Original article

TH1/TH2 chemokines/cytokines profile in rats treated with tetanus toxoid and Euphorbia tirucalli

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A B S T R A C T

Natural products, including their purified materials, play a remarkable role in drug development. The Euphorbiaceae family, mainly Euphorbia tirucalli, is used in some traditional medicine, and has evidence that its latex comprises immunomodulatory properties and cytokine production. This study aimed to measure the in vivo production of chemokines (IL-1α, IL-1β, IL-12, and RANTES), TH1 cytokines (IFN-γ, TNF-α, GM-CSF, and IL-2) and TH2 cytokines (IL-4, IL-6, IL-10, and IL-13) in rats after treatments with ethanol latex extract of E. tirucalli. Vaccine treated and untreated rats were divided into seven groups to assess antimicrobial activities of the extracted components. After completion of the treatment schedule, blood was withdrawn and sera were collected. The results showed that the main component of the extract was a euphol compound. The extract showed antimicrobial activity and had the ability to modulate innate and adaptive immunity. Animals treated with extract for only 7 days before vaccination showed higher levels of antibody production. The extract showed antibacterial and antifungal activities. The extract could stimulate both adaptive and innate immunity. Pre-treatment with the extract increased immune responses in vaccinated animals, indicating the usefulness of the extract before immunization.

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1. Introduction

Infants and neonates remain at high risk of infectious diseases. As estimated by the World Health Organization (WHO), death toll of infants between the age of one and 12 years increase more than 2.5 million infants per year due to infectious diseases (Siegrist, 2001). This high susceptibility of infants and neonates to infections, for the most part, is ascribed to relative immature immune system, which might include several features. Initially, the cellular population of the peripheral lymphoid organs in neonates is lower than adults, and the microarchitecture of secondary lymphoid organs is not mature (Adkins et al., 2004; Ridge et al., 1996). Second important reason is that neonatal immune cells are functionally not well developed as compared to these cells found in adults. Responses of T cell-dependent/independent antibody production are notably low in newborns and children aged <2 years and in the young of experimental animals. In neonates, this response is characterized by languid immunoglobulin isotype
switching with low immunoglobulin affinity maturation and restricted heterogeneity (Ma and Ross, 2005).

In neonates, the responses of T helper 1 (Th1) cells and cytotoxic T lymphocytes are low, leading to increased susceptibility to intracellular microbes (Galiano-Albarrán et al., 2016). In addition, the immaturity of antigen-presenting cells might lead to limited immune responses in newborns, as indicated by the low expression of co-stimulatory molecules, weak antigen-presenting capability, and low interleukin-12 expression following vaccination or infection (Adkins et al., 2004; Levin and Gershon, 1989; Muthukkumar et al., 2000).

At present, early-life vaccination is an essential approach to protect newborns from infectious diseases. In children older than two years of age and adults, traditional vaccines can produce ample amount of specific protective antibodies to counteract infectious agents (or their products), protecting the host from infectious disease. However, in case of neonates, their immature immune system dramatically impedes the production of a protective vaccine response (Siegrist, 2001).

Thus, techniques to improve the efficiency of vaccination in early life are highly required. Many workers demonstrated encouraging results with different approaches by using many adjuvants, like IL-12, to induce the production of Th1 cytokines and cytotoxic T cell responses and to increase antibody production in newborn mice (Arulanandam et al., 2000; Kovarik et al., 1999; Millan et al., 1998). The drawback shown in some of these techniques were weight loss (Kovarik et al., 2000) and adverse reactions resulting from using these adjuvants.

The plant family Euphorbiaceae is one of the largest families and has more than 330 genera and 8000 species (Webster, 1994). Euphorbiaceae family members include simple weeds and woody trees. Members in this family range from inhabitants of hot dry tropical climates to rainforest trees. The family is characterized by the presence of diverse of secondary metabolites, which leads to a broad spectrum of ethnomedicine of Euphorbiaceae (Seigler, 1994). Some members are poisonous and might cause or influence the susceptibility to specific body ailments (Rizk, 1987). Other species are known to be carcinogenic and can promote excessive cell division, leading to tumor growth (Rizk, 1987; Uchida et al., 2007). However, Euphorbiaceae have been used since ancient times in traditional medicine in India and China to cure different ailments, asthma, and cough (Lai et al., 2004; Shlomovitz et al., 2009).

In alternative medicine, teas and fresh latex are used as antiviral (Appendino and Szallas, 1997; Gupta et al., 2007) and in the treatment of diarrheal diseases, asthma, cancer, parasites, leprosy, scorpion bites and syphilis (Elujoba et al., 2006).

In this study, an attempt was made to investigate the in vivo effects of using natural products to enhance the immune system during the expanded program of immunization (EPI) recommended vaccination for infants.

2. Materials and methods

2.1. Euphorbia tirucalli extract preparation and its chemical analysis

The aerial parts of Euphorbia tirucalli were collected from the Aseer region in September 2016. The active ingredients in the latex of the plant were extracted according to Ghramh et al. (2018) with some modifications. The latex of the plant was thoroughly extracted with 80% ethanol. To test its components, the evaporation of plant ethanol extract was performed at reduced pressure of 40°C (rotary evaporator, Buchi, Germany) to get about 2.5 g solid material. Some part of the obtained material was subjected to column chromatography of silica gel 60 (70–230 mesh, Merck) and thin layer chromatography (TLC; 0.25 and 1 mm precoated plates 60 F254, Merck) and chromatographed again over silica gel 60 with hexane/ethyl acetate (8:2.5). The isolated compounds were chemically analyzed using NMR (1H NMR (500.13 MHz) and 13C NMR (125.75 MHz)) on a Bruker DRX 500 spectrometer and identified. The solid material was dissolved again in 80% ethanol to get 1% solution. The ethanol extract was given orally (0.5 mL) to the animal daily through a gastric tube according to specific group schedule. Other part of solid materials was re-dissolved in dimethyl sulfoxide (DMSO) for antibacterial activity.

2.2. Study of antimicrobial efficacy of Euphorbia tirucalli extract

Antimicrobial activity was investigated by the agar well diffusion method using 50 μL of microbial suspension containing 106 CFU/mL of bacteria (gram-positive bacteria including Bacillus subtilis, Staphylococcus aureus, and Micrococcus sp. and gram-negative bacteria including P. aeruginosa, Klebsiella sp., Proteus mirabilis, and Shigella dysenteriae), 104 CFU/mL yeast (Candida albicans) per McFarland standard, inoculated into the Mueller Hinton Agar (Difco). Well of about 6 mm were prepared in agar plates. Aliquots of 25 μL of the plant extract were evenly applied directly to the wells. Petri plates were stored at 4°C for 2 h. The bacterial inoculated petri plates were incubated at 37 ± 0.1°C for 24 h and agar plates inoculated with yeast were incubated at 25 ± 0.1°C for 48 h. The antimicrobial activity found in the extract was assessed by measuring the zone of inhibition (ZOI) against the test organisms (Kirbag et al., 2013). The extract was tested in triplicate and erythromycin (15 μg) disc was used as a positive control and sterile water as a negative control.

2.3. Animals and experimental design

Male Sprague Dawley rats (200–250 g), kindly supplied by the animal house of the university, were housed in cages under proper ventilation and provided ad libitum food and water throughout the experimental period. These animals were looked after at 22–25°C and 40–60% relative humidity with the 12 h light–dark cycles. This investigation was in accordance with the Ethical Principles for Animal Experimentation, utilized University Ethics Committee for Animal Experimentation.

The animals were divided into seven groups with six animals in each group (Table 1) as follows: Group 1 (G1); control, untreated animals received nothing throughout the experiment. Group 2 (G2); animals were vaccinated at the start day with adsorbed Tetanus Toxoid vaccine (Serum Institute of India, India), the dose of the vaccine per rat weight was calculated and adjusted in a volume of 100 μL. The vaccine dose was administered into the two hind femurs. At days 7, 14, and 21, the animals were boosted with the same dose and manner and left for one week without any vaccine treatment before sacrifice. Group 3 (G3); animals received plant extract daily at the start day and continued for 21 days. Group 4 (G4); animals received plant extract daily for 7 days before the

| Table 1: Schedule of animal immunization. |
|------------------------------------------|
| Group        | 7 days before | 21 days treatment | 7 days after |
| G1           | NT           | NT               | NT          |
| G2           | NT           | Vaccine          | NT          |
| G3           | NT           | Extract          | NT          |
| G4           | Extract      | Extract          | Extract     |
| G5           | Extract      | Vaccine          | NT          |
| G6           | NT           | Extract/Vaccine  | NT          |
| G7           | Extract      | Extract/Vaccine  | NT          |

where 7 days before = 7 days before starting vaccine treatment; 21 days treatment = either daily oral administration of plant extract, weekly immunization with vaccine or both; 7 days after = animals left untreated for 7 days before sacrifice or daily oral administration with plant extract; NT = No treatment.
start day and continued until the end of the experiment. Group 5 (G5); animals received plant extract 7 days before the start day and were vaccinated the same way as G2. Group 6 (G6); animals were vaccinated the same way as G2 and received plant extract the same way as G3. Group 7 (G7); animals were treated the same as G6 but also received plant extract 7 days before the start day. After completion of the schedule, all animals were anesthetized, blood was withdrawn, and sera were collected, separated and stored at −80 °C until use.

2.4. Serum anti-tetanus toxoid antibodies analysis

Serum total anti-tetanus toxoid IgG was quantified according to Reder et al. (2008) using an ELISA kit (Abnova) for the quantitative determination of rat anti-tetanus toxoid IgG according to the manufacturer instructions. In addition to the dilution recommended by the manufacturer (5000 fold), sera were 2-fold serially diluted to ensure the measurements and to avoid over reading.

2.5. Th1/Th2 cytokines and chemokines determinations

Sera of animals under different groups were analyzed in duplicate for the cytokines/chemokines interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and Regulated Upon Activation Normal T cell Express Sequence (RANTES, also called CCL5) according to Rao et al. (2012) through a multi-analyte ELISAarray kit (Qiagen) as per manufacturer instructions for revealing the inflammatory cytokines in rats.

2.6. Statistical analysis

Data were expressed as the means ± SE (n = 6 animals/group). Variation in different groups were analyzed with one-way analysis of variance (ANOVA) using SPSS (version 17). Differences with p ≤ 0.05 were considered statistically significant. For comparisons between different cytokines, data were expressed as fold increases/decreases (Chen et al., 2015).

3. Results

3.1. Chemical profile of Euphorbia tirucalli extract

The fraction of E. tirucalli ethanol extract gave a positive Libermann Burchardt test for a triterpene and gave a pink color when it is sprayed with H2SO4/MeOH reagent. The 1H NMR spectrum analysis (Fig. 1A) revealed seven tertiary methyl signal groups at δ 0.79, 0.89, 0.90, 0.98, 1.28, 1.61, and 1.67 and a secondary methyl signal group at δ 0.87 (d, J = 6.5 Hz, 3H-21) with 13C NMR (Fig. 1B) at δ 29.2 (C-29), 25.9 (C-27), 24.9 (C-28), 23.7 (C-19), 20.1 (C-21), 16.3 (C-30), and 16.1 (C-18). The oxymethine signal at δ 3.22 (1H, dd, J = 11.7, 4.5 Hz, H-3) was recognized as H-3, whereas the olefin proton at δ 5.18 (1H, t, J = 7.1 Hz, H-24) was ascribed to H-24, with analogous 13C NMR signals at δ 79.7 and 126.1 respectively. Signals at δ 135.6 and 134.9 designate C-8 and C-9 respectively, and the presence of a triterpene euphol moiety was assumed which was verified by comparing the 13C NMR signals with already published data of euphol.

3.2. Antimicrobial efficacy of Euphorbia tirucalli extract

The results showed that Euphorbia plant extract exhibited the antimicrobial activity of different degrees against the test microbes (Fig. 2, Table 2). The antibacterial and antifungal activity of the plant extracts against the tested microorganisms suggest the presence of antibiotic compounds with broad-spectrum in the plant. The antimicrobial effects of the plant extract were observed to be high against gram-negative bacteria (P. aeruginosa, Klebsiella sp, Proteus mirabilis and Shigella dysenteriae) and moderate to low against gram-positive bacteria (Bacillus subtilis, Staphylococcus aureus and Micrococcus sp) and Candida albicans, as seen in Table 2. The positive control disc (erythromycin 15 μg) showed no inhibitory effect against the test microorganisms, except that B. subtilis showed a 32 mm inhibition zone.

3.3. Cytokine/Chemokine production

3.3.1. Th1 type cytokines

The Th1 cytokines tested in this experiment were IL-2, IFN-γ, TNF-α, and GM-CSF (Fig. 3). In G2, which received vaccine only, IL-2 significantly (p < 0.05) increased. G3 and G4, which received plant extract only without vaccine treatment, also showed significantly increased IL-2 production (p < 0.001) in comparison with control group. Group 3, which received extract only for 21 days, was non-significantly higher than G4, which received extract throughout the experiment. G6 showed a non-significant increase in IL-2.

IFN-γ showed significant increases (p < 0.001) in G5, which received vaccine only preceded by treatment with extract for 7 days, and G6 (p < 0.001), which received both extract and vaccine for 21 days. G5 showed a significant increase (p < 0.05) in IFN-γ
production compared with G6. G4 and G7 showed non-significant increases.

TNF-α exhibited a significant increase ($p < 0.001$) in groups G4 through G6. G6 is significantly ($p < 0.001$) higher than G5, and G5 is significantly ($p < 0.001$) higher than G4.

GM-CSF showed substantial increases ($p < 0.001$) in all treated groups except G7, which showed a non-significant increase, and G2, which showed no change. G6 showed a substantial rise ($p < 0.001$) in GM-CSF production compared with the other groups. G5 was significantly higher than G4, and G4 was significantly higher than G3.

3.3.2. TH2 type cytokines

The TH2 cytokines tested in this experiment were IL-4, IL-6, IL-10, and IL-13 (Fig. 4). IL-4 presented a substantial ($p < 0.05$) rise in G4 and non-significant increases in G3, G5, G6 and G7.

IL-6 and IL-10 showed non-significant increases in all groups except in G6, which showed no change. G6 showed a non-significant increase ($p < 0.001$) in IL-13 only significantly higher ($p < 0.001$) in the G4 group, which received extract throughout the experiment.

3.3.3. Chemokines

The chemokines tested in this experiment were IL-1α, IL-1β, IL-12, and RANTES. The IL-1α rise in groups G3-G7 was non-significant and a non-significant decrease in G2 (Fig. 5).

IL-1β exhibited a significant increase ($p < 0.001$) in G3 and a non-significant increase in G4 and IL1-β displayed a significant decrease ($p < 0.001$) in G2 and G5 ($p < 0.05$).

IL-12 presented a significant rise ($p < 0.001$) only in G6, with a non-significant increase in G7.

RANTES revealed a significant increase ($p < 0.001$) in groups G3-G6. G5 was significantly higher than G4 and G6. The increase in G7 was non-significant, and there was no increase in G2.

3.4. Anti-tetanus toxoid antibodies titer

All groups that received vaccines showed high antibody production (Fig. 6). The antibody production in G5 was significantly ($p < 0.001$) higher than all other groups. G6 showed a significant increase ($p < 0.05$) in antibody as compared to G2 and G7.

4. Discussion

Vaccination or immunization is a fundamental approach to protect newborn health. By the method of vaccination, dozens of serious diseases can be prevented. The failure to vaccinate might lead to putting children at risk of severe and fatal diseases. Infants are particularly susceptible to infections; therefore, it is imperative to protect infants through immunization. Immunization is a significant way to prevent the spread of diseases and protect infants and children from dangerous complications (CDC, 2014).

In this study, an attempt was made to investigate the potential effects of Euphorbia tirucalli sap extract to modulate the adaptive and innate immune system to overcome weak immune responses in infants.

E. tirucalli has been indiscriminately used in popular medicine to treat various illnesses. In the current study, the latex of E. tirucalli was collected and its ethanol extract was prepared. The extract contained mainly functional groups including triterpene (euphol). The tetracyclic triterpene euphol is used in folk medicine against many diseases, but the mechanisms of action remain to be elucidated (Duarte et al., 2009; Lin et al., 2012; Passos et al., 2013; Wang et al., 2013).

The results showed that Euphorbia plant extract exhibited different degrees of antimicrobial activity against the studied microbes. The antimicrobial potential of various Euphorbia species is variable in other research findings (Barla et al., 2007; Kamba and Hassan, 2010; Sudhakar et al., 2006), and these differences might be due to the genetic structure of plant or phytochemical differences among species. In some other studies that focused on
Fig. 3. $\text{T}_{\text{H}1}$ type cytokines level in different treated groups. $^* = p < 0.05$, $^{**} = p < 0.001$.

Fig. 4. $\text{T}_{\text{H}2}$ type cytokines level in different treated groups. $^* = p < 0.05$, $^{**} = p < 0.001$. 
antimicrobial activity, various plant extracts inhibited microbial growth with different ratios (Kirbag et al., 2013).

In our study, there were different immune responses to the extract, the vaccine and combined treatment with both extract and vaccine. In G2, in which animals were vaccinated at the start day with tetanus toxoid vaccine, there were no major variations in the cytokines/chemokines levels except IL-1β, which showed a significant decrease, and a substantial increase in IL-2. IL-1α exhibited no significant decrease. G3 animals received plant extract daily on the day of immunization of G2 started and continued for 21 days. Treatment with the extract led to an increase in IL-1β production. IL-1β is known to be a T helper 1 inducer, so increased IL-1β production might lead to T helper 1 stimulation, indicating the potential of the extract to induce immunity. This finding might explain the increase of IL-2 in our results. IL-1 family cytokines are a group of pro-inflammatory mediators. IL-1 family cytokines work as secreted mediators (Luheshi et al., 2009), and both IL-1α and IL-1β are produced by several cells such as neutrophils, monocytes, macrophages, and hepatocytes (Arend et al., 2008), meaning that the extract could induce these cells to produce these cytokines. In our results, the detection of IL-1β, and not of IL-1α, in the sera of the rats might be because most of the IL-1 present in the circulation is IL-1β. IL-1 encompasses a pleiotropic cytokine family that can have numerous actions (Allan et al., 2005).

There were no significant changes in the TH1 cytokines except IL-2 and GM-CSF. GM-CSF was significantly higher than IL-2. Many cells produce GM-CSF, including activated T cells and macrophages. The cytokine GM-CSF works to induce bone marrow cells to produce dendritic cells and monocytes, resulting in an increase in antigen presentation potential. The Th2 cytokines showed no significant changes, except for IL-1β and RANTES, which showed a significant increase. From these results, it is clear that the extract can make some changes in innate immunity by inducing IL-2 and GM-CSF by Th1 cell cytokines and the chemokines IL-1β and RANTES. Helper T cell subsets differ in some of the chemokine receptors they express, and therefore, different chemokines could promote selective Th1 or Th2 recruitment to inflammatory sites. For example, Th1 cells make expression for CCR5 and CXCR3 and respond to the chemokines IP-10, ITAC, Mip1-α, Mip1β and RANTES, all of which are resulted in response to microbial threats and stimuli produced during innate immune responses to microorganisms (such as the cytokine IFN-γ). In our results, the extract could stimulate RANTES expression, which in turn leads to Th1 stimulation.

Increasing the period of treating animals with the extract in G4 in which animals received daily plant extract only for 7 days before the extract to induce immunity. This finding might explain the increase of IL-2 in our results. IL-1 family cytokines are a group of pro-inflammatory mediators. IL-1 family cytokines work as secreted mediators (Luheshi et al., 2009), and both IL-1α and IL-1β are produced by several cells such as neutrophils, monocytes, macrophages, and hepatocytes (Arend et al., 2008), meaning that the extract could induce these cells to produce these cytokines. In our results, the detection of IL-1β, and not of IL-1α, in the sera of the rats might be because most of the IL-1 present in the circulation is IL-1β. IL-1 encompasses a pleiotropic cytokine family that can have numerous actions (Allan et al., 2005).

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Increasing the period of treating animals with the extract in G4 in which animals received daily plant extract only for 7 days before

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**Fig. 5.** Chemokines level in different treated groups. * = p < 0.05, ** = p < 0.001.

**Fig. 6.** Anti-tetanus toxoid antibody levels in different treated groups. * = p < 0.05, ** = p < 0.001.
immunization of G2 and continuing till the end of the experiment, i.e., animals received extract for a total period of 35 days, lead to significant changes in TH1 cytokines (IL-2, TNF-α and GM/CSF), whereas there was no substantial change in IFN-γ. GM/CSF was considerably higher than IL-2 and TNF-α. TNF-α induces cytokine production, adhesion molecule activation or expression and growth stimulation. The TH2 cytokines (IL-4, IL-6, and IL-10) showed no significant changes, except IL-13, which showed a significant increase. The chemokines (IL-1α, IL-1β and IL-12) exhibited no significant variations, whereas RANTES displayed a major increase. The new cytokine appearing in this group was IL-13. IL-13 mimics the effects of IL-4 on non-lymphoid cells, such as macrophages, but appears to have less of an effect on T or B lymphocytes than does IL-4. IL-13 inhibits the activation of macrophages and antagonizes IFN-γ.

Group 5, which received plant extract 7 days before immunization of G2 and was vaccinated the same way as G2, showed some responses to extract and vaccine treatment. TH1 cytokines showed a significant increase in IFN-γ, TNF-α, and GM/CSF, with no change in the IL-2 level. IFN-γ is a major macrophage-activating cytokine that has essential roles in innate immunity and adaptive immunity (Decker et al., 2002). IFN-γ is also known as immune, or type II, IFN. IFN-γ is a homodimeric protein which is made by NK cells, CD4+ TH1 cells, and CD8+ T cells. In the innate immune response, NK cells discharge IFN-γ in reply of recognition of unidentified components of microorganisms or response to IL-12. In adaptive immunity, T cells yield in IFN-γ in response to antigen, and this production is boosted by IL-12 and IL-18. The reaction order comprising IL-12 and IFN-γ is essential for cellular immunity against intracellular microorganisms. Furthermore, IFN-γ secreted by activated T cells and NK could augment TNF production, explaining the high level of TNF-α in this group. TH2 cytokines (IL-4, IL-6, IL-10, and IL-13) presented no significant changes. Regarding chemokines, IL-1α, and IL-12 exhibited no significant changes, whereas IL-1β revealed a significant decrease. RANTES showed a significant increase. Pre-treatment with the extract favored the stimulation of TH1 cytokines. Stimulation of TH1 cells might cause B cells to produce antibodies.

Group 6 was treated the same way as G2 and with plant extract the same way as G3. The TH1 cytokine IL-2 showed no significant increase, whereas IFN-γ, TNF-α, and GM/CSF exhibited significant increases. GM/CSF was remarkably higher than IFN-γ and TNF-α, and TNF-α was substantially higher than IFN-γ. Regarding TH2 cytokines, a significant increase was shown in IL-6 and IL-10, and there were no changes in IL-4 and IL-13. The family of IL-6 is composed of pleiotropic cytokines that consist of the members IL-6 itself and IL-11 (Kishimoto, 2006). IL-6 is produced by phagocytes, T and B cells, fibroblasts, endothelial cells, hepatocytes in reply to microorganisms and other cytokines, notably IL-1 and TNF (Jucser et al., 1991). The rise in TNF-α in this group might lead to the increase in IL-6 production. IL-6 functions in both innate and adaptive immunity.

IL-10 can be produced by many cell types and can inhibit the production of IFN-γ and IL-2 by TH1 cells; IL-4 and IL-5 by TH2 cells, chemokines (including RANTES) and proinflammatory cytokines (including TNF-α, IL-1α & β, IL-6, and IL-12) (Viallard et al., 1999). These inhibitory effects on the accessory functions are responsible for the inhibition of TH1 and TH2 cytokine production. In our results, the cytokines IL-2, IL-4, IL-13 and the chemokines IL-1α and IL-1β are produced at near normal levels without any increases, perhaps because of the increase in IL-10 production.

There was a significant rise in the chemokines IL-12 and RANTES, and IL-12 was significantly higher than RANTES. IL-1α and IL-1β showed non-significant changes.

Interleukin-12 is an essential cytokine in the initial innate immune response, especially to intracellular microorganisms, and is very important for cell-mediated immunity against microbes. IL-12 was initially recognized as an activator of NK cell cytolytic function, but its prime function is to stimulate IFN-γ production by T cells and by NK cells. This action explains the significant increase in IFN-γ in our results.

Group 7, which received plant extract 7 days before immunization of G2 and was vaccinated the same way as G2 and received plant extract the same way as G3, showed no substantial changes in the levels of cytokines and chemokines except for GM/CSF and RANTES, indicating that prolonged treatment with extract with the vaccine is not recommended.

All groups that received vaccines showed antibody production. The antibody production in G5 was significantly higher than in any other groups. G6 showed a significant rise in antibody production compared with G2 and G7.

The animals in Group 2 showed no significant changes in the cytokines/chemokines levels, except IL-1b, which showed a significant decrease, and a substantial increase in IL-2. The increase in IL-2 is an indicator of TH1 activation. In the time the antibody response was strong, normal or baseline levels of cytokines and chemokines are noticed.

The antibody titer was the highest in G5. G5 animals were vaccinated and received plant extract seven days before immunization. The TH1 cytokine profile in this group showed an increase in TH1 cytokines TNF-α and GM/CSF with no change in the TH2 cytokines and an increase in the chemokine RANTES. The increase in TNF-α is a result of the increase in IFN-γ. TNF-α induces cytokine production, adhesion molecule expression, and growth stimulation (Khader et al., 2007; Tartaglia and Goeddel, 1992). A high level of GM/CSF dramatically increases the production of inflammatory leukocytes. GM/CSF boosts to mature the bone marrow cells into dendritic cells and monocytes, increasing the potential of antigen presenting cells. Increased antigen presentation will lead to adaptive immune responses, including antibody production. Activated T-cells help B-cells to produce antibodies. In addition, an increased level of RANTES, the TH1 activator, might increase the cellular responses, resulting in increased antibody production.

Group 6 was vaccinated and treated with plant extract the same in the same period. There were increases in the TH1 cytokines IFN-γ, TNF-α and GM/CSF and the TH2 cytokines. There were increases in the IL-12 and RANTES chemokines. This profile of cytokines is ideal for B-cells to produce antibodies. The antibody titer in this group was higher than in any other groups and was lower than the group treated for a shorter time with the extract (G5). Group 7 animals, which were treated with the extract for a longer time (28 days), showed no significant increase over the control immunized group (G2).

5. Conclusion

Active products of the Euphorbia tirucalli sap extract could modulate innate and adaptive immunity, as indicated by cytokine/chemokine changes after treatment with the extract. Treating animals with the extract only seven days before immunization gave the best antibody response.

Author disclosure statement

The authors disclose that there are no any commercial associations that might create a conflict of interest in connection with submitted manuscripts.

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