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Research article

Assessing the Antioxidant Activity and Anti-inflammatory Potency on Lipopolysaccharide-induced RAW 264.7 Macrophages of Eleven Methanolic Extracts Indigenous Vegetables

Siriluk Sintupachee[1*], Naunpun Sangphech[2], and Worrawat Promden[3]

[1] Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, 80280, Thailand
[2] Faculty of Allied Health Sciences, Thammasat University, Prathumtani, 12121, Thailand
[3] Faculty of Education, Buriram Rajabhat University, Buriram, 31000, Thailand
* Correspondence: siriluk_sint@nstru.ac.th

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Abstract: Indigenous vegetables have long been part of the traditional diet in southern Thailand, often paired with spicy foods. This study aimed to explore the total phenolic and flavonoid contents of eleven commonly consumed indigenous vegetables in Nakhon Si Thammarat province. It also evaluated their antioxidant and anti-inflammatory properties through methanolic crude extracts. The findings revealed that *Litsea petiolate* (LP) contained the highest amount of flavonoids, with 270.90 ± 6.175 mg QE/g extract, whereas *Anacardium occidentale* (AO) showed the greatest phenolic content at 160.40 ± 1.32 mg GAE/g extract. In terms of antioxidant properties, *Glochidion wallichianum* (GW) demonstrated significant activity through the FRAP assay, yielding 484.08 ± 10.010 μmole TE/g extract and 4,115.36 ± 100.00 μmole Fe^{2+}/g extract. Meanwhile, AO exhibited the most substantial results in the DPPH assay, with an 85.99 ± 0.762 mg/mL capacity. The IC_{50} values of AO (7.39 ± 0.176 mg/mL) and GW (7.70 ± 0.820 mg/mL) surpassed that of ascorbic acid, indicating their high antioxidant potential. Regarding anti-inflammatory effects, *Crassocephalum crepidioides* (CCr) effectively inhibited nitric oxide production at different concentrations. At the same time, the LP extract impacted the viability of the RAW264.7 cell line, a distinction not observed in the other vegetables studied. These results underscore the significant bioactive potential of these indigenous vegetables. This enhances their value in sustainable food systems and highlights their educational importance in promoting knowledge of the plant cycle for future generations.

Keywords: Flavonoids; Herb; Natural resource; Phenols; Phytochemicals

1. Introduction

Plants exhibit captivating qualities that have garnered global interest. Researchers have identified active chemical groups within plants, known as phytochemicals, that are essential for their bioactivity [1]. Among these, antioxidants stand out, ranking highest among the secondary metabolites. The compounds naturally occurring in plants are known for their potential to offer antioxidant and anti-inflammatory benefits [2-4]. These compounds encompass a wide range, including flavonoids, polyphenols, carotenoids, alkaloids, and others [5]. Flavonoids: These are known for their strong antioxidant activity [6,7].

They help neutralize harmful free radicals in the body, reducing oxidative stress and contributing to various chronic diseases [7]. Polyphenols, these compounds are linked to a reduced risk of chronic diseases and help protect cells and DNA from oxidative damage [7].

Antioxidants protect cells from damage caused by free radicals, which are unstable atoms that can lead to oxidative stress and various disorders. They neutralize free radicals by donating electrons, thus reducing cellular harm [8]. Antioxidants can be endogenous (produced by the body) or exogenous (obtained from foods such as fruits, vegetables, nuts, and seeds), including dietary sources like vitamins C and E, beta-carotene, and flavonoids [9,10]. Common methods to evaluate antioxidant activity include 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC), Cupric Ion Reducing Antioxidant Capacity (CUPRAC), Thiobarbituric Acid Reactive Substances (TBARS), and Total Phenolic Content (TPC) assays, which measure the ability of antioxidants to scavenge free radicals, reduce metal ions, or prevent lipid peroxidation, with results typically quantified spectrophotometrically [16]. Multiple methods are often used together for a comprehensive assessment [11]. Anti-inflammatory medicines reduce inflammation and are classified as NSAIDs, corticosteroids, and biologics produced from pharmaceuticals and natural chemicals [12]. They treat arthritis and asthma by blocking inflammatory processes. To assure efficacy and safety, evaluation methods include in vitro assays, in vivo models, and clinical trials [13]. In anti-inflammatory research, cell culture assays and biochemical methods are employed to gauge a substance's anti-inflammatory efficacy. These assays, including cytokine inhibition (e.g., TNF-α, IL-6 ELISA), enzyme inhibition (e.g., COX, 5-LOX), and ROS reduction assays, were aid in identifying and comprehending anti-inflammatory mechanisms [8, 14]. By assessing the impact of substances on cultured cells, these assays provide valuable insights into their biological effects and contribute to the development of novel anti-inflammatory treatments [15].

It's important to note that phytochemicals often work synergistically with other nutrients and compounds in whole foods. A diet rich in fruits, vegetables, whole grains, and herbs can provide various phytochemicals, contributing to overall health and well-being by providing antioxidant and anti-inflammatory benefits [3]. The use of plants as food is a heritage transferred from generation to generation, especially in an area rich in natural resources. They have a unique pattern for using the plant as healing food. The indigenous people of southern Thailand, particularly in Nakhon Si Thammarat Province, have a unique vegetable-eating culture deeply rooted in their folk traditions [16]. This region boasts a diverse geography, encompassing seacoasts and lowland rice fields in the east, while its central areas are characterized by imposing mountains and dense forests [17]. With a consistent year-round rainfall pattern, the region boasts a rich array of natural resources [16, 17]. Consuming seasonal and locally sourced vegetables is a cherished local tradition, serving as a means to adapt to the changing seasons. People in this region incorporate spicy foods with fresh vegetables into their diets, both for their quick and satisfying flavors and their potential to counteract the effects of spiciness [18]. The indigenous vegetables commonly paired with spicy dishes in this area tend to have sour and astringent flavors, often possessing medicinal properties that help alleviate conditions such as flatulence, chest pain, joint discomfort, asthma, and coughs [18]. This study aimed to explore the antioxidant and anti-inflammatory properties of a commonly consumed vegetable that accompanies spicy foods in southern Thailand. The study aims to identify plant species with high antioxidant potential based on FRAP and DPPH assays and extracts with strong anti-inflammatory activity indicated by Nitric oxide inhibition while ensuring their safety through MTT assays. Key findings will include identifying plant species with significant biological activities, minimal cytotoxicity, and critical biomarker values for further studies or therapeutic applications. The investigation incorporates phytochemical screening using HPTLC, measuring phenol and flavonoid levels, and identifying chemical groups with potential medicinal applications. This research emphasizes the value of local vegetables as dietary sources for healing rather than chemical drug production. By highlighting the overlooked potential of local vegetables, often marginalized due to unpopularity, limited cultivation, and natural resource depletion, the study seeks to inspire new generations to preserve and prioritize their entire ecosystem, promoting sustainability and biodiversity.

2. Materials and Methods

2.1 Plant collection and experimental design

All the plants were harvested from a field in Nakhon Si Thammarat province, Thailand, where no chemical treatments were applied during cultivation. The global positioning system (GPS) coordinates are

8°25′7″N and 99°57′49″E. The temperature and relative humidity were 27 ± 2.45°C and 79 ± 4.93%, respectively. The selection of plant parts for the collection was determined by their traditional use as fresh vegetables in spicy dishes, as indicated in the cultural guidelines provided. The experiment involves testing 11 plant crude extracts at various concentrations for FRAP, DPPH, Nitric oxide inhibition, and MTT assays. Each assay will include negative controls (solvent without extracts) and positive controls, such as Trolox and $FeSo_4$ for FRAP and DPPH, Dexamethasone for Nitric oxide inhibition, and DMSO for MTT assay. The groups will be divided based on plant type and extract concentrations. Measurements will be performed in triplicates to ensure reliability. Results will compare the activity of the extracts against the controls for statistical significance.

2.2 Plant preparation and crude extract

The leaves or immature fruit of the 11 plants underwent drying at 50°C for two consecutive nights, followed by grinding into a fine powder. Crude extracts were obtained according to the Sintupachee et al., 2020 [19] method: briefly by mixing 100 mg of the plant powder with 10 mL of methanol and subjecting the mixture to reflux at 70°C using a Syncore®Analyst (BUCHI) for 2 hours. The resulting solution was transferred to a new extraction tube, and the solvents were evaporated for 1 hour. The extract was reconstituted with 2 mL of methanol in a 1.5 mL microtube. After centrifugation at 10,000 rpm, the supernatant was carefully transferred into a fresh 1.5-mL microtube and utilized for phytochemical analysis. The extracts were weighed after solvent evaporation to calculate the yield and dissolved in dimethyl sulfoxide (DMSO) for antioxidant and anti-inflammatory activity assays. The extracts were freshly prepared and used immediately for activity testing after evaporation.

2.3 Preliminary phytochemical screening

Two microliters of the extract were applied to a 10 cm length x 10 cm width TLC Silica Gel 60 F_{254} aluminum plate (Merck) using the CAMAG® Linomat5 and subsequently developed in a TLC tank saturated with a mobile phase consisting of acetonitrile, ethyl acetic acid, and ethanol in a ratio of 35:5:15 (CAMAG® Automatic Developing Chamber 2 (ADC 2)). Relative humidity of the system and pH were 82% and 7.3, respectively. The TLC plate was then examined under 254 nm, 366 nm, and white light transillumination (CAMAG® TLC Visualizer 3). To assess the phytochemical fingerprint, the saturated TLC was derivatized with the specific chemical using the CAMAG® Derivatizer to identify it according to the following methods [20]: For flavonoids, the TLC plate was derivatized by spraying a 1% ethanolic solution of aluminum chloride, heated at 110°C for 5 min, and visualized under 366 nm. For phenol groups, the TLC plate was sprayed with the natural product reagent (NP reagent: 0.5% 2-aminoethyl diphenyl-borinate in ethyl acetate), followed by the polyethylene glycol reagent (PEG reagent: 2.5% polyethylene glycol 400 in dichloromethane), heated at 110°C for 5 min, and visualized under 366 nm.

2.4 Determination of total phenolic content

The total phenolic contents (TPC) were determined in a 96-well microplate with triplicate samples. Each well contained a 200 μL reaction mixture comprising 10 μL of 2 mg/ml crude extract in DMSO, 90 μL of 10% Folin-Cioalteu reagent, and 100 μL of 7% Na_2CO_3 [21]. The plate was then vigorously shaken and incubated at room temperature for 30 minutes. The reaction resulted in a blue coloration indicative of phenols, and the absorbance was measured at 765 nm. Quantity was determined using a calibration curve of gallic acid standards at two-fold dilution concentrations ranging from 62.5 to 500 nM, expressed as milligrams of gallic acid equivalent per gram of crude extract (mg GAE/ g extract).

2.5 Determination of total flavonoid contents

The total flavonoid contents (TFC) were measured in a 96-well microplate [22]. Each well was filled with a 200-μL reaction mixture comprising 10 μL of 2 mg/mL crude extract in DMSO, 40 μL of 0.25 M $NaNO_2$, and 50 μL of 0.15 M aluminum chloride. After incubation at room temperature for 5 minutes, 100 μL of 1M NaOH was added to each well. The assay was carried out in triplicate. The reaction mixture's absorbance was then measured at 506 nm. The amount was expressed as milligrams of quercetin equivalent per 1 gram of crude extract (mg QE/1 g extract), using a two-fold dilution of a calibration curve from 62.5, 125, 250, 500, and 1,000 μM.

2.6 Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP experiment was conducted in a 96-well microplate, where each well accommodated a 200 µL reaction mixture comprising 10 µL of 2 mg/mL crude extract in DMSO and 190 µL of FRAP reagent. The FRAP reagent, outlined by Benzie and Strain (1996) [23], consisted of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM hydrochloric acid, and 20 mM iron (III) chloride in a 10:1:1 ratio. The assay was conducted in triplicate. Subsequently, the reaction mixture was incubated at 37°C for 30 minutes under light-protected conditions. The absorbance of the reaction was measured at 595 nm. The results were quantified as micromoles of Fe^{2+} and Trolox equivalent per gram of extract. Calibration curves were generated using aqueous solutions of $FeSO_4$ and Trolox at a two-fold dilution, with concentrations ranging from 62.5 to 500 µM.

2.7 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging activity was assessed in a 96-well microplate, with each well containing a 200 µL reaction mixture, and conducted in triplicate. The DPPH reaction mixture consisted of 10 µL of 2 mg/mL crude extract in DMSO and 190 µL of 100 µM DPPH (Sigma-Aldrich, St. Louis, MO, US) in methanol. The reaction mixture in the microplate was then incubated at 37°C for 30 minutes. The DPPH free radical scavenging activity was measured at 515 nm absorbance. The radical scavenging activity was reported as a percentage using the equation: antioxidant activity (%) = $[(A_{control} - A_{sample})/A_{sample}] \times 100$, where $A_{control}$ represents the absorbance of the control reaction. A_{sample} denotes the absorbance in the presence of the tested compound [24]. The 50% inhibitory concentration (IC_{50}) values (the sample concentration required to scavenge 50% DPPH free radicals) were derived from a simple regression analysis. The IC_{50} value was calculated as the concentration (µg/mL) of the test sample that causes 50% quenching of the UV absorption of DPPH.

2.8 Cell culture

The RAW 264.7 cell line, a murine macrophage-like cell line, ATCC No. TIB-71 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, US) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, US) and 100 units/mL penicillin/streptomycin (Gibco, US). Cells were generated in a petri dish at 37°C with 5% CO_2 and 95% humidity. The cells were collected at 90% confluence.

2.9 Inhibition of nitric oxide assay

Nitric oxide production by macrophages [25] was determined by detecting nitrite levels in the culture medium using the Griess reagent. The RAW 264.7 cells were seeded overnight in a 96-well culture plate at 1 x 10^5 cells/100 µL. They were activated with lipopolysaccharide (LPS) derived from *Escherichia coli* (Sigma, US) (1 g/mL) in a complete medium and two-fold various concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/mL). The cells were treated with 100 µL of crude extracts at various doses (0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/mL), with methanol (1%) as the vehicle control, which was sustained for 20 hours. Fifty microliters of culture medium were taken to quantify nitric oxide using Griess' test. In this test, 50 µL of 1% w/v sulfanilamide was added and incubated in darkness for 5 minutes. Then, 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 2% phosphoric acid was added and incubated for another 5 minutes in darkness. The absorbance was then measured at 570 nm. The nitric oxide concentration in culture supernatants was measured using a standard curve created with a sodium nitrate solution. The percentage inhibition of nitric oxide production was determined by comparing the crude extract-treated cell with the LPS-treated control (cells without crude extracts), which did not affect inhibition of NO production. The cytotoxicity was also conducted parallelly. To assess cell viability after 20 hours of incubation, 10 µL of 5 mg/mL 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 hours in a 5% CO_2 incubator. The culture supernatant was removed, and the formazan crystals were dissolved in 0.04N HCl. The absorbance at 570 nm was used to calculate cell viability using the following equations:

$$\text{Cell viability (\%)} = (A_{sample} - A_{blank})/ (A_{control} - A_{blank}) \times 100 \quad (1)$$

Cell viability percentages were computed relative to the untreated control; cells without LPS-containing extracts were considered to have 100% vitality. The experiment was duplicated, and the results were combined from three independent experiments.

2.10 Statistical analysis

Data was provided as mean and standard deviation (Mean ± SD) from triplicate trials. The statistical analysis was performed using a one-way analysis of variance (ANOVA) to identify significant differences between groups, followed by a Tukey post hoc test for pairwise comparisons. Regression curve analysis was used to determine the IC_{50} values for antioxidant (DPPH) and anti-inflammatory (Nitric oxide) activities. Data was presented graphically with statistical significance markers; p-values < 0.05 were considered significant. The analysis aims to identify plant extracts with the most potent biological activity.

3. Results and Discussion

3.1 Phytochemical screening

Eleven species of indigenous vegetables, including *Anacardium occidentale* (AO, F. Anacardiaceae, O. Sapindales), *Litsea petiolate* (LP, F. Lauraceae, O. Laurales), *Clausena cambidiana* (CC, F. Rutaceae, O. Aurantioideae), *Glochidion wallichianum* (GW, F. Phyllanthaceae, O. Malpighiales), *Gynura pseudochina* (GP, F. Asteraceae, O. Asterales), *Crassocephalum crepidioides* (CCr, F. Asteraceae, O. Asterales), *Spondias pinnata* (SP, F. Anacardiaceae, O. Sapindales), *Nasturtium officinale* (NO, F. Brassicaceae, O. Brassicales), *Ficus botryocarpa* (FB, F. Moraceae, O. Rosales), *Oenanthe stolonifera* (OS, F. Apiaceae, O. Apiales), and *Baccaurea ramiflora* (BR, F. Phyllanthaceae, O. Malpighiales) (Figure 1), were chosen due to their popularity and use in local cuisine, often paired with spicy dishes. Shoots and leaves are typically eaten fresh, except for FB and BR, which use the immature fruit. These vegetables were harvested from chemical-free plots and distributed to customers around the province.

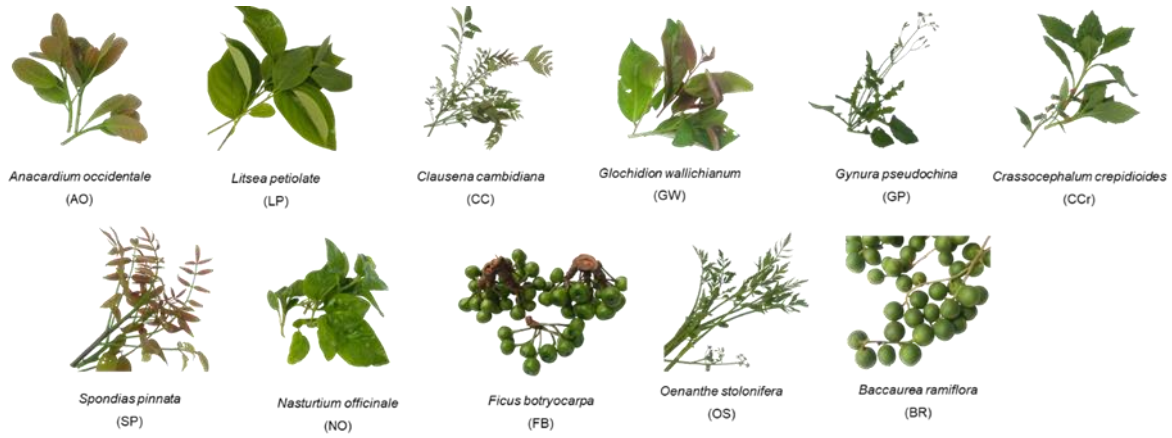
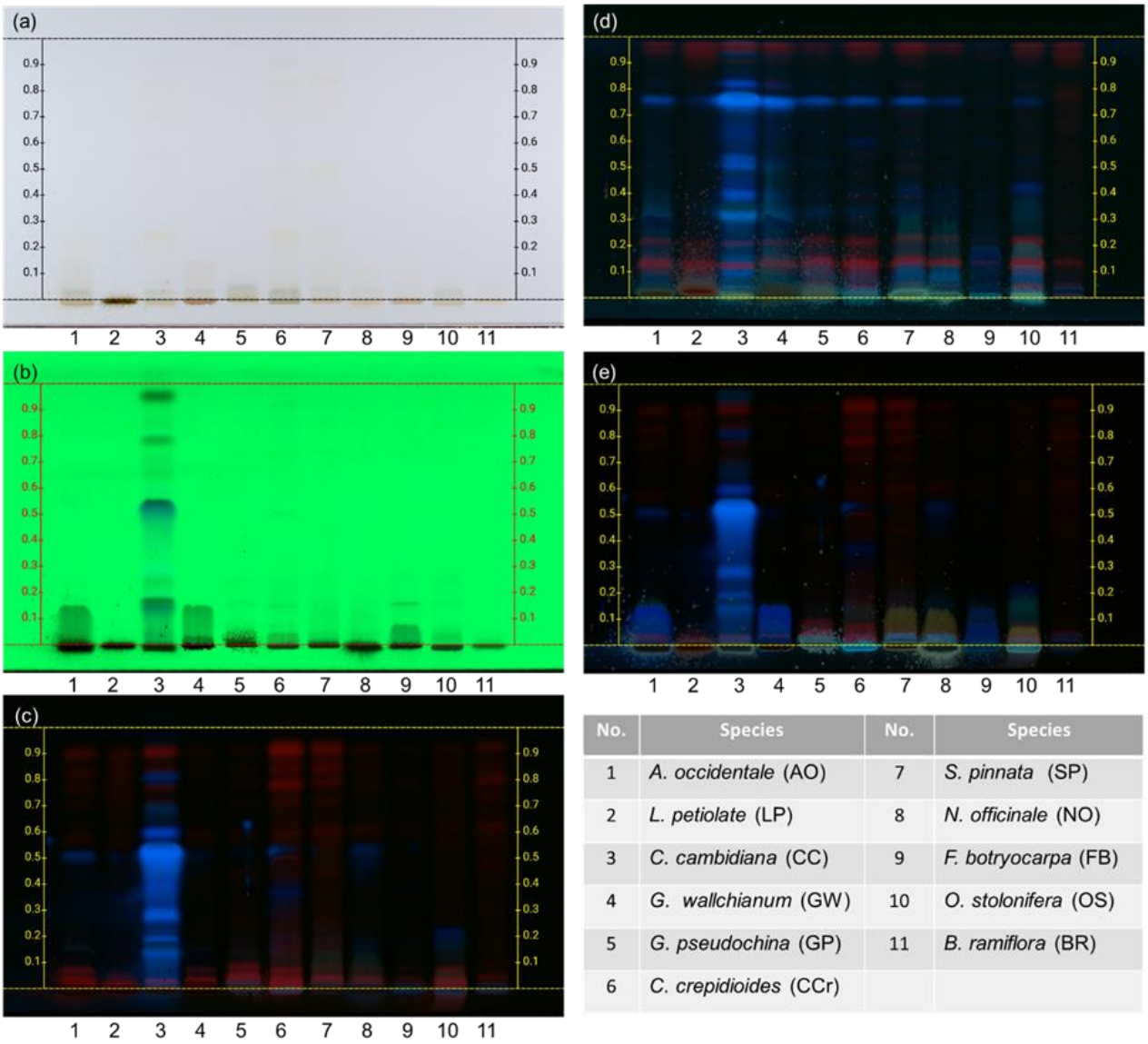


Figure 1 The eleven indigenous vegetable species.

The methanol extracts were evaluated by gram/dry weight (g/DW), 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, 0.234 ± 0.023 g/DW, and 0.234 ± 0.023 g/DW for AO, LP, CC, GW, GP, CCr, SP, NO, FB, OS, and BR, respectively. Methanol extracts were chosen to evaluate antioxidant and anti-inflammatory activity because they easily extract a wide range of bioactive compounds, including phenolics and flavonoids, which are known to have these properties [26]. Methanol's polarity improves the solubility of these compounds, allowing for a more thorough investigation of the extract's biological potential. Furthermore, methanol has been widely used in similar trials, allowing comparisons to previous research. The vegetable species' fingerprints were assessed on TLC under white light and wavelengths of 254 nm and 366 nm (Figures 2a, 2b, and 2c). Phytochemical screening reveals the presence of the flavonoid and phenol groups, represented as yellowish and brown bands on the TLC plate under the 366 nm wavelength (Figures 2d and 2e). Remarkably, both chemical groups exhibited color reactions across all eleven species.



No.	Species	No.	Species
1	*A. occidentale* (AO)	7	*S. pinnata* (SP)
2	*L. petiolate* (LP)	8	*N. officinale* (NO)
3	*C. cambidiana* (CC)	9	*F. botryocarpa* (FB)
4	*G. wallchianum* (GW)	10	*O. stolonifera* (OS)
5	*G. pseudochina* (GP)	11	*B. ramiflora* (BR)
6	*C. crepidioides* (CCr)		

Figure 2 The phytochemical screening of eleven native vegetable species was performed on TLC plates under three different conditions: (a) white light, (b) 254 nm, and (c) 366 nm wavelengths. Subsequently, derivatization was performed for (d) the flavonoid group using $AlCl_3$ and (e) the phenol group using the NP reagent under 366 nm illumination.

3.2 Antioxidant activity

The total phenolic content (TPC) was quantified using the gallic acid calibration curve equation (Y=0.2542X - 0.01070, $R^2 = 0.9924$, Figure 3a) and revealed significant differences among the samples ($F(10, 22) = 1125$, p-value < 0.0001). Among the tested extracts, AO showed the highest TPC, with a 160.40 ± 1.322 mg GAE/g extract value. Similarly, the total flavonoid content (TFC) was quantified using the quercetin calibration curve equation ($Y = 0.03397X + 0.04122$, $R^2 = 0.9946$, Figure 3b), also showing significant differences among the samples ($F(10, 22) = 1086$, $p < 0.0001$). The LP extract recorded the highest TFC at 270.90 mg QE/g extract. In contrast, the NO extract exhibited the lowest TPC and TFC, with values of 4.82 ± 0.295 mg GAE/g extract and 11.68 ± 0.248 mg QE/g extract, respectively (Table 1).

Table 1. Antioxidant activity of methanolic extracts of the eleven indigenous vegetables.

Species	Total Flavonoid Contents (TFC) (mg QE/g extract)	Total Phenolic Content (TPC) (mg GAE/g extract)	FRAP-assay (µmole TE/g extract)	FRAP-assay (µmole Fe^{2+}/g extract)	DPPH-assay (µg/mL)	DPPH-assay- IC_{50} (µg/mL)
AO	67.64 ± 0.490	160.40 ± 1.322**	478.19 ± 6.466**	3,858.26 ± 157.105**	85.99 ± 0.762	7.39 ± 0.176**
LP	270.90 ± 6.175**	63.88 ± 3.966	457.48 ± 5.956	3,852.44 ± 11.637**	78.29 ± 2.040	44.18 ± 1.548
CC	72.06 ± 7.616	30.87 ± 0.375	250.62 ± 3.843	2,188.29 ± 31.421	83.92 ± 0.352	93.02 ± 7.567
GW	76.20 ± 3.158	99.56 ± 6.755	484.08 ± 10.010**	4,115.36 ± 100.00**	80.77 ± 0.907	7.703 ± 0.820**
GP	16.96 ± 0.430	6.01 ± 0.051	70.08 ± 5.907	712.09 ± 48.295	79.23 ± 0.616	ND
CCr	65.21 ± 3.035	26.78 ± 0.952	199.53 ± 12.240	1,770.51 ± 100.081	70.92 ± 3.011	101.60 ± 4.858
SP	30.38 ± 0.889	23.88 ± 0.69	394.30 ± 5.764	3,420.00 ± 261.259**	81.44 ± 0.906	109.40 ± 6.752
NO	11.68 ± 0.248	4.82 ± 0.295	21.82 ± 2.099	311.77 ± 21.529	29.17 ± 1.507	ND
FB	85.62 ± 5.843	25.83 ± 0.265	394.30 ± 5.764	3,363.09 ± 47.131	76.88 ± 1.256	152.90 ± 4.998
OS	46.80 ± 1.781	19.30 ± 1.468	144.73 ± 5.124	1,322.47 ± 41.895	58.96 ± 4.427	ND
BR	29.09 ± 0.248	10.05 ± 0.843	261.19 ± 9.599	2,192.95 ± 320.028	31.11 ± 1.683	ND
Ascorbic acid						11.08 ± 0.623

Data are expressed as means ± SD. The small letters after the means represent significance in the same citrus tissue at 0.05 level. -: not detected. ** statistically significant different, ND= not determined.

Antioxidant activity was evaluated using the FRAP technique, with activity expressed in μmole Trolox equivalent/g extract (Trolox: Y = 0.01428X - 0.06976, R^2 = 0.9954, Figure 3c) and μmole Fe^{2+}/g extract ($FeSo_4$: Y = 0.01648X - 0.03085, R^2 = 0.9964, Figure 3d). Both measurements exhibited statistically significant differences among the crude extracts, with F(10, 22) = 11.76 and *p*-value < 0.0001 for Trolox equivalent activity, and likewise with F(10, 22) = 2.66.1 and *p*-value < 0.0001 for Fe^{2+} activity. GW demonstrated the highest activity with 484.08 ± 10.010 μmole TE/g extract and 4,115.36 ± 100.00 μmole Fe^{2+}/g extract (Table 1). Conversely, NO exhibited the lowest activity with 21.82 ± 2.099 μmole TE/g extract and 311.77 ± 21.529 μmole Fe^{2+}/g extract (Table 1).

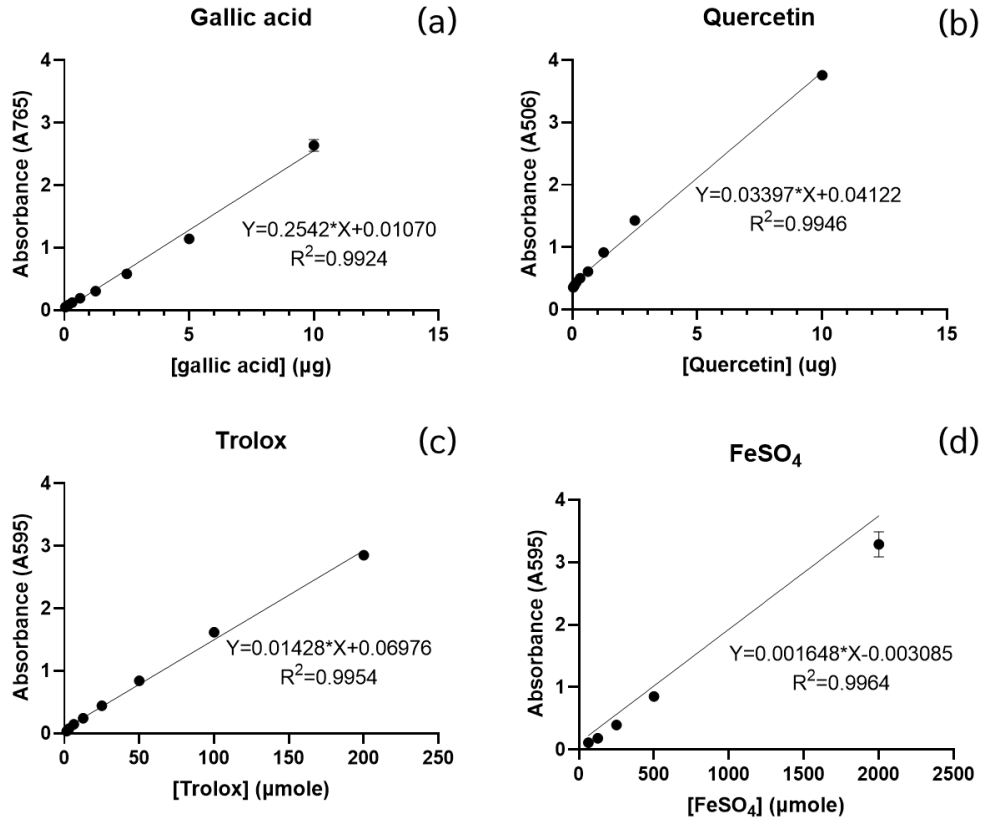


Figure 3 The standard curve of (a) gallic acid, (b) Quercetin, (c) Torox, and (d) $FeSO_4$

In the DPPH scavenging assay, the activity showed statistically significant differences (F(10, 22) = 326.9, *p*-value < 0.0001) among the crude extracts. AO, LP, CC, GW, CCr, SP, and FB exhibited scavenging activity of 85.99 ± 0.762 μg/mL, 78.29 ± 2.040 μg/mL, 83.92 ± 0.352 μg/ml, 80.77 ± 0.907 μg/mL, 70.92 ± 3.011 μg/mL, and 81.44 ± 0.906 μg/mL, respectively, over 50% scavenging (Table 1). Scavenging activity was assessed, revealing that the IC_{50} values of AO and GW were comparable to those of ascorbic acid, the positive control. Specifically, AO exhibited an IC_{50} value of 7.39 ± 0.176 μg/mL, while GW showed a value of 7.70 ± 0.820 μg/mL. In contrast, the IC_{50} value for ascorbic acid was 11.08 ± 0.623 μg/mL (Table 1).

The antioxidant activity of plants is gaining significant attention worldwide, with various methodologies being employed to study it. Our research focused on calculating the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of indigenous vegetables from Nakhon Si Thammarat province in Thailand. To assess the antioxidant activity, we utilized the DPPH scavenging assay and compared the results with the FRAP assay, using ascorbic acid as the control. All eleven plant species exhibited Total Phenolic Content (TPC), ranging from 4.82 to 160.40, and Total Flavonoid Content (TFC) ranging from 11.68 to 270.90.

Similarly, the antioxidant activity measured by the FRAP assay, in terms of Trolox equivalents (TE) and Fe^{2+} equivalents, followed the same trend. Eight species demonstrated more than 50 percent DPPH scavenging inhibition, with two species, AO and GW, outperforming ascorbic acid. Almost all indigenous vegetables used in this study were examined for their antioxidant activity by assessing their total phenolic and/or flavonoid contents, followed by tests for their biological activity. However, some vegetables, including GP, FB, OS, and BR, were new and lacked phytochemical content and biological activity data. Despite this, they were investigated within the same genus. This research reports for the first time on their TPC and TFC and their antioxidant and anti-inflammatory activities. These vegetables' phytochemical content and activity were lower than the others, but this data could serve as a foundation for further studies, particularly into their biological activity. The TPC and TFC of AO were higher than those of other plant groups, with DPPH free radical scavenging activity surpassing ascorbic acid. According to the Brazil research group, AO methanol extract showed 2.32 mg GAE/g and 0.29 mg RE/g (rutin equivalent per gram of extract), respectively [27]. Another study investigated AO leaves, finding TPC and TFC values of 8.5 ± 0.57 mg GAE/g and 0.86 ± 0.05 mg QE/g, respectively. The antioxidant activity measured through DPPH scavenging revealed an IC_{50} value of 4.96 ± 0.12 μg/mL, compared to 4.91 ± 0.43 μg/mL for ascorbic acid [28]. In Nigeria, AO leaves demonstrated DPPH free radical scavenging activity with an IC_{50} of 1.01 ± 0.07 μg/mL, compared to 0.06 ± 0.07 μg/mL for ascorbic acid [29]. Our research found the highest amount of TFC in the methanol extract of LP leaves compared to other groups not investigated for TFC and TPC. The dichloromethane extract of LP leaves from Malaysia exhibited IC_{50} values of 27.36 μg/mL for the DPPH radical scavenging test, while the positive control (quercetin) had an IC_{50} of 3.96 μg/mL [30]. However, in this study, the IC_{50} for ascorbic acid was shown to be 44.18 μg/mL. The TPC in GW in this study aligns with findings from another group in the same province, reporting a total extractable phenolic content of 16 mg/100 g leaf extract, which was also tested for antioxidants in the Sausage Model System [31]. Additionally, another group found the total phenolic and flavonoid content of GW leaf extract to be 373.93 ± 18.67 mg gallic acid equivalent/g DW and 73.03 ± 0.55 mg rutin equivalent/g DW, respectively [32]. The TPC of CCr from Vietnam was 114.3 ± 1.7 mg GAE/g, and the TFC was 145.46 ± 3.1 mg QE/g. The antioxidant activity of CCr leaf extract was investigated using the DPPH free radical-scavenging assay, showing an IC_{50} of 48.0 ± 1.0 μg/mL compared to the control, gallic acid, which had an IC_{50} of 2.9 ± 0.0 μg/mL [33]. In Malaysia, the TPC and TFC of the CCr ethanol extract were 422.22 ± 0.05 mg GAE/g and 3.46 ± 0.00 mg quercetin equivalent/g, respectively. The DPPH assay for the ethanol extract showed an IC_{50} value of 0.30 ± 0.01 μg/mL [34]. Both studies indicate higher TPC and TFC content and greater DPPH scavenging activity compared to our group's CCr methanol leaf extracts. For SP, research on TPC and TFC used the exocarp as a material instead of the leaves used in this study. The exocarp aqueous extracts in India showed TPC and TFC values of 570.20 ± 0.48 mg GAE/g and 115.77 ± 0.89 TE/g, respectively. The DPPH radical scavenging activity was dose-dependent, with all fractions demonstrating strong free radical scavenging activity and low IC_{50} values of 2.10 ± 0.21 μg/mL compared to the control, ascorbic acid, at 4.94 ± 0.21 μg/mL [35]. In contrast, our study found that the IC_{50} values for the leaf extract were about ten times higher than those for ascorbic acid. For NO, a leaf extract from Iran exhibited phenolic and flavonoid contents of 96.6 ± 3.5 mg gallic acid equivalents/g dried extract and 62.3 ± 2.4 mg catechin equivalents/g dried extract, respectively. The EC_{50} values for DPPH scavenging by NO extract and ascorbic acid were 114.7 μg/mL and 3.5 μg/mL, respectively [36]. In another study involving NO leaf extract from micro-shoot cultures of NO, the maximum antioxidant activity was observed with 0.5 mM Trp (day 10) (0.90 mmol TE/100 g DW), which was 1.3 times higher than in control (0.69 mmol TE/100 g DW), showing activity against microaerobic Gram-positive acne strains [37]. However, the NO leaf extract in this research exhibited lower amounts of TPC and TFC and reduced antioxidant properties. However, the geographical differences of a plant can affect the content of its phytochemicals and the strength of its antioxidant activity. The phytochemical content within the same species has been linked to variations in antioxidant activities. The AO extract had a significant amount of phenolic compounds and well-known antioxidants. Phenolics operate as electron donors, scavenging free radicals and chelating metal ions that power oxidative reactions.

3.3 Inhibition of nitric oxide (NO) production

Nitric oxide is an inflammatory mediator generated by activated RAW264.7 cells, which LPS induced. Nitric oxide was measured using Griess reagent in a cell culture medium of nitrite ion (NO_2^-) form. At 24 h, nitric oxide production was significantly decreased by CC extract, and other species had no inhibition signal.

The inhibitory activity of eleven indigenous vegetable species on nitric oxide production by induced RAW 264.7 macrophage cell lines is presented in Figure 4. The CC extract had the best inhibitory activity on nitric oxide production (98.23% inhibition and 96.61% cell viability) at 40 μg/mL. In addition, the potential to inhibit nitric oxide production of CC at 0.4, 0.6, 0.8, and 1.0 μg/mL was significantly different compared to the control. Furthermore, CC up to 40 μg/mL did not exhibit cytotoxicity on RAW264.7 cells (Figure 4).

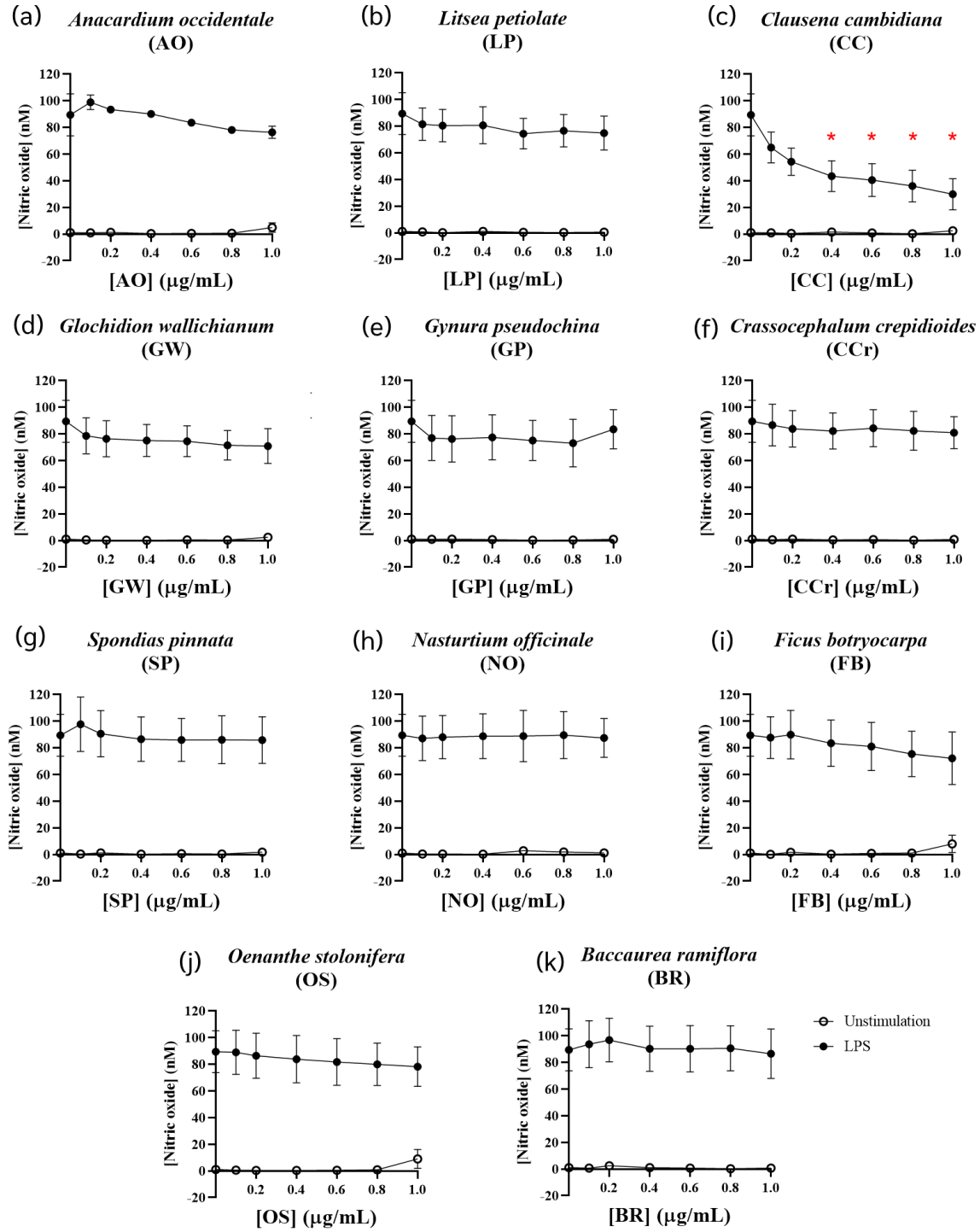


Figure 4 The inhibition of nitric oxide production was assessed using eleven crude extracts, namely: (a) AC, (b) LP, (c) CC, (d) GW, (e) GP, (f) CCr, (g) SP, (h) NO, (i) FB, (j) OS, and (k) BR. These extracts were tested against lipopolysaccharide (LPS) derived from *E. coli*. * on the graph indicates statistical significance at a p-value < 0.05.

The first step in examining the beneficial characteristics of plants is to evaluate their antioxidant properties, followed by their anti-inflammatory properties. The activity is observed in all concentrations of CC and FB at 1 mg/mL, while LP impacts cell viability at all concentrations. The plant's anti-inflammatory properties were studied using mouse macrophage RAW 264.7 cells induced with LPS at various extract concentrations. Of the 11 species tested, two species, LB and FB, exhibited cytotoxic effects on RAW 264.7 cells, while the others showed no inhibition of nitric oxide generation. LB displayed cytotoxicity across all tested concentrations, whereas FB showed 1.0 μg/mL cytotoxicity.

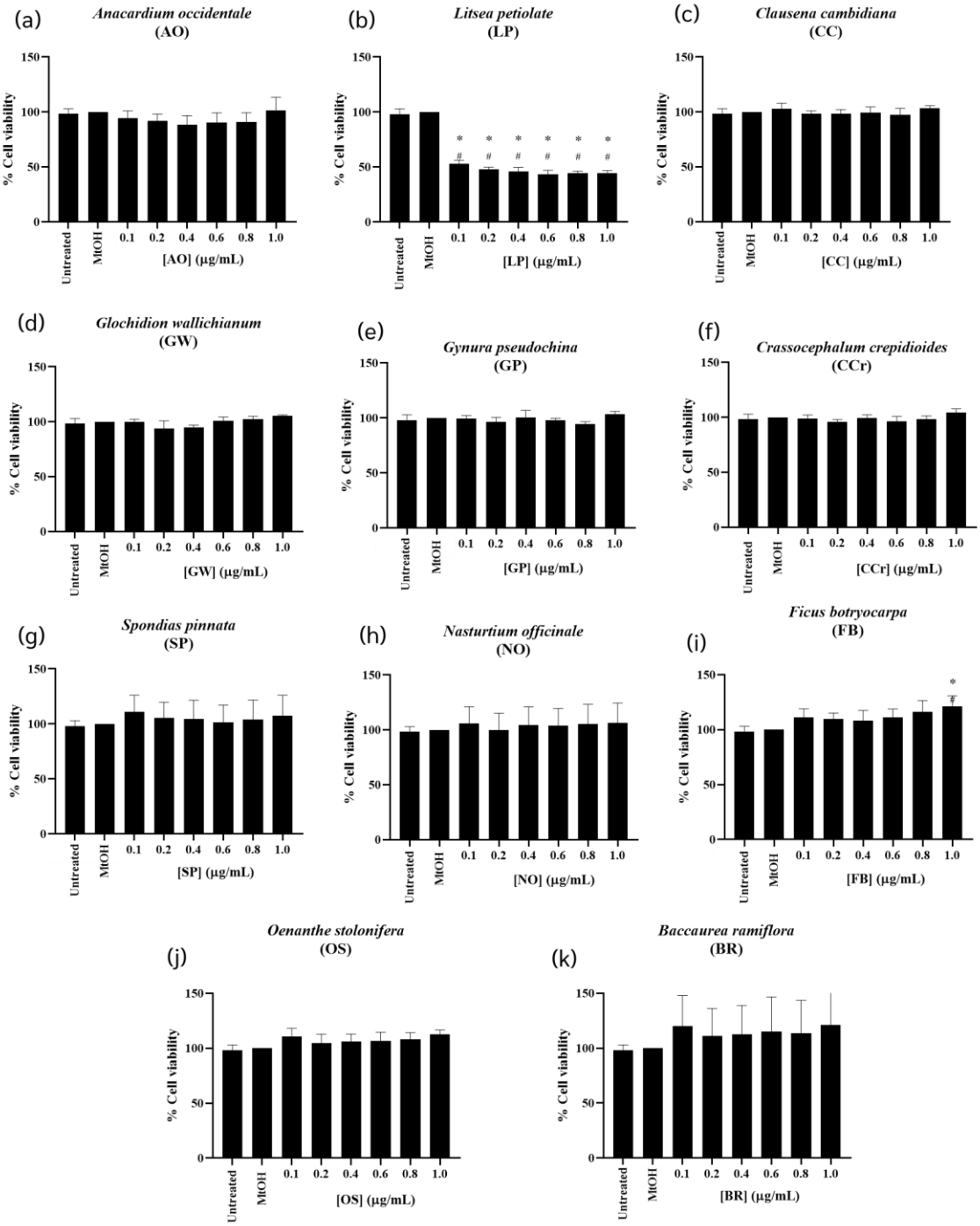


Figure 5 The inhibition of nitric oxide production was assessed using eleven crude extracts, namely: (a) AC, (b) LP, (c) CC, (d) GW, (e) GP, (f) CCr, (g) SP, (h) NO, (i) FB, (j) OS, and (k) BR. These extracts were tested against lipopolysaccharide (LPS) derived from *E. coli*. * on the bar graph indicates statistical significance at a p-value < 0.05.

Plant extracts' phytochemical analysis and antioxidant activity show a strong correlation with their anti-inflammatory properties, notably nitric oxide inhibition. CC methanol extract had the highest nitric oxide inhibitory efficacy among 11 indigenous vegetable species, attaining 98.23% inhibition and 96.61% cell viability at 40 μg/mL. This shows that CC's antioxidant qualities, as suggested by its phenolic and flavonoid concentration, may contribute to its efficacy in suppressing inflammatory reactions. The CC extract's inhibitory effects were concentration-dependent, with considerable nitric oxide inhibition at low concentrations (0.4-1.0 μg/mL) and no cytotoxicity at concentrations up to 40 μg/mL. In contrast, LP, which exhibited the highest total flavonoid content (270.90 mg QE/g), did not show nitric oxide inhibition, and its extract impacted cell viability across all tested concentrations, limiting its potential for anti-inflammatory applications. Similarly, FB showed cytotoxicity at 1.0 μg/mL, while LB displayed cytotoxic effects at all tested concentrations, indicating that their toxicity likely hindered their anti-inflammatory activities. Despite their strong antioxidant activities (e.g., AO and GW), the absence of nitric oxide inhibition in most extracts highlights the complexity of plant bioactivity [38, 39]. Antioxidant potential, while indicating the ability to scavenge free radicals, does not always directly translate to anti-inflammatory effects in cellular models. The findings emphasize that, whereas phenolic and flavonoid compounds (e.g., in AO and LP) contribute considerably to antioxidant activity, their ability to suppress inflammatory mediators such as nitric oxide depends on their interaction with cellular pathways. CC's exceptional anti-inflammatory action and its safety profile highlight its therapeutic promise and call for further exploration as a natural source of anti-inflammatory chemicals [38]. Compared to other research, the AO extract from Basil significantly decreased cell viability within the first 24 hours at a 500 mg/mL concentration. This was observed across tested concentrations of 0.5 mg/mL, 5 mg/mL, and 500 mg/mL [27]. The CCr extract from Vietnam demonstrated anti-inflammatory capacity *in vitro* by reducing NO production in LPS-induced mouse macrophage RAW 264.7 cells at 0.1 μg/mL. Histopathological analysis of 7-day-old wounds indicated that CCr treatment at 50 mg/kg/day reduced inflammatory cell density by 2.8 times [33]. The CCr ethanol extract from Malaysia also inhibited lipoxygenases by reacting with free radicals generated at the enzyme's active site (55.01 ± 0.04 μg/mL) [34]. The *N. officinale* leaf extract from Iran exhibited moderate NO-scavenging activity in the range of 25–400 μg/mL, relative to catechin, and in a dose-dependent manner [36]. However, our study had a drawback in that the effects of the vehicle control (DMSO) were not thoroughly investigated, which could affect the interpretation of the nitric oxide inhibition results and cell viability. DMSO is a popular solvent *in vitro* research because it dissolves polar and nonpolar molecules. However, it may have biological effects on cells, such as changing membrane permeability, lowering oxidative stress, or influencing gene expression. Even at low doses, DMSO can interfere with physiological responses, such as inflammatory signaling pathways, potentially affecting nitric oxide generation and cell survival [40]. In this work, DMSO was used as the vehicle control, with specific effects on RAW 264.7 cells produced with LPS were not separately evaluated. This omission makes it impossible to determine whether the observed decrease in nitric oxide production and changes in cell viability were caused by extracts or were influenced by the solvent. To ensure appropriate interpretation, future research should do a detailed characterization of DMSO effects, such as assessing its influence at equal concentrations. This would confirm that the observed anti-inflammatory action, notably in the CC extract, is due to the extract itself, not vehicle influence. The study does not validate the MTT assay's specificity, which raises issues regarding the veracity of cell viability results. Plant extracts, particularly those high in phenolics or flavonoids, can interfere with the assay by directly decreasing MTT, resulting in an overestimating cell viability. Furthermore, using a single assay without supplementary procedures, such as trypan blue exclusion or flow cytometry, reduces the reliability of the results. To overcome this, future research should use alternative assays and controls to ensure that the observed effects are attributable to cellular responses rather than assay interference.

4. Conclusions

All eleven species of local vegetables exhibited antioxidant activity, with total phenolic contents and total flavonoid contents showing a direct variable relationship. However, two species, AO (*A. occidentale*) and GW (*G. wallichianum*), had a higher percentage of free radical inhibition than ascorbic acid. Further investigation into the anti-inflammatory compounds of these species revealed that the compound with the anti-inflammatory property was CC (*C. cambidiana*). In the cytotoxicity test, only LP (*L. petiolate*) reduced cell

viability at all concentrations, while FB (*F. botryocarpa*) inhibited cell growth only at the highest concentration tested. This presentation aims to highlight the pharmacological properties of these long-used local vegetables and provide scientific evidence to enhance the value of local foods, thereby helping to realize the value chain of the parties involved.

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