Research Article

An Investigation of the Relationship between the Anti-Inflammatory Activity, Polyphenolic Content, and Antioxidant Activities of Cooked and *In Vitro* Digested Culinary Herbs

Magali Chohan, Declan P. Naughton, Lucy Jones, and Elizabeth I. Opara

School of Life Sciences, Kingston University, Penrhyn Road, Kingston upon Thames KT1 2EE, UK

Correspondence should be addressed to Elizabeth I. Opara, e.opara@kingston.ac.uk

There is little research on how cooking and digestion affect the anti-inflammatory activity of culinary herbs. Thus, the aim of this paper was to investigate this activity following cooking and *in vitro* digestion of the common culinary herbs, rosemary, sage, and thyme, and the relationship between their anti-inflammatory activity, polyphenol content, and antioxidant capacity. The anti-inflammatory activity of uncooked (U), cooked (C), cooked and *in vitro* digested (C&D), and standardised (STD, 30 mg/mL) culinary herbs was assessed by measuring their effect on interleukin 8 (IL-8) release from stimulated human peripheral blood lymphocytes (PBLs) and Caco-2 cells. The trolox equivalent capacity (TEAC) and estimated total phenolic content of the herbs were also determined. There was a significant decrease in IL-8 release from PBLs stimulated with H_2O_2 incubated with (U), (C), (C&D), and (STD) herbs and from Caco-2 cells stimulated with TNFα incubated with (C&D) and (STD) herbs. PBLs pre-incubated with (C&D) herbs prior to stimulation (H_2O_2 or TNFα) caused a significant inhibition in IL-8 release. The significant correlations between TEAC and estimated phenolic content and the anti-inflammatory activity suggest a possible contributory role of polyphenols to the anti-inflammatory activity of the culinary herbs investigated.

1. Introduction

There is growing evidence concerning the health-promoting potential of culinary herbs in a dietary context [1, 2], and the group of compounds that appear to be key contributors to this potential are the polyphenols. Polyphenols are major nonnutrient constituents of many common culinary herbs. Multiple studies, both epidemiological and experimental, suggest that polyphenols possess anti-inflammatory and antioxidant activity that may contribute, via the diet, to the prevention of chronic diseases such as cancer, cardiovascular disease, inflammatory bowel disease, and Alzheimer's [2–6]. Some polyphenols (caffeic acid, rosmarinic acid) known to be present in significant amounts in Labiatae culinary herbs, including rosemary, sage, and thyme [7], have been shown to affect the inflammatory response by inhibiting the action of proinflammatory cytokines such as IL-8 [8, 9]. However, their mechanism of action has yet to be fully elucidated [2, 6]. Culinary herbs are traditionally used in small amounts in the flavouring of food. Furthermore they are often cooked prior to consumption and, as with all foods, are affected by digestive processes following ingestion. Therefore, to gain a better understanding of the role that such herbs play in contributing to the dietary intake of polyphenols and their purported health-promoting properties, the impact of both cooking and digestion on the biological properties of these herbs needs to be established. Thus, the aims of this study were to determine the impact of cooking and *in vitro* digestion on the anti-inflammatory properties of the culinary herbs, rosemary, sage, and thyme and to determine whether this activity is associated with their polyphenol content and antioxidant capacity.

2. Material and Methods

2.1. Reagents. Human salivary α-amylase (100 units/mg; 1 unit corresponds to the amount of enzyme that liberates 1 μmol of maltose per minute at pH 6.9 at 25°C), porcine pepsin (800–2.500 units/mg of protein; 1 unit gives a ΔA280 of 0.001 per minute at pH 2 at 37°C), porcine pancreas pancreatin (4x U.S. Pharmacopeia), bile salts, hydrochloric acid (HCl), sodium bicarbonate (NaHCO$_3$), 1,2 ethyl acetate, formic acid, acetic acid, natural product reagent (NP), polyethylene glycol (PEG), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, methanol, rosmarinic acid, sodium hydroxide (NaOH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin Ciocalteu reagent (FC), sodium carbonate (Na$_2$CO$_3$), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate (K$_2$S$_2$O$_8$), catalase (from bovine liver; 1 unit corresponds to the amount of enzyme which decomposes 1 μmol H$_2$O$_2$ per minute at pH 7.0 and 25°C), hydrogen peroxide (H$_2$O$_2$), tumor necrosis factor alfa (TNFα), 12 and 48 well plates (Costar), Millex disposable syringe filter units, (pore size 0.22 μm, Millipore), and grade 1 filter paper (Whatman) were all purchased from Sigma Aldrich, Poole, UK. Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L), 1% nonessential amino acids (NEAA), L-glutamine, RPMI 1640 medium, 100 U/mL penicillin/100 μg/mL streptomycin and heat inactivated fetal bovine serum, 24 well plates (Coning), 15 mL and 25 mL flasks with vented caps (Coning), and Ficoll-Paque Plus were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Caco-2 cells passage 44 were purchased from the European Collection of Cell Culture (ECACC), Health Protection Agency, Salisbury, UK. The Quantikine Elisa kit assay D8000C was purchased from R&D systems, Oxon, UK.

2.2. Culinary Herbs. Rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) dried and certified organic were purchased from Neal's Yard Remedies, Richmond, Surrey, UK and stored in air-tight containers in the dark at room temperature.

2.3. Preparation of Herb Samples. To mimic the cooking of these herbs as closely as possible, quantities of these herbs normally used in the preparation of food were used.

Uncooked herb samples (U) were prepared by adding herbs (1 g) to dark glass vials. These were then covered with water (25 mL, 37°C) infused for 10 minutes, filtered (grade 1 filter paper), and then filter sterilised (pore size 0.22 μm). The mean amounts of plant material in these samples were estimated by allowing the water in these extracts to evaporate under a fan overnight until no moisture was visible and weighing the dry material (rosemary (5.1 ± 0.4 mg/mL), sage (6.5 ± 0.7 mg/mL), and thyme (10.3 ± 0.1 mg/mL), $n = 3$).

Cooked herb samples (C) were prepared by heating herbs (1 g) in a Teflon stir frying pan for 10 minutes (without oil,

as oils are known to possess antioxidant activity [10]); herbs were then prepared as described above.

Cooked and (*in vitro*) digested (C&D) herb samples were prepared by heating the herbs (1 g) in a Teflon stir frying pan for 10 minutes (as described above). The herbs were then passed through a model of *in vitro* digestion with a simulated buccal fluid [11] and a simulated gastric and intestinal fluid [12, 13]. Herbs (C) (1 g) were added to simulated buccal fluid (14 mL, pH 7.0, 37°C) containing α-amylase (1.4 units/mL) for 2 minutes in a preheated glass pestle and mortar. To simulate mastication, herbs were lightly crushed once. For the simulated gastric fluid, each buccal stage mixture was acidified to pH 2.0 with HCl (5 mL, 0.1 mol/L). A solution (1 mL) of pepsin (40 mg/mL) in HCl (0.1 mol/L) was then added, and the mixture was then poured into a glass bottle, with a screw cap, and placed in a water bath (Grant, SS40-2. Fisher, UK) shaking at a rate of 190 strokes per minute so as to mimic the contractions of the human stomach. The mixtures were left in the simulated gastric fluid for 1 hour at 37°C. For the simulated intestinal fluid, the pH of the mixture was raised to pH 5–5.5 by adding NaHCO$_3$(1 M, drop wise). A solution, containing NaHCO$_3$ (5 mL, 0.1 M), pancreatin (2 mg/mL), and bile salts (12 mg/mL), was added and the mixture incubated for 2 hours (37°C) in a water bath shaking at a rate of 190 strokes per minute so as to simulate the human small intestine during digestion. Sodium hydroxide (NaOH, 1 M) was added drop by drop to maintain the pH at 7.5. Control digests were also prepared (no herbs), and all samples were filtered (grade 1 filter paper) and filter sterilised (pore size 0.22 μm).

The pH of (U), (C), and (C&D) herb samples was adjusted to 6.0 ± 0.2 using HCl (0.1 M) (as pH via changes in charge can affect the solubility and chelating capacity of antioxidants [14]) and stored at −80°C.

Preliminary studies carried out using the trypan blue exclusion test showed that the control digest and (C&D) herb samples affected the viability of Caco-2 cells, possibly due to the digestive enzymes from the *in vitro* digestion process. To insure that these enzymes would not interfere with either of the cell assays, digestive enzymes were deactivated for all (C&D) herb samples by placing the entire preparation of each (C&D) herb samples and control digests in a glass test tube and placing this tube in boiled water for 5 minutes.

To gain a better understanding of the impact of the food matrix on the digestion of polyphenols in these culinary herbs, the effect of digestion on rosmarinic acid was carried out. Rosmarinic acid was chosen as has been determined from the literature to be a major polyphenol in the herbs selected for this study [7, 15, 16]. Rosmarinic acid (10 mg in 1 mL solution of 100 μL ethanol and 900 μL distilled water) was digested using the same *in vitro* process described above; however, it was not heated in a Teflon stir frying pan for 10 minutes as this was not practical. It was then compared to nondigested rosmarinic acid (10 mg/mL) to which the amount of distilled water added was equivalent to the amount of digestive fluids used throughout the *in vitro* digestion process.

Preliminary studies showed that the amount of plant material present in the extracts of the herb samples prepared

varied; therefore standardised amounts of plant material (STD) were prepared in order to assess the effect of the same amount of plant material for each herb investigated. This was achieved by adding herbs (1 g) to dark glass vials. The herbs were then covered with water (25 mL, boiled), infused for 10 minutes at room temperature, filtered (grade 1 filter paper), and filter sterilised (pore size $0.22\,\mu m$). The water in these extracts was left to evaporate under a fan overnight until no moisture was visible. The resulting dry material was weighed and resuspended with sterile distilled water at 30 mg of herb material/mL. The pH was then adjusted to pH 6 ± 0.2 and the samples stored at $-80°C$.

2.4. Determination of Antioxidant Capacity and Estimated Phenolic Content of Herb Samples.

The antioxidant capacity of herb samples (U), (C), (C&D), and (STD) was determined using the ABTS radical scavenging capacity assay [17]. Herb samples ($10\,\mu L$) were added to ABTS^{*+} stock solution ($990\,\mu L$) (with an absorbance of $0.700 \pm 0.02\,nM$), and absorbance values were read, after 5 minutes at 730 nm at room temperature, on a Helios β spectrophotometer, (Unicam, UK). Trolox standards, prepared in ethanol, were diluted using PBS ($0–20\,\mu M$) and assayed with the samples, and blanks, to determine the trolox equivalent capacity (TEAC). Gallic acid equivalents (GAEs) were determined for herb samples (U), (C), (C&D), and (STD) using the Folin Ciocalteu (FC) reagent as described previously [18]. However, bearing in mind that this reagent is reactive towards nonphenolic compounds, including ascorbic acid, it has been argued that this assay gives no more than a good "ballpark" estimation of total phenolic content [19]. Thus the values from this assay are expressed as estimated total phenolic content. Diluted herb samples and blanks ($100\,\mu L$) and gallic acid standards (0.5–0.05 mg/mL) were added to 12 well plates (Costar); then FC reagent ($200\,\mu L$) was added, followed by the addition of distilled water (2 mL) and Na_2CO_3 (15%) (1 mL). Solutions were incubated for 2 hours and measured at $25°C$ at 765 nm on a Cary 50 Microplate Reader (Varian Inc, UK).

2.5. Determination of Antioxidant Activity and Polyphenol Profile Using Thin-Layer Chromatography (TLC).

Herb samples, pH 6.0 ($5\,\mu L$), were spotted on to silica TLC plates (10 cm $\times$ 10 cm) and air-dried. The solvent system used was 1,2 ethyl acetate, formic acid, acetic acid, and water (100 : 11 : 11 : 26) [20]. TLC plates were then air dried and sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.2% in methanol) to highlight antioxidant activity or natural product reagent (NP) and polyethylene glycol (PEG) to determine the presence of polyphenols. Yellow areas, against a purple background, corresponded to antioxidant activity. Areas of blue corresponded to phenolic acids, and green/yellow areas corresponded to other polyphenolic compounds. Reference standards ($5\,\mu L$, 500 ppm) of rosmarinic acid were also run.

2.6. Cell Culture.

Ethical approval was obtained from the Ethics Committee of the Faculty of Science, Kingston University, UK. Subjects were recruited once they had read the participant information sheet and had given informed consent. Blood collections and storage procedures were carried out in accordance with the UK Human Tissue Act [21]. Peripheral blood lymphocytes (PBLs) were isolated from heparinised venous whole blood (15 mL) using Ficoll-Paque Plus and then cultured in RPMI media supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin, placed in 15 mL flasks with vented caps (Coning) to incubate at $37°C$ and 5% CO_2 atmosphere for 24 hours and used promptly for cytotoxicity tests or ELISA assays. Caco-2 cell lines were established and maintained by serial passage in a standard sterile tissue culture environment [22]. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (500 mL) containing glucose (4.5 g/L), 1% nonessential amino acids (NEAA), supplemented with 1% L-glutamine, and heat inactivated fetal bovine serum (10%). Cells were placed in 25 mL culture flasks with vented caps (Coning), incubated at $37°C$ and 5% CO_2 and grown for 14 days prior to carrying out cytotoxicity tests or ELISA assays.

2.7. Cytotoxicity Tests on PBLs and Caco-2 Cells Exposed to Herb Samples.

In order to ensure that herb samples were not toxic to the cell lines, cytotoxicity tests were performed. PBLs cells were seeded at a concentration of 1×10^5 cells/mL in 48 well plates (coning) and incubated with herb samples (diluted 1 : 10 in fresh FBS-free RPMI media supplemented with 100 U/mL penicillin/100 μg/mL streptomycin). Caco-2 cells seeded at 2×10^5 in 24 well plates (Coning) were grown for 14 days; the media were replaced every 3-4 days. Cells were washed 3 times in PBS and then incubated with herb samples (diluted 1 : 10 in FBS-free DMEM with glucose (4.5 g/L), 1% nonessential amino acids (NEAA), supplemented with 1% L-glutamine). Both cell lines were placed in an incubator ($37°C$) and 5% CO_2 for 24 hours with herb samples and controls and assessed for cytotoxicity using the trypan blue exclusion test.

2.8. Assay for Catalase-Like Activity.

The potential catalase-like activity for each herb sample was investigated to rule out the potential inactivation of H_2O_2 by the samples through catalase-like activity. The assay was carried out using standard manometry; catalase was used as the positive control [23].

2.9. Investigation of the Anti-Inflammatory Activity of Herb Samples on PBLs and Caco-2 Cells.

The anti-inflammatory activities of the herb samples were assessed in two ways so as to provide insight into how the herb samples affected the action of the proinflammatory factors H_2O_2 and TNF-α. First, PBLs and Caco-2 cells were coincubated with (U), (C), (C&D), and standardised (STD) herb samples, diluted 1 : 10 in respective medium, and H_2O_2 (2 mM) or TNFα (100 μg/mL) for 24 hours. Cells were also preincubated for 3 hours with (C&D) herb samples, diluted 1 : 10 in medium, to determine if the herbs following cooking and *in vitro* digestion were able to protect the cells from proinflammatory
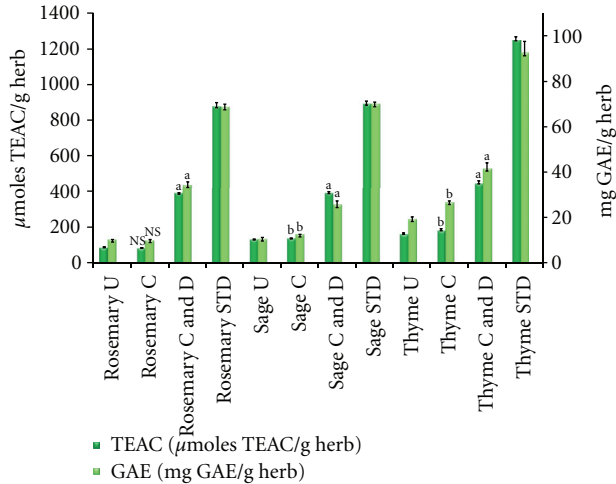
FIGURE 1: Antioxidant capacity (TEAC) and total phenolic content (GAE) of herb extracts. Data are presented as the mean ± SD ($n = 3$). NS: no significant differences between TEAC rosemary (U) and rosemary (C) ($P \geq 0.05$) and no significant differences between GAE rosemary (U) and rosemary (C) ($P \geq 0.05$). [a]$P \leq$ 0.001 (C&D) herb samples significantly different from their (U) & (C) counterparts. [b]$P \leq 0.05$ (C) herb samples significantly different from their (U) counterparts. STD: standardised herb sample (30 mg/mL).

factors. These cells were then exposed to H_2O_2 or TNFα 24 hours at the concentrations stated above. PBL and Caco-2 controls were set up, in their respective media, with H_2O_2 or TNFα only at the concentrations stated above. Spontaneous release of IL-8 was assessed by incubating cells in media only (in the absence of herb samples, H_2O_2 or TNFα). Cells were also exposed to (U), (C), (C&D) blank digest, and (STD) samples (diluted 1 : 10 in their respective media) to determine they if they affected IL-8 release in the absence of H_2O_2 or TNFα.

Media were then collected and their IL-8 concentrations determined using the quantitative sandwich enzyme immunoassay ELISA for interleukin-8 kit (carried out as per manufacturer's instructions) to ascertain if its release was affected. Plate absorbance values were read at 450 nm with a correction at 540 nm using a Cary 50 Microplate Reader (Varian Inc, UK).

2.10. Expression of Data and Statistical Analysis. Data are expressed as means of triplicate analysis ± SEM, ($n = 3$) unless otherwise stated. Statistical analyses were performed using SPSS for windows (Version 17). The antioxidant capacity was expressed in μmoles TEAC/g herb and estimation of total phenolic content in mg GAE/g herb. Mean values for blank digest samples were subtracted from respective TEAC and GAE values for C&D herb samples. ANOVA with post hoc Tukey were used to compare TEAC and GAE of (U), (C), (C&D), and STD herb samples. The independent sample t-test was used to compare the TEAC of rosmarinic acid digested to rosmarinic acid nondigested. The percentage inhibition of IL-8 release from PBLs or Caco-2 cells was

determined using the following equation: ((IL-8 release from respective control cells (stimulated by H_2O_2 or TNFα)- IL-8 release from cells coincubated or preincubated with herb samples and H_2O_2 or TNFα)/IL-8 release from respective control cells (stimulated by H_2O_2 or TNFα)) $\times 100$. As percentages are bound data, the percentages for IL-8 inhibition were checked for normality using the Shapiro-Wilk test. ANOVA with post hoc Tukey was then used to compare the percentage inhibition by herb samples (U), (C), (C&D), and (STD) preincubated with H_2O_2 or TNFα against their respective controls. Pearson's correlation coefficients (r) with level of significance ($P \leq 0.05$) (2-tailed) were used to compare all herb samples irrespective of treatments for TEAC and GAE assay results, and TEAC and GAE with percentage inhibition of TNFα or H_2O_2 stimulated IL-8 release by PBLs or Caco-2 cells.

3. Results

3.1. Antioxidant Capacity and Estimated Total Phenolic Content of Herb Samples. The antioxidant capacity (TEAC) and estimated total phenolic content (GAE) of the (C&D) herb samples were significantly higher than those of their (C) counterparts ($P \leq 0.001$) which were significantly higher than those of the (U) herb samples ($P \leq 0.05$) with the exception of rosemary ($P \geq 0.05$ for both TEAC and GAE assays) (Figure 1). The TEAC and GAE for the (STD) herb samples were significantly higher than (U), (C), and (C&D) samples ($P \leq 0.001$) (Figure 1). (STD) thyme was higher than (STD) sage and rosemary for both assays. For TEAC values for rosmarinic acid there was no statistical difference ($P = 0.692$) between nondigested rosmarinic acid ($2901 \pm 149.1\,\mu$moles TEAC/g) and digested rosmarinic acid ($2973.1 \pm 140.6\,\mu$moles TEAC/g), ($n = 3$).

3.2. Antioxidant Activity and Polyphenol Analysis Using TLC. For TLC plates sprayed with DPPH, all the (C&D) herb samples provided a more intense yellow colour compared to their undigested (U) and (C) counterparts. This difference in colour indicated a higher antioxidant activity (Figure 2(a)). For the TLC plates sprayed with NP and PEG, phenolic acids appeared in shades of blue towards the top of the plates, and other polyphenolic compounds appeared in shades of green and yellow in the centre of the plates (Figure 2(b)). The colours of phenolic acids and other polyphenolic compounds were more intense for the (C&D) herb samples, compared to their (U) and (C) counterparts, indicating higher levels of phenolic compounds. For (C) herbs, there was no difference in colour intensity for rosemary, a marginal increase in colour intensity for sage and an evident increase in colour intensity for thyme when compared with their (U) counterparts (Figures 2(a) and 2(b)).

3.3. Herb Samples Cell Cytotoxicity Tests and Catalase-Like Activity. The viability of both PBLs and Caco-2 cells exposed to (U), (C) (C&D), and (STD) herb samples diluted 1 in 10 in respective media for 24 hours was not significantly different from that of the controls ($P > 0.05$, data not shown).
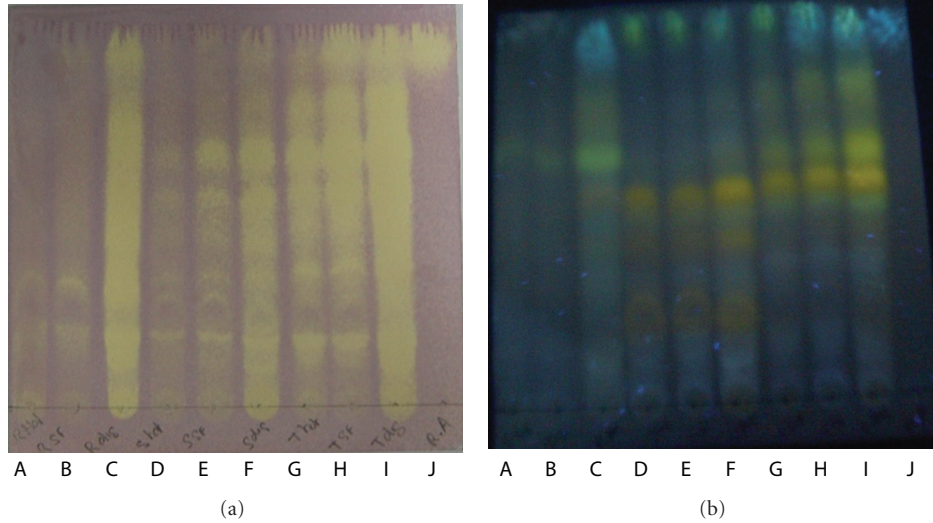
(a)

(b)

FIGURE 2: Percentage inhibition of IL-8 release by H_2O_2 or TNFα exposed PBLs pre-incubated or co-incubated with herb samples. (a) TLC plate spotted with herb samples (5 μL, pH 6) and rosmarinic acid (0.5 mg/mL) and sprayed with 2% DPPH. The yellow areas correspond to antioxidant activity. A: rosemary (U), B: rosemary (C), C: rosemary (C&D), D: sage (U), E: sage (C), F: sage (C&D) G: thyme (U), H: thyme (C), I: thyme (C&D), J: rosmarinic acid (U) uncooked, (C) heated, (C&D) heated, and digested. (b) TLC plate spotted with herb samples (5 μL, pH 6) and rosmarinic acid (0.5 mg/mL) and sprayed with natural product reagent (NP) and polyethylene glycol (PEG). Phenolic acids appear in shades of blue towards the upper part of the plate and other polyphenolic compounds appear in a range of shades of green and yellow in the centre of the plate. A: rosemary (U), B: rosemary (C), C: rosemary (C&D), D: sage (U), E: sage (C), F: sage (C&D) G: thyme (U), H: thyme (C), I: thyme (C&D), J: rosmarinic acid (U) uncooked, (C) heated, (C&D) heated and digested.

None of the herb samples exhibited catalase-like activity indicating that H_2O_2 was not inactivated by these samples.

3.4. Spontaneous Release of IL-8 from PBLs and Caco-2 Cells Compared to TNFα and H_2O_2 Stimulated PBL and Caco-2 Cell Controls.

Spontaneous IL-8 release was detected for both PBLs (855.5 ± 10.7 pg/mL, $n = 8$) and Caco-2 cells (60.4 ± 1.1 pg/mL, $n = 6$). Exposure of cells to TNFα significantly increased IL-8 release compared to nonstimulated cells ($P \leq 0.05$) for both PBL (1011 ± 63.9 pg/mL, $n = 6$) and Caco-2 Cells (183 ± 14.5 pg/mL, $n = 6$). Exposure of cells to H_2O_2 significantly increased IL-8 release compared to nonstimulated cells ($P \leq 0.05$) for both PBLs (1144.6 ± 47.5 pg/mL, $n = 6$) and Caco-2 Cells (91.8 ± 8.2 pg/mL, $n = 6$). None of the herb samples had a significant effect on spontaneous IL-8 release from either cell lines.

3.5. Effect of Coincubation of Herb Samples with H_2O_2 or TNFα.

For (U), (C), and (C&D) herb samples there were decreases in IL-8 release from PBLs but these decreases were only significant for PBLs, stimulated by H_2O_2 ($P \leq 0.05$, Figure 3) with the exception of (C) rosemary. (C&D) herb samples significantly decreased the release of IL-8 from Caco-2 cells stimulated by TNFα ($P \leq 0.001$, Figure 4). (STD) herb samples significantly inhibited the release of IL-8 from PBLs stimulated by H_2O_2 and PBLs stimulated by TNFα ($P \leq 0.001$, Figure 3). (STD) herb samples significantly decreased the release of IL-8 from Caco-2 cells stimulated by TNFα ($P \leq 0.001$, Figure 4). (U), (C), (C&D), and (STD)
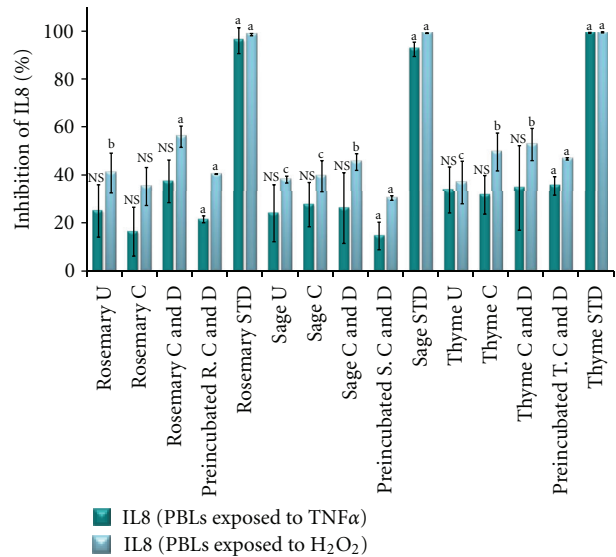

FIGURE 3: Percentage inhibition of IL-8 release by TNFα exposed Caco-2 cells pre-incubated or co-incubated with herb samples. (R.): Rosemary, (S.): Sage, (T.): Thyme. Data are presented as the means ± SEM ($n = 3$). %inhibition of IL-8 = [(IL-8 release from control PBLs (stimulated by H_2O_2 or TNFα)-IL-8 release from PBLS co-incubated/pre-incubated with herb samples)/IL-8 release from control PBLs (stimulated by H_2O_2 or TNFα)-] ×100. Significant differences between IL-8 released from control PBLs and PBLs co-incubated/pre-incubated with herb samples, [a]$P \leq 0.001$, [b]$P \leq 0.01$, [c]$P \leq 0.05$, $n = 3$. NS: nonsignificant difference between IL-8 release for control PBLs and that of PBLs co-incubated with herb samples and TNFα or H_2O_2, or pre-incubated with herb samples.
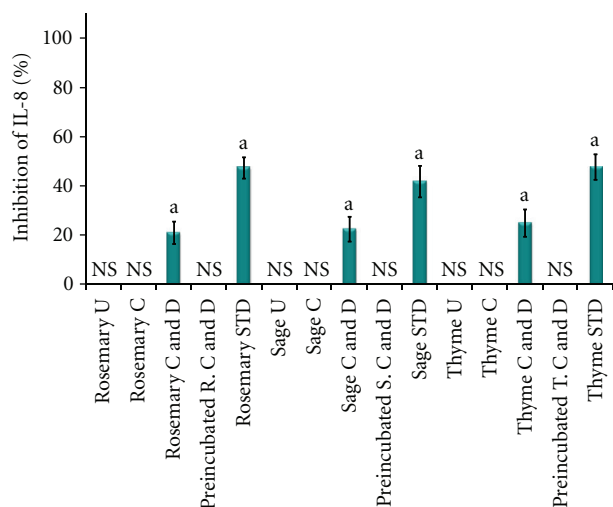
FIGURE 4: Percentage inhibition of IL-8 release by TNFα exposed Caco-2 cells pre-incubated or co-incubated with herb samples. (R.): Rosemary, (S.): Sage, (T.): Thyme. Data are presented as the Means ± SEM ($n = 3$). %inhibition of IL-8 = [(IL-8 release from control Caco-2 cells (stimulated by TNFα)-IL-8 release from Caco-2 co-incubated/pre-incubated with herb samples)/IL-8 release from control Caco-2 cells (stimulated by TNFα)] ×100]. Significant differences between IL-8 released from control PBLs and PBLs co-incubated/pre-incubated with herb samples, $^{a}P \leq 0.001$, ($n = 3$). NS: nonsignificant difference between IL-8 release for Caco-2 cells control (TNFα) and that of Caco-2 cells co-incubated with herb samples and TNFα, or pre-incubated with herb samples.

herb samples had no effect on IL-8 release from Caco-2 cells stimulated by H_2O_2(data not shown).

3.6. Effect of Preincubation with Herb Samples prior to Adding H_2O_2 or TNFα.

Preincubation with (C&D) herb samples caused a significant decrease in IL-8 release from PBLs stimulated by H_2O_2 or TNFα ($P \leq 0.001$, Figure 3). Preincubating the Caco-2 cells with (C&D) herb samples had no effect on IL-8 release when stimulated by H_2O_2 or TNFα (Figure 4).

3.7. Correlations between Antioxidant Capacities, Total Phenolic Contents, and Anti-Inflammatory Activities of Culinary Herbs, Irrespective of Treatments.

There was a strong correlation between TEAC and GAE ($r = 0.989$, $P \leq 0.01$), (Table 1) and between the %decrease in IL-8 release caused by herb samples for H_2O_2 or TNF-α stimulated PBLs and their estimated total phenolic content and antioxidant capacity ($P \leq 0.01$, Table 1). There was a strong and significant correlation between the %decrease in IL-8 release from TNF-α stimulated Caco-2 cells and their estimated total phenolic content and antioxidant capacity ($r = 0.892$, $P \leq 0.01$, Table 1).

4. Discussion

The aims of this study were, first, to investigate what effect cooking and digestion *in vitro* have on the anti-inflammatory activity of the culinary herbs rosemary, sage, and thyme and, second, to investigate if there is an association between the anti-inflammatory activity and the antioxidant capacity and estimated total phenolic content of these herbs.

Based on the co- and preincubation experiments, this study indicates that rosemary, sage and thyme (U), (C), (C&D) and (STD) significantly decrease IL-8 release via inhibition of, and protection against, the action of H_2O_2 or TNFα. The anti-inflammatory action of the herbs was far greater for the PBLs than for the Caco-2 cells. In fact, with the exception of (C&D) and (STD) herbs in TNFα stimulated Caco-2 cells, none of the other herb samples showed any anti-inflammatory action based on the inhibition of IL-8 release for both H_2O_2 or TNFα stimulated Caco-2 cells. The Caco-2 cell line is a transformed human colonic adenocarcinoma; however, the PBLs are mature "healthy" lymphocytes, and these differences might explain the difference observed in IL-8 release [24].

Cooking the herbs did not consistently significantly increase their estimated total phenolic content or antioxidant capacity, compared to (U) herbs in the current study. Other studies that have looked at the impact of cooking on various plant foods concur that differences in polyphenol activity content are due to both the food matrix and the cooking techniques employed [25–27]. Cooking followed by *in vitro* digestion did elicit significant increases in TEAC and GAE compared to (U) and (C) herbs in the current study. In relation to the literature the impact of digestion on polyphenols using *in vitro* models resulted in decreases in activity in fruits juices [28] and either no changes or decreases in herbal teas (infusions from powdered herbs) [29]. These differences may be due to the nature of the food matrix (whole herbs were used in the current study) as well as the type of *in vitro* model of digestion used.

Analysis using TLC showed the presence of phenolic acids and other polyphenolic compounds in the (U) herb samples which agrees well with the literature [16, 20, 30]. However, to the authors' knowledge, no TLC work has been done to determine the polyphenolic profiles of rosemary, sage, and thyme following cooking and digestion *in vitro*. TLC results from the present study did not suggest that new compounds were formed or lost as a result of cooking as the profiles for (C) versus (U) herb samples were identical for rosemary and more intense in colour for sage and thyme but not dissimilar. The *in vitro* digestion process increased the levels of phenolic acids and other polyphenolic compounds. These observations are supported by the increases in the antioxidant capacity, activity (TLC), and estimated total phenolic content data. Rosmarinic acid, measured via the TEAC assay, was not affected by *in vitro* digestion, and although the effect of cooking on rosmarinic acid was not investigated, the lack of difference in TEAC and GAE in (C) compared to (U) herbs strongly indicates that polyphenols in these herbs, including rosmarinic which is a predominant polyphenol in these herbs, were not decreased as a consequence of the cooking method used.

A positive linear correlation has been established between polyphenols and antioxidant capacity of herbs [16]. Polyphenols are thought to be most concentrated in the vacuoles of

TABLE 1: Correlations between antioxidant capacities, estimated total phenolic content, and %decrease in IL-8 release irrespective of treatments.

Correlations	r	P
GAE versus TEAC	0.989	≤ 0.01
%decrease in IL-8 for PBLs exposed to TNFα versus GAE	0.914	≤ 0.01
%decrease in IL-8 for PBLs exposed to TNFα versus TEAC	0.901	≤ 0.01
%decrease in IL-8 for PBLs exposed to H_2O_2 versus GAE	0.922	≤ 0.01
%decrease in IL-8 for PBLs exposed to H_2O_2 versus TEAC	0.905	≤ 0.01
%decrease in IL-8 release for Caco-2 exposed to TNFα versus GAE	0.876	≤ 0.01
%decrease in IL-8 release Caco-2 exposed to TNFα versus TEAC	0.892	≤ 0.01

paraveinal and parenchymal cells below the epidermis [31]. Thus, the increase in antioxidant capacity and estimated phenolic content is likely to be due to the breakdown of the herbs' plant cell walls via the *in vitro* digestion process, facilitating the release of polyphenols. The antioxidant capacity, estimated total phenolic content, and anti-inflammatory effects of (STD) herb samples were by far the greatest (thyme>sage, rosemary). This is most likely to be due to a greater concentration of plant material (7.3 ± 2.4 mg/mL for (U) and 30 mg/mL for (STD) herb samples) and thus a greater concentration of polyphenols resulting in a dose-dependant response. This is further supported by the much greater decrease in IL-8 release that resulted when stimulated cells, (with the exception of H_2O_2 stimulated Caco-2 cells), were coincubated with (STD) herb samples, thus corroborating the role of polyphenols as significant contributors to the anti-inflammatory properties of these herbs.

The mechanism of action to explain the effect of the herbs on IL-8 release is unclear. However, evidence suggests that polyphenols may play a role in the anti-inflammatory activity reported in the current study. Rosmarinic acid is the predominant polyphenol in rosemary, sage, and thyme [7, 15, 16, 30, 32]. The effectiveness of rosmarinic acid in reducing inflammation by inhibiting the proinflammatory PKC/NF-κB pathway was demonstrated in mice [33]. Therefore it is possible to speculate that the rosmarinic acid present in the herbs studied may be responsible, at least in part, for the IL-8 inhibition observed. In addition, caffeic acid, another polyphenol shown to be present in the herbs investigated [7], was shown to inhibit both H_2O_2 and TNFα stimulated IL-8 release from Caco-2 cells [9]. This study indicated that inhibition occurred at the transcriptional and post-transcriptional level.

One clear difference observed in this study was that the inhibition of IL-8 release from PBLs stimulated by H_2O_2 was generally greater (and more statistically significant) than for those stimulated by TNFα. This difference may be because TNFα and H_2O_2 may affect different inflammatory pathways within the cells or may affect the same inflammatory pathways differently [24].

It remains unclear why none of the herb samples inhibited IL-8 release from Caco-2 cells exposed to H_2O_2. Differences in H_2O_2 and TNFα induced release of IL-8 were also reported [9] with caffeic acid, a metabolite of rosmarinic acid. The authors demonstrated that caffeic acid (2.00 mmol/L) was able to inhibit the H_2O_2 or TNFα induced release of IL-8 from Caco-2 cells; however, whilst it was shown that caffeic acid inhibition appeared to occur via the inhibition of their expression of IL-8 mRNA, it did not suppress the TNFα-induced increase in the IL-8 mRNA expression. That study suggests that the mechanisms by which polyphenols suppress the stimulation of IL-8 production and its subsequent release may differ based on the inflammatory stimulant. However, these results are in disagreement with the current study where only TNFα-stimulated IL-8 release was inhibited by the herb samples. As stated above, caffeic acid is a polyphenol found in all three culinary herbs investigated [7], although one must bear in mind that the concentration of polyphenols is affected by numerous factors including seasonal variations [34, 35] and the place of provenance [15, 36], as well as presale processing and storage [37] and also cooking [25, 26, 38].

The strong and significant correlations between percentage inhibition of IL-8 release from the stimulated PBLs with TEAC and GAE, irrespective of treatments, suggest that there is an association between the antioxidant capacity, polyphenol content, and the anti-inflammatory activity of the herbs investigated [5, 9, 39, 40]. In conjunction with literature concerning the anti-inflammatory activity of individual polyphenols, this association does provide compelling evidence that the polyphenols in these culinary herbs are major contributors to the inhibitory effects on IL-8 release reported. However, it is not clear if this action is in any way due to their antioxidant activity. Analytical methods would need to be employed to confirm this hypothesis and to clarify which polyphenols, including their metabolites, are responsible for the biological activities observed.

Whilst (STD) herb samples may not have a physiological relevance in terms of dietary intake, their use removed the impact that the amount of plant material has on antioxidant capacity, estimated total phenolic content, and anti-inflammatory activity when comparing the herbs. In addition, it also helped to demonstrate a dose-dependent relationship between the amount of herb and the anti-inflammatory activity. The dose-response relationship is of particular interest since the amount of herbs used in the preparation of food varies [41] and also varies between individuals. A recent study showed that the consumption of herbs and spices ranged from 0.0 to 10.0 g/person/day [42].

5. Conclusion

In conclusion, that study shows that the culinary herbs rosemary, sage, and thyme, in quantities used for cooking, possess significant anti-inflammatory activity that may be due to their polyphenol content. Further work is required to fully elucidate the polyphenols responsible for this action and their mechanisms of action.

Acknowledgments

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