Modulatory effect of Cardiospermum halicacabum extracts on TLR-2 in PBMC cells

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ABSTRACT

Cardiospermum halicacabum leaves extract were investigated for their effect on expression of toll like receptor 2 (TLR-2) in Peripheral Blood Mononuclear Cells (PBMC) of healthy individual. Cell viability was not affected up to 30 µg of extract which was determined by tryphan blue dye exclusion method. TLR-2 mRNA expression was quantified by of Real time quantitative PCR technique. Ethanolic extract increased the TLR-2 expression by 2.7 fold whereas ethyl acetate extract reduced slightly. The results of the present study suggest that ethanolic extract of C. halicacabum contains lead compounds that could modulate the expression of toll like receptor which is normally expressed in peripheral blood mononuclear cells.

Keywords: Toll like receptor-2, Cardiospermum halicacabum, mRNA, PBMC

1. INTRODUCTION

Toll like receptors (TLRs) belongs to the family of pattern recognition receptors (PRR) [1]. These receptors can recognize the pathogen associated molecular components during infection. TLRs can also play negative roles in autoimmune disorders like rheumatoid arthritis where the expression of toll like receptor is up-regulated [2]. TLRs sense the pathogen associated molecular patterns (PAMPs) and up regulate the transcription of genes involved in inflammatory responses [3]. 11 members of TLRs have been identified of which TLR-1, 2, 4, 5 and 6 are localized to the cell surface and TLR-3, 7, and 8 are intracellularly located on the endosome compartment [4].

TLR2 designated as CD282 (cluster of differentiation 282) plays an important role in immune system. This particular gene is expressed most abundantly in peripheral blood leukocytes and mediates the host response to Gram positive bacteria via stimulation of NF-kB [5]. Lipoproteins, peptidoglycans, porins, lipoarrabinomannans and zymogens are the targets of TLR2 [6]. TLR2 has an important role in innate immune response against Bacillus anthracis due to which TNF alpha secretion was found to be mediated by TLR2 dependent pathway which is a therapeutic use [7]. Cardiospermum halicacabum belonging to Papindacae family grows all over the world especially in Asia and Africa where this plant is commonly known as balloon vine [8]. The leaves are recommended for rheumatism, chronic bronchitis, and stiffness of limbs [9]. Pharmacological validation and toxicological evaluation have proven its safety and not toxic nature of C. halicacabum (40 g/kg in rats). It suppressed the production of TNF alpha and nitric oxide in in vitro experiment with PMBC [10]. Antioxidant, Anti-inflammatory and anti-arthic activities of this herbal extract have already been proven [11]. With this prelude, the present investigation was carried out...
with the primary objective of evaluating the effect of C. halicacabum on TLR-2 expression in normal PBMC.

2. MATERIALS AND METHODS

2.1. Preparation of plant extracts

Cardiospermum halicacabum leaves were collected in and around Coimbatore city and authenticated by Botanical Survey of India, Coimbatore (BSI/SRC/S/23-12-11/CSH 123). Leaves were shade dried under room temperature and 70 g of dry leaf powder was subjected to successive extraction with ethyl acetate and ethanol solvents using soxhlet apparatus and lyophilized. Further, the samples were dissolved in Dimethyl sulfoxide (DMSO) to obtain final concentration of 1µg/µl.

2.2. Isolation of peripheral blood mononuclear cells and treatment

8 ml of blood was directly collected from a healthy individual via venipuncture into cell preparation tube (CPT) tube and centrifuged at 1800 g for 20 minutes at 18 ºC. After centrifugation, CPT tube was brought to a biological safety cabinet and carefully opened. The cells from the middle layer were gently transferred to another falcon tubes. Further centrifugation was carried out to obtain the cell pellet containing peripheral blood mononuclear cells (PBMC). Cells isolated by ficoll-hypaque method were cultured into 3 different flasks. 9 ml RPMI medium, 1 ml Fetal Calf Serum (FCS) (10%) and 50 µl antibiotics were added to the culture flasks. Peripheral blood mononuclear cells, at the concentration of 4.8 x 10⁶ cells, were introduced to each flask and kept in CO₂ incubator at 37°C under 5% CO₂ supply. Cells were allowed to grow for 72 hours. Three different culture flasks were taken for treating with Dimethyl sulfoxide (DMSO), ethyl acetate and ethanol extract respectively. Cells were introduced at the concentration of 1x10⁶ cells to the medium and treated with 30 µg of both ethyl acetate extract and ethanol extract of C. halicacabum. 30 µl of DMSO treated cells served as control as it was used as vehicle to dissolve the herbal extract. Culture flasks were kept in CO₂ incubator at 37°C under 5% CO₂ supply for 2 hours. After incubation was over, the cells were isolated from the medium.

2.3. Quantification of TLR-2 by Real-time PCR technique

Cells were lysed and RNA was extracted using the total RNA isolation kit (TRI reagent method) and purified by PrepEase RNA Spin Kit method (BioRad). Aliquot of RNA (65 ng) was reverse transcribed using virus-reverse transcriptase (RT) and oligo (dT) as primer (iScript select cDNA synthesis Kit-BioRad). The obtained cDNA was diluted and used for amplification. Parameter-specific primer were designed and purchased from Search-LC (Heidelberg, Germany). The details of primer have been furnished in Table 1. The PCR was performed with the CFX96 Real-Time system-SYBR Green-1 kit according to the protocol provided in the kits. A melting curve analysis was performed to monitor the specificity of the amplification products.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Oligo name</th>
<th>Primer Sequence (5'--3')</th>
<th>Length</th>
<th>T_m</th>
<th>GC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TLR2FP</td>
<td>ATTGTGCCCATTGCTTTTCACTGC</td>
<td>25</td>
<td>72.5</td>
<td>48</td>
</tr>
<tr>
<td>2.</td>
<td>TLR2RP</td>
<td>TGAGGGAATGGA GTTTAAGATCC</td>
<td>24</td>
<td>65.0</td>
<td>42</td>
</tr>
<tr>
<td>3.</td>
<td>ACT FP</td>
<td>TCCCTGGA GAA GA GCTACG</td>
<td>19</td>
<td>62.1</td>
<td>58</td>
</tr>
<tr>
<td>4.</td>
<td>ACT RP</td>
<td>GTAGTTTCGT GGA TGCCA CA</td>
<td>20</td>
<td>63.5</td>
<td>50</td>
</tr>
</tbody>
</table>
2.4. Gel electrophoresis

An adequate volume of electrophoresis tank buffer was prepared and filled in the electrophoresis tank. 0.6 g of Agarose was added in 50 ml of 1X TAE buffer to prepare 1.2% gel. Bromophenol blue (front dye) and Xylene cyanol (tracking dye) were used. The gel was allowed to run at 75 volts till the bromophenol blue reaches the ¾th of the gel. The bands were visualized by placing on a UV light source and photographed directly by gel documentation unit.

3. RESULTS AND DISCUSSION

3.1. Effect of leaf extracts on PBMC viability

The PBMC cells were exposed to 30 µg/ml of C. halicacabum extracts for 2 hours incubation to ascertain the effect on survival of PBMC cells. Cell viability was found to be 96.33 ± 0.23 and 95.52 ± 0.43 % after the cells were treated with ethanolic and ethyl acetate extract respectively (Table 2). Since DMSO was used to dissolve the herbal extracts, DMSO treated cells served as control and viability in those cells was found to be 95.33 ± 0.22%. The results suggest that cell death were less in both the herbal extract treated cells when compared with that of DMSO treated PBMC cells (Figure 1). The cytotoxic actions exerted on PBMC were less/no pronounced when they were treated with C. halicacabum. It is worth noting that the extracts are safe to the normal cells in human [12]. The active constituents present in the extracts or fractions should be further investigated extensively to evaluate through in vitro studies [13].

3.2. Modulatory effect of C. halicacabum on toll like receptor-2 expression

TLR-2 gene expression was investigated by quantifying its mRNA level in the cells treated with two different extracts of the herb. Total mRNA was isolated, purified, and reverse transcribed to synthesize cDNA. TLR-2 specific primers were used to subject the synthesized cDNA by real time PCR quantification method. 1500 ng of cDNA was taken for the analysis where beta actin served as housekeeping gene. The cycle threshold (Ct) values for beta actin were found to be 33.74 and 32.46 in ethyl acetate and ethanol extract treatment respectively. The Ct values for TLR-2 gene were 42.16 and 39.10 in the similar treatment. Delta-delta Ct method was used to neutralize and compare the expression of target and reference genes. Melt curve analysis suggest that there is no nonspecific primer-dimer formation during PCR (Figure 2).

From the results, it was observed that ethanol extract increased the TLR-2 expression up to 2.7 fold when compared to the untreated cells. Whereas no significant effect on TLR-2 expression was found in the cells treated with ethyl acetate extract of C. halicacabum (Figure 3). In LPS induced RAW264.7 cells, ethanolic extract of C. halicacabum inhibited the mRNA expression of COX-2, TNF-α, iNOS and COX-2 whereas, no significant effect on expression of COX-1 [14]. It has been previously reported that herbal extract and their bioactive principles tend to activate macrophages, PBMC and B cells. This modulatory effect on mRNA expression is due the activation of membrane receptors and intracellular signaling factors [15]. However, down regulation of pattern recognition receptors and other downstream signaling proteins has also been observed in some studies where French maritime pine extract suppresses Toll-like receptor-4 and further adipose differentiation-related protein in macrophage cells [16]. Further the PCR products were subjected to agarose gel electrophoresis to confirm the amplification of the target gene TLR-2 and reference gene as well. TLR-2 gene of 204 bp size was observed in the gel and the observation proves that ethyl acetate extract of C. halicacabum significantly alters the expression of toll like receptor 2 gene in healthy PBMC cells (Figure 4). The studies prove that plant extracts contain lead molecules and active principles that could either up regulate or down regulate pro-inflammatory genes.

Table 2. Effect of herbal extract treatment on PBMC cell viability

<table>
<thead>
<tr>
<th>Samples</th>
<th>Percentage viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO Treatment</td>
<td>95.33 ± 0.22</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td>95.52 ± 0.43</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>96.33 ± 0.23</td>
</tr>
</tbody>
</table>

All experiments were done in triplicates. All data values are expressed as Mean ± SD
Modulatory effect of *C. halicacabum* extracts on TLR-2 in PBMC cells

**Figure 1.** Cell viability in treated PBMC

**Figure 2.** Melt curve analysis for TLR-2 (A) and Beta-actin (B); Green- DMSO treated; pink- ethyl acetate treated; blue- ethanol treated

**Figure 3.** Expression levels of TLR-2 upon treatment normalized with the housekeeping gene

**Figure 4.** Gel confirmation of TLR2 and beta actin gene products
4. CONCLUSION

Ethanolic extract of Cardiospermum halicacabum enhanced the Toll-like receptor 2 expression around 2.7 fold in the healthy PMBC, whereas, ethyl acetate extract does not show any promising effect. Further fractionization of these extract would lead to development of novel ligands which specifically modulate toll like receptors expression. Studying the effect of different herbal extract on TLR expression would be a major step forward for developing drugs using plant derived compounds which modulate TLRs expression specifically without having any side effects as in case of synthetic drugs. Plant based TLR ligands could be used to modulate the TLR expressions. Since TLR-2 tends to interact with TLR-1 and TLR-6 to form heterodimers, further studies we planned in our laboratory, to study the expression of these two TLRs with C. halicacabum extract in LPS induced cell line models.

Conflict of Interest

The authors declare that they have no conflicts of interest

Acknowledgment

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References


11. Annamalai A, Ponmari G Sathishkumar R and Lakshmi PTV (2011). Effect of Drying Treatment on The Contents of Antioxidants and iNOS expression, which is mediated by	
