Evidence for Anti-inflammatory Effects and Modulation of Neurotransmitter Metabolism by *Salvia Officinalis* L.

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Research Article

Keywords: *Salvia officinalis*, inflammation, cognitive health, Intestinal cells, Blood brain barrier cells, Monoamines, Dopamine, Serotonin, Gut-brain axis, phenotypic drug discovery

Posted Date: February 1st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1271841/v1>

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Abstract

Background: Cognitive health is of great interest to society, with neuroinflammation and systemic inflammation as age-related risk factors linked to declines in cognitive performance. Several botanical ingredients have been suggested to have benefits in this area including sage (*Salvia officinalis*), which has shown anti-inflammatory effects and exhibited promising cognitive improvements in multiple human studies. The current study aims to further investigate sage's anti-inflammatory effects in human cells and uncover other relevant biological activities.

Methods: The release of multiple Cytokine and Chemokines was measured in human primary intestinal epithelial cells treated with sage extract, with or without LPS stimulation, and Blood Brain Barrier (BBB) cells in presence or absence of recombinant IL-17A and/or Human IL-17RA/IL-17R Antibody. Antioxidant effects were also assessed in BBB cells incubated with the extract and H₂O₂. The anti-inflammatory effects of sage extract were further assessed based on clinically-relevant biomarker readouts across twelve human primary cell-based disease models of the BioMAP® Diversity PLUS® Panel.

Results: Sage showed significant attenuation of the release of most cytokines/chemokines into apical media in LPS-stimulated intestinal cells, but small increases of in the release of markers including IL-6, IL-8 in basolateral media; where TNF- α was the only marker to be significantly reduced. Sage attenuated the release of CRP and VCAM-1 from BBB cells under IL-17A induced conditions, as well as the basal level of release of MCP-1, and also decreased H₂O₂ induced ROS overproduction in these cells. Phenotypic profiling with the BioMAP® Diversity PLUS® Panel identified additional anti-inflammatory mediators, and based on a similarity search analysis suggested potential mechanistic similarity to caffeic acid and drugs known to inhibit COMT and MAO activity to modulate monoamine metabolism. Subsequent *in vitro* assessment showed that sage was able to inhibit the activity of these same enzymes.

Conclusions: Sage extract showed anti-inflammatory effects across multiple human cell lines, which could potentially reduce peripheral inflammation and support cognitive health. Sage extract also showed the ability to inhibit enzymes related to the metabolism of monoamine neurotransmitters, suggesting possible dopaminergic and serotonergic effects acting alongside proposed cholinergic effects to mediate acute cognitive performance benefits previously demonstrated for the extract.

Background

The average global life expectancy has increased by more than 6 years between 2000 and 2019; and is predicted to continue increasing on a similar way. By 2050, the global life expectancy is predicted to reach 77.1 years (1). Many theories about normal aging have suggested that aging is associated with the risk of the decline of neurophysiological functions, which often leads to reductions in cognitive performance and capacity (2). Consequently, the promotion of successful cognitive health, particularly in elderly populations, is becoming a pressing concern for individuals, society and public health. The promotion of successful cognitive health involves the prevention of the decline of cognitive function

related to neurodegenerative diseases, as well as the enhancement of the brain capacity and cognitive reserve (3).

The role of chronic, low-grade inflammation in increasing the risk of numerous age-related health conditions from type 2 diabetes (4) to cardiovascular disease (5) is well established; and there has been growing awareness of the role that inflammation plays in increasing the risk of cognitive decline (6). It is widely observed that neurodegenerative diseases including Alzheimer's disease (AD) (7), Parkinson's disease (PD) (8), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (9) are commonly associated with neuroinflammation, which itself is related to systemic inflammation.

Awareness of the role of the intestinal tract in cognitive and mental health is also growing, with a growing body of research into the gut-brain axis: A bidirectional communication between gut microbiota and the brain, rising from the association of dysbiosis with various cognitive disorders (10, 11). The inner lining of the small intestine is covered by a single layer of intestinal epithelial cells. Apart from nutrient absorption, the intestinal epithelium plays a variety of critical roles including maintaining barrier integrity, preventing invasion by microbial commensals and pathogens, and modulating the intestinal immune system. Inflammation can damage the intestinal barrier, which enhances the inflammatory response, leading to what is commonly known as a leaky gut. Recent data indicate that intestinal inflammation contributes to the pathogenesis of PD (12), and increasing numbers of studies imply that PD may start in the gastrointestinal system years before any motor symptoms develop. Indeed, the dysfunction of the intestinal barrier has been associated with several disease states, including obesity and diabetes (13), cancer (14), and other neurodegenerative diseases (15).

A large number of cytokines, including Interleukin-6 (IL-6), Tumour Necrosis Factor- α (TNF- α), IL-18, IL-1 β , and IL-17, are over-expressed in the inflamed gut and have been related to intestinal dysfunction and damage (16). Such markers may also contribute towards systemic effects of inflammation; including neuroinflammation. For example, IL-17A has been highlighted with a role in disruption of the blood-brain barrier (BBB) (17, 18): The protective barrier of the central nervous system (CNS) that separates the blood of the periphery from cerebrospinal fluid (CSF). IL-17A has been shown to induce BBB breakdown by the over-formation of reactive oxygen species (ROS), inflammatory molecules, and decreasing the number of tight junctions (17, 19). The BBB is highly selective and semi-permeable; allowing it to regulate the transport of molecules in and out of the CNS, whilst restricting the entry of pathogens and neurotoxic plasma components. BBB damage and dysfunction can lead to these components leaking into the CNS, and has been associated with several neurodegenerative and autoimmune diseases (20).

Salvia officinalis (sage) has been used for decades as a medicinal plant in treating several diseases. Recent studies have shown promising activity in treating cancer (21) through to heart disease, dementia, and obesity (22); with different classes of active compounds being identified in the plant, including phenolic acids and terpenes (23). In particular, *Salvia* plants have traditional applications in cognitive health dating back hundreds of years (24), and there is a growing body of scientific evidence supporting beneficial effects in this area (reviewed in (25)). This includes several human volunteer studies with

extracts of *Salvia* showing cognitive performance benefits in healthy adults (26–30), as well as in AD patients (31).

Our previous investigation of *S. officinalis* extract supported anti-inflammatory effects in adipose cells, as well as attenuation of the cross-talk between peripheral tissues and nerve tissues (32). The same extract has previously been shown to have cognitive performance benefits in both healthy older and younger adults (26, 27). In addition to anti-inflammatory effects, modulation of the metabolism of acetylcholine (ACh) neurotransmitters, by the inhibition of Acetylcholinesterase (AChE), has been proposed as one mechanism by which *Salvia* extracts impart acute cognitive benefits in humans (26). Among other activities, acetylcholine neurotransmitters play an important role in the encoding of new memories (33), and inhibition of AChE is a mechanism shared by a number of drugs used in the management of symptoms in AD patients (34).

Monoterpenes, including 1,8-cineole and α -pinene have been proposed as driving the AChE inhibition activity from *Salvia* extracts (29, 35), but phenolic acids such as caffeic and rosmarinic acid have also been shown to have this activity (36, 37). Similarly, other neurotransmitters, such as the monoamine neurotransmitters dopamine and serotonin have effects on cognitive performance and mental wellness (38–41), and modulation of these other neurotransmitters may be relevant to the acute cognitive performance benefits demonstrated for *Salvia* extracts.

Building on our previous work showing anti-inflammatory effects of *S. officinalis* extract in human cells (32), we now assess these effects across a broader set of cellular contexts. This includes assessment of the extract through *in vitro* models of the intestinal barrier and blood brain barrier, as well as in the BioMAP® Diversity PLUS® Panel, to gain further insights into the anti-inflammatory activities and other potential mechanisms of action that contribute towards the cognitive performance benefits demonstrated in human volunteers for this extract (26, 27).

Methods

Investigational materials

S. officinalis (sage) extract was provided by Sibelius Ltd. for use in this study. Sage plant material of known and invariant provenance was grown in the United Kingdom, according to defined production protocols and Good Agricultural Practice standards. Sage leaves, certified as *S. officinalis* by HPTLC (Alkemist Labs, CA, USA; Additional file 1) were dried in an artificially heated (gas fired) hot air drier at temperatures less than 70°C and then soaked in ethanol (68% w/w) for 48 h. The resultant solution was then concentrated using a climbing film evaporator and dried in a vacuum oven to a final ratio of approximately 7.5:1 dry plant material to extract. The resulting material was milled using a 45 mesh to produce the final Sibelius™: Sage extract, which is a commercial extract standardised to a minimum level of 2.5% w/w rosmarinic acid (batch number 43625/N0587 and 44878/N0775 used in this study;

certificates of analysis in Additional file 1). The final concentration of DMSO in the media for control and treated cells was 0.04%.

Biochemical analyses

Total phenolic levels were measured using the Folin and Ciocalteu method, based on Thangaraj *et al.*, (42). Extract batch N0587 was measured at 15.4% w/w and batch N0775 was measured at 15.5% w/w total phenolics. Rosmarinic acid and 1,8-cineole levels were measured by GCMS analysis at RSSL (Reading, UK). Briefly, 2g of sample added 20ml of dichloromethane and sonicated for 15 minutes, of which 2ml was passed through a syringe filter into a vial. This sample was then run on a GCMS system and quantitated using selected ions against external standards.

In vitro **assessment of anti-inflammatory activity**

Cell culture

Human primary small intestinal epithelial cells, media and supplements were obtained from Creative Bio-Array, US (cat# CSC-C92295, lot# 1602413) and delivered at a cell density of 0.5×10^6 at passage 3. These cells were isolated from normal human small intestinal tissue. Human small intestinal epithelial cells were cultured in human complete epithelial cell medium (cat#CN-1098X, lot# 1651390). The media was supplemented with 10% FBS (Life Technologies, cat#10500064), Antibiotic-Antimycotic solution (cat# 16B0913), Insulin-Transferrin- Selenium or ITS (#1490), Epidermal growth factor or EGF (#1490), Epithelial cell supplement (cat# C94327) and L-Glutamine (#1413). Cell culture was grown in a T25 coated with 0.1% Gelatin-Based Coating Solution (Sigma UK, cat#SF008) and stored at 37°C and 5% CO₂. The cell media was changed after 24hrs and then every 48 hours until 70-80% confluent. Cells were checked daily and visualised microscopically to check growth progression.

Primary human brain microvascular cells were immortalized with Lenti-SV40 Lentivirus. Cells were delivered at a cell density of 1×10^6 . Cells were cultured in Endothelial cell medium (P60104). The media was supplemented with 5% fetal bovine serum (FBS) (Gibco, Lot no# 08Q508IK), Antibiotic-Antimycotic solution (Lot no# 28663) and Endothelial cell growth supplement (Lot #26387). Media, FBS and supplements were also sourced from Innoprot, Spain. Cells were grown in a T25 flask and stored at 37°C and 5% CO₂. The cell media was changed after 24hrs and then every 48 hours until 70-80% confluent. Cells were checked daily and visualised microscopically to check growth progression.

LPS cell treatment

Human intestinal cells were treated with a low (5µg/ml) and high (20µg/ml) of sage extract (batch N0587) in the presence or absence of LPS (*E.coli*) (#L4391, Sigma-Aldrich) at 100ng/ml and incubated for 24 hours at 37°C and 5% CO₂. Following incubation for 24 hours, the medium was removed from both the top (apical) and bottom (basolateral) of the well inserts. The medium was stored at -20°C until it was required for cytokine analysis.

IL-17A treatment

Human brain cells were treated with sage extract (20µg/ml; batch N0587) in the presence or absence of recombinant human IL-17A protein (R&D Systems, #7955-IL-025) and/or Human IL-17RA/IL-17R Antibody (R&D Systems, #MAB177-100) and incubated for 24 hours at 37°C and 5% CO₂. Following incubation for 24 hours, the medium was removed and stored at -20°C until it was required for cytokine analysis.

Measurement of cytokine release

Protein released into the cell culture medium was measured using MSD Cytokines and Chemokines V-PLEX Human assay kits (Meso Scale Discovery, Gaithersburg, MD, USA). These provide a rapid and convenient method for measuring the levels of protein targets in a small volume sample. MSD plates were pre-coated with capture antibodies on independent and well-defined spots. Samples or calibrators (50µl) were added to each well in duplicate and the plates were sealed and incubated at room temperature and shaken (700–1000 oscillations/min) for 2h. The plates were washed three times with 200µl/well of wash buffer and 25µl of detection antibody solution was added to each well. The plates were then sealed and incubated at room temperature and shaken (700–1000 oscillations/min) for a further 2h. Finally, the plates were washed by the same method again and 150µl of reading buffer was added to each plate and read using a Mesoscale Discovery instrument (MSD SECTOR Imager 2400). The unknown levels of cytokines and chemokines released in the cell culture media were determined by the Meso Scale software. Both raw data statistical analysis (using one way Anova test followed by Dunnett's multiple comparison test), and graphs representation were performed using GraphPad Prism 5 software.

Measurement of ROS production

Human brain cells were seeded in to 96 well plates and treated with various doses of sage extract (1µg/ml, 5µg/ml, 20µg/ml and 50µg/ml) for 24 hours at 37°C and 5% CO₂. Media was then removed and the cells were incubated with 10µM of a Fluorescent Substrate (probe) H₂DCFDA (Millipore, USA, lot no# 3256654) in the dark at 37°C for 45 minutes. Cells were washed with 100µL of phosphate buffered saline (PBS) (Gibco, # 10010-015) before being treated with 1mM H₂O₂ (Sigma, UK, #216765) for 1 hour at 37°C and 5% CO₂. Plates were then read using a fluorescence microplate reader (Spectra MAX Gemini EM, Molecular Devices, California) at Ex/Em 495/519nm. The probe and H₂O₂ were diluted in growth media.

BioMAP® Diversity PLUS®

Phenotypic profiling with the BioMAP® Diversity PLUS® Panel was conducted by Eurofins Discovery (DiscoverX, USA) as described previously (43), with doses of the sage extract (batch N0775) of 2.5µg/ml, 5µg/ml, 10µg/ml, and 20µg/ml (the previous analysis of the sage extract presented in Additional file 3 was with completed with batch N0587). Human primary cell-based disease models in the BioMAP® Diversity PLUS Panel represent a broad set of tissue and disease biology, including: vascular biology (venular endothelial cells) modelled in the Th1 (3C system) and Th2 (4H system) inflammatory environments, as well as in a Th1 inflammatory state specific to arterial smooth muscle cells (CASM3C

system); systemic immune responses are modelled in a Th1 inflammation environment (LPS system) or after T cell stimulation (SAG system; both venular endothelial cells and peripheral blood mononuclear cells [PBMCs]); chronic Th1 inflammation driven by macrophage activation (/Mphg system; macrophages and venular endothelial cells) and the T cell-dependent activation of B cells (BT system; PBMCs and B-cells). Lung inflammation is modelled in the Th1 (BE3C system) and Th2 (BF4T system) inflammatory environments (bronchial epithelial cells, or bronchial epithelial cells and dermal fibroblasts respectively); myofibroblast-lung tissue remodelling is assessed in lung fibroblasts (MyoF system); skin biology is addressed in a Th1 cutaneous inflammation environment in keratinocytes and dermal fibroblasts (KF3CT system); and wound healing and tissue remodelling is assessed in dermal fibroblasts (HDF3CGF system). Biomarker activities are determined to be statistically significant, and are thus annotated, when two or more consecutive concentrations of a test agent change in the same direction relative to vehicle controls, are outside of a significance envelope generated from historical control data, and have at least one concentration of test agent with an effect size > 20% ($|\log_{10} \text{ratio}| > 0.1$).

BioMAP profiles can be compared against a proprietary reference database of > 4,000 BioMAP profiles of bioactive agents (biologics, approved drugs, chemicals and experimental agents) to classify and identify the most similar profiles. Common biomarker readouts are annotated when the readout for both profiles is outside of the significance envelope with an effect size > 20% in the same direction. Concentrations of test agents that have 3 or more detectable systems with cytotoxicity are excluded from similarity analysis. The similarity between agents is determined using a combinatorial approach that accounts for the characteristics of BioMAP profiles by filtering (Tanimoto metric) and ranking (BioMAP Z-Standard) the Pearson's correlation coefficient between two profiles. Profiles are identified as having mechanistically relevant similarity if the Pearson's correlation coefficient is ≥ 0.7 . Briefly, a Pearson's correlation coefficient (r) is first generated to measure the linear association between two profiles that is based on the similarity in the direction and magnitude of the relationship. Since the Pearson's correlation can be influenced by the magnitude of any biomarker activity, a per-system weighted average Tanimoto metric is used as a filter to account for underrepresentation of less robust systems. The Tanimoto metric does not consider the amplitude of biomarker activity, but addresses whether the identity and number of readouts are in common on a weighted, per system basis. A real-value Tanimoto metric is calculated first by normalizing each profile to the unit vector (e.g., $A = \frac{A}{\|A\|}$) and then applying the following formula: $\frac{A \cdot B}{\|A\| + \|B\| - A \cdot B}$, where A and B are the 2 profile vectors. Then, it is incorporated into a system weighted-averaged real-value Tanimoto metric in this calculation: $= \frac{\sum W_i \cdot T_i}{\sum W_i}$. The calculation uses the real-value Tanimoto score for each i th system (T_i) and the weight of each i th system (W_i). W_i is calculated for each system in the following formula: $\frac{1}{1 + e^{-100 \cdot (|r| - 0.09)}}$, where $|r|$ is the largest absolute value of the ratios from the 2 profiles being compared. Based on the optimal performance of reference compounds, profiles are identified as having mechanistically relevant similarity if the Pearson's correlation coefficient (r) ≥ 0.7 . Finally, a Fisher r-to-z-transformation is used to calculate a z-score to convert a short tail distribution into a normal distribution as follows: $z = 0.5 \log_{10} \frac{1+r}{1-r}$. Then the BioMAP Z-Standard, which adjusts for the

number of common readouts (CR), is generated according to the following formula: $Z\text{-Standard} = z \cdot \sqrt{CR - 3}$. A larger BioMAP Z-Standard value corresponds to a higher confidence level, and this is the metric used to rank similarity results.

Enzymatic assays

All of the enzymatic assays were conducted in the sage extract batch N0775. The Catechol-O-methyl transferase (COMT) inhibition assay was conducted by Eurofins Discovery (Panlabs, Taiwan), with activity of COMT from porcine liver measured by spectrofluorimetric quantitation of scopoletin based on the method of Müller-Enoch *et al.*, (44). Monoamine Oxidase-A (MAO-A) and MAO-B inhibition assays were conducted by Eurofins Discovery (Cerep, France). MAO-A activity was measured by photometric detection of 4-OHquinoline using MAO-A enzyme from human placenta based on the method of Weyler and Salach (45), and MAO-B activity was measured by detection of luminescence from methyl ester luciferin using human recombinant MAO-B enzyme based on the method of Tsugeno *et al.*, (46). The Acetylcholinesterase (AChE) inhibition assay conducted by Eurofins Botanical testing (USA), with spectrophotometric detection of 2-nitro-mercaptobenzoate using AChE enzyme from electric eel based on the method of Vintutha *et al.*, (47).

Results

Anti-inflammatory effects in intestinal epithelial cells

Given growing understanding of the importance of peripheral inflammation, gut-health and the gut-brain axis on cognitive health, the effects of sage extract were assessed in human small intestinal epithelial cells as a model of the intestinal barrier. Figures 1 and 2 summarise the levels of cytokines and chemokines released into apical and basolateral cell media respectively by human small intestinal epithelial cells incubated with LPS and sage extract for 24 hours. Compared to the control group, the LPS treatment caused a significant increase in the release of all eight of the protein markers tested in the apical media (Figure 1), and all but CRP and VCAM-1 in the basolateral media (Figure 2); which supports successful induction of pro-inflammatory response by the LPS treatment.

Within the apical media, the 5µg/ml and 20µg/ml doses of the sage extract showed significant ($p < 0.01$) reductions in the levels of CRP after stimulation with LPS; returning it close to the basal level of release (44% and 33% reductions respectively; Figure 1A). The sage treatment also showed a dose-response attenuation of the induction of IL-8 levels in the apical media after LPS stimulation; reducing the release of IL-8 by 20% and 34% for the 5µg/ml and 20µg/ml extract doses respectively (Figure 1E). A significant decrease (22%) in the basal level of IL-8 release was shown for the 20 µg/ml extract dose treatment (Figure 1E), and both sage extract doses caused a significant reduction in the release of IL-6 under LPS induced conditions (16% and 17% respectively; Figure 1C). There was also attenuation of LPS-induced release for SAA, VCAM-1, and ICAM-1 (31%, 20%, and 23% reductions respectively) in response to the 5µg/ml sage extract treatment (Figures 1F, 1G and 1H).

Cytokine and chemokine levels were much lower in the basolateral media than the apical media (Figures 1 and 2). Within the basolateral media, the 5µg/ml sage extract dose showed a significant decrease of basal (69%) and LPS-stimulated levels of TNF-α (35%; Figure 2B). Conversely, there were significant increases in the basal levels of IL-6 for both sage extract doses in the basolateral media, as well as after LPS stimulation for the 20µg/ml sage extract treatment (Figure 2C). Similarly, SAA and IL-8 basal levels ($p<0.001$) were significantly increased by 20µg/ml treatment dose (Figures 2E and F), and the basal levels of ICAM-1 were increased in a dose responsive manner ($p<0.05$ and $p<0.001$; Figure 2G).

The role of cytokines in maintaining and modulating gut barrier function is complex (48, 49) and difficult to model; especially in an *in vitro* setting. However, the reduction of the apical release of cytokines in response to the sage treatment in the intestinal epithelial cell model, would translate to reduced levels within the intestinal lumen. Given that increased levels of inflammation markers within the intestinal lumen is linked to inflammation (50, 51), a reduction of such markers by the sage extract could contribute towards anti-inflammatory effects and a reduction in peripheral inflammation.

Anti-inflammatory effects in human brain microvascular endothelial cells

As well as reducing peripheral inflammation, we were interested in investigating the potential anti-inflammatory effects of the sage extract in reducing neuroinflammation. Integrity of the BBB plays an important role in maintaining homeostasis in the brain microenvironment. Inflammation can have negative effects on the function of the BBB, with IL-17 in particular linked to disruption of the BBB (17, 18). The release of cytokine and chemokines were assessed in human brain microvascular endothelial cells as a model of the BBB. Figure 3 shows the levels of cytokine and chemokine markers released into the media by human brain microvascular endothelial cells incubated with IL-17A, and sage extract (20µg/ml) or IL-17 antibody treatments for 24 hours. Consistent with induction of inflammation, compared to the control treatment the IL-17A treatment significantly increased the release of multiple inflammatory markers including MCP-1, SAA, ICAM-1, IL-6, and IL-8 (Figure 3). Although IL-17A treatment did not significantly increase the levels of CRP, both the sage extract and IL-17 antibody caused a significant reduction in the level of CRP in the IL-17A induced conditions (both 37%; Figure 3B). IL-17 antibody also caused an attenuation of the increase in release of MCP-1 (25%), SAA (32%), IL-6 (39%), and IL-8 (6%), but no effects were shown on VCAM-1 or ICAM-1, and TNF-α showed a significant increase in response to the antibody treatment (Figure 3).

In addition to the same effect on CRP under IL-17A induced conditions, the sage extract also showed a similar effect as the IL-17 antibody treatment on TNF-α levels, all be it in the opposite direction to CRP with both treatments instead showing an increase in release of TNF-α (Figures 3B and H). The sage extract also reduced the release of MCP-1 (16%); although this was only significant under basal conditions and not under IL-17A induced conditions as for IL-17 antibody (Figures 3A). However, conversely, the sage extract showed no effect on SAA, and caused slight increases to IL-6 and IL-8 levels in human brain microvascular endothelial cells, whereas the IL-17 antibody treatment had shown a reduction in these markers (Figures 3C, F and G).

Similar effects of the treatments on CRP, TNF- α , and to some extent MCP-1, suggest that the sage extract is attenuating the IL-17A induction, in a similar way to the IL-17 antibody treatment. However, the opposite effect of the sage and IL-17 antibody treatments on levels of IL-6 and IL-8, suggest that there are also differences in the mechanisms that are responsible. The action of independent mechanisms between the treatments is further supported by the sage extract showing significant reduction in the release of VCAM-1 under both basal and IL-17A-induced conditions (22% and 28% reductions respectively), whereas the IL-17 antibody treatment had no effect on this marker (Figure 3D).

The induction of ROS formation by IL-17A has been proposed as one mechanism leading to disruption of the BBB caused by this cytokine (17). Sage has previously been shown to have antioxidant activity; linked to high levels of phenolic and other active compounds present in the plant (23). Therefore, we also tested the anti-oxidant activity of the sage extract in human brain microvascular endothelial cells. Figure 4 shows the level of ROS production from Human brain microvascular endothelial cells incubated with sage for 24 hours. Treatment of the cells with H₂O₂ significantly increased ROS production after 24 hours compared to the control conditions, and the lowest doses of sage extract (1 μ g/ml and 5 μ g/ml) showed no significant effect on H₂O₂-induced ROS production (Figure 4). However, the 20 μ g/ml and 50 μ g/ml sage extract doses significantly reduced H₂O₂-induced ROS production by 31% and 19%, respectively, supporting anti-oxidant effects for the botanical extract that may contribute to reducing inflammation and maintaining BBB integrity (Figure 4).

BioMAP® Diversity PLUS®

To further investigate the anti-inflammatory effects of the sage across a broader set of cellular contexts, as well as seek new insights into potential mechanisms of action, the extract was assessed using the BioMAP® Diversity PLUS® Panel. This systems biology approach provides 148 biomarker readouts across 12 different primary cell-based disease models, advanced analytics, and a comprehensive reference database for insights on mechanism of action, efficacy and safety. The panel is typically applied in pharmaceutical development, but has been used by the U.S. Environmental Protection Agency, and has previously been shown to successfully work for plant extracts (43). Figure 5 summarises the results of profiling sage extract in BioMAP® Diversity PLUS®.

The extract showed dose-dependent effects on multiple inflammation and immunomodulatory markers across multiple cell systems; biomarker annotations included reductions in ICAM-1, VCAM-1, IL-17 and IL-10 among other markers (Figure 5). In the case of VCAM-1 this was reduced across three different cellular disease models; together with the data from the intestinal epithelial cells and vascular endothelial cells, as well as our previous data (32), this offers strong support for effects on this vascular injury marker. Also consistent with our previous results was the reduction in ICAM-1 in the HDF3CGF panel, and mild increases in IL-8 across the KF3CT (keratinocytes and dermal fibroblasts), MyoF (lung fibroblasts) and /Mphg panels (Macrophages and venular endothelial cells; Figure 5).

The greatest number of annotated activities of the sage extract were shown in the HDF3CGF cell system; a model of wound healing and matrix/tissue remodelling, comprised of dermal fibroblasts stimulated with TNF α , IL-1 β , Interferon-gamma (IFN γ), Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), and Platelet-Derived Growth Factor-BB (PDGF-BB). In addition to the reduction of inflammation markers in this panel, there was also a reduction of Plasminogen activator inhibitor-I (PAI-I), EGF receptor (EGFR), Collagen-I (Col-I) and Col-III; suggesting that the sage extract may also modulate tissue remodelling in response to perturbation (Figure 5). This cell panel, as well as the 3C (Venular endothelial cells stimulated with TNF α , IL-1 β , and IFN γ), and SAg (Venular endothelial cells and peripheral blood mononuclear cells stimulated with TCR ligands) also showed antiproliferative effects (Figure 5), which may relate to the tissue remodelling effects, but also to potential anti-cancer activities proposed for sage previously (23).

In addition to offering independent support for anti-inflammatory effects, and suggesting potential tissue remodelling and antiproliferative effects for the sage extract, the BioMAP® Diversity PLUS® Panel also provides the possibility to map a test agent's response profile against a database of known agents to suggest potential mechanistic similarity with any of these agents. At the top dose tested the sage extract showed above statistically significant ($r \geq 0.7$) threshold hits to antimicrobial and antifungal treatments, which is perhaps not surprising given traditional application in this area (23) (See Additional file 2). However, perhaps more interesting was the above threshold connection shown with caffeic acid at the 10 μ g/ml sage treatment dose, indicating that the sage extract may share some mechanistic similarity with this phenolic acid known to have anti-oxidant, anti-inflammatory and antineoplastic properties (52) (See Additional file 2).

Caffeic acid is synthesized broadly across plant species, including sage. The sage extract assessed in the current study is relatively rich in rosmarinic acid (4.2% w/w), which is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. The extract also contains total phenolic levels of approximately 15.5% (w/w), which is consistent with the possibility that these compounds contribute towards the anti-inflammatory effects observed for the sage extract.

The sage extract assessed has previously been demonstrated to provide cognitive performance benefits in older adults as well as younger adults; which appears to be at least partially due to cholinergic properties through the inhibition of acetylcholinesterase (AChE) (26, 27). It has been proposed that this activity was due to monoterpenes including 1,8-cineole and α -pinene present in high levels in *Salvia* essential oils. These compounds have both been shown to inhibit AChE activity (both with IC₅₀ estimates of approximately 0.67mM) (29, 35). However, phenolic acids provide alternative candidates to modulate this activity; with caffeic acid (IC₅₀ estimates of 23.3 μ M (37)), and rosmarinic acid (IC₅₀ > 300 μ M (36)) having reported inhibition of AChE activity *in vitro*. Such activity shared between the sage extract and caffeic acid might contribute towards the functional similarity suggested between these entities by the BioMAP Similarity Search analysis.

In addition to cholinergic activities, caffeic acid and rosmarinic acid have also been shown to potentially modulate the metabolism of monoamine neurotransmitters through inhibition of Monoamine Oxidase A (MAO-A; rosmarinic acid IC₅₀ 50.1µM, caffeic acid IC₅₀ 138.5µM (36)), MAO-B (rosmarinic acid IC₅₀ 184.6µM, caffeic acid IC₅₀ 247.7µM (36)), and Catechol-O-methyl transferase (COMT; rosmarinic acid IC₅₀ 26.7µM, caffeic acid IC₅₀ 89.9µM (36)). This is perhaps unsurprising given the structural similarity between caffeic acid and monoamines. Consistent with this, a previous analysis of the sage extract on the BioMAP® Diversity PLUS® Panel, run across a slightly different concentration range, showed a below threshold connection to two COMT inhibitor agents among the top three hits: Entacapone and Phenazopyridine (Additional file 3). The other agent in the top three was SR1001, a retinoic acid-related orphan receptor-α (RORα)/RORγ ligand, which could offer a potential mechanism for the IL-17-related effects of sage (53). However, it must be reiterated that these database hits were below the level of significance ($r < 0.7$), so they cannot be considered as sharing mechanistically relevant similarity based on this analysis alone.

Given the significant connection to caffeic acid, and previously demonstrated activities of this compound (36), the ability of the sage extract to inhibit COMT and MOA activity was assessed *in vitro*. Table 1 summarises enzyme inhibitory activity for the sage extract. Assay interference meant that it was not possible to assess the effect on MAO-A, but the sage extract did show inhibition of both COMT and MAO-B, with IC₅₀ values of 31.2µg/ml and 84.2µg/ml respectively (Table 1; also see Additional file 4). Indeed, comparison with the AChE inhibition activity for the sage extract (IC₅₀ 1794.0µg/ml) suggested that the extract had greater potency for inhibition of metabolism of monoamine neurotransmitters than acetylcholine (Table 1).

Table 1
Inhibition of enzyme activity by sage extract

Enzyme	IC ₅₀ (µg/ml)
COMT	31.2
MAO-B	84.2
AChE	1794.0

Discussion

The anti-inflammatory effects of a sage extract have now been demonstrated across multiple cell types and cellular models; supporting results from our previous research (32). Treatment of human cells with the sage extract caused a reduction in a number of inflammation markers including CRP, TNF-α, SAA, VCAM-1, ICAM-1, MCP-1, IL-17, IL-6, and IL-8, but the data also support an increase in the release of some makers; notably IL-6 and IL-8.

VCAM-1 has shown consistent response to treatment with the sage extract in the current study; being reduced in intestinal epithelial cells and microvascular endothelial cells, as well as in venular endothelial cells – alone and in combination with PBMCs – and dermal fibroblasts. This is in addition to human mature adipocytes from our previous analysis (32). Given the reported cognitive benefits of sage, it is interesting to note that studies have identified an inverse relationship between VCAM-1 levels and cognitive function in older adults (54, 55). These studies also showed an inverse relationship between VCAM-1 levels and cerebral blood flow; with atherosclerosis induced by the inflammation marker suggested as a possible cause for this. Reduction in VCAM-1 levels has also been proposed as a mechanism to improve BBB integrity and function, ameliorating age-related neurodegeneration in studies in mice (56).

As noted above, IL-17 has also been linked to BBB function and integrity (17, 18). The sage extract showed some phenotypic similarity to IL-17 antibody treatment in human microvascular cells, suggesting that the treatment may modulate IL-17A signalling. Potentially relevant to this, the pentacyclic triterpene acid ursolic acid, which is known to be rich in sage (57), has previously been shown to suppress IL-17 production via inhibition of ROR γ (58).

Attenuation of VCAM-1 and IL-17 signalling by the sage extract provides a possible mechanism by which the extract may help to maintain cognitive function through supporting healthy BBB function and improved cerebral blood flow. Healthy BBB function appears to be very important in the prevention or delay of onset for neurodegenerative conditions. For example IL-17 has also been shown to play an important role in migration of β -synuclein across the Blood Brain Barrier (BBB) resulting in autoimmune damage to grey matter in a mouse model of MS (59), and defects in BBB function have been suggested as a potential mechanism of increased AD risk associated with the *ApoE4* allele (60). It is therefore possible that supporting BBB function may have contributed towards the beneficial effects seen in studies of a sage extract in AD patients (31).

Whilst the general pattern has been of a reduction in inflammation markers in response to treatment with the sage extract, the induction of the pro-inflammatory cytokines IL-6 and/or IL-8 has now been observed across several different cellular contexts. Although pro-inflammatory effects cannot be ruled out, IL-6 is known to have pleiotropic effects including anti-inflammatory (61, 62), and IL-8 is also involved in promoting resolution of infections (e.g. phagocytosis, oxidative burst) and angiogenesis, which can help resolve inflammatory stimulus and promote healing (63, 64). Therefore, the induction of these two cytokines may actually be involved in supporting resolution of inflammation. It is also possible that the increase in IL-6 in human brain microvascular endothelial cells could contribute towards the anti-oxidant effect shown in these cells; based on induction of the stress response transcription factor Nuclear factor-erythroid factor 2-related factor 2 (NRF2) by the cytokine (65). An NRF2-mediated mechanism might also contribute towards the increase in expression of genes related to NADPH metabolism, and therefore potentially increased glutathione levels, thereby increasing endogenous antioxidant capacity, as suggested for the sage extract by previous analysis of gene expression responses in human mature adipocytes (32).

Reductions in peripheral- and neuro-inflammation, with associated improvements to BBB function and cerebral blood flow, may contribute long-term benefits towards cognitive health. However, such mechanisms seem less likely to explain the acute cognitive benefits of sage extracts; with effects on cognitive performance seen within 1hr of a single dose of the extract (26, 27). Modulation of neurotransmitter levels, however, may provide a mechanism that could explain these rapid effects. Analysis of the BioMAP® data suggested potential pharmacological activities for the sage through regulation of the metabolism of acetylcholine as well as monoamine neurotransmitters, however, these preliminary results need confirmation. Sage has previously been linked to cholinergic effects through inhibition of AChE; with monoterpenes including 1,8-cineole proposed to underlie this effect (29, 35). Essential oils, which are rich in monoterpenes, account for between 1-3% of *S. officinalis* dry weight, and it is therefore very plausible that these compounds contribute to the cognitive enhancing effects observed in studies of *Salvia* essential oils (29, 30, 66). However, monoterpenes are typically found at much lower levels in solid-liquid extracts of sage (57, 67, 68). Although 1,8-cineole was not measured in the sage extract batch utilised in the current study, analysis of an equivalent batch only showed 0.4ppm, which supports very low levels of this monoterpene being present in the Sibelius™:Sage extract; especially when compared to levels as high as 36.4% w/w in the clinically assessed *Salvia* essential oil (29).

As alternatives to or in complement with the monoterpenes, phenolic acids provide very plausible candidates for active compounds in the sage extract. They have been shown to inhibit AChE (36, 37), and as noted above are present at much higher levels in the tested aqueous ethanol sage extract. In addition to cholinergic activities, phenolic acids have also been shown to inhibit COMT, MAO-A and MAO-B (36); and we have now demonstrated similar activities for the sage extract. Indeed comparison of the IC₅₀ values for the extract showed greater potency for COMT and MAO-B than for AChE (Table 1), suggesting that modulation of monoamine metabolism might even be more important to the acute cognitive enhancing effects shown for the sage extract than the cholinergic activity. This greater potency for COMT and MAO-B versus AChE is shared by both the extract and the rosmarinic acid (36), which is consistent with phenolic acids making a significant contribution towards the overall activity shown by the whole extract.

Although it is difficult to directly compare doses between different assays, it must be noted that the IC₅₀ values for COMT and MAO-B inhibition by the sage extract (31.2µg/ml and 84.2µg/ml respectively) were both above the highest concentration tested on the BioMAP® Platform (20µg/ml). However, the data suggested that the sage extract could at least partially inhibit the activity of both COMT and MAO-B at doses of 20µg/ml and below (Additional file 4).

The monoamines are a group of neurotransmitters consisting of an amino group connected to an aromatic ring. MAO and COMT enzymes are involved in their breakdown and recycling: Specifically the subset of catecholamines in the case of COMT; which includes dopamine, epinephrine, and norepinephrine. MAO inhibitors are utilised as anti-depressants based upon increasing levels of serotonin in subjects, whereas COMT inhibitors are typically used in combination with L-Dopa, to increase dopamine levels and aid the management of PD symptoms (69). Serotonin has been demonstrated to

have effects on cognitive function (focus and flexibility) and mood (40, 41), whilst dopamine levels are strongly linked to working memory and attention (38, 39).

Studies of the COMT inhibitor Tolcapone in healthy human volunteers have shown increased levels of dopamine in the Pre-Frontal Cortex, as well as significant effects cognitive performance (70–74). Furthermore, studies of common genetic variants of COMT and MAO, which alter the stability and/or activity of the enzymes, have been strongly linked to elements of cognitive performance (75). It also seems that the effect of COMT alleles on executive function and working memory increases with age (76, 77), which is consistent with known changes to dopamine signalling with ageing (39). Together, such effects are consistent with the benefits observed in multiple studies into the effect of sage extracts on cognitive performance (26–30), and support modulation of the metabolism of dopamine and serotonin by the extract as a strong candidate mechanism contributing towards these effects. It is also worth noting that monoamine and catecholamine neurotransmitters are involved in the inter-play between the gut and gut-microbiome; influencing gut physiology and the innate immune system, and likely playing an important role in the gut-brain axis (78). Thus, modulating the metabolism of these neurotransmitters in other parts of the body might also contribute towards cognitive health benefits.

Modulation of the activity of COMT and MAO might also lead to broader benefits for sage extracts. Allelic variation in COMT and MAO genes has also been linked to Attention deficit hyperactivity disorder (ADHD) (79–81) and PD (69), and it has also been proposed that MAO inhibitors can lead to a reduction in the accumulation of A β plaques in Alzheimer's Disease (AD) through greater monoamine levels (82); a mechanism which alongside the anti-inflammatory effects might contribute towards the benefits observed in AD patients treated with a sage extract (31). Similarly, regulation of glycogen metabolism by catecholamines (83) might also contribute towards metabolic health benefits that have previously been associated with sage extracts (84).

Conclusions

Helping to prevent the decline of cognitive function and onset of neurodegenerative diseases, as well as enhancing the cognitive health and performance in healthy individuals, is of great interest to society. Beyond genetic influences - and prior to pharmaceutical intervention - lifestyle factors including diet and exercise can make a significant contribution towards this; there is also a role for dietary supplements to play. Numerous botanical ingredients have been linked to supporting cognitive health, including species of the *Salvia* genus (25), with an extract of sage assessed in the current study.

The role that chronic and neuro-inflammation plays in increasing the risk of cognitive decline, as well as other health concerns, is well established. We have now demonstrated anti-inflammatory effects for a sage extract across multiple cellular models; with notable effects on VCAM-1 and IL-17 signalling; which could translate to improved cerebral blood flow and BBB function to reduce neuroinflammation and support cognitive health over the longer-term.

In addition to this, we believe that we have also demonstrated inhibition of enzymes related to metabolism of monoamine and catecholamine neurotransmitters by sage for the first time. We propose potential dopaminergic and serotonergic effects, alongside previously proposed cholinergic effects of sage, contributing towards the acute cognitive performance benefits previously shown for this same extract in humans (26, 27). Indeed, given the greater potency of the extract towards COMT and MAO-B enzymes than for AChE, it is possible that the dopaminergic and serotonergic effects are more important than the cholinergic effects to the cognitive benefits.

Abbreviations

AChE: Acetylcholinesterase; AD: Alzheimer's disease; BBB: Blood brain barrier; COMT: Catechol-O-methyltransferase; CNS: Central nervous system; CRP: C-Reactive Protein; ICAM-1: Intercellular Adhesion Molecule-1; IL-1 β : Interleukin-1 Beta; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-17A: Interleukin-17A; LPS: Lipopolysaccharide; MAO: Monoamine Oxidase; MCP-1: Monocyte Chemoattractant Protein-1; PD: Parkinson's disease; ROS: Reactive Oxygen Species; SAA: Serum Amyloid A; TNF- α : Tumour Necrosis Factor alpha; VCAM-1: Vascular Cell Adhesion Molecule-1.

Declarations

Ethics approval and consent to participate

The use of plants in the present study complies with international, national and/or institutional guidelines.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

KE and SK are employees of Sibelius Limited who manufacture and sell SibeliusTM: Sage extract. The authors declare that they have no other competing interests

Funding

All research described in this study was funded by Sibelius Limited.

Authors' contributions

KE and MZ conceived the study and designed experiments and were involved in analysis and interpretation of data. GM designed and performed the *in vitro* studies with human primary small intestinal epithelial cells and human brain microvascular cells, including data analysis, interpretation and presentation. SK contributed reagents/materials for all experiments. NZ was involved in human brain microvascular cells and oxidative stress experiments, including data analysis and interpretation. All authors were involved in the writing of the paper.

Acknowledgements

The authors would like to thank Kate Feeney of Eurofins Discovery for assistance with the enzymatic assays performed by Eurofins Discovery. The authors would also like to thank Alison O'Mahony formerly of Eurofins Discovery (DiscoverX) and Sheryl Denker of Eurofins Discovery (DiscoverX) for assistance with the BioMAP® Diversity PLUS® Panel analysis, as well as with discussion and interpretation of the results.

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Figures

Figure 1

Sage extracts attenuation of cytokine and chemokine release by human small intestinal epithelial cells in apical media after 24-hour treatment with LPS

Levels of CRP, TNF-α, IL-6, IL-1β, IL-8, SAA, ICAM-1 and VCAM-1 released in human small intestinal epithelial apical culture media after 24 hours treatment with 5 or 20µg/mL of sage, and in the presence or

absence of 100ng/ml of LPS. All values are mean± SEM (n=6, in each treated cell group). See inset keys for treatment identification. Statistical analysis were performed using one-way ANOVA test flowed by Dunnett's multiple comparison test and Unpaired t test. Statistical significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 2

Sage extracts attenuation of cytokine and chemokine release by human small intestinal epithelial cells in basolateral media after 24-hour treatment with LPS

Levels of CRP, TNF- α , IL-6, IL-1 β , IL-8, SAA, ICAM-1 and VCAM-1 released in human small intestinal epithelial basolateral culture media after 24 hours treatment with 5 or 20 μ g/mL of sage, and in the presence or absence of 100ng/ml of LPS. All values are mean $\pm$ SEM (n=6, in each treated cell group). See inset keys for treatment identification. Statistical analysis were performed using one-way ANOVA test followed by Dunnett's multiple comparison test and Unpaired t test. Statistical significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 3

Sage extracts attenuation of cytokine and chemokine release by human brain microvascular endothelial cells after 24-hour treatment with IL-17A

Levels of MCP-1, CRP, SAA, VCAM-1, ICAM-1, IL-6, IL-8 and TNF-α released in human brain microvascular endothelial culture media after 24 hours treatment with 20µg/mL of sage or 2µg/ml IL-17 antibody (IL-

17AR), and in the presence or absence of 50ng/ml of IL-17A. All values are mean± SEM (n=8, in each treated cell group). See inset keys for treatment identification. Statistical analysis were performed using one-way ANOVA test flowed by Dunnett’s multiple comparison test and Unpaired t test. Statistical significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 4

Sage extracts attenuation of ROS release by human brain microvascular endothelial cells after 24-hour treatment with H₂O₂

Levels of ROS released in human brain microvascular endothelial culture media after 24 hours treatment with 1, 5, 20 and 50µg/mL of sage, and in the presence or absence of 1mM of H2O2. All values are mean ± SEM (n=8, in each treated cell group). See inset keys for sage dose. Statistical analysis were performed using one-way ANOVA test flowed by Dunnett’s multiple comparison test and Unpaired t test. Statistical significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 5

Assessment of sage impact on translational protein biomarkers using the BioMAP® Diversity PLUS® Panel

Plot showing the biomarker profile of the sage extract. The X-axis lists the quantitative protein-based biomarker readouts across the 12 cell-based disease models (see Methods section for a brief description of each cell system), with log-transformed ratio of the biomarker readouts for the sage-treated sample ($n = 1$) over vehicle controls ($n \geq 6$) presented on the Y-axis at concentrations of 2.5µg/ml (green), 5µg/ml (yellow), 10µg/ml (orange), and 20µg/ml (red). The grey region around the Y-axis represents the 95% significance envelope generated from historical vehicle controls. Biomarker activities are annotated in the plot when 2 or more consecutive concentrations change in the same direction relative to vehicle controls, are outside of the significance envelope, and have at least one concentration with an effect size > 20% ($|\log_{10} \text{ratio}| > 0.1$). Antiproliferative effects are indicated by a thick grey arrow above the X-axis.

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