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Analysis of Polyphenolic Composition, Antioxidant Power and Stress-Response Effects of Fractionated *Perilla* Leaf Extract on Cells In Vitro

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Abstract: Background/Objectives: *Perilla frutescens* has historically been used to protect against inflammation and redox stress. This has been partly attributed to its high polyphenolic content; however, polyphenolic components in *Perilla* extract remain incompletely defined. This study aimed to characterise the polyphenolic composition in *Perilla* extract and evaluate its effect on the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), regulating antioxidant defenses during inflammation and oxidative stress. **Methods**: Hot water extraction from Perilla leaves was followed by fractionation using four solvents of different polarity, namely methanol, butanol, ethyl acetate and ether. The polyphenolic composition of these fractions was analysed using RP-HPLC, and some of these compounds were quantified. The total phenolic, flavonoid, and ortho-diphenolic contents of each *Perilla* fraction were determined. The antioxidant activity was assessed using metal cation reduction and radical scavenging assays. A dual-luciferase assay using a human NQO1 ARE-luciferase reporter plasmid was employed to quantify Nrf2 activation by the *Perilla* fractions. **Results**: HPLC analysis identified 35 polyphenolic compounds, with the highest phenolic content present in the polar fractions and rosmarinic acid being the major constituent. Radical scavenging tests (DPPH and ABTS) confirmed the highest antioxidant capacity in the polar fractions. On cells in vitro, the methanol *Perilla* fraction displayed the strongest antioxidant activity, showing up to a 1.5-fold increase in human NQO1 ARE-luciferase reporter induction. **Conclusions**: This study has shown that Perilla extract contains a diversity of polyphenolic compounds contributing to its potent antioxidant effects, with methanol and butanol being the most efficient extraction solvents. While rosmarinic acid is expected to be the major contributor towards providing protection against inflammation and redox stress, further work is required on the synergystic effects between different polyphenols.

Keywords: *Perilla frutescens*; polyphenols; redox stress; anti-inflammatory response; antioxidant response element

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1. Introduction

Perilla frutescens (L.) Britton is a member of the mint family (Lamiaceae) that is widely distributed across Southeast Asia [1]. The leaves of *P. frutescens* have been used in traditional medicine to treat various ailments [2,3], and ample research has been performed demonstrating the impact of *P. frutescens* on biological processes such as inflammation,

redox stress, allergic reaction and even carcinogenesis [4–14], attributable to its rich phytochemical composition.

The bioactive constituents in *Perilla* extracts fall into a number of different chemical classes, with the ones of greatest relevance to its antioxidant activity being alkaloids, phenylpropane analogues, terpenoids, and polyphenols (phenolic acids, flavonoids including anthocyanins, tannins, stilbenes, and lignans) [14].

These secondary metabolites identified in *P. frutescens* are known to act as antioxidants mainly by neutralising reactive oxygen (such as hydroxyl radical and superoxide ion) and nitrogen (such as nitric oxide, peroxynitrite) radicals via hydrogen donation from their hydroxyl groups [15–17]. They can also chelate transition metal ions such as Fe^{3+} Cu^{2+} involved in the production of free radicals [17]. Synergistic effects have also been observed between various pairs of polyphenols [18], further enhancing their biological effects within natural extracts such as that from *Perilla* leaves.

The polyphenols and flavonoids in *P. frutescens* exhibit diverse pharmacological properties. They present strong interactions with proteins because of the hydrophobicity of their rings and hydrogen-bonding potential of the phenolic hydroxyl groups [19] and have the potential to inhibit some enzymes involved in radical generation, including xanthine oxidase, myeloperoxidase, and lipoxygenase [20], as well as regulation of intracellular glutathione levels [21]. In addition to their antioxidant action, polyphenols also display anti-inflammatory, anti-allergenic, anti-viral, anti-microbial, anti-mutagenic, anti-cancer and cardio-protective properties [22].

Specifically looking at the flavonoids in *P. frutescens*, their free radical scavenging capacity is influenced by their structure, including the number and position of hydroxyl groups, as well as the ability of the hydroxyl groups to donate hydrogen atoms to radicals in order to stabilise them [23]. Flavonoids have also been reported to present interesting anti-cancer properties by modulating several biological signalling pathways, including redox metabolism (detoxification, oxidation and reduction), inflammation, suppression of oncogenes and tumour formation, cell growth and proliferation, cell cycle checkpoints, DNA repair, senescence, intrinsic and extrinsic apoptosis, autophagy, stimulation of the immune system [24,25].

Similarly, phenolic acids present in *P. frutescens* have varying levels of antioxidant activity based on the number and position of hydroxyl groups within these molecules, with higher antioxidant activity upon introduction of a second ortho- or para-hydroxyl group or methoxyl groups [26]. Additionally, they also present anti-cancer properties, such as their ability to inhibit cell proliferation, angiogenic factors, oncogenic signaling cascades, growth and differentiation, preventing cellular migration and metastasis whilst inducing apoptosis [27].

Inflammation and oxidative stress are known to play a role in a variety of human conditions, including auto-immune disorders and cancer [28–31]. A major transcription factor involved in protecting cells against oxidative stress is nuclear factor erythroid 2-related factor 2 (Nrf2). It functions by binding to a motif called the antioxidant response element (ARE) found on the promoter of phase II antioxidant enzymes [32]. The polyphenolic and flavonoid components of P. frutescens have shown potential in activating Nrf2 signaling, highlighting their relevance in therapeutic interventions targeting oxidative stress.

Despite these promising findings about *Perilla* leaf extract, the overall contribution of the polyphenolic, flavonoid and ortho-diphenolic components are within *P. frutescens* and contributing to its bioactive functions, particularly antioxidant activity, remain underexplored [33–36]. There is a need to characterise their roles in metal ion reduction, radical scavenging, and Nrf2 induction.

The overall aim of this study was, first of all, to define the polyphenolic composition of *Perilla* leaf extract by fractionation using solvents of varying polarity followed by HPLC analysis. The biological activity of these components was quantified through the determination of the metal cation reduction activity and their scavenging power. To evaluate the antioxidant and cellular effects of the fractions of the *Perilla* leaf extract, these were then incubated with cells cultured in vitro to quantify the induction of Nrf2.

2. Materials and Methods

2.1. Sample Preparation

The *Perilla* leaf extract used in this study was provided by Amino Up (Sapporo, Japan). Dissimilar to the *Perilla* extract products sold by Amino Up Co., Ltd., the powder used in this study was 100% *Perilla*-water-soluble components. The *Perilla* leaf extract was manufactured on an industrial scale. Dried green Perilla (Shiso) leaves were sourced from several contract farmers in Hokkaido, Japan. Extraction was performed in hot water, and after removing the solid residue, the supernatant was collected and sterilised at 121 °C for 45 min. The extract was concentrated under reduced pressure using an evaporator and powdered by spray drying. Portions of 0.2 g *Perilla* leaf extract were first treated using different pH conditions (pH 2, 4, 7, 9 adjusted using NaOH or HCl) at 70 °C, after which the solution underwent exhaustive extraction using a 1:5 dry weight to solvent ratio in butanol, ethyl acetate (pH 7) and ether (pH 7) or direct extraction from the powder using methanol. The fractions were dried at 30 °C under vacuum, followed by reconstitution in 1 mL of methanol for all the subsequent testing.

2.2. High Performance Liquid Chromatography (HPLC)

The identification and quantification of polyphenolics in the *Perilla* fractions was carried out by a Shimadzu LC-20 AB (Shimadzu Europa GmbH, Duisburg, Germany) HPLC system linked to a binary pump, autosampler (SIL-20AC) (Shimadzu Europa GmbH, Duisburg, Germany), and UV/vis detector (SPD-20AV) (Shimadzu Europa GmbH, Duisburg, Germany) to monitor the 280 nm and 320 nm wavelengths. Samples were centrifuged and syringe filtered through a 0.45 micron PVDF filter. A 20 µL injection volume was used for analysis using an ACE® C18 analytical column (250 × 4.6 mm i.d.) having a 5 µm particle size (Aberdeen, Scotland). The mobile phases were degassed and consisted of (A) water:Acetic acid (95:5, v/v) and (B) methanol:Acetonitrile (1:1, v/v) at a constant flow rate of 1 mL/min. A gradient elution was performed using the following solvent program: 95% (A): 5% (B) 0–30 min; 70% (A): 30% (B) 30–35 min; 50% (A): 50% (B) 35–40 min; 100% (B) 40–50 min and then 95% (A): 5% (B) for the final 2 min as a post-equilibration step. The column temperature was maintained at 35 °C using a Shimadzu CTO-10AC (Shimadzu Europa GmbH, Duisburg, Germany) thermostatically controlled column compartment, whilst the sample chamber was kept at 4 °C to prevent phenolic degradation. Compounds were identified by comparing peaks to standards and their relative retention times.

2.3. Determination of Total Phenolic Content

The Folin–Ciocalteu colourimetric method [37] was employed for the determination of total phenolic content present in the hydroalcoholic fractions derived from the *Perilla* leaf extract against a standard calibration curve made with caffeic acid (Sigma Aldrich, Munich, Germany). The concentrated fractions derived from liquid–liquid extraction were diluted by a factor of 10 using 1:1 methanol to acetonitrile (v/v). Then, 20 µL of the resulting solution was oxidised with 100 µL of Folin–Ciocalteu reagent (Sigma Aldrich, Munich, Germany) diluted 5-fold, followed by neutralisation by adding 80 µL of 7.5% Na_2CO_3 in a 96-well microtiter plate. Following a 2 h incubation at room temperature in the dark, a

microtiter plate reader was used to measure the absorbance at 600 nm. A value in mg caffeic acid equivalents (mg/mL CAE) was produced as the expression of total phenolic content.

2.4. Determination of Total Flavonoid Content

The total flavonoid content was obtained using the protocol by Mabry et al. [38] with minor modifications. For this analysis, 25 µL of the diluted fractions were mixed with 7.5 µL of 10% aluminium chloride, 7.5 µL of 7% *w*/*v* sodium nitrite and 80 µL of distilled water, and incubated for 30 min at room temperature, followed by the addition of 100 µL of 1 M NaOH. Following vigorous shaking, the absorbance at 415 nm was measured. The assay was calibrated against catechin. A value in mg catechin equivalents (mg/mL CE) was produced as the expression of total flavonoid content.

2.5. Determination of Ortho-Diphenolic Content

The *o*-diphenolic content was determined using Arnow's colourimetric method [39] against a pyrocatechol (Sigma Aldrich) standard calibration curve. To perform this determination, 20 µL of 5-fold diluted fractions were added to 20 µL of 1 M HCl in a 96-well microtiter plate. Following mixing, 20 µL of Arnow's reagent (10 g of sodium nitrite and 10 g of sodium molybdate dihydrate in 100 mL 1:1 ethanol to water (*v*/*v*)). Following vigorous shaking, the plate was incubated for 15 min at room temperature, and eventually the addition 80 µL of water and 40 µL of 1 M NaOH. The absorbance at 370 nm was recorded. The ortho-diphenolic content was expressed as mg pyrocatechol equivalents (mg/mL PyCE).

2.6. Determination of 2 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The radical scavenging activity of the phenolic compounds in the *Perilla* fractions was measured using the DPPH assay [40]. On the day of the experiment, 60 µM DPPH in methanol stock solution was prepared (stored in the dark at 4 °C). Initially, 25 µL of phenolic stock solution was pipetted and then two-fold serially diluted down to 7.8 µg/mL in a 96-well microtiter plate. The negative DPPH controls consisted of 100 µL of MeOH per well. Then, 150 µL of methanolic DPPH were pipetted per well and incubated for 30 min in the dark. A microplate reader was used to measure the absorbance at 517 nm. The % of DPPH radicals scavenged by the fractions was calculated using the equation:

$$\%RadicalScavenging = |Absorbance_{DPPHinmethanol}| - \frac{\left|Absorbance_{DPPHinsample}\right|}{|Absorbance_{DPPHinmethanol}|} \times 100 \quad (1)$$

The DPPH EC_{50} value, i.e., the *Perilla* fraction concentration, which reduced the DPPH radical concentration by 50%, was derived from plotting the percentage inhibition against *Perilla* fraction concentration.

2.7. Determination of 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (Abts) Radical Cation Stabilisation

The reaction between 7 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2.45 mM potassium persulfate generated the $ABTS^{\bullet+}$ radicals. The mixture was incubated at room temperature, in the dark, for 12 h before use [41]. Methanol was used to adjust the $ABTS^{\bullet+}$ radical solution absorbance at 734 nm to 0.700 (0.020 ± mean ± SD). Then, 20 µL of sample or solvent was added to 280 µL of the $ABTS^{\bullet+}$ radical solution in a 96-well plate. For each fraction, the stock solution of 500 µg/mL was added into the well and two-fold serially diluted down to the lowest concentration of 7.8 µg/mL. The microplate was incubated at 30 °C for 5 min, and the absorbance at 734 nm was measured. The percentage inhibition of

$ABTS^{\bullet+}$ was calculated as follows:

$$\%RadicalScavenging = |Absorbance_{ABTSinmethanol}| - \frac{\left|Absorbance_{ABTSinSample}\right|}{|Absorbance_{ABTSinmethanol}|} \times 100 \quad (2)$$

The EC_{50} value for the ABTS assay, defined as the *Perilla* fraction concentration, which reduced the $ABTS^{\bullet+}$ radical concentration by 50%, was obtained by generating a plot for percentage inhibition vs. *Perilla* concentration.

2.8. Determination of Cupric Reducing Antioxidant Power

The copper ion-reducing antioxidant capacity assay employs the use of copper (II) neocuproine reagent as the chromogenic oxidising agent [42]. The method was adjusted for microtiter plates whereby 20 µL of diluted fraction was added to 100 µL of 10 mM $CuCl_2$ solution, after which 100 µL of 1 M ammonium acetate buffer (pH 7.0) was added. To the resulting solution, 100 µL of 7.5 mM neocuproine ethanolic solution was added and incubated for 30 min at room temperature. The absorbance at 450 nm was then recorded.

2.9. Determination of Ferric Reducing Antioxidant Power

Ferric-reducing antioxidant power (FRAP) was determined by the spectrophotometric method previously described by Benzie and Strain [43], using the reduction of a ferric tripyridyl triazine (Fe(III)(TPTZ)2) complex at low pH. The FRAP reagent consisted of 25 mL of 300.0 mmol/L acetate buffer, 2.5 mL of 10 mmol/L ferric tripyridyl triazine (TPTZ) solution, and 2.5 mL of 20 mmol/L $FeCl_3$ solution in a 10:1:1 ratio. 200 µL of FRAP reagent and 10 µL of sample were mixed and vigorously shaken. The absorbance of the reduced iron complex was measured at 593 nm, and the concentration was determined from an ascorbic acid calibration curve such that it was expressed as mg/mL ascorbic acid.

2.10. Determination of Nitrous Oxide Radical Scavenging Activity

Sodium nitroprusside was used to generate Nitric oxide (NO) and then measured using the Griess reagent (naphthylethylenediamine). 50 µL of 10 mM sodium nitroprusside in phosphate buffer saline at pH 7 was incubated with 10 µL of the test fractions at room temperature for 180 min. Then, 100 µL of freshly prepared Griess reagent (1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid in a 1:1 ratio prepared immediately before use) were added, and the absorbance was measured at 546 nm [44]. Control samples contained an equal volume of buffer but without an aliquot of the fractions. For coloured fractions absorbing at 540 nm, the reaction was carried out with no sodium nitroprusside and the absorbance was subtracted. The absorbance was read at 540 nm, and the percentage of nitric oxide inhibition by the fractions was calculated using the following equation:

$$\%NitricOxideScavanging = |Absorbance_{Control}| - \frac{\left|Absorbance_{sample}\right|}{\left|Absorbance_{sample}\right|} \times 100 \quad (3)$$

2.11. Determination of Hydrogen Peroxide Scavenging Activity

This assay is based on the reaction of ferrous ions (Fe^{+2}) with 1,10-phenanthroline. The hydrogen peroxide scavenging assay was performed following the method described by Zhang et al. [45], with slight modifications. To determine the hydroxyl radical scavenging activity, 3.6 µL of 1.0 mM $FeCl_3$, 5.4 µL of 1 mM 1,10-phenanthroline, 100 µL of 0.2 M phosphate buffer (pH 7.8), and 9 µL of 0.17 M H_2O_2, were mixed with 90 µL of the test fraction. The reaction was started by adding H_2O_2. The mix was incubated at room

temperature for 5 min, followed by which the absorbance at 510 nm was measured using a spectrophotometer.

2.12. Cell Culture

Human embryonic kidney, Hek293, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Bremen, Germany) containing 10% foetal bovine serum and 1% penicillin/streptomycin and kept at 37 °C, 5% CO_2 and >95% humidity.

2.13. Cell Viability

Hek293 cells were seeded at 5000 cells/well in a 96-well plate in a complete medium and allowed to adhere for 24 h. The cells were then treated with the different *Perilla* fractions (aqueous, methanol, butanol, ethyl acetate and ether) at 1 or 5 mg/mL. The plates were incubated at 37 °C, 5% CO_2 and >95% humidity for 72 h. After 72 h incubation with the various fractions, the cell proliferation assay was performed by adding 20 µL of 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; CellTiter 96®Aqueous One Solution Cell Proliferation Assay; Promega, Southampton, UK) to each well. Absorbance readings were taken after 1 h incubation using the Mithras LB940 microplate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) at 490 nm.

2.14. Construction of the Human NQO1 ARE-Luciferase Reporter

The human NQO1 ARE (GCAGTCACAGTGACTCAGCAGAATCT) was amplified from HepG2 (liver cancer cell line) cDNA using the primers hNQO1-ARE-Forward: CCT-GAGCTCGCTAGCCTCGACAGGGGTGGTGCAGTGGCAT; hNQO1-ARE-Reverse: CCA-GATCTTGATATCCTCGAGGCTCTGGTGCAGTCCGGGG. The PCR involved 5 min initial denaturation at 95 °C, 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 1 min extension at 72 °C repeated 35 cycles and a final 1 min extension at 72 °C. The pGL4.1 luciferase reporter vector (Promega) was digested using the Xho1 restriction enzyme. The 723 bp PCR product was ligated into the linearised plasmid using the In-Fusion HD Cloning Mix (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) following the manufacturer's instructions. A number of colonies that successfully grew on the ampicillin plates were picked and sequenced in order to ensure that the human NQO1 ARE construct did not present any mutations.

2.15. Transfection

Once the Hek293 cells transferred to the wells reached 80% confluency, they were transfected with 100 ng pGL3 reporter plasmid containing the human NQO1 ARE-luciferase fusion and 5 ng pRL-SV40, using a magnetofection procedure (OZ Biosciences, Marseille, France), with a DNA:NeuroMag ratio of 100 ng:0.1 µL per well. After mixing the DNA and NeuroMag, the complex was incubated at RT for 20 min before being added to the cells and incubated on the magnet for 30 min.

2.16. Dual-Luciferase Reporter Assay

The effect of the different solvent extractions of the *Perilla* leaf extract (0.5 mg/mL) on redox stress was determined by using the human NQO1 ARE-luciferase fusion in a pGL3-Enhancer vector (Promega, Southampton, UK) as the reporter. Following a 24, 48 or 72 h incubation, the Hek293 cells in each well were lysed using 20 µL Passive Lysis Buffer. To 5 µL of lysate, 100 µL of LAR II was added, and firefly luciferase activity was measured. Following that, 100 µL of Stop & Glo Reagent (Promega, Southampton, UK) was added, and Renilla luciferase activity was measured. The firefly luciferase values were normalised using the co-transfected Renilla luciferase for triplicate experiments. The

relative fold changes in the averages for each of the extract fractions acting upon the firefly reporter were then generated and compared with untreated, transfected cells.

2.17. Western Blotting

Hek293 cells treated with 0.5 mg/mL of the various fractions for 24, 48 or 72 h were harvested and lysed using urea lysis buffer (8 M urea, 1.5 M thiourea, 0.5 M NaCl). The Bradford Assay was performed to determine protein concentration by using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) following the manufacturer's instructions and measured on a Bio Photometer Plus UV/Vis spectrophotometer (Eppendorf, Wien, Austria). SDS-PAGE was performed to separate 20 µg of protein using an AE-6450 Dual Mini Slab Electrophoresis Kit (Atto Co., Ltd., Tokyo, Japan) at 20 mA constant current and 300 V for 60 min.

Semi-dry electroblotting was carried out using the Trans-Blot SD Semi-Dry Transfer Cell (BioRad) set at 120 mA and 100 V for 60 min. Following electroblotting, transfer success was evaluated using 0.1% Ponceau S solution, and the membrane was then blocked with 5% skimmed milk in Tris Buffer Saline (TBS) for 1 h. The membrane was then incubated overnight at 4 °C with primary antibodies against HSP27 (NBP2-32972, Novus Bio; 1:1000), HSP70 (NB110-61582, Novus Bio; 1:1000), HSP90 (NB110-61640, Novus Bio; 1:1000), Actin (NB100-74340, Novus Bio; 1:1000), mono-methyl lysine (14679, Cell Signaling Technology: 1:1000) and tri-methyl lysine (14680, Cell Signaling Technology; 1:1000), diluted using a 5% Bovine Serum Albumin (BSA) solution in TBS. The membrane was then incubated with IRDye 800CW goat anti-mouse secondary IgG antibody (926-32210, Li-Cor; 1:5000) or IRDye 800CW goat anti-rabbit secondary IgG antibody (926-32211, Li-Cor; 1:10,000) diluted using 5% BSA in TBS. The bands were visualised using the Odyssey (Li-Cor) imaging system.

3. Results

3.1. Qualitative and Quantitative Assessment of Fractions Using HPLC

HPLC (Figures 1 and 2) identified a total of 35 distinct compounds, of which 18 were quantified for each fraction. Table 1 indicates the regression formula obtained and the regression coefficient for each compound based on triplicate injections and 6-point calibration. The table highlights the limits of detection and limits of quantification for each compound.

Table 1. Quantification of different phenolic compounds identified in the *Perilla* extract.

Chemical Compound	Rt	Formula	R^2	LOD	LOQ
Caffeic Acid	10.81	y = 106920x + 179396	0.9846	0.088	0.29
Syringic	12.36	y = 67460x − 355785	0.9919	0.050	0.17
Vanillin	14.59	y = 108318x + 38533	0.9957	0.054	0.18
p-coumaric acid	16.24	y = 118736x − 174419	0.9997	0.034	0.11
Ferulic acid	18.90	y = 73150x − 84158	0.9999	0.048	0.16
Ellagic Acid	20.62	y = 48178x + 25528	0.9968	0.041	0.14
Rosmarinic Acid	23.81	y = 29517x + 164232	0.9946	0.055	0.18
Trans-Cinnamic	28.55	y = 50153x + 36860	0.9998	0.091	0.30
3′,4′,5,7 tetrahydroxyflavone	29.17	y = 57138x + 68835	0.9954	0.041	0.14
Quercetin	29.51	y = 102718x + 116121	0.9994	0.050	0.17
4′,5,7-trihydroxyisoflavone	31.03	y = 77186x + 43243	0.9998	0.077	0.26
5,7-dihydroxy flavone	37.57	y = 170069x − 714585	0.9833	0.172	0.57
Rutin	38.55	y = 194800x + 49409	0.9999	0.077	0.26

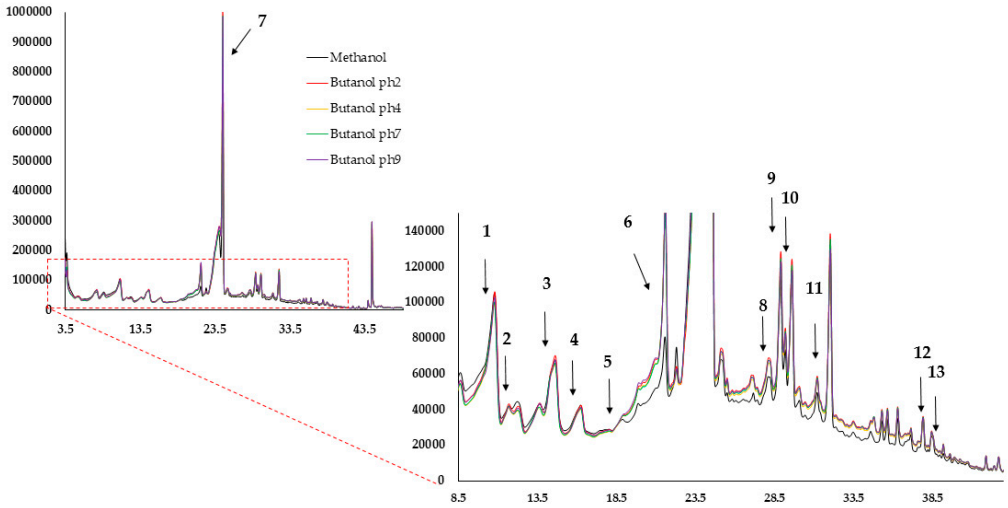

Figure 1. Chromatogram of *Perilla* phenolic extract obtained using methanol, and different hydrolysis conditions observed at 280 nm. Peaks detected at 280 nm are labelled as follows: 1: Caffeic Acid (RT 10.81), 2: Syringic Acid (RT 12.36), 3: Vanillin (RT 14.59), 4: p-Coumaric Acid (RT 16.24), 5: Ferulic Acid (RT 18.90), 6: Ellagic Acid (RT 20.62), 7: Rosmarinic Acid (RT 23.81), 8: Trans-Cinnamic Acid (RT 28.55), 9: 3,4,5,7-Tetrahydroxyflavone (RT 29.17), 10: Quercetin (RT 29.51), 11: 4′,5,7-Trihydroxyisoflavone (RT 31.03), 12: 5,7-Dihydroxyflavone (RT 37.57), 13: Rutin (RT 38.55).

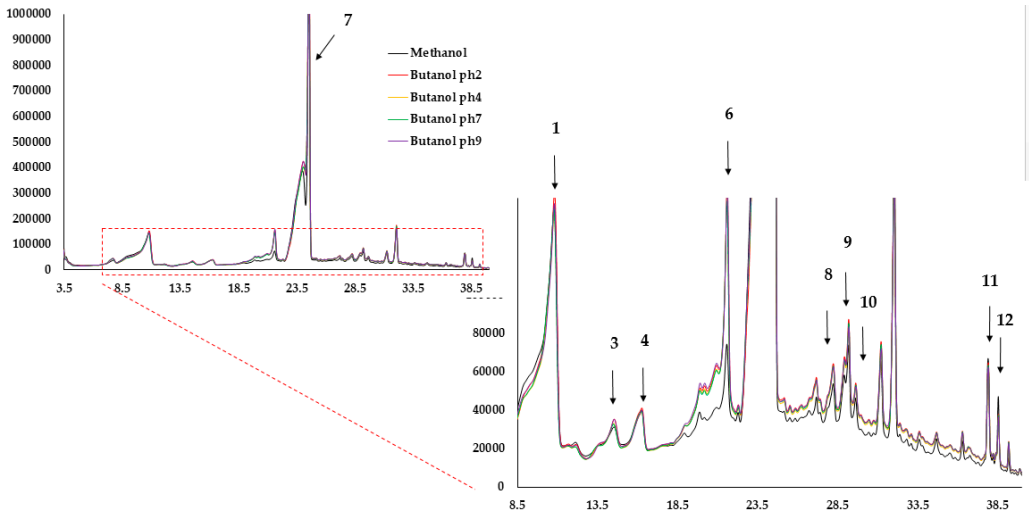

Figure 2. Chromatogram of *Perilla* phenolic extract obtained using methanol, and different hydrolysis conditions observed at 320 nm. Peaks detected at 320 nm are labelled as follows: 1: Caffeic Acid (RT 10.81), 2: Syringic Acid (RT 12.36), 3: Vanillin (RT 14.59), 4: p-Coumaric Acid (RT 16.24), 5: Ferulic Acid (RT 18.90), 6: Ellagic Acid (RT 20.62), 7: Rosmarinic Acid (RT 23.81), 8: Trans-Cinnamic Acid (RT 28.55), 9: 3,4,5,7-Tetrahydroxyflavone (RT 29.17), 10: Quercetin (RT 29.51), 11: 4′,5,7-Trihydroxyisoflavone (RT 31.03), 12: 5,7-Dihydroxyflavone (RT 37.57), 13: Rutin (RT 38.55).

Data comparing the hydrophilic and lipophilic fractions of the *Perilla* extract (Table 2) shows that the 18 polyphenols quantified (out of the 35 identified) are significantly higher in the methanol fraction than in the ether fraction, with rosmarinic acid being by far the major constituent.

The butanol fraction was subjected to 4 different pHs (Table 3). The fractions subjected to acidic pH values had a higher concentration of most phenolic acids, with some acids, including rosmarinic acid, being more abundant at neutral pH, whilst the concentrations of flavonoids and their derivatives were not affected by pH.

Table 2. Chemical comparison of hydrophilic and lipophilic fractions of the *Perilla* extract.

Class	Compound	Methanol Fraction	Ether Fraction
Phenolic acid	Caffeic Acid	39.45 ± 2.61	0.20 ± 0.01
	Syringic Acid	7.13 ± 0.59	ND
	p-coumaric acid	4.87 ± 0.18	0.03 ± 0.00
	Ferulic acid	3.07 ± 0.04	ND
	Ellagic Acid	23.87 ± 0.27	0.33 ± 0.01
	Rosmarinic Acid	786.88 ± 1.51	4.60 ± 0.04
	Trans-Cinnamic Acid	34.02 ± 0.29	0.58 ± 0.17
Phenolic aldehyde	Vanillin	14.85 ± 0.32	0.17 ± 0.00
Flavonoid	3′,4′,5,7-tetrahydroxyflavone	10.20 ± 0.20	0.10 ± 0.04
	Quercetin	7.84 ± 0.06	2.69 ± 4.50
	4′,5,7-trihydroxyisoflavone	8.69 ± 0.08	0.09 ± 0.01
	5,7-dihydroxyflavone	1.09 ± 0.06	1.17 ± 0.02
	4′,5,7-trihydroxyflavone [2]	18.03 ± 0.02	0.26 ± 0.20
	Kaempferol [2]	0.40 ± 0.02	ND
	Pinocembrin [2]	0.56 ± 0.02	0.06 ± 0.00
Flavonoid glycoside	Rutin	1.29 ± 0.03	0.89 ± 0.00
Gallate ester	Methyl 3,4,5-trihydroxybenzoate [1]	8.04 ± 0.49	ND
Amino Acid	Tryptophanol [1]	11.13 ± 0.17	0.02 ± 0.00

[1] Quantified and expressed in terms of caffeic acid equivalent. [2] Quantified and expressed in terms of 5,7-dihydroxyflavone equivalent. ND = not detected.

Table 3. Chemical comparison of butanol fractions of *Perilla* subjected to different hydrolysis conditions.

Compound	Butanol Fraction pH 2	Butanol Fraction pH 4	Butanol Fraction pH 7	Butanol Fraction pH 9
Caffeic acid	38.61 ± 0.39	37.85 ± 0.31	39.70 ± 0.69	38.62 ± 0.00
Syringic acid	5.75 ± 0.01	5.81 ± 0.02	6.30 ± 0.35	6.26 ± 0.02
Vanillin	14.90 ± 0.16	14.65 ± 0.14	15.55 ± 0.28	14.97 ± 0.10
p-coumaric acid	5.23 ± 0.11	5.04 ± 0.07	5.37 ± 0.12	5.03 ± 0.06
Ferulic acid	5.32 ± 0.07	3.92 ± 1.70	1.72 ± 0.11	1.29 ± 0.81
Ellagic acid	20.71 ± 15.92	30.03 ± 6.28	17.47 ± 1.67	19.55 ± 0.07
Rosmarinic acid	905.51 ± 3.21	859.48 ± 51.51	523.96 ± 7.18	482.18 ± 2.17
Trans-Cinnamic acid	40.32 ± 1.33	33.29 ± 10. 47	46.59 ± 0.49	26.46 ± 0.12
3,4,5,7-tetrahydroxyflavone	12.89 ± 0.59	10.35 ± 3.65	14.90 ± 0.05	7.64 ± 0.02
Quercetin	8.51 ± 0.35	7.12 ± 1.97	9.64 ± 0.03	5.80 ± 0.03
4′,5,7-trihydroxyisoflavone	14.57 ± 1.96	9.83 ± 7.98	19.01 ± 0.12	4.09 ± 0.07
5,7di hydroxy flavone	1.01 ± 0.01	0.98 ± 0.04	1.06 ± 0.01	1.00 ± 0.01
Rutin	1.07 ± 0.00	1.06 ± 0.01	1.09 ± 0.01	1.06 ± 0.11
Methyl 3,4,5-trihydroxybenzoate [1]	8.29 ± 0.01	8.14 ± 0.00	8.35 ± 0.18	8.32 ± 0.01
Tryptophanol [1]	9.36 ± 1.03	7.31 ± 3.30	1.24 ± 0.59	1.06 ± 0.13
4′,5,7-trihydroxyflavone [2]	20.66 ± 1.16	18.20 ± 3.88	23.39 ± 0.15	15.54 ± 0.09
Kaempferol [2]	1.98 ± 0.66	1.27 ± 1.63	3.18 ± 0.05	0.12 ± 0.00
Pinocembrin [2]	0.48 ± 0.00	0.47 ± 0.00	0.49 ± 0.00	0.47 ± 0.01

[1] Quantified and expressed in terms of caffeic acid equivalent. [2] Quantified and expressed in terms of 5,7-dihydroxyflavone equivalent.

3.2. Determination of Total Phenolic, Flavonoid and Ortho-Diphenolic Content

These data (Table 4) showed that the highest phenolic, flavonoid and ortho-diphenolic content was present in the polar solvent fractions using methanol and butanol. More specifically, the methanol fractions presented the highest phenolic content compared with all the butanol fractions at different pHs. Total flavonoid content was actually the highest for the butanol fraction at pH 7. There was no observable difference between the flavonoid content in the methanol fractions and the butanol fractions at pH 2 and pH 4, whilst the butanol fraction at pH 9 presented the lowest flavonoid content. Similarly, for the ortho-diphenolic content, the butanol fraction at pH 7 presented the highest value, with no observable difference between the ortho-diphenolic content in the methanol fractions and

the butanol fractions at pH 2 and pH 4, whilst the butanol fraction at pH 9 presented the lowest ortho-diphenolic content. On the other hand, the extraction of phenolic, flavonoid and ortho-diphenolic compounds was extremely inefficient using either ethyl acetate or ether.

Table 4. Total phenolic content (TPC) expressed as mg/L of caffeic acid equivalents (CAE), total flavonoid content (TFC) expressed as mg/L of catechin equivalents (CatE), and total ortho-diphenolic content (TdPC) expressed as mg/L of pyrocatechol equivalents (PyE).

	TPC (mg/L CAE)	TFC (mg/L CatE)	TdPC (mg/L PyE)
Methanol Fraction Replicate 1	1705.43 ± 144.9	1199.43 ±26.5	1401.95 ± 33.2
Methanol Fraction Replicate 2	1865.75 ± 266.7	1240.86 ± 13.8	1580.15 ± 76.1
Ethyl Acetate Fraction Replicate 1	321.83 ± 37.1	208.00 ± 5.2	247.33 ± 9.9
Ethyl Acetate Fraction Replicate 2	371.44 ± 6.1	304.19 ± 14.5	340.41 ± 4.9
Ether Fraction Replicate 1	46.36 ± 8.7	77.52 ± 6.4	6.31 ± 5.4
Ether Fraction Replicate 2	55.96 ± 0.5	87.05 ± 22.4	17.85 ± 2.0
Butanol Fraction pH 2	1267.33 ± 224.3	1253.24 ± 15.9	1496.05 ± 134.9
Butanol Fraction pH 4	1302.89 ± 42.6	1278.48 ± 52.5	1525.15 ± 45.6
Butanol Fraction pH 7	1472.10 ± 94.2	1414.67 ± 38.3	1747.85 ± 63.7
Butanol Fraction pH 9	1289.27 ± 61.9	987.52 ± 51.0	1356.82 ± 106.4

3.3. Determination of the DPPH Radical Scavenging Activity and ABTS Radical Cation Stabilization

These data (Table 5 and Figure 3) showed that the highest percentage inhibition of DPPH radicals was for the methanol fraction, whilst the lowest by far was for the ether fraction. The butanol fractions all clustered together but at double the fraction content. Similarly, these data (Table 5 and Figure 4) showed the highest percentage inhibition of ABTS radicals or the methanol fraction and the lowest for the ether fraction, with a relatively close clustering of the methanol and butanol fractions.

Table 5. Calculated effective concentration of different fractions against DPPH and ABTS radicals.

	EC_{50} DPPH	EC_{50} $ABTS^{\bullet+}$
Methanol fraction replicate 1	2.98 ± 0.0	0.75 ± 0.0
Ethyl Acetate fraction replicate 1	>10	3.24 ± 0.2
Ether fraction replicate 1	>10	>10
Butanol fraction pH2	4.23 ± 0.1	1.08 ± 0.1
Butanol fraction pH4	4.28 ± 0.1	1.19 ± 0.1
Butanol fraction pH7	4.22 ± 0.1	0.86 ± 0.1
Butanol fraction pH9	4.25 ± 0.8	1.27 ± 0.2

3.4. Determination of Cupric and Ferric Reducing Antioxidant Power

These data (Table 6) showed that the highest cupric and ferric-reducing antioxidant power was present in the polar solvent fractions using methanol and butanol. More specifically, the methanol fractions presented the highest cupric and ferric-reducing antioxidant power compared with all the butanol fractions at different pHs. Of all the butanol fractions, the fraction at pH 7 presented slightly higher cupric reducing antioxidant power, while the fractions at pH 7 and pH 9 presented higher ferric reducing antioxidant power. The butanol fractions at pH 2 and pH 4 presented comparable cupric but low ferric reducing antioxidant power, particularly pH 4. On the other hand, the cupric and ferric-reducing antioxidant power was extremely low for both the ethyl acetate and ether fractions.

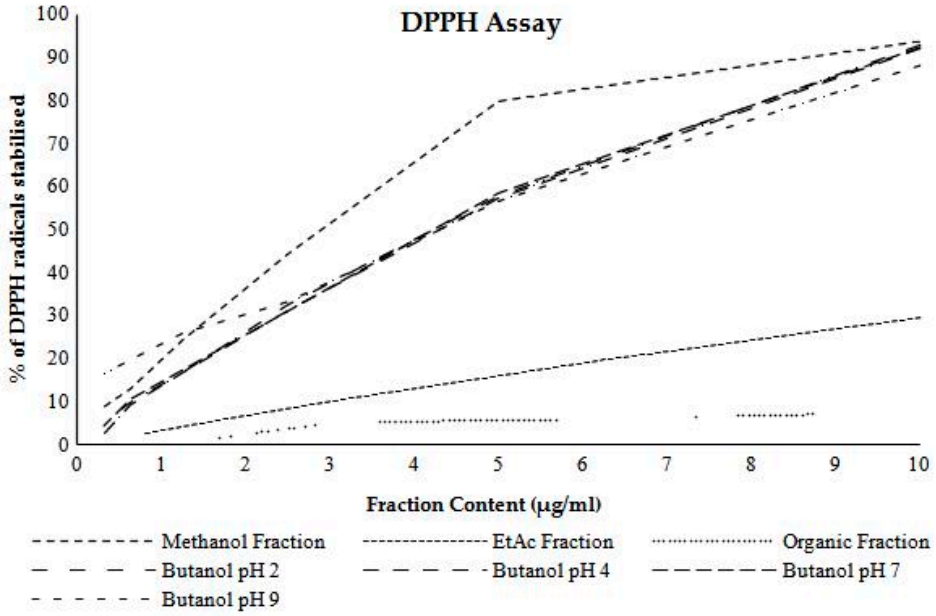

Figure 3. Dose–response curve of various fractions and the % inhibition of DPPH radicals. The solid black line represents the effective concentration at which 50% of the DPPH radicals are stabilised.

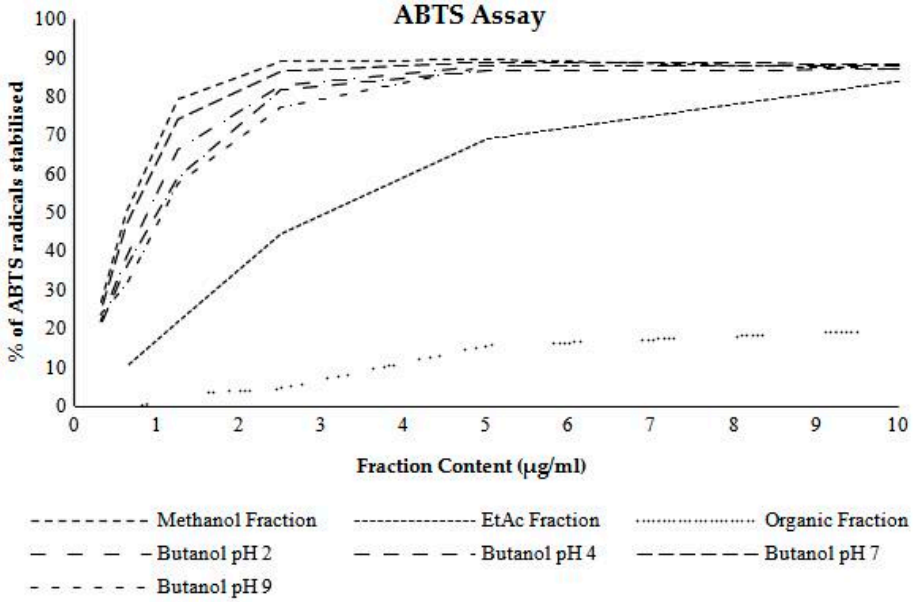

Figure 4. Dose–response curve of various fractions and the % inhibition of ABTS radical cations. The solid black line represents the effective concentration at which 50% of the ABTS radical cations are stabilised.

Table 6. Metal cation Cu^{2+} and Fe^{3+} reduction activity of the various fractions using different solvents expressed as mg/L of caffeic acid equivalents for CUPRAC and mg/L of ascorbic acid equivalents for FRAP.

	CUPRAC (mg/L CAE)	FRAP (mg/L AcE)
Methanol fraction replicate 1	557.96 ± 59.4	1892.22 ± 117.1
Methanol fraction replicate 2	514.44 ± 49.4	2147.78 ± 19.2
Ethyl Acetate fraction replicate 1	109.63 ± 15.2	747.78 ± 96.2
Ethyl Acetate fraction replicate 2	193.15 ± 7.3	847.78 ± 50.9

Table 6. *Cont.*

	CUPRAC (mg/L CAE)	FRAP (mg/L AcE)
Organic fraction replicate 1	54.63 ± 6.1	292.22 ± 69.4
Organic fraction replicate 2	77.96 ± 4.5	281.11 ± 38.5
Butanol fraction pH 2	438.33 ± 24.6	814.44 ± 245.7
Butanol fraction pH 4	475.19 ± 60.0	381.11 ± 279.5
Butanol fraction pH 7	512.59 ± 18.1	1770.00 ± 100.0
Butanol fraction pH 9	487.96 ± 54.8	1792.22 ± 77.0

3.5. Determination of Nitrous Oxide Radical and Hydrogen Peroxide Scavenging Activity

These data (Table 7) showed that the highest nitrous oxide radical and hydrogen peroxide scavenging activity was present in the polar solvent fractions using methanol and butanol. More specifically, the butanol fraction at pH 9 presented the highest nitrous oxide radical scavenging activity, with all other butanol fractions, as well as the methanol fractions being comparable in their nitrous oxide radical scavenging activity. Hydrogen peroxide scavenging activity was highest for the methanol fractions. The ethyl acetate and ether fractions showed comparable nitrous oxide radical scavenging activity to the polar solvent fractions; however, they showed poor hydrogen peroxide scavenging activity.

Table 7. The % of Nitric oxide and hydroxyl radicals scavenging activity of the various fractions using different solvents.

	% NOS	% OH
Methanol fraction replicate 1	76.18 ± 5.1	72.95 ± 1.5
Methanol fraction replicate 2	71.18 ± 3.2	67.32 ± 2.0
Ethyl Acetate fraction replicate 1	75.00 ± 0.9	27.83 ± 1.0
Ethyl Acetate fraction replicate 2	72.65 ± 1.2	34.23 ± 2.1
Organic fraction replicate 1	59.47 ± 0.3	12.87 ± 3.7
Organic fraction replicate 2	58.74 ± 0.5	12.33 ± 0.4
Butanol fraction pH 2	80.19 ± 0.0	55.00 ± 0.9
Butanol fraction pH 4	78.62 ± 1.0	56.73 ± 0.5
Butanol fraction pH 7	71.91 ± 12.1	56.79 ± 1.7
Butanol fraction pH 9	84.80 ± 0.7	50.75 ± 0.6

3.6. Cell Viability

These microscopy data (Figure 5), as well as the cell viability assay data (Figure 6) performed in parallel, showed that the various fractions of the *Perilla* extract presented no toxic effects on the cells at 1 mg/mL but had varying levels of cytotoxicity at 5 mg/mL.

3.7. Dual-Luciferase Reporter Assay

Dual-luciferase assay data (Figure 7) showed that the most effective components for the induction of Nrf2 found in the crude extract are extracted by methanol, as evident by the high activity of the methanol fraction, only second to that of the crude extract. Butanol seems to be less effective in extracting these components as the observed effect is somewhat lower compared with the methanol fraction. Ethyl ethanoate also presents a similarly low activity, which can be considered to be practically negligible. Interestingly, the aqueous leftover fraction initially presents a detrimental effect, which is reduced over time.

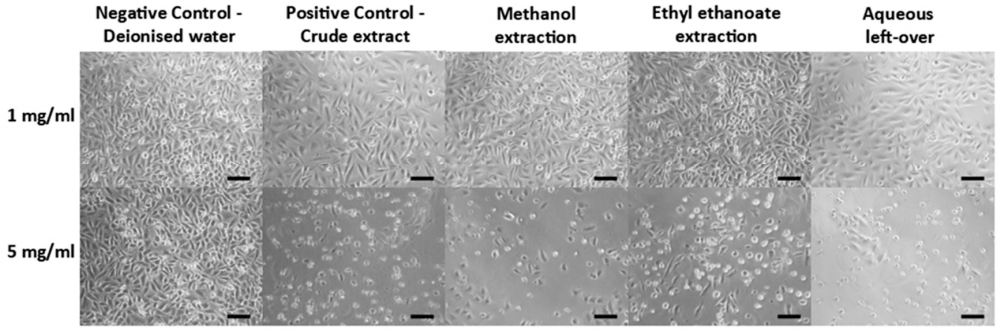

Figure 5. Cell imaging following incubation with the different *Perilla* fractions (Mag ×100; scale bar 50 μm).

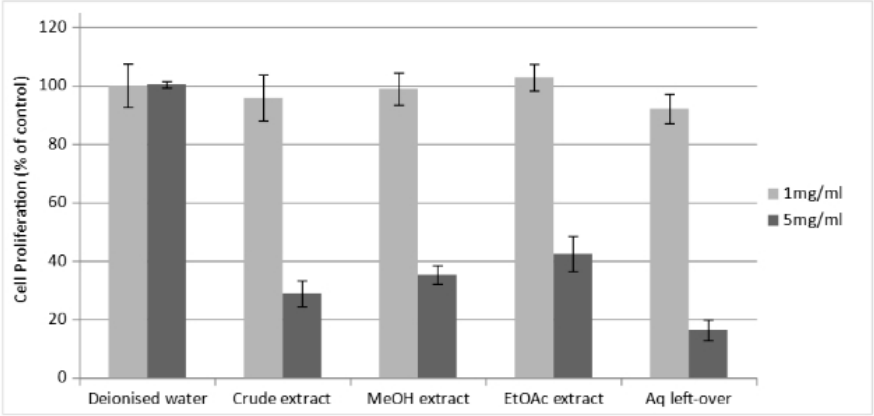

Figure 6. Cell viability assay following incubation with the different *Perilla* fractions.

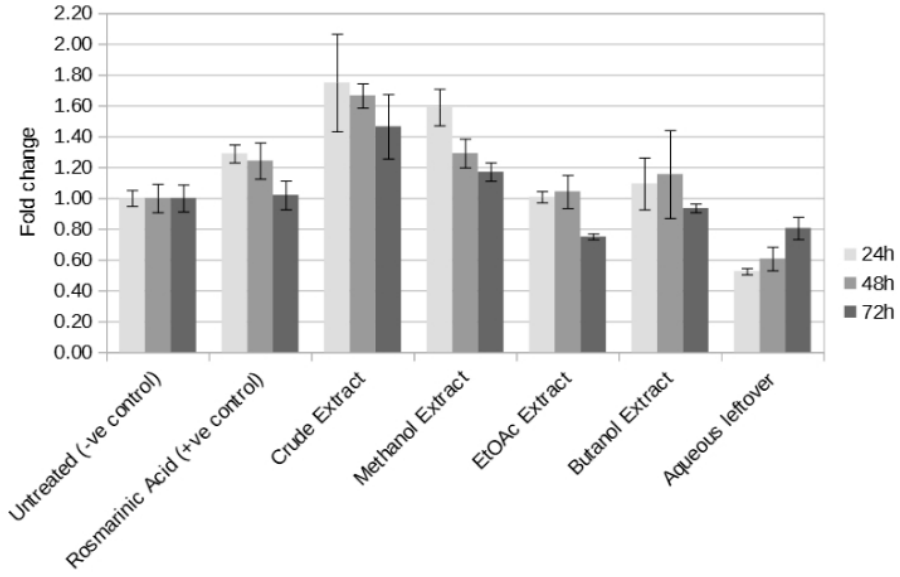

Figure 7. Dual-luciferase assay for the different *Perilla* fractions using the human NQO1 ARE, performed at 24, 48, and 72 h.

3.8. Western Blotting

Western blotting data for HSP70 and HSP27 (Figure 8) show little variation between the different conditions over the 72 h period of testing. Interestingly, the blots for mono-methyl and tri-methyl lysine (Figure 9) show that different doses of the crude *Perilla* extract bring about changes in the band intensities of methylated proteins throughout the 10–250 kDa range, although the band intensity is not always apparent because of the low abundances of such modifications.

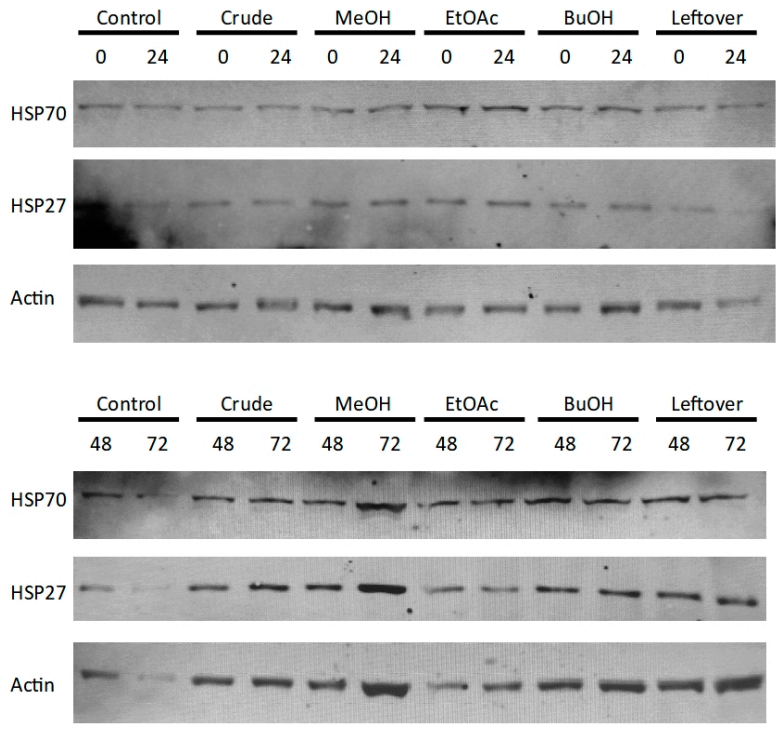

Figure 8. Western blots for HSP70, HSP27 and Actin (as loading control) at 0, 24, 48 and 72 h for the various *Perilla* extract fractions, where: Control = untreated, Crude = whole extract, MeOH = methanol fraction, EtOAc = ethyl-ethanoate fraction, BuOH = n-butanol fraction, Leftover = water-soluble remnant.

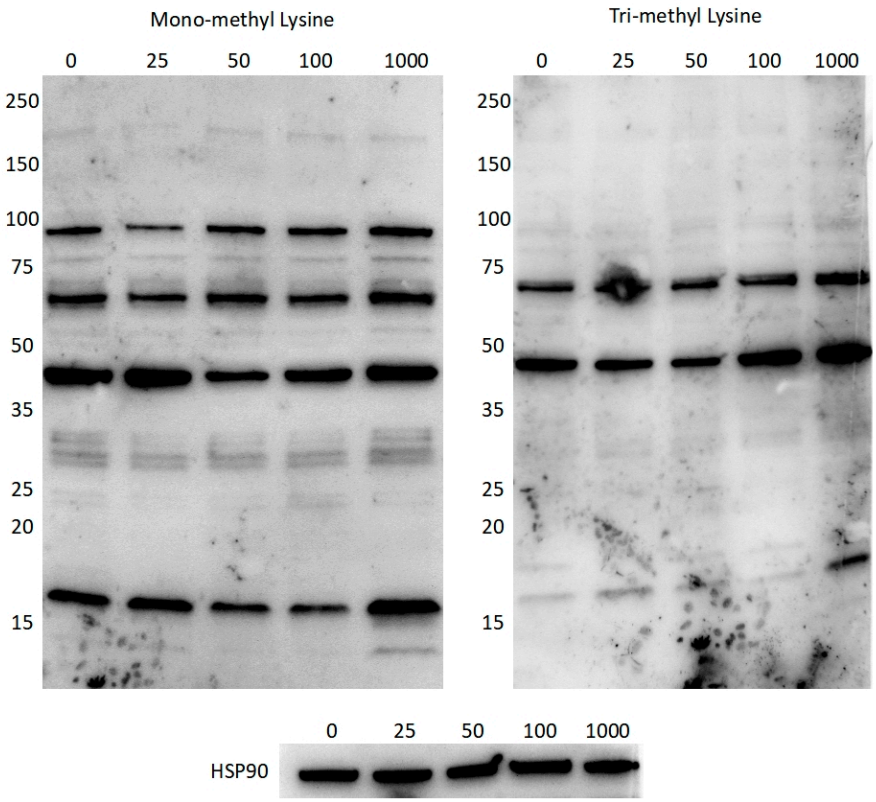

Figure 9. Western blotting for mono-methyl lysine, tri-methyl lysine and HSP90 (as loading control) after 24 h exposure to increasing concentrations (25, 50, 100 and 1000 μg/mL) of the crude *Perilla* extract.

4. Discussion

The identity of the main polyphenolic, flavonoid, and ortho-diphenolic components in the *Perilla* leaf extract and their bioactive functions were investigated so as to obtain a better understanding of the dose, action time, and molecular targets for therapeutic applications. This study presents several novel findings and insights into the antioxidant and cytoprotective potential of *Perilla* leaf extract fractions. First was the identification of 35 polyphenolic compounds and quantification of 18 of these compounds through HPLC analysis, out of which Rosmarinic acid was found to be 20 times more abundant than other polyphenolic components. Whilst methanol and n-butanol have been known to be the most effective solvents for extracting phenolics and flavonoids, investigating n-butanol extraction at different pHs identified pH-specific variations (flavonoid and ortho-diphenolic extractions were found to be most effective at neutral pH). Then, using a human NQO1 ARE reporter assay, variations in antioxidant and cytoprotective effects between fractions were demonstrated (with the crude extract having the highest Nrf2 induction capability, whilst the ethyl acetate and aqueous fractions showed distinct patterns). Another novel aspect of the study was the Western blot analysis of changes in mono-methyl and tri-methyl lysine protein band intensities following treatment with Perilla extract fractions, suggesting potential modulation of post-translational modifications.

To maximise the polyphenolic extraction from the crude *Perilla* leaf extract, this was fractionated using solvents of varying polarity, namely methanol, n-butanol, ethyl ethanoate and ether. The highest phenolic, flavonoid and ortho-diphenolic content were obtained using the polar solvents methanol and n-butanol, with very low recoveries obtained using either ethyl acetate or ether (Table 4). Considering different pHs of n-butanol, phenolic extraction was not pH dependent, whilst flavonoid and ortho-diphenolic extraction were best at neutral pH (pH 7). Acidic pHs (pH 2 and pH 4) presented only slightly lower extraction efficiency, whilst the n-butanol fraction at alkaline pH (pH 9) presented the lowest flavonoid and ortho-diphenolic recovery. Upon performing HPLC analysis on the different solvent fractions, a total of 35 distinct polyphenolic compounds were identified (Figures 1 and 2), and 18 of these were further quantified (Table 1). The comparative quantification of the polyphenolic composition of the hydrophilic (methanol) and lipophilic (ether) fractions identified rosmarinic acid as being the major constituent, 20-fold more abundant than any other component (Table 2). The n-butanol fraction subjected to acidic pH values had a higher concentration of most of the quantified phenolic acids, with some acids, including rosmarinic acid having a higher abundance in the neutral pH fraction. On the other hand, flavonoids and their derivatives showed similar concentrations at all pHs (Table 3).

Other studies that investigated the composition of *Perilla* extract. For example, Ueda et al. [46] identified caffeic acid, rosmarinic acid and 3′,4′,5,7-tetrahydroxyflavone (luteolin) as the main constituents of *Perilla* extract. Izumi et al. [34] identified 2′,3′-dihydroxy-4′,6′-dimethoxychalcone from an ether extract. Kwon et al. [33] identified protocatechuic acid, chlorogenic acid, caffeic acid, 4-methyoxycinnamic acid, oleanolic acid, kaempferol-3-O-rutinoside, rosmarinic acid, luteolin, methyl-rosmarinic acid, apigenin and 4′,5,7-trimethoxyflavone but according to them the EtOAc fraction had the highest activity. Tantipaiboonwong et al. [9] identified rosmarinic acid, chlorogenic acid, caffeic acid, ferulic acid and luteolin as the major constituents, with rosmarinic acid being the predominant compound. Adam et al. [36] identified 18 compounds, with the major components being caffeic acid, syringic acid, cinnamic acid, rosmarinic acid, quercitin, kaemferol, and pinostrobin. The concentrations extracted vary greatly between the studies, mainly because of the different procedures and solvents employed.

Rosmarinic acid has been known to be present in *Perilla* for 30 years [47] and was later proven to be one of the main polyphenolic constituents found in *P. frutescens* leaf extracts [48]. It has also been identified in numerous other medicinal plant species of the Lamiaceae family, including basil, sage, rosemary, and mint [49,50]. Rosmarinic acid is produced from the aromatic amino acids l-phenylalanine and l-tyrosine through the intermediary precursors 4-coumaroyl-CoA and 4-hydroxyphenyllactic acid, respectively, and formed as an ester of caffeic acid and 3,4-dihydroxyphenyllactic [51]. It is well known to suppress inflammation reactions and protect against redox stress [52–60].

Of note is that the yields of the various polyphenolics extracted from *Perilla frutescens* are influenced by several factors. The primary factor is the existence of multiple cultivars of *Perilla frutescens* [36,61]. Additionally, geographical location [62] and growing conditions, including soil type, play a significant role. Consequently the antioxidant effect exhibited is greatly impacted by both the solvent used and the process used for the extraction. This variability arises from the compatibility between the chemical characteristics of the polyphenolic compounds and the polarity of the solvent used, which determines how much of each compound is recovered by a specific solvent [63]. The two key factors related to solvent capacity are the polarity of the solvent used and the solubility of specific polyphenols in the selected solvent. More polar solvents (such as methanol or ethanol) are very efficient at extracting flavonoids and phenolic acids, even better than water due to their ability to solubilise a broader range of polyphenolic structures, resulting in an overall increased yield and diversity in the extract produced [64,65]. On the other hand, the solubility of each individual polyphenolic compound also impacts its extraction efficiency, and in this respect, extraction pH and temperature play a key role, leading to variations in the final concentrations present in extracts produced using different solvents [65,66]. Consequently, the presence of different concentrations of these polyphenolic compounds contributes to the difference in antioxidant capacity or other biological activity properties of the extracts produced.

Particularly with respect to acid hydrolysis, it facilitates the release of polyphenolic compounds by breaking down structural components, including cell walls, that may hinder extraction, thus enhancing extraction yield [67]. The bioactivity of extracted polyphenols, such as their antioxidant capacity, in some cases may be improved by acid hydrolysis, as it increases the availability of active polyphenolic compounds [68]. Furthermore, polyphenols obtained through acid hydrolysis often exhibit greater stability against oxidation, which is essential for preserving their health benefits during storage and use [69]. In this study, upon comparing the chemical composition of acidic (pH 2 and pH 4) versus neutral (pH 7) or alkaline (pH 9) butanol fractions of Perilla (Table 3), the concentration was only higher under acidic conditions for ferulic acid, ellagic acid, rosmarinic acid and tryptophanol. The other quantified polyphenols either did not show a difference in pH or a decrease under acidic conditions.

Further to this, there has been only limited investigation of the extent of antioxidant activity by these compounds as distributed following various solvent extractions from the same starting material. Moreover, when this has been investigated it was performed in very different ways, making comparison difficult. The closest extraction procedure to the one used in this study was that of Hong et al. [55], which reported much lower total phenolic and flavonoid content than the current study.

The biological activity of these components has been generally quantified through the determination of the metal cation reduction activity and their scavenging power. Adam et al. [36] determined that the ethanolic extracts had the highest hydroxyl radical scavenging capacity and superoxide anion radical neutralisation potential, similar to the methanolic fraction of the current study; however, Hong et al. [55] reported that the DPPH radical

scavenging ability was similar between ethyl acetate and n-butanol fraction, while in this study the DPPH radical scavenging ability for the ethyl acetate fraction was found to be lower than that of the n-butanol fraction. Furthermore, Hong et al. [55] reported that the reducing power (determined by the potassium ferricyanide reduction method) was higher for the ethyl acetate fraction than the butanol fraction; however, in the current study, the reducing power of the butanol fraction was twice as high as the ethyl acetate fraction. Yet different conclusions were obtained by Kwon et al. [33] that reported the aqueous fraction had the highest hydroxyl radical scavenging activity, while the ethylethanoate fraction had the highest hydrogen peroxide scavenging activity, nitric oxide scavenging activity, ferric reducing/antioxidant power, ABTS scavenging activity and DPPH scavenging activity, whilst n-butanol had the highest ferrous ion chelating activity of the various fractions prepared.

To quantify the biological significance of the *Perilla* leaf extract fractions, Nrf2 induction was used as an indicator. First, the cytotoxicity of the various fractions of the *Perilla* extract on Hek293 cells was determined to be negligible up to 1 mg/mL but presented varying levels of cytotoxicity at 5 mg/mL (Figure 5). This is in line with a number of other studies that described cytotoxicity at concentrations within the same range. Similar to the current study, Kim et al. [70] found no toxic effects of the aqueous extract on rat hepatocytes up to a concentration of 1 mg/mL. Adam et al. [36] reported cytotoxicity from 0.5 mg/mL on Mg-63 (osteosarcoma) cells and A431 (squamous cell carcinoma) cells.

In this study, Nrf2 induction was highest with the crude extract, followed by the methanol fraction. Butanol and ethyl ethanoate were less effective at extracting the active components (Figure 7). Interestingly, the induction effect seems to be time-dependent, with most fractions showing the highest activation within the first 24 h, followed by a decline. Conversely, the aqueous leftover fraction initially exhibited a cytotoxic or inhibitory effect, which diminished by the 72 h mark. Similarly, Adam et al. [36] demonstrated that different extracts exerted their cytotoxic effects at varying time points over the 72 h experimental period.

The cytoprotective and antioxidant nature of *Perilla* extract has been shown to be mediated through the inhibition of enzymes, including cytochrome P450 isoforms, lipoxygenases, cyclooxygenase, and xanthine oxidase involved in radical generation [71]. This inhibition is achieved by precipitation or the formation of various complexes that hinder enzyme activity [72]. It has been shown that both *Perilla* extracts and rosmarinic acid reduce liver injury induced by d-galactosamine and lipopolysaccharide (LPS) in mice by scavenging superoxide radicals produced by Kupffer cells as well as inhibiting peroxynitrite formation induced by inducible nitric oxide synthase (iNOS) [73]. The anti-inflammatory action of *Perilla* extract is achieved through the inhibition of pro-inflammatory cytokine secretion, including interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α) [10,74]. Furthermore, it downregulates inflammatory pathways involving inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [8–10]. With respect to its protection against oxidative stress, *Perilla* extract upregulates antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [9,75]. It reduces reactive oxygen species (ROS) levels, including H_2O_2 and lipid peroxidation markers such as malondialdehyde (MDA) [9,75]. By modulating inflammation and oxidative stress pathways, particularly through the activation of Nrf2 [9,34,75], *Perilla* extract significantly improves overall redox balance. Kwon et al. [33] described the major antioxidant constituents of ethanolic *Perilla* extracts as being protocatechuic acid, chlorogenic acid, caffeic acid, rosmarinic acid, luteolin, methyl-rosmarinic acid, apigenin and 4′,5,7-trimethoxyflavone.

Western blotting data for both HSP70 and HSP27 (Figure 8) did not show any markable difference in expression of the 72 h of incubation with the extract fractions. This could have been due to the absence of a redox insult or the low-dose induction produced by

the single dose used. That being said, both mono-methyl and tri-methyl lysine (Figure 9) presented changes in the band intensities of methylated proteins throughout the 10–250 kDa range upon incubation with different doses of the crude *Perilla* extract. Low abundances hinder quantification and the large number of overlapping proteins makes identification of the modified proteins impossible. That being said, using shotgun mass spectrometry or Western blotting for specific targets could make the identification of some of these proteins possible, which opens up a whole new avenue for antioxidant research.

Since inflammation is closely associated with elevated levels of reactive species [76], further work could potentially focus on investigating the effect of *Perilla* extract fractions on controlling oxidative stress and redox-related post-translational modifications such as phosphorylation and methylation on inflammatory mediators. The evaluation of which components in the Perilla extract best reduce the expression and activity of iNOS and COX-2 would be mechanistically relevant, given their roles in inflammation and reactive oxygen species production. Another research avenue would be to assess the level of suppression of the production of inflammatory cytokines (TNF-a, IL-1b, and IL-6) using reporter assays or Western blotting, which would quantify the clinical benefit. Furthermore, investigating the effects of *Perilla* extract biomolecules on the activity of NF-κB activation and nuclear translocation together with the inhibition of upstream kinases (e.g., the NF-κB signalling activator IKKβ), regulation of AMP-activated protein kinase (AMPK) phosphorylation status and its downstream targets (e.g., acetyl-CoA carboxylase), and HIF-1α expression could provide mechanistic insight into the processes involved in controlling inflammation by polyphenolics. Such in vitro findings would need to be validated in animal models of inflammatory conditions such as arthritis, colitis, or sepsis, monitoring inflammatory proteins, oxidative stress markers, and histopathological changes.

5. Conclusions

This study has shown that *Perilla* extract contains a large variety of polyphenolic, flavonoid and ortho-diphenolic components, which contribute to its potent antioxidant properties. Using four solvents of varying polarities (methanol, ethanol, acetone, and water), it was possible to fractionate these bioactive components effectively, revealing significant differences in extraction yields. Notably, methanol and butanol proved to be the most efficient solvents, yielding higher concentrations of total polyphenols and flavonoids compared with ethyl acetate and ether.

The antioxidant capacity of each solvent-extracted fraction was confirmed both through chemical analysis (such as DPPH and ABTS radical scavenging tests) as well as through a biological assay involving the induction of the human NQO1 ARE luciferase reporter. The results showed that methanol-extracted fractions exhibited the strongest antioxidant activity. The polarity of the extraction solvent played a crucial role in determining the specific profile and efficacy of the extracted antioxidants.

These findings provide valuable insight into the chemical diversity and biological activity of Perilla extract, offering a strong basis for further investigation of the proteins and molecular pathways influenced by these components. Additionally, a better understanding of the combined effect of multiple polyphenolics could pave the way for future studies focusing on potential therapeutic applications and the synergistic interactions within the *Perilla* extract.

Author Contributions: Conceptualization, F.L. and B.B.; methodology, F.L. and B.B.; validation, F.L. and B.B.; formal analysis, F.L. and B.B.; investigation, F.L. and B.B.; resources, F.L. and B.B.; writing—original draft preparation, B.B.; writing—review and editing, F.L.; visualization, F.L. and B.B.; project administration, B.B. All authors have read and agreed to the published version of the manuscript.

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