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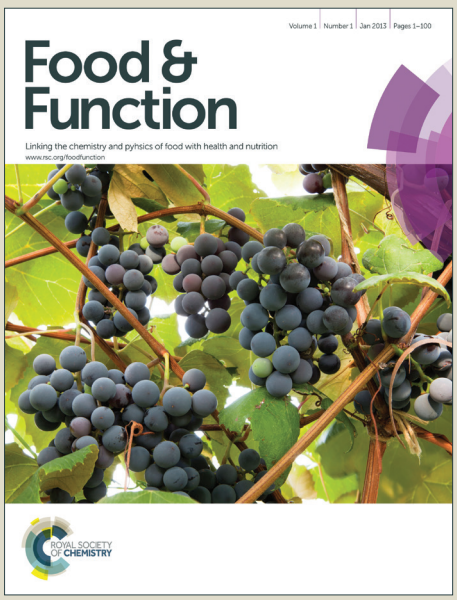

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Prepared for submission to: *Food & Function*

Title: *Bacopa monnieri* (L.) exerts anti-inflammatory effects on cells of the innate immune system *in vitro*

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Running Title: Anti-inflammatory activity of *Bacopa monnieri*

Keywords: Innate immune system; inflammation; *Bacopa monnieri*; Brahmi; memory; cognition; neuroprotection

Abbreviations: AD, Alzheimer's Disease; BM, Bacopa monnieri; CNS, central nervous system; COX, cyclo-oxygenase; CSF, cerebral spinal fluid; IL-*n*, interleukin *n*; IFN-γ, interferon gamma; LPS, lipopolysaccharide; MCI, mild cognitive impairment; PHA, phytohemeagglutinin; TNF-α, tumour necrosis factor alpha;

Abstract

Bacopa monnieri (L., BM) is a traditional Ayurvedic medicinal herb recognised for its efficacy in relieving acute pain and inflammation, as related to selective inhibition of cyclo-oxygenase-2 (COX-2) enzyme and consequent reduction in COX-2-mediated prostanoid mediators. BM is also associated with cognitive enhancing (nootropic) activity including improving memory free recall, observed after prolonged intake (>3 months). It is likely that the timeframe required to exert an effect in the brain reflects regulation by BM of chronic inflammation and oxidative stress associated with aging and chronic diseases, and other polypharmacological effects. We report down-regulation by BM of NO and TNF-α in stimulated RAW 246.7 macrophages and of IFN-γ in stimulated human blood cells. Furthermore, in human blood cells, IL-10 was slightly elevated indicating polarisation towards a regulatory T cell phenotype. These results provide further supportive evidence to justify the clinical evaluation of BM for managing diseases involving chronic systemic and brain inflammation driven by the innate immune system.

Introduction

Bacopa monnieri (L.) Wettst. (*Syn. Bacopa monnieri* (L.) Pennell (Scrophularaicae) (BM) known as Brahmi in traditional Ayurvedic medicine, is a small, creeping herb with numerous branches, small oblong leaves and light purple flowers. It is found throughout the Indian subcontinent in wet, damp and marshy areas. In Ayurvedic medicine, Brahmi is classified as a 'medhya-rasayana', the name given to drugs that promote cognitive functions and memory [1]. An extract of BM is now available in supplement form as 'Membac', and marketed for aiding memory recall and learning retention, in addition to assisting mental clarity, concentration and attention span. Effects of BM on memory and cognitive performance have been substantiated in a small number of randomized, controlled human clinical trials. A systematic review of randomized controlled trials showed that BM improved performance in 9 out of 17 tests in the domain of memory free recall but was not effective in other cognitive domains [2].

Compounds responsible for the pharmacological effects of BM include alkaloids, triterpenoid saponins and sterols. Many active constituents including the alkaloids: Brahmine and herpestine, the saponins: d-mannitol and hersaponin; and other compounds identified as acid A and monnierin have been characterised in India over 40 years ago. Other active constituents have since been identified, including betulic acid, stigmastarol, beta-sitosterol, and a number of triterpenoid saponins identified as bacosides and bacopasaponins [3]. To date, the effects of BM on cognitive function have been attributed to enhancing nerve impulse transmission by the triterpenoid saponins and bacosides [4]. In addition, the bacosides aid in repair of damaged neurons by enhancing kinase activity, neuronal synthesis, and restoration of synaptic activity, and ultimately nerve impulse transmission.

However, the anti-inflammatory properties of BM could also be relevant to observed neuroprotective effects. Chronic low grade inflammation is linked with cognitive decline in Alzheimer's disease (AD) and other neurodegenerative disorders [5, 6]. Both histochemical and blood biomarker evidence [7] as well as imaging studies [8, 9] suggest that release of pro-inflammatory cytokines by activated macrophages in the blood and microglia in the brain contribute significantly to neuronal cell death [7]. These pro-inflammatory cytokines stimulate a cascade of pro-inflammatory cytokines and mediators including TNF-α, prostaglandin E2, nitric oxide and hyper-secretion of cortisol, the plasma levels of which predict rate of cognitive decline [10]. The latter steroid inhibits protein synthesis thereby reducing the synthesis of neurotrophic factors. Thus, chronic inflammation is strongly involved with the pathology of dementia, leading to increased neurodegeneration, reduced neuroprotection and neuronal repair [11].

In addition to neurotrophic activities, BM appears to stabilize mast cells *in vitro,* and inhibit prostaglandin synthesis [12]. The bacopasides exert anti-inflammatory and neuropathic pain-relieving effects by inhibition of cycloxygenase-2 (COX-2), with potential for the typical bacopaside: bacoside A_3 (BA_3) to activate opioid receptors due to their structural similarity to morphine [13]. However, effects of BM on global parameters of inflammation, e.g cytokine release and free radical production have not yet been studied. The aim of this study was therefore to investigate effects of BM on modulating key inflammatory mediators produced by stimulated immune cells.

Materials and methods

The *Bacopa monnieri* was sourced as a bacoside-rich ethanolic extract representing bacosides from 4 g of dried plant solids, contained in each Membac capsule (Keen Mind CDRI 08, Keen Health Pty Ltd, Rozelle, NSW, Australia). . The standardised dried extract contained bacosides A and B accounting for at least 55% of total bacosides [14] and approximately 30% of total capsule solids. Excipients used to formulate the hard capsules are not knownLipopolysaccharide (LPS), phytohemeagglutinin (PHA) and hydrocortisone were obtained from Sigma Chemical Co. (St Louis, MO, USA) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Carlsbad, CA, USA).

Determination of anti-inflammatory activity in RAW 264.7 macrophages

The procedure for determining anti-inflammatory activity in murine RAW 264.7 macrophage cells was previously described [15]. Briefly, the *Bacopa monnieri* sample was dissolved in 10% aqueous ethanol and diluted 10-fold to 10 mg/ml in serum-free DMEM before further serial dilution. Cells were pre-incubated for 1 h with sample (50 µl) before activation with lipopolysaccharide (LPS, 25 µg/ml in DMEM) and IFN-γ (10 U/ml in DMEM), followed by further incubation at 37°C for 24 h. Cells with media alone constituted the negative control, and cells treated with pro-inflammatory stimuli, but no sample, were used as a positive control. After 24 h, cell viability was measured using the Alamar Blue assay involving the cellular reduction of resazurin to resorufin, and supernatants were deployed for analysis of nitrous oxide production by the Griess assay and TNF-α production by commercial ELISA kit (Cat. 900-K54, Peprotech, NJ, USA), according to manufacturer's instructions. At least 3 independent dose response experiments with duplicate determinations from each well, were conducted for each assay.

Determination of anti-inflammatory activity in human whole blood cells

Blood was obtained from healthy adult volunteers following their informed consent, as required for study approval by the CSIRO Food and Nutritional Sciences Human Ethics committee. An assay system utilising diluted whole blood was adapted from Matalka (2003) [16] and described previously [17]. Cells were stimulated into an inflammatory state by addition of LPS and PHA together with test compound, before further incubation at 37°C for 48 h. Hydrocortisone (HC, 200 ng/ml) was used as a positive control. The *Bacopa monnieri* sample was dissolved in 10% aqueous ethanol and diluted in serum-free media before further serial dilution. Samples or positive control were tested in duplicate for each of 4 donors. Supernatants were recovered for analysis of IFN-γ and IL-10 by commercial ELISA kits according to manufacturer's instructions (DuoSet, R&D Systems, Minneapolis, MN, USA). For each cytokine, analysis of each supernatant was conducted in triplicate.

Results

Inflammation prevention model - efficacy of Bacop monnieri macrophages

The experimental method of pre-conditioning of macrophages with BM for 1 h prior to pro-inflammatory stimulation allowed BM to diffuse into the cells to then regulate the subsequent pro-inflammatory challenge with LPS and IFN-γ. The results indicated that BM was effective in lowering NO production (EC_{50} = 0.25 $\pm$ 0.3 mg/ml) but less effective in lowering TNF-α production (EC_{50}= 1.15 $\pm$ 0.1 mg/ml). Loss of cell viability was also observed with an EC_{50} value of 1.32 $\pm$ 0.23 mg/ml (Figure 1).

Inflammation competitive regulation model - efficacy of Bacopa monnieri in whole blood

In the whole blood assay, diluted whole blood from healthy volunteers was co-stimulated with LPS, PHA and BM simultaneously before incubation for 48 hr. The experimental method of co-stimulating blood cells with BM and pro-inflammatory mediators simultaneously therefore represented a model for testing capacity of BM to counter and suppress pro-inflammatory responses in an immediate timeframe. The results indicated that BM was effective in lowering IFN-γ production (EC_{50} = 12.8 ± 1.7 μg/ml) and this co-incided with sustained (trend towards increase) production of IL-10 (1-30 μg/ml) before decline above ~500 μg/ml, associated with apparent toxicity in RAW264.7 cells (Figure 2). The steady state or trend towards increase in IL10 production at ~15 μg/ml) suggested that control of inflammation was associated with elevation of the regulatory T cell population. The results for both IFN-γ and IL-10 suggested that the BM appeared to become toxic to blood cells above ~30 μg/ml (Figure 2).

Discussion

The results from 2 independent cellular model systems suggest that BM is likely to exert an anti-inflammatory effect by the regulation of Th1-polarised immune responses involving suppression of NO (and TNF-α) by macrophages and IFN-γ by innate lymphocytes. Furthermore, sustained production of IL-10 was indicative of neutralisation of Th1 activation in favour of activation of regulatory T cells. These results are supported by concentration-dependent inhibition of TNF-α release in human whole blood after pre-incubation (45 min)

with various solvent extracts of BM and LPS-mediated stimulation [18]. These results indicated that the soluble ethyl acetate extract was more active than the bacoside-enriched extract.

The multiple bioactivities of BM have been attributed to the pentacyclic terpenoid saponins [19] and the related aglycone, betulinic acid [20]. The most studied pentacylclic terpenoid, ursolic acid is found in cranberry and displays significant anti-inflammatory and anti-cancer activities [21]. Anti-inflammatory regulation of TNF-α-induced activation of NF-κB was demonstrated in both Jurkat cells and human T lymphocytes [21]. Likewise, betulinic acid, was reported to suppress pro-inflammatory IL-6 production and NF-κB activation in LPS-stimulated human peripheral blood mononuclear cells [20]. In the rat paw edema model, using histamine, serotonin, bradykinin, arachidonic acid and prostaglandin E2-stimulated edemas, efficacy of BM was 100% for prostaglandin E2 and moderate to insignificant for all other phlogistic agents [22]. These results support that bacopasides of BM are known to exert their anti-inflammatory and neuropathic pain relieving effects by inhibition of cycloxygenase-2 (COX-2), with additional possible pharmacological effects as related to the structural similarity of BA_3 bacopasides to morphine [13].

All activated effector and immune cells can produce pro-inflammatory eicosanoids, specifically, prostaglandins via COX-2 mediated pathway, which are stimulated by pro-inflammatory cytokines including IFN-γ and TNF-α. In addition to selective inhibition of COX-2 by different solvent fractions of BM [18], a minor aglycone, betulinic acid in BM, was also associated with modulating NF-κB translocation via regulating p38 and ERK MAP kinases. In the study by Viji *et al.*, the combination of betulinic acid with specific p38 and ERK MAP kinase inhibitors was more effective at down-regulating NF-κB than either of the p38 and MAP kinase inhibitors alone, suggesting that betulinic acid was acting on an

additional pathway, presumably inhibiting COX-2, and could account for the synergistic anti-inflammatory effect [20]. It is therefore possible that pro-inflammatory activation of macrophages and human blood cells reported here also induced expression of COX-2 and prostaglandin mediators, and that apparent suppression of NO and TNF-α in RAW macrophages (Figure 1) and IFN-γ in human blood cells (Figure 2) could be attributed to upstream inhibition of COX-2.

Apart from pain management reflecting COX-2 inhibitory activity [13], a significant application focus for BM reflects its reputed nootropic efficacy and potential for improving cognitive function in the elderly [23]. A recent systematic review of cognition-enhancing effects concluded that there was some evidence for improvement in the memory domain of free recall associated with long term BM supplementation, but inadequate evidence demonstrating effects in any other cognitive domains [24]. Collectively, reported nootropic effects suggest that metabolites of BM are bioavailable to brain and the apparent range of polypharmacological actions [23] most probably reflect beneficial modulation of the chronically-activated peripheral and CNS immune systems, associated with several chronic diseases and aging [25]. An immune inflammation-centred hypothesis is supported by the need for extended therapy (>3 months) before benefits of BM are evident [23] and that intake of 300 mg of BM does not provide any immediate benefit for cognition in healthy humans [26].

The apparent requirement for long-term use to detect a cognitive benefit for BM is reminiscent of the protective effects associated with long-term use of barbiturate-free, non-steroidal anti-inflammatory drugs (NSAIDs), which was significantly correlated with lowering risk of AD and to a lesser extent, MCI [27]. Furthermore, long-term intervention studies (2 years) with the anti-inflammatory drug, naproxen, in cognitively normal elderly

significantly prevented or delayed onset of AD [6]. In contrast, anti-inflammatory therapies have been consistently ineffective in slowing progression of dementia symptoms in MCI and AD patients [28]. These results suggest that future intervention studies with BM seeking to improve memory should involve long term interventions and also seek to evaluate effects on biomarkers of inflammation. This approach can contribute to the understanding of the role of inflammation in memory decline and progression of dementia.

Acknowledgements

This work was conducted with support from the CSIRO Preventative Health National Research Flagship. Technical assistance of Kiruba Shanmugam and Santina Forsyth are also gratefully acknowledged.

Conflict of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Figure Captions

Figure 1.
Concentration-dependent effects of *Bacopa monnieri* (BM) on the production of pro-inflammatory mediators by RAW264.7 macrophages activated with LPS and IFN-γ.
Percentage change from control (no added BM) of mediator release in the presence of BM (1-1000 μg/ml). Results for cell viability (CV), nitrous oxide (NO) and tumor necrosis factor α (TNF-α) are shown. Data are the mean of 3 independent experiments ± SD.

Figure 2.
Concentration-dependent effects of *Bacopa monnieri* (BM) on the production of pro-inflammatory and regulatory mediators by human blood cells activated with lipopolysaccharide and phytohemeagglutinin**.** Percentage change from control (no added BM) in the presence of BM (1- 1250 μg/ml), showing results for IFN-γ and IL-10 are shown. Positive control was hydrocortisone (not shown). Results are the mean of four donors ± SEM.

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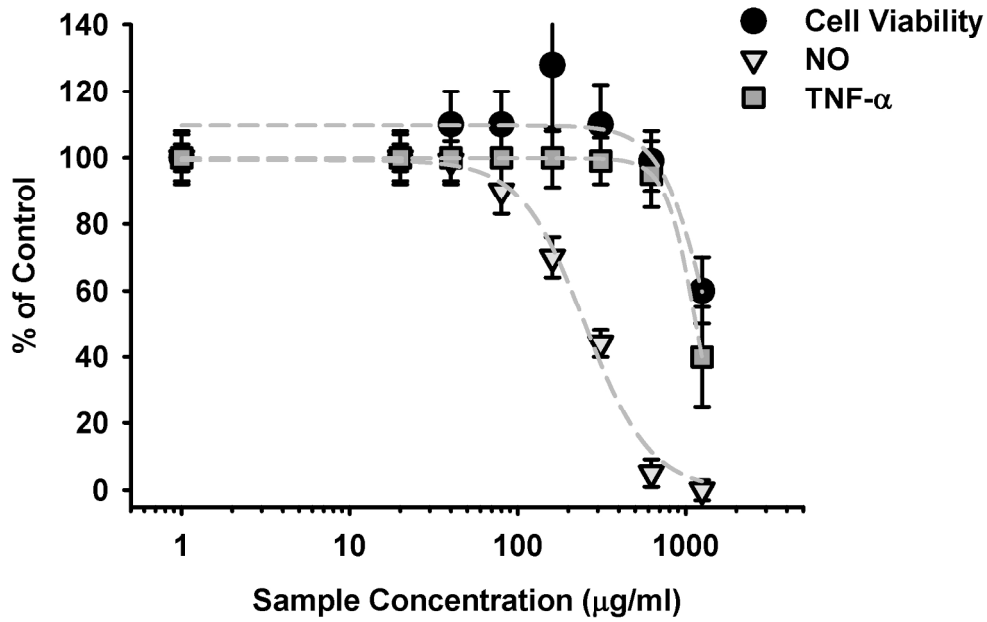

Concentration-dependent effects of Bacopa monnieri (BM) on the production of pro-inflammatory mediators by RAW264.7 macrophages activated with LPS and IFN-γ. Percentage change from control (no added BM) of mediator release in the presence of BM (1- 1000 μg/ml). Results for cell viability (CV), nitrous oxide (NO) and tumor necrosis factor α (TNF-α) are shown. Data are the mean of 3 independent experiments ± SD.
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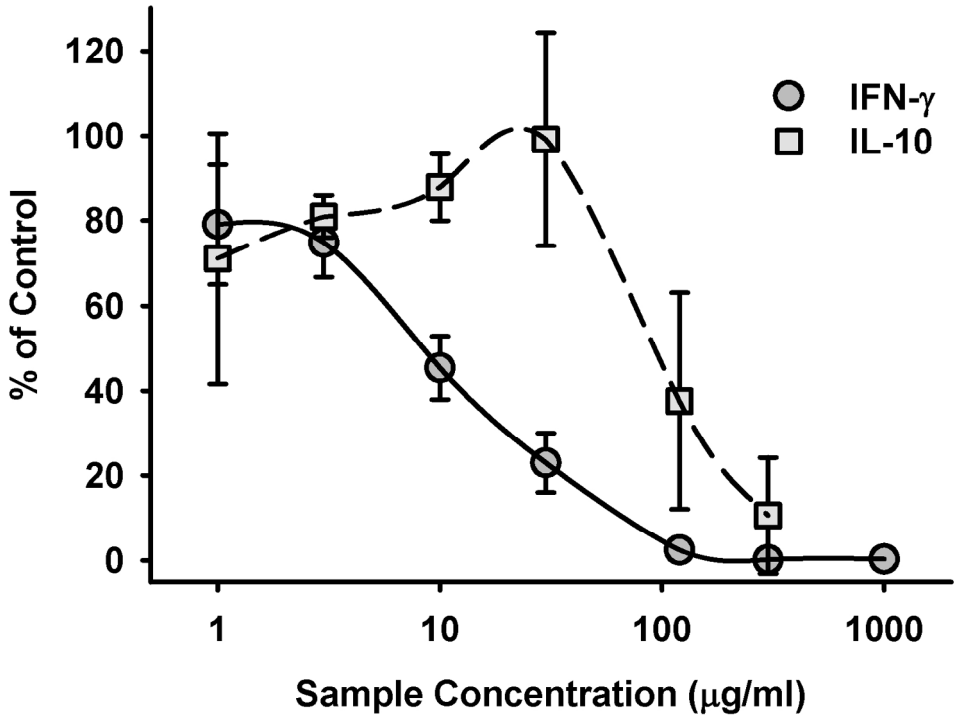

Concentration-dependent effects of Bacopa monnieri (BM) on the production of pro-inflammatory and regulatory mediators by human blood cells activated with lipopolysaccharide and phytohemeagglutinin. Percentage change from control (no added BM) in the presence of BM (1- 1250 μg/ml), showing results for IFN-γ and IL-10 are shown. Positive control was hydrocortisone (not shown). Results are the mean of four donors ± SEM.
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