Characterization and in Vitro Bioactivity of Green Extract from Fermented Soybean Waste

Sulagna Gupta†‡§ and Wei Ning Chen*¶

†Interdisciplinary Graduate School, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore
‡Residues and Resource Reclamation Centre, Nanyang Environment & Water Research Institute, Nanyang Technological University, 1 CleanTech Loop, CleanTech One, #06-08, Singapore 637141, Singapore
§School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459, Singapore

ABSTRACT: Extracts were extricated from raw okara and okara fermented with Rhizopus oligosporus using a clean, green protocol; water was used as the extraction solvent and coupled with ultrasound assistance for enhanced extraction. In vitro anti-oxidant analyses for antioxidant potential and capacity, superoxide scavenging activity, and nitric oxide scavenging activity validated that fermented okara yielded superior bioactive performance compared to raw okara. Fermented okara extracts showed no toxicity to erythrocytes and successfully prevented induced haemolysis. After 48 h incubation at the highest tested concentration (100 mg/mL), fermented okara extracts could inhibit HepG2 cells by 48.47 ± 5.28%, which was significantly different from their effects on NIH 3T3 cells. Gas chromatography–mass spectrometry characterization of extracts validated amino acids to be the chief fraction responsible for the detected bioactivity of the fermented okara extract. The results derived in this study open up the possibility that biofermented okara extract may be a potential novel sustainable nutraceutical.

1. INTRODUCTION

Okara, a prime agri-waste, is the insoluble waste residue generated by soybean processing industries. As it is highly perishable because of its high moisture content, its standard discard methods include incineration and disposal in landfills. However, there exists a potential for recycling and reusing fresh okara, as it has been proven to encompass a variety of nutrients. According to Li et al., per 100 g of dry matter raw okara possesses a protein content of 25.4–28.4 g, fat content of 9.3–10.9 g, dietary fibre content of 52.8–58.1 g, and carbohydrate content of 3.8–5.3 g. van der Riet et al. observed that okara also contains several important minerals and vitamins as well. Further, raw okara also has a low calorific value.

In recent times, solid-state fermentation has garnered a lot of attention for its ability to economically bulk-produce enzymes, organic acids, and bioactive secondary metabolites using industrial agro-wastes as substrates. It is also a viable valorization treatment technique to enrich the nutritional content of the substrate through microbial activity. Currently, there is a growing awareness to curb the usage of petrochemical solvents for extraction purposes. This is because most of the organic solvents are toxic, volatile, flammable, and contribute to environmental pollution and greenhouse effect. Water, being polar, is suitable for the extraction of natural water-soluble products such as flavonoids. An added benefit is that easy manipulation of its dielectric constant is possible through modulating temperature and pressure. This allows water to even be used as an extraction solvent for compounds possessing low polarity. Ultrasound is a clean, green method for extraction of compounds of commercial importance, including but not limited to polysaccharides, proteins, and bioactive molecules. Its ease of use, versatility, and nontoxicity make it a popular economic option in the quest for sustainable green technology extraction methods.

Liver cancer may be triggered by several factors, notable of which include chronic hepatitis B and C infection and exposure to aflatoxins. Hepatocellular carcinoma poses a major challenge to the healthcare sector; there is a persistent quest for finding a safe, efficient economic medication, with little or negligible side effects. Today, there is a lot of focus on discovering natural healthcare products in order to avoid the various side effects caused by the consumption of synthetic antioxidants. This paper has detailed the investigation of in vitro antioxidant and antiproliferative assays carried out using an extract sourced from okara fermented with Rhizopus oligosporus, an FDA-
Figure 1. (a) DPPH radical scavenging potential of raw and fermented okara ($n = 3$; alphabets with different letters represent significant difference between groups at a particular concentration). (b) FRAP potential of raw and fermented okara ($n = 3$; alphabets with different letters represent significant difference between groups at a particular concentration). (c) Superoxide RSA of raw and fermented okara ($n = 3$; alphabets with different letters represent significant difference between groups at a particular concentration). (d) Nitric oxide RSA of raw and fermented okara ($n = 3$; alphabets with different letters represent significant difference between groups at a particular concentration).

approved, GRAS (generally recognized as safe) food-grade fungus using a clean, green method.

2. RESULTS AND DISCUSSION

2.1. Antioxidant Analyses. Oxidative stress is responsible for a plethora of serious health concerns. Biofermentation of okara using R. oligosporus, an FDA-approved, food-grade GRAS microbe, was expected to contribute positive nutritional enhancements in conjecture with the work carried out by Gupta et al.\textsuperscript{10} Natural antioxidants, as evaluated in our study, have the advantage of not having the side effects associated with commercial synthetic antioxidants.

2,2-Diphenylpicrylhydrazyl (DPPH) assay is one of the most commonly used assays for testing radical scavenging activities (RSA) of samples. Antioxidants are able to quench the stable DPPH radical (dark purple) to its nonradical stable form (colourless) as a measure of their free radical scavenging potential. Figure 1a demonstrates the antioxidant potential of dose-dependent fermented and unfermented okara extracts against a positive control of quercetin. As is evident from the results obtained, fermented okara extracts possess significantly higher scavenging activities as compared to unfermented okara extracts at the same concentrations. However, the activities of fermented okara extracts were lesser than the activities of quercetin at the respective concentrations. The results of this experiment warranted a deeper investigation of the antioxidant potential of the fermented okara extract using different antioxidant tests [ferric reducing antioxidant power (FRAP), superoxide radical scavenging activity ($O_2^{−}$) and nitric oxide radical scavenging activity ("NO")].

The results of FRAP assay to estimate antioxidant capacity of fermented okara have been illustrated in Figure 1b. The reducing power of the extracts was studied as a function of their concentration. The mechanism of FRAP is based solely on electron transfer; the ability of the extracts to reduce Fe$^{3+}$ to Fe$^{2+}$ by electron donation was taken as a representation of the potential of the corresponding compound to reduce free radicals in plasma and tissues, thereby modulating the redox state. As seen in Figure 1b, the antioxidant capacities of fermented okara extracts far surpassed those of unfermented okara extracts at their respective equivalent concentrations. However, fermented okara extracts remained less efficient when compared with standard antioxidant quercetin. Nevertheless, the results of this experiment confirmed the presence of preliminary antioxidant activity in fermented okara extracts.

Superoxide anions generated from dissolved oxygen in a riboflavin-light-nitro blue tetrazolium chloride (NBT) system can reduce NBT in the system. In the absence of any antioxidant activity, superoxides can reduce the yellow dye (NBT$^{2+}$) to produce formazan. Antioxidants can prevent this activity. As perceptible from Figure 1c, the superoxide inhibitory activity of extracts rose with increase in concentration of extracts. Fermented okara extracts yielded better results than raw okara extracts. Superoxide scavenging potential is widely relevant to the biological system as superoxides are capable of reducing certain iron complexes (e.g., cytochrome c), causing peroxidation of lipids and generating singlet oxygen and hydroxyl radicals that react with biomacromolecules and thereby induce tissue damage.\textsuperscript{11}

In the reaction system used for evaluating nitric oxide scavenging activity, nitric oxide is generated from sodium nitroprusside (SNP) and reacts with oxygen to form nitrite. The nitrite ions subsequently react with the components of the Griess reagent by diazotizing sulphanilamide and coupling with naphthylethlediamine. These reactions produce a pink color, the formation of which is inhibited when antioxidants scavenge the free radicals. As conspicuous from Figure 1d, the nitric oxide inhibitory activity of extracts intensified with increase in concentration of extracts. Once again, fermented okara extracts yielded better results than raw okara extracts. Nitric oxide scavenging activity is of immense importance to the healthcare industry. Biologically, chronic exposure to nitric oxide has been linked to various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and
ulcerative colitis. Nitric oxide toxicity increases exponentially when it reacts with superoxide radicals to form the highly reactive peroxynitrite anion (ONOO−).

Table 1 depicts the correlation between the different antioxidant analyses for fermented okara extracts. As is evident from the high correlation coefficients ($R^2 > 0.5$), there exists a strong correlation between the different assays performed for evaluation of antioxidant capacities.

2.2. Erythrocyte Lysis Assays. The erythrocyte toxicity assay [Figure 2a] proved that unfermented okara extracts may be considered toxic at concentrations higher than 2 mg/mL. However, fermented okara extracts were observed to be nontoxic to erythrocytes at concentrations even as high as 4 mg/mL. Hence, haemolysis inhibition assay was carried out with the fermented okara extract, to further study its effect on erythrocyte cells.

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) generates free radicals and attacks erythrocytes to induce chain oxidation of proteins and lipids, ultimately leading to haemolysis. As seen from Figure 2b, fermented okara extracts were able to prevent AAPH-induced haemolysis of erythrocyte cells in a dose-dependent manner. On carrying out a t-test, no significant differences were observed between the percentage haemolysis inhibitions at the highest and lowest concentrations of tested fermented okara extract. This attests that even at concentrations as low as 500 ng/mL in phosphate buffered saline (PBS), fermented okara extract is able to prevent AAPH-induced lysis of red blood cells.

2.3. Antiproliferative Assay. Cancer is believed to be a result of reactive oxygen species inducing oxidative damage to biomolecules such as lipids, proteins, carbohydrates, and DNA. According to Sun, free radicals are involved in both the establishment and propagation of multistage carcinogenesis. Antioxidants scavenge free radicals and are therefore considered to be potential anticarcinogens. The results obtained from our antioxidant analyses [Figure 1a−d] gave rise to a strong hypothesis that fermented okara extracts would be able to exhibit antiproliferative activities in cancer cell lines. Recent research has been focused on identification of anticancer agents from soybean.

Figure 3a(i),(ii) exhibit a section of HepG2 cells before and after the extract treatment, respectively. To comparatively ascertain the effects of fermented and unfermented okara extract treatments on HepG2 and NIH 3T3 cells, contour plots were created [Figure 3b(i),(ii)] that illustrate the range of viability changes that occurred in both HepG2 and NIH 3T3 cell lines as a function of extract concentration and time. In both figures, it could be seen that at lower concentrations, the extracts were able to induce cell proliferation, thereby resulting in viability above control cells. This may be explained by the hypothesis that at low concentrations, some drugs are capable of upregulating the viability of cancer cells by accelerating cell proliferation and decreasing apoptosis. It is clear from Figure 3b that fermented okara extracts induced greater loss of viability as opposed to unfermented okara extracts. As may be observed from Figure 3b(i), there was not a significant decrease in viability in HepG2 cells after treatment for 24 h. The treatment for 48 h showed a much more favorable response, as evidenced by Figure 3b(ii); viability of

Table 1. Correlation Chart of the Different Antioxidant Analyses Performed Using Fermented Okara Extracta

|       | SO    | NO   |
|-------|-------|------|
| FRAP  | 0.9401| 0.9342|
| NO    | 0.8501|

a$n = 3$, results have been displayed as the mean of three readings.

Figure 2. (a) Erythrocyte toxicity assay using unfermented and fermented okara extracts ($n = 3$). (b) (i) Percentage of AAPH-induced haemolysis of erythrocytes in the presence of fermented okara extracts ($n = 3$). (ii) Percentage of inhibition of AAPH-induced haemolysis of erythrocytes in the presence of fermented okara extracts ($n = 3$).
HepG2 steadily decreased with analogous increase in concentration of the fermented extract. It may be postulated from this study that the viability losses were due to apoptosis and necrosis mechanisms, triggered by the extracts. Although from Figure 3b(ii), it is evident that the viability differed significantly across the cell lines with respect to the extract treatment, statistical analyses tools were employed to gauge the levels of similarity or unsimilarity. A paired t-test analysis was thus carried out (Table 2). As may be observed, the results validate the implications generated by Figure 3b(ii). Because higher concentration of fermented okara extract yielded desirable inhibition of viability in the HepG2 cell line after 48 h incubation, a comparative analysis was made to justify the usage of the same. It was noted that at corresponding concentrations, a significant difference existed between HepG2 cells treated with fermented extracts versus treated with unfermented extracts. Except for the lowest

Figure 3. (a) (i) Live HepG2 cells (before treatment). (ii) Dead HepG2 cells (after treatment). (i) Contour plot representing viability of cell lines after 24 h extract treatment. Key: HepG2 treated with unfermented okara extract (HepG2 UF); HepG2 treated with fermented okara extract (HepG2 F); NIH 3T3 treated with unfermented okara extract (3T3 UF); NIH 3T3 treated with fermented okara extract (3T3 F). (ii) Contour plot representing viability of cell lines after 48 h extract treatment. Key: HepG2 treated with unfermented okara extract (HepG2 UF); HepG2 treated with fermented okara extract (HepG2 F); NIH 3T3 treated with unfermented okara extract (3T3 UF); NIH 3T3 treated with fermented okara extract (3T3 F). (c) Increased inhibition of the HepG2 cell line after 48 hours treatment with increase in concentration-fermented okara extract; (i) 25 mg/mL extract; (ii) 50 mg/mL extract; (iii) 75 mg/mL extract; and (iv) 100 mg/mL extract.

Table 2. Paired t-test Results as a Function of HepG2 F vs HepG2 UF and HepG2 F vs NIH 3T3 F (* = p < 0.05, ** = p < 0.01; F: Fermented Okara Extract, UF: Unfermented Okara Extract) n = 3, Results Have Been Displayed as the Mean of Three Readings

| concentration (mg/mL) | HepG2 UF  | NIH 3T3 F |
|-----------------------|-----------|-----------|
| 5                     | 0.098848  | 0.256113  |
| 25                    | 0.031167* | 0.011401* |
| 50                    | 0.001777**| 0.040201* |
| 75                    | 0.009315**| 0.042723* |
| 100                   | 0.005864**| 0.041499* |
concentration tested, at all other extract concentrations, the \( p \)-values were lesser than 0.05. A similar trend was observed on comparing the effects of parallel concentrations of fermented extract on HepG2 cells versus NIH 3T3 cells; except for the lowest concentration tested, at all other extract concentrations, the \( p \)-values were lesser than 0.05, thereby indicating significant difference in the viability percentages (Table 2). Thus, it may be stated from the results obtained that the treatment with fermented okara extracts could induce better reduction in the viability of cancer cell line HepG2 as opposed to the treatment with unfermented extracts but had not as great an effect on normal cells NIH 3T3 at analogous concentrations, thereby implying its nontoxicity on normal cells.

Figure 3c displays a stacked column analysis of the variation in inhibition across the four highest concentrations of fermented okara extracts tested on HepG2 and NIH 3T3 cell lines after 48 h incubation. As may be observed from

Figure 4. (a) GC–MS chromatogram of unfermented okara extract. Key: 2 = propanoic acid; 11 = hexadecanoic acid; 14 = \( \alpha \)-D-galactopyranoside; 15 = D-glucose; 16 = D-ribose; IS = internal standard (ribitol). (b) GC–MS chromatogram of fermented okara extract. Key: 1 = alanine; 2 = propanoic acid; 3 = valine; 4 = leucine; 5 = glycine; 6 = butanedioic acid; 7 = serine; 8 = threonine; 9 = proline; 10 = D-glucose; 11 = hexadecanoic acid; 12 = tryptophan; 13 = mannonic acid; IS = internal standard (ribitol). (c) PCA biplot derived from GC–MS data for unfermented (control) and fermented okara samples. Key: unfermented okara control (C); okara fermented with \( R. \) oligosporus (RO).
Figure 3c(i–iv), the percentages of inhibition rise with increase in concentrations of extract used for the treatment. There was an inhibition of 18.49 ± 3.43, 42.07 ± 3.35, 44.47 ± 5.6, and 48.47 ± 5.28% in HepG2 viability, on treating cells with a dose of 25, 50, 75, and 100 mg/mL, respectively. Conversely, NIH 3T3 cells remained less affected by the extract treatment; loss of viability was 0 ± 7.49, 17.28 ± 7.59, 25.81 ± 2.69, and 30.11 ± 2.73% for treatment doses of 25, 50, 75, and 100 mg/mL, respectively. The results obtained heighten the possibility that increasing the incubation time would elicit greater inhibitory response against the tested cancer cell line, at parallel concentrations of the extract treatment. In tandem with our present results, it would be a valid hypothesis that the corresponding treatment would not have a significant effect on normal cell lines.

2.4. Characterization of Extracts Using Gas Chromatography–Mass Spectrometry. A gas chromatography–mass spectrometry (GC–MS) metabolomics approach was used to characterize the extracts used in the antioxidant and antiproliferative analyses, to investigate the bioactive components responsible for the activities. Clearly separated compounds with high abundance have been indicated in Figure 4a,b. The compounds identified were grouped into three parent groups: amino acids, organic acids, and sugars (Table 3). Four essential (valine, tryptophan, leucine, and threonine) and four nonessential (alanine, glycine, serine, and proline) amino acids were detected. Further, as seen from Figure 4a,b, there exists a marked difference in the metabolomic output between fermented and unfermented okara extracts. Hence, a PCA biplot [Figure 4c] was generated to validate the variance between the unfermented and fermented samples. The GC–MS data were analyzed using a correlation matrix with listwise exclusion. The first two principal components (PC), PC1 and PC2, attributed to 91.45 and 6.82% of variance, respectively, and thus cumulatively accounted for 98.27% of the total variance. PC1 being the larger PC, it may be interpreted that the distribution pattern of metabolites detected in unfermented and fermented extracts were significantly different.

The fold-change of characterized compounds after fermentation has been documented in Figure 5. As may be observed,

fermented okara extracts contained significantly increased levels of amino acids and organic acids. On the other hand, sugar concentration seemed to chiefly decrease after fermentation. This may be attributed to microbial bioactivity breaking down complex proteins into their constituent amino acids, while using basic sugars as nutrition.

2.5. Link of Characterized Metabolites with Bioactivity. High levels of sugars are associated with metastasis of malignant cancer. Glucose formed by glycolysis, along with glutamine is able to generate the carbon skeletons, NADPH, and ATP to serve as the building blocks for new cancer cells. These cancer cells are able to survive in hypoxic conditions and subsequently modify the metabolic pathways for cell growth and survival, thus leading to malignancy.15

Butanediolic acid, commonly known as succinic acid, has been studied to have an effect on antioxidant capacities and has been suggested in its implementation as an effective preventive antioxidant.16 Further, it has been observed to increase the activities of several enzymes including superoxide dismutase, catalase, and peroxidase,17 all of which are vital plasma antioxidant enzymes.

Amino acids play an essential role in the maintenance of cancer redox homeostasis. Proline is a well-known stress adaptor molecule. Its metabolism influences various cell signaling pathways, thereby playing a crucial role in triggering tumor suppression and cell survival in animals.18 Further, work carried out by Vaughan et al.19 documented that alanine is capable of suppressing tumour cells. Dietary threonine has been documented to improve levels of superoxide dismutase, catalase, and glutathione peroxidase and complement components C3 and C4.20 Studies indicate that branched-chain amino acids, including valine and leucine, elicit NO scavenging biofunctional responses and inhibit lipid peroxidation.21 Nayak and Buttar22 observed that tryptophan too possesses antioxidant and antiproliferative properties. The bioactivity of serine was observed by Maralani et al.23 through the elevation of several antioxidant factors. Oral administration of glycine has been shown to reduce oxidative stress.24

![Figure 5. Fold change in selected metabolites after fermentation of okara with R. oligosporus.](image-url)
3. CONCLUSIONS

In conclusion, it may be summarized that this work holds substantial potential for employing biofermented okara as a sustainable functional food. Previous studies carried out\(^{10}\) documented that biofermentation proved to not only increase the quantity of several important compounds (amino acids, fatty acids, tannins, and so forth.) but also to generate an increase in antioxidant activity. Further, the effect of the extracts on HepG2 and NIH 3T3 cell lines revealed the possibility of utilization of the fermented okara extract as a potent nutraceutical, as with increased incubation time and at particular concentrations, a greater, more significant loss of viability was noticed in HepG2 cells as compared to NIH 3T3 cells. An important criterion of our study was to propose the extraction of bioactive fractions using green technology. Lastly, characterization of the extracts revealed amino acids to be the chief group of bioactive compounds in the fermented okara extract. This is in tandem with our bioactivity studies as Marcuse\(^{25}\) suggested that amino acids have antioxidant activities and Bonfill, et al.\(^{26}\) observed that essential amino acids can activate apoptosis in cancer cells.

4. MATERIALS AND METHODS

4.1. Materials, Microorganisms, and Cells. Fresh okara was obtained from Unicurd Food Company Pte Ltd (Singapore) and stored at \(-80\) °C until used. Fungal strain \(R.\) oligosporus (DSM 1964) was procured from the Leibniz Institute DSMZ German Collection of Microorganism and Cell Cultures. Fresh single human donor (female) blood stored at \(-80\) °C was transferred to Eppendorf tubes containing 0.5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin at \(37\) °C, 5% CO\(_2\) till 75–80% confluence was reached. Cells were then seeded into 96-well microplates, with each well containing \(10^4\) cells.

4.2. Fermentation and Extract Preparation. A quantity of \(10^5\) g of fresh okara was inoculated with a culture inoculum of \(10^5\) CFU \(R.\) oligosporus and incubated at \(30\) °C for \(48\) h. For extraction of phytochemicals, \(1\) g of the fermented sample (procedure repeated for unfermented sample) was added to \(7\) mL of the extraction solvent (water), macerated and ultrasonicated for \(10\) min. The crude mixture was then incubated at \(40\) °C in the shaking condition for \(4\) h, following which it was centrifuged at \(10,000\) g for \(15\) min. The supernatant was used as the extract for all antioxidant assays. For antiproliferative assays, the supernatant was freeze-dried, redissolved in PBS, and filtered using \(0.45\) \(\mu\)M filter prior to treating the cell lines.

4.3. DPPH Antioxidant Assay. A DPPH antioxidant assay was carried out using the protocol employed by Gupta et al.\(^{10}\) with minor modifications. DPPH solution (500 \( \mu\)L) (0.6 mM in ethanol) was added to \(500\) \( \mu\)L of the aqueous extract at different concentrations. Ethanol (4 \( \mu\)L) was added to this mixture and the tube incubated in dark for 30 min at room temperature. Above solution (1 \( \mu\)L) was read for absorbance at \(515\) nm. Quercetin was used as the standard.

4.4. FRAP Assay. The FRAP was calculated using the methodology employed by Jemli et al.\(^{27}\) with minor modifications. PBS (2.5 \( \mu\)L) and 2.5 \( \mu\)L 1% \( K_2[Fe(CN)_6]\) were added to 1 \( \mu\)L of the extract (at different concentrations), and the mixture was incubated at \(50\) °C for 20 min. Subsequently, 2.5 \( \mu\)L of 10% trichloroacetic acid (TCA) was added and the mixture centrifuged at 3000 rpm for \(10\) min. To 2.5 \( \mu\)L of the supernatant, 2.5 \( \mu\)L of distilled water and 2.5 \( \mu\)L of 0.1% FeCl\(_3\) were added and the absorbance measured at 700 nm. Quercetin was used as the standard.

4.5. Superoxide Radical (\(O_2^-\)) Scavenging Activity. The \(O_2^-\) scavenging activity of the extracts were obtained using the procedure followed by Parimala and Selvan,\(^{28}\) with minor modifications. To \(0.1\) \( \mu\)L of the extract (at different concentrations), 0.1 \( \mu\)L of 1.5 mM NBT, 0.2 \( \mu\)L of 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.05 \( \mu\)L of 0.12 mM riboflavin, and 2.55 \( \mu\)L of 0.067 M PBS were added and the mixture illuminated for 30 min. Absorbance was then measured at 590 nm. Quercetin was used as the standard.

4.6. Nitric Oxide Radical (\(\cdot\)NO) Scavenging Activity. For the erythrocyte toxicity assay, 0.1 \( \mu\)L of fresh erythrocytes were added to an Eppendorf tube containing 300 \( \mu\)L of PBS and 100 \( \mu\)L of sample extracts at different concentrations. The mixtures were allowed to incubate for 3 hours at room temperature in a shaking condition at 200 rpm. Following this, each tube was diluted with 8 \( \mu\)L of PBS and centrifuged at 1041 g. The supernatant was subsequently read at 540 nm. PBS was used as blank. PBS and sterile distilled water were used in place of samples, in the two control tubes, respectively.

The toxicity was calculated according to the formula

\[
\text{Toxicity (\%) } = \left[ \frac{A_s - A_p}{A_W - A_p} \right] \times 100
\]

where \(A_s\) is the absorbance value of the sample, \(A_p\) is the absorbance value of PBS control, and \(A_W\) is the absorbance value of the water control.

The erythrocyte haemolysis inhibition assay was carried out in the same way as the above, except for a minor modification in the experimental setup. Fresh RBCs (100 \( \mu\)L) were added to an Eppendorf tube containing 200 \( \mu\)L of PBS, 100 \( \mu\)L of 0.5 M AAPH, and 100 \( \mu\)L of sample extracts at different concentrations. Vitamin C (100 \( \mu\)L) (0.125 mg/mL) was added in place of the sample in the control tube.

The haemolysis inhibition was calculated according to the formula

\[
\text{Haemolysis inhibition (\%) } = \left[ 1 - \frac{(A_s - A_p)}{(A_W - A_p)} \right] \times 100
\]

where \(A_s\) is the absorbance value of the sample, \(A_p\) is the absorbance value of PBS control, and \(A_W\) is the absorbance value of the water control.

4.7. Erythrocyte Toxicity and Haemolysis Inhibition Assays. Erythrocyte toxicity and haemolysis inhibition assays were performed as described by Cheung et al.,\(^{29}\) with minor modifications.

For the erythrocyte toxicity assay, 100 \( \mu\)L of fresh erythrocytes were added to an Eppendorf tube containing 300 \( \mu\)L of PBS and 100 \( \mu\)L of sample extracts at different concentrations. The mixtures were allowed to incubate for 3 hours at room temperature in a shaking condition at 200 rpm. Following this, each tube was diluted with 8 \( \mu\)L of PBS and centrifuged at 1041 g. The supernatant was subsequently read at 540 nm. PBS was used as blank. PBS and sterile distilled water were used in place of samples, in the two control tubes, respectively.

The haemolysis inhibition was calculated according to the formula

\[
\text{Haemolysis inhibition (\%) } = \left[ 1 - \frac{(A_s - A_p)}{(A_W - A_p)} \right] \times 100
\]
When the wells achieved a confluency of ~70%, 10 μL of the extract was added to each well (except control wells) and the microplates incubated for 24 and 48 h. On completion of their respective incubation periods, the old medium was removed and an equivalent quantity of the fresh medium was added to each well. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μL) (12 mM) was added next, followed by incubating the microplate at 37 °C, 5% CO₂ for 2 hours. Subsequently, 25 μL of solution was allowed to remain and the rest carefully removed without disturbing the cell layer at the bottom of the well. Dimethyl sulfoxide (DMSO) (100 μL) was then added to each well and mixed thoroughly to dissolve the formazan crystals. The microplate was then again incubated for 10 min at 37 °C, 5% CO₂ and finally read at 570 nm.

4.9. GC–MS Analysis of Extract. Sample preparation for GC–MS was done using the method followed by Chen and Chen. A quantity of 50 μL of 20 mg/mL methoxamine hydrochloride in pyridine was added to 1 mL of the dried sample, vortexed for 1 min, and incubated at 37 °C for 1 h. Following this, 100 μL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane was added to the sample. Samples were incubated at 70 °C for 30 min and finally centrifuged at 14,000 rpm for 1 h at room temperature before being transferred to glass vials for GC–MS analysis.

All samples for GC–MS were analyzed within 12 h after derivatization. 5975C inert MSD with the Triple-Axis Detector from Agilent Technologies was used for the process with hexane as the stationary phase. The capillary column was 0.25 μm thick and had dimensions of 30 m × 0.250 mm. A quantity of 1 μL of the sample was injected in the splitless mode; carrier gas helium was maintained at a purge ow of 50 mL per minute. The inlet was sustained at an isothermal temperature of 230 °C. The GC oven was initiated at 75 °C (4 min hold) and ramped to 280 °C at 4 °C per minute, with a final hold time of 2 min at 280 °C. Data were acquired in full scan from 30 to 900 m/z. Metabolites were identified using NIST08 mass spectral library. The chromatographic peaks were normalized according to the internal standard (ribitol) before being subjected to statistical analyses.

4.10. Statistical Analysis. All analyses were carried out in triplicate. Data have been represented as an average of the three trials. Antioxidant and cell culture assays were subjected to the t-test, with p < 0.05 being considered significant. GC–MS data were analyzed by the one-way ANOVA and post-hoc t-test.

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**ABBREVIATIONS**

PBS phosphate buffered saline
K₃[Fe(CN)₆] potassium ferricyanide
TCA trichloroacetic acid
FeCl₃ ferric chloride
NBT nitro blue tetrazolium chloride
EDTA ethylenediaminetetraacetic acid
SNP sodium nitroprusside
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM Dulbecco’s modified Eagle’s medium
FBS fetal bovine serum
FDA U.S. Food and Drug administration
Q. quercetin
DMSO dimethyl sulfoxide
ATCC American Type Culture Collection
AAPH 2,2’-azobis(2-amidinopropane) dihydrochloride
RSA radical scavenging activity

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