

METHODS OF PREPARATION OF ZINGERONE, COMPOSITIONS COMPRISING ZINGERONE, AND USES THEREFOR

RELATED APPLICATION

[0001] This application claims the benefit of United States patent application number 63/446,013 filed on 15 February 2023, the entire contents of which are incorporated herein by reference.

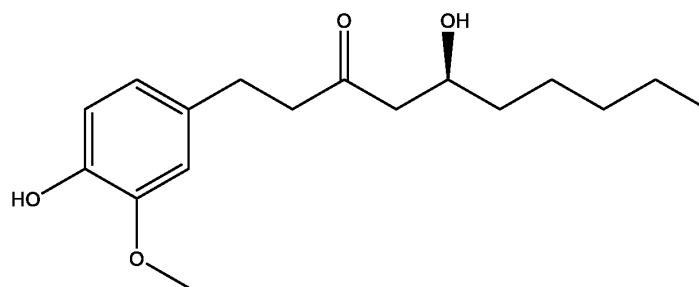
FIELD

[0002] The present disclosure relates to methods for preparing zingerone and compositions comprising zingerone. Specifically noted are beneficial compositions, including pharmaceutical compositions and dietary compositions, and uses for these compositions.

BACKGROUND

[0003] Ginger (*Zingiber officinale*) is a flowering plant whose rhizome is widely used as a spice and in traditional medicine. If consumed in reasonable quantities, ginger has few negative side effects. It is on the FDA's "generally recognized as safe" list.

[0004] The characteristic fragrance and flavour of ginger result from volatile oils that compose 1-3% of the weight of fresh ginger, primarily consisting of zingerone, shogaols, and gingerols with [6]-gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone)



6-gingerol

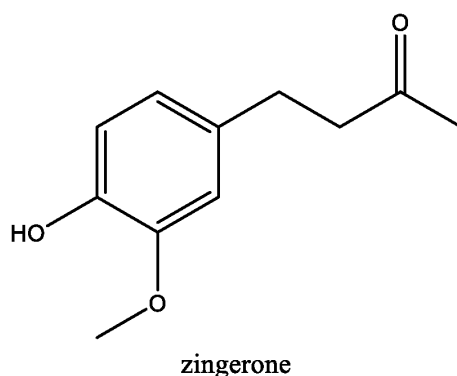
as the major pungent compound.

[0005] Zingerone (also known as gingerone) has been reported as being produced from gingerols during drying or heat treatment at temperatures of about 40

degrees Celsius as reported by Li *et. al.*, 2016, “Chemical Characterisation and antioxidant activities comparison in fresh, dried, stir frying and carbonized ginger”

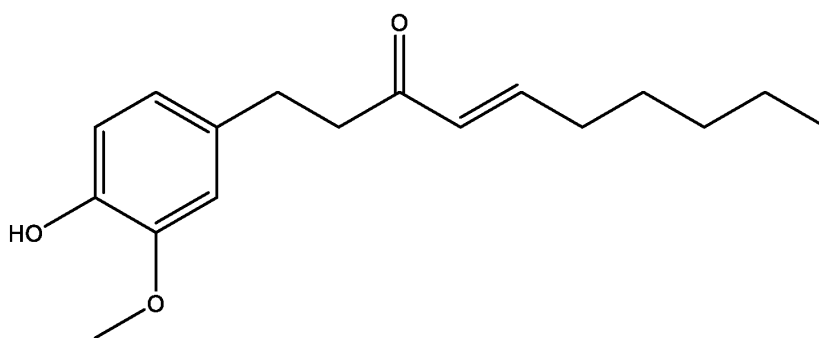
Journal of Chromatography B Analyt. Technol. Biomed. Life Sci. 1011: 223-232.

Zingerone has a lower pungency and a spicy-sweet aroma. Zingerone is also called vanillylacetone and is a crystalline solid that is reported to be sparingly soluble in water and soluble in ether. Zingerone’s water solubility value of 0.57 g/L and LogP value of 2.02 1.92 and logS of -2.5 is described on the FoodB compounds database; see <https://foodb.ca/compounds/FDB010527>.



[0006] Fresh ginger contains minimal zingerone, and it is known to be produced by cooking or drying of the ginger root, which causes dehydration of gingerol through the loss of a water molecule to produce zingerone and hexanal. See, e.g., Gopi *et al.* 2016, “Study on temperature dependent conversion of active components of ginger” *Int. J. of Pharma Sciences* 6(1): 1344-1347.

[0007] Shogaols are more pungent and have higher antioxidant activity and are not found in raw ginger, but are formed from gingerols during heating, storage or via acidity.



shogaol

Shogaol is a dehydrated form of gingerol.

[0008] Zingerone was first isolated from the ginger root in 1917 by Hiroshi Nomura. Nomura identified and later patented (US 1,263,796, issued April 23, 1918) a method for the synthesis of zingerone, in which vanillin and acetone are reacted under basic conditions to form dehydrozingerone. This compound was obtained in about 95% quantity. This reaction was followed by catalytic hydrogenation of the intermediate compound in order to form zingerone, obtained in approximately 100% quantity.

[0009] Ginger compounds have been shown to be active against enterotoxigenic *Escherichia coli* heat-labile enterotoxin-induced diarrhoea. This type of diarrhoea is the leading cause of infant death in developing countries. It has been reported that Zingerone is likely the active constituent responsible for the antidiarrheal efficacy of ginger. The study concluded that ginger's bioactive compounds significantly blocked the binding of enterotoxigenic *Escherichia coli* heat-labile enterotoxin to cell-surface receptor G M1, resulting in the inhibition of fluid accumulation in the closed ileal loops of mice. See, e.g., Chen *et al.*, 2007, "Ginger and its bioactive component inhibit enterotoxigenic *Escherichia coli* heat-labile enterotoxin-induced diarrhoea in mice" *Journal of Agricultural and Food Chemistry* 55 (21): 8390-7.

[0010] Zingerone has been shown to have an anti-inflammatory effect on liver inflammation in a peritonitis mouse model as reported by Kumar *et al.* See Kumar *et al.*, "Zingerone suppresses liver inflammation induced by antibiotic mediated endotoxemia through down regulating hepatic mRNA expression of inflammatory markers in *Pseudomonas aeruginosa* peritonitis mouse model" *PLOS ONE* 9(9): e106536.

[0011] Kumar *et al.* have also reported that zingerone can enhance the susceptibility of *Pseudomonas aeruginosa* cells to antibiotics. See Kumar *et al.*, 2014, *Life*

Sciences 117: 24-32. Kumar *et al.* concluded that zingerone was found to cause alterations in the cell surface properties of *Pseudomonas aeruginosa* thereby increasing the susceptibility of *Pseudomonas aeruginosa* cells to antibiotics.

[0012] Limited research has been performed on optimising the production of zingerone from natural sources. Given the current emphasis on compositions derived from natural sources, there is a need for new compositions, including plant-based compositions, and particularly those with immune-modulating and anti-inflammatory activity. The present application aims to meet these and other needs.

SUMMARY

[0013] In one aspect, this disclosure encompasses a method of producing zingerone by: (i) subjecting ginger root to an alkaline treatment in alkaline solution; (ii) subjecting juice obtained from ginger root to an alkaline treatment in an alkaline solution; or (iii) subjecting juice and marc obtained from ginger root to alkaline treatment in alkaline solution.

[0014] In specific aspects:

[0015] The ginger root is fresh.

[0016] The ginger root is dried.

[0017] The ginger root is dried at about 40 to about 70 degrees Celsius, or at about 40 to about 60 degrees Celsius, or at about 55 to about 65 degrees Celsius, or at about 60 degrees Celsius.

[0018] The juice is obtained by macerating and/or pressing the ginger root.

[0019] The marc is obtained by juicing, macerating, and/or pressing the ginger root.

[0020] The ginger root is diced and subjected to the alkaline treatment.

[0021] The ginger root is diced, dried and subjected to the alkaline treatment.

[0022] The alkaline treatment is carried out at about 40 to about 70 degrees Celsius.

[0023] The alkaline treatment is carried out at about 50 to about 60 degrees Celsius.

- [0024] The alkaline treatment is carried out at about 55 to about 65 degrees Celsius.
- [0025] The alkaline treatment is carried out at about 60 degrees Celsius.
- [0026] The alkaline treatment is carried out for about 1-72 hours.
- [0027] The alkaline treatment is carried out for about 1-48 hours.
- [0028] The alkaline treatment is carried out for about 1-24 hours.
- [0029] The alkaline treatment is carried out for about 1-30 hours, or about 1-20 hours, or about 1-10 hours, or about 1-5 hours.
- [0030] The alkaline treatment is carried out for about 0.5 to about 3 hours, or about 0.5 to about 2 hours, or about 1 to about 2 hours.
- [0031] The alkaline treatment is carried out for about 2 hours.
- [0032] The alkaline treatment is carried out for about 1 hour.
- [0033] Potassium hydroxide (KOH) is used.
- [0034] A liquid form of potassium hydroxide (KOH) is used.
- [0035] About 0.1% to about 6% KOH (v/v) is used. About 0.5% to about 5.5% KOH (v/v) is used. About 1% to about 6% KOH (v/v) is used. About 1.5% to about 5.5% KOH (v/v) is used. About 2% to about 4% KOH (v/v) is used. About 1.5% to about 3.5% KOH (v/v) is used.
- [0036] Calcium hydroxide Ca(OH)_2 is used.
- [0037] About 0.5% to about 4% Ca(OH)_2 (v/v) is used. About 1.5% to about 3.5% Ca(OH)_2 (v/v) is used. About 2% to about 3% Ca(OH)_2 (v/v) is used.
- [0038] After alkaline treatment, the alkaline solution is neutralised.
- [0039] The alkaline solution is neutralised with citric acid.
- [0040] The alkaline solution is cooled during neutralisation to alleviate excess heat.
- [0041] The alkaline solution is neutralised to obtain a pH of about 6.5 to about 7.5 or about 7.0 to about 7.3.
- [0042] The neutralised solution is freeze dried.
- [0043] The neutralised solution is heat dried.

- [0044] The neutralised solution is subjected to extraction of the zingerone.
- [0045] The neutralised solution is dried and optionally subjected to extraction of the zingerone.
- [0046] The drying is at about 50 degrees Celsius to at about 70 degrees Celsius.
- [0047] The drying is at about 55 degrees Celsius to about 65 degrees Celsius.
- [0048] The drying is at about 60 degrees Celsius.
- [0049] The drying is for at least 24 hours.
- [0050] The drying is for about 24 hours to about 28 hours.
- [0051] The dried material is optionally milled.
- [0052] The zingerone is optionally further extracted by one or more alcohol extraction steps.
- [0053] The zingerone is optionally further extracted by one or more ethanol extraction steps.
- [0054] The ethanol extraction is carried at about 35 degrees Celsius to at about 65 degrees Celsius.
- [0055] The ethanol extraction is carried at about 45 degrees Celsius to about 55 degrees Celsius.
- [0056] The ethanol extraction is carried at about 50 degrees Celsius.
- [0057] The ethanol extraction is carried out for at least 7 days.
- [0058] The ethanol extraction is carried out for 24 hours or less.
- [0059] The ethanol extraction is carried out for at least 4 hours.
- [0060] The ethanol extraction is carried out for about 4 to about 8 hours.
- [0061] The ethanolic extract is optionally dried.
- [0062] The zingerone is extracted using supercritical fluid extraction.
- [0063] The zingerone is extracted by a supercritical fluid extraction followed by an alcohol extraction step.
- [0064] The method produces a product, this being a composition that comprises zingerone.

- [0065] The composition is free from or substantially free from aldehydes.
- [0066] The composition is a botanical extract.
- [0067] The composition is an ethanolic extract.
- [0068] The composition is a powder.
- [0069] In one aspect, this disclosure encompasses a method of producing zingerone by subjecting ginger root extract to an alkaline treatment.
- [0070] The ginger root extract is obtained by supercritical fluid extraction of the ginger root.
- [0071] The ginger root extract is obtained by alcohol extraction of the ginger root.
- [0072] The ginger root extract is obtained by juicing the ginger root.
- [0073] The ginger root extract is obtained by juicing the ginger root to obtain a juice and a marc.
- [0074] The juicing includes macerating and/or pressing the ginger root.
- [0075] The alkaline treatment is carried out at about 30 to about 70 degrees Celsius.
- [0076] The alkaline treatment is carried out at about 50 about 60 degrees Celsius.
- [0077] The alkaline treatment is carried out at about 55 to about 65 degrees Celsius.
- [0078] The alkaline treatment is carried out at about 60 degrees Celsius.
- [0079] The alkaline treatment is carried out for about 1-72 hours.
- [0080] The alkaline treatment is carried out for about 1-48 hours.
- [0081] The alkaline treatment is carried out for about 1-24 hours.
- [0082] The alkaline treatment is carried out for about 1-30 hours, or about 1-20 hours, or about 1-10 hours, or about 1-5 hours.
- [0083] The alkaline treatment is carried out for about 0.5 to about 3 hours, or about 0.5 to about 2 hours, or about 1 to about 2 hours.
- [0084] The alkaline treatment is carried out for about 2 hours.
- [0085] The alkaline treatment is carried out for about 1 hour.

- [0086] Potassium hydroxide (KOH) is used.
- [0087] A liquid form of potassium hydroxide (KOH) is used.
- [0088] About 0.1% to about 6% KOH (v/v) is used. About 0.5% to about 5.5% KOH (v/v) is used. About 1% to about 6% KOH (v/v) is used. About 1.5% to about 5.5% KOH (v/v) is used. About 2% to about 4% KOH (v/v) is used. About 1.5% KOH (v/v) to about 3.5% KOH (v/v) is used.
- [0089] Calcium hydroxide Ca(OH)_2 is used.
- [0090] About 0.5% to about 4% Ca(OH)_2 (v/v) is used. About 1.5% to about 3.5% Ca(OH)_2 (v/v) is used. About 2% to about 3% Ca(OH)_2 (v/v) is used.
- [0091] After alkaline treatment, the alkaline solution is neutralised.
- [0092] The alkaline solution is neutralised to obtain a pH of about 6.5 to about 7.5 or about 7.0 to about 7.3.
- [0093] After neutralisation of the alkaline solution, the neutralised material is dried.
- [0094] The dried material is optionally milled.
- [0095] The dried material is optionally further extracted.
- [0096] The zingerone is optionally further extracted by one or more alcohol extraction steps.
- [0097] The zingerone is optionally further extracted by one or more ethanol extraction steps.
- [0098] The ethanol extraction is carried out for at least 7 days.
- [0099] The ethanol extraction is carried out for 24 hours or less.
- [00100] The ethanol extraction is carried out for at least 4 hours.
- [00101] The ethanol extraction is carried out for about 4 to about 8 hours.
- [00102] The ethanolic extract is optionally dried.
- [00103] The zingerone is optionally further extracted using supercritical fluid extraction.
- [00104] The zingerone is optionally further extracted by a supercritical fluid extraction followed by an alcohol extraction step .

- [00105] The method produces a product, this being a composition that comprises zingerone.
- [00106] The composition is free from or substantially free from aldehydes.
- [00107] The composition is a botanical extract.
- [00108] The composition is an ethanolic extract.
- [00109] The composition is a powder.
- [00110] The method comprises (i) subjecting ginger root to an alkaline treatment in alkaline solution; or (ii) subjecting juice obtained from ginger root, and optionally marc obtained from ginger root, to an alkaline treatment in an alkaline solution, wherein the alkaline solution comprises about 1.5% to about 3.5% KOH (v/v), wherein the alkaline treatment is carried out for about 1 to about 2 hours, and wherein following alkaline treatment, the alkaline solution is neutralised to a pH of about 6.5 to about 7.5.
- [00111] Also encompassed is a composition, the composition being a botanical extract comprising zingerone, the zingerone being prepared by a method of any one of the preceding aspects.
- [00112] The composition is free from or substantially free from aldehydes.
- [00113] The composition is an ethanolic extract.
- [00114] The composition is a powder.
- [00115] The composition is formulated for administration as a pharmaceutical composition or a dietary composition.
- [00116] The composition is formulated as a dietary supplement.
- [00117] Additionally encompassed is a composition comprising zingerone, the zingerone being prepared by a method of any one of the preceding aspects.
- [00118] In one aspect, this disclosure encompasses a method of treating or preventing inflammation, comprising: administering to a subject a composition of any one of the preceding aspects, thereby treating or preventing the inflammation.
- [00119] In one other aspect, this disclosure encompasses the use of a composition of any one of the preceding aspects for preparing a medicament for treating or preventing inflammation.

- [00120] Also encompassed is a composition comprising zingerone for treating or preventing inflammation.
- [00121] In various aspects:
- [00122] The composition is obtained by a method of any one of the preceding aspects.
- [00123] The composition is obtained from a ginger root.
- [00124] The composition is obtained from fresh ginger root.
- [00125] The composition is obtained from dried ginger root.
- [00126] The composition is obtained from juice prepared from a ginger root.
- [00127] The juice is prepared by macerating and/or pressing of the ginger root.
- [00128] The composition is obtained using an alkaline conversion step to convert the gingerol in the ginger root, or in the juice from ginger root, to zingerone.
- [00129] The composition is free from or substantially free from aldehydes.
- [00130] The composition is formulated as a powder.
- [00131] The composition is formulated as a tincture.
- [00132] The composition further comprises one or more anti-inflammatory agents.
- [00133] The composition further comprises one or more of: an analgesic compound, antipyretic compound, and psychotropic compound.
- [00134] The composition further comprises one or more of: a cannabinoid compound, mushroom compound, non-steroid anti-inflammatory drug compound (NSAID), opioid compound, salicylate compound, and steroid compound.
- [00135] The composition further comprises one or more of: acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, eptazocinem, betamethasone, cortisone, deflazacort, dexamethasone, ethamethasoneb, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, cannabidiol, cannabigerol, tetrahydrocannabinol, psilocybin, and psilocin.

- [00136] The inflammation requires modulation.
- [00137] The inflammation is acute or chronic inflammation.
- [00138] The inflammation is an inflammatory disorder.
- [00139] The inflammation is inflammation of one or more of: an immune disorder; an arthritic disorder; an infection; a cardiac, circulatory, or pulmonary disorder; a neurological disorder; and a neoplastic disorder.
- [00140] The inflammation is an inflammation affecting one or more of: a joint, skin, eye, ear, nose, mouth, throat, oesophagus, kidney, bladder, liver, spleen, lung, heart, brain, circulatory system, digestive system, endocrine system, genitourinary system, lymphatic system, nervous system, and skeletal system.
- [00141] The inflammation is inflammation of one or more of: Alzheimer's disease, early stage Alzheimer's disease, ankylosing spondylitis, arthritis, asthma, colitis (e.g., ulcerated colitis), Crohn's disease, dementia, early stage dementia, depression, diabetes, fibromyalgia, gout, infection (e.g., microbial infection), immune mediated inflammatory disease, inflammatory bowel disease (IBD), interstitial cystitis, multiple sclerosis (MS), polymyalgia psoriasis, scleroderma, and Sjögren's syndrome, and systemic lupus erythematosus (SLE; lupus).
- [00142] The inflammation is inflammation of one or more of: rheumatoid arthritis, ankylosing spondylitis arthritis, fibromyalgia arthritis, gout arthritis, juvenile idiopathic arthritis (JIA), lupus arthritis, osteoarthritis, polymyalgia rheumatica, psoriatic arthritis, reactive arthritis, scleroderma arthritis, Sjögren's syndrome arthritis.
- [00143] The inflammation is inflammation of one or more of: atherosclerosis, coronary artery disease, pulmonary artery hypertension, hypoxia-induced pulmonary hypertension, pneumonia, acute respiratory distress syndrome, coronavirus respiratory disorder, and cytokine storm syndrome.
- [00144] The inflammation is inflammation of one or more of: breast cancer, leukaemia, multiple myeloma, myelodysplastic syndrome, pancreatic cancer, and prostate cancer.
- [00145] In various aspects for the composition:
- [00146] The composition is formulated for topical administration, or oral administration.

- [00147] The composition is formulated as a solid, semi-solid, or liquid.
- [00148] The composition is formulated as a solution, tincture, gel, jelly, gummy, powder, tablet, or capsule.
- [00149] The composition is provided in a sachet.
- [00150] The composition comprises a dose of about 10 mg to about 3000 mg of zingerone.
- [00151] The composition comprises a dose of about 10 mg to about 1500 mg of zingerone.
- [00152] The composition comprises a dose of about 10 mg to about 1000 mg of zingerone.
- [00153] The composition comprises a dose of about 10 mg to about 500 mg of zingerone.
- [00154] The composition comprises a dose of about 10 mg to about 300 mg of zingerone.
- [00155] The composition comprises a dose of about 10 mg to about 150 mg of zingerone.
- [00156] The composition comprises a dose of about 10 mg to about 100 mg of zingerone.
- [00157] The composition comprises a dose of about 10 mg to about 75 mg of zingerone.
- [00158] The composition comprises a dose of about 10 mg to about 50 mg of zingerone.
- [00159] The composition is formulated for co-administration with one or more anti-inflammatory agents.
- [00160] The composition is formulated for co-administration with one or more of: an analgesic compound, antipyretic compound, and psychotropic compound.
- [00161] The composition is formulated for co-administration with one or more of: a cannabinoid compound, mushroom compound, non-steroid anti-inflammatory drug compound (NSAID), opioid compound, salicylate compound, and steroid compound.

[00162] The composition is formulated for co-administration with one or more of: acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, eptazocinem, betamethasone, cortisone, deflazacort, dexamethasone, ethamethasoneb, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, cannabidiol, cannabigerol, tetrahydrocannabinol, psilocybin, and psilocin.

[00163] Also encompassed is a use of a composition of a preceding aspect for preparing a medicament for treating or preventing inflammation in a subject.

[00164] Also encompassed is a method of treating or preventing inflammation in a subject comprising administering to the subject a composition of a preceding aspect.

[00165] The foregoing brief summary broadly describes the features and technical advantages of certain embodiments of this disclosure. Further technical advantages will be described in the detailed description and examples that follows.

[00166] Novel features that are believed to be characteristic will be better understood from the detailed description when considered in connection with any accompanying figures and examples. However, the figures and examples provided herein are intended to help illustrate what is disclosed or assist with developing an understanding what is disclosed, and are not intended to limit the scope of this disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[00167] Figure 1: Photograph depicting fresh ginger root.

[00168] Figure 2: HPLC UV chromatogram traces (280 nm) for alkaline treated ginger.

[00169] Figure 3: Schematic showing processing comparison.

[00170] Figure 4A: Photograph depicting juicing machine and raw ginger prior to juicing.

[00171] Figure 4B: Photograph depicting juicing of raw ginger in progress.

[00172] Figure 5A: Ginger juice treated with KOH (0.5%) and analysed by HPLC. Peak areas shown for zingerone (Z) and gingerol (G).

- [00173] Figure 5B: Ginger juice treated with KOH (1%) and analysed by HPLC. Peak areas shown for zingerone (Z) and gingerol (G).
- [00174] Figure 5C: Ginger juice treated with KOH (2%) and analysed by HPLC. Peak areas shown for zingerone (Z) and gingerol (G).
- [00175] Figure 6A: Ginger marc produced by pressing.
- [00176] Figure 6B: Ginger juice produced by pressing.
- [00177] Figure 7: Schematic showing ethanolic extraction process and evaporation.
- [00178] Figure 8A: GCMS TIC analysis for ethanolic extract.
- [00179] Figure 8B: Comparison of ethanolic extract with hexanal standard. Shown are 2-7 minute regions of chromatogram.
- [00180] Figure 9: Dose response curve for cytotoxicity assay. Disclosed botanical extract assessed.
- [00181] Figure 10: Dose response curve for nitric oxide assay. Disclosed botanical extract assessed.
- [00182] Figure 11: Dose response curve for IL-6 assay. Disclosed botanical extract assessed.
- [00183] Figure 12: Dose response curve for cytotoxicity, NO, and IL-6 assays. Disclosed botanical extract assessed.
- [00184] Figure 13: Cell viability of RAW264.7 cells was measured with the WST-1 assay. Comparative study for disclosed botanical extract and commercially sourced zingerone.
- [00185] Figure 14: Interleukin (IL)-6 produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS). Comparative study for disclosed botanical extract and commercially sourced zingerone.
- [00186] Figure 15: Interleukin (IL)-10 produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS). Comparative study for disclosed botanical extract and commercially sourced zingerone.

[00187] Figure 16: Tumour necrosis factor (TNF)- α produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS). Comparative study for disclosed botanical extract and commercially sourced zingerone.

[00188] Figures 17A-17B: Both testing methods showed efficacy of dexamethasone as a positive control. Figure 17A shows results for Method 1 analysis. Figure 17B shows results for Method 2 analysis.

[00189] Figures 18A-18B: Both testing methods performed similarly for MTT scoring. Figure 18A shows results for Method 1 analysis. Figure 18B shows results for Method 2 analysis.

[00190] Figures 19A-19D: Disclosed botanical extract showed a cytotoxic effect at 150, 100, 75 and 50 μ M. Data is presented as the mean of three biological replicates $\pm$ SEM. Non-linear regression was computed using GraphPad Prism 9.0. Figure 19A shows results for synthetic zingerone. Figure 19B shows results for acetyl zingerone. Figure 19C shows results for ferulic acid. Figure 19D shows results for disclosed botanical extract.

[00191] Figure 20: Disclosed botanical extract produces a dose dependent inhibition of IL-6 production from stimulated RAW264.7 cells. Data is presented as the mean of three biological replicates $\pm$ SEM.

[00192] Figure 21: Treatment with the disclosed botanical extract at 25 μ M produces significantly less IL-6 compared to treatment with synthetic zingerone, acetyl zingerone, or ferulic acid at 150 μ M. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures One-Way ANOVA with a Turkey Correction for multiple comparisons was computed in GraphPad Prism 9.0. *: $P < 0.05$, ***: $P < 0.001$

[00193] Figure 22: Treatment with the disclosed botanical extract at 25 μ M significantly lowered IL-6 levels compared to vehicle control. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures Two-Way ANOVA with a Sidak correction for multiple comparisons was computed in GraphPad Prism 9.0. *: $P < 0.05$, **: $P < 0.01$.

[00194] Figures 23A-23D: No observable effect on IL-6 production from unstimulated RAW264.7 cells. Data is presented as the mean of three biological replicates $\pm$ SEM. Figure 23A shows results for synthetic zingerone. Figure 23B shows results for

acetyl zingerone. Figure 23C shows results for ferulic acid. Figure 23D shows results for disclosed botanical extract.

[00195] Figure 24: Disclosed botanical extract reduces TNF (TNF- α) production by stimulated RAW264.7 cells. Data is presented as the mean of three biological replicates $\pm$ SEM.

[00196] Figure 25: Treatment with the disclosed botanical extract at 25 μ M produces significantly less IL-6 compared to treatment with acetyl zingerone at 150 μ M. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures One-Way ANOVA with a Turkey Correction for multiple comparisons was computed in GraphPad Prism 9.0. *: $P < 0.05$.

[00197] Figure 26: Reduction of TNF production by stimulated RAW264.7 cells. Ferulic acid at 150 μ M produces a significant reduction of TNF compared to vehicle. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures Two-Way ANOVA with a Sidak correction for multiple comparisons was computed in GraphPad Prism 9.0. *: $P < 0.05$, **: $P < 0.01$.

[00198] Figures 27A-27D: No observable effect on TNF production from unstimulated RAW264.7 cells. Data is presented as the mean of three biological replicates $\pm$ SEM. Figure 27A shows results for synthetic zingerone. Figure 27B shows results for acetyl zingerone. Figure 27C shows results for ferulic acid. Figure 27D shows results for disclosed botanical extract.

[00199] Figure 28: Disclosed botanical extract reduces NO levels in stimulated RAW264.7 cells. Data is presented as the mean of three biological replicates $\pm$ SEM.

[00200] Figure 29: Treatment with the disclosed botanical extract at 25 μ M produces significantly lower NO levels compared to treatment with synthetic zingerone, acetyl zingerone, or ferulic acid at 150 μ M. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures One-Way ANOVA with a Turkey correction for multiple comparisons was computed in GraphPad Prism 9.0.

[00201] Figure 30: Treatment with synthetic zingerone at 150 μ M produces a significantly higher levels of NO compared to treatment with vehicle. Data is presented as the mean of three biological replicates $\pm$ SEM. Repeated Measures Two-Way ANOVA with a Sidak correction for multiple comparisons was computed in GraphPad Prism 9.0.

- [00202] Figure 31: Flow chart for a large scale ginger juicing method.
- [00203] Figure 32: Flow chart for a large scale zingerone extraction method with ginger juice as starting material.
- [00204] Figure 33: Flow chart for a large scale zingerone extraction method with ginger marc as starting material.
- [00205] Figures 34A-34B: Stability of zingerone extract obtained by large scale production. Testing was carried out over two months. Figure 34A shows zingerone content at 5°C and 40°C. Figure 34B shows pH levels at 5°C and 40°C.

DETAILED DESCRIPTION

[00206] The following description sets forth numerous exemplary configurations, parameters, and the like. It should be recognised, however, that such description is not intended as a limitation on the scope of the present disclosure, but is instead provided as a description of exemplary embodiments.

[00207] All references, including patents and patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Nor does discussion of any reference constitute an admission that such reference forms part of the common general knowledge in the art, in New Zealand or in any other country.

Definitions

[00208] Where a range is given in the specification, for example, a temperature range, a time range, or a composition range, all intermediate ranges and subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure. Thus, each range that is specified (e.g., 1 to 10) includes all possible combinations of numerical values between the lowest value and the highest value enumerated (e.g., 1, 1.1, 2, 3, 3.3, 4, 5.5, 6, 7, 8.9, 9 and 10) and also any range of rational numbers within that range (e.g., 2 to 8, 1.5 to 5.5, and 3.1 to 4.9), and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. The numeric values provided in parentheses here are only examples of what is specifically intended and all possible combinations of numerical value between the lowest value and the highest

value enumerated are to be considered to be expressly stated in this disclosure in a similar manner.

[00209] In each instance herein, in descriptions, embodiments, and examples of the present disclosure, the terms “comprising”, “including”, etc., are to be read expansively, without limitation. Thus, unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as to opposed to an exclusive sense, that is to say in the sense of “including but not limited to”.

[00210] As used herein “and/or” means additionally or alternatively.

[00211] In the present description, the articles “a” and “an” are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” can be taken to mean one element or more than one element.

[00212] Throughout this description, the term “about” is used to indicate that a value includes the standard deviation of error for the method being employed to determine the value, for example, levels of compounds or dosage levels, as described in detail herein. In particular, the term “about” encompasses up to a 10% deviation (positive and negative) in the stated value or range.

[00213] The term “comprising”, as used herein, may refer to the presence of zingerone or a zingerone extract in a composition. As exemplifications, the zingerone or zingerone extract may be at least 1%, at least 2%, at least 4%, at least 5%, at least 10%, at least 12%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% by weight of the composition (% w/w). Alternatively, the zingerone or zingerone extract may be at least 1%, at least 2%, at least 4%, at least 5%, at least 10%, at least 12%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% by volume of the composition (% w/v or % v/v).

[00214] The term “substantially free” in relation to aldehydes refers to a product having negligible aldehyde levels. The product may be, for example, a composition as described herein, or may be, for example, a product produced by a method as described herein. As exemplifications, aldehyde levels may be less than 20 ppm, less than 15 ppm, less than 10 ppm, less than 7.5 ppm, less than 5 ppm, less than 2 ppm, less than 1.5 ppm, less than 1 ppm, less than 0.75 ppm, less than 0.5 ppm, less than 0.2 ppm, less than 0.1 ppm, less than 0.05 ppm, less than 0.005 ppm, or less than 0.0005 ppm.

[00215] The term “alkaline treatment” as used herein means the exposure of a sample (e.g., ginger, ginger juice, ginger marc, or any combination thereof) to an aqueous solution containing alkali having a pH greater than 7. Included, without limitation, are solutions comprising, sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide and any combination thereof. It is to be appreciated that the alkaline treatment can occur at a range of temperatures as described herein and that the alkaline solution may be heated prior to or during exposure to the sample comprising ginger or an extract from ginger. The alkaline solution will have a chemically effective amount of alkali present to convert at least some gingerol present in the sample to zingerone. Particular methodologies are described in detail herein.

[00216] Generally speaking, an “extract” of this disclosure will refer to a botanical extract (also referred to as a “botanical drug”). More specifically, an “extract” refers to a composition where one or more liquid, solid, or chemical constituents of a plant or plant part has been isolated or concentrated. For example, a liquid, solid, or semi-solid extract may be obtained. An extract may be obtained by one or more of: juicing, pressing, macerating, mashing, milling, or other standard processes. Solvent-based extraction is also included. Solid extracts are specifically noted, for example, powders obtained from drying or evaporation. As specific exemplifications, an extract may be prepared as a dry form, or may be prepared in the form of a solution. A “zingerone extract” refers to an extract comprising zingerone, as prepared/produced from ginger root (i.e., ginger rhizome, which can also be referred to as “ginger”). Particular extracts and their production methods are described in detail herein.

[00217] The term “composition” as used herein encompasses a product comprising one or more active components (e.g., combinations as set out herein), and one or more suitable excipients comprising other ingredients. These may be physiologically acceptable excipients. Encompassed is any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the active components. In particular aspects, the composition may comprise any suitable solvate or salt of each compound. As specific examples, an extract of this disclosure can be prepared as a composition suitable for administration to a subject, or suitable for formulation for administration to a subject. Various exemplary compositions are described in detail herein.

[00218] A “pharmaceutical composition” refers to a composition administered to a subject, for example, to treat or prevent inflammation. A “dietary composition” refers to a composition to be ingested by a subject, for example, to alleviate or prevent inflammation.

[00219] As used herein, “administration of” or “administering” refers to the providing the disclosed extract or a composition formulated from the disclosed extract to a subject. The disclosed extract and disclosed composition can be administered via any suitable route and via any suitable formulation. In some cases, it may be useful to use different routes of administration and/or different formulations in the same subject. For example, one or more oral formulations may be used, or one or more topical formulations may be used, or one or more oral formulation may be used in conjunction with one or more topical formulations. Non-limiting exemplifications of routes of administration and formulations for administration are provided herein.

[00220] An “anti-inflammatory agent” refers to a constituent that mitigates one or more symptoms of inflammation. Included amongst these are pharmaceutical agents, phytochemical agents, plant components, plant extracts, and essential oils, as well as tisanes and other infusions, along with various other constituents, to assist in the reduction of inflammation. Such may be utilised in combination with the compositions and extracts of this disclosure, and may be used to assist in regulating an immune response.

[00221] “Co-administration” or “co-administering” refers to the combined use of active components, for example, for therapy or for cosmetic enhancement, and includes the administration of co-formulations (i.e., combination formulations), as well as the simultaneous, sequential, or separate administration of distinct formulations. Similarly, “in conjunction” refers to the combined use of one or more active components and a device/procedure. This can include use of the active component(s) preceding use of the device/procedure, simultaneously with the device/procedure, and/or following use of the device/procedure.

[00222] The term “inflammation” includes any degree of inflamed tissue in a subject that persists for any period of time. Particular exemplifications include acute inflammation or chronic inflammation. The inflammation may be associated with pain. The inflammation may be that of the joint, skin, lung, heart, circulatory system, digestive tract, genitourinary tract, etc. These and other types of inflammation are encompassed herein.

[00223] A “symptom” of inflammation includes one or more of: pain, heat, redness, swelling, and loss of function. Also noted as markers of inflammation are the production of proinflammatory cytokines (e.g., IL-6, TNF- α) and/or proinflammatory small molecules (e.g., NO), as well as the activation and/or accumulation of immune cells.

[00224] As used herein, a “subject” may be a human or non-human animal, particularly a mammal, including cattle, sheep, goats, pigs, horses, and other livestock, including, as well, dogs, cats, and other domesticated pets. In particular aspects, the subject is a human being.

[00225] “Preventing” as used herein refers to halting or delaying the onset or progression of inflammation or a disorder involving inflammation. A preventative measure may result in the stoppage or delay of development of the inflammation or the disorder, or its symptom(s), a prevention of progression of the inflammation or the disorder, or its symptom(s), or a lessening of the developed inflammation or disorder, or its symptom(s), if such happen to arise. A preventative measure may also act in supporting, maintaining, and/or protecting of a bodily system. It should be understood that the term “treating or preventing” does not exclude the possibility of obtaining both treatment and prevention of the disorder. A “therapeutic” effect or “therapeutic” method may include treatment, or prevention, or both.

[00226] “Treating” as used herein refers to ameliorating or resolving inflammation or a disorder involving inflammation. A treatment will result in the reduction, e.g., amelioration or resolution, of the inflammation or the disorder, or one or more symptoms of the inflammation or the disorder. Resolution in the context of a treatment includes partial or complete reversal of the inflammation or the disorder, or its symptom(s). Partial or complete healing is encompassed by this, e.g., an improvement in one or more relevant health parameters. A treatment can include a lessening in the expression of the inflammation or the disorder, or its symptom(s). A treatment may also suppress existing inflammation/inflammatory disorder or its symptom(s), or put existing inflammation/inflammatory disorder or its symptom(s) into remission. In the context of “treating”, the healing of wounds and rashes is specifically noted.

[00227] “Alleviation” refers to ameliorating inflammation or a disorder involving inflammation. An alleviation will result in the reduction, e.g., amelioration, of the inflammation or the disorder, or one or more symptoms of the inflammation or the disorder.

Healing is specifically encompassed, e.g., an improvement in one or more relevant health parameters. An alleviation includes a lessening in the expression of the inflammation or the disorder, or its symptom(s). An alleviation may also be an action in suppressing existing inflammation/inflammatory disorder or its symptom(s). Alleviation of the inflammation of wounds and rashes is specifically noted.

[00228] The term “effective amount” refers to a sufficient quantity of the active component(s), in a suitable composition, and in a suitable dosage form to treat or prevent a noted disorder, or at least one symptom thereof. The “effective amount” will vary depending on the component(s) used, the type of therapy, and the species, age, weight, health, etc, of the subject to be treated.

[00229] “Combination” refers to combined use of two or more components (e.g., two or more active components). Usage may be by co-formulated components (i.e., combination formulations), or by simultaneous, sequential, or separate use of components (e.g., via different formulations, same formulations, or co-formulations). These and other specific combinations are encompassed in this disclosure.

Methods of preparing compositions

[00230] The inventors have found that zingerone compositions prepared from ginger root in accordance with the disclosed methods have significant anti-inflammatory activity, which exceed the activity of commercially available zingerone compositions. Therefore, the present disclosure relates generally to a zingerone composition prepared from ginger root, and methods of preparation of such.

[00231] In one aspect, this disclosure provides a method of producing zingerone from ginger root by subjecting the ginger root to an alkaline treatment. The alkaline treatment may include incubation in an alkaline solution as described herein. As starting material, the ginger root may be fresh ginger root. For example, to assist with preparation, it may be helpful to optimise the period of time that the ginger root is retained in the soil prior to harvesting. In this way, the ginger root that is utilised will be fresh and will retain the advantageous characteristics of fresh ginger root.

[00232] The disclosed preparation methods produce a highly efficacious botanical extract. As exemplifications, to optimise freshness, the ginger root may be harvested less than 48 hours before processing, less than 24 hours before processing, or less than 12 hours before processing, or less than 6 hours before processing, or less than 3 hours before

processing. For example, fresh ginger may have a moisture content of about 80% to about 95%, about 81% to about 95%, or about 82% to about 95%, or about 83% to about 95%, or about 85% to about 95% on a wet basis.

[00233] Alternatively, the ginger root may be dried prior to treatment. For example, The ginger root may be dried at about 40° to about 70°C, or at about 55° to about 65°C, or at about 60°C. Drying may be carried out for about 1-72 hours, or about 1-48 hours, or about 1-24 hours, or about 1-20 hours, or about 1-18 hours, or about 1-10 hours, or about 1-5 hours.

[00234] In certain aspects, the ginger root selected for use in the disclosed methods may have a minim level of gingerol, e.g., 6-gingerol. For example, the ginger root (e.g., fresh ginger root) may have about 0.3 to about 10 mg/g, or about 0.3 to about 9 mg/g, or about 0.3 to about 8 mg/g, or about 0.3 to about 7 mg/g, or about 0.3 to about 6 mg/g, or about 0.4 to about 5 mg/g of 6-gingerol. As further exemplifications, the ginger root may have at least 1 mg/g, at least 2 mg/g, at least 3 mg/g, at least 4 mg/g, or at least 5 mg/g of 6-gingerol. Thus, in certain circumstances, it may be advantageous to test levels of gingerol, e.g., 6-gingerol, in the starting material before commencing a method as disclosed herein.

[00235] As one aspect, the method comprises subjecting juice and/or marc from the ginger root to an alkaline treatment. The ginger juice and/or ginger marc may be obtained by macerating and/or pressing. The macerating may comprise homogenising with a blender, food processor, or similar machinery. For pressing, machine or hand presses may be utilised. Screw pressing is specifically noted. The solid material remaining after juicing (ginger marc) may be re-juiced to obtain ginger juice. This may be repeated as needed. The various juice samples and marc samples may be combined before alkaline treatment, e.g., juice sample A + juice sample B, or marc sample A + marc sample B, or juice sample A, B + marc sample A, B.

[00236] Optionally, the ginger marc may be subjected to hot water treatment to obtain a diluted juice. For example, water may be added to the marc at a ratio of about 6 to about 1 (~6:1), or about 5 to about 1 (~5:1), or about 4 about 1 (~4:1), or about 3 to about 1 (~3:1) by weight. The water may be, for example, at about 40°C to about 80°C, or at about 50°C to about 70°C, or at about 55°C to about 65°C, or at about 60°C. The incubation time in the water may be about 5 minutes to about 60 minutes, or about 10

minutes to about 30 minutes, or about 15 minutes to about 20 minutes, or about 15 minutes. The diluted juice samples may then be subjected to alkaline treatment. The diluted juice samples may be combined with other juice samples before alkaline treatment.

[00237] In one aspect, potassium hydroxide (KOH) may be used in the alkaline treatment. For example, a solid form of KOH may be used, e.g., KOH pellets. As an exemplification, the solid form of KOH may be at about 100% starting concentration, or at about 90% to about 100% starting concentration. Alternatively, a liquid form of KOH may be used. As an exemplification, the liquid form of KOH may be at about 50% starting concentration, or at about 40% to about 60%, or at about 45% to about 55%. The concentration of KOH used in the treatment mixture (e.g., final concentration) may be, for example, about 0.1% to about 6%, or about 0.5% to about 5.5%, or about 1% to about 6%, or about 1.5% to about 5.5%, or about 2% to about 4%, or about 1.5% to about 3.5%, or about 2% (v/v). As an alternative to this, calcium hydroxide Ca(OH)_2 may be used in the alkaline treatment. For example, a liquid form of Ca(OH)_2 may be used in the alkaline solution. The concentration of Ca(OH)_2 used in the treatment mixture may be, for example, about 0.5% to about 4%, about 1.5% to about 3.5%, about 1% to about 2%, or about 3.0% (v/v). Liquid forms may include, for example, stock solutions of about 25% to about 65%, or about 30% to about 60%, or about 35% to about 55%, or about 45% to about 55%, or about 50%.

[00238] In certain aspects, the alkaline treatment may achieve a pH level for the treatment solution of about pH 9 to about pH 14, or about pH 9.5 to about pH 13.5, or about pH 10 to about pH 13.5, or about pH 10.5 to about pH 13.5, or about pH 11.5 to about pH 13.5, or about pH 12.5 to about pH 13.5, or a pH of at least 13. The alkaline treatment may be carried out for a sufficient time and at a sufficiently elevated temperature to obtain desired levels of zingerone. For example, the alkaline treatment may be carried out for about 1-72 hours, or about 1-48 hours, or about 1-24 hours. Further examples include treatment for about 1 to about 30 hours, or about 1 to about 20 hours, or about 1 to about 10 hours, or about 1 to about 15 hours, or about 1 to about 7 hours, or about 1 to about 6 hours, or about 1 to about 5 hours, or about 1 to about 4 hours, or about 0.5 to about 3 hours, or about 0.5 to about 2 hours, or about 1 to about 2 hours, or at least 1 hour, or about 2 hours, or about 1 hour. As particular examples, the alkaline treatment may be carried out at about 40° to about 70°C, or about 50° to about 60°C, or about 55° to about

65°C, or about 60°C. It will be understood that lower temperatures can allow for longer treatment periods. For example, alkaline treatments performed at room temperature can be carried out for about 3 days to about 9 days, or for about 5 days to about 9 days, or for about 5 days to about 7 days.

[00239] Following alkaline treatment, the treatment mixture may be further processed, for example, by one or more of: neutralisation, extraction, and drying. For neutralisation, citric acid or other acid composition may be utilised. As exemplifications, neutralisation may achieve a pH of about 6.4 to about 7.4, or about 6.5 to about 7.5, or about 6.6 to about 7.6, or about 6.9 to about 7.4, or about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, or about 7.5. For example, about 10 to about 700 g/L citric acid may be utilised, or about 10 to about 600 g/L, or about 10 to about 500 g/L, or about 10 to about 400 g/L, or about 10 to about 300 g/L, or about 10 to about 200 g/L, or about 10 to about 100 g/L, or about 10 to about 50 g/L, or about 10 to about 40 g/L, or about 10 to about 30 g/L, or about 10 to about 20 g/L, or about 15 to about 16 g/L citric acid may be utilised.

[00240] For extraction, zingerone extraction may be achieved by one or more alcohol extractions, e.g., one or more ethanol extractions. As exemplifications, an alcohol extraction, e.g., ethanol extraction, may be carried out for about 1-72 hours, or about 1-48 hours, or about 1-24 hours, or about 6-24 hours, or about 8-24 hours, or about 12-24 hours, or about 18-24 hours. In one specific aspect, alcohol extraction may be carried out for 24 hours or less, for example, for at least 4 hours, or for about 4 to about 12 hours, or for about 4 to about 8 hours. The alcohol extraction may be carried out, for example, at about 35°C to about 65°C, or at about 45°C to about 55°C, or at least 50°C, or at about 50°C.

[00241] One or more drying steps may be used before and/or after extraction (e.g., alcohol extraction). For example, freeze drying or heat drying may be utilised. In particular, the amount of ethanol in the extract composition may be reduced by air drying, rotary evaporation, lyophilisation, or other techniques. With certain drying methods (e.g., rotary drying), the temperature and pressure can be increased to remove residual liquid(s). For example, drying can be carried out under vacuum, for example, at 50 mBar or less, 45 mBar or less, or 40 mBar or less. As further examples, drying can be carried out at about 35°C to about 50°C, or at about 35°C to about 45°C, or at least 30°C, or at least 35°C, or at least 40°C, or at about 40°C. Where the material is dried after neutralisation but before

alcohol extraction, drying may be carried out, e.g., for at least 18 hours, or at least 24 hours, or at least 28 hours, or at least 30 hours, or about 18 to about 28 hours, or about 24 to about 28 hours. The drying may be carried out, for example, at about 45°C to about 75°C, or at about 55°C to about 65°C, or no more than 60°C, or at about 60°C. Other alternatives for drying are described herein.

[00242] As part of the initial processing, the ginger root may be washed or sterilised. The plant component (e.g., fruit or seed) may be passed through an assembly having one or more roller brushes for removing any adhering foreign matter. Conventional washing techniques may then be employed. For example, it is possible to use a series of spray nozzles to wash the components. Wash additives aiding cleansing or reducing the microbial count on the plant components may be employed according to local regulations and requirements. For example, the plant components may be washed by a chlorine wash and/or an ozone impregnated water wash followed by a fresh water rinse.

[00243] As noted, it may be desirable for a liquid or semi-solid zingerone composition to be prepared from ginger root. As described herein, zingerone components may be extracted by chemical means (e.g., solvent-based extraction). Solvent-based extraction may utilise one or more of: water, methanol, ethanol, or 2-propanol, extraction. Supercritical fluid extraction, for example CO₂ extraction may also be used to extract zingerone. Also suitable are emulsions, pastes, suspensions, and syrups. For example, in certain aspects, it may be desirable to use a paste from the ginger root or from the ginger root component (e.g., zingerone or zingerone extract). As an exemplification, the ginger root may be heated for several hours, strained, and reduced to a thick, concentrated form. Upon thickening, the paste can be spread on a flat sheet, or transferred to a packaging, for example, a bag, tube, jar, bottle, or other container. The paste may be transferred aseptically. It may be desired to prepare the paste from mature plant components. The paste may be a smooth preparation.

[00244] In specific aspects, the present disclosure encompasses mechanical means (e.g., juicing means such as maceration and/or pressing) for extracting zingerone from ginger root. In one embodiment, a pressing assembly may be adapted to perform a pulping or comminution process. Such process can be relatively mild and gentle (soft pulping) compared to conventional fruit pulping techniques. With soft pulping, no significant disintegration or lysis of cells is utilised. The press belts may be multiple loops rotated

about a series of pulleys. The distance separating the press belts may decrease in the direction of travel of the plant component. In this way, increased force may be exerted upon the plant component as it travels along the length of the pressing assembly. In a particular aspect, a pressing assembly or mechanical press may be used to obtain juice from the ginger root, as described herein. Alternatively, or in addition to this, mechanical maceration may be used to obtain juice. For example, commercial juicing equipment may be utilised.

[00245] The ginger root component (e.g., zingerone or zingerone extract) may be processed by a freezing step. This may be followed by or used in conjunction with a drying or evaporation step. In an alternative embodiment, the component is dried or evaporated, and then processed to a powder without an intervening freezing step. Methods involving air drying or heat-assisted drying (e.g., oven drying) may be used. Drying may be obtained, for example, by one or more of: sun or solar drying, hot air drying, batch drying, rotary drying, tunnel drying, belt drying, fluidised bed drying, impingement drying, puff drying, drum drying, spray drying, vacuum drying, freeze drying, or osmotic drying. Exemplary temperatures for drying include about 50°C-70°C, about 55°C-65°C, or at least 50°C, at least 55°C, at least 60°, or at least 65°C. Evaporation may be obtained, for example, by one or more of: pan evaporation, batch evaporation, tube evaporation, rising film evaporation, falling film evaporation, rising-falling film evaporation, or agitated film evaporation. Combinations of various drying and evaporation methods may be used. For example, filtering followed by freeze drying may be used.

[00246] If freezing is used, it may be desirable to freeze the ginger root component (e.g., zingerone or zingerone extract) as soon as possible after it is produced to maintain freshness. However, freezing may be carried out within 24 or 48 hours, as needed. Standard freezing methodologies may be utilised. Blast freezing is particularly desirable for use with the present disclosure. The component may be frozen in standard sized pales, which are used to collect the frozen product after processing. The component, for example, can be stored frozen (e.g., at -18°C) until it is required. Optionally, the component may then be freeze dried, i.e., lyophilised. Freeze drying techniques are widely used. The freeze-drying cycle may be up to 48 hours. In particular aspects, the process may be carried out to such that water formation is avoided, and the moisture content is minimised during processing. It will be understood that freeze drying/lyophilising does not exclude the use of higher

temperatures (i.e., higher than freezing temperatures). For example, higher temperatures may be used for removing residual moisture during the secondary drying phase for lyophilisation/freeze drying procedures.

[00247] The resulting dried or evaporated component from ginger root (e.g., zingerone or zingerone extract) may then be milled into a powder, which can then be utilised as appropriate. Standard milling methods may be utilised. Standard mesh sizes may be used to produce the powder, for example, US 20, US 23, US 30, US 35, US 40, US 45, or US 50 mesh sizes may be used. The sieve size for the powder may range from 1.0 to 0.3 mm; or 0.84 to 0.4 mm; or 0.71 to 0.5 mm; or may be about 1.0 mm, about 0.84 mm, about 0.71 mm, about 0.59 mm, about 0.5 mm, about 0.47 mm, about 0.465 mm, about 0.437 mm, about 0.4 mm, about 0.355 mm, or about 0.3 mm.

[00248] The composition may be prepared as a pharmaceutical composition. The composition can also be prepared as a dietary composition, for example, a functional food or beverage, a natural ingredient (e.g., a natural additive), or a natural supplement (e.g., a dietary supplement). In various aspects, the composition may be prepared in liquid or solid form, or semi-solid form. Various formulations are encompassed in this disclosure. In certain aspects, it may be desirable to formulate the composition into a powder. The powder may be provided in free flowing form or as a solid cake. The composition may be provided as a powder for forming a suspension, powder for forming a solution, bulk granules, or bulk powder. The powder may be prepared as tablets or capsules, or other formulations, as described in detail herein.

[00249] It will be understood that, for any liquid or semi-solid product obtained from ginger root, the liquid/semi-solid may be used in this form or may be dried or evaporated to obtain a powder form for use as a pharmaceutical composition or dietary composition, as described herein. In the same way, it will be understood that, for any solid product obtained from ginger root, the solid may be used as such (e.g., with milling, sieving, or other processing), or may be re-suspended to obtain a liquid or semi-solid form for use as a pharmaceutical composition or dietary composition, as described herein.

Compositions

[00250] The inventor has found that zingerone compositions prepared from ginger root in accordance with the methods disclosed herein have significant anti-inflammatory and immunomodulating properties that are useful for reducing cellular inflammation

markers, reducing tissue damage, antioxidant action, as well as for treating or preventing inflammation and inflammatory disorders in a subject.

[00251] The composition of the present disclosure may be prepared as one or more formulations, including pharmaceutical compositions and dietary compositions. As non-limiting examples, the percentage of zingerone or zingerone extract in the composition may be about 0.01% to about 30%, or about 1% to about 30%, or about 1% to about 15%, or about 1% to about 10%, or about 1% to about 9%, or about 1% to about 8%, or about 0.1% to about 7%, or about 0.1% to about 6%, or about 0.1% to about 5%, or about 0.1% to about 4%, or about 0.1% to about 4%, or about 0.1% to about 3%, or a percentage of at least about 1%, at least about 4%, at least about 5%, at least about 6%, at least about 10%, at least about 12%, at least about 15%, at least about 20%, at least about 23%, at least about 25%, at least about 30%, at least about 40%, or at least about 50%, or a percentage of about 6.25%, about 12.5%, or about 25%, these percentages being representative of v/v values for a liquid composition, or w/w values for a solid composition, or w/v values for a liquid or semi-solid composition. In any of the various forms disclosed herein (e.g., liquid, solid, semi-solid, etc), the composition may be free from or substantially free from aldehydes.

[00252] As exemplifications, a solid composition may include about 0.5 to about 300 mg/g zingerone, about 1 to about 150 mg/g zingerone, about 1 to about 100 mg/g zingerone, or about 1 to about 80 mg/g, or about 1 to about 60 mg/g, or about 1 to about 50 mg/g, or about 1 to about 40 mg/g, or about 1 to about 20 mg/g, about 1 to about 15 mg/g, or about 1 to about 10 mg/g, or about 10 to about 60 mg/g zingerone, or about 10 to about 50 mg/g zingerone, or about 10 to about 40 mg/g zingerone, or about 10 to about 30 mg/g zingerone, or about 10 to about 20 mg/g zingerone, or about 10 to about 15 mg/g zingerone, or at least about 50 mg/g zingerone, or at least about 40 mg/g zingerone, or at least about 10 mg/g zingerone (w/w).

[00253] Similarly, as further exemplifications, a liquid or semi-solid composition may include about 0.5 to about 300 mg/ml zingerone, about 1 to about 150 mg/ml zingerone, about 1 to about 100 mg/ml zingerone, or about 1 to about 80 mg/ml, or about 1 to about 60 mg/ml, about 1 to about 50 mg/ml, or about 1 to about 40 mg/ml, or about 1 to about 20 mg/ml, or about 1 to about 15 mg/ml, or about 1 to about 10 mg/ml, or about 10 to about 60 mg/ml zingerone, or about 10 to about 50 mg/ml zingerone, or about 10 to about 40 mg/ml zingerone, or about 10 to about 30 mg/ml zingerone, or about 10 to about

20 mg/ml zingerone, or about 10 to about 15 mg/ml zingerone, or at least about 50 mg/ml zingerone, or at least about 40 mg/ml zingerone, or at least about 10 mg/ml zingerone (w/v).

[00254] In various aspects, topical compositions may be prepared, for example: for use on hands (e.g., hand creams), pre-operative tissue (e.g., surgical preparations for skin), mucous membranes (e.g., treatments for bladder, urethral, or vaginal inflammation, or cleansing of these cavities prior to medical procedures), wounds or burns (e.g., ointments, bandages, or dressings), mouth or throat (e.g., mouthwashes or lozenges), or eye (e.g., eye drops or ointments).

[00255] As non-limiting examples, topical compositions may include one or more of: diluents (e.g., ethanol or other alcohol), emollients (e.g., PEG-45, palm kernel glycerides, or isopropyl myristate), humectants (e.g., glycerine or methylpropanediol), carriers (e.g., one or more oils), occlusive agents (e.g., mineral oil or dimethicone) other conditioning agents (e.g., behentrimonium methosulfate or polyquaternium-7), and surfactants (e.g., mild surfactants (e.g., amphotoacetate, isethionate, sulfosuccinate, in particular, sodium lauroamphoacetate, sodium cocoyl isethionate, disodium oleoamido sulfosuccinate, sodium lauryl sulfate, sodium C14-16 olefin sulfonate). Exemplary oils include olive oil, coconut oil (e.g., coconut-derived MCT oil), palm oil (e.g., palm kernel-derived MCT oil), any other MCT oil (medium-chain triglyceride oil), and any combination thereof. Other possible carriers include lecithin (e.g., liquid form) and propylene glycol. Any combination of the carriers set out herein is also noted.

[00256] In yet other aspects, the compositions may be prepared for various routes of administration, including oral formulas. Also included are compositions prepared for other routes of enteral or parenteral administration. Enteral formulations include but are not limited to: oral, rectal, sublingual, sublabial, and buccal preparations. Parenteral formulations include but are not limited to: nasal, intraocular, vaginal, intralesional, transdermal, and transmucosal preparations. Standard methods are available for formulating pharmaceutical compositions. See, e.g., *Remington: Essentials of Pharmaceutics*, 2013, Pharmaceutical Press, London.

[00257] In particular aspects, the composition of this disclosure may be prepared as a powder, or in any other suitable dosage form. Topical formulations may be prepared, for example, as aerosols, balms, creams, dressings, drops, emulsions, films, foams, gels,

jellies, liquids, lotions, masks, oils, ointments, pastes, powders, salves, soaps, sprays, suspensions, solutions, tinctures, and vapours. Further topical formulations include bandages, dressings, patches, pads, sponges, strips, tapes, and others noted herein.

[00258] As described herein, the composition may be formulated as a semi-solid or liquid composition, for example, for oral administration (e.g., taken directly by mouth or via inclusion in capsules or in other forms), or for enteral or parenteral administration (e.g., taken by injection, feeding tube, or in other forms). Alternatively, the composition may be formulated as a powder to be encapsulated, tableted, or added to or incorporated in other products.

[00259] Oral formulations may be prepared, for example, as draughts, drops, elixirs, emulsions, liquids, linctuses, solutions, sprays, suspensions, syrups, tonics, or, as films, gels, jellies, gummies, lozenges, nuggets, pastes, purees, pomaces, powders, pills, or strips. In other aspects, oral formulations may be prepared as a tablets or as capsules, for example, with liquid, semi-solid, or solid contents. Oral formulations may be provided in sachet form, for example, a powder sachet, or a gel or jelly sachet. Included also are oral formulations comprising thin strips, or comprising solids in a capsule to mix with food or drink. The oral formulation may be provided as shooters or shots (to be consumed by mouth), for example, liquid shots, gel or jelly shots, paste shots, or powder shots.

[00260] Particularly encompassed are delayed release formulas, extended release formulas, as well as formulas for rapid disintegration. Capsules, for example gel capsules, are specifically encompassed, as well as sachets and chewable tablets. Additionally, included are combination formulas, which include the powder of the present disclosure mixed with other beneficial agents, e.g., one or more anti-inflammatory agents. Other formulas are also possible, as described herein.

[00261] The dissolution time for an oral formulation can be modified for a rapid effect or for sustained release. Oral formulations may also contain a mixture of slow and fast release particles to produce rapid and sustained absorption in the same dose. Special coatings can be used with oral formulations such as tablets and capsules to impart resistance to stomach acids. Oral formulations can also be coated with sugar, varnish, or wax to improve taste.

[00262] Thus, tablets may be prepared as rapid dissolve tablets and capsules may be prepared as extended release capsules. The tablets may be scored tablets, chewable

tablets, effervescent tablets, orally disintegrating tablets, or tablets for forming a suspension. The capsules may be gel capsules, for example, and may include solid, semi-solid, or liquid contents. This includes gel capsules made by single piece gel encapsulation and two piece gel encapsulation. Hard shell capsules and soft shell capsules are specially noted. Non-gelatine capsules are also noted, as well as caplets.

[00263] It will be understood that certain formulations will be suitable for topical or other applications. Particular formulations of interest are: eye formulas (e.g., drops, ointments), ear formulas (e.g., drops, ointments), nasal or airway formulas (e.g., drops, sprays, insufflation compositions, inhalation compositions, nebulisation compositions), skin formulas (e.g., soaps, sprays, aerosols, gels, pastes, lotions, creams, ointments, pads, patches, tapes, bandages, dressings, sponges, vapours) throat or mouth formulas (e.g., drops, lozenges, mouthwashes, toothpastes, sprays, pastes, gels, jellies, gummies), mucous membrane formulas (e.g., sprays, aerosols, gels, pastes, lotions, creams, ointments, pads, dressings, sponges).

[00264] Solid, semi-solid, and liquid compositions can combine zingerone or a zingerone extract with one more compounds to ensure a stable and active composition. For example, an oral formulation, such as a tablet or capsule, may include: about 5 to about 50% w/w zingerone or zingerone extract; up to about 80% w/w of one or more fillers, lubricants, glidants, or binders; and up to about 10% w/w of compounds to ensure easy disintegration, disaggregation, and dissolution of the tablet in the stomach or the intestine. In one particular exemplification, an extract of this disclosure (e.g., ethanolic tincture) may be mixed with one or more carrier substances such as glycerol, glyceryl esters, hydrogenated oils, polyethylene glycols, poloxamers, etc, and included in a capsule (e.g., gel capsule).

[00265] Thus, the composition may contain various excipients, for example, one or more: solubilizers, stabilizers, buffers, tonicity modifiers, bulking agents, thickening agents, viscosity enhancers/reducers, emollients, surfactants, chelating agents, adjuvants, anti-adherents, anti-caking agents, binders, coatings, disintegrants, lubricants, glidants, flow agents, sorbents, flavours, flavour masking agents, colours, sweeteners, or preservatives.

[00266] As exemplifications, the composition may include less than 1% of a preservative, for example, about 0.005% to about 0.5%, or about 0.05% to about 0.15%,

or may include about 0.04%, about 0.06%, about 0.08%, about 0.1%, about 0.12%, about 0.14%, about 0.16%, about 0.18%, or about 0.2% of a preservative, these percentages being representative of w/v values or w/w values. Useful preservatives include but are not limited to sorbic acid, sodium sorbate, potassium sorbate, citric acid, ascorbic acid, malic acid, tartaric acid, propionic acid, and benzoic acid, for example, in the form of its sodium salt, e.g., sodium benzoate.

[00267] Other useful excipients include but are not limited to: stearin, magnesium stearate, and stearic acid; saccharides and their derivatives, e.g., disaccharides: sucrose, lactose; polysaccharides and their derivatives, e.g., starches, cellulose or modified cellulose such as microcrystalline cellulose and cellulose ethers such as hydroxypropyl cellulose; sugar alcohols such as isomalt, xylitol, sorbitol and maltitol; proteins such as gelatin; polysaccharides such as pectin; gums and other thickeners, e.g., acacia gum, gellan gum, guar gum, locust bean gum, xanthan gum, agar, arrowroot carrageenan, gelatine, glycerine, kudzu, lecithin, starch; synthetic polymers such as polyvinylpyrrolidone, polyethylene glycol; fatty acids, plant based surfactants; e.g., sunflower lecithin, waxes, shellac, plastics, and plant fibres, e.g., corn protein zein; hydroxypropyl methylcellulose; cross linked polymers, e.g., cross linked polyvinylpyrrolidone (crospovidone), and cross linked sodium carboxymethyl cellulose (croscarmellose sodium); sodium starch glycolate; silicon dioxide, fumed silica, talc, and magnesium carbonate. Any combination of excipients may be utilised.

[00268] A wide array of delivery systems may be utilised, for example, nanoparticle delivery systems such as polymeric nanoparticles (e.g., PEG, PLGA, PLA, chitosan, etc), lipid-based nanoparticles (e.g., liposomes, micellar nanoparticles, phytosomes etc), nanocrystals/nanoshells, and inorganic nanoparticles (e.g., metal nanoparticles, dendrimers, etc). For example, phytosomes, which may include lecithin, may be used to increase absorption of the zingerone or zingerone extract, both topically and orally.

[00269] Liquid compositions may be stored, for example, in vials, bags, ampoules, cartridges, or prefilled syringes. The composition may also be transferred from a vial to a larger container and mixed with other materials. Dried or evaporated compositions may be stored, for example, in vials, cartridges, dual chamber syringes, or prefilled mixing systems. Before administration, a dry-form composition may be reconstituted as a liquid before being administered.

[00270] Exemplary unit dosages of the composition include: about 0.1 mg to about 1000 mg zingerone or zingerone extract, about 1 mg to about 500 mg zingerone or zingerone extract, about 1 mg to about 250 mg zingerone or zingerone extract, about 1 mg to about 200 mg zingerone or zingerone extract, about 1 mg to about 100 mg zingerone or zingerone extract, about 1 mg to about 50 mg zingerone or zingerone extract, or about 1 mg to about 25 mg zingerone or zingerone extract. The dosage may be formulated for administration once per week, twice per week, three times per week, every other day, once per day, twice per day, or three times per day, or more as needed. The dosage may be adjusted for paediatric, geriatric, overweight, underweight, or other patients, where required. Dosage modification can be made in accordance with standard methods. It is to be appreciated therefore that a wide range of unit dose forms may be envisioned and prepared.

Methods of using the compositions

[00271] As noted above, the disclosed compositions can be used to treat or prevent inflammation and various health conditions associated with inflammation. For example, the disclosed compositions may be utilised to reduce proinflammatory cytokine or proinflammatory small molecule levels in a subject. In particular aspects, the proinflammatory cytokine may be an interleukin cytokine such as IL-6 and/or IL-10. The proinflammatory cytokine may be a tumour necrosis factor such as TNF. The proinflammatory small molecule may be nitric oxide.

[00272] In certain aspects, the composition may comprise zingerone or a zingerone extract, as produced by the methods set out herein. The composition of the present disclosure may also be prepared as one or more pharmaceutical forms. In addition, or as an alternative to this, the composition can be prepared as one or more dietary forms, for example, a functional food or beverage, a natural ingredient (e.g., a natural additive), or a natural supplement (e.g., a dietary supplement).

[00273] In various aspects, the disclosed compositions may be used to target one or more inflammatory disorders. The inflammation is an inflammation affecting one or more of: a joint, skin, eye, ear, nose, mouth, throat, oesophagus, kidney, bladder, liver, spleen, lung, heart, brain, circulatory system, digestive system, endocrine system, genitourinary system, lymphatic system, nervous system, and skeletal system.

[00274] Disorders of interest include but are not limited to: immune system disorders (e.g., autoimmune disorders) such as Alzheimer's disease (e.g., early stage Alzheimer's disease), ankylosing spondylitis, arthritis, asthma, colitis (e.g., ulcerated colitis), Crohn's disease, dementia (e.g., early stage dementia), depression, disease, diabetes, fibromyalgia, gout, immune mediated inflammatory disease, infection (e.g., microbial infection), inflammatory bowel disease (IBD), interstitial cystitis, multiple sclerosis (MS), polymyalgia psoriasis, scleroderma, and Sjögren's syndrome, and systemic lupus erythematosus (SLE; lupus); arthritic disorders such as rheumatoid arthritis, ankylosing spondylitis arthritis, fibromyalgia arthritis, gout arthritis, juvenile idiopathic arthritis (JIA), lupus arthritis, osteoarthritis, polymyalgia rheumatica, psoriatic arthritis, reactive arthritis, scleroderma arthritis, Sjögren's syndrome arthritis; cardiac, circulatory, and pulmonary disorders such as atherosclerosis, coronary artery disease, pulmonary artery hypertension, hypoxia-induced pulmonary hypertension, pneumonia, acute respiratory distress syndrome, and coronavirus (e.g., Covid-19) respiratory disorders, cytokine storm syndrome; and neoplastic disorders including various cancers and tumours, such as breast cancer, leukaemia, multiple myeloma, myelodysplastic syndrome, pancreatic cancer, and prostate cancer.

[00275] Other disorders of interest include musculoskeletal disorders (e.g., disorders of the bones, cartilage, digits, joints, limbs, muscles, tendons, etc), and back disorders (e.g., conditions of the spine or soft tissue of the back). Also noted are inflammatory disorders associated with infections, e.g., gingival inflammation. Noted specifically are anti-inflammatory therapies for the skin. As non-limiting examples, the disclosed compositions may be used for one or more of: blisters, dermatitis, eczema, hives, lesions, papules, plaques, psoriasis, rashes, rosacea, ulcers, and wounds. Wounds may be acute or chronic (e.g., non-healing or recurring wounds). Surgical wounds and scarring are specifically noted.

[00276] In particular aspects, the disclosed compositions may be used together with one or more anti-inflammatory agents. For example, the composition may be prepared as a combined formulation with one or more anti-inflammatory agents. Alternatively, the composition may be utilised as a separate formulation along with one or more anti-inflammatory agents. Where two or more actives are used (e.g., a zingerone composition and an anti-inflammatory agent), it is possible to coordinate use by simultaneous,

sequential, or separate administration. In addition, a composition as described herein may be used in conjunction with various medical or non-medical procedures. Use of the composition may be carried out prior to, during, or after the procedure(s), or any combination thereof.

[00277] As examples, anti-inflammatory agents may include one or more constituents obtained from plants, for example, one or more plant compounds, concoctions, extractions, and/or oils. These include constituents from: manuka (e.g., *L. scoparium*), houhere (e.g., *Hoheria angustifolia*, *Hoheria glabrata*, *Hoheria lyallii*, *Hoheria populnea*, *Hoheria sexstylosa*), horopito (e.g., *Pseudowintera colorata*), kawakawa (e.g., *Piper excelsum*), koromiko (e.g., *Hebe stricta*, *Hebe salicifolia*, or *Hebe elliptica*), poroporo (e.g., *Solanum aviculare*), pukatea (e.g., *Laurelia novae-zelandiae*), and others. Extracts from manuka (e.g., manuka oil) and kawakawa (e.g., leaf extraction) are specifically noted. Also noted are constituents from *Psilocybe* spp., such as *P. azurescens*, *P. semilanceata*, and *P. cyanescens*, as well as those from *Cannabis* spp., such as *C. sativa*.

[00278] Also included are essential oils, such as those from basil, bergamot, chamomile (e.g., Roman chamomile), clary sage, clove, copaiba, eucalyptus, fennel, frankincense, helichrysum, hops, lavender, marjoram (e.g., sweet marjoram), patchouli, peppermint, rose, rosemary, tea tree, thyme, and turmeric. Further included are honey (e.g., manuka honey), arnica (e.g., arnica oil, cream, or gel), activated charcoal, capsaicin, sesame, yarrow (e.g., for various skin formulas), and comfrey (e.g., for ointments or creams).

[00279] As further examples, anti-inflammatory agents may include one or more drug compounds. Included amongst these are analgesic agents, antipyretic agents, and psychotropic agents. Exemplifications include acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, and others. NSAID compounds and salicylate compounds are specifically noted. Noted as well are opioid compounds (e.g., KOR inhibitors) and steroid compounds (e.g., corticosteroids). Exemplifications include butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, and eptazocinem. Further exemplifications include betamethasone, cortisone, deflazacort, dexamethasone, ethamethasoneb, hydrocortisone, methylprednisolone, prednisolone,

prednisone, and triamcinolone. Cannabinoid compounds and mushroom compounds are also noted. Exemplifications include cannabidiol, cannabigerol, and tetrahydrocannabinol. Further exemplifications include psilocybin and psilocin.

[00280] Various anti-inflammatory agents may be adapted and utilised in accordance with this disclosure. Any combination of anti-inflammatory agents can be utilised. Any medical devices and procedures may also be used in conjunction with the disclosed compositions and extracts.

[00281] As described herein, compositions of the present disclosure are useful as anti-inflammatory formulations. In particular aspects, compositions can be used in methods to reduce or delay inflammation in certain tissue. This tissue includes: a joint, skin, ear, eye, nose, mouth, gums, throat, digestive, cardiac, circulatory, pulmonary, vaginal, and urinary tract tissues, and others noted herein. The compositions may be applied, for example, to burns, to lessen the chance of inflammation, or to the skin before surgery, to combat inflammation on the skin around the operation site. The compositions may be used as hand cleansers (e.g., soaps or hand sanitisers), to be applied with or without water. The compositions may be used for minor skin irritations, cuts, or grazes. The compositions may be used as mouthwashes or gargles, for example, to combat inflammation from mouth sores or gingival inflammation. Compositions may also be utilised as lozenges and throat sprays, for example, to relieve a sore throat. Eye drops or ointments may be used to combat inflammation of the eye, including the eye lid.

[00282] The compositions of the present disclosure also find use as formulations, which can be used in methods for treating or preventing inflammations and inflammatory disorders, as described herein. The inflammation may affect one or more physiological components, including one or more parts of: the articular system, circulatory system, respiratory system, digestive system, renal system, excretory system, reproductive system, integumentary system, nervous system, lymphatic system, endocrine system, muscular system, skeletal system, and sensory system.

[00283] Various routes of administration may be used for the compositions, including parenteral (e.g., topical) and enteral (e.g., oral) administration, as described herein. Enteral administration may be by duodenal tubing or gastric tubing, including nasogastric tubing, or other standard means. Oral administration may be by tablets, capsules, sachets, drops, elixirs, linctuses, solutions, emulsions, suspensions, draughts,

purees, pastes, pomaces, syrups, gels, jellies, gummies, tonics, or various other means. Topical administration may be by drops, sprays, ointments, soaps, pads, sponges, dressings, bandages, or various other means. Standard modes of administration may be utilised by a skilled person. The compositions disclosed herein are not limited to a particular form for administration.

[00284] As exemplary dosages, the compositions may be administered at a dose of about 1 to about 3000 mg zingerone or zingerone extract, or about 100 to about 3000 mg zingerone or zingerone extract, or about 100 to about 2500 mg zingerone or zingerone extract, or about 100 to about 2000 mg zingerone or zingerone extract, or about 1 to about 1800 mg zingerone or zingerone extract, or about 100 to about 1600 mg zingerone or zingerone extract, or about 100 to about 1400 mg zingerone or zingerone extract, or about 100 to about 1200 mg zingerone or zingerone extract, or about 100 to about 1000 mg zingerone or zingerone extract. Additional exemplifications include about 10 to about 300 mg dose of zingerone or zingerone extract, or about 10 to about 200 mg dose of zingerone or zingerone extract, or about 10 to about 150 mg dose of zingerone or zingerone extract, or about 10 to about 100 mg dose of zingerone or zingerone extract, or about 10 to about 80 mg dose of zingerone or zingerone extract, or about 10 to about 60 mg dose of zingerone or zingerone extract, or about 10 to about 55 mg dose of zingerone or zingerone extract, or about 10 to about 50 mg dose of zingerone or zingerone extract, or about 10 to about 40 mg dose of zingerone or zingerone extract. These ranges of dosages are particularly useful for a ginger component (e.g., zingerone or zingerone extract) that is dried and milled to a powder.

[00285] The European Food Safety Authority (EFSA) has classified zingerone safe for animal consumption (e.g., rats) with No Observed Adverse Effects (NOAEL) based on a dose of up to 128 mg/kg/day (EFSA, 2016, 14(8):4557). Exemplary dosages may be determined, for example, for an average 70 kg human subject. Such exemplary dosages may include, for example, about 1 mg/kg to about 50 mg/kg, or about 5 mg/kg to about 50 mg/kg, or about 5 mg/kg to about 45 mg/kg, or about 5 mg/kg to about 40 mg/kg, or about 5 mg/kg to about 45 mg/kg, or about 5 mg/kg to about 35 mg/kg, or about 5 mg/kg to about 30 mg/kg, or about 5 mg/kg to about 25 mg/kg, or about 5 mg/kg to about 20 mg/kg, or about 5 mg/kg to about 15 mg/kg for zingerone or zingerone extract. Additional exemplary dosages may include, for example, about 0.1 mg/kg to about 20 mg/kg, or about 0.1 mg/kg

to about 15 mg/kg, or about 0.1 mg/kg to about 10 mg/kg, or about 0.1 mg/kg to about 8 mg/kg, or about 0.5 mg/kg to about 6 mg/kg, or about 0.1 mg/kg to about 4 mg/kg, or about 0.1 mg/kg to about 2 mg/kg, or about 0.1 mg/kg to about 1 mg/kg, for zingerone or zingerone extract.

[00286] The dosages as indicated herein may be administered once per week, every other day, once per day, twice per day, three times per day, or less or more, as needed. Administration may be made with food, or before a meal. The appropriate dosage and dosage form will be readily determined by skilled persons.

EXAMPLES

[00287] The examples described herein are provided for the purpose of illustrating specific embodiments and are not intended to limit this disclosure in any way.

Example 1: Preparation of Zingerone

[00288] An initial sample of fresh ginger (400 gm) as shown in Figure 1 was sourced from New Zealand and cleaned of dirt and soil. The cleaned ginger was then diced or chopped finely and subjected to alkaline treatment (800 g of 0.5% potassium hydroxide in distilled water). The resulting mixture was stirred and left in an oven at 60°C for 22 hours. It is to be appreciated that the resulting mixture could also be placed in a water bath and maintained at around 60°C for the desired time period.

[00289] The pH of the mixture was then adjusted to pH 7 by addition of concentrated citric acid and the treated plant material was spread out on a metal tray and dried in an oven at 60°C for 20 hours. The resulting dried material weighed 35 grams resulting in a dry yield of 8.75%. The dried material was then scraped into a flask and covered with 95% ethanol for extraction (210 ml). The flask was shaken and placed in an oven at 40°C for 16 hours. The extract (Extract 1) was filtered off using a glass funnel with a glass wool plug. The remaining plant material was extracted again with 95% ethanol (Extract 2) and then twice more with 50% ethanol (Extracts 3 and 4).

[00290] The extracts were analysed directly for zingerone content. A 294 mg chopped sample of the fresh ginger was also extracted with 2 ml of ethanol and this extract was also analysed. The results are shown in Table 1.

Table 1. Extract composition

Extract	Volume (mL)	Weight (g)	Total zingerone (mg)	Total 6-gingerol (mg)	*Dried Weight (g)
1	142	119	67	94	4.04
2	140	116	14	21	1.18
3	112	93	8	15	
4	104	86	3	9	
Totals			92	139	

* The dry weight of Extract 1 and Extract 2 was estimated by drying a 5 ml portion of each extract.

Results

[00291] The 400 g fresh ginger root supplied was shown to contain 260 mg of 6-gingerol (i.e. 0.65 mg/g). This would mean the theoretical maximum yield of zingerone would be in the order of 171 mg from the treated material (loss of weight due to lower molecular weight of zingerone vs 6-gingerol). About 50% of the 6-gingerol was unconverted. It is envisioned that further studies could be used to increase the alkaline conversion of 6-gingerol to zingerone.

[00292] The ethanolic extraction was done using the minimal volume needed to cover the treated plant material. This meant that Extract 1, the most concentrated one, had a zingerone content of 0.47 mg/mL. It is envisioned that this concentration could, in principle, be increased using either a multi-steeping process or by evaporation of some of the ethanol. Reducing the volume of ethanol by 80% would yield a solution with 2.35 mg zingerone/mL.

[00293] It is envisioned that drying the extract fully would give a concentration of approximately 16 mg/g of solid extract. Higher concentrations would be expected if starting with a higher initial content of 6-gingerol and/or with more complete conversion. A theoretical 25 mg dose, for example, could then potentially be achieved by formulation of the dried extract directly into an oil or glycerol carrier to give the required zingerone dose in one or two 500 mg capsules.

[00294] The majority of the zingerone was extracted in the first extraction. There are situations where double extraction would be beneficial. The third and fourth extraction increased the totals by modest amounts. Overall, ethanol extraction was seen to be highly effective, and very efficient and inexpensive as a preparative method.

Example 2: Preparation of Zingerone

[00295] Overview: These studies show that an aqueous alkaline treatment followed by freeze-drying of fresh ginger greatly improved conversion, reaching a ~1% zingerone content in the dry ginger. The treated ginger was then extracted with supercritical CO₂ and CO₂ + ethanol co-solvent with a combined extraction yield of 3% and an average concentration of zingerone in the extracted oleoresin of approximately 12%. In addition, it was found that drying of fresh ginger at a moderate temperature (60°C) followed by supercritical CO₂ extraction resulted in an extraction yield of 4.6%. The extracted oleoresin was then alkaline treated and the final product contained approximately 15% zingerone.

[00296] Drying: Samples of the fresh ginger material imported from Fiji were sliced into 2-5 mm slices and placed on a single layer on perforated oven trays. Drying was performed in a forced convection. Drying was carried out at a moderate temperature (60°C) with the goal of eliminating moisture without conversion of gingerol. This process was considered finished when the moisture content of the ginger reached 7%. The dried ginger obtained was stored refrigerated until it was used in the extraction trials.

[00297] Catalysed conversion: Small scale, preliminary trials were performed by treating around 1.6 g of fresh chopped Fijian sourced ginger with aqueous solutions of 0.5% KOH (pH 14), 1% Ca(OH)₂ (pH 11.6) and 1% sodium carbonate (pH 10.5). The volume:weight ratio of reagent added:ginger was around 3:1. The samples were then shaken and placed in a fumehood at room temperature, or ovens at 37°C or 60°C, overnight, before being analysed. It is to be appreciated that suitable water baths could be used to keep the samples at the desired temperature for the desired period of time.

[00298] Once the alkaline treatment had been selected, 5.2 kg of fresh ginger was minced using a vertical cutter mixer (RobotCoupe R45). The ginger was then mixed with a 3:1 liquid:solid ratio (volume: weight) of KOH 0.5% (~0.1 N), resulting in a pH ~ 12.5. The mixture was manually stirred and placed in an oven at 60°C for 24 hours. After this time, the mixture was neutralised by adding concentrated citric acid (625 g/L) to a pH ~ 7.2. The neutralised mixture was then freeze-dried, and the ginger obtained in this process was stored refrigerated until it was used in the extraction trials.

[00299] Catalysed conversion results: As noted, different alkalis were tested: 0.5% potassium hydroxide or KOH (pH 14), 1% calcium hydroxide or Ca(OH)₂ (pH 11.6), and 1% sodium carbonate or Na₂CO₃ (pH 10.5). The content of zingerone and 6-gingerol was

quantified for these experiments (Table 2), and it was concluded that a treatment with KOH at 60°C was the most efficient and gave the highest zingerone concentration. Table 2 shows the amounts of 6-gingerol mg/g and zingerone (mg/g) obtained for the respective conditions, with results expressed on a wet basis. HPLC traces are shown in Figure 2. It can be seen from the HPLC traces that Ca(OH)₂ at 1% and KOH at 0.5% perform similarly. At 60°C for KOH 0.5% the ratio of zingerone:6-gingerol is 6.5. At 60°C for Ca(OH)₂ 1.0% the ratio of zingerone:6-gingerol is 4.0.

Table 2. Peak areas at 280 nm for zingerone and 6-gingerol in treated samples

Alkali	Temperature	6-gingerol (mg/g)	Zingerone (mg/g)
KOH	Room temperature	1.4	0.2
KOH	37°C	1.0	0.3
KOH	60°C	0.4	0.7
Na ₂ CO ₃	Room temperature	1.4	0.1

[00300] This treatment was applied to a larger sample of fresh ginger (5.2 kg) and the resulting treated ginger was then neutralised and freeze-dried. The yield of the freeze-dried ginger was 17%, i.e., 17 g of treated, freeze-dried ginger per 100 g of minced raw ginger. The zingerone and 6-gingerol content of the freeze-dried ginger was measured at 10.2 and 3.3 mg/g, respectively (dry basis). See Table 2-1. The zingerone content was at least 10 times higher than that of the oven dried ginger.

Table 2-1: Composition of treated and freeze-dried ginger

		Wet basis	Wet basis	Dry basis	Dry basis
Treatment	Moisture	6-Gingerol (mg/g)	Zingerone (mg/g)	6-Gingerol (mg/g)	Zingerone (mg/g)
KOH 0.5% at 60°C + freeze-dried	4.5%	3.2	9.8	3.3	10.2

[00301] Extraction: Supercritical extraction trials were carried out using the alkaline treated ginger. The alkaline treated, freeze-dried ginger was lightly crushed by hand and placed in a 2 L extraction vessel with sintered filter discs at both ends, filling the vessel completely. The extraction was carried out as described above until a sharp decrease in the extraction rate was observed, corresponding with a CO₂:feed ratio of 13:1. At this point, the ethanol co-solvent pump was started, and ethanol was added to the CO₂ stream with a ratio of approximately 10 wt% (i.e. 10 g ethanol per 100 g CO₂). The ethanol pump

was stopped after a 2:1 ethanol:feed had been introduced (2 g ethanol per g of feed). CO₂ was then circulated to flush out any ethanol remaining in the bed. When the extraction was finished, the plant was depressurized, and the residual marc was allowed to degas overnight before being unloaded. The ethanol present in the extract was removed by rotary evaporation under vacuum. Extraction parameters are listed in Table 3.

Table 3. Summary for extraction parameters

	Extraction 1	Extraction 2
Feed material	Untreated ginger (dried at 60°C)	Alkaline treated ginger (freeze-dried)
Solvent phase	CO ₂	CO ₂ => CO ₂ + ethanol
Extraction pressure	300 bar	300 bar
Extraction temperature	40°C	40°C
Separator pressure	55 bar	55 bar
Separator temperature	40°C	40°C
Feed mass	358.6 g	322.41 g
Average CO ₂ flow rate	2.8 kg/h	2.8 kg/h
Average ethanol flow rate		4.5 g/min
Ethanol %		10%
Ethanol to feed ratio		2:1
CO ₂ to feed ratio	37.5:1	55:1

[00302] Analysis: Samples were prepared for analysis by addition of methanol after neutralisation as needed. Extracts were dissolved directly in methanol. Analysis was by HPLC with an acetonitrile/0.1% formic acid gradient. Detection was performed at 280 nm, and the column used was a Phenomenex Kinetex C18 (150 X 2.1 mm). Zingerone eluted at around 2 minutes and 6-gingerol at 5.2 minutes. Quantification of zingerone and 6-gingerol was obtained from a standard curve prepared using analytical standards of these compounds.

[00303] Extraction results: As noted, the alkaline treated and subsequently freeze-dried ginger was extracted with CO₂ followed by CO₂+ethanol co-solvent. No free water was observed in the CO₂ extract, and the ethanol obtained in the CO₂+ethanol extract was removed by rotary evaporation under vacuum. The extracts had a sweet, caramelized fragrance. The yield obtained with CO₂ was 1.5%. Addition of 10% ethanol co-solvent allowed for extraction of an additional 1.5%. The composition of the different fractions is shown in Table 4 and Table 5. See, also, Figure 3.

Table 4. Composition of extracts obtained from alkali treated ginger

		6-Gingerol (mg/g)	Zingerone (mg/g)
untreated	CO ₂ extract	290	0
treated	CO ₂ extract	103	153
treated	CO ₂ extract + ethanol	52	91
treated	Marc	1.2	5.4

Table 5. Zingerone (Z) mass balance for the extraction of alkali treated ginger

Feed			CO ₂ extract			CO ₂ extract + EtOH extract			Marc			Mass balance
g	Z mg/g	Z mg	g	Z mg/g	Z mg	g	Z mg/g	Z mg	g	Z mg/g	Z mg	
322	9.8	3143	4.8	153	735	4.7	91	428	312	5.4	1687	90.6%

[00304] It can be seen from the results that for the alkali treated sample, the CO₂ extract contained 153 mg/g of zingerone (15.3%) and 103 mg/g (10.3%) 6-gingerol, with a zingerone/gingerol ratio of approximately 1.5. The CO₂+ethanol extract contained 91 mg/g of zingerone and 52 mg/g of gingerol (zingerone/gingerol ratio of approximately 1.75). The marc or residual ginger post extraction was also analysed, and found to contain 5.4 mg/g of zingerone. When the mass of feed, extracts and marc is taken into account (Table 4), the zingerone mass balance can be calculated at 90.6%. This indicates that 90.6% of the zingerone present initially in the feed is accounted for in the extracts and marc. The difference could be caused by degradation during extraction or in the ethanol removal step. The zingerone extraction yield (i.e., grams of zingerone extracted per 100 g of zingerone in the feed) was only 37% when calculated based on the extracts. However, 54% of the initial zingerone present in the feed remains unextracted in the marc, so the zingerone extraction yield can also be calculated as 46% when based on the marc results. This accounts for the “missing” zingerone. Since the proportion of unextracted zingerone is significant, the extraction process could be further improved to reduce this. The extraction yield of gingerol is higher than that of zingerone (82% in the untreated sample and 71% in the treated sample), since it is more soluble in CO₂.

[00305] In another experiment, a CO₂ extract obtained from untreated ginger root was subjected to an alkaline treatment to study the conversion of gingerol into zingerone. Overnight treatment with both 0.1 N and 1 N KOH at 60°C worked well, with a resulting concentration of zingerone in the treated ginger of around 15%. The resulting material appeared to be much cleaner than the alkaline treated crude ginger, so is an interesting alternative process that could provide an even more cost effective extraction process

overall as the conversion process is on a smaller volume of material. In fact, for the same amount of starting fresh ginger (100 kg), and based on the results obtained in this work, the extraction of untreated ginger followed by alkaline treatment of the extract would yield almost twice as much zingerone in the final product than the alternative process (see comparison in the table below). However, even in this case, the overall zingerone yield of the process is ~0.1% (0.1 kg zingerone per 100 kg fresh ginger). It is expected that further optimisation is possible.

[00306] In another experiment a sample of CO₂ extract of untreated ginger was mixed with KOH and converted to a zingerone rich extract. The post-treatment neutralisation step involved the separation of the zingerone as a zingerone enriched resin from the aqueous reaction mixture. 3 grams of oleoresin was taken in duplicate and 9 ml of 1 N KOH was added to the samples in a plastic vial. The samples were then shaken and left overnight in a 60 degrees oven. The treated samples were then neutralised by the addition of 10 ml of 1 N HCl. This was a small excess of acid to ensure that all of the KOH was neutralised. The addition was performed in two steps with mixing after each step. The samples were centrifuged at 2000 rpm to separate the water from the oleoresin. After centrifugation the bulk of the water was then removed by pipette. The resultant resin was then removed. Absolute alcohol was added to one of the resin samples (5 ml ethanol in approximately 3 grams resin) to produce a tincture (Sample 1). The other sample was kept in resin form (Sample 2). Sample 1 and Sample 2 were analysed for zingerone content. The concentration of zingerone in the tincture (Sample 1) was calculated at 23 mg/g of tincture while that of the treated resin (Sample 2) was 52 mg/g.

[00307] These values for both Samples 1 and 2 were lower than those obtained with earlier treatments for which 150 mg/g zingerone content was estimated. In this work the resin Sample 2 had been separated from the water and analysed. Subsequently the separated water was also analysed and estimated to contain another 50-55 mg (approximately 25%) of zingerone for each 3 g batch. Zingerone has been reported as having quite limited water solubility so this result was quite unexpected. Because it appears that a significant portion of the zingerone is staying in the water and not separating out with the resin, this method is contrasted to direct addition of alcohol to the crude neutralised product.

[00308] Because the water is able to extract some of the zingerone from the resin it may also be possible to produce a high yield of zingerone after treatment by drying down the total neutralised alkaline treatment product. Further process optimisation is to be undertaken to improve yields given the finding that after alkaline treatment zingerone appears to be more soluble in water than previously reported.

Example 3: Further reaction methods and comparisons

[00309] Overview: Zingerone is not naturally present in ginger; it is a conversion product from gingerol through a treatment process. In these studies, a sample of alkaline treated and dried ginger was received from Samoa and extracted with ethanol using two different sets of extraction conditions: extraction at 40°C for 20 h (Extract A) and at room temperature for 7 days (Extract B). The resulting zingerone concentration of the extracts was found to be 41.4 mg/g for Extract A and 43.8 mg/g for Extract B.

[00310] Extraction A: The treated ginger produced by SROS (Scientific Research Organisation of Samoa) was supplied in two separate plastic bags. The contents of both bags were combined, frozen at -80°C and subsequently milled using a knife mill (Wiley) with a 2 mm mesh attached. The milled material (782.3 g) was then placed in a round bottom flask along with food grade ethanol with a ratio of approximately 5:1 by weight. The flask was then placed in a water bath at 40°C and 5 rpm stirring overnight (total extraction time 20 hours). After this time, the mixture was filtered under vacuum and the ethanol was removed by rotary evaporation under vacuum to produce 43.8 g of highly viscous, dark brown resin with a characteristic ginger aroma. Extraction yield was 5.6%. 10 g of this resin (Extract A) were taken out and stored refrigerated and under nitrogen flush for possible future bioassays.

[00311] Extraction B: The ginger was frozen and milled as described above. The milled material (785.9 g) was placed in a bucket along with food grade ethanol with a ratio of approximately 5:1 by weight. The ginger was macerated in ethanol at room temperature (22-29°C) over 7 days. Samples were taken on days 1, 3 and 7. After 7 days, the mixture was filtered under vacuum and the ethanol was removed by rotary evaporation under vacuum to produce 49.3 g of highly viscous, dark brown resin with a characteristic ginger aroma (Figure 1), very similar to the one obtained in Extraction A. 10 g of this resin

(Extract B) were taken out and stored refrigerated and under nitrogen flush for possible future bioassays. Extraction yield was 6.3%.

[00312] Zingerone and aldehyde analysis: Quantification of zingerone was carried out by HPLC in the starting material (i.e., treated ginger) and the final two resins, as well as in the samples on days 1, 3 and 7 for Extraction B (note that the liquid sample from day 7 is equivalent to the final resin sample). The HPLC quantification method included methanol addition and grinding the sample before analysis. Zingerone content for all fractions is shown in Table 6. Zingerone mass balance and yield are shown in Table 7.

Table 6. Comparing extraction conditions – zingerone content for different fractions

	Treated ginger	Day 1 sample	Day 3 sample	Day 7 sample	Final resin
Zingerone extract A	3.1 mg/g				41.4 mg/g
Zingerone extract B	3.1 mg/g	0.38 mg/ml	0.44 mg/ml	0.4 mg/ml	43.8 mg/g

Table 7. Zingerone mass balance and yield

	Feed (g)	Zingerone in feed (mg/g)	Zingerone in feed (g)	Extract (g)	Zingerone in extract (mg/g)	Zingerone in extract (g)	Zingerone yield	Extraction Yield
Ext A.	782.3	3.1	2.4	43.8	41.4	1.8	75%	5.6%
Ext B.	785.9	3.1	2.4	49.3	43.8	2.2	89%	6.3%

[00313] As indicated above, Extract A was obtained by treatment at 40 degrees, duration 20 hrs, while Extract B was obtained by treatment at room temperature, duration 7 days. The results for Tables 6 and 7 show that higher levels of zingerone were obtained by longer treatment at room temperature, although high yields were also obtained by a temperature increase to 40°C.

[00314] To determine zingerone content in the starting material, this was first extracted into a suitable solvent. In one process, this extraction was carried out with ethanol resulting in a lower zingerone content (1.44 mg/g). In a second process, this was carried out using methanol and grinding the ginger along with the solvent in a mortar and pestle. This resulted in a higher zingerone content (3.1 mg/g). For reference, the zingerone content reported by the Samoan lab for this material was 1.86 mg/g.

[00315] Based on this, the zingerone yield (i.e., the amount of zingerone in the extract relative to the zingerone in the feed) was estimated at 75% for Extraction A and 89% for Extraction B. The 6-gingerol peak seen in the HPLC analysis when determining

zingerone content was consistently observed at around 1/8th the peak area of zingerone. This suggests that extraction had little effect on the zingerone to gingerol ratio.

[00316] A sample of both final resins was taken up in ethanol and examined by GCMS for aldehyde analysis. A very low level of hexanal was seen (too low to quantify). Hexanal is a side product of the reaction to form zingerone but is somewhat volatile. The identity of the hexanal peak was confirmed by library matching of MS data and a separate injection of a hexanal standard.

Discussion

[00317] Each of the studies described herein was effective in producing zingerone. A comparison of the results from Examples 2 and 3 is provided in Table 8.

Table 8. Comparison of zingerone yields

	Zingerone	Difference to fresh ginger
Fresh ginger	0.1 mg/g	Baseline
Dried ginger (180°C, 10 hr)	0.7 mg/g	7-fold higher
KOH 0.5% at 60°C + freeze-dried (Example 2)	10.2 mg/g	102-fold higher
Disclosed processing method Samoan ginger (Example 3)	43.8 mg/g	438-fold higher
Disclosed processing method Fijian ginger (Example 2)	310 mg/g expected	3100-fold higher
Disclosed processing method Fijian ginger (Example 6)	88.9 mg/g	889-fold higher
Disclosed processing method Fiji ginger (Example 5)	177.8 mg/g expected	1778-fold higher

[00318] Similar values for fresh and dried ginger were obtained by Li *et al.* See Li *et al.*, 2016, “Chemical characterization and antioxidant activities comparison in fresh, dried, stir-frying and carbonized ginger” *J Chromatogr B Analyt. Technol. Biomed. Life Sci.* 1011: 223-32. Example 2, as described above, advances well beyond a standard retro-aldol reaction. Example 3, as described directly above, provides further advancements, utilising temperature and pH adjustments, and extraction. Further advancements are provided in Examples 5, 6, and 12, as described herein.

[00319] Regarding the Samoan ginger, it is noted that the ginger was not harvested at the requested time (9 months in the ground), and this affected the level of gingerol

present in the ginger and subsequently the zingerone content in the end product. Therefore, it is expected that further gains can be obtained. Regarding the Fijian ginger, this was substantially higher in zingerone compared to the Samoan ginger ($10.2 : 1.44 = 7.08 \times$ higher). This means that the total yield can be extrapolated for the Fijian ginger as 310 mg/g, if the experimental conditions of Example 3 were to be applied. That is: 43.8 mg/g (amount obtained from Samoan ginger in Example 3) $\times 7.08$ (higher starting content in Fijian ginger) = 310 mg/g.

[00320] Table 5 in Example 2 shows the output of the pH treated ginger followed by CO₂ extraction. It was found that 322 grams of fresh ginger provides 153 mg/g zingerone. In comparison to this, Example 3 utilises 785.9 grams (2.4 times more compared to amount of product used in Example 2) and provides 43.8 mg/g zingerone. However, this lower yield can be explained by the lower levels in the starting material from Samoa (1.44 mg/g zingerone).

Example 4: Processing methods using juicing and alkaline treatment

[00321] Summary: Ginger root was mechanically juiced and the levels of 6-gingerol were determined for juice and remnant solids. The majority of the 6-gingerol was present in the juice. Treatment of the juice with alkali showed that effectively all of the 6-gingerol was converted to zingerone in 5-6 hrs at 60°C.

[00322] Overview for juicing: Fresh ginger (500 to 1000 g) was pre-treated by blending/macerating and pressing the ginger. The liquid fraction was retained, and the marc was further washed with warm water (4 parts water to 1 part ginger) at a temperature of 55-60°C for a period of 10-15 minutes. This was done in a covered vessel. This was then pressed again. Each fraction was analysed for 6-gingerol content (a total of 5 analyses), namely: 1) Fresh ginger sample immediately prior to processing; 2) Liquid fraction following initial blending/maceration; 3) Ginger marc following initial blending/maceration; 4) 2nd liquid fraction collected following further washing of initial marc; 5) Final ginger marc following washing then pressing as above. A moisture content reading was made on each lot of ginger marc, after final pressing (i.e. samples 3) and 5) above).

[00323] Overview for alkaline treatment: An alkaline treatment was performed on the liquid fractions to establish the conversion to zingerone. Samples were taken at a range of timepoints and analysed for zingerone content. The samples were subjected to small

scale treatments with KOH (5% as previously). Treatment was carried out using 1 ml samples at room temperature, 30°C and 60°C samples at 1, 2, 3 and 5 hours. Additionally, one sample was treated at 60°C for 24 hours. Analysis of up to 15 samples was carried out.

[00324] Juicing method: Two samples of fresh ginger root were obtained, one locally (organic, from Evi the in Petone) and a second received from Phil Rasmussen in Auckland. Both samples appeared to be plumper/juicier than normal supermarket ginger root. The moisture content was determined by slicing up approximately 10 g of each root, freezing with liquid air and then freeze drying. The 6-gingerol content was determined extraction of root with methanol. For this, around 5 g of each sample was cut into 4-5 pieces and crushed using a small kitchen garlic press. The crushed root and juice were extracted with methanol (2 x 15 ml) at 60°C for 20 minutes. This was followed by HPLC analysis with detection at 280 nm. The results are shown in Table 9, below.

Table 9. Moisture content of gingers

Sample	% solid after freeze drying	6-gingerol content (mg/g)
Auckland sample	16	1.46
Petone sample	5.3	1.58

[00325] The Auckland sample was chosen for the juicing work. For this, 635 g ginger root was processed using a home juicer. This contained a rotating screw drive with mesh juice filter and adjustable solids nozzle (see Figures 4A-4B). The juicer removed 516.3 g (81%) of liquid (juice 1, J1) with 101.5 g of solid (marc 1, M1) collected. Some of the dry solid was removed for analysis and 90.5 g extracted with 360 ml of hot tap water. This was allowed to sit for 15 minutes before running this material through the juicer. From this step, 350 g of juice (juice 2, J2) was recovered along with 70.2 g of solid (marc 1, M2).

[00326] The two liquids were refrigerated overnight. Both were cloudy with settled solids. These samples were shaken up prior to analysis or treatment. The juice samples were analysed for 6-gingerol content by mixing a sample with ethanol (1:1), centrifuging and direct injection of the supernatant. The solid content of the two pressed solids M1 and M2 were 37% and 26.3% respectively.

[00327] The results are shown in Table 10, below. The gingerol values were multiplied by weight to give the total amounts of gingerol in each material. The juice was found to contain 81.6% of the measured gingerol. The total gingerol calculated for the feed

was lower than the recovered amount suggesting a partial extraction of gingerol from the root. The percentage gingerol was based on the total measured gingerol for the marc and juices (rather than the feed measurement).

Table 10. Weights and gingerol content

sample	weight (mg)	6-gingerol (mg/g)	total 6-gingerol (mg)	% 6-gingerol
Feed	635	1.46	928	
Juice 1	516.3	1.58	814	75
Juice 2	350	0.2	72	6.6
Marc 1	101.5	1.95	198	16.3
Marc 2	90.5	1.816	146	13.4
Sum of Juice 1, 2 + Marc 2			1085	

[00328] Alkaline treatment: This work was performed on the first juice (J1) recovered from the juicing process (above). Samples of juice (after shaking to suspend all solids) were allowed to react with KOH at RT, 30 or 60°C for 1, 2, 3, 5, and also at 60°C for 24 hours. Three concentrations of KOH were also trialled, 0.5, 1.0 and 2.0%. A solution of 2 N KOH was prepared (5.6 g KOH in 50 ml water). To generate 0.5%, 1% and 2% KOH concentrations in each sample, 0.25, 0.5 or 1 ml of the 2 N KOH was added to 5.5 ml of juice and shaken. The samples were then placed at RT (lab), 30°C (water bath) or 60°C (drying oven). Sampling for HPLC analysis was done by taking 200 µl from each sample, adding 200 µl of 1 N HCl, and then 500 µl of ethanol. After centrifugation the sample was directly injected into the HPLC. The peak areas of zingerone and gingerol were compared (see below).

[00329] These results demonstrated that treatment with 2% KOH was able to achieve complete conversion to zingerone within 5 hours. See Figure 5C. Incubation at 60°C was particularly favourable. See Figure 5C. The results are presented as peaks areas for zingerone (Z) and gingerol (G). It was noted that the KOH treated samples had solid present which settled in the tube. For analysis, the tubes were shaken and a sample was taken with a wide bore tip to avoid plugging.

Example 5: Additional processing methods using juicing and alkaline treatment

[00330] Overview: The aim is to produce an extract from ginger having the 6-gingerol converted to zingerone by an alkaline-catalysed reversed aldol reaction. The current process seeks to reduce treatment time and water use. This process was trialled at a scale of roughly 40 kg before further production at a 200 kg scale will be undertaken.

[00331] In brief, fresh ginger was received and processed by treating it in alkali followed by freeze-drying to produce a treated ginger powder. This powder was extracted with ethanol at room temperature for 3 days, with samples taken at 24, 48 and 72 hours to assess the progress of the extraction. The ethanol extraction is detailed in Example 6.

[00332] Methodology: Alkaline pre-treatment and drying of imported fresh ginger were carried out. For this, fresh ginger (36.67 kg) was pressed in a Vincent Corporation CP-4 screw press to make two streams: a ginger juice and a marc. Screw Press settings were a VSD speed of 50%, and a cone air pressure of 2 bar. The marc from the initial press was pressed a second time to remove any remaining juice.

[00333] The two juices were combined. The juice was heated to 60°C and KOH was added to a final concentration of 2% w/w. The alkalised juice was held at 60°C for 5 hours to convert gingerol to zingerone. The juice was neutralised to a pH of 7.2 by addition of anhydrous citric acid. The juice, now containing zingerone, was freeze-dried and ground.

[00334] Samples were taken of: the fresh ginger (ZINGO); the two marcs (GMARC and GMARC2); the juice before KOH additions (GKOH0); the juice after 2, 3, and 5 hours of treatment (GKOH2, GKOH3, GKOH5); the final dried extract (GPE).

[00335] In addition, a sample of GMARC2 was extracted with hot water as follows. Water was added to GMARC2 at a ratio of 5:1 w:w. The mixture was heated to 60°C and held at this temperature for 15 minutes. The extract was separated from the solids by screw pressing with the same settings as above. Samples were taken of: the extract (GMARC2 HWEX); the marc (HW MARC). Each sample was analysed for total solids (LOD, 16 hrs 100°C) and gingerol or zingerone content by HPLC.

[00336] Sample analysis: Analysis was performed using HPLC with UV detection at 280 nm. Sample preparation was as follows: (1) liquid samples such as juice were diluted 1:1 with ethanol and centrifuged. In-process liquid samples with alkali present were diluted with 1 N HCl and ethanol 1:1:1 (2) solid samples such as raw ginger or ginger marc were extracted by double extraction with ethanol (ultrasonication, heat at 60°C for 20 minutes, vortexed and centrifuged) and combining supernatants. Solid extracts (from approximately 5 g) were generally made up to 50 mL for analysis. The raw fresh ginger was roughly chopped and then blended with ethanol using an ULTRA-TURRAX® type

mixer. Quantification was performed by comparison to a standard curve prepared using zingerone. A molecular weight correction was made for gingerol.

[00337] Pressing: 36.67 kg of raw Fijian ginger was received and pressed. Pressing was effective, producing a large volume of light green juice, and a fibrous marc. 28.92 kg of juice was recovered from first press. 6.6 kg of marc was then pressed a second time to recover an additional 2.18 kg of juice. Total yield of juice was 31.1 kg, equivalent to 86% of the incoming raw ginger by mass. The final marc mass recovered was 4.05 kg. There was typically 1-2 kg of holdup in the screw press at the end of a run. This gave rise to a minor difference noted between the feed mass and the combined mass of marc and juice.

[00338] Hot water extraction: 3.62 kg of GMARC2 and 18.1 kg of water were heated to 60°C and then separated by screw pressing after 15 minutes of extraction at 60°C. Next, 21.18 kg of mixture was pressed. Noting that approximately 500 g of water was lost as evaporation during the extraction. From this, 17.62 kg of extract was recovered and subsampled for analysis. In addition, 2.69 kg of marc was recovered.

[00339] Alkaline treatment: For this, 1.236 kg of 50% KOH solution was added to the 31.1 kg of ginger juice, to reach a target KOH concentration of 2%. The pH after KOH addition was 12.18. On addition of KOH the colour of the juice changed for a light green to a reddish brown. The juice was held at 60°C for 5 hours, and then neutralised by the addition of 500 g anhydrous citric acid. The pH after citric acid addition was 7.23.

[00340] Freeze drying: The treated juice was transferred to freeze dryer trays and frozen overnight before transferring to the Cuddon FD80 freeze dryer. A total of 28.51 kg of juice was loaded onto trays and dried. Approximately 3 kg of juice was lost prior to freezing and drying as a result of manual handling. After drying, 2.86 kg of dried extract was collected- a total of 10% of the mass of juice dried. This was ground and subsampled. After grinding and subsampling and handling losses a total of 2.19 kg was packed into foil bags for storage until further processing. Approximately 1.6 kg of this was sent for ethanol extraction (see Example 6).

[00341] Mass balance summaries are shown below.

Juicing

Inputs	fresh ginger	100 kg
Outputs	ginger juice	85.79 kg
	ginger marc	14.21 kg

Alkaline treatment

Inputs	ginger juice	85.79 kg
	50% KOH solution	3.37 kg
	anhydrous citric acid	1.36 kg
Outputs	treated ginger juice	90.53 kg

Freeze drying

Inputs	treated ginger juice	90.53 kg
Outputs	dried ginger extract	9.08 kg

[00342] Gingerol and zingerone levels were measured as described above. The results are shown in Table 11, below.

Table 11. Gingerol and zingerone levels

Sample	6-Gingerol (mg/g)	Zingerone (mg/g)	Basis
ZING0 (feed)	0.522	N/A	Wet
GMARC 1	1.08	N/A	Wet
GMARC 2	1.99	N/A	Wet
MARC HW	0.22	N/A	Wet
GKOH-0	6.90	0.00	Dry
GKOH-2	0.93	5.76	Dry
GKOH-3	0.90	5.74	Dry
GKOH-5	0.79	5.32	Dry
GPE	N/A	5.70	Dry

[00343] This table shows the gingerol content of different fractions during pre-treatment and the treatment process. From these measurements, there was a total of 19.1 g of 6-gingerol in the 36.67 kg of raw ginger feed. The dried ginger extract (GPE) had a zingerone content of 5.7 mg/g. Therefore, the total zingerone in the 2.86 kg of dried powder (before losses and milling losses), was 16.30 g. The concentration of gingerol in the juice before conversion (GKOH-0) was 6.9 mg/g on a dry basis. When converted back to a wet basis, using a solids concentration of 6.67% (before the addition of KOH and anhydrous citric acid increased the total solids content to approximately 10%), the total gingerol in the juice was 14.32 g. The GKOH-2, -3, -5 values for 6-gingerol were to be confirmed.

[00344] The mass balances between the gingerol in the juice, and the zingerone in the final powdered extract are not precisely aligned. This may be due to variations in measurements. The marc numbers appear to be somewhat elevated, in view of the total mass balance calculations. When the marc was extracted with hot water, the extract had a

6-gingerol content of 0.22 mg/g, corresponding to a total of 5.7 g of 6-gingerol in this extract, roughly 25% of the 6-gingerol in the feed. For water extraction, this required a total of 18 kg of water. This in turn would increase the mass of KOH and citric acid, and the drying loads by an additional 58%. Thus, in certain circumstances, it may be desirable to omit water extraction.

[00345] Notably, these experiments showed that almost total recovery of 6-gingerol (in the form of zingerone) in the final product was achieved. The samples taken during the conversion reactions - GKOH2, GKOH3, and GKOH5 - show that the conversion from 6-gingerol to zingerone occurred during the first two hours of treatment, with no significant increase in zingerone levels in samples taken after 2 hours of treatment. Given that the conversion appears to be complete after 2 hours, this incubation time (or even shorter incubation times) will be sufficient.

[00346] It was concluded that the processing method, including pressing followed by KOH treatment in the juice phase, was an effective production method. This is an advancement well beyond standard alkaline-catalysed reversed aldol reactions. Further experiments will employ more than 200 kg ginger as starting material. 6-Gingerol levels in this batch were 1.1 mg/g and this was expected to reflect in the corresponding zingerone levels.

Example 6: Ethanol extraction process and analysis

[00347] Overview: Fresh ginger was received and processed by treating it in alkali followed by freeze-drying to produce a treated ginger powder (see Example 5, above). This powder was extracted with ethanol at room temperature for 3 days, with samples taken at 24, 48 and 72 hours to assess the progress of the extraction.

[00348] Methodology: The treated ginger produced as above (Example 5) was stored refrigerated until used. Approximately half of the received ginger was extracted with XNS food grade ethanol at room temperature. The treated ginger, along with ethanol (using a ginger:ethanol ratio of 1:5 by weight), was placed in a 10 L glass vessel equipped with an overhead stainless-steel stirrer. The mixture was stirred for 72 hours, with a stirring speed sufficient as to not allow sedimentation of solids at the bottom.

[00349] After 24 hours had passed, the stirrer was turned off and the solids were allowed to settle for 10 minutes before taking a 50 mL sample from the top. After another 24 hours had passed, a second sample was taken out using the same procedure. After a

total 72 hours had passed, stirring was stopped and the mixture was filtered under vacuum using filter paper. Samples were taken of the cake and a final tincture sample, representing 72 hours, was taken from the filtrate. The remaining filtrate was labelled ZINGOEE. A ~300 mL sample of ZINGOEE was taken and stored refrigerated in a glass bottle. In a further step, all ZINGOEE was evaporated to create a total resin volume of 43.5 g at a value of 88.9 mg/g zingerone.

[00350] The remaining ethanolic tincture was evaporated under vacuum using a Buchi R220SE rotary evaporator operating at 50 mbar and 40°C until a volume reduction of approximately 31-fold was achieved. The resulting concentrated extract (ZINGOCE) was then analysed and once the zingerone content was confirmed, a small sample of this concentrated extract was used to produce a standardised tincture containing ~12 mg zingerone per gram by diluting it with food grade ethanol. Two separate samples of this standardised tincture were sent to SCU (Australia) for analysis, and a third sample was retained onsite for zingerone analysis.

[00351] Results: The process and results are set out in Figure 7. For these experiments, 801.5 g of the received treated ginger was used in the ethanol extraction, along with 4007.3 g of food grade ethanol. After 72 hours at room temperature (16-20°C) under stirring, the mixture was filtered and 3556.7 g of a clear brown, aromatic ethanol tincture was obtained [ZINGOEE], as well as 1033.4 g of cake (i.e., spent ginger solids). Approximately 140 g of ethanol was lost due to evaporation during the extraction. The total weight of ethanolic tincture produced, including the samples taken at 24 and 48 hours, was 3631.7 g.

[00352] Quantification of zingerone was carried out by HPLC in the starting material (i.e., treated ginger, GPE) and the samples after 24, 48 and 72 hour ethanol extraction at room temperature. The spent ginger solids (i.e., the cake from the filtration process) was also analysed. Zingerone content for all fractions is shown in Table 12. HPLC results showed little difference between the three extraction times, indicating that 24 hours is sufficient for extraction.

Table 12. Zingerone content (mg/g) for samples

Treated ginger [GPE]	24 h sample	48 h sample	72 h sample [ZINGOEE]	Spent ginger solids [CAKE]
5.7	1.07	1.08	1.1	0.6

[00353] The filtrate [ZINGOEE] had a zingerone concentration of 1.1 mg/g, i.e., 3.88 g zingerone are present in the liquid, or 85% of the starting zingerone, indicating reasonable recovery. The cake had a zingerone concentration of 0.61 mg/g, i.e., 0.63 g of zingerone in the cake. However, it was noted that the cake had certain amount of ethanolic solution still present. In future processing, the cake could be washed with clean ethanol to flush out as much extract as possible.

[00354] The zingerone mass balance for the ethanol extraction process is 98.5%. This is based on 3.88 g in the extract, plus 0.63 g in the cake, divided by 4.58 g in the feed. It was determined that room temperature extraction provided advantageous recovery of zingerone. It is possible that increasing the extraction temperature could lead to higher zingerone recovery, but room temperature extraction is clearly effective.

[00355] After taking ~300 mL sample of ZINGOEE, the remaining extract (3249 g) was evaporated under vacuum to produce 104.9 g of concentrated extract [ZINGOCE] with a zingerone content of 33 mg/g (3.46 g of zingerone). This concentrated extract had a total solids content of 37.1% (measured as loss on drying at 110°C), indicating that the final oleoresin weight that could be achieved if all ethanol were removed would be 38.9 g. If this number is extrapolated to the total amount of ZINGOEE produced, the resulting extraction yield for the process is around 5.4%. A standardised tincture containing 12 mg/g of zingerone was prepared by combining 25.5 g of ZINGOCE with 44.5 g of food grade ethanol. Samples of this tincture were sent for further testing.

[00356] In addition, further analysis showed that no aldehydes were present in the final product. See Figures 8A-8B. For these assessments, a sample of an ethanolic extract of treated ginger was analysed using GCMS for the presence of aldehydes. If present, this would be expected as (predominantly) hexanal derived from 6-gingerol. It is expected that the powder preparations (e.g., pre-extraction powders, as in Examples 4 and 5, above) will also lack aldehydes. Next steps will include evaporation of the ethanolic tincture to produce a thick ethanolic paste.

[00357] Conclusions: The proposed method which included screw pressing followed by KOH treatment in the juice phase, neutralisation with citric acid, and then freeze drying, proved to be highly effective, with almost total recovery of 6-gingerol in the juice phase achieved, as well as total conversion to zingerone after 5 hours of treatment with 2% KOH at 60°C. The pre-processed dried powder currently contained 5.32 mg/g

zingerone, which is at least two times more efficient than previous manufacturing techniques.

[00358] The ethanol extraction at room temperature for 24 hours is an optional step achieving at least 85% recovery of zingerone. The recovery could be further increased by washing the solids with fresh ethanol after extraction. In these methods, the ethanolic extract [ZINGOEE] was evaporated to achieve a significant volume reduction, followed by reconstitution of the concentrated extract [ZINGOCE] with fresh ethanol in order to produce a standardised tincture containing the target dose of 12 mg/g zingerone.

[00359] For the extraction process, the overall Z mass balance (out/in) was 98.5%. The zingerone content in the filtrate (ZINGOEE) was 1.1 mg/g, and the zingerone content in the concentrated extract (ZINGOCE) was 33 mg/g. The method produced 104.9 g of ZINGOCE at 33 mg/g, leaving 3.46 g zingerone. This number is lower than the 3.88 g in ZINGOCE because ~400 g of ZINGOCE was removed before evaporation (for testing, plus ~300 mL retention sample). Taking this into account, the calculations fit very well. The overall zingerone mass balance before evaporation was calculated as 98.5% ($= (0.63 + 3.88)/4.58$).

[00360] The results obtained in this work indicate that 100 kg of fresh ginger with a 6-gingerol content of 0.5 mg/g would yield 3.6 kg of standardised tincture with a zingerone content of 12 mg/g. From this, it can be taken that higher 6-gingerol level should produce a higher yield of zingerone.

Example 7: Anti-inflammatory activity for zingerone composition

[00361] Overview: These studies were carried out to determine the anti-inflammatory activity of the disclosed botanical extract (ethanolic extract (tincture); see Example 6) standardised to zingerone content. Nitric oxide (NO) and IL-6 levels were assessed.

Methodology: The anti-inflammatory activity was determined in lipopolysaccharide (LPS) stimulated murine macrophages, RAW264.7 cells cultured in standard cell culture media (DMEM, foetal bovine serum 5%) and incubated in the presence or absence of different test compounds/extracts and controls. The production of inflammatory mediators, including NO and IL-6 were measured by established methods using commercial ELISA kits (suppliers listed in Table 13). Each sample was tested with at least 6 concentrations (from 20 µg/mL to 0.6 µg/mL). This was done using 3 replicates (maximum concentration

was 40 μ M zingerone) (n=9), with relevant internal controls. In addition, the cytotoxicity of each sample tested was determined by MTT assay (tetrazolium dye MTT, which is chemically 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The assay parameters for each assay are summarised in Table 13.

Table 13. Anti-inflammatory assays and positive controls

Assay	Plating time	Stimulation	Incubation time	Kit supplier	Positive control
NO	48 hours	LPS 50 ng/ml	18 hours	Griess Reagent	1400W (Cayman)
IL-6	48 hours	LPS 50 ng/ml	18 hours	Peprtech	Dexamethasone (Sigma-Aldrich)

[00362] The tincture from Example 6 was utilised for these studies. For the NO and IL-6 assays the cultured RAW264.7 cells were counted and plated (0.8×10^5 cells/well) in 96 well plates and incubated for 48 hours. The medium was then aspirated and replaced with fresh medium followed by the addition of the test compounds. The compounds were incubated for 1 hour prior to the addition of the stimulant. The plates were then incubated for 18 hours and the supernatant analysed for the mediator of interest, the remaining cell viability was determined by MTT.

[00363] The positive controls were selected based on their widespread use in similar assays, including N-(3-(aminomethyl)benzyl)acetamidine (1400W), a slow, tight binding inhibitor of inducible nitric-oxide synthase (iNOS) (Garvey et al., 1997, J Biol Chem 272(8):4959-63) and dexamethasone, a commonly used cytokine inhibitor. The data were presented as means and standard error of means (sem) (n=9).

[00364] The dose response curves were fit using Graph Pad Prism. The 95% confidence intervals (CI) were calculated using the whole data set and gave an estimate error in the IC_{50} value. The 95% CI for the IC_{50} where the IC_{50} was not reached in the testing concentrations, require graphical extrapolation to calculate the IC_{50} . Where the IC_{50} has been estimated by graphical extrapolation, “~” is used to indicate that it is an estimate and not experimentally determined, unless otherwise stated. The graphical extrapolation results in a wider 95% confidence interval.

[00365] Results: The tincture from Example 6 was assayed to determine anti-inflammatory activity. The anti-inflammatory activity was assessed using key mediators

of inflammation: NO, and IL-6. The cytotoxicity of each sample was also determined by MTT assay.

[00366] Cytotoxicity: The effect of the compounds on the viability of the cells was determined by MTT. Potency was monitored to avoid false positives, as dead cells do not produce inflammatory mediators. Cytotoxicity was determined spectrophotometrically as mitochondrial dehydrogenase present in viable cells cleaves the tetrazolium ring of MTT to yield a purple MTT formazan. All doses were tested up to 40 μ M zingerone. The results are shown in Figure 9. From the cytotoxicity assay, the EC₅₀ for the disclosed tincture was determined as 40 μ M zingerone.

[00367] NO assay: NO is a radical metabolite, which has been shown to have numerous physiological functions both as a signalling molecule and as a toxic agent in inflammation (Coleman, 2001). The inhibition of iNOS and reduction in NO levels secreted by immune cells may be a contributing factor to anti-inflammatory activity. The disclosed tincture was therefore assayed to determine if they showed inhibition on the inflammatory signalling molecule NO. The dose dependent effects of the tincture on NO are shown in Figure 10. From the NO assay, the IC₅₀ for the disclosed tincture was determined as 9.2 μ M zingerone.

[00368] IL-6 assay: IL-6 is considered a pro-inflammatory cytokine. IL-6 is secreted by T cells and macrophages, which stimulates an immune response. IL-6 is responsible for the increased production of neutrophils in the bone marrow. It supports the growth of B cells and is antagonistic to the differentiation of T cells into regulatory T cells. It can cross the blood-brain barrier and initiating synthesis of PGE2 in the hypothalamus, thereby changing the body's temperature set point (Banks, Kastin, & Gutierrez, 1994). The inhibition of IL-6 release by immune cells points to anti-inflammatory activity. The dose dependent effects of the disclosed tincture on IL-6 are shown in Figure 11. From the IL-6 assay, the IC₅₀ for the tincture was determined as 4.6 μ M zingerone.

[00369] It can be seen from Figures 10, 11, and 12, that the disclosed tincture inhibited NO and IL-6 in a dose dependent manner. The effective inhibitory levels for NO and IL-6 were significantly lower than the levels required for cytotoxicity, validating the observed anti-inflammatory effects of the tincture.

Example 8: Comparative anti-inflammatory testing for zingerone compositions

[00370] Overview: These studies were carried out to determine the anti-inflammatory bioactivity of the disclosed botanical extract (ethanolic extract (tincture); see Example 6) as compared to commercially sourced zingerone in RAW264.7 macrophages.

[00371] Methodology: An extract (ethanolic extract (tincture); Rx7/22/161) was prepared as described in Example 6. The extract was standardised at 33 mg of zingerone per mL of extract. Commercially available zingerone powder (Vigon #500938) was obtained and dissolved fresh to 33 mg/mL in ethanol on the day of use. Samples in ethanol were further diluted in cell media immediately prior to addition to cell culture. An ethanol solvent control was included in cell culture experiments to exclude any potential solvent effect.

[00372] RAW264.7 cells in growth media (DMEM, 10% FBS, PSN, 2 mM L-glutamine) were plated at 0.8×10^5 cells/well in 96-well tissue culture treated plates and incubated at 37°C / 5% CO₂ in a humidified environment for 48 hours. The spent media was then aspirated and replaced with media containing 5% FBS. Treatments were added to give final concentrations of 0.625 – 40 µM zingerone (or equivalent dilutions for ethanol solvent control). The cells were incubated with the treatments for 1 hour prior to the addition of 50 ng/mL lipopolysaccharide (LPS; from Escherichia coli O111:B4).

[00373] The cells were co-incubated with treatments and LPS for 18 hours before conditioned media was harvested, centrifuged and cell-free supernatants collected. The viability of the remaining cells was determined by WST-1 assay. Controls included were unstimulated (cells with no LPS), LPS (cells exposed to 50 ng/mL LPS), Dex (cells exposed to 1 or 10 µg/mL dexamethasone before addition of 50 ng/mL LPS), and media (media containing no cells or samples).

[00374] For the cytotoxicity assay, an equal volume of a 1:5 mixture of WST-1:media was added to each cell well and incubated for 10 minutes. Immediately prior to measurement of absorbance, an equal volume of DPBS was added and absorbance read at 440 nm (and 620 nm for removal of background). Hydrogen peroxide was included as a cytotoxic positive control. Results were normalised to LPS-stimulated control and expressed as percentage cell viability. Sample concentrations where cell viability is below 80% of the unstimulated control were deemed cytotoxic.

[00375] For the IL-6 assay, Interleukin 6 (IL-6) was analysed in cell-free conditioned media that was collected after treatment and LPS-stimulation using a bead-based multiplex assay panel (Legendplex MU Th1/Th2 8-plex panel; BioLegend #741054) and measured using a Cytex Aurora Spectral 3 laser flow cytometer. As a follow-up to the IL-6 assay, assays were performed to measure IL-10 and TNF.

[00376] Statistical analysis was conducted using Minitab 18.0. Student's paired t-tests were conducted to statistical differences between treatments at each timepoint. Data are presented as mean $\pm$ standard error of the mean (SEM). Three replicate cell wells were included in each experiment, and three separate cell experiments were performed.

[00377] Results: The viability of the cells after incubation with the compounds/extracts was measured. In the present assay, WST-1 reacts with a mitochondrial enzyme to form a coloured dye which can be measured by absorbance. As such, WST-1 is a measure of cellular metabolism, and a reduction in the WST-1 reaction rate can be indicative of cellular death. Hydrogen peroxide (H_2O_2) is used as a positive control to induce cell death and ensure the validity of the assay.

[00378] Results for cytotoxicity are shown in Figure 13. This figure depicts cell viability levels following 18 hour treatment of cells with 50 ng/mL lipopolysaccharide (LPS) and LPS with the disclosed botanical extract or zingerone (0.6 to 40 μM) and equivalent ethanol controls. Data are means $\pm$ SEM from three independent experiments ($n = 3$). Assay validation was determined with hydrogen peroxide (H_2O_2).

[00379] In the left-hand panel, a clear cytotoxic dose-dependent response is seen in the H_2O_2 -exposed cell samples, confirming the assay is working as expected. WST-1 values of $<80\%$ of the untreated control (dashed horizontal line) are considered cytotoxic. Cytotoxicity was assessed in RAW264.7 cells after 18 hour of exposure to the disclosed botanical extract or commercially sourced zingerone. Concentrations ranged from 0.3 to 40 μM zingerone. No apparent cell death was detected by the WST-1 assay in RAW264.7 cells exposed to either the ethanolic extract or commercially sourced zingerone at the concentrations tested (Figure 13). That is, none of the data points fell below 80% of WST-1 response of the untreated LPS-stimulated control. This validated the results from the cytokine assay (results below).

[00380] The cell model used for IL-6 assessments explores the pro-inflammatory response by a mouse macrophage cell line (RAW264.7) when exposed to a bacterial

endotoxin (lipopolysaccharide; LPS). Mechanistically, LPS interacts with the membrane-bound TLR4 receptor, triggering a signalling cascade within the cell to activate NF- κ B, a transcription factor that regulates the expression of many proinflammatory cytokines, including IL-6.

[00381] Results for IL-6 measurements are shown in Figure 14. This figure depicts IL-6 levels produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS), LPS + 10 μ g/mL dexamethasone (Dex) and LPS + disclosed botanical extract or commercially sourced zingerone (0.6 to 40 μ M) for 18 hours. Data are means $\pm$ SEM from three independent experiments ($n = 3$). The asterisk denotes significantly lower IL-6 production from zingerone at the corresponding concentration ($p < 0.05$). Treatments with no error bars at 20,000 pg/mL indicate values $\geq 20,000$ pg/mL.

[00382] It was found that the exposure of RAW264.7 cells to LPS for 18 hours significantly induced IL-6 secretion to concentrations above the assay's upper limit of accuracy of 20,000 pg/mL (Figure 2). IL-6 secretion by RAW264.7 cells following co-treatment with equivalent ethanol concentrations present in the disclosed botanical extract and commercially sourced zingerone dilutions evaluated were also above the assay's upper limit. Co-treatment of RAW264.7 cells with LPS + disclosed botanical extract or LPS + commercially sourced zingerone at concentrations ranging from 0.6 to 10 μ M were similarly above the assays upper limit of accuracy, making it difficult to assess the anti-inflammatory bioactivity of these samples at these concentrations.

[00383] Notably, co-treatment of RAW 264.7 macrophages with LPS and disclosed botanical extract or commercially sourced zingerone at the highest concentrations evaluated (20 and 40 μ M zingerone), resulted in measurable reductions in IL-6 that was within the limits of the assay's accuracy. In particular, co-treatment of RAW264.7 with the disclosed botanical extract at 20 and 40 μ M zingerone resulted in significantly ($p < 0.05$) lower IL-6 production compared with co-treatment with commercially sourced zingerone. These findings indicate that the disclosed botanical extract is more efficacious than commercially sourced zingerone in reducing IL-6 production at these concentrations.

[00384] Specific reductions were also seen for IL-10. Figure 15 depicts IL-10 levels produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS), LPS + 10 μ g/mL dexamethasone (Dex) and LPS + disclosed botanical extract or LPS + commercially sourced zingerone (1.25 to 40 μ M) for 18 hours. Data are means $\pm$ SEM

from three independent experiments ($n = 3$). Carrot denotes significantly lower IL-10 production compared with corresponding ethanol control ($p < 0.05$). Asterisk denotes significantly lower IL-10 production from zingerone at the corresponding concentration ($p < 0.05$). Treatments with no error bars at 15,000 pg/mL indicate values 215,000 pg/mL and are above the assays limit of accuracy.

[00385] The results showed that exposure of RAW264.7 macrophages to LPS for 18 hours induced a significant increase in IL-10 that was attenuated by co-treatment with dexamethasone, a known immune suppressant. Co-treatment of cells with 10 and 20 μ M zingerone equivalence of the disclosed botanical extract, but not commercially sourced zingerone, significantly attenuated LPS-induced IL-10 secretion by RAW264.7 ($p < 0.05$). Additionally, IL-10 secretion by RAW264.7 co-treated with the disclosed botanical extract at 20 and 40 μ M zingerone was significantly lower compared with commercially sourced zingerone at these concentrations ($p < 0.05$).

[00386] Results for TNF are shown in Figure 16. This figure depicts TNF levels produced by RAW264.7 cells after treatment with lipopolysaccharide (LPS), LPS + 10 μ g/mL dexamethasone (Dex) and LPS + disclosed botanical extract or LPS + commercially sourced zingerone (1.25 to 40 μ M) for 18 hours. Data are means $\pm$ SEM from three independent experiments ($n = 3$). Asterisks denotes significantly lower IL-6 production from zingerone at the corresponding concentration ($p < 0.05$). Treatments with no error bars at 15,000 pg/mL indicate values 215,000 pg/mL and are above the assays limit of accuracy.

[00387] The results showed that exposure of RAW264.7 macrophages to LPS for 18 hours induced a significant increase in TNF that was attenuated by co-treatment with dexamethasone, a known immune suppressant. Co-treatment of cells with the disclosed botanical extract or pure zingerone compound had no significant effect in attenuating LPS-induced TNF secretion ($p > 0.05$).

[00388] Overall, the anti-inflammatory activities of the disclosed botanical extract (with naturally occurring zingerone) are significant and notably superior to the activities of commercially sourced zingerone, particularly in relation to IL-6 and IL-10 levels. Future assays will be carried out with overall lower induction levels to allow more accurate comparisons.

[00389] On further review of these studies, it was noted that the concentration of zingerone in the disclosed botanical extract (tincture) was noted incorrectly by the researchers performing the experiments. Although indicated in these studies as 33 mg/mL (0.170 M), the concentration of zingerone in the tincture was actually 33 mg/g (0.134 M). As such, the zingerone concentrations for the tincture dilutions have been incorrectly noted as 0.625, 1.25, 2.5, 5, 10, 20, and 40 μ M. Based on the correct starting concentration of 33 mg/g, the zingerone concentrations for the tincture dilutions can be correctly calculated as 0.493, 0.986, 1.97, 3.95, 7.89, 15.8, and 31.6 μ M, respectively.

[00390] This means that the results shown for each of IL-6, IL-10, and TNF have all been underestimated in regard to the activity of the disclosed botanical extract, since the commercially sourced zingerone was used at significantly higher concentrations (0.625, 1.25, 2.5, 5, 10, 20, and 40 μ M) than the disclosed botanical extract (0.493, 0.986, 1.97, 3.95, 7.89, 15.8, and 31.6 μ M). In any case, these initial results are extremely positive, and the studies are currently being repeated using the correct calculations.

Table 14. Summary of results for IL-6 inhibition

	Method 1	Method 2
LPS stimulated	6641.11 $\pm$ 503.45 pg/mL	13930.0 $\pm$ 340.48 pg/mL
LPS + dexamethasone 100 nM	1874.44 $\pm$ 394.56 pg/mL	8896.67 $\pm$ 241.14 pg/mL
LPS + absolute ethanol	5685.56 $\pm$ 309.32 pg/mL	13985.56 $\pm$ 401.08 pg/mL

[00391] The MTT assay was used to identify cytotoxic effects of compound treatment on the RAW264.7 cells. No cytotoxic effect was identified for any compound tested. In the Method 1 conditions, there was a notable drop in the MTT absorbance relative to the unstimulated Method 2 conditions, and both stimulated and unstimulated Method 2 conditions (Figure 18A: Method 1 stimulation; Figure 18B: Method 2 stimulation). This drop is expected as IFN- γ 's mechanism involves stopping cellular proliferation, resulting in less cells present compared to (-) IFN- γ conditions. In particular, fewer cells means fewer mitochondria to metabolise the MTT to formazan, resulting in lower absorbance.

[00392] Conclusions: Dexamethasone at 100 nM was shown to be an effective control due to IL-6 inhibitory activity. The Method 2 set up was chosen for the remaining experiments, as this method showed compatibility to other data obtained.

Example 9: Comparing methods for assessing anti-inflammatory activity and viability

[00393] Overview: These studies were carried out: (1) to identify the most effective positive control agent; and (2) to compare two different assessment methods for inflammatory activity. The following outputs were produced for this experiment: viability, measured by MTT; and IL-6 production, measured by ELISA.

[00394] The assessment methods are summarised as follows:

Stimulated	Day 0	Day 1	Day 2	Day 3	Day 4
Method 1		Plate cells IFN- γ Prime	LPS Stim + Treatment	Harvest	MTT and ELISA finish
Method 2	Plate cells		LPS Stim + Treatment	Harvest	MTT and ELISA finish

Unstimulated	Day 0	Day 1	Day 2	Day 3	Day 4
Method 1		Plate cells Add media	Media + Treatment	Harvest	MTT and ELISA finish
Method 2	Plate cells		Media + Treatment	Harvest	MTT and ELISA finish

[00395] Methodology: Dexamethasone and synthetic zingerone were obtained from Sigma-Aldrich. For these studies, dexamethasone (100 nM) was used as the positive control; absolute ethanol was used as the vehicle control; and synthetic zingerone (20 μ M) was used for test treatments. The testing included: (1) IL-6 ELISA – measures production of IL-6 from stimulated/unstimulated RAW264.7 cells; (2) MTT assay – identifies potential cytotoxicity by measuring metabolism of MTT to formazan.

[00396] On Day 0, the flask dedicated to Method 2 conditions was retrieved from 37°C /5% CO₂ incubator. The growth media was decanted. Next, 10 mL CTCM was added and the cells were washed. The growth media was again decanted. Then, 10 mL CTCM was added and cells were harvested using a rubber scraper. The resulting cell suspension was transferred to a 50 mL falcon tube. This was centrifuged at 400 x g for 5 minutes. Cells were counted after applying a 1 in 10 trypan blue dilution. Samples were then diluted to 1 x 10⁶ cells/mL. Cells were then plated or added at 80 μ L/well (80,000 cells/well). For stimulated Method 2 samples, 120 uL media was added. This step was repeated for unstimulated Method 2 samples. The cells were returned to the 37°C /5% CO₂ incubator and left for 48-hours.

[00397] On Day 1, the drug dilutions were prepared (see below). The flask dedicated to Method 1 conditions was retrieved from the 37°C /5% CO₂ incubator. The growth media was decanted. Next, 10 mL CTCM was added and the cells were washed. The growth media was again decanted. Then, 10 mL CTCM was added and cells were harvested using a rubber scraper. The resulting cell suspension was transferred to a 50 mL falcon tube. This was centrifuged at 400 x g for 5 minutes. Cells were counted after applying a 1 in 10 trypan blue dilution. Samples were then diluted to 1 x 10⁶ cells/mL. Cells were plated at 50,000/well (50 µL). For stimulated Method 1 samples, 50 µL IFN-γ was added (final concentration 20 U/mL). For unstimulated samples, cells were plated and well volume was made up to 200 µL.

[00398] On Day 2, LPS and treatment conditions were prepared for Method 2 samples. For stimulated Method 2 samples, 50 µL LPS was added (final concentration 20 ng/mL) and 50 µL treatments were added. For unstimulated Method 2 samples, these were centrifuged and 50 µL of CTCM was removed from treatment wells. To these wells, 50 µL of treatment was added. For stimulated Method 1 samples, these were centrifuged and 100 µL media was removed from the wells. To these wells, 50 µL of LPS was added (final concentration 50 ng/mL) and 50 µL treatments were added. For unstimulated Method 1 samples, these were centrifuged and 50 µL of media was removed from the wells. To these wells, 50 µL of treatment was added. The set ups for IL-6 ELISA captures were started.

[00399] On Day 3, ELISA plates were washed and blocking was started. The supernatants for each sample were harvested (170 µL). Next, 50 µL of warmed CTCM was added to wells. Next, 20 µL of 5 mg/mL MTT solution was added. The plates were returned to the 37°C /5% CO₂ incubator for 45 minutes. While the MTT was incubating, ELISA captures were wash off and blocking solution was added. After 45 minutes, 10 µL MTT solubilizer was added. The ELISA process was continued. Cell counting was carried out for four fields. The cell numbers per field were averaged as follows: (48+58+67+62)/4 = 58.75 cells in 10 µL. This was then calculated as 58.75 * 10 * 10⁴ = 5875000 cells/mL. This was then calculated as 5875000 * 10 = 58750000 cells in 10 mL. Therefore, to make a 1 x 10⁶ cells/mL solution, a cell suspension up to 58.75 mL was prepared.

[00400] To prepare dilutions: (1) IFN-γ stock solution was 5,000,000 U/mL. This was diluted to 24,000 U/mL in CTCM; (2) LPS stock solution was 1,000,000 ng/mL. For

Method 1, a 80 ng/mL solution was required (final concentration 20 ng/mL in the wells). For Method 2, a 200 ng/mL solution was required (final concentration 50 ng/mL in the wells); (3) Synthetic zingerone stock solution was prepared by adding 2 μ L of zingerone into 198 μ L CTCM . This gave a 1544.6401 μ M stock. From this stock, 25.89 μ L was added to 474.11 μ L CTCM. This gave an 80 μ M stock. The 80 μ M stock (50 μ L) was added to samples (along with LPS) to achieve a final concentration 20 μ M (0.013% ethanol).

[00401] To prepare drug dilutions: (1) Dexamethasone stock solution was prepared by introducing 2 μ L of dexamethasone into 998 μ L CTCM . This gave a 5096 nM stock solution. From the 5096 nM stock solution, 78.49 μ L was added to 921.51 μ L CTCM. This gave a 400 nM stock solution. The 400 nM stock solution (50 μ L) was added to samples (along with LPS) to achieve a final concentration of 100 nM (0.003% ethanol); (2) Ibuprofen stock solution was prepared by introducing 2 μ L ibuprofen into 579.704 μ L CTCM. This gave a 1000 μ M ibuprofen stock solution. From the 1000 μ M ibuprofen stock solution, 400 μ L was added into 600 μ L CTCM to make a 400 μ M stock solution. The 400 μ M stock solution (50 μ L) was added to the samples (along with LPS; 0.03% ethanol); (3) Risperidone stock solution was prepared by introducing 2 μ L of risperidone into 248 μ L CTCM. This gave a 584.67 μ M stock solution. From the 584.67 μ M stock solution, 342.1 μ L was added to 157.9 μ L CTCM. This gave a 400 μ M stock solution. The 400 μ M stock solution (50 μ L) was added to the samples (along with LPS) to achieve a final concentration of 100 μ M (0.14% ethanol).

[00402] For ELISA measurement of IL-6: Reagents from BD Biosciences. Capture: purified NA/LE rat anti-Mouse IL-6; Cat. No. 554398, clone MP5-20F3; Standard: recombinant mouse IL-6; Cat. No. 554582; Detection: biotin rat anti-Mouse IL-6 antibody; Cat. No. 554402, clone MP5-32C11; SA-HRP: Streptavidin HRP; Cat. No. 554066; TMB: BD OptEIA™ TMB substrate reagent set; Cat. No. 555214. Two full ELISA plates (each with 96 wells) were utilised when including standards. A total of 11 mL volume was prepared for 50 μ L/well samples and a total of 22 mL volume was prepared for 100 μ L/well samples. For capture, overnight incubation was carried out at 4°C. For the capture antibody, stock was at 1000 μ g/mL, with a final concentration desired at 1 μ g/mL. The following calculation was performed: $1000 \mu\text{g/mL} * V_i = 1 \mu\text{g/mL} * 11,000 \text{ uL}$. From this calculation, V_i equating to 11 μ L rat anti-mouse IL-6 was added into

11 mL pH 9.0 capture buffer. This was introduced to the plates at 50 µL/well. After capture, washing was performed four times.

[00403] To perform blocking, a 2 hour incubation was carried out at room temperature. For this, 100 µL of 10% FCS was added to each well. This was calculated as: 10% FCS = 5 mL FCS in 50 mL 1X PBS. After blocking, washing was performed three times. The plates were incubated for 2 hours at room temperature. The stimulated samples were diluted as 10 µL in 100 µL 5% FCS. 5% FCS, equated to 2.5 mL FCS into 50 mL 1X PBS. This was introduced to the plates at 50 µL/well. Standards were prepared as follows: 16 µL was added into 784 µL 5% FCS. The highest concentration was 4 ng/mL. This was serially diluted by diluting 100 µL into 100 µL down to 7.8125 pg/mL. A sample with 0 pg/mL also included. The serial dilutions were produced in duplicate. After standard preparation, washing was performed four times.

[00404] For the initial detection step, a 1 hour incubation was carried out at room temperature. Stock was at 500 µg/mL, with a final concentration desired at 0.5 µg/mL. The following calculation was performed: $500 \text{ µg/mL} * V_i = 0.5 \text{ µg/mL} * 11,000 \text{ µL}$. From this calculation, the V_i equating to 11 µL biotin rat anti-mouse IL-6 was added into 11 mL 5% FCS/PBS. This was introduced to the plates at 10 µL/well. After this initial detection step, washing was performed six times. Incubation with streptavidin-horseradish peroxidase (SA-HRP) was carried out for 1 hour at room temperature. For this, a 1 in 2000 dilution for SA-HRP was prepared. The calculation was performed as follows: $11,000 / 2000 = 5.5 \text{ µL}$. From this calculation, 5.5 µL SA-HRP was added into 11 mL 5% FCS/PBS. This was introduced to the plates at 50 µL/well. After the SA-HRP step, washing was performed eight times.

[00405] For the final detection step, incubation with TMB (3, 3', 5, 5'-tetramethylbenzidine) was carried out. The TMB was prepared by introducing 11 mL TMB-A and 11 mL TMB-B into separate 15 mL falcon tubes covered in tinfoil. After the washing following SA-HRP incubation, the TMB-A and TMB-B samples were combined and this was introduced to the plates at 100 µL/well. Colour was allowed to develop. Colour development was stopped by adding 100 µL H₂SO₄ to wells. The plates were read on a plate reader.

[00406] Results: Interleukin 6 (IL-6) was produced in response to LPS in both experimental set ups, with greater levels produced using the Method 2 set up (Figure 17A:

Method 1 stimulation; Figure 17B: Method 2 stimulation). The unstimulated conditions for both set ups did not noticeably change with any treatment. Dexamethasone (100 nM) was the only control compound capable of inhibiting IL-6 in either set up. In the Method 1 set up, dexamethasone had a greater inhibition of IL-6 (29.32% of the stimulated/untreated condition and 33.42% of the vehicle) compared to the Method 2 set up (64.03% of the stimulated/untreated condition and 63.62% of the vehicle). A summary of the IL-6 levels is presented in Table 14, below.

Example 10: Anti-inflammatory activity and cell viability analysis in comparative testing

[00407] Overview: The methodology as noted in the previous example was used to assess various markers, including: (1) viability: Identifies concentrations which have a cytotoxic effect, measured by MTT assay; (2) IL-6: Proinflammatory cytokine, measured by ELISA; (3) TNF: Proinflammatory cytokine, measured by ELISA; (6) Nitric oxide: Proinflammatory small molecule, measured by Griess assay.

[00408] Certain data has been presented at a log of the concentration tested to make data visualisation easier and to perform non-linear regression where appropriate. The testing concentrations and their corresponding log₁₀ value are listed below.

Table 15. Testing concentrations and calculated Log₁₀ concentrations

Concentration (μM)	Log ₁₀ Concentration
3.125	0.49485
6.25	0.79588
12.5	1.09691
25	1.39794
50	1.69897
75	1.875061
100	2
150	2.176091

[00409] Methodology: Anti-inflammatory activity and viability was assessed as follows.

[00410] At Day 0, a dedicated T75 flask was retrieved for replicate from incubator. In the laminar flow cabinet, the flask was gently tapped to detach any non-adherent cells. The growth media was decanted and replaced with 10 mL CTCM. The flask was gently swayed to wash the cells. The wash media was decanted and replaced with 10 mL CTCM.

Using a rubber scraper, cells were detached. The suspension was transferred to a 50 mL Falcon tube. The cells were centrifuged at $400 \times g$ for 5 minutes. The cells were resuspended in 10 mL CTCM. Cells were counted after applying a 1 in 10 trypan blue dilution. Cells were diluted to 1×10^6 cells/mL. Cells were plated at $80 \mu\text{L}/\text{well}$. To this, we added $120 \mu\text{L}$ to bring the volume to $200 \mu\text{L}$. The plate was returned to the incubator. Cells were continued to be plated as indicated. In this way, plates were dedicated to stimulated and unstimulated samples. Plates were left for 48 hours before any treatment occurred.

[00411] Round 1 counting (stimulated/unstimulated): Average number of cells in four fields = 54 $\rightarrow \times 10 \times 10^4$ to give 5,400,000 cells in 1 mL, which equated to 54,000,000 cells in 10 mL. For the dilution, 44 mL CTCM was added to bring to 1 million cells/mL.

[00412] Round 2 counting (stimulated): Average number of cells in four fields = 15.7 $\rightarrow \times 10 \times 10^4$ to give 1,570,000 cells in 1 mL, which equated to 15,700,000 cells in 10 mL. For the dilution, 5.70 mL CTCM was added to bring to 1 million cells/mL.

[00413] Round 2 counting (unstimulated): Average number of cells in four fields = 25.75 $\rightarrow \times 10 \times 10^4$ to give 2,575,000 cells in 1 mL, which equated to 25,750,000 cells in 10 mL. For the dilution, 15.75 mL CTCM was added to bring to 1 million cells/mL.

[00414] Round 3 counting (stimulated): Average number of cells in four fields = 25 $\rightarrow \times 10 \times 10^4$ to give 2,500,000 cells in 1 mL, which equated to 25,000,000 cells in 10 mL. For the dilution, 15 mL CTCM was added to bring to 1 million cells/mL.

[00415] Round 3 counting (unstimulated): Average number of cells in four fields = 32 $\rightarrow \times 10 \times 10^4$ to give 3,200,000 cells in 1 mL, which equated to 32,000,000 cells in 10 mL. For the dilution, 22 mL CTCM was added to bring to 1 million cells/mL.

[00416] At Day 1, the treatments were prepared. Synthetic zingerone, acetyl zingerone, and ferulic acid were utilised in stock solutions at 360,416.0231 μM . Acetyl zingerone was obtained from Sytheon Ltd. Synthetic zingerone and ferulic acid were obtained from Sigma-Aldrich. To generate the required dosages, 1199.4 μL CTCM was added to a 2 μL aliquot of each stock solution. This produced a 600 μM test solution of each compound. When added to the cells the concentration was 150 μM – 50 μM in 200 μL . The disclosed botanical extract was utilised in a stock solution at 134,000 μM . To generate the required dosages, 6 μL of the extract was added with 1334 μL CTCM. This produced a 600 μM test solution. See summary tables, below.

Zingerone, Acetyl Zingerone, Ferulic Acid							
Cf (Well)	Cf (stock)	Ci	Vi	Vf	V CTCM	EtOH %	EtOH % in well
150	600	360416	2	1201.4	1199.4	0.166472	0.041618112
100	400	600	433.33333	650	216.6666667	0.110982	0.027745408
75	300	600	325	650	325	0.055491	0.013872704
50	200	400	325	650	325	0.027745	0.006936352
25	100	300	216.66667	650	433.3333333	0.009248	0.002312117
12.5	50	200	162.5	650	487.5	0.002312	0.000578029
6.25	25	100	162.5	650	487.5	0.000578	0.000144507
3.125	12.5	50	162.5	650	487.5	0.000145	3.61268E-05

Botanical Drug Extract							
Conc (Well)	Conc to make	Ci	Vi	Vf	V CTCM	EtOH %	EtOH % in well
150	600	134000	6	1340	1334	0.447761	0.111940299
100	400	600	433.3333	650	216.6667	0.298507	0.074626866
75	300	600	325	650	325	0.149254	0.037313433
50	200	400	325	650	325	0.074627	0.018656716
25	100	300	216.6667	650	433.3333	0.024876	0.006218905
12.5	50	200	162.5	650	487.5	0.006219	0.001554726
6.25	25	100	162.5	650	487.5	0.001555	0.000388682
3.125	12.5	50	162.5	650	487.5	0.000389	9.71704E-05

[00417] At Day 2, stimulation and treatments were started. The plates were retrieved from the incubator. The plates were centrifuged at 400 x g for 5 minutes. The media (150 μ l) was removed and replaced with 50 μ L CTCM. The test solution for each treatment was added (50 μ l) to the appropriate wells. The plates were returned to the incubator for 1 hour. An LPS solution was prepared. For this, a 2 μ L aliquot (1,000,000 ng/mL) was added to 9,998 μ L CTCM to give a 200 ng/mL solution (final concentration 50 ng/mL). The LPS solution was added at 50 μ L/well. For unstimulated conditions, the treatments were added at 50 μ L/well followed by 50 μ L of CTCM. The plates were returned to the incubator for 18 hours. All ELISA plates were coated. The MTT reagent was prepared. This was done by mixing 50 mg MTT into 10 mL 1X PBS. This produced a 5 mg/mL reagent solution for use with 6 plates.

[00418] At Day 3, supernatant harvesting, MTT assays, and ELISA were carried out. For supernatant harvesting, plates were centrifuges for 5 minutes at 400 x g. A multichannel pipette was set to 170 μ L. This was used to remove supernatant. The supernatant was transferred to a labelled UB-96WP. For the MTT assays, CTCM was prewarmed in a 37°C water bath. After harvesting supernatant, 50 μ L of warmed CTCM was added to cells. Next, 20 μ L of MTT reagent solution was added to cells. The plates

were returned to the incubator. After MTT development, 100 μ L MTT solubilising solution was added. This was incubated overnight.

[00419] ELISA reagents: From BD Biosciences. Capture: purified NA/LE rat anti-Mouse IL-6; Cat. No. 554398, clone MP5-20F3; Standard: recombinant mouse IL-6; Cat. No. 554582; Detection: biotin rat anti-Mouse IL-6 antibody; Cat. No. 554402, clone MP5-32C11; SA-HRP: Streptavidin HRP; Cat. No. 554066; TMB: BD OptEIA™ TMB substrate reagent set; Cat. No. 555214. Solutions included: (1) ELISA capture buffer pH 6: 14.196 Na₂HPO₄ in 1 L mqH₂O. The pH was adjusted with HCl. (2) ELISA capture buffer pH 9: 14.196 Na₂HPO₄ in 1 L mqH₂O. The pH was adjusted with NaOH. (3) ELISA wash solution: 1 mL Tween®20, 200 mL 10X PBS, and 1800 mL mqH₂O. (4) 5% FCS in PBS: 2.5 mL FCS in 47.5 mL 1X PBS. (5) 10% FCS in PBS: 5 mL FCS in 45 mL 1X PBS. (6) 0.18 M H₂SO₄: 9.78 mL concentrated M H₂SO₄ in 1 L mL H₂O.

[00420] ELISA assays: ELISA plates were coated with a capture antibody suspended in an ELISA capture buffer at 50 μ L/well. The pH was adjusted based on the cytokine being tested (see summary tables, below). The coated ELISA plates were incubated at 4°C. overnight. The following day, the plates were retrieved from the refrigerator. The plates were washed four times in ELISA wash buffer. Next, 100 μ L/well blocking solution was added to the plates. The plates were incubated at room temperature for 2 hours. The plates were washed three times. The supernatant was diluted depending on the cytokine being tested. The test sample was added along with a standard curve of the cytokine to quantify at 50 μ L/well (see summary tables, below). The plates were incubated for 2 hours at room temperature or overnight at 4°C. For sequential ELISA, the supernatant was transferred to another plate before washing. Washing was carried out four times.

[00421] For detection, a biotinylated detection antibody was suspended in a solution of FCS and PBS and added to the plates at 50 μ L/well. The plates were incubated for 1 hour at room temperature. The plates were washed six times. Streptavidin-horseradish peroxidase was suspended in a solution of FCS and PBS. This was introduced to the plates at 50 μ L/well. The plate was incubated at room temperature, in darkness, for 1 hour. The required volumes of TMB-A and TMB-B were placed into separate falcon tubes. These were stored at room temperature, in darkness, during the SA-HRP incubation. The plates were washed eight times. The TMB solution was introduced to the plates at 100 μ L/well. Colour was allowed to develop. To stop the reaction, 0.18 M H₂SO₄ solution was added at

100 μ L/well. See tables, below. Plates were read using a PerkinElmer EnSpire® plate reader at 450 nm.

Stim	IL-6 - pH 9 Capture			
	Stock	Final	Vf (μ L)	Vi (μ L)
Capture (μ g/mL)	1000	1	15000	15
Block (mL)	10% FCS		50 mL	5 mL
Samples (μ L)	1 in 10 dil		100	10
Standard (ng/mL)	200	4	1200	24
Detection (μ g/mL)	500	0.5	15000	15
SA-HRP	1 in 2000		15000	7.5
Dilutents	Samples, Standard, Detection SA-HRP Diluted in 5%			

Stim	TNF - pH 6 Capture			
	Stock	Final	Vf (μ L)	Vi (μ L)
Capture (μ g/mL)	500	4	15000	120
Block (mL)	10% FCS		50 mL	5 mL
Samples (μ L)	1 in 5 dil		100	20
Standard (ng/mL)	400	8	1200	24
Detection (μ g/mL)	500	1	15000	30
SA-HRP	1 in 2000		15000	7.5
Dilutents	Samples, Standard, Detection SA-HRP Diluted in 5%			

Stimulated conditions		
	IL-6 Capture	TNF Capture
Day 0		
Day 1: 8:00am		
10:00 am	4X Wash Add block (10% FCS)	4X Wash Add block (10% FCS)
12:00 pm	3X Wash Add IL-6 dilution and standards	3X Wash Add TNF dilution and standards
1:00 pm		
2:00 pm	4X Wash Add detection	4X Wash Add detection
3:00 pm	6X Wash Add SA-HRP	6X Wash Add SA-HRP

4:00 pm	8X Wash Add TMB reagent. Add H ₂ SO ₄ .	8X Wash Add TMB reagent. Add H ₂ SO ₄ .
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Unstimulated conditions		
	TNF Capture	IL-6 Capture
Day 0		
Day 1: 8:00am	4X Wash Add block (10% FCS)	
10:00 am	3X Wash Samples and Standards	4X Wash Add block (10% FCS)
12:00 pm	4X Wash Add detection	3X Wash Add IL-6 sample and standards
1:00 pm	6X Wash Add SA-HRP	
2:00 pm	8X Wash Add TMB reagent. Add H ₂ SO ₄ .	4X Wash Add detection
3:00 pm		6X Wash Add SA-HRP
4:00 pm		8X Wash Add TMB reagent. Add H ₂ SO ₄ .

[00422] MTT assays: On the day preceding the assay, MTT powder was dissolved in 1X dPBS to a concentration of 5 mg/mL. Each plate required 20 µL/well. For 96 wells, this required 1920 µL MTT solution to be prepared. The concentration was 5 mg of MTT per mL of solution. This equated to 10 mg MTT per 96 well plate. On the day of the assay, CTCM was warmed in a 37°C water bath. While media was warming, cell cultures (RAW264.7) were centrifuged at 400 x g for 5 minutes. Using a multichannel pipette set to 180 µL, supernatant was removed and transferred to a labelled U-bottom 96 well plate. The supernatant was stored at -20 °C until analysis. To the remaining cells, 60 µL warmed CTCM was added to bring the volume to 80 µL/well. To this, 20 µL MTT solution was added. The plate was returned to the 37°C/5% CO₂ incubator for 45 minutes. After 45 minutes, 50 µL MTT solubilizing solution was added. This included 10% SDS (w/v), 0.01 M HCl – pH adjusted to 4.0 with sodium hydroxide; or 10% SDS (w/v), 45% DMF – pH adjusted to 4.0 with acetic acid. The plate was covered with tinfoil and left overnight. After

overnight incubation, the plate was read using a PerkinElmer EnSpire® plate reader at 580 nm.

[00423] NO assays: A Griess reaction protocol was utilised. NO production was measured via NaNO_2 product in a 96 well format. Reagents included: (1) Greiss solution A (50 mL): 1% (w/v) sulphanilamide (500 mg), 2.5% phosphoric acid, stored at 40°C and protected from light; (2) Greiss solution B (50 mL): 0.1% (w/v) N-(1-naphthyl)ethylenediamine (50 mg), 2.5% phosphoric acid, stored at 40°C and protected from light; (3) 2.5% phosphoric acid (100 mL): 2.94 mL 85% phosphoric acid, 97.6 mL ddH₂O. For the plate standards, rows 1 and 2 (across) of each plate included 1:1 dilutions of NaNO_2 from 500 μM to 0 μM . In rows A and B (down), 50 μL of culture media was added to each well. The first well in each row received 95 μL . To the first wells, 5 μL of 10 mM NaNO_2 was added. From these wells, 50 μL was taken and dilutions made down the row excluding the last well of each row (base line). For the test samples, 170 μL supernatant was removed from the wells without disturbing the cellular monolayer on the bottom of each well. From this 170 μL sample, 50 μL samples (in triplicate) were transferred to a flat-bottomed 96 well plate. For the Griess reaction, equal volumes of Greiss solution A and Greiss solution B were mixed to the desired volume (about 5 mL total per 96 well plate). The mixed Greis solution A+B was added to each well (50 μL per well). Any bubbles were removed using a hair dryer. Absorbance was read at 570 nm.

[00424] Results – viability: The MTT assay was used to identify any drops in metabolism which may be indicative of a cytotoxic effect of any of the compounds. The only compound found to have a cytotoxic effect was the disclosed botanical extract at and above 50 μM . The data is summarised in Figures 19A-19D and Tables 16 and 17, below.

Table 16. IC₅₀ Calculation for the MTT analysis

Extract	Stimulated	Unstimulated
IC ₅₀ (μM)	50.52	48.53
R ²	0.978	0.936

Table 17. Closest concentration to IC₅₀

Botanical Extract	Stimulated	Unstimulated
Viability at 50 μM	64.69 ± 11.31%	51.61 ± 21.94 %

[00425] Results – IL-6: IL-6 is a common proinflammatory cytokine produced by macrophages upon stimulation with LPS. IL-6 is involved in propagating the early innate immune response. The only substantial inhibition of IL-6 production from stimulated RAW264.7 macrophages was from the disclosed botanical extract (Figure 20). The most potent IL-6 inhibition was seen with the botanical extract at 25 μ M, reducing the IL-6 production to 41% of the absolute ethanol control (Figure 20). Considerable inhibition of IL-6 production was also seen with the botanical extract at 6.25 μ M and 12.5 μ M (Figure 20). Surprisingly, the botanical extract at 25 μ M showed significantly greater inhibition of IL-6 than the synthetic zingerone at 150 μ M (Figures 20 and 21). A summary of the dose dependent inhibition by the disclosed botanical extract is set out in Table 18, below. Potential inhibition was observed from treatment with synthetic zingerone or acetyl zingerone at 150 μ M. This treatment with synthetic zingerone or acetyl zingerone reduced IL-6 to 64% and 69% of the absolute ethanol control respectively. These results are summarised in Table 18, below. Ferulic acid did not have an effect on IL-6 production at any concentration tested. Concentrations of the disclosed botanical extract associated with cytotoxicity were omitted to avoid confounding data.

Table 18. Summary of results for IL-6 inhibition

Compound	Concentration	IL-6 (% Vehicle) $\pm$ SEM
Botanical extract	25 μ M	41.66 $\pm$ 7.94 %
	12.5 μ M	61.58 $\pm$ 2.51 %
	6.25 μ M	76.64 $\pm$ 1.23 %
Synthetic zingerone	150 μ M	64.15 $\pm$ 4.01 %
Acetyl zingerone	150 μ M	69.90 $\pm$ 2.64 %
Dexamethasone (control)	100 nM	54.32 $\pm$ 11.03 %

[00426] To determine if RAW264.7 cells treated with the disclosed botanical extract had significantly lower levels of IL-6 compared to cells treated with synthetic zingerone, acetyl zingerone, or ferulic acid, a One-Way Analysis of Variance (One-Way ANOVA) was used. LPS-Stimulated RAW264.7 cells treated with the disclosed botanical extract at 25 μ M had significantly lower levels of IL-6 compared to treatment with synthetic zingerone, acetyl zingerone, or ferulic acid at 150 μ M (Figure 21). A Two-Way ANOVA was also performed to determine if the IL-6 levels observed in the compound treated conditions were significantly different from the vehicle treated conditions. Treatment with the disclosed botanical extract at 25 μ M or synthetic zingerone/acetyl

zingerone at 150 μM produced significantly lower IL-6 compared to treatment with the respective vehicles (Figure 22).

[00427] Under unstimulated conditions (Figures 23A-23D), the levels of IL-6 were very low relative to the stimulated conditions which was expected. Due to the low levels of cytokine produced in unstimulated conditions no trend was able to be observed. This provides confidence that the testing set is not inducing the production of IL-6. Because the levels of IL-6 in unstimulated conditions were low and often at 0 pg/mL, this data is not normalised to the absolute ethanol control.

[00428] Results – TNF: TNF is also a proinflammatory cytokine produced by macrophages when stimulated with LPS. The greatest inhibition of TNF from stimulated RAW264.7 cells was observed after treatment with ferulic acid at 150 μM . This treatment reduced TNF to 61% of the absolute ethanol control (Figure 24). At concentrations of 50 μM , the inhibition from ferulic acid treatment tapered off to the level of the absolute ethanol control. Synthetic zingerone had an apparently modest inhibitory effect on TNF production at 150 μM , reducing the level of TNF to around 80% of the absolute ethanol control. The disclosed botanical extract also had a modest inhibitory effect on TNF, reducing the level of TNF to around 80% of the absolute ethanol control at 25, 12.5 and 6.25 μM . acetyl zingerone had no effect on TNF production. Concentrations of the disclosed botanical extract associated with cytotoxicity were omitted to avoid confounding data. Results are summarised in Table 19, below.

Table 19. Summary of results for TNF inhibition

Compound	Concentration	TNF (% Vehicle) $\pm$ SEM
Ferulic acid	150 μM	61.09 $\pm$ 8.99 %
	100 μM	73.33 $\pm$ 11.49 %
Synthetic zingerone	150 μM	78.95 $\pm$ 8.04 %
Botanical extract	25 μM	80.44 $\pm$ 3.94 %
	12.5 μM	75.24 $\pm$ 4.11 %
	6.25 μM	79.31 $\pm$ 2.67 %
Dexamethasone (control)	100 nM	12.49 $\pm$ 2.05 %

[00429] No significant differences were observed from treatment with the disclosed botanical extract at 25 μM when compared to treatment with synthetic zingerone or ferulic acid at 150 μM (Figure 25). A statistically significant difference was observed between treatment with the disclosed botanical extract at 25 μM and treatment with acetyl zingerone

at 150 μ M. A Two-Way ANOVA was also performed to determine if the TNF levels in the compound treated conditions were statistically significant from the vehicle treated conditions. From this analysis, treatment with ferulic acid at 150 μ M showed significantly lower TNF levels compared to treatment with vehicle (Figure 26).

[00430] In unstimulated conditions (Figures 27A-27D), the levels of TNF were much lower and often not above the background reading of the ELISA. No trend in TNF production was observed in the unstimulated conditions. This provides confidence that the testing set is not inducing the production of TNF. Because the levels of TNF in unstimulated conditions were low and often at 0 pg/mL, this data is not normalised to the absolute ethanol control.

[00431] Results – NO: Nitric oxide (NO) is a small molecule released by macrophages during the inflammatory response which is capable of killing invading pathogens. Only the disclosed botanical extract had an inhibitory effect on NO, reducing it to 77% of the absolute ethanol control (Figure 28). Synthetic zingerone caused elevations at the higher range of concentrations tested (50, 75, 100 and 150 μ M) when compared to the absolute ethanol control. Acetyl zingerone at 150 μ M also caused a slight elevation in NO levels. Ferulic acid did not appear to alter NO production. Concentrations of the disclosed botanical extract associated with cytotoxicity were omitted to avoid confounding data. Results are summarised in Table 20, below.

Table 20. Summary of results for NO production

Compound	Concentration (μ M)	% NO Production $\pm$ SEM
Synthetic zingerone	150	137 $\pm$ 14.87 %
	100	140.44 $\pm$ 14.41 %
	75	135.95 $\pm$ 9.78 %
	50	128.64 $\pm$ 3.55 %
Acetyl zingerone	150	125.22 $\pm$ 7.69 %
Botanical extract	25	77.40 $\pm$ 6.64 %
Dexamethasone (control)	100	90 $\pm$ 3.98 %

[00432] Noting that nitric oxide measurement was only able to be performed in stimulated conditions due to the volume of supernatant required to measure it.

[00433] To determine if RAW 264.7 cells treated with the disclosed botanical extract at 25 μ M had significantly lower NO levels compared to the other treatment conditions, a One-Way ANOVA was used. Treatment with the disclosed botanical extract

at 25 μ M produced significantly lower levels of NO compared to treatment with synthetic zingerone, acetyl zingerone and ferulic acid at 150 μ M (Figure 29). To determine if the changes in NO observed were significantly different from its vehicle, a Two-Way ANOVA was used. Treatment with the disclosed botanical extract at 25 μ M did not produce significantly lower NO compared to treatment with vehicle. However, treatment with synthetic zingerone at 150 μ M produced significantly greater NO levels than treatment with vehicle (Figure 30).

[00434] Conclusions: Regarding the disclosed botanical extract, cytotoxicity was observed at concentrations about 50 μ M. The disclosed extract showed the greatest inhibition of IL-6 from stimulated RAW264.7 cells. Treatment with the disclosed botanical extract at 25 μ M reduced IL-6 levels to below 50% of the absolute ethanol control. This inhibition of IL-6 by the disclosed extract was statistically significant when compared to the vehicle control and when compared to treatment with synthetic zingerone, acetyl zingerone, or ferulic acid. Treatment with the disclosed botanical extract also produced a modest inhibition of TNF from stimulated RAW264.7 cells. In addition, the disclosed botanical extract at 25 μ M inhibited NO production from stimulated RAW264.7 cells. This inhibition of NO by the disclosed botanical extract was statistically significant when compared to treatment with synthetic zingerone, acetyl zingerone, or ferulic acid.

[00435] The other test compounds produced mixed results and, in most cases, substantially lower efficacy. Treatment with synthetic zingerone or acetyl zingerone at 150 μ M produced a modest inhibition of IL-6. These reductions were statistically significant when compared to treatment with vehicle. Treatment with synthetic zingerone at 150 μ M produced a modest reduction in TNF levels. Ferulic acid at 150 μ M also inhibited TNF production, reducing levels to around 60% of the absolute ethanol control. This reduction was deemed to be statistically significant when compared to treatment with vehicle. Ferulic acid showed no effect on NO production. Synthetic zingerone and acetyl zingerone both increased NO production. The increase in NO by synthetic zingerone was deemed to be statistically significant when compared to treatment with vehicle.

[00436] In summary, it was observed that the disclosed botanical extract significantly out-performed the commercially available compounds tested. The botanical extract showed potent inhibition of proinflammatory cytokine expression and proinflammatory small molecule production when compared to commercially sourced

zingerone and commercially sourced acetyl zingerone. These results demonstrate the superior treatment efficacy of the botanical extract for blockade of/interference with inflammatory pathways.

[00437] It should be recognised that the superior inhibitory activity of the botanical extract is particularly surprising, especially given that the comparative testing utilised the botanical extract at significantly lower concentrations (e.g., 25 μ M) than the commercially available compounds (e.g., 100 μ M or 150 μ M).

Example 11: Preliminary testing in animals

[00438] Overview: Zingerone administration was investigated in an encephalomyelitis (EAE) animal model.

[00439] Materials and methods: Six groups of female C57BL/6J mice (9-11 weeks, 5 per group) were administered via oral gavage a vehicle control, zingerone (10 mg/kg), or other test compounds, alongside a healthy control group. EAE induction was initiated on day 1. Test compounds were administered daily from days 5-30.

[00440] Results: EAE was not successfully induced in this study (data not shown). However, body weights of the mice were monitored as an indication of safety and tolerability. On average, body weight increased by 0.3 g over the treatment period, with an average weight of 19.7 g at the beginning of the treatment period and an average weight of 20.0 g at the end of the treatment period. No significant change in body weight was observed for the zingerone treatment, which indicated the dosage used was safe and tolerable.

Example 12: Large scale production process

[00441] Overview: A large scale production process has been carried out. This allows up to 700 ± 50 kg raw ginger rhizome per run as outlined below.

[00442] Step 1: Initial juicing of ginger rhizome.

[00443] Introduction: A total of 1360 kg raw ginger was delivered to Phytex to Synergy Food group manufacturing plant in Brookvale, Sydney. Material was required to be stored under refrigerated conditions ($4^{\circ}\text{C} \pm 3^{\circ}\text{C}$) when stored overnight. In processing, material was transferred back to refrigerated conditions to the extent possible to reduce the risk of degradation. Ginger juice was collected in 200 L blue poly drums for transport back to Phytex facility. The solid marc was collected in a separate 200 L blue poly drum (see,

e.g., Figure 31). Equipment and juicing areas were cleaned and maintenance/calibration was performed on equipment. Clean tags or cleaning logs were used as needed.

[00444] Run A details: Ginger rhizomes were divided into two equal ~700 kg runs. The second 700 kg batch was stored under refrigeration until use in the following week. The first 700 kg batch was passed through the belt press juicer (Voran EBP500). All juice obtained from the juicer was collected into 200 L blue HDPE drums (provided by Phytex) on a wooden pallet. All solid/marc obtained from the juicer was placed into a separate 200 L blue HDPE drum (provided by Phytex). The solid marc was passed through the belt press juicer (Voran EBP500) a second time to remove further liquid from the solid marc. The liquid from the second press was combined with the original 200 L blue HDPE drums containing the first-pressed liquid.

[00445] Run B details: The following week, juicing was commenced for the second 700 kg batch. The second 700 kg batch was passed through the belt press juicer (Voran EBP500). All juice obtained from the juicer was placed into 200 L blue HDPE drums (provided by Phytex) on a wooden pallet. All solid/marc obtained from the juicer was collected into a separate 200 L blue HDPE drum (provided by Phytex). The solid marc was passed through the belt press juicer (Voran EBP500) a second time to remove further liquid from the solid marc. The liquid from the second press was combined with the original 200 L blue HDPE drums containing the first-pressed liquid.

[00446] The total amounts of ginger juice and solid marc were transferred for in-process sampling and readiness for Ultrafiltration (UF). The mass balance and percentage yield were calculated and recorded. At the end of Step 1, approximately 600 L $\pm$ 50 L ginger juice and 200 kg + 50 kg ginger marc/solid was obtained from each batch of 700 kg + 50 kg ginger rhizome.

[00447] Step 2 (i): Alkaline treatment of ginger juice and ginger marc. See, e.g., flowchart in Figure 32.

[00448] Introduction: Run A and Run B were performed in two equal treatments with KOH. The extraction vessel had maximum capacity of 400 L, so each run (A or B) was performed in two parts (run A1/A2 and run B1/B2). Equipment maintenance/calibration and cleaning status was checked before proceeding with treatment. Clean tags or cleaning logs were used as needed.

[00449] Processing of ginger juice (300 L per run): The volume of ginger juice obtained in the multiple 200 L HDPE drums was measured. Net weights were recorded for all HDPE drums. The ginger juice/settleable solids remaining in each 200 L HDPE drum were mixed using a 1 m plastic mixing paddle to ensure solids were resuspended. A clamp was attached to the decanting lid (lid with 25 mm poly valve) to each 200 L HDPE drum. Using the drum lifter all solid fractions were poured into the extraction vessel to combine with the UF concentrate. A wall mounted stirrer purple propeller was inserted into the extraction vessel with 2 x 100 mm stainless steel propellers attached. Propeller heights were set to the appropriate height with to mix the juice volume. The wall mounted stirrer was connected on utilities board and stirring was commenced in a clockwise direction at 50 Hz. The settleable sludge from the bottom of each drum was scooped out and transferred to the extraction vessel (to be dissolved by the heat and alkaline treatment). Each drum was hosed with 1 L water to remove all residual ginger juice, and this was added to the extraction vessel.

[00450] Adding potassium hydroxide to ginger juice: Stirring being commenced, KOH was added at 2.0% w/w. This was based on the volume of total juice obtained from ginger rhizome (raw material). Before KOH addition, the volume for the ginger juice and the initial pH level were recorded. A blue HDPE drum was tared on the 150 kg scale. The total amount of ginger juice was weighed for inclusion in the alkaline treatment. The pH electrode was calibrated with pH 7.0 and pH 4.0 buffer solutions, and calibration was checked with a pH 2.0 buffer solution. In the extraction vessel, 50% KOH was added to the ginger juice extract at the rate of: $\text{wt. KOH (kg)} = \text{wt. ginger juice (kg)} \times 0.03 \text{ kg KOH (50\%)}$. Addition at this rate increased the pH to 13.0 ± 1.0 . All pH reading adjustments were incorporated with automatic temperature compensation (Mettler Toledo pH meter). When the pH range was reached, stirring was continued for a further 15 minutes (minimum). The final pH and volume of added KOH was recorded.

[00451] Alkaline treatment procedure for ginger juice: A stainless steel steam coil was inserted into the extraction vessel and connected to the steam inlet on the utilities wall using the dedicated steam hose. The steam valve was turned on at the wall to allow steam to heat the coil. The pH electrode was calibrated with pH 7.0 and pH 4.0 buffer solutions, and calibration was checked with a pH 2.0 buffer solution. The ginger juice (pH greater than 13) was heated to $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with stirring for 60 minutes. The steam flow was

adjusted to ensure the alkaline liquid remained with the temperature range ($60^{\circ}\text{C} \pm 2^{\circ}\text{C}$). A sample was taken from the stirred alkaline ginger juice (20 mL) to monitor conversion of 6-gingerol to zingerone. The volume of the alkaline ginger juice was recorded.

[00452] Neutralisation procedure for ginger juice: The alkaline ginger juice was stirred for another 5 minutes before neutralising the solution with citric acid >98.0%. The SS coil within the extraction vessel was connected with cooling water and the cooling water was circulated. The addition of citric acid (>98%) to the alkaline juice represented a strong acid-base reaction, hence being exothermic and therefore generating heat. The cooling water was applied to alleviate excess heat from the solution. Prior to use, the citric acid was prepared. This was based on a raw material starting weight of 1340 kg. Citric acid granules (18.0 kg) were weighed out into a 20 L purple polythene bucket. Extra citric acid aqueous solution (50% w/v) was prepared by dissolving 1.0 kg citric acid granules into 1.0 L purified water in a 20 L purple polythene bucket. This was dissolved with a stainless steel hand stirrer. Whilst alkaline ginger juice was stirring in the extraction vessel, citric acid granules were added. This was done by adding 10 kg and waiting for the granules to dissolve. The pH of the solution was monitored during this process. The slow addition of citric acid was continued using 500 ml scoops at a time and waiting 30 seconds before the adding the next scoop. Once all of the citric acid was added and dissolved (18.0 kg), a pH reading was obtained. Then the 50% citric acid aqueous solution was added until a pH range of 7.0-7.3 was obtained. When this desired range was reached, stirring was continued for a further 15 minutes. All pH reading adjustments were incorporated with automatic temperature compensation (Mettler Toledo pH meter). The resulting solution was then noted as “zingerone liquid”. The final pH, volume of added citric acid (>98%), and volume of zingerone liquid was recorded. A sample was taken from the zingerone liquid (20 mL) to monitor conversion of 6-gingerol to zingerone.

[00453] Step 2 (ii): Alkaline treatment of ginger marc. See, e.g., flowchart in Figure 33.

[00454] Processing ginger marc/solids (50 kg per run): The wall mounted stirrer was inserted into the extraction vessel with three large 150 mm propellers attached. Stirring was commenced at 50 Hz. From the 200 L HDPE drum, 50 kg total solid marc was added to the extraction vessel. It was ensured that the solid was continually drawn into the liquid

and the solution was moving freely with the stirrer. If the solution became too viscous, an addition 20 L of water was added.

[00455] Adding potassium hydroxide to ginger marc/solids: KOH was added at 2% w/w. This was based on based on weight of the volume of total juice extract obtained from ginger rhizome raw material. A blue HDPE drum was tared on the 150 kg scale. The total amount of marc was weighed for inclusion in the alkaline treatment. The pH electrode was calibrated with pH 7.0 and pH 4.0 buffer solutions, and calibration was checked with a pH 2.0 buffer solution. In the extraction vessel, 50% KOH was added to the ginger juice extract at the rate of: $\text{wt. KOH (kg)} = \text{wt. ginger marc (kg)} \times 0.03 \text{ kg KOH (50\%)}$. The calculations accounted for the weight of ginger marc (50 kg) and water added (20 kg). Addition at this rate increased the pH to 13.0 ± 1.0 . When the pH range was reached, stirring was continued for a further 15 minutes (minimum). The final pH and volume of added KOH was recorded.

[00456] Alkaline treatment procedure for ginger marc/solids: The SS steam coil was s inserted into the extraction vessel and connected to the steam inlet on the utilities wall using the dedicated steam hose. The steam valve was turned on at the wall to allow steam to heat the coil. The pH electrode was calibrated with pH 7.0 and pH 4.0 buffer solutions, and calibration was checked with a pH 2.0 buffer solution. The ginger marc (pH greater than 13) was heated to $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with stirring for 60 minutes. The steam flow was adjusted to ensure the alkaline liquid remained with the temperature range ($60^{\circ}\text{C} \pm 2^{\circ}\text{C}$). A sample was taken from the stirred alkaline ginger marc (20 mL) to monitor conversion of 6-gingerol to zingerone. The volume of the alkaline ginger marc was recorded.

[00457] Neutralisation of ginger marc/solids: The alkaline ginger marc was stirred for another 5 minutes before neutralising the solution with citric acid >98.0%. The SS coil within the extraction vessel was connected with cooling water and the cooling water was circulated. This was applied to alleviate excess heat from the exothermic reaction (acid + base). Prior to use, the citric acid was prepared as noted above. Whilst alkaline ginger marc was stirring in the extraction vessel, citric acid granules were added. This was done by adding 5 kg and waiting for the granules to dissolve. The pH of the solution was monitored during this process. The slow addition of citric acid was continued using 500 ml scoops at a time and waiting 30 seconds before the adding the next scoop. Once all of the citric acid

was added and dissolved (18 kg, total), a pH reading was obtained. Then the 50% citric acid aqueous solution was added until a pH range of 7.0-7.3 was obtained. When this desired range was reached, stirring was continued for a further 15 minutes. All pH reading adjustments were incorporated with automatic temperature compensation (Mettler Toledo pH meter). The resulting solution was then noted as “zingerone marc”. The final pH, volume of added citric acid (>98%), and volume of zingerone marc was recorded. A sample was taken from the zingerone marc (20 mL) to monitor conversion of 6-gingerol to zingerone. The mass balance and percentage yield were calculated.

[00458] Step 3: Production of zingerone powder by drying and milling.

[00459] Oven drying: Equipment maintenance/calibration and cleaning status were confirmed. The drying over was set up with calibrated COMARK digital logger with stainless steel probe. Time was recorded for each drum of zingerone concentrate to be taken from the cold room to prepare for drying. The Brix% and theoretical zingerone content were noted. The oven was loaded with all trays having Teflon mats for drying. The drum was mixed using a polypropylene paddle before using a 4 L jug to remove a volume of liquid. This was subsequently transferred to a 2 L jug to allow placement into each tray. Next, 3 L was poured onto the Teflon mat for each tray. The trays were loaded onto each shelf to ensure a gap between the trays (not less than 30 mm) and allow for airflow and to allow breaking up of dried materials. The oven temperature was set at 60°C. After 24 hours, each tray was inspected and scraped to release the softer portions off the Teflon mat as needed. The underside of the product was positioned on the top of the flipped tray. The product was gently broken up using the plastic spatula to facilitate drying. The trays were loaded back into the oven and dried for a further 4 hours (28 hours total). When sufficient dried (at 24 or 28 hours), the product was crushed manually with a stainless steel spatula and collected in 100 micron poly bag. Each bag was weighed and recorded and placed into a 100 L square mobile tub. The square mobile tub was weighed and recorded. The product was then ready for milling.

[00460] Milling: Prior to milling, the stainless steel hammer mill was checked as clean and fit with a clean 1 mm mesh screen (“fine zingerone screen”). The lid was fitted with a 3 mm rubber mat seal and the thumb screw was firmly tightened to lock the lid in place. The hammer mill was run for 10 seconds to ensure correct rotation and clearances. A 100 micron poly bag was fitted to the outlet of the mill chamber and tightened with an

adjustable clamp. The overhead dust extractor was activated. Slowly, the coarse dry zingerone material was transferred into the feed chute using the poly scoop. Two scoops at a time were emptied into the chute and the inlet flap was opened every few seconds. All the material in the chute was passed through the stainless steel screen and collected in a poly bag collector. All bags were weighed and calculations were made to determine mass balance and percentage yield. A representative sample (20 g) was removed from each bag and assessed for percentage moisture determination. All 20 kg bags were placed into an additional 100-micron poly bag. The remaining air void was evacuated before sealing with a double seal using an Impulse heat sealer. The bags were stored in the cold room. Quality analysis was performed for the final product (zingerone powder). See Table 21, below. Quality indicators were found to be excellent.

Table 21. Zingerone extract (powder) quality assessment

Content of zingerone (HPLC)	0.2 to 0.4%
Pesticide residues	Less than limit of reporting
Aflatoxins	Less than limit of reporting
Total aerobic microbial count (cfu/g)	180 cfu/ml
Total yeast and mould count (cfu/g)	< 10 cfu/ml
<i>E. coli</i> and salmonella	Absent
Elemental impurities	Cu = 5.4 mg/kg Zn = 31 mg/kg Less than limit of reporting for As, Cd, Cr, Hg, Ni, Pb
Acid insoluble ash	1%
Water soluble ash	35.8%
Total ash	37.1%
Gingerol content	≤ 0.1%
Water determination	3.8%

[00461] Step 4: Ethanol extraction of zingerone powder.

[00462] Introduction: Up to 75 kg dry powder was able to be extracted in a single extraction step as outlined below. Equipment maintenance/calibration and cleaning status (including extraction vessel) was confirmed. The 40 mm stainless steel strainer was attached to the outlet in the bottom of the extraction vessel. This was hand tightened into the bottom 25 mm thread to strain out the solids after extraction stirring. The dedicated stainless steel heating coil was fitted inside the extraction vessel for connection to steam later in this step. The glass condenser coil was attached to the lid of the extraction tank and the inlet/outlet was connected to the cooling water inlet/outlets on the wall. The cooling water was circulated through the glass condenser coil.

[00463] Single extraction: For the extraction, 95% ethanol was added to the clean extraction vessel with the lid fitted. The following addition rate was utilised: vol 95% ethanol = amt. zingerone powder (kg) x 5 kg. The purple WMS shaft was inserted through the bearing gland in the lid of the extraction vessel. Stirring was commenced at 200-300 rpm to facilitate complete mixing of the ethanol solution. Slowly, 75 kg of dry zingerone powder (from Step 3) was added to the extraction vessel through the 100 mm access port on the lid of the vessel. Stirring was continued during this addition. Once all the powder was added, the access port was closed and the lid was sealed. All initial temperatures were recorded before heating. Then, the steam supply was connected to the stainless steel coil in the extraction vessel. Whilst stirring, the temperature of the ethanolic solution was monitored and maintained at a minimum of 50°C for at least 4 hours. The stirrer was turned off and the solution was allowed to stand for 20 minutes. The final temperature was recorded prior to filtration of the ethanolic extract. A sample was removed from the ethanolic extract (20 mL) and tested for zingerone.

[00464] Filtering of residual solid from the ethanolic extract: The contents of the ethanolic extract were drained into two clean 200 L HDPE drums. Stirring was continued while draining. The ethanolic extract was allowed to sit for 30 minutes, to allow any solids to settle. The hopper plate filter was set up with a Z1 pad (5 micron). This was fitted and compacted with a stainless steel plunger tool. The upper layer of the ethanolic extract was decanted through the hopper plate filter using the drum lifter. The decanted solution was poured thorough a 55 micron pre-filter and collected into buckets. The filtered ethanolic extract was collected from the hopper plate filter into two clean blue HDPE drums. When the hopper plate filter was filled with solid components, a full vacuum was applied to the unit for 10 minutes, until all the liquid was collected. The hopper plate filter was emptied after every vacuum filter and set aside in clean poly buckets. This was repeated until all of the ethanolic extract had been filtered through the Z1 pad. The volume of 95% ethanol wash was recorded. A sample was removed from the combined filtered ethanolic extract (20 mL) and tested for zingerone. The two drums with filtered ethanolic extract were stored in the cold room.

[00465] Rotary evaporation of the ethanolic extract: Three 15 L buckets of filtered ethanolic extract were removed from the cold room and allowed to equilibrate to room temperature. A Heidolph round bottom flask was prepared, dried, and weighed. The

Heidolph 20 L rotary evaporator was set up with 1 L fresh 95% ethanol added to the RBF. The cooling water inlet/outlet lines was connected to appropriate valves on the wall and the valve was adjusted to alter flow rate as required. The rotary evaporator was connected to the Heidolph vacuum pump. A white 4 mm HDPE tube, submerged in 95% ethanol, was connected to the inlet tap on the rotary evaporator. Circulation of cooling water was commenced through the glass condenser. Residual water was removed during the evaporation stage by running at a minimum 50 mBar and 40°C. Accordingly, the vacuum was set and slow rotation of the round bottom flask was commenced (approximately 60 rpm). As each bucket was emptied, the inlet tube was transferred to the next bucket until all three buckets (45 L total) had been bled into the rotary evaporator and collected in the condenser flask. Observations were made for pressure, colour, etc.

[00466] Standardisation of ethanolic extract: For each rotary evaporator run (45 L filtered ethanolic extract in three buckets), the volume was condensed down to the 4 L volume line marked on the outside of the RBF. This line represented roughly the standard mark that 45 L Ethanol Extract is condensed down to 4 L (12.5% mg/ml). At that point, a sample of the concentrated tincture (10 mL) was removed and tested for zingerone. If the concentration of zingerone was greater than 12.5 mg/ml, 1 L filtered combined ethanolic extract from Step 4 was added and evaporation was continued until the correct concentration was achieved. If the concentration of zingerone was less than 12.5 mg/ml, evaporation was continued until the correct concentration was achieved. Twelve amber 4 L glass bottles were rinsed with fresh 95% ethanol and left to drain for 30 minutes. When standardised to 12.5 mg/ml, the RBF was removed from the rotary evaporator and placed into a 15 L bucket. The standardised zingerone tincture was decanted into the rinsed and drained bottles, leaving 30 mm from the bottom thread on the bottle. The filled bottles were capped with poly lids having Teflon seals and hand tightened. Parafilm was wrapped around the bottle neck and lid. Sealed bottles were placed into 100 micron poly bags and double heat sealed. These were placed in a box and the box was put into the cold room. Quality analysis was performed for the final product (ethanolic extract). See Table 22, below. Quality indicators were found to be excellent. Stability of the final product was assessed and confirmed after 2 months of storage under accelerated conditions (40°C ±2°C/75% ± 5% RH), and independently, after 2 months of storage at cooling conditions (5°C). Stability was found to be extremely high. See Tables 23 and 24, below. Due to the small sample size tested at accelerated conditions (less than 2 g), some

variability was seen at 1 month time point (Table 23). It is understood that larger sample sizes in storage act to enhance stability of the product by reducing container headspace. Good stability is expected for at least 6 months storage.

Table 22. Zingerone extract (tincture) quality assessment

Content of zingerone (HPLC)	12.8 mg/ml $\pm$ 10%
Pesticide residues	Less than limit of reporting
Total aerobic microbial count (cfu/g)	< 1 cfu/ml
Total yeast and mould count (cfu/g)	< 1 cfu/ml
Limit of non-volatile residues	440 mg
Total ash	3.0%
Specific gravity	0.88
Gingerol content	$\leq$ 1%
Alcohol content	67%

Table 23. Zingerone extract (tincture) stability assessments

Batches / Conditions	Time point (months)	Zingerone (% w/w)	Zingerone (mg/ml)	Gingerols Shogaols (% w/w)	Gingerols + Shogaols (mg/ml)	Gingerols (% w/w)	Gingerols (mg/ml)	Shogaols (% w/w)	Shogaols (mg/ml)
40°C / 75% RH	0	1.62	13.58	0.18	1.51	0.07	0.58	0.11	0.93
	1	1.41	12.39	0.19	1.45	0.07	0.54	0.10	0.91
	2	1.59	13.21	0.17	1.45	0.07	0.58	0.10	0.88

Table 24. Zingerone extract (tincture) stability assessments

ANALYSES /SPECS		Zingerone Assay (HPLC)		pH	Microbial Enumeration Tests			Appearance (Visual)	
STORAGE (months)	CONDITION	Assay (mg/mL)		Report and trend	TAMC	Yeasts & Mould		Describe, note changes	
0	5° C	AIM: NLT 11.5	mg/mL		NMT 10000 cfu/g	NMT 1000 cfu/g			
1	5° C	12.8		7.48	<1	<1			
2	5° C	12.6		7.65	<10	<10		Dark brown liquid with a strong smell of ginger	
		12.8		7.67	<10	<10		Dark brown liquid with a strong smell of ginger	
ANALYSES /SPECS		Zingerone Assay (HPLC)		pH	Microbial Enumeration Tests			Appearance (Visual)	
STORAGE (months)	CONDITION	Assay (mg/mL)		Report and trend	TAMC	Yeasts & Mould		Describe, note changes	
0	40° C/75% RH	AIM: NLT 11.52	mg/mL		NMT 10000 cfu/g	NMT 1000 cfu/g			
1	40° C/75% RH	12.8		7.48	<1	<1			
		12.5		7.67	<10	<10		Dark brown liquid with a strong smell of ginger	

[00467] Persons of ordinary skill can utilise the disclosures and teachings herein to produce other embodiments and variations without undue experimentation. All such embodiments and variations are considered to be part of this disclosure.

[00468] Accordingly, one of ordinary skill in the art will readily appreciate from the present disclosure that later modifications, substitutions, and/or variations performing substantially the same function or achieving substantially the same result as embodiments described herein may be utilised according to such related embodiments. Thus, the present disclosure is intended to encompass, within its scope, the modifications, substitutions, and variations to processes, manufactures, compositions of matter, compounds, means, methods, and/or steps disclosed herein.

[00469] The description herein may contain subject matter that falls outside of the scope of the claimed invention. This subject matter is included to aid understanding of the invention.

[00470] In this specification, where reference has been made to external sources of information, including patent specifications and other documents, this is generally for the purpose of providing a context for discussing the features of this disclosure. Unless stated otherwise, reference to such sources of information is not to be construed, in any jurisdiction, as an admission that such sources of information are prior art or form part of the common general knowledge in the art.

CLAIMS

- 1 A method of producing zingerone, comprising:
 - (i) subjecting ginger root to an alkaline treatment in an alkaline solution; or
 - (ii) subjecting juice and/or marc obtained from ginger root to an alkaline treatment in an alkaline solution;thereby producing zingerone.
- 2 The method as claimed in claim 1 wherein the ginger root is fresh.
- 3 The method as claimed in claim 1 or claim 2, wherein:
 - (a) the ginger root of (i) is chopped;
 - (b) the ginger root of (i) is chopped and dried; or
 - (c) the juice and/or marc of (ii) is obtained via macerating and/or pressing ginger root.
- 4 The method as claimed in any one of claims 1 to 3, wherein:
 - (a) potassium hydroxide (KOH) is used in the alkaline solution; or
 - (b) a liquid form of potassium hydroxide (KOH) is used in the alkaline solution.
- 5 The method as claimed in claim 4, wherein the alkaline solution comprises:
 - (a) about 1% to about 6% KOH (v/v);
 - (b) about 1.5% to about 5.5% KOH (v/v);
 - (c) about 2% to about 4% KOH (v/v);
 - (d) about 1.5% to about 3.5% KOH (v/v); or
 - (e) about 2% KOH (v/v).
- 6 The method as claimed in any one of claims 1 to 3, wherein calcium hydroxide ($\text{Ca}(\text{OH})_2$) is used in the alkaline solution.
- 7 The method as claimed in claim 6, wherein the alkaline solution comprises:
 - (a) about 0.5% to about 4% $\text{Ca}(\text{OH})_2$ (v/v);
 - (b) about 1.5% to about 3.5% $\text{Ca}(\text{OH})_2$ (v/v); or
 - (b) about 2.0% to about 3.0% $\text{Ca}(\text{OH})_2$ (v/v).
- 8 The method as claimed in any one of claims 1 to 7, wherein:

- (a) the alkaline treatment is carried out at about 40 to about 70 degrees Celsius;
 - (b) the alkaline treatment is carried out at about 50 to about 60 degrees Celsius;
 - (c) the alkaline treatment is carried out at about 55 to about 65 degrees Celsius; or
 - (d) the alkaline treatment is carried out at about 60 degrees Celsius.
- 9 The method as claimed in any one of claims 1 to 8, wherein:
- (a) the alkaline treatment is carried out for about 1-30 hours, about 1-20 hours, about 1-10 hours, or about 1-5 hours;
 - (b) the alkaline treatment is carried out for about 0.5 to about 3 hours, or about 0.75 to about 2.5 hours, or about 1 to about 2 hours; or
 - (c) the alkaline treatment is carried out for about 1 hour, or about 2 hours.
- 10 The method as claimed in any one of claims 1 to 9, including a further step of:
- (a) neutralising the alkaline solution;
 - (b) neutralising the alkaline solution and then drying; or
 - (c) neutralising the alkaline solution, optionally drying, and then carrying out one or more additional extraction steps.
- 11 The method as claimed in claim 10, wherein the one or more additional extraction steps include:
- (a) one or more ethanol extraction steps;
 - (b) supercritical fluid extraction; or
 - (c) a supercritical fluid extraction followed by one or more ethanol extraction step.
- 12 The method as claimed in any one of claims 1 to 11, wherein the method produces a composition comprising zingerone.
- 13 The method as claimed in any one of claims 1 to 12, wherein the method produces a composition that is free from aldehydes or substantially free from aldehydes.
- 14 A composition comprising zingerone, wherein the zingerone is obtained by the method of any one of claims 1 to 13.
- 15 The composition as claimed in claim 14, wherein the composition is formulated as a pharmaceutical composition or a dietary supplement.

- 16 The composition as claimed in claim 14 or claim 15, wherein the composition is formulated as a liquid, a solid, or a semi-solid.
- 17 The composition as claimed in any one of claims 14 to 16, which is formulated for topical administration or oral administration.
- 18 The composition as claimed in any one of claims 14 to 17, which is formulated as a solution, tincture, gel, jelly, gummy, powder, tablet, or capsule.
- 19 The composition as claimed in any one of claims 14 to 18, which is formulated to include:
 - (a) a dose of between about 10 mg to about 1500 mg of zingerone;
 - (b) a dose of between about 10 mg to about 1000 mg of zingerone;
 - (c) a dose of between about 10 mg to about 150 mg of zingerone;
 - (d) a dose of about 10 mg to about 100 mg of zingerone; or
 - (e) a dose of about 10 mg to about 50 mg of zingerone.
- 20 The composition as claimed in any one of claims 14 to 19, which is:
 - (a) formulated for co-administration with a further anti-inflammatory agent;
 - (b) formulated for co-administration with one or more of: an analgesic compound, antipyretic compound, and psychotropic compound;
 - (c) formulated for co-administration with one or more of: a cannabinoid compound, mushroom compound, non-steroid anti-inflammatory drug compound (NSAID), opioid compound, salicylate compound, and steroid compound;
 - (d) comprising one or more of: acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, eptazocinem, betamethasone, cortisone, deflazacort, dexamethasone, ethamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, cannabidiol, cannabigerol, tetrahydrocannabinol, psilocybin, and psilocin.
- 21 The composition as claimed in any one of claims 14 to 20 for use in treating or preventing inflammation.

- 22 The composition as claimed in claim 21, wherein the inflammation is acute or chronic inflammation.
- 23 The composition as claimed in claim 22, wherein:
- (a) the inflammation is an inflammatory disorder;
 - (b) the inflammation requires modulation;
 - (c) the inflammation is associated with an immune disorder;
 - (d) the inflammation is associated with an arthritic disorder;
 - (e) the inflammation is associated with an infection;
 - (f) the inflammation is associated with a cardiac, circulatory, or pulmonary disorder;
 - (g) the inflammation is associated with a neurological disorder; and/or
 - (h) the inflammation is associated with a neoplastic disorder.
- 24 The composition as claimed in any one of claims 21 to 23, wherein the inflammation is of one or more of: a joint, skin, eye, ear, nose, mouth, throat, oesophagus, kidney, bladder, liver, spleen, lung, heart, brain, circulatory system, digestive system, endocrine system, genitourinary system, lymphatic system, nervous system, and skeletal system.
- 25 The composition as claimed in any one of claims 21 to 24, wherein the inflammation is associated with one or more of: Alzheimer's disease, early stage Alzheimer's disease, ankylosing spondylitis, arthritis, asthma, colitis, Crohn's disease, dementia, early stage dementia, depression, diabetes, fibromyalgia, gout, infection, immune mediated inflammatory disease, inflammatory bowel disease, interstitial cystitis, multiple sclerosis, polymyalgia psoriasis, scleroderma, and Sjögren's syndrome, and systemic lupus erythematosus.
- 26 The composition as claimed in any one of claims 21 to 24, wherein the inflammation is associated with one or more of: rheumatoid arthritis, ankylosing spondylitis arthritis, fibromyalgia arthritis, gout arthritis, juvenile idiopathic arthritis (JIA), lupus arthritis, osteoarthritis, polymyalgia rheumatica, psoriatic arthritis, reactive arthritis, scleroderma arthritis, and Sjögren's syndrome arthritis.
- 27 The composition as claimed in any one of claims 21 to 24, wherein the inflammation is associated with one or more of: atherosclerosis, coronary artery disease, pulmonary artery hypertension, hypoxia-induced pulmonary hypertension,

pneumonia, acute respiratory distress syndrome, coronavirus respiratory disorder, and cytokine storm syndrome; or from one or more of: breast cancer, leukaemia, multiple myeloma, myelodysplastic syndrome, pancreatic cancer, and prostate cancer.

- 28 The composition as claimed in any one of claims 21 to 24, wherein the inflammation is associated with one or more of: a blister, dermatitis, eczema, hive, lesion, papule, plaque, psoriasis, rash, rosacea, ulcer, and wound.
- 29 Use of a composition as claimed in claim 14 for preparing a medicament for treating or preventing inflammation in a subject.
- 30 The use as claimed in claim 29, wherein the medicament provides for reducing or slowing progression of the inflammation.
- 31 The use as claimed in claim 29 or claim 30, wherein:
- (a) the medicament is formulated as a solid, semi-solid, or liquid; and/or
 - (b) the medicament is formulated for topical administration or oral administration.
- 32 The use as claimed in any one of claims 29 to 31, wherein the medicament is formulated as a solution, gel, jelly, gummy, powder, tablet, or capsule.
- 33 The use as claimed in claim 32, wherein the medicament is formulated to include:
- (a) a dose of between about 10 mg to about 1500 mg of zingerone;
 - (b) a dose of between about 10 mg to about 1000 mg of zingerone;
 - (c) a dose of between about 10 mg to about 150 mg of zingerone;
 - (d) a dose of about 10 mg to about 100 mg of zingerone; or
 - (e) a dose of about 10 mg to about 50 mg of zingerone.
- 34 The use as claimed in any one of claims 29 to 33, wherein:
- (a) the composition is formulated for co-administration with a further anti-inflammatory agent;
 - (b) the composition is formulated for co-administration with one or more of: an analgesic compound, antipyretic compound, and psychotropic compound;
 - (c) the composition is formulated for co-administration with one or more of: a cannabinoid compound, mushroom compound, non-steroid anti-inflammatory drug

compound (NSAID), opioid compound, salicylate compound, and steroid compound;

(d) the medicament comprises one or more of: acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, eptazocinem, betamethasone, cortisone, deflazacort, dexamethasone, ethamethasoneb, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, cannabidiol, cannabigerol, tetrahydrocannabinol, psilocybin, and psilocin.

35 The use as claimed in any one of claims 29 to 34, wherein the inflammation is acute or chronic inflammation.

36 The use as claimed in claim 35, wherein:

- (a) the inflammation is an inflammatory disorder;
- (b) the inflammation requires modulation;
- (c) the inflammation is associated with an immune disorder;
- (d) the inflammation is associated with an arthritic disorder;
- (e) the inflammation is associated with an infection;
- (f) the inflammation is associated with a cardiac, circulatory, or pulmonary disorder;
- (g) the inflammation is associated with a neurological disorder; and/or
- (h) the inflammation is associated with a neoplastic disorder.

37 The use as claimed in claim 35 or claim 36, wherein the inflammation is inflammation of one or more of: a joint, skin, eye, ear, nose, mouth, throat, oesophagus, kidney, bladder, liver, spleen, lung, heart, brain, circulatory system, digestive system, endocrine system, genitourinary system, lymphatic system, nervous system, and skeletal system.

38 The use as claimed in any one of claims 35 to 37, wherein the inflammation is associated with one or more of: Alzheimer's disease, early stage Alzheimer's disease, ankylosing spondylitis, arthritis, asthma, colitis, Crohn's disease, dementia, early stage dementia, depression, diabetes, fibromyalgia, gout, infection, immune mediated inflammatory disease, inflammatory bowel disease, interstitial cystitis,

multiple sclerosis, polymyalgia psoriasis, scleroderma, and Sjögren's syndrome, and systemic lupus erythematosus.

- 39 The use as claimed in any one of claims 35 to 37, wherein the inflammation is associated with one or more of: rheumatoid arthritis, ankylosing spondylitis arthritis, fibromyalgia arthritis, gout arthritis, juvenile idiopathic arthritis (JIA), lupus arthritis, osteoarthritis, polymyalgia rheumatica, psoriatic arthritis, reactive arthritis, scleroderma arthritis, and Sjögren's syndrome arthritis.
- 40 The use as claimed in any one of claims 35 to 37, wherein the inflammation is associated with one or more of: atherosclerosis, coronary artery disease, pulmonary artery hypertension, hypoxia-induced pulmonary hypertension, pneumonia, acute respiratory distress syndrome, coronavirus respiratory disorder, and cytokine storm syndrome; or from one or more of: breast cancer, leukaemia, multiple myeloma, myelodysplastic syndrome, pancreatic cancer, and prostate cancer.
- 41 The use as claimed in any one of claims 35 to 37, wherein the inflammation is associated with one or more of: a blister, dermatitis, eczema, hive, lesion, papule, plaque, psoriasis, rash, rosacea, ulcer, and wound.
- 42 A method of treating or preventing inflammation, the method comprising administering to a subject a composition as claimed in claim 15, thereby treating or preventing the inflammation.
- 43 The method as claimed in claim 42, wherein the administration reduces or slows progression the inflammation.
- 44 The method as claimed in claim 42 or claim 43, wherein:
- (a) the composition is administered as a solid, semi-solid, or liquid; and/or
 - (b) the composition is administered by topical administration or oral administration.
- 45 The method as claimed in any one of claims 42 to 44, wherein the composition is administered as a solution, gel, jelly, gummy, powder, tablet, or capsule.
- 46 The method as claimed in claim 45, wherein the composition is administered at:
- (a) a dose of between about 1 mg to about 5000 mg of zingerone;
 - (b) a dose of between about 1 mg to about 1500 mg of zingerone;
 - (c) a dose of between about 5 mg to about 500 mg of zingerone;

- (d) a dose of about 1 mg to about 15 mg of zingerone; or
 - (e) a dose of about 1 mg to about 10 mg of zingerone.
- 47 The method as claimed in any one of claims 42 to 46, wherein:
- (a) the composition is co-administered with a further anti-inflammatory agent;
 - (b) the composition is co-administered with one or more of: an analgesic compound, antipyretic compound, and psychotropic compound;
 - (c) the composition is co-administered with one or more of: a cannabinoid compound, mushroom compound, non-steroid anti-inflammatory drug compound (NSAID), opioid compound, salicylate compound, and steroid compound;
 - (d) the composition is co-administered with one or more of: acetaminophen, aspirin, celecoxib, diclofenac, diflunisal, etodolac, etoricoxib, felbinac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, lidocaine, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, tenoxicam, butorphanol, nalbuphine, levorphanol, levallorphan, pentazocine, phenazocine, eptazocinem, betamethasone, cortisone, deflazacort, dexamethasone, ethamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, cannabidiol, cannabigerol, tetrahydrocannabinol, psilocybin, and psilocin.
- 48 The method as claimed in any one of claims 42 to 47, wherein the inflammation is acute or chronic inflammation.
- 49 The method as claimed in claim 48, wherein:
- (a) the inflammation is an inflammatory disorder;
 - (b) the inflammation requires modulation;
 - (c) the inflammation is associated with an immune disorder;
 - (d) the inflammation is associated with an arthritic disorder;
 - (e) the inflammation is associated with an infection;
 - (f) the inflammation is associated with a cardiac, circulatory, or pulmonary disorder;
 - (g) the inflammation is associated with a neurological disorder; and/or
 - (h) the inflammation is associated with a neoplastic disorder.
- 50 The method as claimed in claim 48 or claim 49, wherein the inflammation is inflammation of one or more of: a joint, skin, eye, ear, nose, mouth, throat,

oesophagus, kidney, bladder, liver, spleen, lung, heart, brain, circulatory system, digestive system, endocrine system, genitourinary system, lymphatic system, nervous system, and skeletal system.

- 51 The method as claimed in any one of claims 48 to 50, wherein the inflammation is associated with one or more of: Alzheimer's disease, early stage Alzheimer's disease, ankylosing spondylitis, arthritis, asthma, colitis, Crohn's disease, dementia, early stage dementia, depression, diabetes, fibromyalgia, gout, infection, immune mediated inflammatory disease, inflammatory bowel disease, interstitial cystitis, multiple sclerosis, polymyalgia psoriasis, scleroderma, and Sjögren's syndrome, and systemic lupus erythematosus.
- 52 The method as claimed in any one of claims 48 to 50, wherein the inflammation is associated with one or more of: rheumatoid arthritis, ankylosing spondylitis arthritis, fibromyalgia arthritis, gout arthritis, juvenile idiopathic arthritis (JIA), lupus arthritis, osteoarthritis, polymyalgia rheumatica, psoriatic arthritis, reactive arthritis, scleroderma arthritis, and Sjögren's syndrome arthritis.
- 53 The method as claimed in any one of claims 48 to 50, wherein the inflammation is associated with one or more of: atherosclerosis, coronary artery disease, pulmonary artery hypertension, hypoxia-induced pulmonary hypertension, pneumonia, acute respiratory distress syndrome, coronavirus respiratory disorder, and cytokine storm syndrome; or from one or more of: breast cancer, leukaemia, multiple myeloma, myelodysplastic syndrome, pancreatic cancer, and prostate cancer.
- 54 The method as claimed in any one of claims 48 to 50, wherein the inflammation is associated with one or more of: a blister, dermatitis, eczema, hive, lesion, papule, plaque, psoriasis, rash, rosacea, ulcer, and wound.