccine formulations and therefore, there is a major unmet need for a safe efficacious adjuvant capable of boosting both cellular and humoral immunity [O, Hagan et al., 2001]. While exploring the novel plant based immunoadjuvants (Sharma et al., 1994; Gupta et al., 2006), it was found that many glycoconjugates such as picroside-I, picroside-II, catalpol, agnuside and negundoside possess promising dose dependant immune potentiation ability as indicated by B and T cell proliferation (Atal 1986; Puri et al., 1992). However, inspite of all their immunological traits (Khajuria et al., 2007) our detailed evaluation revealed several drawbacks of these molecules viz., lack of immune memory and depot formation. This prompted us to explore the possibility to further develop these molecules as alternate plant based immune adjuvants for vaccines through appropriate structural modifications.

Keeping in view the important characteristics of an ideal adjuvant, semisynthetic modification of these iridoid glycosides have been proposed, through the introduction of micellar structure selectively on some of the hydroxyl groups, which greatly enhances the immunomodulatory potential of these molecules. The rationale behind the introduction of lipophilic groups is to achieve fine-tuning of hydrophilic and lipophilic balance (HLB) which is very crucial for adjuvant activity. In this direction, the design and synthesis of lipophilic structures has been planned to impart desired level of HLB on the adjuvant candidate.

2.1.1. Synthesis of novel Picroside-II based immune adjuvants:-

Picrorhiza kurroa was identified as a potential source of immunomodulatory compounds (Labadie et al., 1989), and its modulatory effects on complement activation and the production of chemiluminescence by activated neutrophils were investigated (Simons 1989). Crude preparation of Picrorhiza kurroa (Kirtikar and Basu 1935; Dey et al., 1980; Chaturvedi and Singh 1966: Handa and Sharma 1986; Dorsch et al., 1991) has been used in the Ayurvedic system of traditional medicine to cure various immune related diseases. Several
Iridoid glycosides enriched fraction (e.g. kutkin, Picroliv and RLJ-NE-299A) as well as pure iridoid glycosides from *P. kuroa* have been standardized and well characterized. Preliminary screening of fractions of *Picrorhiza kurroa* for immune-adjuvant activity revealed that iridoid glycosides enriched fraction have potential immunomodulatory properties (Atal 1986; Puri et al., 1992). Despite the long term human use of secondary metabolite enriched fractions of *Picrorhiza kurroa* (Subedi 2000; Dhuley 1997; Kitagawa et al., 1971; Weinges et al., 1972) as potential immunomodulator in traditional medicines (Puri et al., 1992), there had been no central publication regarding the adjuvant activity of the molecular constituents of this valuable plant. Recently Khajuria *et al* reported the adjuvant activity (Khajuria *et al.*, 2007; Khajuria *et al.*, 2007) of enriched fraction of *P. kurroa* in vaccine formulations. The single molecules derived from these fractions revealed varying degrees of adjuvant activity. The enriched fractions [RLJ-NE-299A, a mixture of picroside-I (PK-I) and picroside-II (PK-II)] derived from this plant exhibited promising adjuvant activity without significant sustained immune memory or depot formation properties, which restricted their use as plant based immune-adjuvants. This prompted us to explore the possibility to develop these molecules as alternate plant based immune adjuvants for vaccines. Even though several iridoid glycosides in their native form are often acylated at specific hydroxyl groups of their sugar or aglycon moiety, we envisaged the need for improved structures derived from these iridoid glycosides with proper hydrophilic-lipophilic balance (Liu *et al.*, 2002) that can be readily fine tuned through acylation with lipid groups of varying chain length and branching. Three major iridoid glycosides *viz.*, picroside-I (PK-I, 1), picroside-II (PK-II, 2) and catalpol (3) ([Fig 1](#)) of *P. kurroa* has been taken up for semi-synthetic moodification. Structural investigation revealed that in PK-I, there has been cinnamoyl group at C6’ of glucose and in case of PK-II a vanilloyl group at the C6 of aglycan moiety, varying from each other by position and nature of acyl group. In the preliminary screening of PK-I, PK-II and the completely deacylated iridoid moiety *i.e.*, catalpol on OVA stimulated splenocyte proliferation ([Fig 2](#)), only PK-II showed moderate enhancement while catalpol was inactive. These results indicated that the presence of lipophilic group may be critical for immune adjuvant activity along with nature and position of acyl group.

Based on these results, we embarked upon a programme to rationally modify PK-II. Due to overwhelming number of hydroxyls present on the molecule against its lipophilic part,
the molecule becomes more hydrophilic with poor HLB thereby rendering the molecule less prone to depot formation. In this section, a structural modification of PK-II is presented. Both chemical and enzymatic lipidation strategies have been adopted to convert the scaffold into improvised structures with significant adjuvant activity.

2.1.1.1. Rational behind structural modification of Picroside-II:-

Picroside-II is polyhydroxylated compounds and acylation of these polyhydroxylated natural compounds not only increase the structural diversity, but also changes their physical and chemical properties, which may result in improved pharmacological and pharmacokinetic properties. Semi-synthetic modification of these natural products through the introduction of lipophilic or micellar structure selectively on some of the hydroxyl groups greatly enhances the immunomodulatory potential of these molecules. In nature iridoid glycosides often appear acylated at specific hydroxyl groups of their sugar or aglycon moiety, either it is at the primary hydroxyl position of glycan moiety or at the secondary hydroxyl position of aglycan part (Fig 1). We envisioned the need for improved structures derived from these iridoid glycosides with optimal HLB that can be readily fine tuned through regio-selective acylation with acyl groups of varying chain length and topology.

Taking hints from the SAR of QS-21 (Liu et al., 2002), three important guidelines, viz., a) degree of acylation. b) site of acylation, c) nature of acyl group has been taken into account while designing the semi-synthetic modifications since these features play very important role in determining the adjuvanticity of a molecules. Based on the above points the iridoid glycosides were acylated and novel lipidated analogs with balanced hydrophilicity and lipophilicity were tested for adjuvant activity. However, direct chemical acylation of iridoid glycosides to achieve this objective is still a distant target because of the present lack of suitable reagents and protocols to discriminate among specific hydroxyl groups of the same molecule and to acylate regio-selectively one over several hydroxyl groups. Furthermore, these molecules are highly sensitive to acidic and thermal conditions. The acid sensitive protection-deprotection strategies cannot be employed while designing regioselective of these molecules. So we are left either with conventional acylation method on un-protected sugar using carbodimide coupling or enzymatic trans-esterification using lipase and a suitable labile ester as acyl transfer agent. Both the methods have their own merits and demerits, which are discussed below in detail.
**Fig 1:** Iridoid glycosides from *Picrorhiza kurroa*

**Fig 2:** Effect of PK-I, PK-II and Catalpol on OVA-stimulated splenocyte proliferation *ex-vivo*

Splenocytes isolated from ova immunized mice on day 15 were cultured with ova and various concentrations of Picroside-I, Picroside-II, Catalpol for cell proliferation. Cell proliferation was done after the 72h incubation using MTT assay. The difference between the control and treated groups is determined by Bonferroni multiple comparison test. *P* < 0.05, **P** < 0.01 where alum and drug treated cultures were compared with ovalbumin only treated cultures.
2.1.1.2. Random acylation of Picroside-II using DCC/ DMAP:

As picroside-II has many hydroxyl groups, conventional method of its acylation results in a mixture of products i.e., mono, di, tri, tetra acylated derivatives (Fig 3). Eventhough it is rather difficult to separate and isolate each one of these derivatives in pure form through conventional chromatography, some of the major acylated products i.e., mono and di-substituted derivatives of picroside-II were successfully isolated in pure form through a flash column chromatography. Due to afore mentioned problems associated with the application of various protection/deprotection strategy on iridoid glycosides, the carbodiimide method can be visualized as the feasible non-enzymatic method by which we can synthesize such acyl derivatives.

All these acylated derivatives (Scheme 1) were prepared through simple DCC/ DMAP coupling reaction of various aliphatic acids to picroside-II in dry THF according to literature procedure and were characterized by IR, $^1$H, $^{13}$C-NMR/ DEPT and mass spectral analysis. The site of acylation has been confirmed by the down field shift of –CH- and- CH$_2$- signal after acylation on the $^1$H/$^{13}$C NMR.

![Fig 3: Site of acylation](image)

In case of mono-acylated product of PK-II obtained through carbodimide coupling, the acylation is found to take place either at primary hydroxyl position of glycan moiety or at phenolic hydroxyl of vanilloyl group. While, in case of diacylated product of PK-II, the acylation is found to take place at the primary hydroxyl position of glycon moiety and phenolic hydroxyl of vanilloyl group. The position of acylation was confirmed by $^1$H NMR. Preliminary screening results revealed that mono acylated product shown better activity as compared to di-acylated product. Moreover, the acylated product at primary hydroxyl of PK-II
had shown better activity. In this direction, our approach is to develop regio-selective acylation method for introduction of lipophillic chains of varying length on the primary hydroxyl of PK-II to further explore the extent of chain length require and deriving optimum adjuvant activity.

\[ R = n\text{-heptyl}, n\text{-pentadecyl} \]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product (4)</th>
<th>( R_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK-II-R1</td>
<td>( R_2 )</td>
<td>( R_1 ) H</td>
</tr>
<tr>
<td>PK-II-R2</td>
<td>H</td>
<td>( R_2 )</td>
</tr>
<tr>
<td>PK-II-R3</td>
<td>( R_2 )</td>
<td>( R_1 ) H</td>
</tr>
<tr>
<td>PK-II-R1</td>
<td>( R_2 )</td>
<td>( R_1 ) H</td>
</tr>
<tr>
<td>PK-II-R2</td>
<td>H</td>
<td>( R_2 )</td>
</tr>
<tr>
<td>PK-II-R3</td>
<td>( R_2 )</td>
<td>( R_1 ) H</td>
</tr>
</tbody>
</table>

Scheme 1: Random acylation of PK-II with fatty acids in the presence of DCC/DMAP

2.1.1.3. Lipase catalysed trans-esterification of Picroside-II: Enzyme optimization study:

Lipases are superior bio-catalysts, which are able to accept a wide array of complex molecules as substrates and catalyze hydrolysis and trans-esterification reactions with high enantio and regio-selectivities (Bjorkling et al., 1991; Crosby 1991; Martin et al., 1992; Chen et al., 1994; Otta et al., 1998). Lipases have been extensively used for the regio-selective acylation of polyhydroxylated natural products such as flavaonoids, saponins and other
polyphenolic compounds (Riva et al., 2002; Kontagianni et al., 2001; Intra et al., 2004; Wang et al., 2004).

In this context, we initiated a research programme directed towards the development of novel amphiphiles and biosurfactant mimics of iridoid glycosides. Initially, picrosides-II has been chosen for lipase catalyzed regio-selective acylation studies using \( p \)-nitrophenyl alkanoate derived from various carboxylic acids of varying chain lengths as acyl donors. Regio-specific acylation of these picrosides was studied using a various commercially available lipases.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Candida rogusa</td>
<td>PK-II (2)</td>
<td>15</td>
</tr>
<tr>
<td>II</td>
<td>Lipase acrylic resin from Candida antarctica (lipase B)</td>
<td>PK-II (2)</td>
<td>50</td>
</tr>
<tr>
<td>III</td>
<td>Candida antarctica recombinant from Aspergillus oryzae (lipase B)</td>
<td>PK-II (2)</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>Candida cylinderracia</td>
<td>PK-II (2)</td>
<td>15</td>
</tr>
<tr>
<td>V</td>
<td>Porcine pancreatic lipase</td>
<td>PK-II (2)</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experimental conditions: 0.1 mmol PK-II (2); 0.2 mmol \( p \)-nitrophenylalkanoate; 20% weight equivalent enzyme; dry THF; rt.

**Table 1: Enzymatic trans-esterification of picroside-II (2) by commercial lipases**

Five commercial lipases viz., Candida rogusa lipase, lipase acrylic resin from Candida antarctica (lipase B), Candida antarctica recombinant from Aspergillus oryzae (lipase B), Candida cylinderracia lipase and porcine pancreatic lipase were used to study the regio-selective acylation of various lipophilic moieties of varying chain lengths on picroside-II using various labile esters such as \( p \)-nitrophenyl alkanoate in dry organic solvent (THF/DMF). The selection of enzyme being one of the most important parameters for enzyme-catalyzed reactions, in order to choose the more efficient and suitable enzyme for the trans-esterification of iridoid glycosides, both soluble lipases and immobilized lipases on macroporous resin were screened to evaluate their potential in terms of selectivity and yields using \( p \)-nitrophenyl alkanoate as acyl donor in dry THF at room temperature. The results were compared and presented in Table 1 which clearly shows that resin bound lipase exhibits its unique advantage to catalyze the trans-esterification reaction, while the soluble lipases reveal the lower catalytic activity.

In case of lipases/substrates studied here, only single regio-isomeric acylated product was formed, indicating high regio-selective nature of the lipases-catalyzed trans-esterification with iridoids. These commercial lipases were screened for the regio-selective acylation using
several solvents such as diethyl ether, dichloromethane, n-hexane, diisopropyl ether, DMF and THF. Owing to the poor solubility of substrates in less polar solvents, polar solvents viz., DMF and THF were found to be suitable for such study. Optimal conversions in the range of 12-21% could be achieved using soluble lipases, whereas higher conversion up to 50% could be achieved with with resin bound immobilized Candida antarctica lipase-B/THF combinations. Result of screening revealed that resin bound immobilized Candida antarctica lipase B was the enzyme of choice when used along with p-nitrophenyl alkanoate as acyl donor and this combination was chosen for all further studies.

2.1.1.4. Regio-selective acylation of picroside-II using resin bound immobilized Candida antarctica lipase B:-

Regioselective acylation of picroside-II (Weinges and Künstler 1977; Weinges et al., 1972; Kitagawa et al., 1971; Wang et al., 1993), a polyhydroxylated iridoid glycosides was achieved with resin bound immobilized Candida antarctica lipase B in the presence of various acyl donors, the acylation occurred only at the primary hydroxyl group of glycan moiety with no reaction at the aglycan part. Immobilized lipase catalyzed trans-esterification of picroside-II gave moderate yields with varying structural types of acyl groups such as acetyl, butanoyl, octanoyl, decanoyl, dodecanoyl, palmitoyl and micellar structure (glyceric acid, triethylene glycol) derived p-nitrophenylalkanoates (Scheme 2). Various acylated analogs of picroside-II thus obtained were purified by flash chromatography and their structure determined by mass, $^1$H/$^{13}$C NMR spectra and by comparison with spectra of their respective substrates. As anticipated the enzymatic trans-esterifications were clean and the unreacted starting compounds were recovered during chromatographic separations. Furthermore, lipase immobilized on resin viz., Candida antarctica lipase B gave moderate yields of esters ranging from 23-52% with various substrates. Comprehensive mass, $^1$H and $^{13}$C NMR assignments have been listed in the experimental section and the key diagnostic signals leading to the structural assignments are summarized below.

In case of picroside-II butanoate ester, $^1$H NMR signal at $\delta$ 4.43 ($J = 11.9, 1.9$) and 4.31 ($J = 11.9, 6.2$) assigned to H-6’ (a’ and b’) of the glucose moiety appeared at a down field (0.50 and 0.66 ppm) compared to that of the picroside-II molecule ($\delta$ 3.93, $J = 11.9, 1.9$ and 3.65, $J = 11.9, 6.7$) shown in Fig 4. Moreover, in the $^{13}$C NMR the signal at $\delta$ 62.8 was
assigned to the C-6’ of the glucose moiety in picroside-II butanoate molecule. Downfield shift of this signal by 1.4 ppm in comparison with that of the picroside II molecule (δ 61.4) suggested the presence of an ester bond on the C-6’ of the sugar moiety of picroside-II shown in Fig 5. Similar, structural assignments were made for other regio-selective esterification products of picroside-II obtained with various other acyl donors (Scheme 2). All new analogs of PK-II thus generated were tested against weak antigen OVA to estimate their adjuvanticity following standard protocols viz., measurement of OVA specific antibody titre, lymphocyte proliferation and estimation of Th1 cytokines.

2.1.1.5. Biological activity: Evaluation of adjuvant activity PK-II analogs

In order to evaluate the acylated analogs of picroside-II on immunomodulatory activity, preliminary splenocyte proliferation ex vivo studies were performed. Based on the preliminary results, detailed immune adjuvant activity of active analogs in the presence of weak antigen ovalbumin were studied by stimulating anti OVA IgG titre, neutralizing antibody (IgG1 and IgG2a) titer as well as the production of soluble mediators of a Th1 (IL-2 and IFN-γ)/Th2 response (IL-4) and proliferation of T lymphocytes sub-sets (CD4/CD8). In all 12 acylated analogs of picroside-II for their possible immunomodulatory activity were tested. Out of these, two acylated analog of picrosides-II viz., PK-II-3 and PK-II-6 exhibited potential immune adjuvant activity.

2.1.1.6. Results and Discussion:-

Effect of acylated analogs of picroside-II on cell proliferation ex vivo

To observe the effect of acylated analogs of picroside-II on cell proliferation, splenocytes isolated from ova sensitized mice were cultured with ova and acylated analogs of picroside-II (0.01, 0.1, 1 and 10 µg) as shown in Table 2. Among all the analogs, three compounds viz., PK-II-2, PK-II-3 and PK-II-6 significantly enhanced OVA-stimulated splenocyte proliferation compared to OVA treated group and Ova/Alum was taken as positive control. PK-II-2, PK-II-3 and PK-II-6 were taken for detailed immune-adjuvant activity of weak antigen ovalbumin.
Scheme 2: Regio-selective acylation of picroside-II

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product (4)</th>
<th>Reaction time (h)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK-II-1</td>
<td>( R_1 )</td>
<td>30</td>
<td>52</td>
</tr>
<tr>
<td>PK-II-2</td>
<td>( 4' )</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>PK-II-3</td>
<td>( 6'' )</td>
<td>48</td>
<td>38</td>
</tr>
<tr>
<td>PK-II-4</td>
<td>( 10'' )</td>
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<td>38</td>
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<tr>
<td>PK-II-5</td>
<td>( 12'' )</td>
<td>49</td>
<td>35</td>
</tr>
<tr>
<td>PK-II-6</td>
<td>( 16'' )</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>PK-II-7</td>
<td>( 14'' )</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>PK-II-8</td>
<td>( 8''' )</td>
<td>50</td>
<td>23</td>
</tr>
</tbody>
</table>

Scheme 2: Regio-selective acylation of picroside-II
Fig 4: A). $^1$H NMR of Picroside-II: Red arrow showed the $^1$H NMR signal for the primary CH$_2$ group of glycon moiety before acylation. B). $^1$H NMR of 6'-O-Butanoyl-picroside-II: Red arrow showed the $^1$H NMR signal for the primary CH$_2$ group of glycon moiety after acylation.

Fig 5: A). $^{13}$C NMR of Picroside-II: Red arrow showed the $^{13}$C NMR signal for the primary CH$_2$ group of glycon moiety before acylation. B). $^{13}$C NMR of 6'-O-Butanoyl-picroside-II: Red arrow showed the $^{13}$C NMR signal for the primary CH$_2$ group of glycon moiety after acylation.
Effect of acylated analogs of picroside II on OVA-specific antibody titre

The OVA-specific IgG, IgG1, and IgG2a antibody titers in the serum were measured by indirect Elisa as shown in Fig 6 (A-C). The serum IgG titer in OVA-immunized mice was significantly enhanced on day 28 by PK-II-3 and PK-II-6 at a dose of 10 µg with p < 0.01(**) when compared with OVA only treated group. OVA-specific serum IgG titre in mice group immunized with OVA/alum and OVA/PK-II-2 were almost similar. PK-II-3 and PK-II-6 at a dose 10 µg significantly enhanced the serum IgG1 titers in OVA immunized mice with p < 0.01(**). Significant enhancements in OVA-specific serum IgG2a titers were also observed with PK-II-3 and PK-II-6 immunized mice at a dose of 10 µg with P < 0.01(**) as compared with OVA only treated group. However, there were no significant differences in the serum IgG2a levels between mice groups immunized with Ova/alum and OVA alone. The results were more promising in case of PK-II-3 and PK-II-6. These findings indicated that PK-II-3 and PK-II-6 significantly enhanced serum OVA-specific antibody production in mice immunized with OVA.

Estimation of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines in serum

The effect of acylated analogs of picroside II at a dose range of 1, 10, 30 and 100 µg on the release of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines in serum were shown in Fig 7 (A-C). Results indicate that compound PK-II-3 and PK-II-6 significantly stimulated Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokine release in a dose-related manner. PK-II-3 and PK-II-6 enhanced the Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines at a dose of 10 µg with P < 0.01(**) where as PK-II-2 only significantly enhanced IFN-γ at a dose of 100 µg with P < 0.01(**). Out of three molecules, the results were more promising in case of PK-II-3 and PK-II-6, which enhance both Th1 and Th2 type of immune response.

Immunophenotyping of lymphocytes by flow cytometry

The overall percentage of T-cell subsets CD4 and CD8 in the splenic lymphocyte population was estimated in the presence of PK-II-2, PK-II-3 and PK-II-6 (Fig 8). The effects of PK-II-2, PK-II-3, and PK-II-6 treated subpopulations were dose dependently increased compared to untreated group. It was observed that the effect of PK-II-3 and PK-II-6 at lower doses was more pronounced compared to that of PK-II-2. The presence of PK-II-3 and PK-II-6 significantly enhanced the percentage of T-cell populations at the dose of 10 µg, however PK-II-2 elicit significant enhancement in T-cell population at the dose of 100 µg.
Safety studies

No weight loss was observed for mice when recorded on days 0, 15 and 28 post-injection. Mice were kept under observation for 28 days post injection for any toxic manifestation and no visible symptoms such as inflammatory, allergic response or any other undesirable effects like edema, induration, granuloma etc. was observed after the treatment of PK-II and its analogues. Although the metabolic fate of iridoid glycosides have not been studied here, the enzyme labile linkages of PK-II, both ester and glycosidic bonds should eventually degrade the molecule into glucose, lipid and and aglycone units that are compatible and harmless to mammalian tissues. Use of the crude extract of Picrorhiza kurroa containing the key precursor of these molecules (PK-II) as immune potentiator in traditional ayurvedic medicine from time immemorial indicates that lipidated iridoid glycosides like PK-II analogs presented in this section may become ideal and safe adjuvants for human vaccine trials.

Discussion: Recently, there has been an increasing emphasis on the development of novel immune-adjuvant/ immunopotentiator to improve efficacy of vaccine formulations. Therefore, the development of plant based immuneadjuvants is one of attractive approach in this direction. Adjuvants have significant effects on the nature of the immune responses, and can tilt the immune system in favor to Th1 or/and Th2 type response. Furthermore, depot formation and cytosol trafficking is also one of the important characteristics for immune-adjuvants, but most of the adjuvants lack these traits. Our aim is to design molecules which not only activate the immune responses but also help in depot formation and cytosol trafficking for internalization and expression of antigen. Recently, Liu (Liu et al., 2002) reported the presence of long lipophilic chain is critical for adjuvant activity. Hence, our effort is to develop a single molecule based immunoadjuvants bearing some of the above mentioned structural features.

In the present study, we also tried to correlate the acyl group at C6/C6’ position of picrosides and their immune responses. In order to examine the effect of various lipophilic groups at C6/C6’ of picroside on the derived adjuvant activity, we synthesized novel acylated analogs at C6’ of picroside-II through regio-selective trans-esterification in the presence of Candida antarctica lipase using p-nitrophenyl alkanoate of varying chain length as acyl donor as shown in Scheme 1 & 2. Novel acylated analogs of picroside-II were prepared through chemo-enzymatic method to examine the minimum structural requirement for
deriving optimal adjuvant activity with balanced humoral and cell mediated immune responses. All the novel acylated analogs of picrosides were evaluated for inducing Th1 or Th2 immune responses in mice against ovalbumin. The results of the present study provide evidence for the interaction of picroside with the immune system and highlight its potential for vaccine adjuvant development. To observe the effect of acylated analogs of picroside-II on cell proliferation, splenocytes isolated from OVA sensitized mice were cultured with OVA and acylated analogs of picroside-II (0.01, 0.1, 1 and 10 µg). As shown in Table 2, among all the compounds, three analogs viz., PK-II-2, PK-II-3 and PK-II-6 significantly enhanced the OVA-stimulated splenocyte proliferation (ex-vivo) in comparison with OVA control group at 1 µg, 1 µg and 0.1 µg respectively. The results indicated that PK-II-2, PK-II-3 and PK-II-6 could significantly enhance the T and B cell population. The above results demonstrate that the varying chain lengths of lipophillic substitution lead to varying degree of immune adjuvant activity. These results were further confirmed by detailed in vivo experiments. The detailed investigation of immune adjuvant activity of acylated analogs of picroside-II viz., PK-II-2, PK-II-3 and PK-II-6 has been carried out with weak antigen (OVA) and the results are summarized in Fig 6-8. The length of the acyl chain on picroside-II derivatives also played a major role, compounds with long chain alkyl group viz., C8 or C16 gave high humoral and cell-mediated immunity in comparison with the parent natural product. Analogues with shorter (C4) fatty acid esters at this position were less effective.

In in-vivo experiments, acylated analogs of picroside-II were evaluated in the presence of OVA with or without aluminum hydroxide in BALB/c mice. Analogues of PK-II viz., PK-II-2, PK-II-3 and PK-II-6 strongly induce antigen-specific cellular and humoral immune responses shown in Fig 7-9. Apart from cellular responses, strong protein-specific humoral responses induced by PK-II-3 and PK-II-6 indicate its potency as immune adjuvant in vaccines directed against extracellular pathogens. The addition of PK-II-2, PK-II-3 and PK-II-6 at a suitable dose to the OVA immunized group increased the potency of the humoral immune response when compared to the OVA only treated group, determined by OVA-specific IgG titre and neutralizing antibody titers (IgG1 and IgG2a). The OVA-specific serum IgG, IgG1 and IgG2a antibody titers in the OVA-immunized mice were shown in Fig 6. The various optimum dose for the adjuvant effect of PK-II-2, PK-II-3 and PK-II-6 on antibody responses suggested that PK-II-3 and PK-II-4 were active towards humoral response with P <
0.01(**). PK-II-3 and PK-II-6 gave optimal response which suggests that lipophilic group at C6’ of picroside-II is favorable for immune adjuvant activity.

We also determined the possible effect of PK-II-2, PK-II-3 and PK-II-6 on soluble mediators of Th1 and Th2 response. PK-II-2, PK-II-3 and PK-II-6 could enhance the Th1 and Th2 immune responses to OVA immunized mice when given together with OVA. As shown in Fig 7, out of the above three analogues, PK-II-3, PK-II-4 significantly increased the production of Th2 (IL-4) and Th1 cytokines (IL-2 and IFN-γ) in the OVA immunized mice with p < 0.01(**). These results lead us to assume that presence of acyl group at C6’ position of PK-II had a significant effect on the Th1 and Th2 immune responses. Furthermore, we evaluated the effect of PK-II-2, PK-II-3 and PK-II-6 on the population of cell surface marker like CD4/CD8. As shown in Fig 8, the PK-II-3 and PK-II-4 significantly enhanced the population of CD4/CD8, but the population of CD4 increased more as compared to CD8.

Lipids and carbohydrates, either used separately or on a single construct have known history as effective vaccine adjuvants with often low toxicity. Usefulness of such moieties as adjuvant is evident by their immune potentiating activities and several such moieties are in their advanced clinical trials. Even though we do not have concrete evidence to establish the possible interaction of iridoid glycosides on some of the carbohydrate receptors on the DC cell, the fact that there has been significant improvement in the antibody titre and CD4+/CD8+ population indicating enhanced immune memory, a hallmark of adjuvant activity. A key feature of PK-II analgs as adjuvants may be explained through their dual ability to target the antigen to APCs through possible cytosol trafficking enhancement, while concomitantly enhancing the co-stimulation of APCs and evoke cytokine production. Such lipitated organic molecules with improved HLB have long been known as the carriers for antigen delivery to APC, as they have potential, depending upon their lipid content (degree and site of lipidation), to lipid membrane fusion with the plasma membrane to deliver their content, or as particulate matter to be phagocytosed, Thus, based on the prolonged antigen presentation facilitated by the lipitated PK-II analogs when injected subcutaneously, we speculate that these modified iridoid glycosides may provide depot effect associated with slow clearance at the injection site. Nevertheless, local edema is not seen following subcutaneous injection of PK-II analogs, a desirable adjuvant characteristic.
<table>
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<tr>
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Table 2: Effect of picroside-II analogs on OVA-stimulated cell proliferation *ex-vivo*

Splenocytes were isolated from immunized mice on day 15 and cultured with different concentration (0.01, 0.1, 1 and 10 µg) of PK-II analogs along with OVA 10 µg/ml for 72 h. Splenocyte proliferation was measured by the MTT method. The optical density was measured at 570 nm. The difference between the untreated and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
Fig 6-A: Effect of PK-II-2, PK-II-3 and PK-II-6 on OVA-specific IgG antibody
Groups of ten Balb/C mice were immunized subcutaneously with OVA (100 µg) on days 1 and 15 along with PK-II-2, PK-II-3 and PK-II-6 (1, 10, 30 and 100 µg). Group of animals treated with Alum (200 µg) along with Ova served as positive control. Sera were collected on day 15, 28 and 60 respectively after 1st immunization to observe the effect on IgG antibodies by ELISA. The values are presented as mean ± S.E. (n = 10). The difference between the ova only and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
Fig 6-B: Effect of PK-II-2, PK-II-3 and PK-II-6 on OVA-specific IgG1 antibody

Groups of ten Balb/C mice were immunized subcutaneously with OVA (100 μg) on days 1 and 15 along with PK-II-2, PK-II-3 and PK-II-6 (1, 10, 30 and 100 μg). Group of animals treated with Alum (200 μg) along with OVA served as positive control. Sera were collected on day 15 and 28 respectively after 1st immunization to observe the effect on IgG1 antibodies by ELISA. The values are presented as mean ± S.E. (n = 10). The difference between the ova only and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
**Fig 6-C: Effect of PK-II-2, PK-II-3 and PK-II-6 on OVA-specific IgG2a antibody**

Groups of ten Balb/C mice were immunized subcutaneously with OVA (100 µg) on days 1 and 15 along with PK-II-2, PK-II-3 and PK-II-6 (1, 10, 30 and 100 µg) for two weeks. Group of animals treated with Alum (200 µg) along with Ova served as positive control. Sera were collected on day 15 and 28 respectively after 1st immunization to observe the effect on IgG2a antibodies by ELISA. The values are presented as mean ± S.E. (n = 10). The difference between the ova only and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
Fig 7 A-C: Effect of PK-II-2, PK-II-3 and PK-II-6 on concentration of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines in mouse serum

The effect of PK-II-2, PK-II-3 and PK-II-6 on the expression of IFN-γ, IL-2 and IL-4 were determined on 28 days after 1st immunization. Cytokine estimation was done by ELISA. Values are means ± S.E. of ten mice; *P < 0.05, **P < 0.01 when compared with ova only group determined by one-way ANOVA (Bonferroni correction multiple comparison test).
Fig 8: Effect of PK-II-2, PK-II-3 and PK-II-6 on T cell sub population CD4 and CD8 in the spleen cells
Mice immunized with ova were given graded doses of PK-II-2, PK-II-3 and PK-II-6 along with Ova. Splenocyte isolated on 28 days after 1st immunization from the PK treated and untreated mice were analyzed by flow cytometry for the expression of various T cell sub populations. A typical bivariate analysis of splenocytes for the expression of cell surface markers and the percent gated population corresponding to each phenotype marker CD4 and CD8 are shown. Cells incubated with anti-mouse FITC-labeled CD8 and PE conjugated CD4 monoclonal antibodies for 30 mints, were washed with PBS before FACScan analysis. Data are representative of one of the two experiments.
2.1.2. Synthesis of Agnuside and Negundoside based immune adjuvants:

Our continued interest in the development of plant based immune adjuvants, lead us to work on other plant based iridoid glycosides such as agnuside (6) and negundoside (7) isolated from *Vitex negundo* (Fig 9). Crude extracts of all parts of *Vitex negundo* have been used extensively in Chinese herbal, Ayurvedic and Unani medicine system from time immemorial to cure various ailments (Hansal *et al.*, 1965; Sehgal 1982; Prabhakar *et al.*, 2004, Vishal *et al.*, 2008; Tandon and Gupta 2006; Telang *et al.*, 1999; Tasduq *et al.*, 2008).

![Agnuside (6) and Negundoside (7)](image)

**Fig 9: Iridoid glycosides from *Vitex negundo***

As part of this study, the semi-synthetic modification of agnuside and negundoside as immune adjuvant for vaccines has been taken up.

2.1.2.1. Rationale behind structural modification of agnuside and negundoside:-

Rationale behind the choice of agnuside and negundoside (Sehgal *et al.*, 1982; Dutta *et al.*, 1983) is to know the minimum structural requirement for iridoid glycosides to act as immune adjuvant as well as importance of presence of lipophilic chains. In agnuside, lipophilic part was attached to primary hydroxyl position of aglycan moiety and in negundoside lipophilic part was attached to secondary hydroxyl of glycon moiety. Our aim is to study iroidoid glycosides *viz.*, agnuside and negundoside in natural form as well as their lipidated analogs for immune adjuvant activity. In this direction, we developed methodologies to introduce regio-selectively lipophilic chains on iridoid glycosides by enzymatic trans-esterification approach. Some of these new semi-synthetic modified compounds have shown better adjuvant activity.
2.1.2.2. Regio-selective acylation of agnuside and negundoside:

Regio-selective acylation of agnuside was studied using various p-nitrophenylalkanoates of varying chain lengths as acyl donor for enzyme catalyzed trans-esterification in the presence of Candida antarctica lipase B. TLC monitoring revealed that agnuside was acylated regioselectively to form a single product, which was purified on silica gel column and characterized as mono-acylated product 8 (6-O-alkanoyl agnuside). $^1$H NMR spectra revealed that esterification occurred at the secondary hydroxyl of aglycon moiety, as evidenced by the downfield shift of signal of H-6 of aglycon moiety of compound AG-2, to δ 5.26 ($J = 2.0$) as compared to agnuside (δ 4.48, $J = 2.1$). Moreover, in $^{13}$C NMR compared with agnuside, C-6 of the compound AG-2 shifted downfield to δ 83.5. All these results suggested that agnuside was regio-selectively acylated only at C-6 of aglycon moiety without acylating the sugar moiety although there is a primary –OH group in agnuside which is generally more reactive in other glycosides such as picroside-II and negundoside. Further, it was observed that acyl donors of varying chain length also afford single acylated product (Scheme 3) with same regioselectivity.

It is noteworthy that the acylation of agnuside occurred preferentially on the secondary –OH at the C-6 of the aglycon moiety even though this molecule consists of a free primary hydroxyl group on the sugar moiety which may be attributed to molecular over-crowding between p-hydroxybenzoate entity of aglycon ester and the primary hydroxyl of sugar unit, which might be creating hindrance to the binding of substrate to the binding domain of lipase and their subsequent interaction with the catalytic site to enable effective transesterification at this site. This is further confirmed by the facile lipase mediated transesterification of negundoside at the primary hydroxyl of sugar, which is devoid of such steric overcrowding and the results are discussed below.

In case of negundoside, esterification occurred at the primary hydroxyl group of sugar. Negundoside also gave moderate yields irrespective of type of acyl groups. As in case of Picroside-II, $^1$H NMR, $^{13}$C NMR of negundoside showed significant downfield shift of the methylene signals of primary hydroxyl group of the sugar moiety which suggested that compound 7 was regio-selectively acylated only at C-6’ of sugar moiety. Acylation using various other nitrophenyl alkanoates of varying chain lengths gave the product without any deviation in the regio-selectivity (Scheme 4).
Candida antarctica, THF Molecular sieves (4A)

R = methyl, n-propyl, n-heptyl, n-nonyl, n-pentadecyl

<table>
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<th>Isolated Yield (%)</th>
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<tr>
<td>AG-5</td>
<td>n-pentadecyl</td>
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Scheme 3: Regio-selective acylation of Agnuside

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<tr>
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<td>28</td>
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</table>

Scheme 4: Regio-selective acylation of Negundoside
In case of iridoid glycoside with free primary hydroxyl group at sugar moiety (except agnuside), the acylation occurred only at the primary hydroxyl group of the sugar moiety. All the lipidated analogs of agnuside and negundoside synthesized were also tested for immune adjuvant activity as discussed in biological activity section below.

2.1.2.3. Biological activity:- Evaluation of adjuvant activity of lipidated analogs of agnuside and negundoside

Iridoid glycosides viz., agnuside and negundoside and their lipidated analogs were examined to explore minimum structural requirement for the iridoids to act as immune adjuvants. All new lipidated analogs of agnuside and negundoside thus generated were tested against weak antigen OVA to estimate their adjuvanticity following standard protocols.

2.1.2.4. Results and Discussion:-

Effect of acylated analogs of agnuside and negundoside on cell proliferation ex vivo

To observe the effect of acylated analogs of agnuside and negundoside on cell proliferation, splenocytes isolated from OVA sensitized mice were cultured with ova and acylated analogs (0.01, 0.1, 1 and 10 µg) as shown in Table 3. Among all the molecules, only AG-5 shown significant cell proliferation at the dose of 10 µg with a p < 0.01 (**). Ova/Alum was taken as positive control. Therefore, AG-5 was taken for detailed immune adjuvant activity.

Effect of AG-5 on OVA-specific IgG, IgG1 and IgG2a antibody titre

The OVA-specific IgG, IgG1, and IgG2a antibody titers in the serum were measured by indirect Elisa as shown in Fig 10 (A)-(C). The serum IgG titer in OVA-immunized mice was significantly enhanced by AG-5 at a dose of 100 µg with p < 0.01(**) when compared with OVA only treated group.

AG-5 at a dose 100 µg also significantly enhanced the serum IgG1 titers in OVA immunized mice with p < 0.01(**). Significant enhancements in OVA-specific serum IgG2a titers were also observed with AG-5 immunized mice at a dose of 100 µg with P < 0.01(**) as compared with OVA only treated group. These findings indicated that AG-5 significantly enhanced serum OVA-specific antibody production in mice immunized with OVA.

Estimation of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines in serum
The effect of AG-5 at a dose range of 1, 10 and 100 µg on the release of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines in serum were shown in Fig 11. Results indicate that compound AG-5 significantly stimulated Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokine release in a dose-related manner. AG-5 enhanced the Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines at a dose of 100 µg with P < 0.01(**). AG-5 has been found to enhance both Th1 and Th2 type of immune response.

**Immunophenotyping of lymphocytes by flow cytometry**

The overall percentage of T-cell subsets CD4 and CD8 in the splenic lymphocyte population was estimated in the presence of AG-5 (Fig 12). The effects of AG-5 treated subpopulations were dose dependently increased compared to untreated control. The presence of AG-5 significantly enhanced the percentage of T-cell populations at the dose of 100 µg.

**Discussion:** In the present study, we tried to correlate the position of acyl group on iridoid glycosides and their immune responses. Agnuside and negundoside (Fig 9) vary from each other with respect to basic skeleton as well as position of acyl group. In agnuside, there is a p-hydroxybenzoyl group at C10 of algycan moiety and in case of negundoside there is p-hydroxybenzoyl group at the C2’ of glycan moiety. Furthermore, in agnuside acylated analogs, both the acyl groups are present at aglycan moiety and in case of negundoside acylated analogs, both the acyl group are present at glycan moiety. In order to evaluate the immune-adjuvant activity of agnuside and negundoside and their acylated analogs, splenocyte proliferation in ex vivo was evaluated. The results shown in Table 3 indicate that only AG-5 treated groups significantly enhanced the the OVA-stimulated splenocyte proliferation at a dose of 10 µg with P < 0.01 as compared to all other molecules. The above results demonstrate that the position of lipophillic substitution lead to varying degree of immune adjuvant activity. The splenocyte proliferation by AG-5 and careful investigation of structure of all the molecules revealed that the presence of acyl group at C6 position of aglycan moiety is crucial for immune-adjuvant activity. The position of acyl group is also very critical for immune-adjuvant activity as revealed by the splenocytes proliferation. Based on the preliminary studies, AG-5 was taken for detailed in vivo experiments.

In *in-vivo* experiments, AG-5 was tested in the presence of OVA with or without aluminum hydroxide in BALB/c mice. AG-5 strongly induces antigen-specific cellular and humoral immune responses shown in Fig 10-12. We determined the possible effect of AG-5
on humoral immune response by measuring OVA-specific IgG titre and neutralizing antibody titers (IgG1 and IgG2a). The OVA-specific serum IgG, IgG1 and IgG2a antibody titers in the OVA-immunized mice were shown in Fig 10. AG-5 significantly increased the production of IgG titre IgG1 and IgG2a titre in the OVA immunized mice at a dose of 100 µg with p < 0.01. The addition of AG-5 at a suitable dose to the OVA immunized group increased the potency of the soluble mediators of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) response when compared to the OVA only treated group. As shown in Fig 11, AG-5 significantly increased the production of Th2 (IL-4) and Th1 cytokines (IL-2 and IFN-γ) in the OVA immunized mice at a dose of 100 µg with p < 0.01(**). Furthermore, we evaluated the effect of AG-5 on the population of cell surface marker like CD4/CD8. As shown in Fig 12, the AG-5 significantly enhanced the population of CD4/CD8, but the population of CD4 increased more as compared to CD8. To sum up, the position of acyl group on agnuside is critical for immune adjuvant activity. Acylated analogs of agnuside AG-5 bearing palmitoyl group at C6 position significantly enhanced both Th2 responses (characterized by the production of IgG and IL-4) and Th1 response-(characterized by the production of IgG2a, IFN-γ and IL-2) against OVA in mice. Furthermore, these analogs also significantly enhanced the population of cell surface marker CD4/CD8. Thus, AG-5 is potent enhancers of antigen specific humoral and cell mediated immune responses but at a higher dose of 100 µg/ml.
Table 3: Effect of agnuside & negundoside and their analogs on OVA-stimulated cell proliferation ex-vivo

Splenocytes were isolated from immunized mice on day 15 and cultured with different concentration (0.01, 0.1, 1 and 10 µg) of agnuside & negundoside and their analogs along with OVA 10 µg/well for 72 h. Splenocyte proliferation was measured by the MTT method. The optical density was measured at 570 nm. The difference between the untreated and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
Fig 10: Effect of AG-5 on OVA-specific IgG, IgG1 and IgG2a antibody
Groups of ten Balb/C mice were immunized subcutaneously with OVA (100 µg) on days 1 and 15 along with AG-5 (1, 10 and 100 µg). Group of animals treated with Alum (200 µg) along with Ova served as positive control. Sera were collected on day 15 and 28 respectively after 1st immunization to observe the effect on IgG antibodies by ELISA. The values are presented as mean ± S.E. (n = 10). The difference between the ova only and drug treated groups is determined by Bonferroni multiple comparison test. * P < 0.05, **P < 0.01.
Fig 11: Effect of AG-5 on concentration of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines in mouse serum
The effect of AG-5 on the expression of IFN-γ, IL-2 and IL-4 were determined after 28 days as described in materials and methods. Cytokine estimation was done by ELSIA. Values are means ± S.E. of ten mice; *P < 0.05, **P < 0.01 when compared with ova only group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

Fig 12: Effect AG-5 on T cell sub population CD4 and CD8 in the spleen cells
Mice immunized with ova were given graded doses of AG-5. Splenocyte isolated after 28 days from the AG-5 treated and untreated mice were analyzed by flow cytometry for the expression of various T cell sub populations. A typical bivariate analysis of splenocytes for the expression of cell surface markers and the percent gated population corresponding to each phenotype marker CD4 and CD8 are shown. Cells incubated with anti-mouse FITC-labeled CD8 and PE conjugated CD4 monoclonal antibodies for 30 minutes, were washed with PBS before FACScan analysis. Data are representative of one of the two experiments.
2.1.3. Synthesis of acyl donors:

1. Synthesis of p-nitrophenylalkanoate

Various acyl donors used for the trans-esterification were prepared in the laboratory following literature methods (Neises and Steiglich 1990). Lipophilic chain containing esters such as p-nitrophenylethanoate, butanoate, octanoate, decanoate, undecanoate and palmitate (C-2, C-4, C-8, C-10, C-12 and C-16) were prepared by the coupling of their fatty acid with p-nitrophenol in the presence of DCC and DMAP under dry conditions.

\[ \text{R} = \text{methyl, } n\text{-propyl, } n\text{-heptyl, } n\text{-nonyl, } n\text{-undecyl, } n\text{-pentadecyl} \]

Scheme 5: Synthesis of p-nitrophenylalkanoate

2. Synthesis of triethylene glycol donor

Triethylene glycol based donor 11 was prepared from triethylene glycol (Scheme 7). Compound 11a was synthesized by a known procedure (Kunz et al., 1984; Seitz and Kunz 1996). The reaction of 11a with perchloric acid/ acetic anhydride gave an intermediate 11b (90 %) which on coupling with p-nitrophenol (1 equivalent to 11b) in the presence of DCC (2 equivalent to 11b) and DMAP (catalytic amount) under dry conditions (Neises and Steiglich 1990) gave the crude product. The crude product on purification by column chromatography (silica gel, 60 – 120 mesh, eluent; n-hexane/EtOAc gradient) gave compound 11 as a yellow liquid in 65 % yield.

3. Synthesis of glyceric acid based donor

For the synthesis of glyceric acid based donor (12), ter-butyl acrylate (2 g, 15 mmol) was treated with aqueous KMnO₄ which resulted in the formation of 2,3-dihydroxy-propionic acid t-butyl ester 12a (45 %). Compound 12a obtained as above, on reaction with n-octanoic acid in the presence of DCC (4 equivalent to 12a), DMAP (catalytic amount) under dry conditions gave diacylated product 12b (90 %). Compound 10b on deprotection using TFA gave compound 12c, which on coupling with p-nitrophenol (1 equivalent to 12c) using the same procedure as described above (Neises and Steiglich 1990), gave crude product. Column
chromatographic purification of the crude product (silica gel, 60–120 mesh, eluent; n-hexane/EtOAc gradient) gave compound 12 as a yellowish semi-solid (1.68 g, 70%).

Scheme 6: Synthesis of triethylene glycol based acyl donor

Scheme 7: Synthesis of glyceric acid based acyl donor
Our literature survey revealed that, this is the first report where lipases were used for the regio-selective acylation on various types of iridoid glycosides using a bulkier acyl group through corresponding nitrophenyl esters as acyl transfer agents (labile esters).

2.1.4. Conclusion:-

Regio-selective acylation of these polyhydroxylated iridoid glycosides could be achieved using resin bound immobilized *Candida antarctica* lipase B in the presence of various acyl donors. Thus, lipase catalyzed acylations of picroside-II, agnuside and negundoside proceeded in good yields with various acyl donors. Resin bound immobilized *Candida antarctica* lipase B is selective towards both primary and secondary hydroxyl groups of sugar or aglycon moieties respectively, depending on the structure of the iridoid glycosides enabling the synthesis of regio-selectively lipidated iridoids which are otherwise difficult to access by conventional methods. Immunological screening results revealed that the structure as well as position of lipophilic chain on iridoid glycosides is critical for immune adjuvant activity. Among all the derivatives of picroside-II, picroside-II derivatives bearing octanoyl and palmitoyl group at C6’ position *viz.*, PK-II-3 and PK-II-4 significantly enhanced both Th2 responses (characterized by the production of IgG1 and IL-4) and Th1 response-(characterized by the production of IgG2a, IFN-γ and IL-2) against OVA in mice. Furthermore, these analogs also significantly enhanced the population of cell surface marker CD4/CD8. Among acylated analogs of agnuside and negundoside, AG-5 bearing palmitoyl group at position C6 also significantly enhanced the Th2 and Th1 response against OVA but at a higher dose compared to PK-II-3 and PK-II-4. Thus, PK-II-3 and PK-II-4 and AG-5 are potent enhancers of antigen specific humoral and cell mediated immune responses, thus showing promise for possible application as immune adjuvant for vaccines against intracellular infectious agents such as viruses, bacteria, protozoa and against cancer. Further immunization and challenge studies with these test compounds adjuvanted with other specific antigens can be performed to establish clinically relevant proof of principle. Derivatives of picrosides are well tolerated in mice, and are of no toxicological consequences and may find useful application clinically.
2.1.5. Experimental section: - Synthesis

Lipase from Candida rugosa (1,410 U/mg solid), porcine pancreas type II (100 - 400 units/mg protein, using olive oil), lipase acrylic resin from Candida antarctica (lipase B, ≥ 10,000 U/g, recombinant) and lipase B Candida antarctica, recombinant from Aspergillus oryzae (~ 9 unit/mg) were purchased from Sigma and Candida cylindracea (2.8 U/mg) from Fluka. All the commercial enzymes were used as such. Enzymatic reactions were carried out on IKA digital shaker at room temperature and 120 rpm. Melting points were recorded on Buchi melting point apparatus D-545; IR spectra (KBr discs) were recorded on Bruker Vector 22 instrument. NMR spectra were recorded at 200 MHz and 500 MHz on Bruker DPX200 instrument in CD$_3$OD/DMSO/CDCl$_3$ with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values were mentioned in δ (ppm) and coupling constants were given in Hz. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument. The progress of all reactions was monitored by TLC (2 x 5 cm pre-coated silica gel 60 F254 plates of thickness of 0.25 mm) and was visualized under UV 254 - 366 nm and iodine.

A. Synthesis of mono- and di-acylated derivatives of picroside-II using conventional DCC/ DMAP method

In a typical procedure, to a solution of picroside-II (0.512 g, 1 mmol) in dry THF (10 ml) was added fatty acid (octanoic acid or palmitic acid, 4 mmol), DCC (5 mmol), DMAP (10 mg) and stirring was continued at ambient temperature for 24 h. The reaction was monitored using T.L.C. After the completion of reaction, solvent was evaporated in vacuo and the crude product was subjected for column chromatography (silica gel, 100-200 mesh, elution; n-chloroform/MeOH gradient) to afford pure mono and di acylated products. The pure products were characterized on the basis of IR, $^1$H NMR, $^{13}$C NMR, DEPT and mass spectrometry as given below.
Compound characterization:

1. 6''-O-octanoyl-picoside II (PK-II-R1): \([\alpha]_D^{25} -86.0\) (c 1.0, CH$_3$OH).

\[\text{H NMR (CDCl}_3, 500\,\text{MHz)}: \delta 0.98\,\text{(m, 3H, H-8''')}, 1.39\,\text{(m, 8H, H-7''' to H-4'''}, 1.70\,\text{(m, 2H, H-3''')}, 2.56\,\text{(m, 4H, H-2'''), H-5, H-9), 3.10-3.45\,\text{(m, 3H, signal obscured with solvent peak, H-4', H-2', H-3'), 3.51-3.90\,\text{(m, 5H, H-5', H-7, H-10, H-6'), 3.95\,\text{(s, 3H), 4.20\,\text{(d, 1H, J = 13.34, H-10), 4.60-4.91\,\text{(m, 2H, signal obscured with solvent peak, H-1', H-1), 5.20\,\text{(m, 2H, H-4, H-6), 6.35\,\text{(d, 1H, J = 5.4, H-3), 7.19\,\text{(d, 1H, J = 8.8, H-5''), 7.69\,\text{(s, 1H, H-6''}, 7.70\,\text{(br s, 1H, H-2'').}}}

\[\text{C NMR (CDCl}_3, 125\,\text{MHz)}: \delta 14.4, 23.7, 26.0, 30.0, 30.1, 32.9, 34.7, 36.8, 43.2, 56.6, 60.2, 61.2, 63.0, 66.8, 71.8, 74.9, 77.7, 78.7, 82.3, 95.1, 99.7, 102.8, 114.4, 116.0, 123.8, 124.1, 142.5, 145.8, 153.2, 167.8, 173.4.

\[\text{IR (KBr, cm}^{-1})\): 1027, 1037, 1069, 1106, 1221, 1284, 1631, 1655, 1705, 2856, 2924.

\[\text{MS (EI, 70eV)}\): 661 (M$^+$ + Na).

\[\text{Elemental analysis calcd. for C}_{31}\text{H}_{42}\text{O}_{14}, \text{C} = 58.30\%, \text{H} = 6.63\%. \text{Found C} = 58.16\%, \text{H} = 6.56\%.

2. 6'-O-octanoyl-picoside II (PK-II-R2): \([\alpha]_D^{25} -80.0\) (c 1.0, CH$_3$OH).

\[\text{H NMR (CDCl}_3, 500\,\text{MHz)}: \delta 0.85\,\text{(t, 3H, J = 6.8, H-8'''), 1.29\,\text{(m, 8H, H-7''' to H-4'''}, 1.63\,\text{(m, 2H, H-3''')}, 2.36\,\text{(t, 2H, J = 7.5, H-2'''), 2.67\,\text{(m, 2H, H-5, H-9), 3.31\,\text{(m, 1H, signal}}}

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obscured with solvent peak, H-4’), 3.36 (d, 1H, J = 9.4, H-2’), 3.42 (d, 1H, J = 9.4, H-3’), 3.51 (m, 1H, H-5’), 3.74 (br s, 1H, H-7), 3.78 (d, 1H, J = 13.1, H-10), 3.91 (s, 3H), 4.19 (d, 1H, J = 13.1, H-10), 4.31 (dd, 1H, J = 11.9, 6.1, H-6’), 4.43 (dd, 1H, J = 11.7, 1.8, H-6’), 4.79 (d, 1H, J = 7.9, H-1’), 4.92 (d, 1H, signal obscured with solvent peak, H-1), 5.02 (dd, 1H, J = 5.8, 3.8, H-4), 5.05 (d, 1H, J = 6.9, H-6), 6.39 (d, 1H, J = 5.7, H-3), 6.87 (d, 1H, J = 8.3, H-5’), 7.57 (br s, 1H, H-2”), 7.59 (dd, 1H, J = 9.2, 1.6, H-6”).

$^{13}$C NMR (CDCl$_3$, 125 MHz): $\delta$ 14.4, 23.6, 26.2, 30.1, 30.2, 32.8, 35.1, 36.7, 43.1, 56.5, 60.1, 61.4, 64.2, 66.8, 71.6, 74.7, 75.9, 77.5, 81.7, 95.3, 99.8, 103.1, 113.6, 116.0, 122.0, 125.3, 142.5, 148.8, 153.2, 167.8, 175.4.

IR (KBr, cm$^{-1}$): 1027, 1036, 1068, 1106, 1220, 1284, 1631, 1655, 1707, 2856, 2924.

MS (El, 70eV): 661 (M$^+$ + Na).

Elemental analysis calcd. for C$_{31}$H$_{42}$O$_{14}$, C = 58.30%, H = 6.63%. Found C = 58.16%, H = 6.56%.

3. $6',6''$-di-O-octanoyl-picroside II (PK-II-R3): $[\alpha]_D^{25}$ = -92.0 (c 1.0, CH$_3$OH).

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 0.98 (m, 6H, H-8’’, H-8’’’), 1.30 (m, 16H, H-7’’’ to H-4’’’, H-7’’’’ to H-4’’’’), 1.71 (m, 4H, H-3’’’, H-3’’’’), 2.35 (m, 2H, H-2’’’’), 2.65 (m, 4H, H-5, H-9, H-2’’’’), 3.31-3.48 (m, 3H, signal obscured with solvent peak, H-4’, H-2’, H-3’), 3.60-3.87 (m, 3H, H-5’, H-7, H-10), 3.93 (s, 3H), 4.19 (d, 1H, J = 13.2, H-10), 4.27 (d, 1H, J = 11.9, H-6’), 4.46 (d, 1H, J = 11.7, H-6’), 4.79-4.92 (m, 2H, signal obscured with solvent peak, H-1, H-1’), 5.1 (m, 2H, H-4, H-6), 6.40 (d, 1H, J = 5.9, H-3), 7.19 (d, 1H, J = 8.7, H-5’’), 7.569 (s, 1H, H-6”), 7.71 (br s, 1H, H-2’”).
13C NMR (CDCl3, 125 MHz): δ 14.6, 23.8, 26.2, 26.4, 30.2, 30.3, 30.4, 30.5, 33.1, 34.9, 35.3, 36.7, 43.1, 56.8, 60.2, 61.4, 64.2, 67.1, 71.8, 75.0, 76.2, 77.8, 82.6, 95.5, 100.1, 103.2, 114.7, 124.0, 124.3, 129.8, 142.7, 145.9, 153.0, 167.3, 173.1, 175.5.

IR (KBr, cm⁻¹): 1023, 1065, 1106, 1240, 1285, 1631, 1655, 1708, 2860, 2920.

MS (EI, 70eV): 787 (M⁺ + Na).

Elemental analysis calcd. for C39H56O15, C = 61.24%, H = 7.38%. Found C = 62.08%, H = 7.48%.

4. 6''-O-palmitoyl-picroside II (PK-II-R4): [α]D²⁵ -89.0 (c 1.0, CH₃OH).

1H NMR (CDCl3, 200 MHz): δ 0.90 (m, 3H, H-16'''), 1.25 (m, 24H, H-15''' to H-4'''), 1.65 (m, 2H, H-3'''), 2.40 (m, 4H, H-2'''', H-5, H-9), 3.12-3.45 (m, 3H, signal obscured with solvent peak, H-4', H-2', H-3'), 3.51-3.89 (m, 5H, H-5', H-7, H-10, H-6'), 3.93 (s, 3H), 4.20 (d, 1H, J = 13.34, H-10), 4.60-4.90 (m, 2H, signal obscured with solvent peak, H-1', H-1), 5.20 (m, 2H, H-4, H-6), 6.35 (d, 1H, J = 5.4, H-3), 7.20 (d, 1H, J = 8.8, H-5'''), 7.69 (s, 1H, H-6'''), 7.70 (br s, 1H, H-2'''').

13C NMR (CDCl3, 125 MHz): δ 14.8, 24.1, 26.6, 30.2, 30.6, 31.2, 31.4, 33.5, 35.6, 37.1, 43.5, 56.6, 60.4, 61.2, 63.0, 66.8, 71.7, 74.9, 77.8, 78.9, 82.3, 95.1, 99.7, 102.8, 114.4, 116.0, 123.8, 124.1, 142.5, 145.8, 153.2, 167.8, 173.4.

IR (KBr, cm⁻¹): 1017, 1038, 1068, 1109, 1221, 1287, 1632, 1655, 1706, 2854, 2926.

MS (EI, 70eV): 773 (M⁺ + Na).

Elemental analysis calcd. for C39H58O14, C = 62.38%, H = 7.79%. Found C = 62.24%, H = 7.71%.
5. 6'-O-palmityl-picroside II (PK-II-R5): [α]D$^25$ -89.0 (c 1.0, CH₃OH).

$^1$H NMR (CDCl₃, 500 MHz): δ 0.91 (t, 3H, J = 6.7, H-16″″), 1.25 (m, 24H, H-15″″ to H-4″″), 1.62 (m, 2H, H-3″″), 2.38 (m, 2H, H-2″″), 2.70 (m, 2H, H-5, H-9), 3.31 (m, 1H, signal obscured with solvent peak, H-4′), 3.36 (d, 1H, J = 9.4, H-2′), 3.42 (d, 1H, J = 9.4, H-3′), 3.51 (m, 1H, H-5′), 3.74 (br s, 1H, H-7), 3.78 (d, 1H, J = 13.1, H-10), 3.91 (s, 3H), 4.19 (d, 1H, J = 13.1, H-10), 4.31 (dd, 1H, J = 11.9, 6.1, H-6′), 4.43 (dd, 1H, J = 11.7, 1.8, H-6″), 4.79 (d, 1H, J = 7.9, H-1′), 4.92 (d, 1H, signal obscured with solvent peak, H-1), 5.02 (dd, 1H, J = 5.8, 3.8, H-4), 5.05 (d, 1H, J = 6.9, H-6), 6.41 (d, 1H, J = 5.9, H-3), 6.88 (d, 1H, J = 8.1, H-5″″), 7.61 (br s, 1H, H-2″″), 7.63 (dd, 1H, J = 8.3, 1.6, H-6″″).

$^{13}$C NMR (CDCl₃, 125 MHz): δ 14.9, 24.1, 26.6, 30.7, 30.8, 31.0, 31.2, 33.5, 35.6, 37.1, 43.5, 55.2, 60.5, 61.9, 64.7, 67.2, 72.1, 75.1, 76.3, 77.9, 82.1, 95.7, 100.2, 103.5, 114.1, 116.4, 122.4, 125.8, 142.9, 149.2, 153.6, 168.2, 175.8.

IR (KBr, cm⁻¹): 1015, 1037, 1068, 1107, 1221, 1287, 1631, 1655, 1707, 2856, 2924.

MS (EI, 70eV): 773 (M⁺ + Na).

Elemental analysis calcd. for C₃₉H₅₈O₁₄, C = 62.38%, H = 7.79%. Found C = 62.24%, H = 7.71%.

6. 6′,6″-di-O-palmityl-picroside II (PK-II-R6): [α]D$^25$ -96.0 (c 1.0, CH₃OH)
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1\(^1\)H NMR (CDCl\(_3\), 200 MHz): \(\delta\) 0.91 (m, 6H, H-16'''', H-16'''''), 1.27 (m, 48H, H-15'''' to H-4''''', H-15'''' to H-4'''''), 1.65 (m, 4H, H-3'''', H-3'''''), 2.40 (m, 6H, H-2'''', H-2'''''', H-5, H-9), 3.12-3.46 (m, 3H, signal obscured with solvent peak, H-4', H-2', H-3'), 3.51-3.89 (m, 3H, H-7, H-10), 3.93 (s, 3H), 4.20 (d, 1H, \(J = 13.3\), H-10), 4.25 (d, 1H, \(J = 11.9\), H-6'), 4.45 (d, 1H, \(J = 11.6\), H-6'), 4.60-4.92 (m, 2H, signal obscured with solvent peak, H-1', H-1), 5.21 (m, 2H, H-4, H-6), 6.34 (d, 1H, \(J = 5.4\), H-3), 7.20 (d, 1H, \(J = 8.8\), H-5'''), 7.69 (s, 1H, H-6'''), 7.70 (br s, 1H, H-2''').

1\(^3\)C NMR (CDCl\(_3\), 125 MHz): \(\delta\) 14.9, 15.1, 24.1, 24.4, 26.6, 26.8 30.7, 30.5, 30.8, 31.0, 31.2, 31.8, 33.5, 33.4, 33.8, 34.9, 35.1, 35.2, 35.6, 37.1, 43.5, 56.8, 60.2, 61.4, 64.2, 67.1, 71.8, 75.0, 76.2, 77.8, 82.6, 95.5, 100.1, 103.2, 114.7, 123.8, 124.3, 130.1, 142.7, 145.9, 153.0, 167.3, 173.4, 175.9; IR (KBr, cm\(^{-1}\)): 1017, 1037, 1109, 1221, 1288, 1633, 1655, 1707, 2856, 2924.

**MS (EI, 70eV):** 1011 (M\(^+\) + Na).

**Elemental analysis calcld.** for C\(_{55}\)H\(_{88}\)O\(_{15}\), C = 66.77%, H = 8.97%. Found C = 66.98%, H = 9.01%.

**B. Synthesis of acyl donors**

**Synthesis of p-nitrophenyl alkanoate (10):**

To a solution of p-nitrophenol (10 mmol) and fatty acid (10 mmol) in dry THF was added DCC (15 mmol) and DMAP in catalytic amount and stirred over the period of 8 - 10 h. After completion of the reaction, as monitored by TLC, solvent was evaporated *in vacuo* and the crude was subjected for column chromatography (silica gel, 100 - 200 mesh, elution; \(n\)-hexane/ethyl acetate gradient) to afford pure \(p\)-nitrophenyl alkanoate in quantitative yield. The pure product was characterized on the basis of 1\(^1\)H NMR, and mass spectrometry.

a) **\(p\)-Nitrophenyl ethanoate:** Yield: 90 %.

1\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 2.1 (s, 3H), 7.32 (d, 2H, \(J = 7.8\) Hz), 8.20 (d, 2H, \(J = 7.9\) Hz).

**ESI-MS:** 181 (M\(^+\)).

b) **\(p\)-Nitrophenyl butanoate:** Yield: 89 %;

1\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 0.89 (t, 3H, \(J = 7.4\) Hz), 1.57 (m, 2H), 2.25 (t, 2H, \(J = 7.3\) Hz), 7.33 (d, 2H, \(J = 7.8\) Hz), 8.22 (d, 2H, \(J = 7.9\) Hz).

**ESI-MS:** 209 (M\(^+\)).
c) *p*-Nitrophenyl octanoate: Yield: 90%.

\[
^1H\text{ NMR (200 MHz, CDCl}_3\]: \delta 0.85 (t, 3H, \textit{J} = 6.7 Hz), 1.30 (m, 8H Hz), 1.63 (m, 2H Hz), 2.61 (t, 2H, \textit{J} = 7.3 Hz), 7.21 (d, 2H, \textit{J} = 9.1 Hz), 8.32 (d, 2H, \textit{J} = 9.1 Hz).
\]

ESI-MS: 265 (M').

d) *p*-Nitrophenyl decanoate: Yield: 87%.

\[
^1H\text{ NMR (200 MHz, CDCl}_3\]: \delta 0.86 (t, 3H, \textit{J} = 6.7 Hz), 1.30 (m, 12H Hz), 1.65 (m, 2H), 2.39 (t, 2H, \textit{J} = 7.5 Hz), 7.25 (d, 2H, \textit{J} = 7.6 Hz), 8.33 (d, 2H, \textit{J} = 7.7 Hz).
\]

ESI-MS: 293 (M').

e) *p*-Nitrophenyl dodecanoate: Yield: 85%.

\[
^1H\text{ NMR (200 MHz, CDCl}_3\]: \delta 0.90 (t, 3H, \textit{J} = 6.9 Hz), 1.35 (m, 16H), 1.66 (m, 2H), 2.40 (t, 2H, \textit{J} = 7.7 Hz), 7.20 (d, 2H, \textit{J} = 7.6 Hz), 8.30 (d, 2H, \textit{J} = 7.7 Hz).
\]

ESI-MS: 321 (M').

f) *p*-Nitrophenyl hexadecanoate: Yield: 82%.

\[
^1H\text{ NMR (200 MHz, CDCl}_3\]: \delta 0.90 (t, 3H, \textit{J} = 6.7 Hz), 1.25 (m, 24H), 1.62 (m, 2H), 2.38 (m, 2H), 7.21 (d, 2H, \textit{J} = 7.6 Hz), 8.22 (d, 2H, \textit{J} = 7.7 Hz).
\]

ESI-MS: 377 (M').

**Synthesis of triethylene glycol based donor (11)**

a) **Synthesis of acid (11b):**

To a solution of anhydrous triethylene glycol (12.8 ml, 0.094 mol) in 50 mL of THF were added 0.02 g (0.87 mmol) of sodium. When the sodium was dissolved, tert-butyl acrylate (4.8 ml, 0.33 mol) was added. The solution was stirred for 20 h and neutralized with 1 ml of 1 M HCl. After removal of the solvent, the residue was suspended in brine and extracted three times with ethyl acetate. The combined organic layers were washed with brine and dried over Na2SO4 before the solvent was removed. The resulting colorless oil was dried \textit{in vacuo} to give 7 g (85%) of 11a. The compound 11a was further treated with perchloric acid (500 µl)/acetic anhydride (25 ml) and stirred for 2 h. After the completion of reaction, reaction was quenched by adding ice cooled H2O. The aqueous layers were extracted with 50 ml of chloroform
followed by extracted twice with 80 ml of saturated NaHCO$_3$-solution. The organic layer on evaporation gave an oily residue. The residue after chromatography (using silica 60-120 mesh size, $n$-hexane:ethyl acetate gradients) gave 6.3 g (90 %) of 11b as light yellow oil.

b) Coupling of acid (11b) with $p$-nitrophenol:

\[
\begin{align*}
\text{To a solution of the acid } & 11b \ (6 \text{ g}) \text{ and } p\text{-nitrophenol } (3.8 \text{ g, 1.2 equivalent to } 11b) \text{ in} \\
25 \text{ ml of dry CH}_2\text{Cl}_2 & \text{ were added DCC (9.3 g, 2 equivalent to } 11b) \text{ and DMAP (50 mg) at } 0^\circ\text{C. After stirring for 30 min, the mixture was allowed to warm up to room temperature and was stirred for additional 3-4 h. The urea which precipitated upon cooling to } 0^\circ\text{C was filtered off. The solvent was removed, and the residue was treated with additional 20 ml of acetone to complete precipitation of urea. The filtrate was concentrated in vacuo and crude material on chromatography (using silica 60-120 mesh size, } n\text{-hexane:ethyl acetate gradients) gave } 3.8 \text{ g of } 11 \text{ as yellowish, highly viscous oil was obtained.}
\end{align*}
\]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 2.1 (s, 3H), 2.93 (t, 2H, $J$=6.1 Hz), 3.75 (m, 10H), 3.93 (m, 2H, $J$ = 6.1 Hz), 4.3 (m, 2H), 7.37 (d, 2H, $J$ = 7.1 Hz), 8.40 (d, 2H, $J$ = 7.1 Hz).

ESI-MS: 385 (M$^+\)).

Synthesis of glyceric acid based donor (12)

a) Synthesis of ter-butyl-2,3-dihydroxypropanoate (12a):

A solution of ter-butyl acrylate (10 g) in aqueous KMnO$_4$ (50 ml) was stirred for 2-3 h at room temperature. After completion of reaction, the compound was extracted with chloroform (3 x 50 ml). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated in vacuo. Chromatography (using silica 60-120 mesh size, $n$-hexane:ethyl acetate gradients) yielded 4.5 g (45 %) of a clear and highly viscous oil.

b) Synthesis of 2,3-bis(octanoyloxy)propanoic acid (12c):
To a solution of the *ter*-butyl-2, 3-dihydroxypropanoate (12a, 4 g) and *n*-octanoic acid (7.8 g, 2.2 equivalent to 12a) in 40 ml of dry CH$_2$Cl$_2$ were added DCC (15.5 g, 3 equivalent to 12a) and DMAP (30 mg) at 0 °C. After stirring for 30 min, the mixture was allowed to warm up to room temperature and was stirred for additional 3-4 h. The urea which precipitated upon cooling to 0 °C was filtered off. The solvent was removed, and the residue was treated with 20 ml of diethyl ether to complete precipitation of urea. The filtrate was concentrated *in vacuo*. The material was further treated with 20 % TFA in CH$_2$Cl$_2$ (20 ml) and stirred for 1 h. After completion of reaction, TFA was evaporated *in vacuo*, and the residue was dissolved in CH$_2$Cl$_2$ and washed with 1M HCl. The aqueous phase was re-extracted with chloroform. The combined organic layers were dried over Na$_2$SO$_4$ and concentrated *in vacuo*. Chromatography (using silica 60-120 mesh size, *n*-heaxne:ethyl acetate gradients) yielded 7 g (90 %) of 2,3-bis(octanoyloxy)propanoic acid.

$^1$H NMR (200 MHz, CDCl$_3$): δ 0.97 (m, 6H), 1.47 (m, 16H), 1.75 (m, 4H), 2.49 (m, 4H), 4.50 (m, 2H), 5.25 (m, 1H).

ESI-MS: 358 (M$^+$).

c) Synthesis of *p*-nitrophenyl-2,3-bis(octanoyloxy)propanoate (12):

To a solution of the carboxylic acid 12c (5 g) and *p*-nitrophenol (2.3 g, 1.2 equivalent to 12c) in 25 ml of dry CH$_2$Cl$_2$ were added DCC (5.8 g, 12 equivalent to 12c) and DMAP (50 mg) at 0 °C. After stirring for 30 min, the mixture was allowed to warm up to room temperature and was stirred for additional 3-4 h. The urea which precipitated upon cooling to 0 °C was filtered off. The solvent was removed, and the residue was treated with 20 ml of
acetone to complete precipitation of urea. The filtrate was concentrated in vacuo and crude material on chromatography (using silica 60-120 mesh size, n-hexane:ethyl acetate gradients) gave 4.5 g of 12 as yellowish viscous material.

**1H NMR (200MHz, CDCl₃):** δ 0.98 (m, 6H), 1.47 (m, 16H), 1.65 (m, 4H), 2.45 (m, 4H), 4.49 (m, 2H), 5.45 (m, 1H), 7.37 (d, 2H, J = 7.1 Hz), 8.40 (d, 2H, J = 7.1 Hz).

**ESI-MS:** 479 (M⁺).

### C.Synthesis of mono acylated analogs of picroside-II using enzymatic method

**General Procedure:**

Substrate (Picroside-II or agnuside or negundoside, 0.1 mmol) was dissolved in dry organic solvent (THF/DMF) in the presence of the lipase (*Candida antarctica* lipase B, 20 % weight equivalent), an acyl donor (p-nitrophenylalkanoate, 0.2 mmol), pre-activated molecular sieves (4 Å) and left for shaking on digital shaker at 120 rpm. The reaction was monitored by TLC and terminated (by filtering off the lipase) after the highest conversion was achieved. The acylated product was purified by flash chromatography and the structure was determined by MS and NMR spectra (¹H, ¹³C, and by comparison with spectra of their respective substrate).

**Compound characterization:**

1. **6'-O-Acetyl-picroside-II (PK-II-1):** [α]D²⁵⁻⁰６·⁶ (c 0.1, CH₃OH).

**1H NMR** (CDCl₃, 500 MHz): δ 2.01 (s, 3H, H-2”), 2.66 (m, 2H, H-5, H-9), 3.30 (m, 1H, signal obscured with solvent peak, H-4’), 3.34 (d, 1H, J = 9.5, H-2’), 3.41 (d, 1H, J = 9.0, H-3’), 3.53 (m, 1H, H-5’), 3.71 (br s, 1H, H-7), 3.77 (d, 1H, J = 13.1, H-10), 3.89 (s, 3H), 4.18 (d, 1H, J = 13.1, H-10), 4.32 (dd, 1H, J = 11.9, 6.2, H-6’), 4.41 (dd, 1H, J = 11.7, 1.9, H-6’), 4.78 (d, 1H, J = 7.9, H-1’), 4.91 (d, 1H, signal obscured in solvent peak, H-1), 5.00 (dd, 1H, J
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= 5.7, 2.2, H-4), 5.04 (d, 1H, J = 6.9, H-6), 6.39 (d, 1H, J = 5.6, H-3), 6.87 (d, 1H, J = 8.3, H-5’), 7.57 (br s, 1H, H-2’’), 7.59 (dd, 1H, J = 8.3, 1.9, H-6’’).

$^{13}$C NMR (CDCl$_3$, 125 MHz): δ 19.4, 35.3, 41.7, 55.0, 58.7, 60.0, 63.5, 65.3, 70.2, 73.3, 74.5, 76.1, 80.3, 93.8, 98.4, 101.6, 112.2, 114.6, 120.6, 123.9, 141.0, 147.4, 151.8, 166.5, 173.8; IR (KBr, cm$^{-1}$): 1015, 1038, 1070, 1104, 1221, 1289, 1629, 1655, 1707, 2924.

MS (EI, 70eV): 577 (M$^+$ + Na).

Elemental analysis calc'd. for C25H30O14, C = 54.15%, H = 5.45%. Found C = 54.01%, H = 5.62%.

2. 6’-O-Butanoyl-picroside-II (PK-II-2): $[\alpha]_{D}^{25}$ -93.0 (c 0.8, CH$_3$OH).

$^1$H NMR (CDCl$_3$, 500 MHz): δ 0.89 (t, 3H, J = 7.4, H-4’’’), 1.57 (m, 2H, H-3’’’), 2.25 (t, 2H, J = 7.3, H-2’’’), 2.57 (m, 2H, H-5, H-9), 3.21 (m, 1H, Signal obscured with solvent peak, H-4’), 3.26 (d, 1H, J = 9.5, H-2’), 3.31 (d, 1H, J = 9.0, H-3’), 3.45 (m, 1H, H-5’), 3.63 (br s, 1H, H-7), 3.67 (d, 1H, J = 13.1, H-10), 3.81 (s, 3H), 4.08 (d, 1H, J = 13.1, H-10), 4.31 (dd, 1H, J = 11.9, 6.2, H-6’), 4.43 (dd, 1H, J = 11.9, 1.9, H-6’), 4.72 (d, 1H, J = 7.9, H-1’), 4.82 (d, 1H, Signal obscured with solvent peak, H-1), 4.92 (dd, 1H, J = 5.7, 2.2, H-4), 5.09 (d, 1H, J = 6.9, H-6), 6.28 (d, 1H, J = 5.6, H-3), 6.76 (d, 1H, J = 8.3, H-5’’), 7.47 (br s, 1H, H-2’’), 7.49 (dd, 1H, J = 8.3, 1.9, H-6’’).

$^{13}$C NMR (CDCl$_3$, 125 MHz): δ 12.6, 18.2, 35.3, 35.6, 41.7, 55.0, 58.7, 60.0, 62.8, 65.3, 70.2, 73.3, 74.5, 76.1, 80.3, 93.8, 98.4, 101.6, 112.2, 114.6, 120.6, 123.9, 141.0, 147.4, 151.8, 166.5, 173.8.

IR (KBr, cm$^{-1}$): 1017, 1036, 1068, 1104, 1221, 1287, 1631, 1655, 1707, 2856, 2924.

MS (EI, 70eV): 605 (M$^+$ + Na).

Elemental analysis calc'd. for C$_{27}$H$_{34}$O$_{14}$, C = 55.67%, H = 5.88%. Found C = 55.56%, H = 5.83%.
3. 6'-O-Octanoyl-picroside-II (PK-II-3): $[\alpha]_D^{25} - 80.0$ (c 1.0, CH$_3$OH).

$^1$H NMR, $^{13}$C NMR, MS are discussed above in PK-II-R2

4. 6'-O-Decanoyl-picroside-II (PK-II-4): $[\alpha]_D^{25} - 77.0$ (c 1.0, CH$_3$OH).

$^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 0.91 (t, 3H, $J = 6.7$, H-16''), 1.25 (m, 12H, H-10'' to H-4''), 1.63 (m, 2H, H-3''), 2.40 (m, 2H, H-2''), 2.72 (m, 2H, H-5, H-9), 3.32 (m, 1H, signal obscured with solvent peak, H-4'), 3.36 (d, 1H, $J = 9.3$, H-2'), 3.42 (d, 1H, $J = 9.8$, H-3'), 3.50 (m, 1H, H-5'), 3.75 (br s, 1H, H-7), 3.80 (d, 1H, $J = 13.2$, H-10), 3.92 (s, 3H), 4.19 (d, 1H, $J = 13.3$, H-10), 4.31 (dd, 1H, $J = 11.9$, 6.1, H-6'), 4.44 (dd, 1H, $J = 11.6$, 1.8, H-6'), 4.80 (d, 1H, $J = 7.9$, H-1'), 4.92 (d, 1H, signal obscured with solvent peak, H-1), 5.02 (dd, 1H, $J = 5.8$, 3.8, H-4), 5.07 (d, 1H, $J = 6.9$, H-6), 6.41 (d, 1H, $J = 5.9$, H-3), 6.88 (d, 1H, $J = 8.1$, H-5''), 7.61 (br s, 1H, H-2''), 7.63 (dd, 1H, $J = 8.2$, 1.6, H-6'').

$^{13}$C NMR (CDCl$_3$, 125 MHz): $\delta$ 14.5, 23.6, 23.9, 26.2, 30.1, 30.2, 30.8, 32.3, 35.2, 36.8, 43.4, 55.2, 60.5, 61.9, 64.7, 67.2, 72.1, 75.1, 76.3, 77.9, 82.1, 95.7, 101.2, 103.5, 114.1, 116.5, 122.4, 125.8, 142.9, 149.2, 153.6, 168.2, 175.9.

IR (KBr, cm$^{-1}$): 1017, 1038, 1068, 1108, 1221, 1287, 1361, 1655, 1707, 2856, 2925

MS (EI, 70eV): 689 (M$^+$ + Na).
Elemental analysis calcd. for C_{33}H_{46}O_{14}, C = 59.45%, H = 6.95%. Found C = 60.04%, H = 7.11%.

5. 6’-O-Dodecanoyl-picroside-II (PK-II-5): [α]_{D}^{25} -75.0 (c 1.0, CH_{3}OH).

{\textbf{H NMR}} (CDCl_{3}, 500 MHz): δ 0.92 (t, 3H, J = 6.1, H-12’’’), 1.24 (m, 16H, H-12’’ to H-4’’’), 1.61 (m, 2H, H-3’’’), 2.39 (m, 2H, H-2’’’), 2.70 (m, 2H, H-5, H-9), 3.31 (m, 1H, signal obscured with solvent peak, H-4’), 3.39 (d, 1H, J = 9.1, H-2’), 3.41 (d, 1H, J = 9.1, H-3’), 3.51 (m, 1H, H-5’), 3.73 (br s, 1H, H-7), 3.78 (d, 1H, J = 13.1, H-10), 3.91 (s, 3H), 4.19 (d, 1H, J = 13.1, H-10), 4.30 (dd, 1H, J = 11.9, 5.9, H-6’), 4.43 (dd, 1H, J = 11.7, 1.7, H-6’), 4.79 (d, 1H, J = 7.9, H-1’), 4.92 (d, 1H, signal obscured with solvent peak, H-1), 5.02 (dd, 1H, J = 5.6, 3.5, H-4), 5.05 (d, 1H, J = 6.9, H-6), 6.41 (d, 1H, J = 5.9, H-3), 6.88 (d, 1H, J = 8.1, H-5’’’), 7.61 (br s, 1H, H-2’’’), 7.64 (dd, 1H, J = 8.3, 1.6, H-6’’’).

{\textbf{C NMR}} (CDCl_{3}, 125 MHz): δ 14.5, 23.6, 23.9, 26.2, 27.4, 30.1, 30.2, 30.8, 32.0, 32.3, 35.2, 36.8, 43.4, 55.2, 60.5, 61.9, 64.6, 67.4, 72.1, 75.3, 76.3, 77.9, 82.4, 95.7, 101.2, 103.5, 114.1, 116.4, 122.5, 125.8, 142.9, 149.2, 153.6, 168.2, 175.4.

{\textbf{IR (KBr, cm}^{-1})): 1028, 1036, 1068, 1107, 1220, 1285, 1631, 1655, 1707, 2856, 2926.

{\textbf{MS (EI, 70eV)}}: 717 (M^{+} + Na).

Elemental analysis calcd. for C_{35}H_{50}O_{14}, C = 60.51%, H = 7.25%. Found C = 60.88%, H = 7.71%.
6. **6′-O-Palmitoyl-picroside-II (PK-II-6):** \([\alpha]_D^{25} -89.0\) (c 1.0, CH$_3$OH).

![Image](image-url)

$^1$H NMR, $^{13}$C NMR, MS are discussed above in **PK-II-R5**

7. **6′-O-[3-{2-[2-(Acetox-y-ethoxy)-ethoxy]-ethoxy]-propanoyl]-picroside-II (PK-II-7):**

\([\alpha]_D^{25} -78.0\) (c 0.5, CH$_3$OH).

![Image](image-url)

$^1$H NMR (CDCl$_3$, 500 MHz): \(\delta\) 2.00 (s, 3H, H-15″′), 2.58 (m, 4H, H-5, H-9, H-12″′), 3.31 (m, 1H, signal obscured with solvent peak, H-4′), 3.36 (d, 1H, \(J = 9.4\), H-2′), 3.42 (d, 1H, \(J = 9.4\), H-3′), 3.51 (m, 1H, H-5′), 3.74 (m, 11H, H-7, H-5′′′, H-6″, H-8′′′, H-9′′′, H-11″′), 3.78 (d, 1H, \(J = 13.1\), H-10), 3.91 (s, 3H), 3.99 (t, 2H, \(J = 6.2\), H-2″′) 4.19 (m, 3H, H-10, H-3″′), 4.31 (dd, 1H, \(J = 11.9\), 6.1, H-6′), 4.43 (dd, 1H, \(J = 11.7\), 1.8, H-6′), 4.79 (d, 1H, \(J = 7.9\), H-1′), 4.92 (d, 1H, signal obscured with solvent peak, H-1), 5.00 (dd, 1H, \(J = 5.8\), 3.8, H-4), 5.03 (d, 1H, \(J = 6.9\), H-6), 6.27 (d, 1H, \(J = 5.9\), H-3), 6.83 (d, 1H, \(J = 8.3\), H-5″′), 7.47 (s, 1H, H-2″′), 7.57 (dd, 1H, \(J = 8.3\), 1.6, H-6″′).

**IR (KBr, cm$^{-1}$):** 1007, 1035, 1104, 1221, 1631, 1657, 1707, 2856, 2924.

**MS (EI, 70eV):** 781 (M$^+$ + Na).

**Elemental analysis calc.** for C$_{34}$H$_{46}$O$_{19}$, C = 53.82%, H = 6.11%. Found C = 53.58%, H = 6.08%.
8. 6′-O-(2′′′,3′′′′Di-octanoylpropanoyl)-picroside-II (PK-II-8): [α]D 25 -100.8 (c 1.0, CH₃OH).

1H NMR (CDCl₃, 500 MHz): δ 0.77 (t, 6H, J = 4.4, H-8′′′, H-8′′′′), 1.20 (m, 16H, H-7′′′′ to H-4′′′′, H-7′′′′ to H-4′′′′), 1.52 (m, 4H, H-3′′′′, H-3′′′′), 2.26 (m, 4H, H-2′′′′, H-2′′′′), 2.57 (m, 2H, H-5, H-9), 3.21 (m, 1H, signal obscured with solvent peak, H-4′), 3.26 (d, 1H, J = 9.5, H-2′), 3.31 (d, 1H, J = 9.0, H-3′), 3.45 (m, 1H, H-5′), 3.63 (br s, 1H), 3.67 (d, 1H, J = 13.1, H-10), 3.81 (s, 3H), 4.08 (d, 1H, J = 13.1, H-10), 4.20 (d, 2H, J = 5.3, H-3′′′), 4.23 (dd, 1H, J = 11.9, 6.2, H-6′), 4.35 (dd, 1H, J = 11.7, 1.9, H-6′), 4.72 (d, 1H, J = 7.9, H-1′), 4.82 (d, 1H, signal obscured with solvent peak, H-1), 4.92 (dd, 1H, J = 5.7, 2.2, H-4), 5.21 (d, 1H, J = 6.9, H-6), 5.23 (m, 1H, H-2′′′′), 6.29 (d, 1H, J = 5.7, H-3), 6.76 (d, 1H, J = 8.07, H-5′′′), 7.48 (s, 1H, H-2′′′), 7.52 (dd, 1H, J = 8.0, 1.6, H-6′′′).

13C NMR (CDCl₃, 125 MHz): δ 13.0, 22.2, 23.5, 24.6, 24.7, 28.6, 28.8, 31.4, 33.6, 35.3, 43.4, 55.1, 59.3, 61.6, 63.5, 65.7, 67.2, 73.1, 75.0, 76.4, 78.1, 80.0, 82.8, 93.9, 99.5, 103.0, 113.5, 117.0, 121.0, 124.1, 141.0, 148.3, 152.4, 166.1, 174.1, 176.2.

IR (KBr, cm⁻¹): 1017, 1036, 1068, 1104, 1221, 1287, 1631, 1655, 1707, 2856, 2924.

MS (EI, 70eV): 875 (M⁺ + Na).

Elemental analysis calcd. for C₄₂H₆₀O₁₈, C = 59.14%, H = 7.09%. Found C = 59.08%, H = 6.98%.

9. 6-O-Acetyl-agnuside (AG-1): [α]D 25 -85.0 (c 0.5, CH₃OH).
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$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 2.20 (s, 3H, $J = 7.3$, H-2""""), 2.82 (m, 1H, H-5), 2.99 (m, 1H, H-9), 3.24-3.45 (m, 4H, signal obscured with solvent peak H-2', H-4', H-5', H-3'), 3.54 (dd, 1H, $J = 11.7$, 5.6, H-6'), 3.77 (dd, 1H, $J = 11.7$, 1.9, H-6"'), 4.60 (d, 1H, $J = 7.8$, H-1''), 4.92 (m, 2H, signal obscured with solvent peak, H-10), 4.98 (m, 1H, H-1), 5.04 (d, 1H, $J = 6.3$, H-4), 5.28 (d, 1H, $J = 2.2$, H-6), 5.74 (m, 1H, H-7), 6.24 (dd, 1H, $J = 6.1$, 1.8, H-3), 6.74 (d, 2H, $J = 8.5$, H-3''', H-5'''), 7.81 (d, 2H, $J = 8.5$, H-2'', H-6'').

$^{13}$C NMR (CDCl$_3$, 125 MHz): $\delta$ 19.4, 41.2, 51.1, 61.4, 61.9, 70.4, 73.4, 76.5, 76.8, 83.5, 95.7, 99.6, 103.4, 114.9, 120.5, 126.6, 131.5, 140.6, 145.2, 162.3, 166.8, 173.8.

IR (KBr, cm$^{-1}$): 623, 1115, 1276, 1609, 1660, 1710, 2853, 2924, 3352.

MS (EI, 70eV): 531 (M$^+$ + Na).

Elemental analysis calcd. for C$_{24}$H$_{28}$O$_{12}$: C = 56.69%, H = 5.55%. Found C = 56.78%, H = 5.96%.

10. 6-O-Butanoyl-agnuside (AG-2): $[\alpha]_D^{25}$ -82.0 (c 0.5, CH$_3$OH).

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 0.81 (t, 3H, $J = 7.4$, H-4''), 1.50 (m, 2H, H-3'''), 2.20 (t, 2H, $J = 7.3$, H-2'''), 2.81 (m, 1H, H-5), 2.98 (m, 1H, H-9), 3.24-3.44 (m, 4H, signal obscured with solvent peak H-2', H-4', H-5', H-3'), 3.54 (dd, 1H, $J = 11.9$, 5.6, H-6'), 3.76 (dd, 1H, $J = 11.9$, 1.9, H-6"'), 4.59 (d, 1H, $J = 7.8$, H-1''), 4.92 (m, 2H, signal obscured with solvent peak, H-10), 4.98 (m, 1H, H-1), 5.04 (d, 1H, $J = 6.3$, H-4), 5.26 (d, 1H, $J = 2.0$, H-6), 5.74 (m, 1H, H-7), 6.23 (dd, 1H, $J = 6.1$, 1.8, H-3), 6.74 (d, 2H, $J = 8.7$, H-3''', H-5''''), 7.81 (d, 2H, $J = 8.7$, H-2'', H-6'').

$^{13}$C NMR (CDCl$_3$, 125 MHz): $\delta$ 12.4, 18.0, 35.6, 41.1, 49.1, 61.3, 61.9, 70.0, 73.4, 76.5, 76.8, 83.5, 95.6, 98.6, 103.4, 114.9, 120.5, 126.6, 131.5, 140.6, 144.9, 162.3, 166.3, 173.9; IR (KBr, cm$^{-1}$): 620, 1113, 1275, 1607, 1659, 1709, 2853, 2924, 3350.

MS (EI, 70eV): 559 (M$^+$ + Na).

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Elemental analysis calcd. for C_{26}H_{32}O_{12}, C = 58.20%, H = 6.01%. Found C = 58.14%, H = 5.96%.

11. **6-O-Octanoyl-agnuside (AG-3):** [α]_{D}^{25} -80.0 (c 0.5, CH_{3}OH):

![Chemical Structure](image)

^{1}H NMR (CDCl_{3}, 200 MHz): δ 0.86 (t, 3H, J = 6.7, H-8'''), 1.25 (m, 8H, H-7''' to H-4'''), 1.57 (m, 2H, H-3'''), 2.27 (t, 2H, J = 7.1, H-2'''), 2.94 (m, 1H, H-5), 3.09 (m, 1H, H-9), 3.23-3.44 (m, 4H, signal obscured with solvent peak H-2', H-4', H-5', H-3'), 3.64 (dd, 1H, J = 11.9, 5.9, H-6'), 3.85 (dd, 1H, J = 11.8, 1.9, H-6'), 4.68 (d, 1H, J = 8.0, H-1'), 4.92 (m, 2H, signal obscured with solvent peak, H-10), 5.00 (m, 1H, H-1), 5.11 (d, 1H, J = 6.3, H-4), 5.36 (d, 1H, J = 2.0, H-6), 5.83 (m, 1H, H-7), 6.33 (dd, 1H, J = 6.0, 1.8, H-3), 6.83 (d, 2H, J = 8.7, H-3'', H-5''), 7.91 (d, 2H, J = 8.7, H-2'', H-6'').

^{13}C NMR (CDCl_{3}, 125 MHz): δ 14.7, 20.1, 22.51, 26.4, 29.3, 31.7, 35.3, 41.8, 49.1, 61.8, 62.2, 71.1, 74.3, 76.2, 76.8, 83.6, 96.7, 98.5, 103.3, 114.8, 120.5, 126.4, 132.4, 141.2, 144.7, 162.2, 167.2, 174.0; IR (KBr, cm^{-1}): 618, 1113, 1275, 1607, 1659, 1707, 2853, 2924, 3353. MS (EI, 70eV): 615 (M^{+} + Na).

Elemental analysis calcd. for C_{30}H_{40}O_{12}, C = 60.80%, H = 6.80%. Found C = 60.91%, H = 6.86%.

12. **6-O-Decanoyl-agnuside (AG-4):** [α]_{D}^{25} -84.0 (c 0.5, CH_{3}OH):
1H NMR (CDCl₃, 200 MHz): δ 0.89 (t, 3H, J = 6.6, H-10’’’), 1.27 (m, 12H, H-9’’’ to H-4’’’), 1.58 (m, 2H, H-3’’’), 2.27 (t, 2H, J = 6.9, H-2’’’), 2.95 (m, 1H, H-5), 3.11 (m, 1H, H-9), 3.24-3.44 (m, 4H, signal obscured with solvent peak H-2’, H-4’, H-5’, H-3’), 3.64 (dd, 1H, J = 11.9, 5.8, H-6’’), 3.85 (dd, 1H, J = 11.9, 1.9, H-6’’), 4.68 (d, 1H, J = 7.9, H-1’’), 4.92 (m, 2H, signal obscured with solvent peak, H-10), 5.08 (m, 1H, H-1), 5.14 (d, 1H, J = 6.7, H-4), 5.36 (d, 1H, J = 2.0, H-6’), 5.83 (m, 1H, H-7), 6.33 (dd, 1H, J = 6.5, 1.8, H-3), 6.83 (d, 2H, J = 8.9, H-3’’’, H-5’’’), 7.91 (d, 2H, J = 8.9, H-2’’, H-6’’’);

13C NMR (CDCl₃, 50 MHz): δ 14.7, 20.1, 20.8, 22.51, 26.4, 27.2, 29.3, 31.7, 35.3, 41.8, 49.1, 61.9, 62.2, 71.1, 74.3, 76.2, 76.8, 83.6, 96.7, 98.5, 103.3, 114.8, 120.5, 126.4, 132.4, 141.2, 144.7, 162.2, 167.2, 174.0.

IR (KBr, cm⁻¹): 620, 1115, 1270, 1609, 1660, 1709, 2853, 2924, 335

4MS (EI, 70eV): 643 (M⁺ + Na)

Elemental analysis calcd. for C₃₂H₄₄O₁₂, C = 61.92%, H = 7.15%. Found C = 61.98%, H = 7.50%.

13. 6-O-Palmitoyl-agnuside (AG-5): [α]D₂₅ -90.0 (c 1.0, CH₃OH);

1H NMR (200 MHz, CD₃OD): δ 0.90 (t, 3H, J = 6.6, H-16’’’), 1.28 (m, 24H, H-15’’’ to H-4’’’), 1.58 (m, 2H, H-3’’’), 2.31 (t, 2H, J = 7.3, H-2’’’), 2.92-3.01 (m, 2H, H-5, H-9), 3.24-3.45 (m,
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4H, signal obscured with solvent peak H-2', H-4', H-5', H-3'), 3.64 (dd, 1H, J = 11.9, 5.6, H-6'), 3.85 (dd, 1H, J = 11.9, 1.9, H-6'), 4.67 (d, 1H, J = 7.9, H-1') 4.93 (m, 2H, signal obscured with solvent peak, H-10), 5.00 (m, 1H, H-1), 5.09 (d, 1H, J = 6.2, H-4), 5.37 (d, 1H, J = 1.9, H-6), 5.84 (m, 1H, H-7), 6.34 (dd, 1H, J = 6.0, 1.8, H-3), 6.84 (d, 2H, J = 8.7, H-3'', H-5''), 7.92 (d, 2H, J = 8.7, H-2'', H-6'').

\(^{13}\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 14.9, 24.1, 26.6, 30.7, 30.8, 31.0, 31.2, 33.5, 35.6, 37.1, 41.1, 49.8, 61.4, 61.9, 70.2, 73.4, 76.5, 77.1, 83.4, 95.6, 98.6, 103.4, 114.9, 120.5, 126.6, 131.5, 140.6, 144.9, 162.3, 166.3, 173.9.

IR (KBr, cm\(^{-1}\)): 619, 1100, 1277, 1609, 1659, 1707, 2853, 2923, 3355.

MS (ESI): 727 (M\(^{+}\) + Na).

Elemental analysis calcd. for C\(_{38}\)H\(_{56}\)O\(_{12}\), C = 64.75%, H = 8.01%. Found C = 64.58%, H = 7.96%.

14. 6'-O-Acetyl-negundoside (NG-1): \([\alpha]_D^{25}\) -98.0 (c 0.5, CH\(_3\)OH).

\(^1\)H NMR (500 MHz, CD\(_3\)OD): \(\delta\) 1.15 (s, 3H, H-10), 1.21 (m, 1H, H-6), 1.49 (m, 1H, H-6), 1.55 (m, 3H, H-3'', H-7), 2.01 (s, 3H), 2.10 (m, 2H, H-9, H-7), 2.92 (m, 1H, H-5), 3.37 (t, 1H, J = 9.4, H-3'), 3.54 (m, 1H, H-5'), 3.62 (t, 1H, J = 8.9, H-4'), 4.16 (dd, 1H, J = 11.9, 5.5, H-6'), 4.40 (dd, 1H, J = 11.9, 1.9, H-6'), 4.89 (d, 1H, J = 8.1, H-1'), 4.92 (d, 1H, signal obscured with solvent peak, H-2') 5.21 (d, 1H, J = 3.7, H-1), 6.72 (dd, 2H, J = 9.6, 2.6, H-3'', H-5''), 7.02 (s, 1H, H-3), 7.76 (dd, 2H, J = 11.4, 1.0, H-2'', H-6'').

\(^{13}\)C NMR (125 MHz, CD\(_3\)OD): \(\delta\) 19.9, 24.6, 30.7, 31.7, 40.5, 52.2, 64.2, 72.2, 74.9, 75.4, 75.8, 80.3, 95.3, 97.8, 113.8, 116.2, 122.2, 133.0, 151.2, 163.7, 167.4, 170.2, 175.1.

IR (KBr, cm\(^{-1}\)): 803, 1077, 1170, 1263, 1610, 1712, 2965.
MS (ESI): 538 (M^+).

Elemental analysis calcd. for C_{25}H_{30}O_{13}, C = 55.76%, H = 5.62%. Found C = 57.43%, H = 5.84%.

15. **6'-O-Butanoyl-neungudoside (NG-2):** \([\alpha]_D^{25} = -100.0 \text{ (c 0.5, CH}_3\text{OH)}\).

\[\text{\textsuperscript{1}H NMR (500 MHz, CD}_3\text{OD):} \delta 0.86 (t, 3H, J = 7.4, H-4'''), 1.15 (s, 3H, H-10), 1.21 (m, 1H, H-6), 1.49 (m, 1H, H-6), 1.55 (m, 3H, H-3''', H-7), 2.05 (m, 2H, H-9, H-7), 2.25 (t, 2H, J = 7.3, H-2'''), 2.90 (m, 1H, H-5), 3.38 (t, 1H, J = 9.4, H-3'), 3.53 (m, 1H, H-5'), 3.62 (t, 1H, J = 8.9, H-4'), 4.17 (dd, 1H, J = 11.9, 5.5, H-6'), 4.39 (dd, 1H, J = 11.9, 1.9, H-6'), 4.89 (d, 1H, J = 8.1, H-1'), 4.92 (d, 1H, signal obscured with solvent peak, H-2') 5.21 (d, 1H, J = 3.7, H-1), 6.72 (dd, 2H, J = 9.6, 2.6, H-3'', H-5''), 7.01 (s, 1H, H-3), 7.76 (dd, 2H, J = 11.4, 1.0, H-2'', H-6'').

\[\text{\textsuperscript{13}C NMR (125 MHz, CD}_3\text{OD):} \delta 14.1, 19.5, 24.6, 30.7, 31.7, 36.9, 40.9, 52.2, 64.2, 71.6, 74.8, 75.7, 75.8, 80.2, 95.3, 97.8, 113.8, 116.2, 122.1, 133.0, 151.2, 163.7, 167.3, 170.2, 175.3; \text{IR (KBr, cm}^{-1})\): 802, 1079, 1169, 1262, 1609, 1712, 2964.

MS (ESI): 566 (M^+).

Elemental analysis calcd. for C_{27}H_{34}O_{13}, C = 57.24%, H = 6.05%. Found C = 57.13%, H = 5.94%.

16. **6'-O-Octanoyl-neungudoside (NG-3):** \([\alpha]_D^{25} = -97.0 \text{ (c 1.0, CH}_3\text{OH)}\).
$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 0.88 (m, 3H, H-8‴′), 1.25 (m, 11H, H-10, H-7‴′′ to H-4‴′′), 1.29 (m, 1H, H-6), 1.47 (m, 1H, H-6), 1.63 (m, 3H, H-7, H-3‴′′), 2.04 (m, 2H, H-7, H-9), 2.23 (t, 2H, $J = 7.3$, H-2‴′′), 2.89 (m, 1H, H-5), 3.30 (t, 1H, $J = 9.5$, H-3′), 3.56 (m, 1H, H-5′), 3.62 (t, 1H, $J = 8.9$, H-4′), 4.26 (dd, 1H, $J = 11.8, 5.7$, H-6′), 4.42 (dd, 1H, $J = 11.9, 1.9$, H-6″), 4.88 (d, 1H, $J = 8.0$, H-1′), 4.92 (d, 1H, signal obscured with solvent peak, H-2′), 5.20 (d, 1H, $J = 3.7$, H-1), 6.78 (dd, 2H, $J = 7.6, 1.8$, H-3‴′, H-5‴′′), 7.07 (s, 1H, H-3), 7.83 (dd, 2H, $J = 7.2, 1.0$, H-2‴′, H-6‴″).

$^{13}$C NMR (125 MHz, CD$_3$OD): $\delta$ 13.0, 19.1, 22.0, 22.4, 25.5, 29.4, 29.6 29.4, 30.7, 36.1, 40.1, 50.0, 63.1, 69.3, 72.4, 74.9, 75.2, 79.2, 92.5, 96.6, 113.5, 115.6, 122.0, 130.9, 148.0, 161.8, 166.0, 167.9, 176.1.

IR (KBr, cm$^{-1}$): 800, 1077, 1169, 1262, 1609, 1715, 2960.

MS (ESI): 622 (M$^+$).

Elemental analysis calcd. for C$_{31}$H$_{42}$O$_{13}$, C = 59.80%, H = 6.80%. Found C = 59.86%, H = 6.68%.

17. $6′$-O-Palmitoyl-negundoside (NG-4): [$\alpha$]$_D^{25}$ -86.0 (c 0.8, CH$_3$OH):
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$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 0.78 (t, 3H, $J = 6.74$, H-16′′′′), 1.21 (m, 27H, H-10, H-15′′′′ to H-4′′′′), 1.47 (m, 2H, H-6), 1.63 (m, 3H, H-7, H-3′′′′′), 2.04 (m, 2H, H-7, H-9), 2.23 (t, 2H, $J = 7.3$, H-2′′′′), 2.89 (m, 1H, H-5), 3.20 (t, 1H, $J = 9.5$, H-3′), 3.56 (m, 1H, H-5′), 3.62 (t, 1H, $J = 8.9$, H-4′), 4.26 (dd, 1H, $J = 11.8$, 5.7, H-6′), 4.42 (dd, 1H, $J = 11.9$, 1.9, H-6′), 4.88 (d, 1H, $J = 8.0$, H-1′), 4.92 (d, 1H, signal obscured with solvent peak, H-2′), 5.17 (d, 1H, $J = 3.7$, H-1), 6.70 (dd, 2H, $J = 8.6$, 1.5, H-3′′, H-5′′′′), 7.01 (s, 1H, H-3), 7.75 (dd, 2H, $J = 8.6$, 1.6, H-2′′, H-6′′′′).

$^{13}$C NMR (125 MHz, CD$_3$OD): $\delta$ 13.1, 22.2, 23.2, 25.0, 29.3, 29.4, 32.5, 33.6, 35.0, 39.9, 51.1, 63.9, 70.9, 74.4, 75.0, 79.9, 89.0, 94.5, 96.7, 113.1, 115.0, 121.2, 132.5, 149.5, 162.5, 166.1, 168.1, 174.9.

MS (ESI): 734 (M$^+$).

IR (KBr, cm$^{-1}$): 803, 1075, 1168, 1262, 1610, 1715, 2960, 2970, 2982.

Elemental analysis calcd. for C$_{39}$H$_{58}$O$_{13}$, C = 63.74%, H = 7.96%. Found C = 63.58%, H = 7.37%.

18. **6′-O-(2′′′′, 3′′′′ Di-octanoylpropanoyl)-negundoside (NG-5):** $[\alpha]_D^{25}$ –42.5 (c 1.0, CH$_3$OH):

$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 0.78 (m, 6H, H-8′′′′′, H-8′′′′′), 1.20 (m, 19H, H-10, H-7′′′′′′ to H-4′′′′′, H-7′′′′′′ to H-4′′′′′), 1.29 (m, 1H, H-6), 1.46 (m, 1H, H-6), 1.63 (m, 5H, H-7, H-3′′′′′, H-3′′′′′′′), 2.04 (m, 2H, H-7, H-9), 2.30 (m, 4H, H-2′′′′′′, H-2′′′′′′′), 2.93 (m, 1H, H-5), 3.21 (t, 1H, $J = 9.5$, H-3′), 3.45 (m, 1H, H-5′), 3.56 (t, 1H, $J = 8.9$, H-4′), 4.20 (d, 2H, $J = 5.5$, H-3′′), 4.26 (dd, 1H, $J = 11.9$, 5.8, H-6′), 4.42 (dd, 1H, $J = 11.9$, 1.8, H-6′) 4.88 (d, 1H, $J = 8.0$, H-1′), 5.91 (d, 1H, signal obscured with solvent peak, H-2′), 5.21 (m, 1H, H-2′′′′′), 5.26 (d, 1H, $J = 3.7$, H-
1), 6.70 (dd, 2H, $J$ = 8.5, 1.6, H-3′′, H-5′′), 7.01 (s, 1H, H-3), 7.75 (dd, 2H, $J$ = 8.6, 1.6, H-2′′, H-6′′).

$^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 12.8, 13.0, 22.2, 24.5, 24.6, 28.7, 29.3, 31.4, 33.5, 33.6, 42.0, 50.0, 62.7, 64.1, 72.1, 73.8, 74.9, 75.2, 80.0, 82.7, 95.4, 98.9, 113.1, 117.9, 121.9, 133.2, 149.3, 162.1, 166.1, 168.5, 173.5, 173.7, 175.1.

IR (KBr, cm$^{-1}$): 805, 1077, 1169, 1262, 1609, 1715, 2960, 2970, 2982.

MS (ESI): 836 (M$^+$).

**Elemental analysis calc.** for C$_{42}$H$_{60}$O$_{17}$, C = 60.28%, H = 7.23%. Found C = 60.16%, H = 7.08%.

2.1.6. **Biological assays:-**

**Immunizations and treatment**

Male BALB/c mice were divided into twenty groups, each consisting of ten mice. Animals were immunized subcutaneously with OVA 100 µg alone dissolved in saline on day 1 and day 15. Alum (200 µg) along with OVA 100 µg has been used as positive control throughout the study. In case of *ex vivo* studies to identify the potential immunomodulator, splenocytes from immunized mice were isolated and treated with various concentrations (0.01, 0.1, 1 and 10 µg/ml) of PK-I, PK-II and catalpol and the derivatives of PK-II along with ovalbumin. For *in vivo* studies of the derivatives of PK-II, mice were treated with various concentrations (1, 10, 30 and 100 µg/ml) of PK-II-2, PK-II-3 and PK-II-4 along with OVA on the days of immunization. Saline-treated animals were included as untreated group. To estimate the expression of cytokine and immunoglobulins, serum samples were taken at various time periods. Effect on Th1/Th2 cytokine expression and T cell proliferation (CD4, CD8) was observed on 28 days after 1$^{st}$ immunization while immunoglobulin isotypes were estimated on day 15, 28 and 60 after 1$^{st}$ immunization to observe initial as well as memory recall response. Experiments were repeated two times.

**Splenocyte isolation and proliferation assay *ex vivo***

Spleen collected from the immunized BALB/c mice under aseptic conditions and suspended in complete medium RPMI containing 10% FCS for further use (Malik *et al.*, 2007). Splenocytes were seeded into 4–5 wells of a 96-well flat-bottom microtiter plate (Nunc) at 5 x 10$^6$ cell/ml in 100 µl complete medium, thereafter OVA (final concentration
10 µg/well), or RPMI-1640 medium were added giving a final volume of 200 µl. The plates were incubated at 37 °C in a humid atmosphere with 5 % CO₂. After 72 h, 20 µl of MTT solution (5 mg/ml) were 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 200 µl of a DMSO working solution (180 µl DMSO with 20 µl, 1 N HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

**Measurement of OVA-specific antibody titre**

OVA-specific IgG, IgG1 and IgG2a antibodies in serum were detected by an indirect ELISA (Sun et al., 2004). In brief, microtiter plate wells (Nunc) were coated with 100 µl OVA solution 50, 25 and 50 µg/ml for IgG, IgG1 and IgG2a antibodies, respectively in 50 mM carbonate-bicarbonate buffer, pH 9.6 for 24 h at 4 °C. The wells were washed three times with PBS containing 0.05 % (v/v) Tween 20 (PBS/Tween) and then blocked with 5 % FCS/PBS at 37 °C for 1 h. After three washings, 100 µl of diluted serum sample (IgG, 1: 400; IgG1, 1: 100; IgG2a, 1: 200) or 0.5 % FCS/PBS as control was added to triplicate wells. The plates were then incubated for 1 h at 37 °C, followed by three times of washing. Aliquots of 100 µl of rabbit anti-mouse IgG horseradish peroxidase conjugate diluted 1: 50,000, or goat anti-mouse IgG1 peroxidase conjugate 1: 16,000 or IgG2a peroxidase conjugate 1: 8000 with 0.5 % FCS/PBS were added to each plate. The plates were further incubated for 1 h at 37 °C. After washing, the peroxidase activity was assayed as following: 100 µl of substrate solution (10 mg of o-phenylenediamine and 37.5 µl of 30 % H₂O₂ in 25 ml of 0.1 M citrate-phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37 °C and enzyme reaction was terminated by adding 50 µl/well of 2 N H₂SO₄. The optical density (OD) was measured in an ELISA reader at 450 nm.

**Determination of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines by ELISA**

Serum was collected on 28 day after 1st immunization. The Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines were measured with an enzyme linked immunosorbent assay (R&D Systems Quantikine) according to the instructions of the manufacturer.

**Lymphocyte immunophenotyping in spleen**

The spleen (1/3 of the organ) was placed in PBS buffer (without Mg²⁺ and Ca²⁺) and stored on ice prior to preparation of single cell suspensions. Splenic erythrocytes were lysed with red blood cell lysing buffer (BD Pharmingen). For each sample, 2×10⁶ cells were stained
with conjugated anti-CD8 FITC (clone L3T4) and CD4 PE (clone Ly-2) antibodies. After staining with antibodies, 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) [Malik et al., 2007].

**Safety studies**

Safety of the proposed adjuvants with weak antigen OVA were proven by subcutaneous injection to mice. BALB/c mice were divided into groups of 10 animals each and were inoculated with variable doses of test compounds, which had been re-suspended in PBS. Untreated mice, received PBS. After injection, mice were observed daily for a period of four weeks. Deviations from their normal behavior were recorded.
References:-


