

# Photoimmunological properties of borage in bovine neutrophil *in vitro* model



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## ABSTRACT

Borage (*Echium amoenum fisch*) is one of the most commonly used medicinal plants, and has long been used as a traditional herbal medicine for many (non)infectious diseases in Iran. Study on photoredox and photoimmunology of borage is little. Natural immunomodulatory plants with minimal adverse/toxic effects could help boost animal health and, ultimately, public health. To determine the effect of borage on the functions of key circulating innate immune cells, effects of borage extract (BE) on bovine neutrophils (PMN) photoredox and phagocytosis events were evaluated using an *in vitro* model system. Blood PMN isolated from healthy high yielding dairy cows ( $n = 8/\text{treatment}$ ) were pre-incubated with BE and the impact on phagocytosis-dependent-and-independent cellular chemiluminescence (CL), phagocytosis, killing of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), fluorescence-based PMN  $\text{H}_2\text{O}_2$  production and necrosis were assessed. Relative to control (no BE) PMN, treatment with BE significantly increased phagocytosis-dependent-and-independent PMN CL (>10–15% increase). While BE also led to increased PMN  $\text{H}_2\text{O}_2$  production, necrosis was also surprisingly higher in these cells. Phagocytosis and killing of both *E. coli* and *S. aureus* by PMN treated with BE was substantially higher than that by control PMN. The increased photoimmunobiological events especially intracellular CL, intracellular  $\text{H}_2\text{O}_2$  formation, and phagocytic capacity of BE-treated PMN support the potential immunotherapeutic implications of borage and its components for particularly immunocompromised animals and humans.

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## 1. Introduction

Medicinal plants have played an important role in the treatment of many diseases [1–3]. Owing to its medicinal/nutraceutical properties, borage (*Echium amoenum fisch*) has been used as an effective herbal medicine [4,5] for many (non)infectious diseases, worldwide. Despite its promising implication in traditional medicine, little *in vivo* or *in vitro* experimental evidence is available about photoimmunology, photoredox and immunomodulatory properties of borage in animal/human models.

Neutrophils (PMN) are pivotal circulating effector innate immune cells that are key to the phagocytosis and removal of pathogens [6–11]; their appropriate functions are vital for the host [12–14]. Mechanistically, PMN are capable of producing large amounts of various substances, especially [intracellular] reactive oxygen species (ROS), to efficiently kill engulfed microbes [10,13,15–17]. Any substance that disturbs PMN function can

compromise innate immunity [7–9]. Despite antibiotic application and advances in nutrition, peri-partum mammals, especially high yielding food animals, are very susceptible to environmental microbes; in many cases, an underlying reason for this is functional impairment of the host PMN [6,12,13,15–17]. Similar phenomena have also been documented in humans [11,14,18].

Nutritional immunotherapists have been investigating whether and how well-known medicinal plants like borage might potentially be of use in improving innate immune functions in particularly immunocompromised hosts. As such, in the study here, bovine PMN have been selected as the model for analyzing the potential photoredox and immunomodulatory effects of borage (more precisely, a borage extract [BE]) *in vitro*. In these studies, effects of BE on PMN phagocytosis-dependent-and-independent cellular chemiluminescence (CL), phagocytosis, killing of pathogens (i.e., *Staphylococcus aureus* and *Escherichia coli*), necrosis, and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production were examined. The data from these studies would reveal new insights into the photoimmunological properties of borage and its components, and so provide additional information concerning potential functional,

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nutraceutical, prophylactic, and therapeutic uses for borage in immunocompromised and diseased mammalian hosts, especially human.

## 2. Materials and methods

### 2.1. Blood neutrophil (PMN) preparation

A group of eight healthy pregnant lactating Holstein cows were used as the source of PMN for the assays herein. According to our previous studies on bovine neutrophil functions [6,7,12,13], physiological conditions of dairy cows, such as age, stage of lactation, parity and health status are key attributable to neutrophils functions; in our study in order to maximally exclude the contribution of the error term to our analyses we tried to maximally restrict those cows' contributable factors to our neutrophil functional assays. As such, in our study a group of clinically healthy primiparous Holstein dairy cows from one of industrial dairy cattle farms (Mashhad, Iran;  $n = 8$ ) were selected and used as a source of PMN for experimental assays. Experiments were performed in mid-September. The dairy cows (age  $35.05 \pm 0.46$  months) were all pregnant ( $87.38 \pm 6.08$  days of pregnancy) and in mid lactation ( $147.38 \pm 7.06$  days in milk). Blood samples were aseptically collected from the external jugular vein into heparinized vacutainer tubes. After first differentiating the relative levels (%) of nucleated blood cells from blood smears [6], PMN were isolated from the entire blood sample by an initial hypotonic lysis of erythrocytes present followed by gradient centrifugation [6]. From the resulting cell pellet, the total number of isolated PMN was quantified with a MEK 6450K Coulter counter (Nihon Kohden Celltac, Tokyo, Japan). These procedures yielded >98% granulocytes (PMN + eosinophil; predominantly PMN [>87%]) with a viability of >98% [6,8]. To ensure reproducibility between days and animals, each functional assay was performed with the same number of calculated viable PMN. For use in each study, PMN suspensions were adjusted to  $5 \times 10^6$  viable PMN/ml with Dulbecco's phosphate-buffered saline (DPBS; Sigma, Deisenhofen, Germany). Use of the animals for these non-clinical studies (i.e., blood sampling for the *in vitro* cell culture assays) was in accordance with the local human/animal welfare regulations and were approved by the ethical committee of Ferdowsi University of Mashhad.

### 2.2. Borage extraction

Fresh borage flowers were collected from the Alborz Mountains south of the Caspian Sea (~2800 m above sea level; Roodsar, Iran). The samples were desiccated in the dark and then samples (~6 g) were soaked in pyrogen-free DPBS [at 2% w/v] at ~85 °C for 10 min so as to release the extract (BE) [1,8]. The extract was then passed through a 0.2- $\mu$ m filter and held at 4 °C for later use in the various *in vitro* assays outlined below. The yields were routinely 15.4% (g extract/g dried borage). Aliquots of filtered water extracts were also assessed for the presence of endotoxin using stress-responsive magnetoelastic sensors [19]. These assays indicated a negligible presence of endotoxin in the extracts (i.e., <0.01 endotoxin unit (EU)/ml, equaling  $\approx <1$  pg LPS/ml).

### 2.3. Effect of borage on PMN phagocytosis dependent-and-independent chemiluminescence

Isolated PMN were exposed to BE (0.1 dilution of 2% [w/v] water extract in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml [all from Sigma, Deisenhofen, Germany]) in 12-well culture plates for 12 h in a humidified 37 °C atmosphere containing

5% CO<sub>2</sub>. Wells without BE served as controls. The use of this dose was based on pilot experiments to determine minimal BE concentrations to use that imparted non-cytotoxic effects on the PMN.

CL assays were performed in 96-well white flat-bottom microtiter culture plates (Nunc, Wiesbaden, Germany) with each well containing  $4 \times 10^5$  PMN/200  $\mu$ l. For the non-phagocytosis-dependent CL assays, phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO) at a final concentration of 200 ng/ml was used as the PMN activator. For the phagocytosis-dependent CL assays, either polystyrene beads (0.76  $\mu$ m diameter,  $4 \times 10^{11}$  particles/ml; Sigma) were added at 130/PMN, or opsonised *S. aureus* (Pansorbin<sup>®</sup>, Calbiochem, Carlsbad, CA) at 25/PMN. In all cases, immediately after addition of the PMN activator, luminol (at final concentration of 0.3 mM; Sigma) was introduced into each well (60  $\mu$ l/well) and measures of CL were then performed continuously over a period of 30 min in a Mithras LB 940 luminometer (Micro-Win, Bad Wildbad, Germany). The area under the curve (AUC) was calculated for registered impulse rates (counts/min) over the entire 30 min period.  $T_{max}$ , which is expressed as time when peak intracellular ROS generation happened during 30 min of CL measure, was also quantified during the CL assays. All CL measures were carried out at pH of 7.2 and at 37 °C. Ultimately, the CL response was corrected for 1000 viable PMN in each sample.

### 2.4. Effect of borage on flow cytometry-based H<sub>2</sub>O<sub>2</sub> production and necrosis of PMN

Modified H<sub>2</sub>O<sub>2</sub> production tests were performed in 96-well round-bottom microtiter plates (Corning, NY) [8,20]. Each well was pre-filled with 100  $\mu$ l isotonic Percoll (Pharmacia, Freiburg, Germany) to avoid adherence and loss of activated PMN. For the detection of H<sub>2</sub>O<sub>2</sub> produced, non-fluorescent dihydro-rhodamine (DHR 123, Mobitec, Goettingen, Germany) dye at a final concentration of 750 ng/ml was added to each Percoll cushion. In this case, the DHR 123 is oxidized to green-fluorescent rhodamine 123 due to the catalytic action of PMN MPO (myeloperoxidase). PMN (50  $\mu$ l of  $10^7$ /ml) suspensions were overlaid on the Percoll cushions in a total volume of 200  $\mu$ l/well; thereafter, 20  $\mu$ l BE (at ~300  $\mu$ g/ml) was added to dedicated wells. After 12 h incubation at 37 °C, the relative amount of H<sub>2</sub>O<sub>2</sub> generated by the 100 nM PMA-activated PMN was measured by flow cytometry (FACScan<sup>®</sup>, Becton Dickinson, Heidelberg, Germany). For this, a determination of relative (mean) green fluorescence intensity of gated PMN populations was done after acquisition of 10,000 events/sample.

For determination of necrosis, isolated PMN ( $2 \times 10^5$ /well of 96 round-bottom microtiter plate) were incubated for 12 h at 37 °C in the presence of the aforementioned concentrations of BE in culture medium. After flow cytometric acquisition of the PMN incubated with and without BE, the absolute numbers of viable PMN were determined by a standard cell dilution assay [8,21]. The procedure was adapted for the bovine PMN with slight modifications as previously described [22]. Reduced numbers of viable PMN compared to controls indirectly indicated necrotic PMN.

### 2.5. Effect of borage on flow cytometry-based PMN phagocytosis

In these experiments, the BE treatment was a dose of ~300  $\mu$ g BE/ml for 12 h prior to assaying activity. The use of this dose was based on pilot experiments to determine minimal BE concentrations to use that imparted non-cytotoxic effects on the PMN. Accordingly [23,24], to determine the effect of BE on PMN functional activity, 10<sup>7</sup> fluorescein isothiocyanate (FITC)-loaded polystyrene microparticles (1.0- $\mu$ m, 20 beads/PMN; Sigma) were

added to  $10^6$  PMN post-treated with and without BE and incubated for 12 h at 37 °C in the humidified 5% CO<sub>2</sub> atmosphere in six-well cell culture plates. PMN were then harvested on ice, washed with ice-cold DPBS and internalization of the particles assessed by flow cytometry with a minimum event/count of 10,000. Results were recorded as mean fluorescence intensity (MFI, intensity of phagocytosed FITC-labeled microparticles); the number of FITC<sup>+</sup> PMN reflected the number of PMN that could phagocytize microparticles and so reflected phagocytic ability of the PMN.

### 2.6. Effect of BE on PMN-pathogen interactions and microbicidal activity

Phagocytosis of and bactericidal activity against *E. coli* (P4:032) and *S. aureus* Newbould 305 [25] by the PMN were assessed in a bactericidal assay described in [7]. In brief, the PMN were exposed to the BE (at a dose of  $\approx 300$   $\mu\text{g/ml}$ ) or medium only for 12 h. The dose was chosen to avoid cytotoxic effects on PMN. At the same time, the *E. coli* and *S. aureus* were cultured in nutrient broth at 37 °C for 18 h before the levels of the bacteria in each culture were roughly estimated by measures of turbidity [with optical density (OD) of >1]; the actual concentrations of each organism was assessed using 10-fold serial dilutions and then plating (in triplicate) on Columbia sheep blood agar to provide accurate estimates of the infecting levels to be used in the uptake/killing assays. To prevent any further growth of the organisms prior to the actual experiment, each original media containing the specific bacteria was placed at 4 °C for 24 h until used in the protocol. Samples of each media were re-plated to confirm that the bacterial concentrations had not changed dramatically from the day before.

For the PMN-bacteria co-incubations, into 1.5 ml microtubes (in final volume of 1 ml) was placed 100  $\mu\text{l}$  *S. aureus* or *E. coli* ( $5 \times 10^7$  cfu/ml), 500  $\mu\text{l}$  BE-exposed (or non-exposed) PMN ( $5 \times 10^6$  viable cells in HBSS), and 400  $\mu\text{l}$  pooled heat-inactivated bovine serum (5% [v/v]). Control tubes (C0, C60; expressed as cfu/ml) contained bacteria, HBSS, and serum without PMN. The microtubes were then rotated end-over-end at 37 °C for 60 min. Samples (25  $\mu\text{l}$ ) were taken from the mixture assay (Ma0, Ma60; expressed as cfu/ml) at 0 and 60 min, diluted in 1 ml ddH<sub>2</sub>O and kept at 0 °C for 3 h to disrupt the PMN. Extracellular (EC; expressed as cfu/ml) bacteria were separated from the PMN by centrifugation (100g, 1 min, 4 °C). A 25  $\mu\text{l}$  sample of the supernatant from Ma60 was taken and diluted as for the mixture assay samples.

Ten-fold serial dilutions of C0, C60, EC, Ma0, and Ma60 were performed and the last dilutions spread onto Columbia sheep blood agar. After overnight incubation at 37 °C, bacterial colonies were counted and the net change relative to the original bacterial suspension levels calculated. Results from the bactericidal assay were expressed as % phagocytosis and killing of bacteria using the reported formulas [7,11].

### 2.7. Statistical analyses

The phagocytosis-and-non-phagocytosis-dependent CL results and the flow cytometry results for phagocytosis, necrosis, and H<sub>2</sub>O<sub>2</sub> production assays were presented as mean  $\pm$  SEM. After performing tests of normality, comparisons of means between BE-treated and control groups was performed using an independent sample *t*-test. A *p*-value  $\leq 0.05$  was accepted as significant.

## 3. Results

### 3.1. Effect of borage on PMN phagocytosis dependent-and-independent chemiluminescence

The PMN phagocytosis-(in)dependent CL/(non)particle-stimulated CL results showed a significant enhancement in

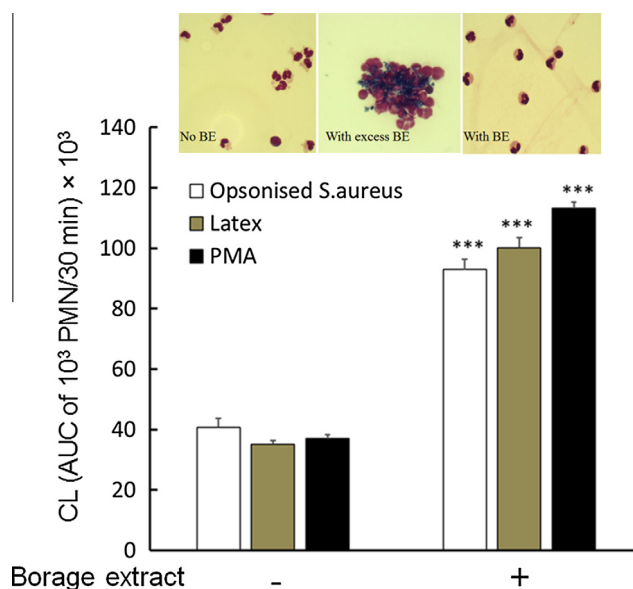
phagocytic activity by BE-exposed PMN (Fig. 1). Similar patterns of increase were noted in PMA-stimulated CL assay. Among PMN stimulated with PMA, latex beads and Pansorbin<sup>®</sup>, the AUC for the BE-exposed PMN were, respectively, 32%, 35%, and 43% greater than observed in respective counterpart control cells. Further, the  $T_{\text{max}}$  in the BE-treated PMN stimulated with PMA, latex, and Pansorbin<sup>®</sup> was increased by 24%, 38%, and 30%, respectively (Table 1), relative to the values for the controls.

### 3.2. Effect of borage on flow cytometry-based PMN H<sub>2</sub>O<sub>2</sub> production and necrosis

The flow cytometry-based measures of H<sub>2</sub>O<sub>2</sub> production by freshly-isolated PMN pre-exposed to BE revealed there was a higher percentage of DHR 123-labeled PMN (i.e., PMN responded with clear shift in fluorescence intensity, indicating enhanced respiratory burst activity and/or increased intracellular H<sub>2</sub>O<sub>2</sub> production) among the BE-exposed PMN (Fig. 2, left panel). In contrast, in the FACS assay to determine percentage PI<sup>+</sup> PMN (i.e., PMN necrosis), a surprisingly increased percentage of necrosis was observed among the BE-exposed PMN (Fig. 2, right panel).

### 3.3. Effect of borage on PMN phagocytic and killing capacity

The flow cytometry-based PMN phagocytosis assay revealed there was a significant increase in phagocytosis of FITC-labeled microparticles by post-BE treated PMN (Fig. 3, right panel). This increase in function was consistent with the results of the PMN-pathogen interaction assays (Fig. 3, left panel). Indeed, BE caused PMN to be significantly more efficient/potent at killing of two human/animal pathogens (*S. aureus* and *E. coli*). The rate of killing was greatly higher ( $\sim 10\%$  in phagocytosis and  $\sim 15\%$  in killing activity) in the BE-exposed PMN relative to in/by control cells.



**Fig. 1.** Luminol-dependent chemiluminescence (CL) in bovine blood neutrophils (PMN) exposed for 12 h with and without borage extract (BE at a dose of  $\approx 300$   $\mu\text{g/ml}$  or medium) the PMN were then stimulated by PMA (black bars), latex beads (gray bars) and opsonized Pansorbin<sup>®</sup> (white bars). Results clearly show positive effects of BE on bovine PMN photoredox activity and function. Insert figures representatively confirm the high purity and similar light microscopic appearance of PMN in both groups, though excess BE led to a different microscopic appearance. Values are expressed as area under the curve (AUC) of relative photon/light units of 1000 viable PMN during 30 min. Data shown are mean  $\pm$  SEM ( $n = 8$ ). \*\*\**p* < 0.001 vs. control.

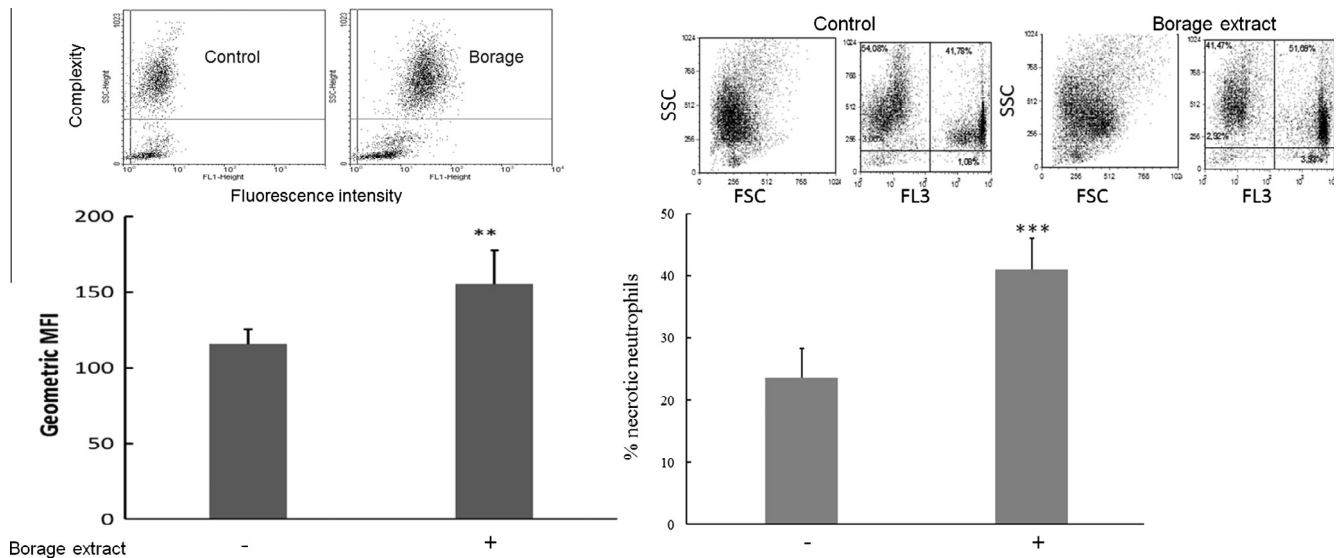
**Table 1**

$T_{\max}$  of luminol-dependent chemiluminescence (CL) in stimulated bovine blood PMN. Control and borage extract (BE)-treated PMN were stimulated by phorbol-12-myristate-13-acetate (PMA), latex beads, or opsonized *S. aureus* (Pansorbin®). Values shown are mean  $\pm$  SEM ( $n = 8$ ).

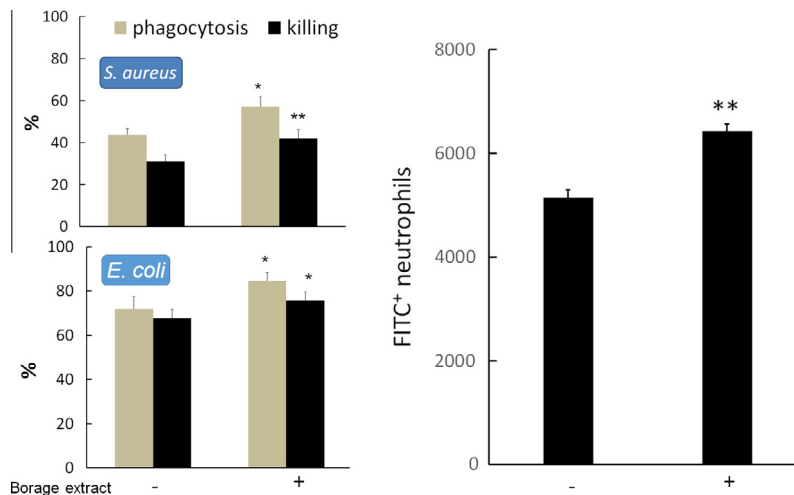
	PMA		Latex beads		Pansorbin®	
	Control	BE	Control	BE	Control	BE
$T_{\max}$	3.92 $\pm$ 0.51	16.38 $\pm$ 1.10**	5.90 $\pm$ 0.44	19.40 $\pm$ 0.61**	7.5 $\pm$ 0.85	24.8 $\pm$ 0.95**

$T_{\max}$  is expressed as time when peak intracellular ROS generation happened during 30 min of CL measure.

\*\*  $p < 0.01$  vs. control.



**Fig. 2.** Representative results of flow cytometric determinations of neutrophils (PMN)  $H_2O_2$  production (left upper portion of figure) and necrosis (right upper portion of figure). Left half of figure presents borage extract (BE)-(non)treated for 12 h with and without (BE at a dose of  $\approx 300$   $\mu\text{g}/\text{ml}$  or medium) PMN ROS production in terms of mean green fluorescence intensity (MFI). Dihydrorhodamine added to show amount of ROS production. Right half of figure presents results of BE-(non)treated overall necrotic neutrophils (%) (lower panel). In both cases, data shown are mean  $\pm$  SEM ( $n = 8$ ). Data are significantly different (\*\* $p < 0.01$  or \*\*\* $p < 0.001$ ) vs. corresponding control value.



**Fig. 3.** Borage extract (BE) boosted phagocytic and killing capacity of the bovine neutrophils (PMN). Left panels: phagocytosis/killing of *S. aureus* and *E. coli* after incubation with isolated PMN exposed to BE or medium only for 12 h (BE at a dose of  $\approx 300$   $\mu\text{g}/\text{ml}$  or medium). Right panel: phagocytosis of 1- $\mu\text{m}$  fluorescent microparticles by PMN exposed to BE or medium. Data shown in all cases are mean  $\pm$  SEM ( $n = 8$ ). Data are significantly different (\* $p < 0.05$  or \*\* $p < 0.01$ ) vs. corresponding experiment control.

#### 4. Discussion

While there has been some investigation of the effects of borage on immune cells [26–29], photoredox effects on PMN are not well known/poorly documented; this is especially so in the case of animal/human's PMN's key functions, phagocytosis and intracellular killing capacity. *Note to Readers:* Technically, the introduction of

colored agents (like BE) into culture media usually is problematic for luminometry- and flow cytometry-based assays [6,8]. As such, the assays in the present study used BE levels based on pilot experiments wherein the minimal amounts of BE that would cause interference with the luminescent/fluorescent endpoints assayed and also imparted minimal cytotoxicity toward the test PMN were determined. Based on these test concentrations, these studies



clearly showed that BE-exposed PMN underwent functional changes, and that the PMN were still able to efficiently kill pathogenic bacteria in part by increases in intracellular photoredox activation and thus formation of intracellular ROS, including H<sub>2</sub>O<sub>2</sub> production. Indeed, the luminol-dependent CL used here provided significant information about intracellular H<sub>2</sub>O<sub>2</sub> production capacity of these cells [3,7,12,13,17,30,31]. Nonetheless, in order to evaluate if the increase of ROS was more dangerous (in the case of extracellular) or really helpful (in the case of intracellular) for the mammals, with various CL probes (e.g., lucigenin, isoluminol, peroxyoxalate, ortho-phenanthroline, indoxyl-beta-glucuronide etc., [6,7,30,31]) it is worth examining the differences between intracellular and extracellular ROS generation of post BE-exposed PMN as well as quantifying various ROS in cell-free and cellular CL systems. It should also be taken into account that the inevitable co-existence of neutrophils (~>87%) and eosinophils (~<13%) in our isolated PMN and practically little elegant approach is available to remove eosinophils from neutrophils during PMN isolation procedure; it is therefore more precise to mention semi-purified batch of PMN rather than absolutely pure PMN.

The results of the phagocytosis dependent and independent CL assays, demonstrating increases in  $T_{max}$  values (time period [length] of maximal ROS production by PMN), supported the hypothesis here that BE could efficiently enhance the antimicrobial potential of PMN. Photoimmunologically, the increase in  $T_{max}$  from 6 to 19 min reflects how the treated PMN could potentially also be more potent/efficient against invading pathogens *in situ* [7,8,15,16]. Similar changes were reflected in the changes in MFI among BE-exposed PMN; these, in turn, also reflected enhancements of overall ROS production by the cells. Consistent with these changes were also the results from the CL- and flow cytometry-based analyses of PMN functions; these revealed consistent increased phagocytosis-dependent CL, phagocytosis of microparticles and the uptake/killing of *S. aureus* and *E. coli* by BE-exposed PMN. Such outcomes were in line with many of the previously documented beneficial modulatory effects of borage (and its extract) [4,5,7].

Purposeful immunomodulation, especially using an approach that strengthens PMN (and/or other innate immune cell) photoimmunological functions with materials other than antibiotics (for example, medicinal plants like borage), can hopefully help to reduce the incidence of (non)infectious disease in mammals, especially those used directly/indirectly for generation of food products, including high-yielding dairy cows. This is most important in the instances when such animals are more likely to be immunosuppressed (i.e., during peripartum and early lactation periods) [6,7,15,16,32]. It is believed that not only diseases like mastitis and metritis are common in postpartum, but also other infectious diseases like salmonellosis and paratuberculosis can be clinically observed. As such, natural immunomodulatory plants/compounds with minimal adverse/toxic effects could help boost animal health and, ultimately, public health [2].

On the other hand, while borage also led to increased PMN H<sub>2</sub>O<sub>2</sub> production, necrosis was also surprisingly higher in these cells. The observed increase in post BE-exposed PMN necrosis might partially weaken therapeutic and nutraceutical applications of borage in animals and human. This challenging issue should be further examined whether the necrosis is resulted from apoptosis pathways. Nevertheless, the potential positive perspective for what concerns an increase in PMN necrosis would be the challenging concepts that one of the medicinal anticancer properties of any medicine/natural compounds is directing the cancerous cells toward necrosis/apoptosis. We have recently found the immunosuppressive effects of aflatoxins in human and animals with slight anti-necrosis/apoptosis affects [8,28], and this toxin is categorized as hazardously highly carcinogen, and its carcinogenicity might

partly be from its inhibitory effects on necrosis/apoptosis. As such, the pronecrotic properties of borage would also be regarded as helpful/positive perspective for borage's broad therapeutic (i.e., anti-inflammatory/anti-cancer) implications.

Because this study was undertaken to obtain an understanding of the general effects of borage/BE on key circulating innate immune cells, we did not seek to specifically identify constituents within the BE that might directly be impacting on the PMN functions. Indeed, there are many known chemical agents/classes of agents like flavonoids, bisphenols, polyphenols and phenolic compounds, alkaloids, anthocyanidine and rosmarinic acid [1,28] that are known to be present in borage/BE. Based on the current findings, determining which of the various agents (alone or in combination) are responsible for the impact on PMN functions (increased PMN's photoredox activity) are clearly worth examining. Further, it is worth performing borage/BE's impact on other immune cells in other species and eventually human *in vitro/situ/vivo*, and eventually as food additives to bolster immunity of immunocompromised animals/humans. Such studies are already underway in our laboratories.

Apart from obtaining clarity as to which are the active immunomodulatory constituents in the borage/BE, more studies on the potential toxic dose range for borage/BE in mammals also need to be undertaken (i.e., determine LD<sub>50</sub>/LC<sub>50</sub> for untoward immunotoxic/adverse effects with conducting experiments using a different time course and a different concentration approaches.) before more widespread use of this material can be undertaken. In addition, potential synergistic effects of borage/BE with other commonly used supplements and/or antibiotics need to be examined. In spite of those items that still need to be done, the findings here highlight the concept of the potential use of borage (or more precisely, its extract) in nutritional immunology as a prophylactic for use in therapeutic regimens for immunocompromised animals and eventually, potentially, humans.

### Conflict of interest

The authors declare no conflict of interest.

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