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MODULATION OF LIPOPOLYSACCHARIDE - INDUCED CYTOKINES RESPONSE IN MICE BY GRAVIOLA LEAF POWDER

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Abstract:

Cytokine response in sepsis involves two encompassed phases, the pro-inflammatory and anti-inflammatory cytokine response. To exam the impact of Graviola on mice cytokine response during experimental sepsis, firstly, LPS sepsis dose and Graviola protective dose against sepsis were determined. Animals were short term pretreated with the protective dose of Graviola leaf powder orally before single sepsis dose of LPS intraperitoneal challenge. Levels of cytokines such as IL-4,IL-6,IL-10 and TNFα were estimated in animal sera at different times after LPS challenge by ELISA. Short term Pretreatment of animals with the protective dose of Graviola leaf powder diminished serum levels of TNF-α and IL-6 whereas IL-4 and IL-10 were slightly affected, although decreased, serum levels of these cytokines were not significantly different from experimental sepsis mice which challenged with LPS alone.

Key words: Lipopolysaccharide, Sepsis, Graviola, Murine model, Cytokine response, IL-4,IL-6,IL-10, TNFα

Introduction

The development of septic shock and associated multiple organs failure are the common and serious consequences of gram negative bacterial infections (Bone, 1991, Martin, 1991). These infections are of frequent sequale associated with mortality rate of about 50-75% (Cohen, 2002). Gram negative bacteria have lipopolysaccharide (LPS), the major component of the outer membrane. It is a glycolipid consists of lipid A, core polysaccharide and side chain polysaccharide. Lipid A moiety is responsible for toxic activity of this huge molecule (Wang and Quinn, 2010).

In order to design effective anti- sepsis therapy, it is essential to understand the host- LPS interaction.

The interaction of endotoxin (LPS) with immune cells such as macrophages is the key for earliest cell mediated events. The LPS- binding proteins (LBP) and signaling receptors in the blood act to enhance the effects of LPS leading to the activation of immune cells. LBP is an acute phase protein, raises in plasma due to bacterial sepsis and binds to LPS through lipid transferring it to the CD14 to develop immune response (Theofan *et al.,* 1994) .

Two distinct phases are involved in septic inflammatory response, the systemic inflammatory response (SIR) and the compensatory anti-inflammatory response (CAIR). the balance between these phases influences host survival whereas imbalance can result in host damage either by, inflammation or immune dysfunction. Temporary increase in pro-inflammatory cytokines like Tumor necrosis factor-α (TNF-α), Interleukin 1 (IL-1), Interleukin 6 (IL-6) and Interleukin 8 (IL-8), is involved in the initial sepsis clinical signs: TNF-α has the ability to activate macrophages, neutrophils and monocytes and stimulate the production of acute phase protein. The increase in IL-1 beta levels correlated to TNFα but acts as inhibitor to cytokines cascade. IL-6 controls the inflammatory response protecting animals against sepsis whereas anti-inflammatory cytokines IL-4, IL-10 are inhibitory for proinflammatiry cytokines and act to reduce the sepsis mortality. The activation of pro-inflammatory cascade and the anti-inflammatory response result in circulatory alterations and cellular disturbance leading to incompatible tissues function and metabolic reactions (Waage *et al.,* 1987, Barton and Jackson, 1993, Casey *et al.,* 1993, Pinsky *et al.,* 1993). These events combined together to develop multiple organs dysfunction (MOD) and consequently, death.

Gene expression of the regulatory proteins: cytokienes such as IL-1, IL-2, IL-6, IL-12,TNF-α; Acute phase

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proteins and other chemokines is regulated by transcription factor nuclear factor kappa B (NF- κB). Activation of NF- κB factor is associated to production of various immune mediators responsible for inflammation (Attiq *et al.,* 2017).

Both pro-inflammatory and anti-inflammatory agents might have a space in targeting the therapeutic treatment of sepsis but the typical targeting is the good understanding of inflammatory events.

As insight point to herbalism, NF- κB factor inhibitors are promising as phytotherapeutics (Attiq *et al.,* 2017).

Because of their easy uptake, inexpensiveness and safety, dietary phytochemicals extracts or even their dried powders have been widely used for treatment of human diseases (Gullett *et al.,* 2010, VanWyk, and Wink, 2004).

The beneficial activity of dietary phytochemicals or plant derived agents remains a fertile area for investigation and researches to design phytotherapeutic agents.

Actually, medicinal plants interfere with certain aspects of adaptive immune response or host defense mechanisms against external microbial invaders and foreign molecules, thus they have been extensively studied. Additionally, their biologically effective components and their modes of action have been further elucidated (Mishra *et al.,* 2006, Kim *et al.,* 2016).

Annona murticata or Graviola is a tropical always green fruity plant (Cijo George *et al.,* 2012, Zorofchian Moghadamtousi *et al.,* 2014). All portions of Graviola tree are beneficial but the most traditional medically used are leaves. Beside the anticancer, analgesic, antinociceptive, antioxidant, antidiabetic, antibacterial, antifungal anti-parasitic, Graviola extracts and dried portions are known to be anti-inflammatory and immunomodulatory (Somsak *et al.,* 2016, Rady *et al.,* 2018).

More than 200 biologically active components in Graviola (Coria-Téllez *et al*., 2018). The chemical analysis of graviola extracts indicated the presence of alkaloids, cyclopeptides, polyphenols, essential oils and flavonoids (Matsushige *et al.,* 2012). Although Graviola contains swanisonines the neurotoxic alkaloids, which characterized by their potent neurotoxicity through inhibition of α-aminosidase of lysosomes, it is considerable to notify that the neurotoxic swanisinins present in high concentrations in seeds and fruits of the plant (Molyneux *et al.,* 1994, Dictionary of Natural Products 2007). Up taking of *Annona murticata* fruits for long period develop pathological disorders of neurons in mice leading to symptoms same to Parkinsonism. but alkaloids remain the most important biologically active constituent of Graviola beside to acetogenins (Haraguchi *et al.,* 2003, Caparros-Lefebvre *et al.,* 2005, Rottscholl *et al.,* 2016).

Rady *et al*. (2018) reported that *Annona murticata* extracts can control the inflammation and pain by targeting the molecular aspects with few contraindications.

Several published literatures elucidate the role of Graviola extracts in treatment of inflammatory diseases. The analgesic and anti-inflammatory properties of *Annona murticata* fruit extract were concluded by Ishola *et al.* (2014). de Souasa *et al.* (2010) reported that when rat were orally treated with ethanolic extract of Graviola leaves showed various changes in the tested inflammatory parameters.

Hamid *et al.* (2012) suggested the anti- inflammatory activity of ethanolic extract of Graviola leaves in rodent model arthritis, their results confirmed that treatment of animals with this extract reduced edema an arthritis and showed inhibited levels of the proinflammatory mediators IL-1β and TNF-α.

The anti-inflammatory activity of Graviola was also indicated by the results of Laksmitawati *et al.* (2016), who concluded that treatment of LPS-induced rodent macrophages (RAW264.7) with ethanolic extract of the plant leaves, cells remain viable at concentration reach to 50μg/ml.

We designed this study to elucidate the effect of Graviola on sepsis and the inflammatory process that mediated by cytokines especially those of controlling properties and to explain the regulatory interactions occur.

Materials and Methods

LPS: Lipopolysaccharide of *E .coli* serotype O111:B4 (Chemcruz: Santa Cruz Company) was used for induction of animal sepsis.

Graviola: *Annona muricata* leaf ground to fine powder (Biotech.), Which demonstrated for human dietary supplement.

Lab. animals: Pathogen free male Balb/c mice, 6-8 weeks old weighing 20-25 gm, were used. Mice were allowed to acclimatize for about week under standardized conditions, receiving human care, fed standard chow formula and filtered water ad libitum.

Determination of septic dose of LPS:

LPS concentration adequate for induction of sepsis in mice was determined according the procedure explained by Soromou *et al*. (2014).

Determination of protective dose of Graviola:

The protective concentration of Graviola was determined according to the protocol of Hong *et al.* (2012)

by treatment of mice orally with (0.5, 0.75, 1, 1,25, 1.5, and 1.75) mg/ mouse of Graviola leaf powder dissolved in DW daily for 5 days. On day five and 1 h after the last oral dose, mice were challenged with the septic dose of the lipopolysaccharide. Animals were monitored for 7 days and survival numbers were recorded

Experiment design:

Mice were grouped as follow:

Groups 1 and 2: Mice were orally given normal saline as controls. Groups 3 and 4: Mice were received Graviola leaf powder orally by gavage. The protective dose was (1.5 mg/ mouse dissolved in DW). Animals were given Graviola leaf powder for 5 days. On day five, after 1hr of the last oral pretreatment, mice of groups 1 and 3 were injected intraperitoneally (IP) with single dose of LPS 200 µg/ mouse. Group 2 and 4 were injected with normal saline. Animals were scarified at times (0, 2, 4,6, 12, 24 and 48) after LPS injection. Blood was collected by heart puncture then serum obtained.

Measurement of cytokine serum levels:

IL-4, IL-6, IL-10 and TNF-α levels were evaluated in animal sera by Enzyne Linked Immunosorbent Assay using the mouse specific commercial Kit Cloud Clone Corp (USA) and according to manufactural instructions.

Ethical issues:

In the case of dealing with animals, care was taken during the research period, with appropriate experimental conditions and when the animals were killed, euthanasia was followed**.**

Statistical analysis:

The experiment was designed as CRD and ANOVA test was used for analysis of data that presented as mean ± SD.

Results and Discussion:

Sepsis is the state of inflammatory disruption which rises when host fail to overwhelm the infection (Buras *et al.,* 2005). The complex and multifactorial events makes difficulties to study the septic shock and to design therapeutic agent for such states.

Murine model has been designed for the induction of septic shock by bacterial lipopolysaccharide, mice were intraperitoneally challenged with (50, 100, 125, 150, 200, 250) µg/mouse of bacterial lipopolysaccharide and the septic dose was 200 µg/ mouse, where 83.3% of mice were killed. For our purpose to complete the next part of the experiment, a single dose of 200 µg/ mouse was used in the induction of animal sepsis.

Table 1: Protection of mice against lethality of LPS by Graviola leaf powder.

Graviola leaf powder mg/mouse	LPS concentration µg/mouse	Number of mice dead/ total
No pretreatment	50	1/8
	100	3/8
	125	4/8
	150	5/8
	200	7/8*
	250	8/8
0.5	Septic dose 200 µg/mouse	7/8
0.75		6/8
1		3/8
1.25		2/8
1.5		0/8**
1.75		0/8

*The sepsis dose of LPS which killed 83.3% of mice.
**The protective dose of Graviola leaf powder, all mice survived.

Endotoxemia or septic shock in the experimental animals appeared early after 12 h of LPS challenge, which clearly characterized by sepsis symptoms.

Pretreatment of mice with Graviola leaf powder was significantly reduced the mortality of mice injected with the septic dose of LPS. As shown in (table 1), mice pretreated with Graviola leaf powder orally by gavage survived after LPS injection.

The protective activity of Graviola leaf powder is dose dependent, that is about 1.5mg/mouse oral daily dose reduced LPS septic mice mortality. This result indicated that short term pretreatment of animals with Graviola improved their survival under LPS septic dose.

The safety of Graviola for mice was also demonstrated. Graviola alone was nontoxic for animals where no alteration in animal behavior were shown and no lethality were observed in groups that orally administrated Graviola leaf powder alone even in high concentrations (Data not shown). Somsack *et al*. (2016) reported that LD_{50} of Graviola leaf water extract was =2500 mg/Kg.

On binding of bacterial lipopolysaccharide molecules to their receptors on macrophage cell, events of cytokine response initiate (Chow *et al.,* 1999). Certain cytokines encompass the inflammatory response to LPS and there is a dramatic interaction among them. Furthermore the balance change of both SIR and CARS have critical role on host immune response thus, as it is known, LPS stimulates the production of pro-inflammatory cytokines from immune cells that mediate the inflammatory response causing sepsis and tissue damage (Pfeffer *et*

al., 1993, Pinsky *et al.,* 1993 and Casey *et al.,* 1993). Animals exposure to the septic dose of LPS caused acute systemic inflammatory response which characterized by various cytokines time course increasing.

The transcription factor NF- κB has a critical role in the down regulation of the inflammatory process. This factor is activated by several stimuli such as cytokines (TNF-α, IL-1β), growth factor, LPS and others. When cell is unstimulated, NF- κB present in the cytoplasm as homodimers and heterodimers, the inactive forms, which interact with the inhibitory protein I κB. On activation, I κB is phosphorylated and degraded releasing the NF-κB homodimers and heterodimers which translocated to nucleus and consequently activates the transcription of the corresponding genes. The activation of NF- κB is triggered by series of signals, the first one is the binding of LPS to TLR4 by which the cell is activated and produces the pro-inflammatory cytokines (Akira, 2003, Hayden and Ghosh, 2004, Hayden and Ghosh, 2008, Hoffmann and Baltimore, 2006, Brown *et al.,* 2008, De *et al.,* 2014).

Their the initiation of cytokine transcription begins where TNF-α produced, this cytokine has the ability to potentiate the inflammatory response (Chow *et al.,* 1999). Our data confirmed that the level of TNFα significantly raised (p= 0.05) and reached the peak at 2 h after the treatment of mice with (200 µg/ mouse) of LPS alone. The next cytokine response phase is the CARS through which IL6, IL10, IL4 elevated where the mean level of IL6 significantly elevated (p= 0.05) and reached the peak at 4 h Figure (1, 2). Our results were in line with Newton *et al*. (2000), who studied early cytokine response which is characterized by elevated levels of IL-1 β, IL-6 and TNF-α within 24 h.

IL6 is essential in the eradication of the inflammation due to induce the production of acute phase proteins in the liver (Xing *et al.,* 1998). Because of dualism, the property of IL-6 by which it carry adverse effects and the correlation of elevated levels of this cytokine with sever sepsis, many researchers referred to as pro-inflammatory cytokine (Damas *et al.,* 1992).

On the other hand IL-4 and IL-10 levels significantly increased (p= 0.05) and reached their peaks at 6-12 h after LPS challenge Figure (1:c,d). The anti- inflammatory cytokine IL10 inhibits the production of the pro-inflammatory cytokines and inhibits the mortality of endotoxic shock whereas the potentiality of IL-4 in reducing the mortality of sepsis by the inhibition of pro-inflammatory cytokines synthesis (Malefyt *et al.,*1991, Takeshita *et al.,* 1996, Shreedhar *et al.,* 1998). Furthermore IL-10 showed biphasic response after LPS injection which in line with Barsig *et al*. (1995).

In our in vivo model the association of the increased levels of TNF-α with septic shock was concluded. Zinyama *et al*. (2001) denoted that TNFα was early stimulated by LPS and the peak occurs at 6h whereas IL 10 increases late but the maximum of all response occurs at 48 h.

There were clear differences between levels of cytokines in mice pretreated with Graviola leaf powder and mice received LPS alone.

Pretreatment of animals with the protective dose of Graviola leaf powder significantly diminished levels of TNF-α and IL-6 whereas slightly affected IL-4 and IL-10 and the decrease in IL-10 levels also showed biphasic response fig. 1.

With regard to the dual behavior of various cytokines which produced in septic shock, any material can skew the balance of these mediators may impact sepsis process, for example controlling of TNF-α levels is critical to avoid the pathological events (Chan *et al.,* 2012).

The mechanism of protection against sepsis is not illustrated yet. Beside the known activity of IL-4 and IL-10 as suppressors for TNF-α by which control the harmful effects of the acute phase inflammatory response. Attiq *et al*. (2017) referred that various species of Annonaceae and their extracts have inhibitory effects on NF- κB. They may inhibit the induction of TNFα via inhibiting NF- κB activation pathway or may block binding of NF- κB to DNA transcription factor and subsequently reduce the production of corresponding cytokines (Orlikova *et al.,* 2013).

Kim *et al*. (2016) suggested that active components of Graviola extracts are potent boosters for immunity by MAP kinase activation. They treated RAW 264.7 Macrophages with the bioactive ingredients of Graviola. A slight increase (Low levels) of TNF-α, IL-1 β and Nitric Oxide Synthase (iNOS) were obtained. The increase was not significant when compared to those of cells treated with LPS alone.

Current work showed slight decrease in IL4 and IL10 levels when mice pretreated with Graviola leaf powder orally, which indicated the protective activity of these cytokines against septic shock. As it is indicated by Van Dissel, *et al.* (1998) that mice lacking IL10 were more susceptible to death by LPS challenge. On the other hand, Takeshita *et al.* (1996) confirmed that pretreatment of mice with IL4 before LPS challenge result in less mortality.

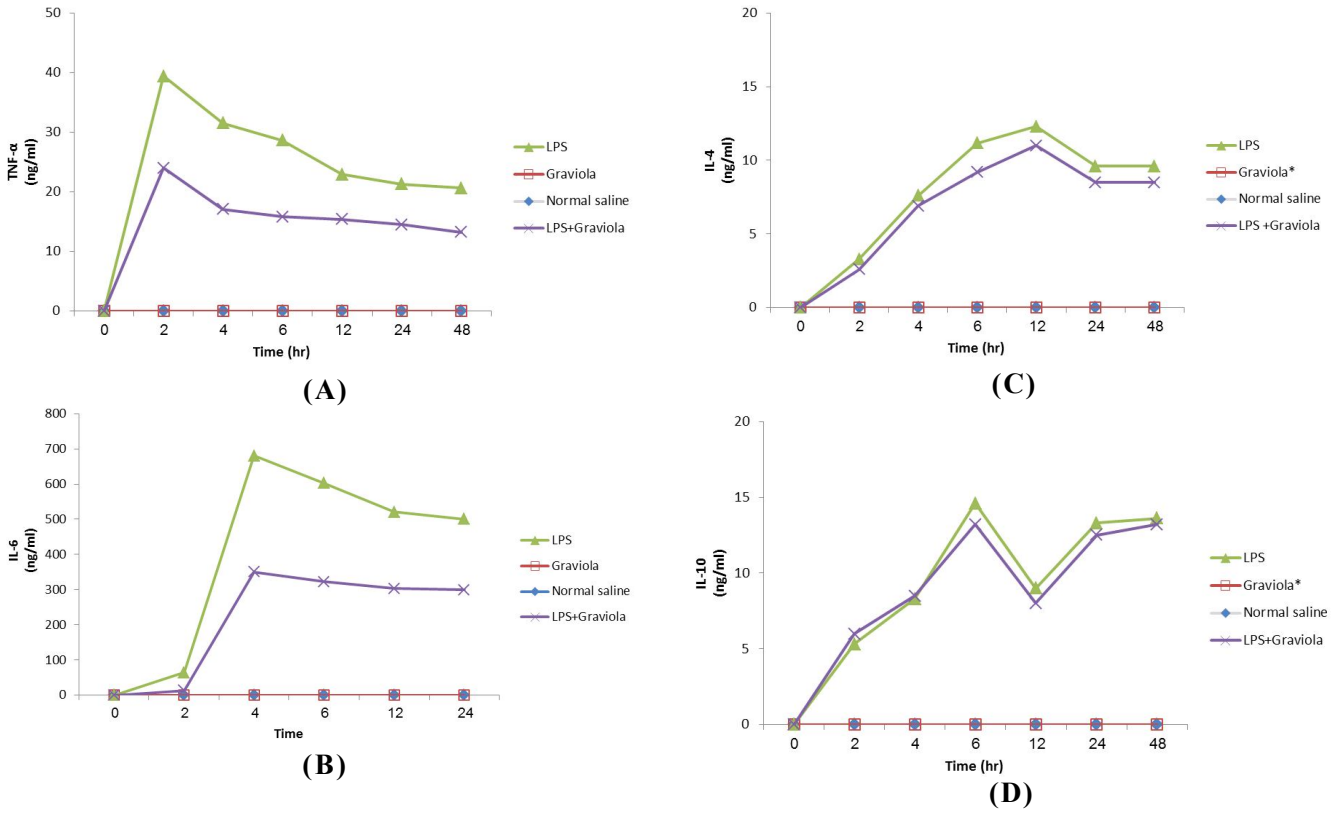

Fig. 1: Serum TNF-α, IL-6, IL-4 and IL-10 levels of mice groups (*n*= 8 mice /group), treated as: (▲ single dose LPS challenge for sepsis induction), (× Graviola leaf powder + LPS), (□ Graviola leaf powder alone) and (◇ Normal saline as negative control). Animals were given Graviola leaf powder as short term pretreatment prior to sepsis induction with LPS. Serum levels of TNF-α, IL-6, IL-4 and IL-10 were estimated at times 0, 2, 4, 6, 12, 24 and 48 hr after LPS challenge. In (a and b): both TNF-α and IL-6 serum levels decreased by mice pretreatment with Graviola leaf powder. In (c and d): a slight inhibition in serum levels of IL-4 and IL-10 occurs which was not significant.

* There were very slight increase in serum levels of IL-4 and IL-10 when mice treated with Graviola leaf powder alone were not distinguished from control groups.

It is necessary to mention, as our data showed, Graviola leaf powder has protective and immune modulating activity when taken orally. To sum up, Graviola leaf powder might be beneficial for protection against LPS- induced septic shock due to its ability to modulate the levels of circulating cytokines.

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