***Punica granatum* Leaf Lipophilic Fraction Favours Mortality by Attenuating Inflammation in Mice with Polymicrobial Sepsis**

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Abstract: *Punica granatum* L. possesses known antimicrobial and anti-inflammatory actions. Preparations of different parts of this plant have been commonly used in the folk medicine to prevent and treat infections; and studies have supported the use of *P. granatum* extracts for this purpose. However, little is known of the effects of *P. granatum*-derived fractions in severe infectious diseases such as sepsis. Here, we investigated the actions of the hexane fraction (HF), rich in lipophilic compounds, obtained from the leaves of this plant, in a murine model of polymicrobial sepsis. Oral pre-treatment with HF increased mortality in septic mice. The same animals presented with lower levels of interleukin-6, nitric oxide and hydrogen peroxide in their peritoneal lavage samples than vehicle mice. HF also increased polymorphonuclear cell accumulation in to the peritoneum of mice with sepsis. Macrophage-derived nitric oxide upon lipopolysaccharide stimuli was markedly reduced by incubation with HF. Diterpenes, triterpenes, phytosterols, vitamin E and ascorbyl palmitate were detected in the HF. These results indicate that *P. granatum* leaf lipophilic fractions may worsen sepsis outcome. This effect may be associated with its bioactive compounds which act synergistically or not, increasing sepsis mortality *in vivo*.

Key words: *P. granatum*, infection, inflammation.

1. Introduction

The emergence of bacteria that are increasingly

capable of acquiring genes related to drug resistance, virulence and immune system evasion has led to an increase in the number of sepsis cases [1, 2]. This syndrome affects millions of patients every year and has different clinical manifestations that can be influenced by several factors such as the etiologic

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agent, initial site of infection, the health status of patient, diagnosis efficiency and timing of treatment [3]. The high mortality rate (up to 70%), the long stays in intensive care units and the morbidity associated with sepsis are responsible for a great health care burden [4].

In sepsis, as a result of microbial recognition by immune cells, the production of inflammatory mediators such as nitric oxide (NO), hydrogen peroxide (H₂O₂), cytokines, amongst others, is increased [5]. However, the excessive release of inflammatory mediators can lead to tissue damage and failure of vital organs, such as the liver, kidney and pancreas, which can often result in death [6]. To counteract this, anti-inflammatory molecules are produced in an attempt to promote the resolution of this exacerbated inflammation characteristic of sepsis (see for review: [7, 8]).

Despite the high prevalence and mortality of sepsis, in addition to the recent advances in understanding its underlying mechanisms, there is a lack of novel effective drugs to treat this syndrome. The available therapeutic interventions aim to improve organ functions and bacteria eradication by the administration of intravenous fluids, antibiotics, oxygen, anti-inflammatory and anticoagulant agents [4, 9, 10], which are not always effective. Therefore, the search for new drugs able to control sepsis lethality is extremely necessary. In this context, therapies with combined antimicrobial and anti-inflammatory actions represent an interesting approach to improve sepsis outcome.

Traditionally, several medicinal plants have been used to treat inflammatory disorders including those of infectious nature [11-13]. *Punica granatum* L. (Punicaceae) is a medicinal plant widely used in different countries for the treatment of inflammatory conditions [12, 14]. The antimicrobial activity of extracts and compounds obtained from different parts of this plant has been also demonstrated [15-18] and recent studies have suggested the potential of *P.*

granatum extracts and/or compounds to treat infectious diseases [19-23]. However, the effects of *P. granatum* lipophilic-rich fractions have not yet been addressed. Here, we evaluated the effects of the hexane fraction (HF) obtained from the hydroalcoholic extract of *P. granatum* leaves in a murine model of sepsis induced by cecal ligation and puncture. HF increased mouse mortality; an effect found to be associated with reduced levels of anti-inflammatory mediator release by peritoneal cells.

2. Method and Materials

2.1 Plant Material and Preparation of the Hexane Fraction from P. granatum Leaf Hydroalcoholic Extract

The leaves of *P. granatum* were collected in September at Santa Luzia, Maranhão, Brazil (4°4'8" S, 45°41'24" W). A voucher specimen (No. 01002) was deposited in the herbarium Ático Seabra of the Universidade Federal do Maranhão, São Luís, Brazil. The collected leaves were washed in running water and were then, dried under forced air circulation at 45 °C. The dried leaves were triturated, and the resulting powder was macerated for 7 days in ethyl alcohol (70%; Sigma-Aldrich, St. Louis, MO, USA) at room temperature. The mixture was filtered through cellulose filter paper (Whatman No. 4, GE Healthcare UK, Amersham, UK) and evaporated to dryness under reduced pressure using a rotary evaporator (Eyela N-1200BV-W, Tokyo, Japan) at 40 °C. The residual solvent was removed under vacuum at 40 °C to yield crude hydroalcoholic extract. The extract yield was of 90 g/L.

The hydroalcoholic extract was further submitted to a liquid-liquid extraction using hexane. For this, a sample (10 g) of dry extract was resuspended in 100 mL of a hydromethanolic solution (water: methanol = 1:9 v/v) and mixed with 100 mL of n-hexane. After 24 h, the hexane phase was collected and concentrated under vacuum at 40 °C to yield *P. granatum* dry HF. The extraction yield of HF was of 29% in relation to

the crude hydroalcoholic extract, i.e., 290 mg/g.

2.2 Chemical Characterization by GC-MS

Analysis was conducted as described by Bombo et al. [24] with modifications. Aliquots of 200 μ L of samples (100 mg of the extract previously lyophilized and diluted in approximately 2 mL of ethanol) were saponified with 1 mL of KOH (1 M in ethanol; Merck, Brazil) at 60 °C for 1 h, mixed with 1 mL of Milli-Q water and extracted twice with 2 mL of hexane. The extract was concentrated at 30 °C and derivatized with a solution of pyridine (100 μ L) and N,O-Bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (all from Sigma-Aldrich). Samples were incubated for 1 h at 60 °C.

HF content was analyzed in a QP 2010 SE GC-MS (Shimadzu Corporation, Kyoto, Japan) equipped with a PTE-5-Supelco column 30 m $\times$ 0.25 mm, 0.25 μ m film (Supelco Inc., Bellefont, PA, USA), using He as the carrier gas. The injector temperature was 100 °C and injection volume was equal to 1 μ L. The column temperature was programmed to increase from 100 °C (3 min) to 280 °C at a rate of 30 °C/min. Column was then maintained at 280 °C for 30 min. Ion source and interface temperatures were set to 250 °C. The analysis used a split mode with a scan range of 40-1000 m/z. The total running time of GC-MS was 39 min. The relative percentages of the identified compounds were expressed as normalized peak area. Interpretation of GC-MS mass spectra was conducted using the database of the National Institute Standard and Technology (NIST) library [25].

2.3 *Escherichia coli* Infection in *Tenebrio molitor* Larvae

For initial screening of the effects of *P. granatum* hexane fraction on sepsis, *T. molitor* larvae were injected with 10 μ L of a suspension containing *Escherichia coli* ATCC 25922 ($\sim \times 10^6$ colony forming units; CFU) in phosphate-buffered saline (PBS) in to their last proleg, in aseptic conditions. Larvae were

then, incubated for 2 h, at 37 °C. Following incubation, the larvae received either vehicle (3% dimethyl sulfoxide (DMSO) in saline) or *P. granatum* (30-300 mg/kg) via the proleg route. Larvae survival rates were registered for 72 h.

2.4 Mice

Non-fasted male C57BL/6 mice (3-month old) were used. Mice were obtained from the animal facility of the Universidade CEUMA (UNICEUMA) and were kept in a climatically controlled environment (room temperature of 22 ± 2 °C and humidity around 60%) under a 12:12 h light-dark cycle (lights on at 07:00 h). Animals received either vehicle (3% DMSO in saline) or HF (100 mg/kg) *per os* (p.o.), 1 h prior to surgery. All procedures were approved by the Ethics Committee of UNICEUMA and were carried out in accordance with the Brazilian Society for Animal Welfare (SBCAL).

2.5 Sepsis Induction in Mice

Experiments in mice with cecal ligation and puncture (CLP) were designed with a pre-mortality end point (at 20 h) according to the method described by Fernandes et al. [26], with minor modifications. Animals received a single i.m. injection containing midazolam (2 mg/kg; Dormire®, Cristália, Brazil), 15 min before the surgery. During the surgical procedure, anaesthesia was maintained with ketamine (50 mg/kg) and xylazine (1 mg/kg). Surgery was performed using aseptic techniques. In brief, an incision of ~ 2 cm was made in the ventral surface of the abdomen and the cecum was exposed through the incision. The cecum was ligated at its base (without causing bowel obstruction) with silk 4-0 suture (Shalon; Sertix) and perforated with a 22-gauge needle resulting in two holes. Sham-operated animals were used as controls. Both sham and CLP mice were sutured with absorbable suture (Vicryl; Shalon; Sertix) and received 1 mL of saline 0.9% (s.c.) for post-surgery hydration. Animals were killed 20 h after surgery by

cervical dislocation. A laparotomy was performed and the peritoneal cavity was washed with 3.0 mL of PBS. The peritoneal exudate lavage fluid (PELF) was collected for further analysis [26].

In a separate series of experiment, the CLP was induced and mouse survival rates were registered over a 72-h period.

2.6 Inflammatory Cell Accumulation in the Peritoneal Cavity

Inflammatory cell migration into the peritoneal cavity was evaluated according to Costa and collaborators [27]. Total cell counts were performed with a Neubauer chamber and microscope (Zeiss Axio ImageReady Z2) after diluting a sample of the collected fluid from the peritoneal cavity with Türk's solution (1:10). Cellular smears were prepared with an aliquot (50 μ L) of the PELF to determine the differential leukocyte population. The slides were stained with Instant-Prov (Newprov, Brazil); then the analysis was carried out under an immersion objective (Zeiss Axio ImageReady Z2).

2.7 PELF Concentrations of Nitrite (NO_2^-) and Nitrate (NO_3^-) Anions

The $\text{NO}_2^-/\text{NO}_3^-$ content (as NO-end products) in PELF and supernatant samples was measured by the Griess assay as an indicator of NO production in the samples as previously described [28]. NO_3^- was reduced to nitrite (NO_2^-) by incubating 80 μ L of sample with 20 μ L of 1 U/mL nitrate reductase and 10 μ L of 1 mM NADPH for 30 min at 37 °C in a 96-well plate. Next, 100 μ L Griess reagent (5% v/v H_3PO_4 containing 1% w/v sulfanilic acid and 0.1% w/v N-1-naphthylethylenediamine) was added and incubated for 15 min at 37 °C. Absorbance at 550 nm was immediately measured. After subtraction of background readings, the absorbance in each sample was compared with that obtained from a sodium nitrite (0-100 μ M) standard curve and expressed as NO_x concentrations (μ M).

2.8 PELF Cytokine Measurements

TNF α , IL-6 and IL-10 levels were measured in the PELF samples by using mouse cytometric bead array (CBA) cytokine kits (BD Biosciences, Brazil) according to the manufacturer's instructions. Analysis was performed on a BD Accuri 6 flow cytometer (BD Biosciences-Immunocytometry Systems). Results were calculated using the CBA FCAP Array software (BD Biosciences, Brazil) and expressed as pg/mL.

2.9 Peritoneal Macrophage Cell Culture

Peritoneal cells were collected from mice injected i.p. with 1 mL of phosphate-buffered saline (PBS, Sigma-Aldrich, Brazil) containing 1% oyster glycogen (Sigma-Aldrich, Brazil). Briefly, 18 h following injection, the peritoneal cavity was washed with 10 mL of cold PBS and the peritoneal cells were harvested, centrifuged (10 min, 4 °C), and resuspended (final concentration of 2×10^6 cells/mL) in DMEM-Glutamax® (Life Technologies, Brazil) containing 10% FCS (v/v, Life Technologies, Brazil) and 1 $\times$ penicillin-streptomycin (Sigma-Aldrich, Brazil).

Cells (6×10^5 /well) were incubated in 96-well plates, at 37 °C under 5% CO_2 , and after 2 h, the non-adhered cells were removed and the adherent cells (macrophages) were incubated with either HF (1-300 μ g/mL) or vehicle (1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Brazil) in PBS), and after 15 min, stimulated with LPS (100 ng/mL in PBS, *Escherichia coli* O111:B4; Sigma-Aldrich, Brazil) or PBS for 24 h. After this period, the supernatant was collected and stored at -80 °C for further analysis of $\text{NO}_2^-/\text{NO}_3^-$ (NO end products; NO_x) concentrations. For analysis of macrophage viability, the remaining cells were incubated with PrestoBlue® reagent (1:10, Life Technologies, Brazil) for 90 min, and then, the absorbances were read at 550 and 650 nm. Results were calculated according to the manufacturer's instructions and are expressed as absorbance in percentage (%) of cell viability in relation to vehicle/PBS-treated cells.

2.10 Statistical Analysis

The results are presented as the mean $\pm$ SEM. The percentages of inhibition are reported as mean $\pm$ SEM relative to the control samples. Statistical comparisons of the data were performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls and unpaired or paired *t* test when appropriate. For comparison of survival rates between groups, data were analysed by the Mantel-Cox test. *P* values < 0.05 were considered significant.

3. Results and Discussion

Sepsis is a complex syndrome characterized by multi-organ failure; and symptom heterogeneity between patients makes hard the implementation of specific and effective treatments [29]. In this study, we investigated the effects of a lipophilic fraction obtained from the leaf hydroalcoholic extract of the well-known medicinal plant *P. granatum*, on sepsis.

We initially assessed the effects of different doses of HF on the survival rates of *E. coli* infected *T. molitor* larvae. Infection induced larvae mortality (70-90%) over 72 h (Figs. 1A and 1B). When administered after the injection of *E. coli*, HF only slightly affected larvae survival rates in comparison with those observed for vehicle-treated larvae (~ 81% in HF versus 87% in vehicle larvae; Fig. 1A). On the other hand, pre-treatment with HF considerably diminished larvae survival (Fig. 1B). Survival rates in HF pre-treated larvae were 18 % and 27 % lower at 48 h and 72 h, respectively, in comparison with those which received vehicle. HF effects on larvae survival were independent of dose, as similar survival rates were observed for all tested ones (30-300 mg/kg).

Of importance, the *P. granatum* leaf hydroalcoholic extract was found to attenuate LPS-induced peritonitis in rats, when administered as a single oral pre-treatment at the dose of 250 mg/kg [21]. Herein, HF yield was of 290 mg/g (~ one third) of *P.*

granatum leaf hydroalcoholic extract. Therefore, the dose of 100 mg/kg was chosen for testing the effects of HF pre-treatment in mice. The induced polymicrobial sepsis caused mortality in mice over the 72 h observation period. Vehicle-treated mice presented a survival rate of 76% and 42%, at 48 h and 72 h, respectively. Mice orally pre-treated with HF exhibited lower survival rates at 48 h and 72 h post-sepsis induction (63% and 27%, respectively) than those which received vehicle (Fig. 1C).

P. granatum potential to modulate infectious diseases was previously suggested in different studies [19-23]. The results obtained from both *T. molitor* larvae and mice indicate for the first time that the pre-administration of HF favours sepsis-induced mortality. In agreement, the preventive treatment with a *P. granatum* fruit extract increased mortality in rats with CLP, an effect that was associated with inhibition of the inflammatory response and increased bacterial load in these animals [30].

We next assessed the ability of HF pre-treatment to influence inflammation in mice with CLP (20 h following sepsis induction). Analysis of PELF samples demonstrated that HF does not affect total and mononuclear cell numbers (Fig. 2A and B). On the other hand, the numbers of PMN were increased by 3.7-fold, in HF-treated septic mice in comparison with sham- and CLP-vehicle controls (Fig. 2C).

Fig. 3 (A and B) depicts the production of NO and H₂O₂ in PELF samples of CLP and sham mice treated or not with HF. Both NO and H₂O₂ were raised (5.7- and 2.8-fold increase, respectively) in PELF samples of CLP mice administered with vehicle in comparison with those obtained from vehicle-treated sham controls (Fig. 3A and B). Both mediators were found to be diminished in HF-administered septic mice, with inhibitions of 64.8% and 43.6% for NO and H₂O₂, respectively (Fig. 3A and B). Similarly, *in vitro* incubation of HF reduced NO release (57%) by LPS-treated peritoneal macrophages (Fig. 4B), without

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Fig. 1 Effect of the hexane fraction of *P. granatum* leaves on septic larvae and mice. Survival rates in septic larvae (A) post- and (B) pre-treated with HF (30-300 mg/kg). (C) Survival rates in septic mice pre-treated with HF. HF was administered at different doses (100 mg/kg). Vehicle (3% DMSO in saline)-treated animals were used as controls. Sepsis survival was monitored over a 72-h period. Data were obtained from two independent experiments ($n = 20$ larvae/group and 10 mice/group).

Fig. 2 Peritoneal cell counts in septic mice treated with the hexane fraction of *P. granatum* leaves. *In vivo* cell migration to the peritoneal cavity was evaluated in mice treated with either vehicle (3% DMSO in saline) or *P. granatum* (100 mg/kg, p.o., 1 h prior), 20 h after surgery for (A) total, (B) mononuclear and (C) polymorphonuclear (PMN) cell counts. Bars represent the mean $\pm$ SEM of all mice per group obtained from three independent experiments. # $p < 0.05$ compared with CLP-operated WT mice treated with vehicle.

Fig. 3 Peritoneal NO and H₂O₂ concentrations measured in septic mice treated with the hexane fraction of *P. granatum* leaves. (A) NO (as NO_x) and (B) H₂O₂ release from the peritoneal lavage cells 20 h after CLP induction in mice treated with either vehicle (3% DMSO in saline) or *P. granatum* (100 mg/kg, p.o., 1 h prior). Bars represent the mean ± SEM of all mice per group obtained from three independent experiments. [#]*p* < 0.05 compared with CLP-operated WT mice treated with vehicle.

Fig. 4 Effect of hexane fraction of *P. granatum* leaves on peritoneal macrophage viability and nitric oxide production. (A) Cell viability and (B) nitric oxide (NO_x) production were quantified in cultured peritoneal macrophages pre-treated with HF (1-300 μg/mL, in 3% DMSO in saline) and stimulated with LPS (100 ng/mL) for 24 h. Vehicle-treated cells were used as controls. Bars represent the mean ± SEM of all mice per group obtained from three independent experiments (*n* = 4/group). ^{*}*p* < 0.05 compared with vehicle-treated cells; [#]*p* < 0.05 compared with LPS-treated cells.

significantly affecting their survival (Fig. 4A). of note, HF had no effects on control cells; i.e., those not stimulated with LPS (Figs. 4A and B).

Different anti-inflammatory effects were previously reported for *P. granatum* extracts or compounds. Indeed, *P. granatum* fruit decoction was found to inhibit NO production in RAW 267.4 macrophage cells stimulated with toxins derived from *Gambierdicus* spp., a pathogen causative of infections in fish [31]. Similarly, *in vitro* LPS-induced NO production by microglial cells was found to be reduced by incubation with *P. granatum* seed oil [32].

Herein, increased concentrations of TNF α (2.5-fold increase), IL-6 (26.1-fold increase) and IL-10 (354.1-fold increase) were detected in PELF samples obtained from vehicle-treated septic mice in comparison with the sham-operated groups (Figs. 5A-C). IL-6 was the only cytokine found to be

reduced (56%) in HF-treated septic mice in comparison with the CLP-controls (Figs. 5A-C).

Additional anti-inflammatory actions of *P. granatum* in cytokine production were also reported in both *in vitro* and *in vivo* models. In a recent study, the hydroalcoholic extract obtained from the leaves of this plant was found to diminish the levels of peritoneal TNF α and to reduce the accumulation of cells (including neutrophils) in to the peritoneum of mice with LPS-induced peritonitis [21]. Similar effects on TNF α production were also observed *in vitro* for *P. granatum* seed oil and methanol extract in microglial cells [32, 33] and for its leaf hydroalcoholic extract in LPS-injected rats [21] and in a rat model of CLP-induced sepsis [20].

The results gathered in our study indicate that septic animals pre-treated with HF present with lower concentrations of IL-6, NO and H₂O₂ in their PELF in

Fig. 5 Effect of the hexane fraction of *P. granatum* leaves on cytokine generation in the peritoneum during sepsis. (A) TNF α , (B) IL-6 and (C) IL-10 generation in the peritoneum of mice treated with either vehicle (3% DMSO in saline) or HF (100 mg/kg, p.o., 1 h prior), 20 h after CLP induction. Bars represent the mean $\pm$ SEM of all mice per group obtained from three independent experiments. * p < 0.05 compared with sham-operated, # p < 0.05 compared with CLP-operated WT mice treated with vehicle.

comparison with those observed for vehicle-controls. However, the *in vivo* treatment with HF increased the accumulation of PMNs in to the peritoneum of the same mice. Discrepancies between our study and those showed decreases in leukocyte accumulation and in cytokine production may be related to the different models and samples used to assess *P. granatum* anti-inflammatory effects (rats versus mice; *in vitro* versus *in vivo*; LPS versus CLP; PELF versus liver tissue samples).

Differences in the chemical profiles of the extracts/oils of *P. granatum* investigated in these studies may also impact the observed results. Therefore, a GC-MS analysis was performed to identify the compounds composing the lipophilic fraction of *P. granatum* leaves. Diterpenes, triterpenes and phytosterols were detected in the HF Phytosterols included γ -sitosterol ($C_{29}H_{50}O$), stigmast-4-en-3-one ($C_{29}H_{48}O$), stigmast-5-en-3-ol, oleate ($C_{47}H_{82}O_2$) and stigmasta-5,22-dien-3-ol-acetate ($C_{31}H_{50}O_2$). The identified triterpenes included α -amyirin (ursane skeleton; $C_{30}H_{50}O$) and β -amyirin (oleanane skeleton; $C_{30}H_{50}O$). Tocopherol (vitamin E; $C_{29}H_{50}O_2$), ascorbyl palmitate (vitamin C; $C_{22}H_{38}O_7$), phytol acetate (3, 7, 11, 15-tetramethyl- 2-hexadecenyl acetate; $C_{22}H_{42}O_2$) and phytol (3, 7, 11, 15-tetramethyl-2- hexadecen-1-ol; $C_{20}H_{40}O$) were also observed (Table 1).

Coumarins, flavonoids and phenolic acids were

previously detected in the crude leaf hydroalcoholic extract of *P. granatum*, with flavonoids suggested to be its major anti-inflammatory molecule [21]. Other compounds (elagic acid, punicalagin and gallic acid) found in *P. granatum* ethyl acetate fractions were also suggested to underlie the anti-inflammatory actions of this plant [19, 20, 22, 23, 33-35]. Here, the chromatographic analysis of the HF revealed the presence of diterpenes (phytol and phytol acetate), triterpenes (α -amyirin and β -amyirin), phytosterols (γ -sitosterol, stigmast-4-en-3-one, stigmast-5 -en-3-ol, oleate; and stigmasta-5, 22-dien- 3-ol-acetate), tocopherol and ascorbyl palmitate. These compounds were previously shown to be not only antimicrobials, but also to present anti-inflammatory properties.

Indeed, α -amyirin and β -amyirin have been pointed as potent inhibitors of inflammation and hepatic oxidative stress [36-38]. Recently, both α -amyirin and β -amyirin were found to reduce NO production by RAW 264.7 macrophages stimulated with LPS without affecting their viability *in vitro* [39, 40]. β -amyirin also inhibited the secretion of IL-6 by LPS-treated human peripheral blood mononuclear cells [41]. Although the effects of these compounds have not been previously addressed in sepsis survival, it was shown that pre-treatment with these compounds prevents liver injury-associated mortality in mice [42].

The pharmacological actions of phytosterols have also been studied. Of importance, phytosterols such as

Table 1 Detection of bioactive phytochemicals in the hexane fraction obtained from the leaf hydroalcoholic extract of *P. granatum*.

Name of the compound	Molecular formula	Molecular weight	Peak area (%)
Phytol	$C_{20}H_{40}O$	296.5300	0.26
Phytol, acetate	$C_{22}H_{42}O_2$	296.3079	0.25
Gamma, Sitosterol (3b,24S)-Stigmast-5-en-3-ol)	$C_{29}H_{50}O$	414.7100	5.67
Stigmast-4-en-3-one	$C_{29}H_{48}O$	412.702	6.16
Stigmast-5-en-3-ol, oleate	$C_{47}H_{82}O_2$	679.171	4.13
Stigmasta-5,22-dien-3-ol, acetate, (3.beta)	$C_{31}H_{50}O_2$	454.739	3.49
Vitamin E (α -tocopherol)	$C_{29}H_{50}O_2$	430.717	6.41
α -amyirin	$C_{30}H_{50}O$	426.729	7.58
β -amyirin	$C_{30}H_{50}O$	426.729	6.65
Ascorbyl palmitate	$C_{22}H_{38}O_7$	414.539	4.20

β -sitosterol were found to inhibit the production of pro-inflammatory cytokines (TNF α , IL-1 β and IL-6) and NO by cultured LPS-stimulated RAW 264.7 macrophages, in a NF κ B-dependent manner [43-46]. Additionally, they were found to inhibit LPS signaling in J774A.1 and intestinal macrophages [47, 48]. β -sitosterol, at a concentration of 200 μ g/mL, was suggested to cause toxicity in cultured macrophages [49], an effect that may be concentration-dependent. Here, we assessed HF effects in cultured peritoneal macrophages at doses ranging from 1-300 μ g/mL; with increased survival observed in these cells.

Phytol also presented anti-inflammatory responses. A study by Silva and collaborators [50] showed this compound inhibits PMN cell migration and activation, in addition to reduce oxidative stress and cytokine generation in mouse carragenan-injected paws. However, we were not able to establish a link between the observed PMN accumulation in HF-treated septic mice and the alterations in inflammatory mediator production in the same mice.

Ascorbyl palmitate and vitamin E are potent antioxidants suggested to be protective in hyper-inflammatory states [50-53]. Similarly, vitamin E derivatives ETS-GS and 2-(α -D-glucopyranosyl) methyl-2,5,7,8- tetramethylchroman-6-ol are found to protect against sepsis in rats, increasing their survival and attenuating lung injury [54, 55] in a macrophage-dependent manner [54]. Here, HF enhanced mortality in septic mice. Data obtained allow us to suggest that the inhibition of IL-6, H₂O₂ and NO production associated with subsequent decrease in survival of septic mice may be linked to the ability of all the above mentioned compounds to suppress NO and cytokine-related pathways such as the activation of NF κ B and iNOS.

4. Conclusions

Overall, we show that the HF obtained from *P. granatum* leaves increases mortality in septic mice by attenuating the release of key inflammatory mediators

in a macrophage-dependent manner. This action may be attributed to the presence of different bioactive phytoconstituents detected in this fraction, which may either synergistically or additively, contribute to the lethal activity of HF in sepsis.

Conflicts of Interest

The authors declare no conflicts of interest.

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