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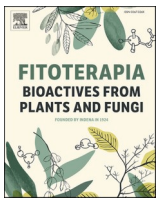

Research Paper

# Oral *Salvia officinalis* phenolics abolish mortality and mitigate inflammation in acute TNBS murine colitis

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ABSTRACT

*Background:* This study is the first to evaluate a phenolic extract of *Salvia officinalis* in the TNBS murine model of colitis. Inflammatory bowel disease (IBD) involves transmural oxidative and nitrosative stress that up-regulates inducible nitric-oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). Although phenolic constituents of *S. officinalis* are anti-inflammatory in vitro, their efficacy has never been tested in the trinitrobenzene-sulphonic acid (TNBS) model of colitis.

*Methods:* Aqueous-ethanolic sage leaves were extracted and standardized [(total phenolics = 6208 ± 200 mg gallic acid equivalent (GAE) $L^{-1}$; rosmarinic acid = 1.4 g/L). Male CD-1 mice received intracolonic TNBS (2.5 % *w/v*, 100 μL). Two hours later, and once daily for four days, they were gavaged with vehicle or sage extract (15 mg phenolic acids $kg^{-1}$). Disease-activity index, colon length, macroscopic and histological scores, iNOS/COX-2 immunostaining, and survival (Kaplan-Meier) were recorded.

*Results:* Extract markedly attenuated TNBS injury: diarrhea score fell from 3.0 ± 0.0 to 0.3 ± 0.2, ulcer length from 3.6 ± 0.1 cm to 0.8 ± 0.2 cm, and colon shortening was prevented (11.8 ± 0.2 to 12.9 ± 0.2 cm). Mortality dropped from 36 % to 0 %. Histology improved (score 3 to 1) and iNOS, COX-2 over-expression was normalized (4.25 to 2.19-fold and 2.48 to 1.03-fold, respectively). Ancillary paw-edema and cell-migration assays confirmed anti-inflammatory activity without indicating anti-metastatic effects.

*Conclusions:* The extract given orally affords the first demonstrated protection against acute TNBS colitis, normalizing key inflammatory markers and abolishing procedure-related mortality. Sage phenolics indicate promising multitarget leads for IBD therapy, meriting chronic-model and pharmacokinetic evaluation.

## 1. Introduction

*Salvia officinalis* L., commonly known as sage, is a perennial herb traditionally used for its culinary and medicinal properties in Southern Europe and the Mediterranean region. Despite its extensive use, the revelation of its biological effects is less well-documented, with most studies focusing on its antimicrobial and antioxidant properties [1–3].

Salvia (*Lamiaceae*) species have been primarily used as flavoring agents in foods, and there is growing interest in these plants from both the pharmaceutical and cosmetic industries due to their distinctive aroma and ease of cultivation [4]. While the medicinal uses of *Salvia officinalis* L. are not as consistently reported as other aromatic herbs, it has been traditionally employed to treat gastrointestinal (GI) disorders such as indigestion, bloating, and diarrhea, owing to its carminative and spasmolytic properties [5]. Additionally, sage's antimicrobial capabilities have prompted research into its application in managing oral and dental infections, which can lead to periodontal inflammation [6,7].

Research has largely been focused on the *Salvia officinalis* L. essential oil, particularly its industrial, culinary, and medicinal applications, with comparatively fewer studies addressing the pharmacological effects of its phenolic compounds [8]. However, there is an increasing interest in exploring the pharmacological roles of phenolic compounds from aromatic plants [9]. Prior studies have also characterized the phenolic content of *Salvia officinalis* L., identifying phenolic acids as key

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constituents with a chemical profile akin to those examined by this team [10–12].

The biological effects of *Salvia officinalis* L. aqueous extracts are mainly linked to high levels of phenolic compounds, such as rosmarinic and salvianolic acids, which contribute notable antioxidant activity due to their catechol groups [13–16]. Terpenic compounds are also present in hydroalcoholic extracts [17,18]. Margetts et al. [19] found that these extracts reduce cytokine and chemokine release in LPS-stimulated human intestinal cells and lower C Reactive Protein and VCAM-1 in blood-brain barrier cells exposed to IL-17 A. Additionally, sage mitigates ROS production in $H_2O_2$-treated blood-brain barrier cells, alleviates inflammation, restores mediator balance, reduces lipid peroxidation, and enhances antioxidant enzymes, helping prevent extraintestinal complications [20].

Inflammatory Bowel Disease (IBD), which includes Crohn's Disease and Ulcerative Colitis, is marked by chronic, relapsing inflammation of the intestinal tract [21]. Although extensive research has been conducted, the precise mechanisms and pathophysiological causes of IBD remain unclear. It is generally accepted that IBD is driven by a genetically mediated dysregulation of the immune system, which leads to an abnormal immune response triggered by environmental factors [22,23].

One significant complication for IBD patients is an elevated risk of colorectal neoplasia, including dysplasia and colorectal cancer (CRC) [24–26]. This risk is largely attributed to the damage caused by chronic inflammation [27,28]. Reactive oxygen species are a major factor in the inflammatory pathways that connect IBD to CRC [29,30]. According to the latest GLOBOCAN data, CRC is among the most prevalent cancers globally, with over 1.8 million cases and 881,000 deaths in 2018, ranking third in incidence and second in mortality [31].

Given the substantial impact of CRC on morbidity and mortality, it is crucial to develop strategies to prevent neoplastic initiation and progression. Modulating chronic inflammation in IBD patients presents an opportunity to reduce CRC risk [32,33]. Depending on its composition, diet may either mitigate or exacerbate CRC risk [34].

To date, investigations of *Salvia officinalis* in experimental colitis have been confined to dextran-sulphate-sodium (DSS) or acetic-acid models, both of which provoke primarily superficial mucosal damage that heals spontaneously and captures only a subset of Crohn-like pathology. Moreover, those studies employed essential oils, crude decoctions or un-standardized leaf powders, leaving the contribution of phenolic constituents unresolved. Here we deliberately shift to the trinitro-benzene-sulphonic-acid (TNBS) model, which produces deep, segmental transmural lesions, robust neutrophil infiltration and fibrosis-features that more faithfully resemble the human disease spectrum while allowing survival analysis [35,36]. By administering a chemically characterized, rosmarinic-acid-rich phenolic fraction, the present work therefore provides the first evaluation of sage phenolics in a clinically relevant, transmural colitis model and establishes a direct link between defined phytochemicals and in-vivo anti-inflammatory efficacy, addressing a critical gap in the pre-clinical evidence base for *S. officinalis*.

The goal of this study was to characterize the chemical composition and antioxidant effects of a *Salvia officinalis* extract and evaluate its anti-inflammatory effects in both acute and chronic models, which could be relevant for pharmacological intervention in IBD and the prevention of CRC development. We screened the acute anti-inflammatory effects in a paw edema animal model and further assessed the effects of the extract in a TNBS-induced colitis mouse model.

## 2. Results

### 2.1. Total phenolic and total flavonoid content

The initial chemical characterization of the sage extract was conducted using the Folin-Ciocalteu method. The total phenolic and flavonoid content of the extract is presented in Table 1 below.

**Table 1**
Total phenolic and total flavonoid content of sage extract. The values displayed are mean ± standard deviation of triplicates.

| Total phenolics compounds (mg GAE/L) | (mg GAE/g DW) |
|---|---|
| 6208 ± 200 | 49.84 ± 1.61 |
| Total flavonoids (mmol CE/L) | (μmol CE/g DW) |
| 17.271 ± 0.522 | 138.7 ± 4.2 |

GAE-Gallic Acid Equivalents; CE-Catechin Equivalents; DW-Dry Weight.

#### 2.1.1. High-performance liquid chromatography (HPLC-DAD)

The aqueous extracts of *Salvia officinalis* L. contained hydroxycinnamic acids, flavonols, flavones, flavanols and proanthocyanidins often in the form of their glycosidic derivatives were also detected (Fig. 1). Considering the distribution by functional groups, the peaks of hydroxycinnamic acids, flavonols and flavones accounted for 45.7 %, 21.1 % and 11.9 % respectively, of total chromatographic area therefore were the three types of phenolic components more abundant in the extract (Fig. 2). Rosmarinic acid, a dimer of caffeic acid, was the major phenolic component with a concentration of 1.4 g/L, what corresponds to 22.6 % of total phenolics in the extract.

#### 2.1.2. Antioxidant capacity

The antioxidant capacity of the sample under study (Table 2), applying the CUPRAC technique were 672,64 ± 44,19 μmol EAA/g dry plant, with the FRAP technique were 546,6 ± 13,1 μmol de $Fe^{2+}$/g dry plant weight, with DPPH assay were 49,80 ± 0,749 mg EAA/g dry plant weight and with Superoxide anion radical-scavenging assay were 450 ± 99 μmol GAE/g plant dry.

#### 2.1.3. In vitro anti-proliferative cell assays evaluation

*2.1.3.1. HT29 cell culture and cell proliferation assay.* The action of phenolic compounds of *Salvia officinalis* L. on the growth of HT29 cells was tested using the successive dilution method, which allowed the determination of Minimum Inhibitory Concentrations (MICs) for the extract (the values were obtained by determining the growth of HT29 in the presence of successive dilutions of the sample).

The MICs of the sage extract was 250.3 $mg.L^{-1}$ which corresponds to the minimum concentrations (in $mg.mL^{-1}$) necessary to inhibit the growth of HT29, compared to the control.

*2.1.3.2. Wound healing assay.* The capacity for cellular invasion is normally associated with the activity of MMPs and allows an assessment of the potential to inhibit the formation of metastases. Fig. 3 shows the invasion rates of HT29 cells in the presence of tested sample. The invasion (% of initial cut) of the control was 85.0 ± 13.46 and 500 μg/mL sample was 77.1 ± 9.0.

The wound assay method consists of making a cut in a monolayer of cells and then measure the percentage of closure of the cut after 48 h of growth. Under normal conditions, HT29 cells will tend to invade the bare area of the cut. A compound that inhibits wound invasion must have the potential to inhibit the formation of metastases in living tissues; therefore, an inhibition of the wound assay invasion rate is indicative of anti-tumor potential.

The figure indicates that the phenolic extract of Salvia did not significantly inhibit the growth of HT29 cells. However, compared to the control, the extract did show a minor, yet detectable, effect, suggesting a potential—albeit modest—capacity to inhibit metastasis in living tissues.

In previous studies conducted by this group, more potent effects were observed with extracts from other plants, such as pennyroyal [37]. Conversely, certain plants like parsley exhibited no inhibitory effects (unpublished data).

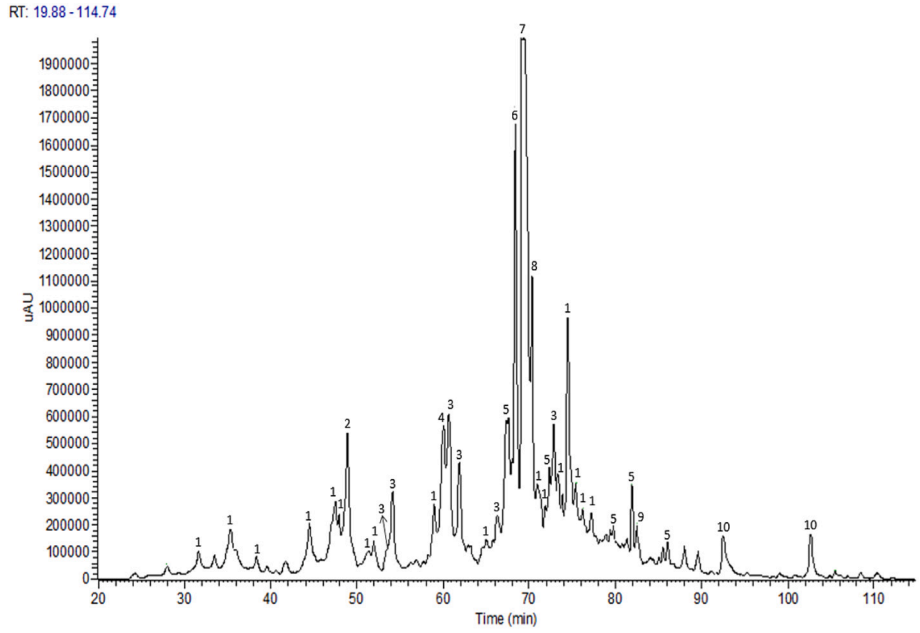


**Fig. 1.** Chromatographic profile of the aqueous extract of *Salvia officinalis* L. analyzed by HPLC-DAD with selective detection at 280 nm. Functional groups or components identified: 1. Hydroxycinnamic acids; 2. Flavanol; 3. Flavonol glucoside; 4. Flavone glucoside; 5. Flavone; 6. Salvianolic acid B; 7. Rosmarinic acid; 8. Salvianolic acid A; 9. Flavonols; 10. Proanthocyanidins.

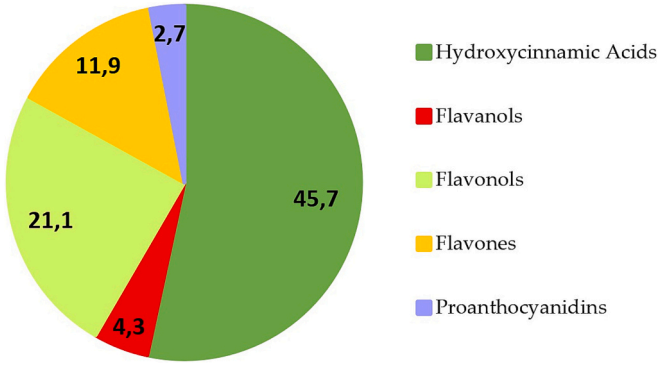


**Fig. 2.** Functional group distribution (%) of the phenolic components present in the aqueous extract of *Salvia officinalis* as determined in the HPLC-DAD profile with selective detection at 280 nm.

**Table 2**
Antioxidant capacity test results.

| Antioxidant assay | mL extract | dry plant |
|---|---|---|
| CUPRAC | 83.78a ± 5.50 (μmol AAE/mL) | 672.64 ± 44.19 (μmol AAE/g) |
| FRAP | 68.07a ± 1.64 (μmol Fe2+/mL) | 546.6a ± 13.1 (μmol Fe2+/g) |
| DPPH | 6202 ± 0.093 (mg AAE/mL) | 49.80 ± 0.749 (mg EAA/g) |
| Superoxide anion radical-scavenging assay | 55.99 ± 12.34 (μmol AGE/mL) | 450 ± 99 (μmol AGE/g) |

AAE – Ascorbic Acid Equivalents; AGE – Gallic Acid Equivalents; DW - Dry Weight.

*2.1.3.3. Evaluation of inhibition of matrix metalloproteinases (MMPs) gelatinolytic activity.* Since the inhibition of the activity of MMP-9 and MMP-2 is associated with the inhibition of invasion, the minimum concentrations of the extract necessary to inhibit the activity of the two gelatinases in the HT-29 medium were determined. The MIC (Minimum

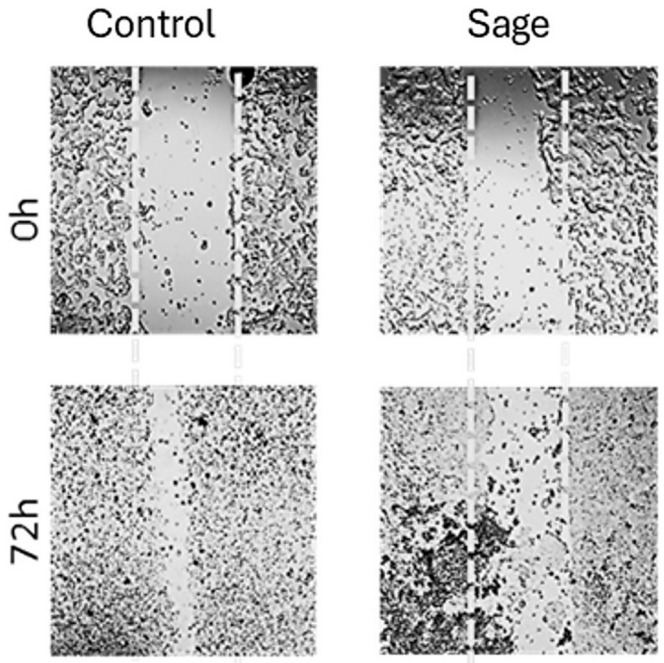


**Fig. 3.** The invasion of HT29 cells in the presence of tested sample during 72 h. The percentage of closure of the cut after 48 h of growth, resulting invasion with 500 μg/mL sample.

inhibitory concentrations) on gelatinase activity in the extracellular environment obtained with sage extract was 3,9 mg.L$^{-1}$ that correspond to the minimum concentrations necessary to inhibit the activity of MMP-9 and MMP-2, compared to control.

Although a modest (9 %) slowing of scratch closure and a reduction in gelatinase activity were observed, these in-vitro changes are insufficient to infer anti-metastatic efficacy and are therefore presented as ancillary findings.

### 2.1.4. In vivo experiments

*2.1.4.1. Carrageenan-induced paw oedema in rat.* Intradermal injection of carrageenan predictably led to paw edema, as indicated by a 70 % increase in paw volume, 6 h post-injection compared to animals injected with saline, thereby validating this model of acute local inflammation. The oral administration of sage extract resulted in a statistically significant reduction in edema formation (Fig. 4). Comparing these results with known anti-inflammatory and antioxidant compounds (indomethacin, trolox, and tempol) demonstrated that a dose of 15 mg/kg of phenolic acids from the sage extract reduced paw edema to the same extent as the positive controls, which was around only 20 % of paw volume increase when compared to the carrageenan control group.

*2.1.4.2. TNBS-induced ulcerative colitis model in mice.* Analysis of results from both the Sham and Ethanol Groups revealed that the animals displayed no visible signs of colonic lesions and had a 0 % mortality rate. In contrast, colitis induction in the TNBS/EtOH Group led to statistically significant changes, including reduced colon length, increased extent of ulceration, and greater severity of diarrhea, along with a 40 % mortality rate (Fig. 5, Fig. 6, Table 3). In groups treated with the *Salvia officinalis* L. extract, these indicators of colonic injury were significantly less severe when compared to the untreated animals with colitis.

Furthermore, mortality was significantly lower in the group treated with sage extract (18,2 %) at the end of experiment (day 5) compared to the TNBS group (36,4 %), as shown in the Kaplan-Meier analysis in Fig. 7.

Throughout the five-day study, the 15 mg/kg phenolic-acid dose of the *Salvia officinalis* extract proved innocuous. Mice that received the extract, either alone (sham + sage) or after TNBS challenge, displayed normal coat condition, posture, mobility and stool consistency; no lethargy, piloerection or other untoward clinical signs were recorded at any point. Body-mass trajectories in the sage-only group overlapped those of untreated healthy controls, and in the colitic cohort sage actually mitigated the transient weight loss induced by TNBS. Importantly, survival was 100 % in both sage-treated groups, whereas TNBS controls showed 75 % survival by day 5.

Post-mortem examination corroborated the absence of systemic toxicity: liver, kidneys, spleen, lungs and small intestine were macroscopically normal, and the colon itself exhibited markedly less damage when sage was co-administered. Histological sections of liver and kidney from representative sage-only animals revealed intact architecture without necrosis or inflammatory infiltrates. Together, these findings confirm that the hydro-ethanolic sage extract, delivering 15 mg/kg total phenolic acids, is non-cytotoxic and well tolerated under the conditions of the present experiment.

*2.1.4.2.1. Histology and immunohistochemistry.* The histological findings (Fig. 8) revealed that while sham mice (without colitis induction) samples exhibit a normal colon with no lesions, mucosa of uniform thickness, normal crypt architecture and no signs of inflammation; the TNBS-induced colitis samples exhibit a severe ulceration with crypts destruction and a marked neutrophils infiltration (score 3). Interestingly, the samples from animals treated with sage extract show partial slight mucosal erosion and small disperse superficial ulcers, without crypt loss although with mononuclear cell infiltration (arrow, score 1).

As shown in Fig. 9 colitis induction led to a marked increase of COX2 (2.48-fold, $p < 0.05$) and even more of iNOS expression (4.25-fold, $p < 0.01$) along the remaining crypts indicated by brown color when compared with control samples. Samples of mice treated with sage extract showed no expression of COX2 (1.03-fold, $p < 0.05$ vs colitis) and a markedly reduced staining for iNOS (2.19-fold, $p < 0.05$ vs colitis), indicative of a decreased inflammatory status.

## 3. Discussion

Sage has been recognized for its culinary and medicinal uses for many centuries, yet scientific literature evaluating its health benefits is surprisingly limited compared to other herbs. Recent studies have highlighted a variety of pharmacological properties attributed to sage, including antioxidant [5], anti-inflammatory [23], antimicrobial [17], and anticancer [38] effects. Research indicates that both the essential oils and the aerial parts of sage contribute significantly to these benefits, particularly in traditional medicine practices across the Mediterranean region [1,39]. Continued investigation is essential to fully elucidate the mechanisms underlying these effects and to standardize its possible applications in health care.

The biological activities of *Salvia officinalis* L. aqueous extracts have been attributed to the presence of significant concentrations of phenolic components, in particular, rosmarinic and salvianolic acids [13–15]. In fact, our study confirmed a high content of phenolic compounds in sage and when characterizing these compounds by HPLC, rosmarinic acid was detected in greater quantities.

High antioxidant activity is attributed to these components and related to the presence of multiple catechol groups in their molecular structures [16].

Terpenic compounds have also been identified in *Salvia officinalis* L. hydroalcoholic extracts [17,18]. These components were not detected in the *Salvia officinalis* L. extracts used in the present work, because their limited solubility in aqueous solutions led to their precipitation after elimination of ethanol during the extract concentration procedure.

Salvia's phenolic compounds may be primarily responsible for both

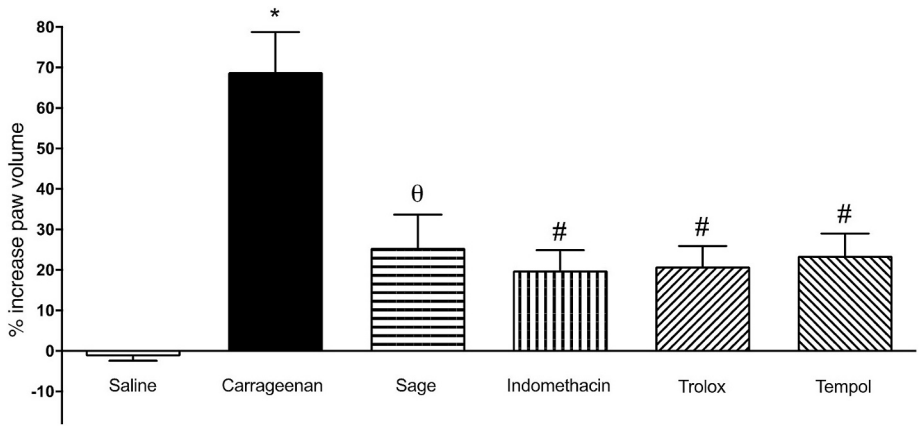


**Fig. 4.** Effect of sage extract administration on the paw edema volume induced by carrageenan. Data is presented as mean ± SEM of 7 mice per group. One-way ANOVA followed by Bonferroni post-hoc; $p < 0.05$ vs TNBS. * $p < 0.001$ vs. Control; # $p < 0.001$ vs. Carrageenan. Saline group ($n = 7$); Carrageenan Group (n = 7); Sage group (n = 7); Indomethacin group (n = 7); Trolox group (n = 7); Tempol group (n = 7). Abbreviations: Sage, phenolic fraction of *S. officinalis*.

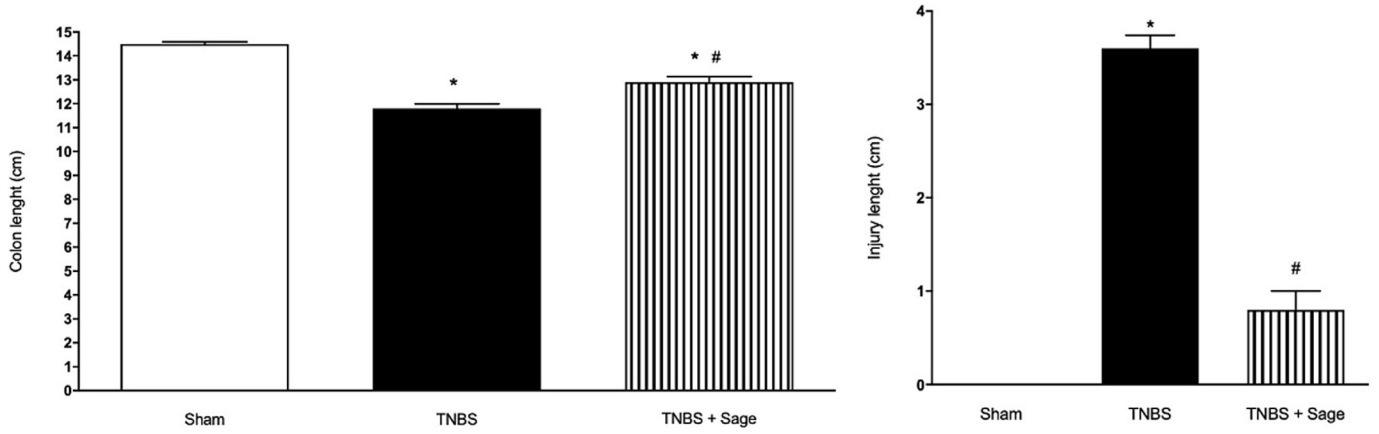


**Fig. 5.** Effect of sage extract administration on the length of the colon (cm) and length of injury. Data is presented as mean ± SEM of n mice per group. One-way ANOVA followed by Bonferroni post-hoc; $p < 0.05$ vs TNBS * $p < 0.001$vs. Sham; # p < 0.001 vs. TNBS. Sham Group ($n = 7$); TNBS Group ($n = 10$); TNBS + Sage Group (n = 10). Abbreviations: TNBS, trinitrobenzene-sulphonic acid; Sage, phenolic fraction of *S. officinalis*.

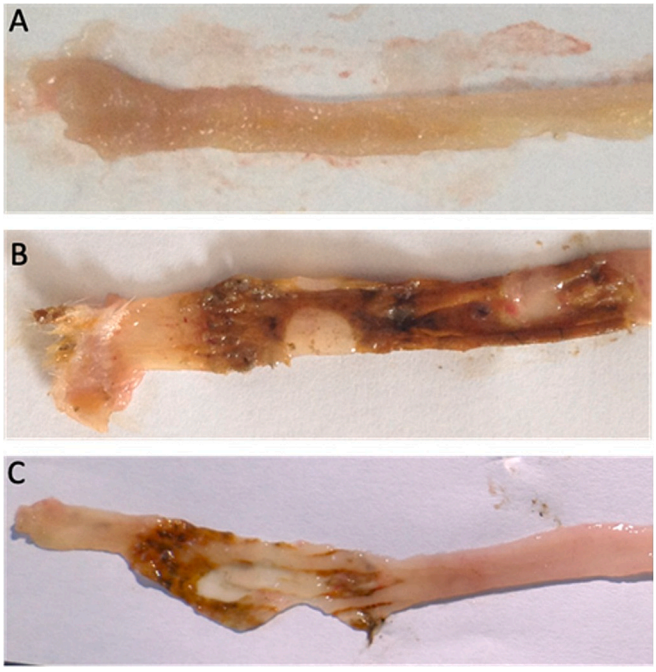


**Fig. 6.** Effect of oral administration of sage extract, on the macroscopic evaluation of colon. (A) Sham group (n = 7), (B) TNBS group ($n = 10$), (C) TNBS + Sage extract ($n = 10$). Abbreviations: TNBS, trinitrobenzene-sulphonic acid; Sage, phenolic fraction of *S. officinalis*.

**Table 3**
Morphological and functional observations of the colon, immediately after collection. Data is presented as mean ± SEM of n mice per group. One-way ANOVA followed by Bonferroni post-hoc; #p < 0.05 vs Sham; *p < 0.05 vs TNBS.

| | Length of colon (cm) | Extent of injury | Presence/Consistency of diarrhea | Mortality (%) |
|---|---|---|---|---|
| Sham | 14.5 ± 0.082 | 0 | 0 | 0 |
| TNBS | 11.8 ± 0.19 # | 3.6 ± 0.14 # | 3 # | 40 |
| TNBS + Sage | 12.9 ± 0.2 * | 0.8 ± 0.2 * | 0.29 * | 0 |

Abbreviations: TNBS, trinitrobenzene-sulphonic acid; Sage, phenolic fraction of *S. officinalis*.

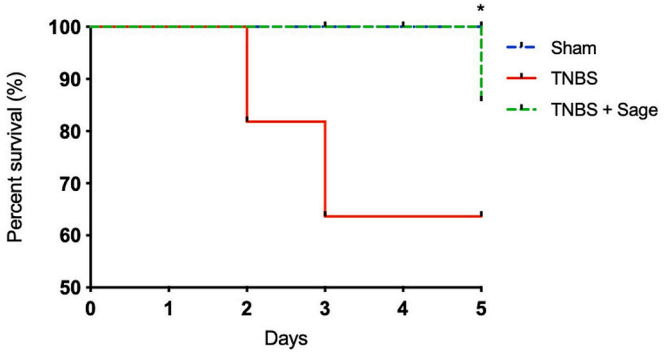


**Fig. 7.** Kaplan–Meier survival analysis of TNBS-challenged mice treated with sage phenolic extract. CD-1 mice received intracolonic TNBS (2.5 % *w/v*, 100 μL) and were gavaged 2 h later, and daily for four days, with vehicle (TNBS + Veh, $n = 10$) or the phenolic extract of *Salvia officinalis* (TNBS + Sage, 15 mg phenolic acids $kg^{-1}$, $n = 10$). Survival was monitored for 5 days. Day-5 survival was 63.6 % (95 % CI 35.4–84.8 %) in the vehicle group versus 100 % (95 % CI 69.1–100 %) in the sage-treated group. Curves were compared with the log-rank (Mantel–Cox) test: $\chi^2 = 4.38$, $P = 0.036$ ($\alpha = 0.05$). Abbreviations: TNBS, trinitrobenzene-sulphonic acid; Veh, vehicle (0.5 % carboxymethylcellulose); Sage, phenolic fraction of *S. officinalis*; CI, confidence interval.

the antioxidant activity demonstrated by this aromatic herb and also for its acute anti-inflammatory capacity, such as that studied in the model of paw oedema caused by carrageenan, as well as in chronic inflammatory disease of the colitis also studied and described in this work. One of the studies that aimed to correlate the antioxidant effect of *Salvia officinalis* L. with its anti-inflammatory properties focused on the evaluation of the potential of an ethanolic extract of sage, rich in bioactive compounds, to protect against liver damage caused by the chemotherapeutic agent cisplatin in rats [40]. Cisplatin treatment led to significant liver damage, increased liver enzymes, oxidative stress, and liver tissue damage. Pre-treatment with a sage extract significantly mitigated these effects, suggesting that its antioxidant and/or anti-inflammatory properties can protect against chemotherapy-induced hepatotoxicity.

In a study by Margetts et al. [19] the impact of *Salvia officinalis* L. extract on the release of various cytokines and chemokines was evaluated in human primary intestinal epithelial cells, both with and without LPS stimulation, and in Blood Brain Barrier (BBB) cells, with or without recombinant IL-17 A and/or Human IL-17RA/IL-17R Antibody. Additionally, the antioxidant effects were tested in BBB cells treated with the extract and $H_2O_2$. The extract significantly reduced cytokine and chemokine release in LPS-stimulated intestinal cells and decreased C

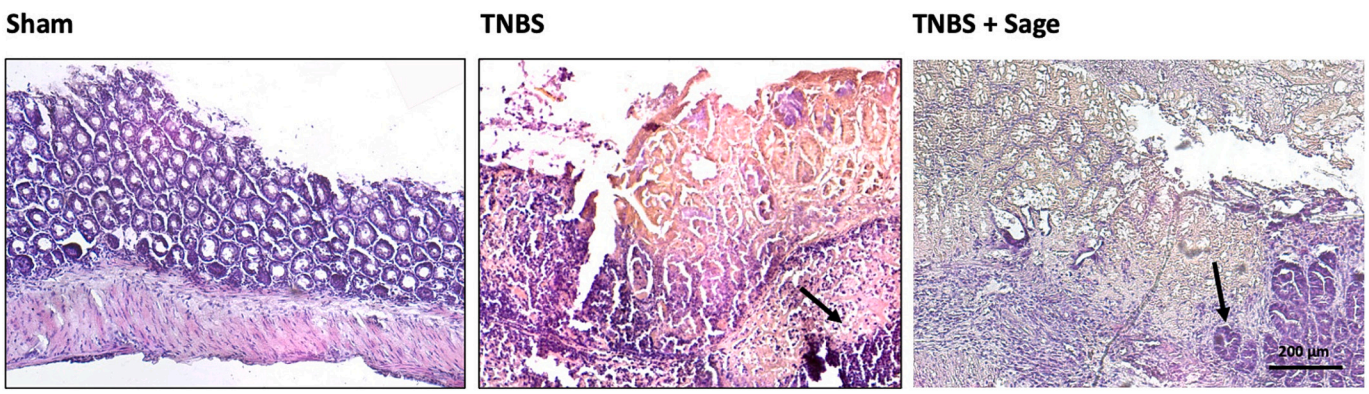


**Fig. 8.** Histological findings in the TNBS-induced colitis model. Original magnification ×100. Scale bar equals 200 µm.

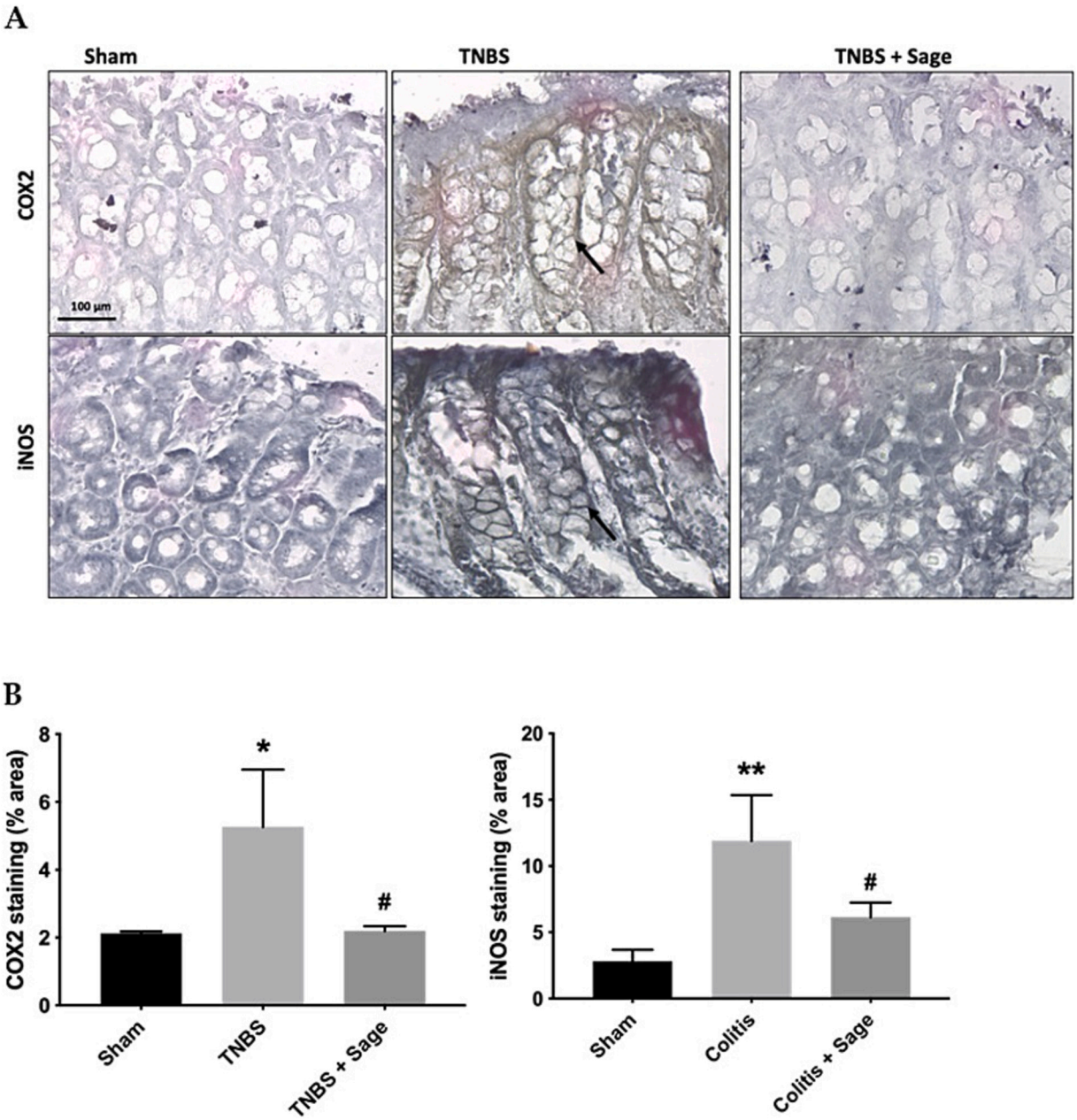


**Fig. 9.** (A) Evaluation of COX-2 and iNOS expression in the TNBS-induced colitis model. Original magnification ×100. Scale bar equals 100 µm. (B) Percentage of staining revealing COX2 and iNOS expression for each group tested.

Reactive Protein and VCAM-1 levels in blood-brain barrier cells under IL-17 A conditions. Sage also demonstrated antioxidant effects by reducing ROS production in $H_2O_2$-treated blood-brain barrier cells.

In this study, the team aimed to first assess the anti-inflammatory effects of sage extract, by investigating the impact of a single oral dose in an acute paw edema model. Measurement of paw volumes after carrageenan-induced edema showed that sage extract reduced paw volume by 43.4 % compared to the vehicle group. Furthermore, the reduction was not statistically different from positive control groups treated with known antioxidants and anti-inflammatory agents (49 % for indomethacin, 48 % for Trolox, and 45.5 % for tempol), suggesting potential benefits of sage extract in treating inflammation-related conditions.

Following the confirmation of sage's anti-inflammatory effects in an acute local inflammation model, was further evaluated the possible benefits of the extract in a mouse model of experimental colitis. Treatment with sage extract in mice with TNBS-induced colitis led to a reduction in various indicators of colon damage and inflammation: it prevented the shortening of the colon, decreased the extent of injury, lessened the severity of diarrhea, and lowered the mortality rate. Furthermore, both visible and microscopic signs of colon inflammation were reduced in mice treated with sage extract compared to those with colitis.

Jedidi et al. [41] showed that a simple water-decoction of *Salvia officinalis,* rich in rosmarinic acid, and seven other phenolics quantified by the same HPLC-DAD methodology employed in the present study, pre-emptively attenuated 4 % acetic-acid colitis in rats. Jedidi et al.'s extract, given for seven days before the insult, lowered ulcer scores and lipid peroxidation while restoring endogenous antioxidant enzymes, leading the authors to ascribe protection chiefly to redox modulation and metal chelation. The study, however, targeted a superficial, self-limiting ulcerative-colitis model and did not report survival or systemic safety data.

The present work advances these findings on several fronts. Using a chemically characterized hydro-ethanolic fraction delivering 15 mg phenolic acids $kg^{-1}$ (62 $\pm$ 3 mg $g^{-1}$ RA), this team intervened after disease induction in the more severe, transmural TNBS model in mice. Therapeutic dosing once daily for four days not only condensed classical damage indices (disease-activity score, ulcer length, and colon shortening) but also normalized iNOS and COX-2 expression and, crucially, abolished TNBS-related mortality (100 % survival versus 63 % in controls). Comprehensive clinical observation, biochemistry and histology confirmed the absence of systemic toxicity at this dose, supporting its translational relevance. Therefore, while both studies underscore the anti-inflammatory value of RA-rich sage phenolics, this current study's data extends the mechanistic scope beyond antioxidant defense to include direct suppression of pro-inflammatory enzymes and demonstrate life-saving efficacy in a clinically tougher model, thereby providing a stronger rationale for developing standardized *S. officinalis* phenolic preparations as co-adjuvant therapeutics for inflammatory bowel disease.

Only three publications have focused on the effects of sage in colitis models, although none with the TNBS-induced model used in this study. A study aimed to investigate the protective effects of *Salvia officinalis* L. leaf decoction extract on liver and kidney damage caused by acetic acid-induced colitis in Wistar albino rats [20]. Rats were pretreated with the extract at doses of 50, 100, and 200 mg/kg orally for 10 days, followed by acute rectal administration of acetic acid. The findings demonstrated that *S. officinalis* L. treatment mitigated liver and kidney injuries, as evidenced by normalized plasma transaminase activities and preserved tissue structures. Treatment also restored high-density lipoprotein-cholesterol levels, significantly reduced lipid peroxidation, and enhanced both enzymatic and non-enzymatic antioxidant defenses in liver and kidney tissues. Furthermore, it protected against inflammation and corrected intracellular mediator imbalances, suggesting that *S. officinalis* L. effectively reduces lipid peroxidation and boosts antioxidant enzyme activity, thereby preventing extraintestinal complications [20]. This study however did not evaluate the effects in the colon and rather focused on the systemic effects associated with this model.

In a different study, ethanolic and methanolic extracts of sage were tested for their potential to treat ulcerative colitis induced by acetic acid in rats [42]. Both ethanolic and methanolic extracts of sage showed significant improvement in colitis symptoms, with the methanolic extract being particularly effective in these experimental conditions. In these circumstances, treatment with sage extracts reduced inflammation and oxidative stress, as indicated by lower levels of myeloperoxidase and malondialdehyde, and improved tissue damage.

In comparison to present work, it is important to already consider that while the acetic acid model is a valid tool for studying colitis in mice it is much simpler in its pathophysiology and suitable only for acute inflammation studies, while the TNBS model is more complex and better suited for studying chronic inflammatory processes and immunological mechanisms, involving both innate and adaptive immune responses in the gut, making it more representative of human IBD [43].

The third study investigated the effectiveness of a phytopharmaceutical blend of sage and bitter apple in treating chronic colitis induced by dextran sulfate sodium (DSS) in mice [44]. In the DSS colitis model, this blend alleviated symptoms in a dose-dependent manner, leading to improved tissue healing as evidenced by a lower histopathological score. The treatment reduced neutrophil counts and the expression of the chemokine CXCL-1/KC, while promoting macrophage recruitment. Additionally, inflammatory marker levels were decreased, and the anti-inflammatory cytokine interleukin-10 was elevated in the colon tissue. However, the authors were not able to quantify the contribution of this effect to the two components of the treatment.

And again, although the DSS model is suitable for studying mucosal inflammation and it is easier to administer, it however lacks the transmural inflammation seen in chronic colitis, a very clinically relevant characteristic of ulcerative colitis, which can be represented in the TNBS model used in the present study [35,43].

This work demonstrated that rosmarinic acid was the principal compound suggesting a relevant role of this phenolic acid in the beneficial effect. An important function of rosmarinic acid was identified in the study of Rocha et al. [45] in the modulation of several critical pathways of inflammation in liver and lung inflammation models, in which administration of rosmarinic acid led to reduction of nuclear factor-kappa B (NF-kB) activation, as well as inhibition of the phosphatidylinositol 3-kinase (PI3k)/protein kinase B (Akt) and Glycogen synthase kinase 3beta (GSK-3beta) pathways [45].

Rosmarinic acid was identified as the main compound in several studies, and as observed in the current research, highlighting its significant role in the detected positive effects. Previously, this team compared rosemary extract and isolated rosmarinic acid in a local inflammation model, finding rosmarinic acid to be primarily responsible for the anti-inflammatory effects [45]. In that study, rosmarinic acid played a crucial role in modulating key inflammation pathways in liver and lung models, leading to reduced activation of nuclear factor-kappa B (NF-kB) and inhibition of the phosphatidylinositol 3-kinase (PI3k)/protein kinase B (Akt) and Glycogen synthase kinase 3beta (GSK-3beta) pathways. In 2017, Jin and colleagues published a study examining the effects of rosmarinic acid on dextran sulfate sodium (DSS)-induced colitis and its potential mechanisms [46]. Their findings showed that administering rosmarinic acid (30 or 60 mg/kg/day, orally) significantly alleviated colitis severity, decreased the production of inflammatory cytokines (IL-6, IL-1β, and IL-22), and reduced the protein levels of COX-2 and iNOS. Additionally, rosmarinic acid inhibited the activation of NF-kB and STAT3, leading to a reduction in the activity of pro-survival genes. In line with these results, this current study observed that experimental colitis induced the expression of iNOS and COX-2, but treatment with sage extract effectively reduced these levels and in the case of COX-2 was able to normalize the levels when compared to sham

animals without colitis induction.

To this regard, a study was published in which assessment of the anti-inflammatory and antioxidant effects of *S. officinalis* L. in a lipopolysaccharide (LPS)-induced inflammation model was performed. Wistar rats receiving 1 mg/kg LPS intraperitoneally and were given 10 and 30 mg/kg of *S. officinalis* L. extract orally. Findings showed higher MDA levels in the inflammation group compared to controls, while superoxide dismutase, catalase, and glutathione peroxidase activities were significantly lower while treatment groups exhibited reduced NO, NF-κB, and TNF-α levels, suggesting that its beneficial effects on this LPS-induced inflammation and oxidative stress model could in part be explained by mechanisms mediated by iNOS [47].

Given that a significant long-term consequence for IBD patients is the heightened risk of developing CRC [28,48], largely due to chronic inflammation [49,50], the beneficial effects reported in this study are particularly noteworthy. Research has underscored that chronic inflammation, along with the severity, extent, and duration of IBD, is the main factor linking IBD to the increased risk of CRC [51].

In this study, regarding the inhibitory effect on the growth of HT29 cells, the extract of *Salvia officinalis* L. required a minimal concentration of 250 mg/L, which is relatively low compared to other aromatic herbs, such as mint and coriander, which showed no inhibition [37,52]. MIC were evaluated not only for cell growth but also for gelatinase activity, because cellular invasion is often linked to the activity of matrix metalloproteinases (MMPs), which are crucial for assessing metastatic potential. This study measured MMP-9 and MMP-2 gelatinolytic activity to determine the minimum concentration of sage extract needed to inhibit MMP-9 and MMP-2 activity in the extracellular environment. MIC determinations show that gelatinolytic activity is inhibited with 3.9 mg/L, indicating inhibition of MMP-9 and MMP-2 activity that is associated with reduced invasion capacity. The modest slowing of scratch closure ($\leq$ 9 %) and the $\approx$ 15 % decrease in MMP-9 gelatinolytic activity produced by the sage hydro-ethanolic extract indicate only a weak anti-migratory effect in vitro. These changes fall well below the magnitude usually considered biologically meaningful for anti-metastatic lead compounds and were not corroborated by complementary extracellular-matrix invasion results. Therefore, the authors view them as ancillary evidence of the extract's general anti-inflammatory profile rather than proof of anti-metastatic potential. Robust assessment of metastatic inhibition will require dedicated in-vivo models that capture the multi-step nature of tumor dissemination.

Inflammatory processes, rather than traditional cancer initiation and progression mechanisms, appear to play a crucial role in regulating colitis-associated cancer, disrupting intestinal microbiota, and interacting with multiple signaling pathways [53]. Thus, a pleiotropic approach might be a more effective pharmacological strategy, targeting both colitis-associated inflammation and the mechanisms driving cancer development and progression. Numerous studies have demonstrated that plant extracts high in rosmarinic acid have anticancer effects across various in vitro and in vivo models of different cancer types [54–56].

One study investigated the effects of rosmarinic acid in a rat model of 1,2 dimethylhydrazine (DMH)-induced colorectal cancer (CRC) [57]. Rosmarinic acid was administered at a dose of 5 mg/kg throughout the CRC induction period, resulting in tumor reduction, decreased oxidative stress markers, lower mucosal bacterial enzyme activity, regulation of xenobiotic metabolizing enzymes, and upregulation of apoptotic factors. In another study, rosmarinic acid inhibited activator protein-1 (AP-1)-dependent activation of COX-2 expression in HT-29 colon cancer cells [58]. The inhibition of COX-2 and iNOS expression observed in this work with sage extract underscores its beneficial effects not only on the pathogenesis of IBD but also on disease progression. The roles of COX-2 and iNOS in angiogenesis, apoptosis, metastatic processes, and resistance to chemotherapy/radiation are well-documented and crucial for IBD and CRC patients [59–63]. Consequently, targeting COX-2 and iNOS in intestinal inflammation has been recognized as a promising strategy for chemoprevention of colon cancer [63].

Although the antiproliferative effects of the essential oil of *Salvia officinalis* L. have been studied, little is known about the phenolic fraction.

A hydrodistilled essential oil from Sicilian *Salvia officinalis, r*ich in α-thujone, 1,8-cineole and camphor, was profiled using gas chromatography identifying 34 constituents, and tested on three human colon-cancer cell lines. After 72 h the oil significantly suppressed tumor-cell proliferation and altered cell-cycle distribution, yet left normal colonic epithelial cells unaffected, indicating selective antiproliferative activity [64].

Water extracts of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO), together with their main phenolic, rosmarinic acid (RA), were tested on two colon-cancer lines with distinct signaling mutations (HCT15 and CO115). All three treatments triggered apoptosis in both lines, but only the sage extracts curbed proliferation in HCT15. Mechanistically, SF, SO and RA suppressed ERK phosphorylation in HCT15, while leaving Akt signaling in CO115 unchanged, pointing to MAPK/ERK pathway inhibition as the key antiproliferative mechanism [55].

## 4. Conclusion

In conclusion, these findings, to the best of our knowledge, demonstrate for the first time that a phenolic extract of sage positively impacts experimental IBD by reducing its severity and mortality, as well as by decreasing key injury markers and inflammatory mediators such as COX-2 and iNOS, which play crucial roles in intestinal inflammation. While the extract ameliorated TNBS-induced colitis and down-regulated iNOS and COX-2, translation to clinical settings will require pharmacokinetic studies and validation in chronic and spontaneous colitis models. No claims of colorectal-cancer prevention can be made from the present data.

The bioavailability of salvia's bioactive compounds remains unexplored, and it is not a food typically consumed in large quantities. Therefore, determining the appropriate dosage of these bioactive compounds could be highly beneficial for their use in controlled doses as a dietary supplement. Another potential approach could be encapsulating these compounds to enhance their absorption and bioavailability.

## 5. Materials and methods

### 5.1. Reagents and chemicals

Unless otherwise stated, all reagents and chemicals were obtained from Sigma-Aldrich (Portugal). Noted exceptions are ketamine (Imalgene® 1000) and xilazine (Rompun® 2 %) acquired from Bio2 *Produtos Veterinários* (Lisboa, Portugal).

### 5.2. Plant material and extract preparation

Fresh *Salvia officinalis* L. samples were sourced locally from cultivated plants in Lisbon, Portugal. The collected plant material, consisting of both leaves and stems, was first thoroughly rinsed with water to remove any debris or contaminants. After washing, the material was cut into thin slices to increase the surface area for extraction.

For the extraction process, 15 g of the sliced plant tissue were immersed in 100 mL of 70 % ethanol (ethanol diluted with water in a 70:30 ratio by volume). The mixture was kept in a dark environment at room temperature and continuously stirred for 24 h. This method helps to maximize the extraction of bioactive compounds by preventing light-induced degradation and ensuring consistent exposure of all plant material to the solvent.

Following the extraction, the mixture was filtered using Whatman no.1 filter paper to separate the liquid extract from the solid plant residues. To further purify the extract, each portion of 30 mL of filtrate was washed with 100 mL of 70 % ethanol, which aids in removing impurities and enhances the concentration of the desired compounds.

After purification, the ethanol solvent was removed using a rotary evaporator set to 40 °C (Heidolph LABOROTA 4001 model), which allows the solvent to evaporate gently without damaging temperature-sensitive components. The concentrated extract was then subjected to centrifugation at 6000*g* for 15 min at 4 °C (using a Sigma 4 K-15C centrifuge) to separate any remaining solid particles. The clear supernatant obtained after centrifugation was carefully collected.

Finally, the resulting supernatant was divided into 1 mL aliquots and stored at −50 °C until further analysis. This careful storage at ultra-low temperature helps preserve the integrity and bioactivity of the extract for subsequent experimental procedures.

### 5.3. Total phenolic and total flavonoid content

Total phenolic compounds and total flavonoid content were determined according to previously described techniques [65,66] and adapted the following [67]. Results of total phenolic content were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of fresh fruit and per mL of the extract. Total flavonoid content was expressed in μmol catechin equivalents of (CE) per mL of extract and per gram of fresh fruit.

### 5.4. High-performance liquid chromatography (HPLC-DAD)

The technique described by Rocha et al. (2019) was followed without any modifications [37]. Spectra acquisition was made in the range of 190 nm to 700 nm with selective detection at 280, 320 and 360 nm. Identification of the main functional groups present in the extract was performed by comparison of their UV spectra with those of representative standards analyzed in the same conditions. Standard curves were determined for rosmarinic and ferulic acids by analyzing the corresponding standards in the concentration range of 0.05 to 1 mg/mL.

### 5.5. Antioxidant capacity

#### 5.5.1. Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC assay was performed following the sample measurement procedure previously described [68]. Results were expressed as μmol ascorbic acid equivalents (AAE) per mL of extract.

#### 5.5.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried according to the procedure described in [69]. A calibration curve of ferrous sulphate (0–1.25 mM) was used and results were expressed as μmol Fe2+ per mL of extract.

#### 5.5.3. DPPH radical-scavenging assay

The DPPH assay was carried according to the procedure previously described [70]. Results were expressed as mg ascorbic acid equivalents (AAE) per mL of extract.

#### 5.5.4. Superoxide anion radical-scavenging assay

The superoxide anion radical-scavenging assay was performed according to the procedure previously described [71]. Results were expressed as μmol equivalents of gallic acid per mL of extract.

### 5.6. In vitro anti-proliferative cell assays evaluation

#### 5.6.1. HT29 cell culture and cell proliferation assay

The HT29 colon adenocarcinomas from *Homo sapiens sapiens*, cell line ECACC, no 91072201, was used in in vitro experiments, as previously described [72]. HT29 cell proliferation assay was performed according to other authors [73] with modifications previously described by us [72].

#### 5.6.2. Wound healing assay

The phenolic extract of sage corresponding to an EC50 level of activity was assessed for its inhibitory activities in HT29 colon adenocarcinoma cells using standard cell migration analysis (wound healing assay) as previously described by us [72]. Data is presented as the mean ± SD.

#### 5.6.3. Evaluation of inhibition of matrix metalloproteinases (MMPs) gelatinolytic activity

MMP-inhibition was tested as previously described [74]. The Minimal Inhibitory Concentrations (MICs) were assessed using the method previously described [72].

### 5.7. In vivo experiments

Experiments were performed according to the Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London, UK, and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication no. 85–23, revised 1996) and according to the most updated EC regulations (Directive 2010/63/EU). ARRIVE Guidelines for Reporting Animal Research summarized at http://www.nc3rs.org.uk were strictly followed and respected during the studies. Protocol was submitted to and approved (CEEE-002/16) by the Ethics Committee for Animal Experiments (CEEA) of the Faculty of Pharmacy—University of Lisboa in February 2016.

#### 5.7.1. Carrageenan-induced paw oedema in rat

The paw edema study was carried out using 42 male Wistar rats (150–200 g) (Harlan - Spain). All rats had free access to water and food until 12 h of the study. Following our previous publications [45], "paw edema was induced by sub-plantar (intradermal) injection of 100 μL of a λ-carrageenan solution (1% in saline) into the rat left hind paw. Paw volume measurements were performed as described: V0 or basal volume is the volume of the hind paw measured immediately after carrageenan injection and V6 is the volume at 6h post carrageenan administration. The increase in paw volume was measured as edema and expressed as relative percentage of the increase in the volume at 6h compared to the initial volume, according to the following formula: % paw volume increase = [(V6-V0) / V0] x 100.". Animals were randomly allocated into six groups: (a) Control group - animals were subjected to the edema protocol described previously, except for the administration of 100 μL of sterile saline instead of carrageenan. Animals were subjected to administration of water (1 mL/kg) by oral gavage ($n$ = 7); (b) Carrageenan group - animals with paw edema induction as described previously and administered with 1 mL/kg of water by oral gavage (n = 7); (c) Sage group - animals with paw edema induction as described previously and administered with Sage extract (15 mg of phenolic acids/kg by oral gavage) 30 min before injection of carrageenan (n = 7); (d) Indomethacin group - animals with paw edema induction as described previously and administered with indomethacin (10 mg/kg by oral gavage) 30 min before injection of carrageenan (n = 7); (e) Tempol group - animals with paw edema induction as described previously and administered with tempol (30 mg/kg by oral gavage) 30 min before injection of carrageenan (n = 7); (f) Trolox group - animals with paw edema induction as described previously and administered with Trolox (10 mg/kg by oral gavage) 30 min before injection of carrageenan (n = 7). The dose of the extract was selected according to previous studies by our group and the dose of 15 mg/kg of phenolic acids has generated consistent results in similar experimental settings and is also within the range of possibility for clinical translation and use, considering a human adult of 70 kg [37,72,75–77].

#### 5.7.2. TNBS-induced ulcerative colitis model in mice

Male mice (CD-1 strain), weighing 28–33 g (5–6 weeks of age) (Harlan, Spain), were maintained according to the standard housing guidelines with free access to water and food, in a room with

environmental conditions automatically controlled (22 ± 1 °C with a 12/12 h light/dark cycle) at the Animal Facility of the Faculty of Pharmacy - University of Lisbon. Induction of colitis was performed by administration of TNBS as previously described [37]. In brief, "a 50% ethanolic solution of TNBS (2.5% m/v) was administered by intracolonic administration (4 cm above the anus). At day 4 post-induction, blood samples were collected by cardiac puncture under surgical anesthesia, followed by euthanasia by cervical dislocation and subsequent necropsy. The colon was removed and was observed for classification of diarrhea severity. Furthermore, the colon was washed with PBS for a macroscopic observation of lesions and fixed in PFA for histological studies." [37]. Animals were randomized into: (a) Sham group ($n = 7$): the colitis induction protocol was followed as described previously except for the intracolonic administration being performed with 100 μL of saline solution instead of the alcoholic TNBS solution. Animals were administered with 10 mL/kg of water by oral gavage throughout the four days of the experiment; (b) Ethanol group (n = 7): the colitis induction protocol was followed as described previously except for the intracolonic administration being performed with 100 μL of 50 % (*v*/v) ethanol solution instead of the alcoholic TNBS solution. Animals were administered with 10 mL/kg of water by oral gavage throughout the four days of the experiment; (c) TNBS group ($n = 10$): the colitis induction protocol was followed as described previously, with the administration of 100 μL of a TNBS solution (2.5 % TNBS in 50 % ethanol). Animals were administered with 10 mL/kg of water by oral gavage throughout the four days of the experiment; (d) TNBS + Sage group (n = 10): the colitis induction protocol was followed as described in the previous experimental group. Animals were administered with Sage extract (15 mg/kg of phenolic acids by oral gavage) throughout the four days of the experiment.

Regarding the macroscopic evaluation of colitis severity, diarrhea severity was classified by an observer blinded to the experimental groups according to Table 4. A microscope observation of the tissue was performed followed by measurement of the entire colon and injury extent.

Histology and immunohistochemistry procedures, including Hematoxylin & Eosin (H&E) staining, as well as the immunohistochemistry studies, for measurement of COX-2 and iNOS expression, were performed as previously described [72]. In brief, "colon histological damage was scored as follows: score 0 - normal colon with no lesions, the mucosa is of uniform thickness, and the crypts are straight, normal crypt architecture, there is no cellular infiltration, edema, or exudate; score 1 - colon with mild lesions, there are mucosal erosion and small superficial ulcers scattered along the length of the colon, with slight crypt loss and mononuclear cell infiltration; score 2 - colon with moderate lesions, intestines have extensive erosion and ulceration, with moderate crypt loss and neutrophil infiltration; score 3 - colon with very severe ulceration, much of the mucosa is thin with loss of crypts and markedly increased infiltration of neutrophils and acute inflammatory exudate. The intensity of the protein staining is relatable to the level of expression of iNOS and COX-2. The level of iNOS or COX-2 staining was quantitatively evaluated by determining the percentage of tissue area that was stained in brown, using the ImageJ (Fiji Is Just) software" [72].

The animal dose-selection rationale and pilot in rat colitis models, Lamiaceae extracts rich in phenolic compounds (including spearmint and pennyroyal) were protective at 12–18 mg/kg phenolic acids when injury was induced with DSS or acetic acid [37,52]. This study used a dose within this range to allow for direct comparison with earlier research while incorporating the TNBS model with S. officinalis for the first time.

**Table 4**
Score of diarrhea severity.

| Score | Feces consistency |
|---|---|
| 0 | Normal (hard pellets) |
| 1 | Slightly mucous |
| 2 | Soft |
| 3 | Liquid |

A pilot tolerability study, as a preliminary 7-day assessment (at doses of 5, 15, and 30 mg/kg phenolic acids; n = 4 per sex) indicated no observable clinical or biochemical toxicity at any dose. However, rats administered 30 mg/kg phenolic acids showed approximately 4 % reduction in body weight and lower food intake. Based on these findings, 15 mg/kg was selected as the highest dose without observed adverse effects, remaining within the effective range. No adverse outcomes were noted in the main TNBS experiment.

For human relevance, the chosen rat dose of 15 mg/kg is equivalent to approximately 2.4 mg/kg in humans via body-surface-area conversion. For a 70 kg adult, this equates to roughly 170 mg phenolic acids, which is below the typical daily intake from culinary sage infusions (~300 mg phenolics $day^{-1}$), supporting the potential for translation and safety.

In the in vivo animal experiments the results were expressed as mean ± standard error of the mean (SEM) of n observations (n representing the number of animals). Comparison of results was performed by a one-factorial ANOVA test, followed by a Bonferroni's post hoc test (Prism 6.0 software – GraphPad). Statically significances were considered for *P* values less than 0.05. In the in vitro and ex vivo studies with the HT-29 cells all experiments were executed as triplicates (a 3 independent experiments) and results were expressed as the mean ± standard deviation (SD). Comparison of results was performed using the software SigmaPlot (12.5 version), with one-factorial ANOVA test followed by a Tukey test for comparison between groups. Statistical differences were considered significant when $p < 0.05$.

## CRediT authorship contribution statement

**Rosa Direito:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **João Rocha:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Conceptualization. **Inês Alves de Melo:** Investigation. **Margarida Gonçalves:** Investigation. **Maria Paula Duarte:** Investigation. **Adelaide Fernandes:** Investigation. **Bruno Sepodes:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Conceptualization. **Maria-Eduardo Figueira:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Conceptualization.

## Informed consent statement

Not applicable.

## Institutional review board statement

Experiments were performed according to the Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London, UK, and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication no. 85–23, revised 1996) and according to the most updated EC regulations (Directive 2010/63/EU). ARRIVE Guidelines for Reporting Animal Research summarized at http://www.nc3rs.org.uk were strictly followed and respected during the studies. Protocol was submitted to and approved (CEEE-002/16) by the Ethics Committee for Animal Experiments (CEEA) of the Faculty of Pharmacy—University of Lisboa at February 2016.

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## Declaration of competing interest

The authors declare no conflicts of interest.

## Data availability

Data will be made available on request.

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