

Original article

Emu Oil and Saireito in combination reduce tumour development and clinical indicators of disease in a mouse model of colitis-associated colorectal cancer

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ABSTRACT

Background: Emu Oil (EO) previously demonstrated therapeutic potential in a mouse model of colitis-associated CRC (CA-CRC). Saireito, a traditional Japanese medicine, has not been investigated in CA-CRC.

Aim: To determine whether EO and Saireito could be therapeutic in an azoxymethane (AOM)/dextran sulphate sodium (DSS) model of CA-CRC.

Methods: Female C57BL/6 mice were assigned to groups (n = 10/group): 1) saline control, 2) saline+Saireito, 3) saline+EO, 4) saline+EO/Saireito, 5) AOM/DSS control, 6) AOM/DSS+Saireito, 7) AOM/DSS+EO and 8) AOM/DSS+EO/Saireito. Mice were intraperitoneally injected with saline or AOM (7.4 mg/kg) on day 0 and underwent three DSS/water cycles (2%w/v DSS for 7 days, 14 days water). Mice were orally-gavaged with either water (80 µL), Saireito (80 µL), EO (80 µL) or EO/Saireito (160 µL; 80 µL EO + 80 µL Saireito) thrice weekly. Daily bodyweight and disease activity index (DAI) were recorded and colonoscopies performed on days 20, 41 and 62. Mice were euthanized on day 63. p < 0.05 was considered statistically significant.

Results: AOM/DSS induced significant bodyweight loss throughout the trial (max -36%), which was attenuated by Saireito (max +7%), EO (max +5%) and EO/Saireito (max +14%; p < 0.05). AOM/DSS increased DAI compared to saline controls (p < 0.05), which was reduced by Saireito, EO and EO/Saireito (p < 0.05). All treatments reduced colonoscopically-assessed colitis severity (days 20 and 41; p < 0.05). EO/Saireito further decreased colitis severity compared to Saireito and EO alone (day 20; p < 0.05). Finally, EO and EO/Saireito resulted in fewer colonic tumours compared to AOM/DSS controls (p < 0.05).

Conclusion: Combined EO and Saireito reduced disease and tumour development in AOM/DSS mice, suggesting therapeutic potential in CA-CRC.

1. Introduction

Ulcerative colitis (UC) is a debilitating and lifelong inflammatory bowel disease (IBD) that presents as continuous damage and inflammation of the large intestine [1]. IBD pathogenesis is complex and not fully understood, however, a combination of immunological dysregulation, environmental, diet, lifestyle and psychological wellbeing can

contribute to disease onset [1,2]. Furthermore, 8–14% of UC patients have a family history of IBD, highlighting a genetic predisposition. UC has historically been most common in Western societies, although prevalence is now increasing in Asian and Latin American countries [2]. There is currently no cure for UC and patients must resort to anti-inflammatory and immunomodulatory drugs and potent steroids to manage symptoms and maintain remission [2]. Unfortunately, 15% of

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UC patients will require surgery to remove segments of the colon, or sometimes the entire colon, approximately 20 years following diagnosis [2]. Furthermore, cancer is the major cause of mortality for IBD patients, with UC patients being at an increased risk of developing colitis-associated colorectal cancer (CA-CRC) due to uncontrolled and long-term chronic inflammation [3,4].

Recently, a disruption of the epithelial barrier and intestinal microbial biodiversity has been a focus of UC and CA-CRC research. Treatments are now centring on restoring intestinal integrity, impeding migration of immune cells and controlling microbial populations via transplants from healthy donors [2,5,6]. Naturally-sourced compounds derived from animals or plants have also gained interest as adjunct therapies for gastrointestinal conditions [7–10].

Emu Oil, derived from the subcutaneous and retroperitoneal adipose tissue of the native Australian Emu (*Dromaius novaehollandiae*) has been investigated as an orally-administered anti-inflammatory agent in pre-clinical settings of gastrointestinal disorders including UC, Crohn's disease, intestinal mucositis and CA-CRC [11–21]. Traditionally, Emu Oil has been used topically by Indigenous Australian people to alleviate pain and promote wound healing [12]. Although the mechanisms are still not fully understood, the anti-inflammatory and anti-oxidant properties of Emu Oil are attributed to the fatty acid composition and the uncharacterised 2% non-triglyceride fraction of the oil [7,12,22]. Previously in a study of CA-CRC in mice, Emu Oil application resulted in decreased clinical indicators of disease and reduced small colonic tumours compared to disease controls over a 9-week experimental period [14]. However, the overall tumour number was unaffected by Emu Oil treatment. Combining Emu Oil with other naturally-sourced compounds could therefore be one means to increase its potency and elicit a more pronounced effect on tumour development in pre-clinical CA-CRC.

Kampo medicines are Traditional Japanese Herbal formulations that comprise various ratios of plant-based constituents. Kampo is widely accepted in Japan with 148 Kampo extractions being covered by national health insurance and approximately 85% of practitioners regularly prescribing these traditional medicines [23]. Kampo is used in conjunction with Western medicines to enhance immunity and relieve adverse drug reactions for a plethora of conditions including gastrointestinal diseases and cancer [24]. Specifically, Daikenchuto and Juzen-taihoto have been widely investigated in pre-clinical settings of UC and CRC, whereby these Kampo formulations inhibited tumour growth, decreased levels of inflammatory cytokines (TNF- α and IL-1 β) and increased the abundance of natural killer cells [24–26]. Saireito, a combination of two Kampo medicines (Shosaikoto and Goreisan) is composed of 12 herbs; *Bupleuri radix*, *Pinelliae tuber*, *Alismatis rhizoma*, *Scutellariae radix*, *Ginseng radix*, *Poria*, *Polyporus*, *Astractylodis lanceae rhizoma*, *Zizyphi fructus*, *Glycyrrhizae radix*, *Cinnamomi cortex* and *Zingiberis rhizome*. Saireito has displayed anti-inflammatory and immunomodulatory effects in clinical and experimental investigations of UC, chronic hepatitis and rheumatoid arthritis [27–29]. However, Saireito is yet to be investigated in the setting of CA-CRC.

The aim of the current study was to investigate whether Emu Oil and Saireito in combination could reduce the severity of chronic colitis and inhibit the subsequent development of colorectal tumours in an azoxymethane (AOM)/ dextran sulphate sodium (DSS) mouse model of CA-CRC. Furthermore, the anti-inflammatory action of Emu Oil and Kampo medicines is well understood; however, the mechanism of action and effect on inflammatory cytokines has not yet been defined, prompting further analyses *in vitro*.

2. Materials and methods

2.1. Dendritic cell maturation and ELISA

The potential anti-inflammatory effect of Emu Oil and/or Saireito was determined using dendritic cell (DC) model as described previously (Hardardottir, Olafsdottir et al. [30]). In short, CD14⁺ monocytes were

isolated from peripheral blood mononuclear cells that were obtained from buffy coat from healthy blood donors (Blood Bank, Landspítali - the National University Hospital of Iceland, Reykjavik, Iceland) using CD14 Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The CD14⁺ monocytes were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS) (both from Gibco, Thermo Fisher Scientific, Paisley, UK) and 12.5 ng/ml interleukin (IL)-4 and 25 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; both from R&D Systems, Bio-technie, Abingdon, UK) for differentiation into immature dendritic cells (imDC). The 48-well tissue culture plate was incubated at 37 °C with 5% CO₂ and 100% humidity for seven days. On day seven, the imDCs were harvested and activated into matured dendritic cells (mDC) by culturing them in 48-well culture plates for 2 days in RPMI medium supplemented with 10% FCS and 10 ng/ml IL-1 β , 50 ng/ml tumour necrosis factor (TNF)- α (both from R&D Systems, Bio-technie, Abingdon, UK), and 500 ng/ml lipopolysaccharides (LPS) (Sigma-Aldrich, trading as Merck, Darmstadt, Germany). At the same time 10 μ L of 100 μ g/ml Saireito, sonicated Emu Oil and combined Emu Oil/Saireito were added to three wells each (final concentration 2 μ g/ml) and three wells left without addition. mDCs were harvested and centrifuged (300 g for 10 min at 4 °C) and supernatant collected and stored at – 80 °C until used [30].

The maturation of DCs was determined by measuring the concentration of the pro-inflammatory cytokine IL-12p40 and anti-inflammatory cytokine IL-10 in the cell supernatants using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Results are presented as secretion index (SI), indicating the concentration of the cytokine in supernatant from cultured DCs cultured with Saireito, sonicated Emu Oil and combined Emu Oil/Saireito, divided by the concentration of the cytokine in supernatant from DCs cultured without any addition.

2.2. Experimental timeline

Animal studies were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committees of The University of Adelaide and Children, Youth and Women's Health Service (AE 1079/3/21). Female C57BL/6 mice (C57BL/6JArc, n = 80) were sourced from the Animal Resource Centre (Perth, Western Australia, Australia) at 8 weeks of age and were group-housed in standard open-top cages (polypropylene; 470 mm x 175 mm x 120 mm; Crestware Industries) at room temperature with a light/dark cycle of 14:10 h. Mice were fed standard mouse chow (meat-free mouse diet; Specialty Feeds, Glen Forrest, Western Australia, Australia) and were provided with enrichment items including shredded paper, polycarbonate 'houses' and cardboard toilet paper rolls for the duration of the trial. Plain drinking water was also provided *ad libitum* throughout the trial, except when precluded by group allocation.

Mice (n = 10/group) were randomly allocated to eight treatment groups (displayed as intraperitoneal (i.p.) injection + *ad libitum* + oral gavage); 1) Saline + Water + Water, 2) Saline + Water + Saireito, 3) Saline + Water + Emu Oil, 4) Saline + Water + combined Emu Oil and Saireito (Emu Oil/Saireito), 5) AOM + DSS + Water, 6) AOM + DSS + Saireito, 7) AOM + DSS + Emu Oil and 8) AOM + DSS + Emu Oil/Saireito. On day 0, all mice were injected (i.p.) with either saline or the carcinogen AOM (7.4 mg/kg; Sigma-Aldrich, Castle Hill New South Wales, Australia) and underwent three DSS/water cycles, each comprising of seven days DSS (2%w/v; 2 g/100 ml distilled water; MP Biomedicals LLC, Santa Ana California, USA) followed by 14 days of plain water recovery. Starting immediately, all mice were administered thrice weekly via oral gavage with 80 μ L of either water, Saireito (1 g/kg bodyweight of mouse; Tsumura & Co., Tokyo, Japan), Emu Oil (100% v/v; Emu Tracks, Marleston, South Australia, Australia) or a combination of Emu Oil and Saireito (160 μ L; 80 μ L Emu Oil + 80 μ L Saireito) for the duration of the trial. All animals were euthanized on day 63 via CO₂

asphyxiation and cervical dislocation. Blood was collected *via* cardiac puncture from groups 1 and 5–8 for intestinal permeability analyses. Visceral organs (heart, liver, lungs, spleen, thymus, kidney, stomach and caecum) were weighed and gastrointestinal organs (small and large intestine) measured and weighed. Sections of the proximal and distal colon were also collected for histological analysis.

2.3. Daily measurements

Bodyweight and disease activity index (DAI) were measured daily during routine morning monitoring over the experimental period. DAI was calculated from bodyweight loss, general condition, stool consistency and rectal bleeding as described [11]. Each parameter was scored from 0 to 3 with increasing severity and then totalled to calculate the DAI score for each mouse on each day, with a maximum obtainable score of 12.

2.4. Colonoscopy

At the end of each three-week DSS/water cycle (days 20, 41 and 62), disease progression was monitored *via* colonoscopy using a high resolution colonoscope (Karl Storz, 1.9 mm outer diameter, Tuttlingen, Germany). Mice were individually placed on a heating pad and anaesthetised using isoflurane inhalant (AbbVie Pty Ltd, Mascot New South Wales, Australia) for the duration of the colonoscopy procedure. Immediately following the procedure, mice were transferred to a recovery cage on a heating pad and closely and continuously monitored until their behaviour returned to normal. Mice were then returned to their home cage. Videos obtained from the colonoscopy procedures were subsequently scored in a blinded fashion for colitis severity and tumour development as described by Becker et al. [31]. Colitis severity was calculated from five parameters (scored 0–3 with increasing severity) including, thickening of the colon, vasculature pattern, fibrin, granularity of the mucosal surface and stool consistency and summed for a total colitis severity score, with a maximum attainable score of 15 [31]. Colonic tumours were also counted from colonoscopy videos for each mouse at each time-point.

2.5. Burrowing analyses

Burrowing behaviour was analysed during the experimental period as a measure of wellbeing and affective state [32]. At baseline (day –1) and at the end of each DSS/water cycle (days 19, 40 and 61), nearing their dark cycle, mice were placed in the dark for at least one hour to acclimatize. In the dark, mice were then placed in individual cages containing an attached burrow with 400 g of pre-weighed pebbles (400 g; kitty litter ‘pebbles’; Black and Gold, Australian Asia/Pacific Wholesalers Pty Ltd, Australia). Mice were then left to burrow for an hour. After this time, mice were removed from the burrowing cages and burrows were re-weighed to determine the amount burrowed for each mouse at each time-point.

2.6. Nesting behaviour

Nest building is a normal behaviour that is carried out by mice in their home cage. Mice were provided with shredded paper and tissues in their home cage and nesting was analysed twice per week (every 3–4 days), 24 h after home cages were changed and cleaned. Scores of 0 or 1 were determined for each treatment group at each time-point, where 0 indicated no nest was built and 1 represented positive nesting behaviour [33,34].

2.7. Intestinal permeability (FITC-dextran assay)

Three hours prior to sacrifice, mice from groups 1 and 5–8, were orally-gavaged with a 500 mg/kg dose of fluorescein isothiocyanate

(FITC)-dextran (mol wt 4000, 75 mg/ml; Sigma, Castle Hill, New South Wales, Australia). Blood was then collected at kill *via* cardiac puncture. Samples were centrifuged (11,000 g at 23 °C) for 12 min and serum collected. Serum samples were diluted 1:3 with 0.2 M PBS and FITC-dextran was quantified using a BioTek Synergy Mx Microplate Reader (BioTek, Winooski, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (0.001–100 µg/ml).

2.8. Tumour photographs

At time of kill, colons were removed and opened longitudinally to visualise tumours. Photographs of longitudinally-opened mouse colons (Canon 5D Mark IV with 17–40 mm lens) were analysed in a blinded fashion using Olympus Soft Imaging Solutions GmbH computer software analysis version 5.2 (Tokyo, Japan). The number of tumours was determined and categorised into sizes as described previously [14,17]. Colonic tumours with a diameter < 2 mm were determined as ‘small’, 2–3 mm as ‘medium’ and ‘large’ tumours were those with a diameter > 3 mm.

2.9. Histological analysis

Sections of the proximal and distal colon were routinely processed and embedded in paraffin wax following collection. Section (4 µm) were then stained with haematoxylin and eosin (H&E) and mounted on plain glass microscope slides. Histologically-assessed damage severity was assessed in a blinded-fashion using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan) as previously described [35]. Six parameters including goblet cell reduction, crypt and crypt cell disruption, polymorphonuclear infiltration and thickening/oedema of the submucosa and muscularis externa were scored from 0 to 3 with increasing severity for four cross sections of colonic tissue per mouse. Median scores for each parameter were then calculated and summed to determine a final severity score per mouse per colonic section, with a maximum attainable score of 18.

2.10. Statistical analyses

Statistical analyses were completed using SPSS, version 25 for Windows (SPSS Inc. Chicago, Illinois, USA). Data were tested for normality using a Shapiro–Wilk test. Bodyweight, DAI, burrowing activity, colonoscopically-assessed colitis score and tumour number were analysed by repeated measures ANOVA with least significance difference (LSD) to compare among and within a group. ELISA results were analysed by two samples *t*-test (assuming equal variance) at a 95% confidence interval. Nesting behaviour was analysed with a non-parametric Kruskal–Wallis test. FITC-dextran data were logarithmically transformed and analysed using linear regression. Organ data and histologically-assessed severity scores were analysed using a one-way ANOVA with a Tukey’s *post hoc* test. For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cytokine secretion by dendritic cells *in vitro*

Culturing dendritic cells in the presence of Emu Oil or in the presence of the combination of Emu Oil and Saireito decreased their secretion of IL-12p40 compared to that when they were cultured in the absence of the nutraceuticals ($p < 0.05$; Fig. 1). However, culturing dendritic cells in the presence of Saireito alone did not significantly impact their IL-12p40 secretion compared to the negative control. None of the treatments affected dendritic cell secretion of IL-10 (Fig. 1).

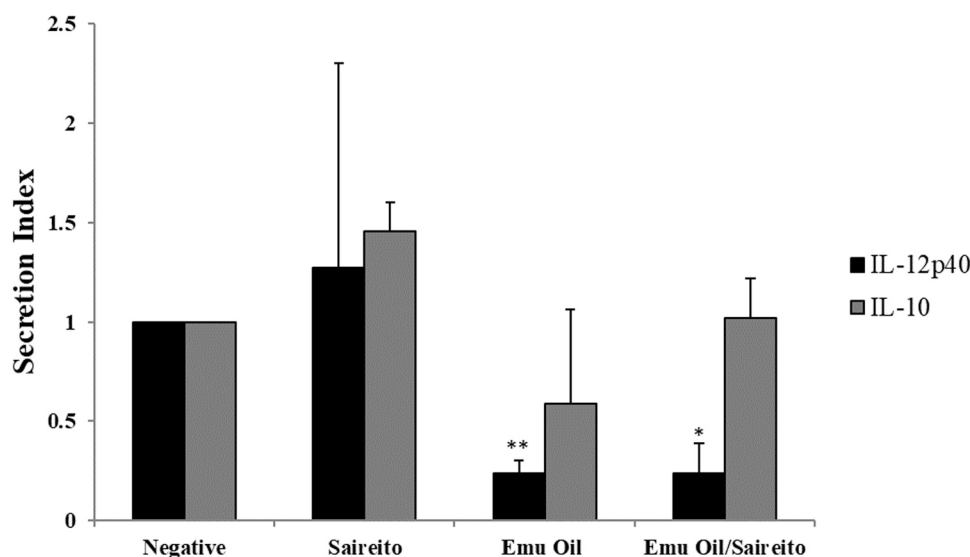


Fig. 1. Secretion of IL-12p40 and IL-10 by mature dendritic cells. Dendritic cells were matured with IL-1 β , TNF- α and LPS for 24 h in the absence (negative) or presence of Saireito, Emu Oil or the combination of Emu Oil and Saireito at the concentration of 2 μ g/ml. The concentrations of IL-12p40 and IL-10 in the supernatants were determined by ELISA. The data are presented as SI, i.e. the concentration of each cytokine in the supernatant of cells in the presence of the nutraceutical divided by the concentration of the cytokine in the supernatant of cells in the absence of the nutraceutical. Data are expressed as mean $\pm$ SEM (n = 2 donors). **p < 0.01, *p < 0.05 compared to negative.

3.2. Bodyweight

In normal mice, Saireito (day 63) and Emu Oil (day 18) slightly increased bodyweight compared to saline controls, with no effect exhibited by the treatments on other days ($p < 0.05$; Fig. 2a). AOM/DSS induced significant bodyweight loss on most days of the experimental trial compared to saline controls (days 4–16, 25–33, 46–56 and 61; $p < 0.05$; Fig. 2b). Saireito-administration increased bodyweight of AOM/DSS mice on day 50 and slightly decreased bodyweight on days 41 and 43 compared to AOM/DSS controls ($p < 0.05$; Fig. 2c). Additionally, Emu Oil increased bodyweight on days 15, 57, 58, 62 and 63 of the trial compared to AOM/DSS controls ($p < 0.05$; Fig. 2c). However, Emu Oil slightly decreased bodyweight of AOM/DSS mice on days 41–43 compared to AOM/DSS controls ($p < 0.05$; Fig. 2c). The combination of Emu Oil/Saireito attenuated bodyweight loss in AOM/DSS mice on days 6–12, 29–32 and 47–51 compared to AOM/DSS controls ($p < 0.05$; Fig. 2c). Finally, the combination of Emu Oil and Saireito further attenuated bodyweight loss of AOM/DSS mice compared to Saireito alone (days 6–11, 29–32, 34, 37, 38, 43, 47 and 48) and Emu Oil alone (days 7–18, 20–21, 25–27, 30–35, 38, 46–58 and 60–63; $p < 0.05$; Fig. 2c).

3.3. DAI

In normal mice, administration of Saireito (day 48), Emu Oil (day 57) and Emu Oil/Saireito (day 6) slightly increased DAI scores compared to saline controls ($p < 0.05$; Fig. 3); however, this result was not consistently observed throughout the nine-week trial. As expected, AOM/DSS controls displayed significantly increased DAI scores compared to saline controls at most time-points (days 4–19, 21–33, 35–36 and 38–63; $p < 0.05$; Fig. 3). Saireito (days 6, 9, 12, 13, 15, 17, 19, 20, 22, 24, 25, 27, 29–32, 39, 40, 42, 44–46, 50–56, 59–61 and 63) and Emu Oil (days 6, 9, 12, 16, 17, 19, 21, 22, 25, 27–29, 31, 32, 39, 40, 44–47, 51, 53, 54, 56 and 59–63) significantly decreased DAI scores of AOM/DSS mice, compared to AOM/DSS controls ($p < 0.05$; Fig. 3). Moreover, the combination of Emu Oil/Saireito in AOM/DSS mice decreased DAI scores in comparison with AOM/DSS controls on days 5–32, 34, 36–40, 42–57 and 59–63 ($p < 0.05$; Fig. 3). Finally, the Emu Oil/Saireito combination further decreased DAI scores compared to Saireito alone (days 4, 6–10, 24–26, 28, 32, 34, 43 and 46–48) and Emu Oil alone (7–10, 15, 18, 23–26, 30, 38, 40, 42, 45–48, 50 and 55; $p < 0.05$; Fig. 3).

3.4. Colonoscopically-assessed parameters

AOM/DSS mice presented with severe colitis as determined by colonoscopy on days 20, 41 and 62 ($p < 0.05$; Fig. 4a). Saireito, Emu Oil and the combination of Emu Oil/Saireito significantly reduced colitis severity scores on days 20 and 4, compared to AOM/DSS controls, with no effect exhibited by treatments on day 62 ($p < 0.05$; Fig. 4a). Furthermore, Emu Oil/Saireito in combination further decreased colitis severity scores in comparison with both Saireito and Emu Oil alone on day 20 ($p < 0.05$; Fig. 4a).

Colonic tumours were evident in all AOM/DSS mice. AOM/DSS controls presented with the highest number of colonic tumours at all time-points of the trial ($p < 0.05$; Fig. 4b). Emu Oil-administration significantly decreased the number of tumours on day 20 compared to AOM/DSS controls ($p < 0.05$; Fig. 4b). Saireito alone did not significantly impact tumour development at any time-point; however, the combination of Emu Oil/Saireito significantly reduced tumour burden on day 41 of the trial ($p < 0.05$; Fig. 4b), with no effect observed on other days. Finally, Emu Oil/Saireito further decreased tumour number compared to Saireito alone on days 20 and 62 ($p < 0.05$; Fig. 4b); with no further effect compared to Emu Oil alone ($p > 0.05$).

3.5. Burrowing

In normal mice, Saireito, Emu Oil or combined Emu Oil/Saireito did not significantly impact burrowing ability on the days analysed ($p > 0.05$). AOM/DSS impaired burrowing activity on days 19 and 40 compared to saline controls ($p < 0.05$; Fig. 5), with no effect exhibited on day 61. Administration of Saireito alone significantly increased burrowing activity of AOM/DSS mice on day 19 compared to AOM/DSS controls ($p < 0.05$; Fig. 5). Emu Oil and the combination of Emu Oil/Saireito did not affect burrowing behaviour of AOM/DSS mice during the experimental trial.

3.6. Nesting behaviour

In normal mice, positive nesting behaviour was evident in all treatment groups (1 ± 0). Furthermore, AOM/DSS did not affect nesting ability as all mice successfully building nests (1 ± 0). Administration of Saireito and Emu Oil, alone and in combination, had no effect on nest building compared to AOM/DSS controls (1 ± 0 ; $p > 0.05$).

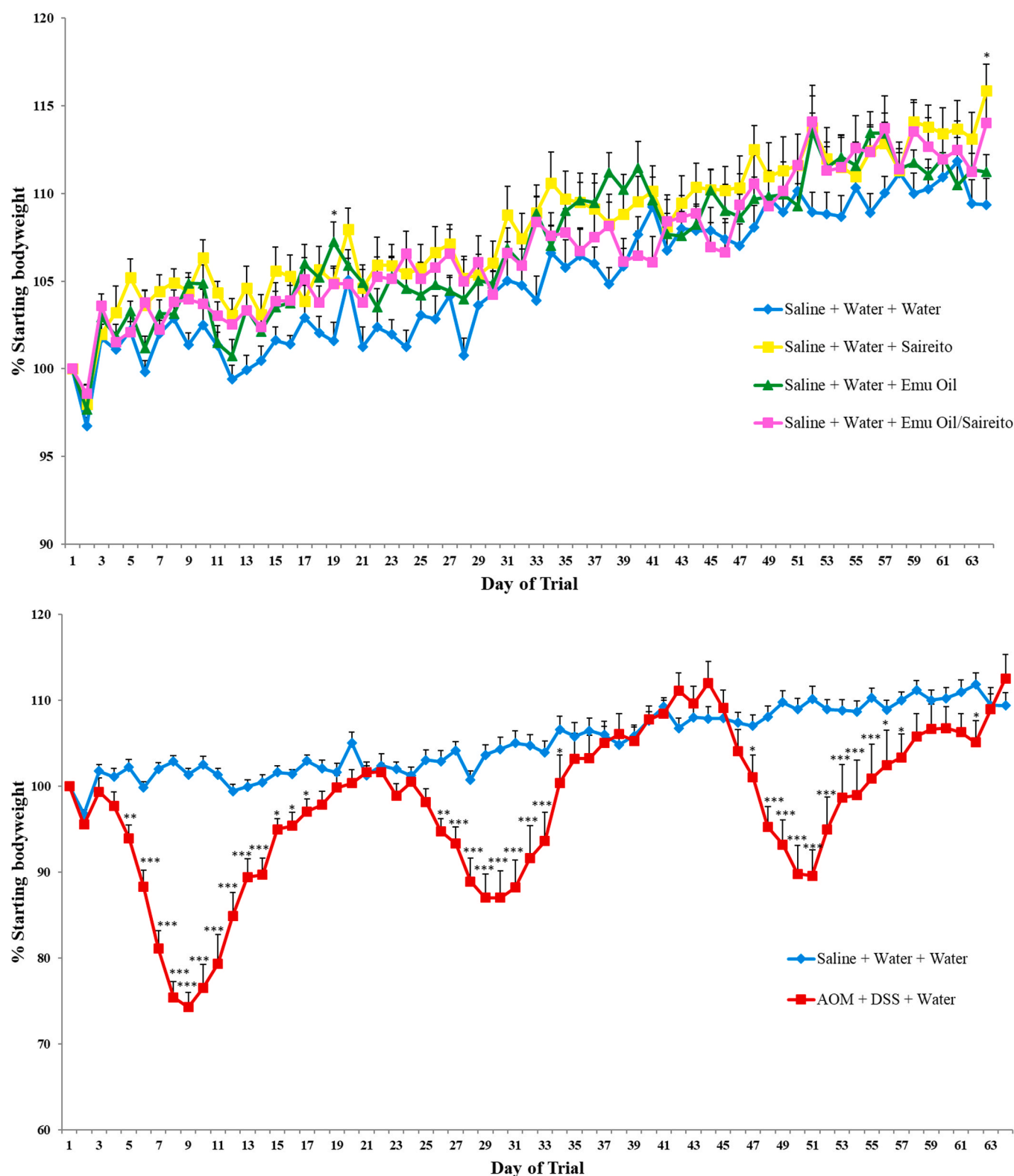


Fig. 2. Daily bodyweight change of (a) Saline mice, (b) Saline and AOM/DSS controls and (c) AOM/DSS mice. Data are expressed as mean (% starting bodyweight) $\pm$ SEM (n = 10/group). ***p < 0.001, **p < 0.01, *p < 0.05 compared to Saline + Water + Water; ~~~p < 0.001, ~p < 0.01, ^p < 0.05 compared to AOM + DSS + Water; ###p < 0.001, ##p < 0.01, #p < 0.05 compared to AOM + DSS + Saireito; \$\$\$p < 0.001, \$\$p < 0.01, \$p < 0.05 compared to AOM + DSS + Emu Oil on the same day.

3.7. Intestinal permeability (FITC-dextran)

FITC-dextran concentrations ($\mu\text{g/ml}$) were unchanged in AOM/DSS

mice compared to normal controls ($p > 0.05$; Fig. 6). Moreover, Saireito, Emu Oil and combined Emu Oil/Saireito did not impact intestinal permeability in comparison with AOM/DSS controls ($p > 0.05$; Fig. 6).

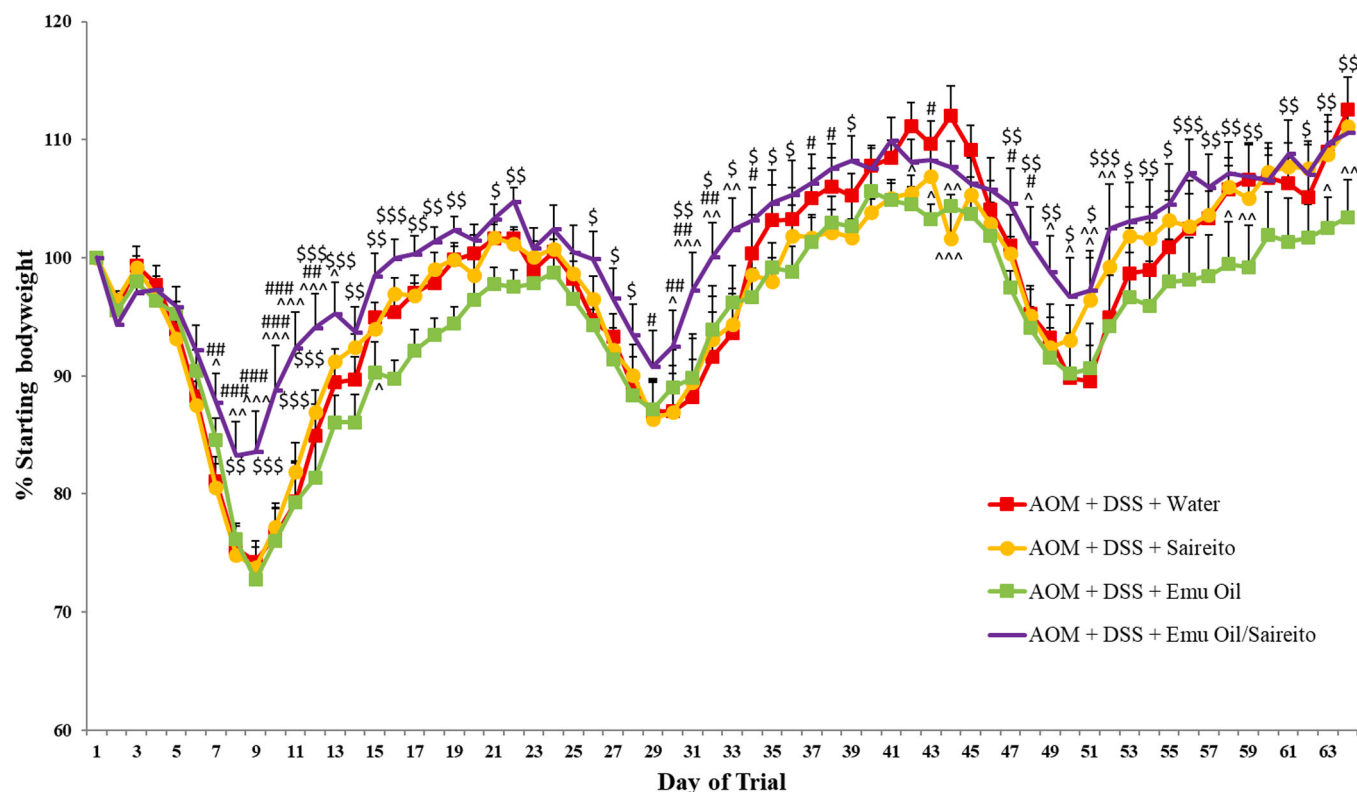


Fig. 2. (continued).

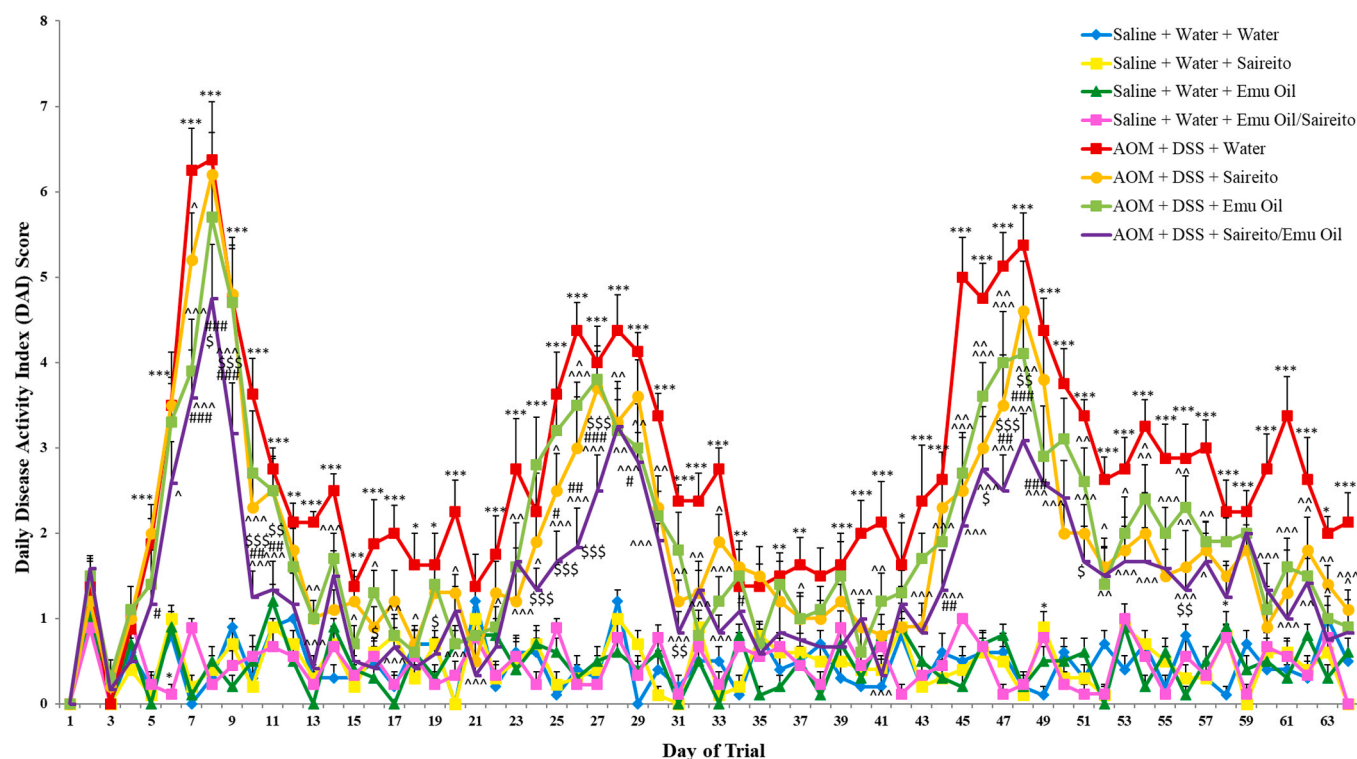


Fig. 3. Daily Disease Activity Index (DAI) score. Data are expressed as mean $\pm$ SEM (n = 10/group). ***p < 0.001, **p < 0.01, *p < 0.05 compared to Saline + Water + Water; ~~~p < 0.001, ~p < 0.01, ^p < 0.05 compared to AOM + DSS + Water; ###p < 0.001, ##p < 0.01, #p < 0.05 compared to AOM + DSS + Saireito; \$\$\$\$p < 0.0001, \$\$\$p < 0.001, \$\$p < 0.01, \$p < 0.05 compared to AOM + DSS + Emu Oil on the same day.

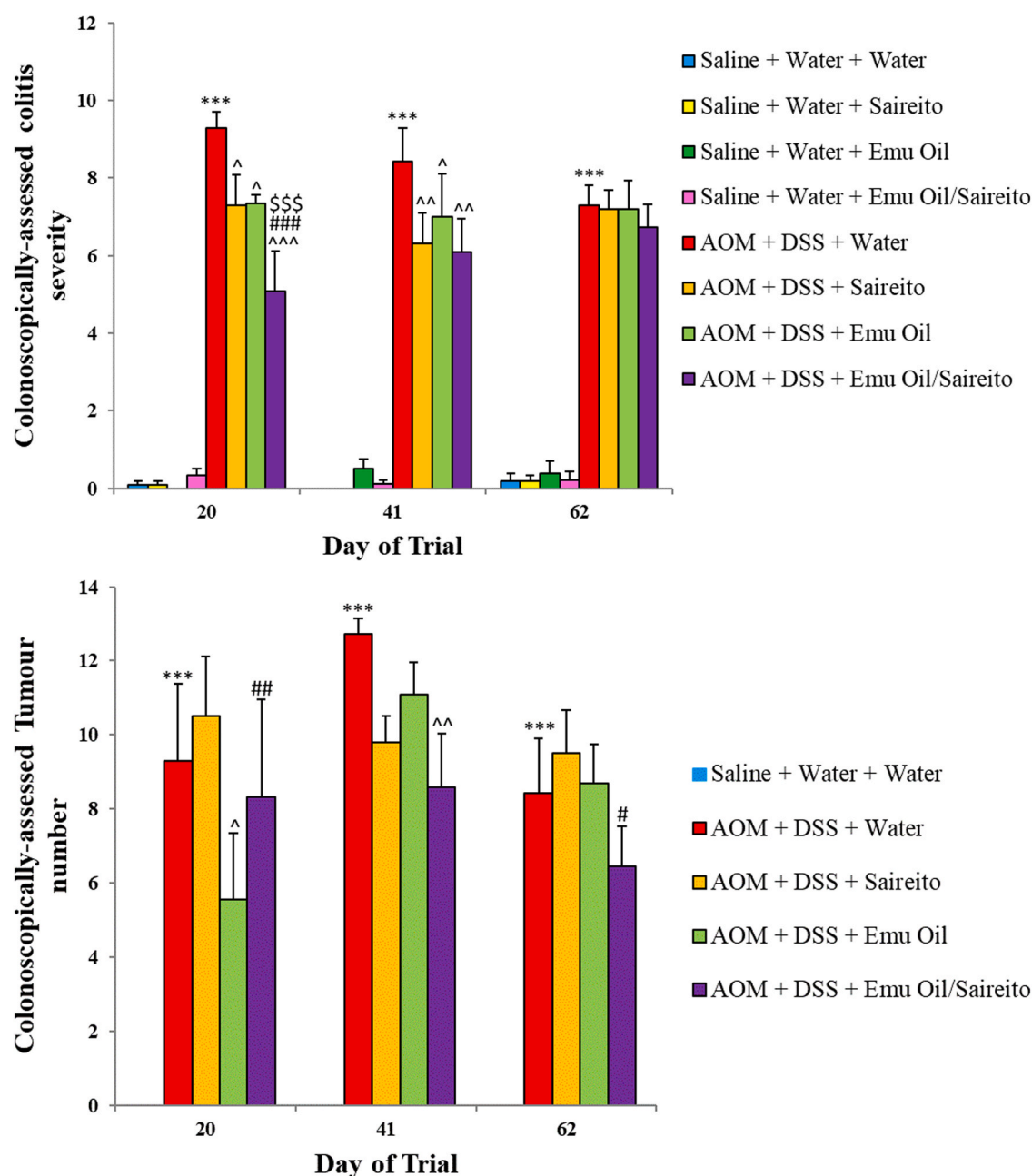


Fig. 4. Colonoscopically-assessed (a) colitis severity and (b) tumour development. Data are expressed as mean (colitis severity score or tumour number) $\pm$ SEM (n = 10/group). ***p < 0.001, compared to Saline + Water + Water; ^~p < 0.001, ^p < 0.01, ^p < 0.05 compared to AOM + DSS + Water; ###p < 0.001, ##p < 0.01, #p < 0.05 compared to AOM + DSS + Saireito; \$\$\$p < 0.001 compared to AOM + DSS + Emu Oil on the same day.

3.8. Intestinal and visceral organ measurements

In normal animals, weights of the visceral organs and intestinal lengths/weights remained unaffected by administration of Emu Oil, Saireito and the combination of Emu Oil/Saireito ($p > 0.05$; Table 1). AOM/DSS mice presented with significantly increased colonic weights compared to saline controls ($p < 0.05$; Table 1), with no effect exhibited by Emu Oil, Saireito or the combined treatment ($p > 0.05$). Furthermore, colonic shortening was evident in AOM/DSS controls in comparison with saline controls ($p < 0.05$; Table 1). Finally, weights of the liver and spleen were increased in AOM/DSS control mice compared to saline controls ($p < 0.05$; Table 1), with no effect exhibited by Emu Oil, Saireito or the combination of Emu Oil/ Saireito ($p > 0.05$). All other visceral organ weights remained unaffected in AOM/DSS mice (Table 1).

3.9. Gross tumour morphology

Mice treated with AOM/DSS presented with significant distal colonic tumours (Fig. 7). At kill (day 63) Emu Oil and Saireito-treatment alone did not significantly impact tumour sizes or overall tumour number ($p > 0.05$; Fig. 8). The numbers of large tumours in Emu Oil, Saireito and the combination of Emu Oil/Saireito treatment groups were less than in AOM/DSS controls; however, this did not achieve statistical significance. Finally, the combination of Emu Oil/Saireito significantly reduced the number of small colonic tumours (diameter < 2 mm) and the overall number of tumours compared to AOM/DSS controls ($p < 0.05$; Fig. 8).

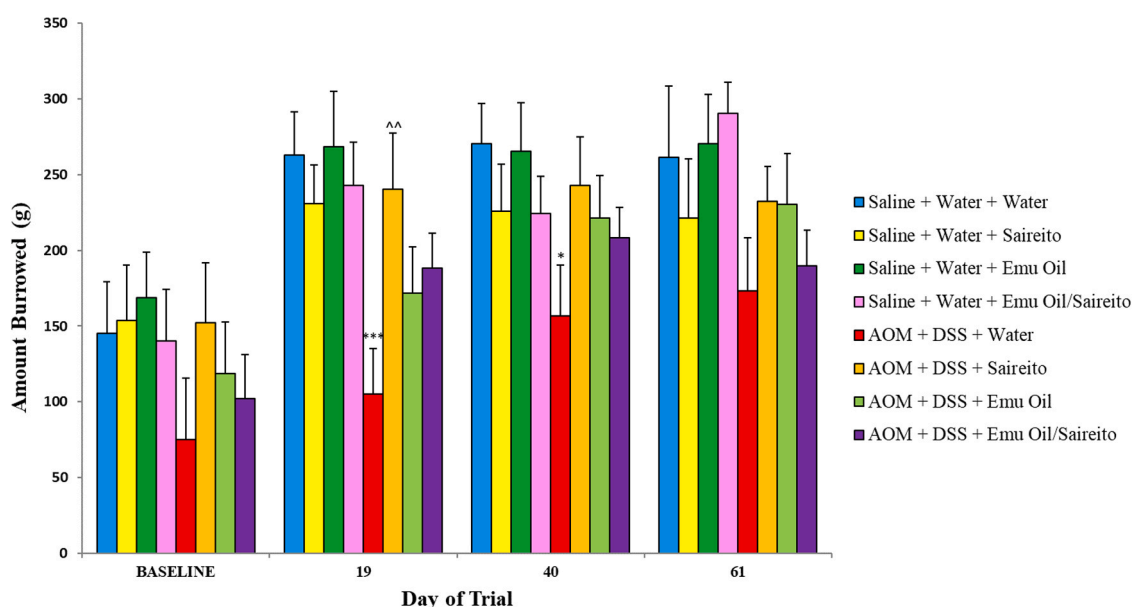


Fig. 5. Burrowing ability of mice. Data are expressed as mean (amount burrowed) $\pm$ SEM ($n = 10$ /group). *** $p < 0.001$, * $p < 0.05$ compared to Saline + Water + Water; ^ $p < 0.01$ compared to AOM + DSS + Water on the same day.

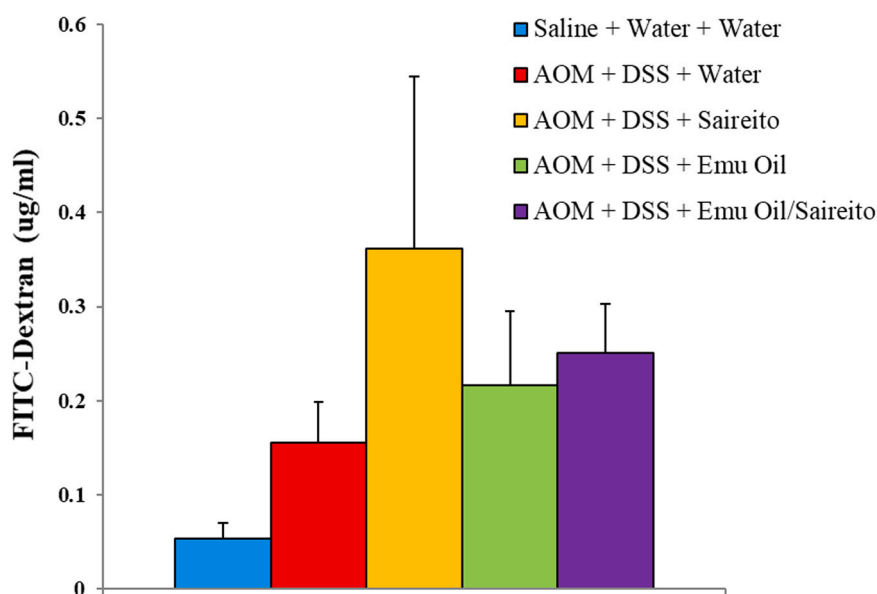


Fig. 6. Intestinal permeability assessed by FITC-dextran levels. Data are expressed as mean serum FITC-dextran ($\mu\text{g/ml}$) $\pm$ SEM ($n = 10$ /group).

3.10. Histologically-assessed disease severity

In the proximal colon, there were no significant differences in histologically-assessed severity scores across experimental groups ($p > 0.05$; Fig. 9). However, AOM/DSS controls presented with significant damage and increased histological severity scores in the distal colon compared with normal controls ($p < 0.05$; Fig. 9). Neither Emu Oil nor Saireito-treatment impacted histologically-assessed severity scores in the distal colon ($p > 0.05$).

4. Discussion

In the current study, the combination of Emu Oil and the herbal formula Saireito decreased disease severity in mice with experimentally-induced CA-CRC. Clinical indicators including bodyweight attenuation, DAI, colonoscopically-assessed inflammation and tumour burden were

improved by the combined treatment to a greater extent than Emu Oil or Saireito alone. Importantly, in normal mice, administration of Emu Oil/Saireito or each nutraceutical alone, did not result in any adverse side-effects during the nine-weeks of treatment. Minor weight gain and a slightly increased DAI score was evident in normal control mice treated with Emu Oil and Saireito alone; however, this was only observed on a few days throughout the 63-day study and was therefore deemed to be of negligible significance.

Colonoscopy procedures are crucial in the diagnosis and monitoring of humans with IBD and CRC; however, only in the past twenty years has it been executable in pre-clinical mouse models [36,37]. Additional to mimicking both human monitoring and diagnosis techniques, the colonoscopy procedure is advantageous during long-term mouse trials as it allows researchers to obtain greater amounts of data during *in vivo* studies with a singular end-point. Furthermore, colonoscopies may also identify when pathogenesis is progressing rapidly or severely, early

Table 1
Intestinal and visceral organ measurements.

	Saline + Water + Water	Saline + Water + Saireito	Saline + Water + EO	Saline + Water + EO/Saireito	AOM + DSS + Water	AOM + DSS + Saireito	AOM + DSS + EO	AOM + DSS + EO/Saireito
Visceral Organ Weights (% relative to bodyweight and *10⁻²)								
Heart	0.50 ± 0.01	0.50 ± 0.02	0.52 ± 0.03	0.52 ± 0.02	0.50 ± 0.03	0.53 ± 0.03	0.56 ± 0.02	0.54 ± 0.02
Liver	4.62 ± 0.12	4.52 ± 0.14	4.64 ± 0.16	4.31 ± 0.15	5.34 ± 0.16**	5.18 ± 0.17	5.76 ± 0.33	5.57 ± 0.11
Spleen	0.35 ± 0.01	0.38 ± 0.01	0.40 ± 0.01	0.37 ± 0.13	0.86 ± 0.07***	0.77 ± 0.07	0.80 ± 0.06	0.82 ± 0.08
Thymus	0.21 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.19 ± 0.02	0.18 ± 0.03	0.24 ± 0.03	0.23 ± 0.03	0.24 ± 0.01
Lung	0.68 ± 0.04	0.75 ± 0.03	0.73 ± 0.02	0.75 ± 0.02	0.68 ± 0.02	0.72 ± 0.02	0.73 ± 0.04	0.71 ± 0.01
L Kidney	0.48 ± 0.01	0.49 ± 0.01	0.48 ± 0.02	0.47 ± 0.02	0.49 ± 0.02	0.52 ± 0.01	0.55 ± 0.01	0.55 ± 0.02
R Kidney	0.51 ± 0.02	0.51 ± 0.02	0.55 ± 0.02	0.50 ± 0.01	0.55 ± 0.01	0.54 ± 0.01	0.53 ± 0.04	0.58 ± 0.03
Stomach	0.79 ± 0.03	0.78 ± 0.03	0.84 ± 0.05	0.83 ± 0.04	0.71 ± 0.03	0.73 ± 0.01	0.69 ± 0.06	0.76 ± 0.02
	Saline + Water + Water	Saline + Water + Saireito	Saline + Water + EO	Saline + Water + EO/Saireito	AOM + DSS + Water	AOM + DSS + Saireito	AOM + DSS + EO	AOM + DSS + EO/Saireito
Intestinal Weights (% relative to bodyweight and *10⁻²)								
Colon	1.03 ± 0.03	0.89 ± 0.03	0.94 ± 0.04	0.88 ± 0.04	1.71 ± 0.18***	1.88 ± 0.17	1.82 ± 0.38	1.81 ± 0.22
Duodenum	0.51 ± 0.04	0.53 ± 0.02	0.53 ± 0.02	0.54 ± 0.03	0.51 ± 0.04	0.55 ± 0.03	0.46 ± 0.03	0.54 ± 0.00
Small Intestine	3.55 ± 0.14	4.79 ± 1.47	3.52 ± 0.06	3.42 ± 0.12	3.61 ± 0.16	3.64 ± 0.12	3.38 ± 0.20	3.76 ± 0.11
Intestinal Lengths (cm)								
Colon (cm)	8.01 ± 0.15	7.16 ± 0.30	7.58 ± 0.28	7.2 ± 0.33	5.55 ± 0.24***	6.95 ± 0.23	6.33 ± 0.17	6.25 ± 0.43
Duodenum (cm)	3.96 ± 0.07	3.47 ± 0.11	3.51 ± 0.10	3.84 ± 0.20	4.05 ± 0.17	3.98 ± 0.12	3.8 ± 0.15	3.7 ± 0.20
Small Intestine (cm)	29.2 ± 0.53	28.7 ± 0.45	28.8 ± 0.36	27.1 ± 0.61	30.25 ± 1.11	29.33 ± 0.33	29.67 ± 1.45	30.75 ± 0.48

p<0.01 compared to Saline + Water + Water, *p<0.001.

enough for interventions to be implemented without impacting animal welfare. In the current study, and previous AOM/DSS investigations [14, 38–40], colonoscopies were performed at three time-points at the end of each DSS/water cycle. The nutraceuticals administered in the current study were protective against colitis, with the combination of Emu Oil and Saireito offering greater protection compared to the treatments alone. Interestingly, these protective effects were only observed at the first two colonoscopy time-points (days 20 and 41), with no effect determined on the final procedure (day 62). However, this was not an unexpected finding as the parameters defining colitis severity score assess tissue void of tumours, which inevitably becomes progressively sparse as CRC development is more pronounced [31]. At the final colonoscopy time-point, tumours are often very large and occupy a large circumference of the video diameter, and in severe cases, are almost unpassable. Nonetheless, colonoscopically-assessed colorectal tumours were significantly decreased by Emu Oil and the combination of Emu Oil and Saireito, with the combination resulting in the greatest reduction in tumour number. Notably, these results were reflected in tumour assessment at time of euthanasia, whereby the combination of Emu Oil and Saireito decreased the number of small colonic tumours and overall tumour development. Additionally, future studies should aim to characterise the tumours formed to indicate whether they are hyperplastic polyps, adenomatous polyps, polyps with dysplasia or other, as this may assist in preventing neoplasia.

Although Emu Oil has previously been investigated in CA-CRC [14], the present study is the first to investigate the Japanese Kampo formula, Saireito, in the AOM/DSS model. Gastrointestinal tumours are the most common cause of cancer-related death in Japan and Kampo formulae, including Juzentaihoto, Daikenchuto, Hocheuekkito and Shosaihoto, have been investigated in randomized clinical trials of such cancers [41–43]. Kato et al. investigated the efficacy of Saireito in 5-Fluorouracil (5-FU)-induced mucositis in comparison with Daikenchuto [44]. Similar to that observed in the current study, Saireito-administration significantly protected against bodyweight loss and diarrhoea compared to 5-FU controls [44]. Furthermore, twice-daily administration of Saireito dose-dependently (100–1000 mg/kg) improved histologically-assessed morphological changes, including villus shortening and crypt disruption that were induced by 5-FU [44]. Daikenchuto was also effective at improving these histological parameters but to a lesser effect than Saireito. However, in the current study, there were no significant impacts of Saireito or Emu Oil on histologically-assessed disease severity, although villus height and crypt disruption were not investigated as individual parameters. 5-FU also caused an upregulation of TNF-alpha and IL-1beta mRNA that was attenuated by Saireito at a dose of 1000 mg/kg [44]. Furthermore, in the Kato study, tumour growth following implantation was reduced by 5-FU without a specific effect of Saireito on tumourigenesis. Importantly, Saireito significantly decreased diarrhoea and bodyweight loss caused by the chemotherapeutic agent [44], an effect that was also observed in the present study. Additionally, analysis of the microbiome collected from faecal samples using shotgun metagenomics would be beneficial in understanding the mechanism of action and the effect that Emu Oil and Saireito have on species and abundance of microbiota in AOM/DSS mice. Faecal samples collected as part of routine daily measurements could also be investigated for lipocalin2, a marker of inflammation throughout the 9-week experimental timeline.

The Chinese herbal medicine *Panax notoginseng* and American ginseng (*Panax quinquefolius* L.) have previously increased the anti-tumour effects of chemotherapy and been investigated in AOM/DSS induced CA-CRC [45,46]. American ginseng decreased colitis severity, DAI and histological severity during the acute pre-neoplastic stage of the study, and later suppressed tumour multiplicity [45]. Wang et al. [45] highlighted that tumour suppression was linked to attenuation of the inflammatory-associated cytokines, IL-1α, IL-1β and IL-6. IL-6 is proposed to be involved in the pathogenesis of IBD and CRC by direct promotion of tumour cell proliferation (*in vitro* and *in vivo*) and survival

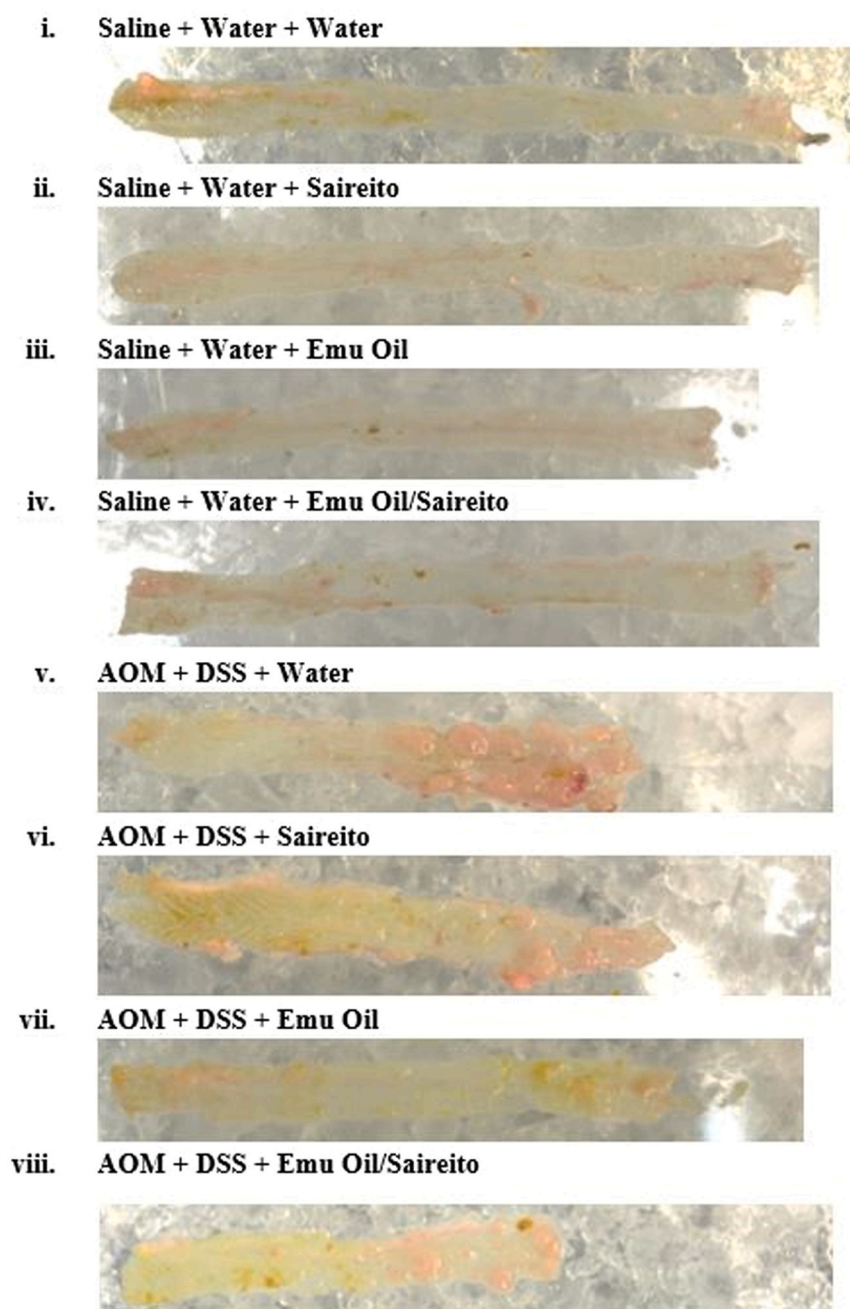


Fig. 7. Photos of longitudinally-opened mouse colons taken at time of euthanasia.

through activation of the STAT3 pathway, thus creating a tumour-promoting microenvironment [47,48]. This is largely supported by a study whereby IL-6 knockout mice presented with significantly reduced tumour growth in a STAT3 dependent manner [49]. Additionally, IL-12 and lack of IL-10 have been implicated in the pathogenesis of colitis and specifically, IL-10 deficient mice can develop UC and CRC just weeks after birth [50]. In the current study, the effects of Emu Oil and Saireito on secretion of IL-12p40 and IL-10 from mDCs were analysed *in vitro*. Cells treated with Emu Oil and the combination of Emu Oil and Saireito exhibited potent anti-inflammatory potential by reducing the secretion of the pro-inflammatory cytokine IL-12p40; an effect that was not observed following treatment of Saireito alone. Moreover, the anti-inflammatory cytokine IL-10 was unaffected by nutraceutical treatment. Future investigations should determine the effects of the selected nutraceuticals on IL-12, IL-10 and IL-6 in intestinal tissue

collected as biopsies during colonoscopy procedures or at time of kill in CA-CRC animal studies. Novel therapies could then be developed to target IL-6 as a primary mediator for inflammation-associated carcinogenesis. Furthermore, *in vitro* techniques including the MTT-assay for cell viability of Caco-2 (colorectal cancer) and healthy intestinal cells, would provide evidence of any direct toxic effect of neoplastic cells by Emu Oil and Saireito.

Techniques that monitor pain, stress and daily-living behaviour are important in laboratory animal studies in order to evaluate how disease or treatments are impacting overall animal well-being [51]. Recently, DAI, facial grimace and burrowing were assessed and compared in CA-CRC mice [52]. It was concluded, that DAI was the most reliable method of welfare evaluation in this model of chronic gastrointestinal disease [52]. In the present study, burrowing and nesting techniques were utilised, whereby there was a significant improvement in

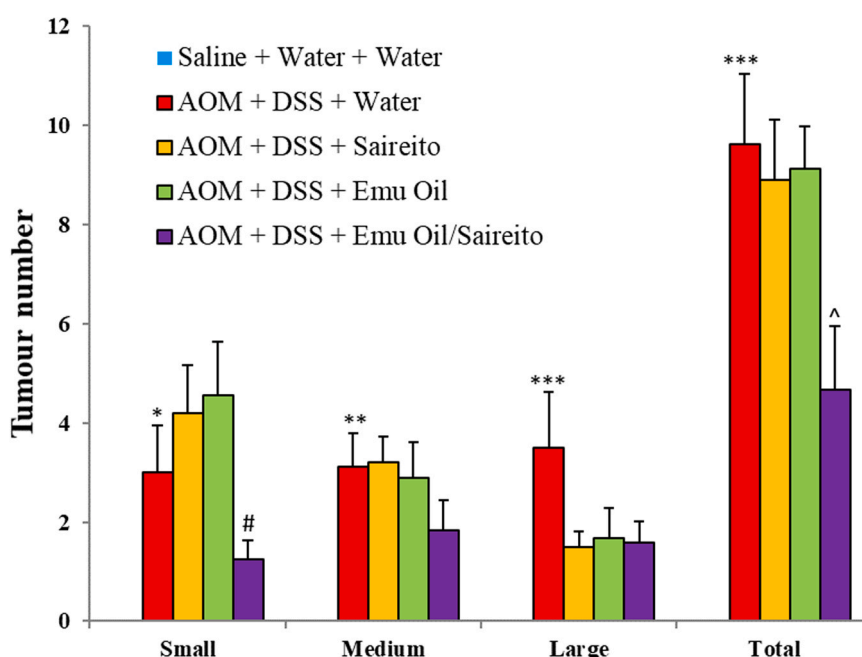


Fig. 8. Tumour number obtained from longitudinally-opened colons. Data are expressed as mean (tumour number) $\pm$ SEM (n = 10/group). Colonic tumours with a diameter < 2 mm were determined as 'small', 2–3 mm as 'medium' and a diameter of > 3 mm was 'large'. ***p < 0.001, **p < 0.01, *p < 0.05 compared to Saline + Water + Water; #p < 0.05 compared to AOM + DSS + Water; ^p < 0.05 compared to AOM + DSS + Saireito.

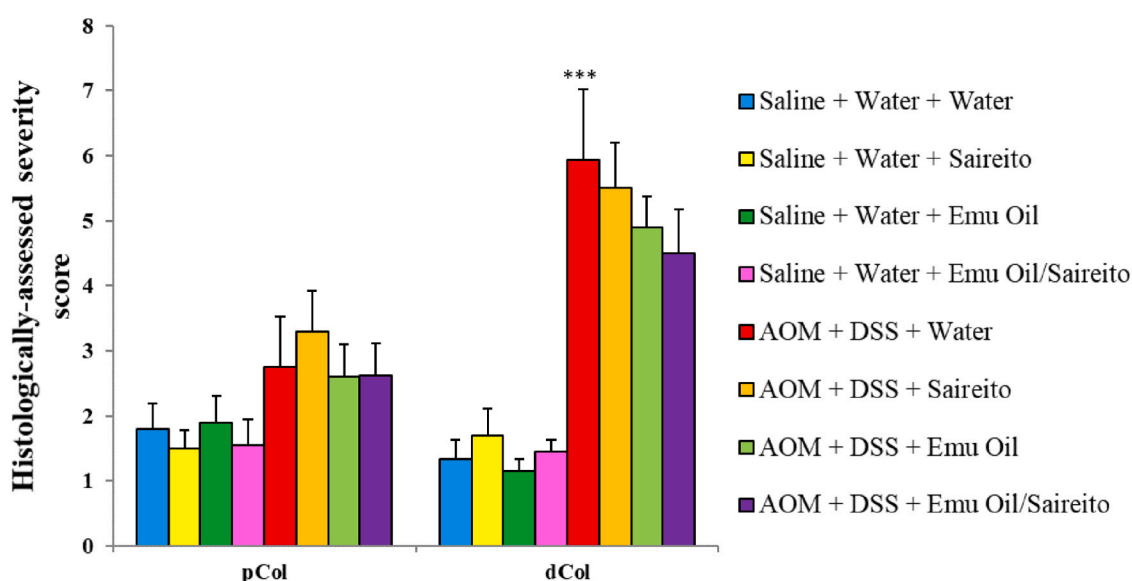


Fig. 9. Histologically-assessed disease severity scores of the proximal and distal colon. Data are expressed as mean (severity score) $\pm$ SEM (n = 10/group). ***p < 0.001 compared to Saline + Water + Water.

burrowing activity of AOM/DSS mice treated with Saireito on day 19 of the trial. However, nesting behaviour was not impacted in AOM/DSS mice compared to normal controls or by nutraceutical treatment. Although the present study scored nesting using the simplest scoring method of a zero for no nest and a one for a successful nest built, future studies in the AOM/DSS model could evaluate nesting in a more complex format using methods described by Deacon et al. and Paumier et al. [53,54]. Moreover, hoarding and judgement bias techniques may also be of interest in the AOM/DSS model [53,55].

In conclusion, the combination of Emu Oil and Saireito represents a promising adjuvant treatment to conventional CA-CRC treatments. This combination improved clinical indicators of CA-CRC while reduced numbers of colonic tumours. However, further investigation into the

effects of these nutraceuticals on inflammatory-associated cytokines, such as IL-6, linked to the pathogenesis of CA-CRC and microbial analysis of faecal samples is crucial to understand the mechanism by which Emu Oil and Saireito exert their beneficial action in combination.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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