Original Article

ANTIOXIDANT AND INHIBITORY EFFECT OF SELECTED GHANAIAN VEGETABLES ON NITRIC OXIDE EXPRESSION IN LIPOPOLYSACCHARIDE-INDUCED MACROPHAGE CELLS

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ABSTRACT

Objective: Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation. Inhibitors of NO may be useful candidates for the treatment of inflammatory diseases. The study aimed to determine the antioxidant and inhibitory effect of commonly used Ghanaian vegetables, namely *Corchorus olitorius (CO), Solanum melongena (SM), Solanum torvum (ST), Xanthosoma sagittifolia (XS)* and *Abelmoschus esculentus (AE)* on NO expression in a Lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cell line.

Methods: The cytotoxic effects of the vegetables on the cell line were determined using a tetrazolium-based colorimetric assay. The inflammatory activity was determined by measuring the inhibition of NO production in LPS-induced RAW 264.7 macrophage cells. Total antioxidant activity, total phenolic, flavonoid, and reduced glutathione contents were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, Folin-Ciocalteu, aluminium chloride, and O-Phthalaldehyde methods, respectively.

Results: Our results showed that *CO* and *ST* significantly inhibited NO production in a concentration-dependent manner with good cell viability. *Solanum torvum* also exhibited strong antioxidant activity (IC_{50}= 0.466±0.23 mg/ml) with total phenolic content of 230.73±1.84 mg/g GAE, while *CO* showed high flavonoid content (291.45±2.14 mg/g QUE). *Abelmoschus esculentus* recorded the highest glutathione content (58.6 µg/g GSH. Saponins, alkaloids, tannins, terpenoids, and cardiac glycosides were present in all the samples except *SM* and *AE,* which lacked terpenoids.

Conclusion: These findings suggest that *CO* and *ST* possess anti-inflammatory and antioxidant activities that could be explored as potential therapeutic remedies for inflammatory disorders.

Keywords: Antioxidant, Nitric oxide, Lipopolysaccharide, Inflammation

INTRODUCTION

Inflammation is an important host response to a foreign challenge or tissue injury, which leads to the restoration of tissue structure and function [1, 2]. During the process, the activation of immune cells induced by pro-inflammatory cytokines up-regulates the inflammation [3]. It is well known that macrophages, together with neutrophils and dendritic cells, play an important role in the innate immune response [4]. Key inflammatory mediators such as inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) can be released by activated macrophages [2, 5].

In mammalian cells, nitric oxide (NO) is synthesized by three different isoforms of NO synthase (NOS), namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS [6]. Although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ (IFN-γ), lipopolysaccharide (LPS), and a variety of proinflammatory cytokines [5]. Lipopolysaccharide, a cell wall component of gram-negative bacteria, has been reported to activate macrophages to produce inflammatory mediators such as iNOS, TNF-α, and COX-2, mimicking the inflammatory reaction *in vivo* [2, 3]. Nitric Oxide production is controlled by NOS. Most importantly, iNOS is highly expressed in macrophages. Its activation leads to organ destruction in some inflammatory and autoimmune diseases [6, 7].

Non-steroidal anti-inflammatory drugs (NSAIDs) are very effective in the alleviation of pain, fever, and inflammation. However, most of the available drugs induce adverse effects such as gastrointestinal irritation and allergies. Plants are a major source of several drugs, including anti-inflammatory drugs. Hence, the search for alternative anti-inflammatory drugs from plant sources is warranted [8, 9].

Phytochemical antioxidant constituents, including phenolics, flavonoids, and reduced glutathione of plant origin, have been reported as scavengers of reactive oxygen species (ROS) that are implicated in inflammatory and autoimmune diseases [10]. Thus, these phytochemicals are seen as promising therapeutic agents for free radical pathologies [11]. These phytochemicals are usually part of the everyday diet, and some are also used as medicines or food supplements [12, 13].

In Ghana, commonly consumed vegetables include *Corchorus olitorius, Solanum melongena, Solanum torvum, Xanthosoma sagittifolia, and Abelmoschus esculentus.* The aim of this study was to assess the antioxidant capacity of common vegetables and investigate their inhibitory effects on nitric oxide expression in LPS-induced RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's (DMEM) culture medium, Fetal Bovine Serum (FBS), Penicillin-streptomycin, Trypsin, EDTA, 3-(4,5-Dimethylythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sulfanilamide and LPS (*Escherichia coli* Serotype 055: B5) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Naphthylethylenediamine dihydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). The murine macrophage cell line RAW264.7 was obtained from the RIKEN BioResource Center Cell Bank (Japan). All other reagents were obtained from standard suppliers.

Sample collection

The vegetables (table 1) were purchased from the local market at Madina, Accra, Ghana, and transported to the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. The plant samples were identified at the Ghana Herbarium, Department of Plant and Environmental Biology, University of Ghana, where voucher specimens have earlier been placed [14].

Table 1: Common vegetables used in ghana

Vegetable name	Code	Family	Local name	Part used	Voucher No.
Corchorus olitorius	CO	Tiliaceae	Adeimey	Leaves	AF0227001
Solanum ethiopicum	SE	Solanaceae	Nyaadoa	Fruit	AF0227002
Xanthosoma sagittifolia	XE	Araceae	Kontomire	Leaves	AF0227003
Solanum torvum	ST	Solanaceae	Nsusoa	Fruit	AF0227004
Abelmoschus esculentus	AE	Malvaceae	Nkruma	Fruit	AF0227005

The parts of vegetables used in the study are edible. Table adapted from [14]

Sample preparation

The vegetables were prepared using a procedure like that used to prepare these vegetables for consumption in Ghana [14]. Each fresh vegetable (10 g) was boiled in 100 ml of distilled water for 10 min, allowed to cool, and blended with the water used for boiling. The samples were freeze-dried (using Labconco Free Zone 6.0; Labconco Corporation, Kansas City, MO, USA) and kept at-20 °C. The percentage yield of the samples was calculated using the following equation:

$$\% \text{ yield} = \frac{\text{Weight of freeze} - \text{dried vegetable}}{\text{Weight of fresh vegetable}} \times 100\%$$

Cell culture and viability assay (MTT assay)

RAW 264.7 macrophage cells were cultured in a humidified incubator at 37 °C with 5% CO_2 in a DMEM culture medium with 10% FBS. Cell viability was determined by MTT assay [3]. Briefly, RAW 264.7 cells (2×10^4 cells/well) were plated into a 96-well plate and incubated for 24 h at 37 °C. The cells were treated with freeze-dried vegetables (concentration range, 25 to 100 µg/ml) or vehicle before exposure to 50 ng/ml lipopolysaccharide (LPS). After a 12 h incubation period, 20 µl MTT solution (2.5 mg/ml in PBS) was added to each well and the cells were incubated for 4 h at 37 °C. The insoluble formazan product formed after incubation was dissolved by the addition of 150 µl acidified isopropanol (Isopropanol: HCl (500:7)) containing 1% Triton-X and incubation for 24 h. The amount of formazan formed was determined by measuring the absorbance of the well content at the wavelength of 570 nm with the microplate reader. All experiments were performed in triplicates and the percentage viability of cells at each concentration of the extracts was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{Absorbance of control}} \times 100\%$$

Nitric oxide assay

Nitrite concentration was determined using a dilution of sodium nitrite as a standard [3]. Briefly, RAW 264.7 cells (3×10^5 cells/well) were plated in 24-well plates and pretreated with or without freeze-dried vegetables (25-100 µg/ml) for 1 h prior to stimulation with 50 ng/ml of LPS for 24 h. The supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) and then incubated at room temperature (25 °C) for 10 min. The absorbance was measured at the wavelength of 540 nm with the Tecan Infinite microplate reader. The amount of nitrite was calculated from the sodium nitrite standard curve.

Phytochemical constituent and antioxidant activity of vegetables

Estimation of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method with slight modification [15]. Stock solutions of 5 mg of freeze-dried vegetables dissolved in distilled water were prepared and 2-fold serial dilutions were made to obtain the concentration range of 0.0396 to 5.0 mg/ml. Fifty microliters of Folin-Ciocalteu reagent were added, mixed, and incubated at room temperature for 8 min. One hundred and fifty microlitres (150 µl) of 0.09 M sodium carbonate solution was added and the mixture was incubated for 2 h at room temperature and absorbance was measured at the wavelength of 760 nm. The experiment was carried out in triplicate. The total phenolic was calculated using a calibration curve for gallic acid. The total phenolic content was expressed in terms of Gallic acid equivalent (GAE).

Estimation of total flavonoid content

The total flavonoid content was determined by the aluminium chloride method [15]. The reaction mixture comprising 100 µl freeze-dried vegetable (concentration range 0.0396 to 5.0 mg/ml) and 100 µl of 2% aluminium chloride (in methanol) was incubated at room temperature for 20 min before measuring absorbance at 415 nm. The experiment was carried out in triplicate. Quercetin was used as a positive control. The total flavonoid content was calculated using a calibration curve for quercetin. The total flavonoid content obtained was expressed in quercetin equivalent (QUE).

Estimation of reduced glutathione content

Estimation of total reduced glutathione (GSH) content was done using the O-Phthalaldehyde method as described with slight modification [16]. The reaction mixture consisted of 180 µl of GSH buffer (100 mmol NaH_2PO_4, 5 mmol EDTA, pH 8.0 with 1 N NaOH), 10 µl freeze-dried vegetable (concentration range 0.0009 to 2000 µg/ml) prepared in distilled water and 10 µl of 0.75 mmol O-Phthalaldehyde (OPT). This mixture was incubated for 15 min at room temperature and fluorescence was measured at an excitation wavelength of 350 nm and emission wavelength of 420 nm. The experiment was carried out in triplicate. The total glutathione content was calculated using a glutathione calibration curve.

Determination of phytochemical constituents

Phytochemical constituents of the freeze-dried vegetables were assessed qualitatively as described [17] with slight modifications.

Saponins

Five milliliters (5 ml) of distilled water were added to 0.10 g of the freeze-dried vegetable in a test tube and shaken vigorously. A stable persistent (≥ 1 min) froth indicated the presence of saponins.

Alkaloids

Ten milliliters (10 ml) of 10% HCL was added to 0.10 g of the sample, boiled for 2 min, and filtered. Two milliliters of diluted (10%) ammonia solution were added and filtrated. To extract the alkaloidal base, 1 ml of chloroform was added and gently shaken. Subsequently, 10 ml of acetic acid and 100 µl Dragendorff's reagent were added. A reddish-brown precipitate indicated the presence of alkaloids. Quinidine was used as a positive control.

Tannins

Ten milliliters of distilled water were boiled with 0.5 g of sample briefly and filtered. One hundred microliters of 0.1% $FeCl_3$ were added to the filtrate. A brownish-green or blue-black coloration indicated the presence of tannins. Gallic acid was used as a positive control.

Terpenoids

Two milliliters of chloroform were added to 0.1 g of the sample and then 1 ml of concentrated sulphuric acid was added. A reddish-brown color at the interface between chloroform and concentrated sulphuric acid indicated the presence of terpenoids. Ursolic acid was used as a positive control.

Cardiac glycosides

Two milliliters of glacial acetic acid were added to 0.1 g of a sample containing 100 µl of 0.1% ferric chloride and then mixed with 1.0 ml of concentrated sulphuric acid. A brown ring that occurred at the interface between glacial acetic and concentrated sulphuric acid indicated the presence of cardiac glycosides.

DPPH antioxidant activity

The free radical scavenging activity was measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [14]. Three-fold serial dilutions of stock solutions of 10 mg/ml freeze-dried vegetables were prepared with distilled water to obtain 7 different concentrations per vegetable. A reaction mixture consisted of 100 μl of 0.5 mmol DPPH in absolute methanol and 100 μl of the extract (concentration range 0.0023 to 10 mg/ml). After incubation at room temperature for 10 min in the dark, the absorbance was measured at a wavelength of 517 nm. Butylated hydroxytoluene (BHT) was used as a positive control. The experiment was carried out in triplicate. Percentage antioxidant activity was calculated from the formula below:

$$\text{Percentage Antioxidant} = \frac{A - B}{A} \times 100\%$$

Where A = optical density of blank and B = optical density of the sample.

A plot of percent inhibition versus log concentration of the extract was used to determine EC_{50} values.

RESULTS

Sample yield after freeze-drying

The percentage yields of *CO, SM, ST, XS,* and *AE* were 11.70, 9.18, 16.34, 17.62, and 11.10, respectively.
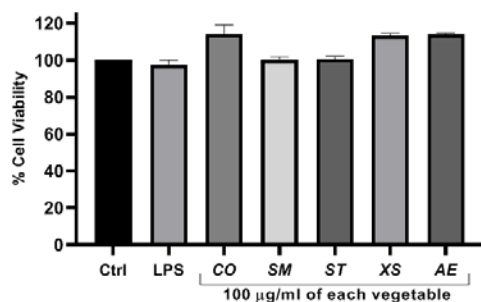

Fig. 1: Effect of vegetables on the viability of RAW 264.7 cells. RAW 264.7 cells were treated with the highest vegetable concentration (100 μg/ml) for 30 min before incubating with LPS (50 ng/ml). The data represent means±SD of three independent experiments. There was no significant difference between the control and each treatment.

Effect of vegetable extract on LPS-simulated RAW 264.7 cells

Fig. 1 shows the effects of the vegetables on LPS-stimulated RAW 264.7 cells. The vegetables did not cause a significant cytotoxic effect on the RAW 264.7 cells. The increases in cell viability caused by *CO, XS* and *AE* were marginal.

Inhibition of nitric oxide

The inhibitory effect of the vegetable extracts on NO production is shown in fig. 2. Concentration-dependent decreases in NO levels were found with *CO, ST,* and *XS.* However, no significant decreases were observed with *SM* and *AE.*
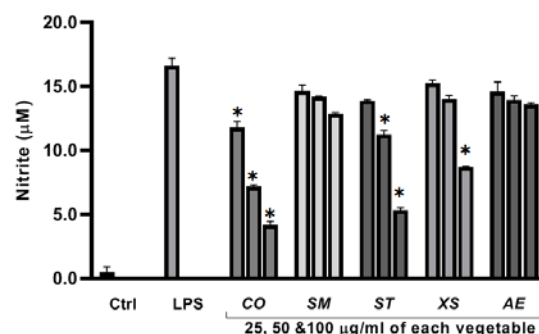

Fig. 2: The effects of the vegetables on the production of nitrite in LPS-stimulated RAW 264.7 cells which were untreated (C), treated with only 50 ng/ml LPS (LPS), pre-treated with vegetables at concentrations of 25 to 100 *μ*g/ml. The data represent means+SD of three independent experiments (y=0.0225x+0.0104 R^2=0.9998). *P<0.05 represents a significant difference in NO compared to the cells treated with extracts and only LPS

Phytochemical constituent and antioxidant activity of vegetables

Phenolics, flavonoids, and glutathione content

Phenolics, flavonoids, and glutathione were present in all the vegetables. *Solanum torvum, Corchorus olitorius* and *Abelmoschus esculentus* recorded the highest levels of phenolic, flavonoid, and glutathione contents, respectively (table 2).

Table 2: Total phenolic, flavonoid, and glutathione contents in vegetables

Plant name	Total phenolic content[a] (mg/g GAE)	Flavonoid content[b] (mg/g QUE)	Glutathione[c] (μg/g GSH)
CO	174.93±1.86	291.45±2.14	6.22±0.500
SM	129.40±0.54	13.42±1.21	55.49±0.600
ST	230.73±1.84	84.62±1.62	17.903±1.10
XS	163.67±1.10	278.50±2.5	10.19±0.72
AE	100.14±0.85	147.02±1.60	58.65±2.01

Each value is presented as mean±SD (n=3). Gallic Acid, quercetin, and glutathione standard curves were used to calculate the values. The following are the equations of the curves: GAE (y=0.2794x+0.0375 R^2=0.9971), QUE (y = 0.3211x-0.037 R^2 = 0.9831), and GSH (y=3951x+29.996 R^2=0.9999).

Table 3: Phytochemical constituent of vegetables

Phytochemical constituent	CO	SM	ST	XS	AE
Saponin	++	+++	+++	++	+
Alkaloid	++	+	++	+	+++
Tannin	+++	+++	+	+++	+++
Terpenoid	+++	-	+	+++	-
Cardiac Glycoside*	+	+	+	+	+

Absent (-) low concentration (+) moderate concentration (++) High Concentration (+++) Quinidine, gallic acid, and ursolic acid were standard compounds used for alkaloid, tannin, and terpenoid, respectively. All standards were scored as (+++). Asterisk (*) indicates no standard compound is used for Cardiac Glycoside.

DPPH scavenging activity

The effective concentration (EC$_{50}$) value of free radical (DPPH) scavenging activity was calculated from a plot of percentage inhibition against the log concentration of the sample (table 4). *Solanum torvum* exhibited the strongest antioxidant activity of 0.466±0.09 mg/ml.

Table 4: Free radical scavenging activity

Plant name	EC$_{50}$ (mg/ml)
Corchorus olitorius	3.048±0.20
Solanum melongena	2.212±0.58
Solanum torvum	0.466±0.09
Xanthosoma sagittifolia	4.734±0.56
Abelmoschus esculentus	3.253±0.66
BHT	0.048±0.006

Each value is presented as mean±SD (n=3). Butylated hydroxytoluene (BHT) was used as a positive control.

DISCUSSION

In the present study, we investigated the antioxidant activity of vegetables commonly used in Ghana and their effects on nitric oxide expression in LPS-induced RAW 264.7 macrophage cells. Phytochemical constituents of the vegetable extracts were also assessed. In murine macrophage RAW 264.7 cells, LPS stimulation alone has been demonstrated to induce iNOS transcription and its protein synthesis, with a corresponding increase in NO production [18-20]. Nitric oxide inhibitors represent important therapeutic agents in the management of inflammatory diseases [21]. Inflammation is associated with heat, redness, swelling and pain and eventually causes several diseases and conditions such as cancer, rheumatoid arthritis, and atherosclerosis [9].

Our results showed that all five vegetables tested inhibited NO production at a concentration of 100 µg/ml without any cytotoxic effect on the cell line. *Corchorus olitorius* and *S. torvum* significantly inhibited NO production in a concentration-dependent manner. In another study, *C. olitorius* and *S. melongena* chloroform extracts inhibited NO expression significantly [22]. The inhibition of NO may be attributed to components of the chloroform extract which are similar to those present in the vegetable samples in the present study. Homemade vegetables are prepared with water and the extracts were also prepared with water. Hence, these results are representative of the potential of consumed vegetables to inhibit NO. A review of earlier studies showed similar anti-inflammatory potentials of medicinal plants [23, 24]. Likewise, turmeric from *Curcuma longa* has been shown to have interesting anti-inflammatory activity [25].

Flavonoids and phenolic compounds are known to act as antioxidants [26]. In addition, flavonoids and phenolics play important roles in the control of cancer, inflammation, and other human diseases [27, 28]. GSH is also one of the most important cellular antioxidants, indeed the major scavenger of electrophiles in humans since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced [29-31]. GSH is part of the glutathione-ascorbate cycle that helps to prevent or minimize damage caused by reactive oxygen species [32-34]. *Solanum torvum*, *C. olitorius*, *S. melongena*, and *X. sagittifolia* have antioxidant, analgesic, and anti-inflammatory agents in different traditional medicinal systems [14, 35-38]. In this study, *S. torvum* exhibited strong antioxidant activity with an EC$_{50}$ value of 0.466±0.23 mg/ml and a high total phenolic content of 230.73±1.84 mg/g GAE, while *C. olitorius* similarly showed high flavonoid content (291.45±2.14 mg/g QUE). On the other hand, *A. esculentus* showed the highest GSH content. Hence, the abilities of *C. olitorius* and *S. torvum* to inhibit NO could be partly attributed to their phenolic and flavonoid contents. Phytochemicals such as saponins, alkaloids, tannins, terpenoids, and cardiac glycosides were present in all the samples except for *S. melongena* and *A. esculentus,* which lacked terpenoids. The presence of these phytochemical constituents in the samples might have contributed to their antioxidant activity and NO inhibition.

CONCLUSION

This study has shown that *C. olitorius, S. melongena, S. torvum, X. sagittifolia,* and *A. esculentus* samples contain flavonoid, phenolic, glutathione, and other phytochemical compounds that may contribute to their antioxidant activity and nitric oxide inhibition properties. Reduction in the level of NO in LPS-stimulated RAW264.7 cells by *S. torvum* and *C. olitorius* samples could partly be due to the high flavonoid and phenolic contents as well as the free radical scavenging activity. The findings of the present study provide evidence that supports the use of Ghanaian vegetables as a source of antioxidant and anti-inflammatory agents. Further studies are warranted to determine the active principle(s) in these samples.

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AUTHORS CONTRIBUTIONS

EOA conceived the study, participated in the *in vitro* assays and data analysis, and drafted the manuscript. AA participated in cell cultures, *in vitro* assays, and data analysis. RAO and AG supervised the study and contributed to manuscript writing. All authors read and approved the manuscript.

CONFLICT OF INTERESTS

Declared none

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