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Evaluation of the antioxidant activity and nitric oxide production effect of formulated crispy vegetables from thermal processing of *Amaranthus viridis* and *Sauropus androgynous*

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ABSTRACT

Background: *Amaranthus viridis* and *Sauropus androgynous* are common edible vegetables consumed by the natives of Asia. This research aimed to carry out a comparative evaluation on the antioxidant activity and nitric oxide production effect of crispy vegetables formula of *A. viridis* and S. *androgynous* made by thermal processing.

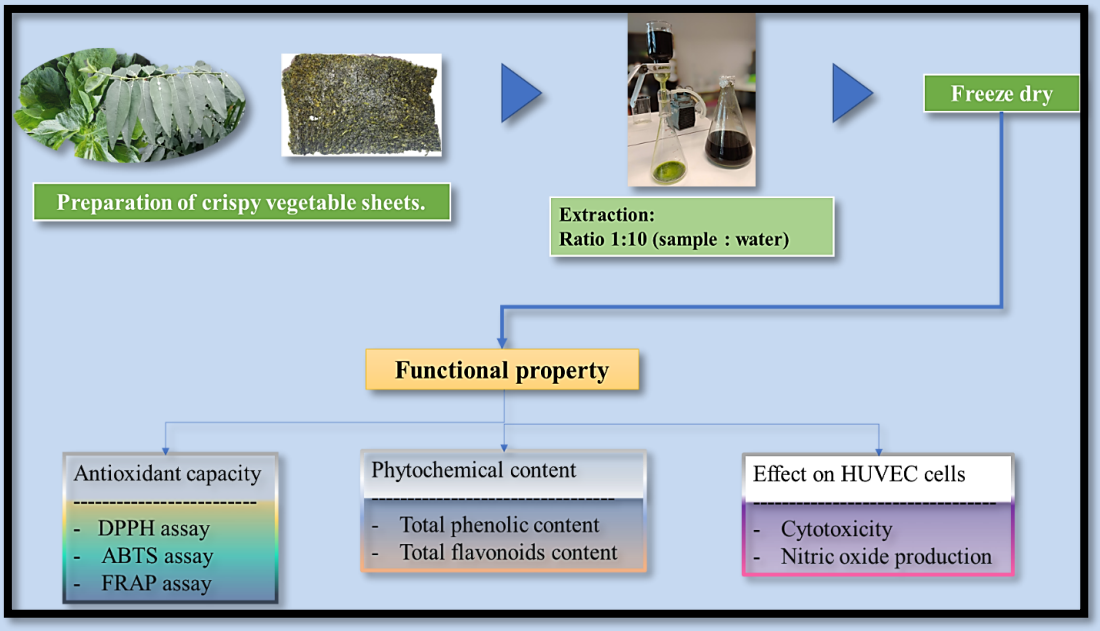
Methods: *A. viridis* and *S. androgynous* fresh vegetable leaves were dried, processed and formulated into crispy vegetables: *A. viridis* crispy vegetables, *S. androgynous* crispy vegetables and 50:50% (w/w) mixture of *A. viridis* and *S. androgynous* crispy vegetables. Total phenolic and flavonoids contents of the three crispy vegetables were evaluated using Folin Ciocalteu colorimetric and aluminum chloride assay.

Results: S. *androgynous* crispy vegetable had higher phenolic (36.55 ± 0.01 mg GAE/g) and flavonoid (81.17±0.00 mg QE/g) contents than *A. viridis* crispy vegetables (16.55±0.00 mg GAE/g and 40.58±0.01 QE/g respectively) and

50:50% mixed crispy vegetable formula of *A. viridis* and *S. androgynous* (26.01 ± 0.00 mg GAE/g; 59.38±0.00 QE/g respectively). *S. androgynous* crispy vegetable sheet also demonstrated a better antioxidant activity against the radical effect of DPPH (15.43±0.07%), ABTS (34.04±0.05%) and FRAP (1.73±0.01) than *A. viridis* (13.45±0.11%, 26.35±0.17 &; 1.56±0.01 respectively) and was comparable with mixed formula (14.23±0.43%; 32.31±0.12%, 1.81±0.03 respectively). A significant dose-dependent cytotoxic effect was induced by the three crispy vegetables with IC_{50} of 8.33±0.69, 7.84±0.35, 7.86±0.33 respectively on HUVECs. The results also showed that mixed crispy vegetables (50:50%) promoted endothelial nitric oxide production.

Conclusion: The study concludes that the antioxidant activity of the crispy vegetables reflects the total phenolic and total flavonoid content as their functional properties. Our findings also suggest that thermal processing of foods could have significant effect on the antioxidant activity of functional foods; however, *A. viridis* and *S. androgynous* prepared in crispy form may pose no potential threat to the body like the fresh vegetables.

Key Words: *Amaranthus viridis*, *S. androgynous*, crispy vegetables, antioxidant, nitric oxide, hypertension, Thailand



Graphical Abstract

INTRODUCTION

Oxidative stress has been implicated in the pathogenesis of several diseases [1,2] including hypertension. This phenomenon induces cell, tissue, protein, and DNA damage due to generation and accumulation of free radicals either in the form of reactive oxygen species (ROS) or reactive nitrogen species (RNO) in the living cell. These free radicals, also called oxidants, have unpaired or free electrons which upon binding with other molecules of the body gain stability and induce oxidative stress thus resulting into metabolic and clinical conditions such

as inflammation, diabetes mellitus, hypertension, etc. For instance, superoxide radical (O^{2-}) a ROS, can impede the availability of nitric oxide (NO) in the endothelium via oxidative stress to cause endothelial dysfunction and subsequently heart-related diseases like myocardial ischemia and hypertension.

NO is produced in the endothelium through reaction that involves the conversion of L-arginine to L-citrulline; catalyzed by the enzyme endothelial nitric oxide synthase (eNOs). The availability of NO in moderate amount in endothelial cells plays a protective role against the development of cardiovascular diseases (CVD) like hypertension by acting as vasodilator [3]. Hypertension is a major risk factor for the development of CVD as it accounts for 45% cardiac attack and 51% strokes in human [4].

Compounds capable of activating and enhancing eNOS expression are considered potential candidates for preventing hypertension and other related CVD [3]. For many decades, plants have been used as an alternative remedy particularly in areas devoid of health care facilities to treat many oxidative stress-related diseases like hypertension. It is believed that plants as natural resources contain metabolites e.g. polyphenols capable of counteracting the effect of oxidants or ROS by different mechanisms one of which is through their antioxidant properties. Based on this reason, there is increasing demand in the consumption of foods rich in antioxidants, especially fruits and vegetables. Polyphenols such as flavonoids and phenolics among others constitute the major antioxidant secondary metabolites in fruits and vegetables. They combat oxidative effects induced by oxidants on cells and DNA by acting as reductants where they donate electrons to the oxidants or free radicals and convert them to non-radical forms. Phenols and flavonoids are also responsible for the organoleptic characteristics of fruits and vegetables such as color, taste properties, and as well contribute to their nutritional qualities [5] .

Amaranthus viridis is a species of the genus *Amaranthus* commonly called Green Amaranth and belongs to the family *Amaranthaceae*. *A. viridis* and other several species of the same genus are largely consumed conventionally as edible vegetables around the globe [6,7]. Studies from literature on the phytoconstituents of this plant revealed flavonoids and phenolic compounds to be present in appreciable amount [8] and are responsible for the various therapeutic values attributed to the plant among which are antimicrobial, anti-inflammatory, anti-malarial, anti-diabetic, anti-carcinogenic and hepato-protective activities [7,9–13] .

Sauropus androgynous vegetation is highly distributed across Asia and Australia [14]. It is a shrub that grows under extreme temperatures and humid conditions. *S. androgynous* commonly called Multivitamin plants is consumed among natives of the South and South-east Asia including Thailand [15] as vegetables, curry, salads, and soup [16]. The plant has anti-microbial, analgesic, anticancer, anti-diabetic, antioxidant, anti-inflammatory, anti-malaria, and anti-anemic activities [15,17,18]. These activities have also been attributed to the presence of flavonoids, saponins, phenolic acids, and tannins present in the plant [15]. Several antioxidant studies have been carried out on different extracts of *A. viridis* and *S. androgynous* [10–13].

In Thailand, cereal produce is the staple food consumed by the natives. Most dishes served across the Thai restaurants are often garnished or spiced with some fruits and vegetables usually by frying them to make them crispy. *A. viridis* and *S. androgynous* are among the common vegetables used as recipes to make crispy dishes in South-east Thailand. Crispy foods are considered unhealthy because they are a potential risk for developing CVD due to their high saturated and trans-fat contents. Due to the aforementioned claim, this study is carried out to evaluate the antioxidant activity and nitric

oxide production effect of *A. viridis* and S. *androgynous* prepared as crispy vegetables by thermal processing with respect to their functional properties. This could be used as fundamental data to achieve the selection of formulations based on consumer sensory acceptance testing.

MATERIALS AND METHODS

Chemicals: Reagents used for the antioxidant capacity study such as quercetin, gallic acid, 1,1-diphenyl-2-picryl-hydrazyl,2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonicacid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as well as other chemicals used for the cytotoxicity assay were purchased from Sigma-Aldrich, Germany and Invitrogen™, USA. Human umbilical vein endothelial cells (HUVEC) and reagents used for determination of nitric oxide production were purchased from the American Type Culture Collection (ATTC®). All other reagents used for this study were purchased commercially and were prepared according to standard methods.

Collection of *A. viridis* and *S. androgynous* and preparation into crispy vegetable sheets: Fresh vegetables of *A. viridis* and *S. androgynous* were collected from Hat Yai, the South-eastern part of Thailand in 2022 at early morning hours (9.00-11.00) and were authenticated at Prince of Songkla University, Hat Yai, Thailand. The thermal process employed in preparing *A. viridis and S. androgynous* leaves into crispy vegetable sheets is summarized in Figure 1. Each harvested vegetable was washed under running tap water to remove dirt then brunched in boiling water for 3 min. Each sample of the blenched leaves was soaked in cold water, drained, and cut into 1 cm pieces and ground. Cornstarch 28% (w/v) was added as a binding material to the vegetable-powdered samples which were then spread on a Teflon sheet and baked in an oven into crispy vegetable sheets at 70 ºC until when dried. The crispy vegetable sheets were finely crushed and homogenized with distilled water (1:10) and then stirred for 45 min at room temperature (25°C). They were filtered through chess cloth and centrifuged at 6000 × *g* (Avanti® J-E, Beckman coulter, USA) and the clear supernatants were further filtered with Whiteman paper (No.1), freeze-dried, and stored until use.

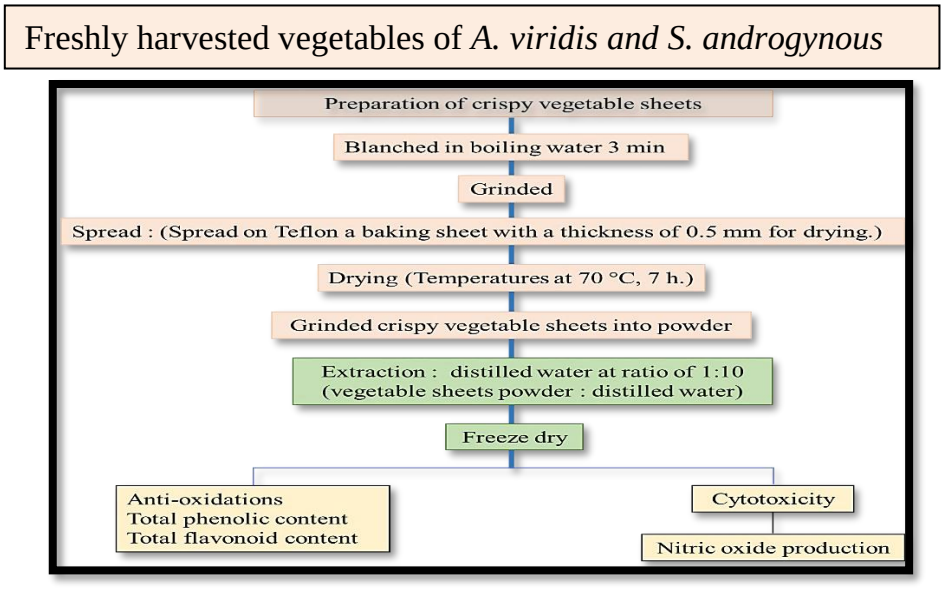


Figure 1: Flow diagram of preparation of *A. viridis and S. androgynous* crispy vegetables

Determination of total phenolic content: The total phenolic contents (TPC) for the three crispy vegetable sheets (*A. viridis* crispy sheet, *S. androgynous* crispy sheet and 50:50% mixed crispy formula of *A. viridis* and *S. androgynous* were determined by the Folin-Ciocalteu colorimetric method of Aksoy *et al.* [19] with slight modification. In brief, 1g each of lyophilized single and mixed crispy vegetables formula of *A. viridis and S. androgynous* was dissolved in 50 ml distilled water. 1 ml of each was mixed with 1 ml of diluted Folin-C and incubated for 4 min. Following the incubation, 0.8 ml of 7.5% (w/v), $NaCO_3$ was added and the mixture vortex for 10 s and further incubated for 1 h. Absorbance was read with UV spectrophotometer (VLBLOTD1, SINGAPORE) at 765 nm against a blank. Gallic acid of varying (0, 100, 200, 300, 400, 500 mg/l) concentrations was used to prepare the standard curve and results were expressed in milligrams of gallic acid equivalents per gram of sample dry weight (mg GAE/g DW). The process was performed in triplicate.

Total flavonoid content: The method described by Chang *et al.* [20] was employed. Briefly, 0.25 ml each of the crispy vegetables was dissolved with 1.25 ml of deionized water and 75 µl of 5% sodium nitrite ($NaNO_2$) in a test tube wrapped with aluminum foil. The mixture was allowed to stand for 6 min and thereafter 150 µl of 10% (w/v) $AlCl_3$ was introduced and further allowed to stand for 5 min before adding 0.5 ml of 1 M NaOH and 275 µl of deionized water. Paraffin was used to cover the tip of the test tube and vortex for 10 s followed by the absorbance reading at 510 nm against the blank. Quercitin of varying (0, 100, 200, 300, 400, 500 mg/l) concentrations was used to prepare the standard curve and the results expressed in milligrams of quercitin equivalents per gram of sample dry weight (mg QE/g DW). The process was repeated in triplicate.

***In vitro* antioxidant study**: The antioxidant activity of the three crispy vegetables sheet was studied for 1,1-diphenyl-2-picryhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP)

2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging activity assay: The ABTS radical scavenging assay was performed based on the method of Re *et al*.[21]. Briefly, 3 ml of ABTS solution was mixed with 300 μl of the crispy vegetable. The decrease in the absorbance was measured at the exact time of 1 min after mixing the solution until it reached 6 min. The procedure was repeated in triplicate. The final absorbance was recorded at 745 nm and the inhibition of ABTS radical was calculated by the expression:

% inhibition of ABTS = [Abs control –Abs sample / Abs control] x 100

DPPH scavenging assay: The method of [22] was used for the DPPH assay. Each crispy vegetable sheet at a concentration of 1 mg/ml was dissolved in 95% methanol. 20 µl of each dissolved crispy vegetable sheet was mixed with 180 µl of DPPH solution (0.1mM); shaken and incubated in the dark for 30 min. Absorbance was measured at 517 nm. This process was repeated thrice and the inhibition of DPPH radical by the crispy vegetable samples was calculated using the expression:

% inhibition of DPPH = [Abs control –Abs sample / Abs control] x 100

Ferric reducing antioxidant (FRAP) assay: The reducing power of each crispy vegetable for ferric was determined by FRAP assay of [23]. In brief, 1 ml solution of each crispy vegetables extract (1 mg/ml in methanol) was added to 2.5 ml of Na_2SO_4 (0.2 M; pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide ($K_3Fe(CN)6$) solution. The mixture was thoroughly shaken and incubated at 50°C for 20 min followed by the addition of 2.5 ml 10% (w/v) trichloroacetic acid and was centrifuged at 3000 rpm. Finally, 2.5 ml deionized water and 0.5 ml 0.1% (w/v) ferric chloride was added to 2.5 ml of the supernatant and mixed. The absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture is an indication of high reducing power.

Cell cultures: HUVECs were obtained from umbilical cord veins using type I collagenase (0.1 %) and digestion in endothelial cell basal medium (EBM-2) supplemented with endothelial cell growth medium EGM-2 at 37°C under 5% CO_2 atmosphere. HUVECs were identified through the cobblestone morphology of the typical endothelial cell and positive expression of von Willebrand factor and CD31 in immunocytochemistry. The culture medium was changed every other day until the cells reached confluence and HUVECs with passage between 4 and 6 were used for the study.

Nitric oxide production assay: The HUVECs were grouped into four groups: control (Negative control), treatment with 180 µM hydrogen peroxide (H_2O_2) to induce oxidative stress (positive control); treatment with 0.31 mg/ml 50:50% mixed crispy vegetables formula, treatment with 0.63 mg/ml 50:50 mixed crispy vegetables formula and 180 µM H_2O_2.

Procedure: In brief, 50 µl of the culture medium was diluted with 35 µl of assay buffer and mixed with 10 µl of nitrate reductase and 10 µl NADH. After incubating for 20 minutes to convert nitrate to nitrite, absorbance at 540 nm was taken to measure the total nitrite with Griess reagents. All treatments lasted for 24 hours. NO production was calculated by the expression:

Average Net OD = Average OD $_{sample}$ – Average OD $_{blank}$

OD= optical density absorbance at 540 nm

Cytotoxicity test on crispy vegetable sheets: Cytotoxicity activity (cell viability) of the crispy vegetable sheets made from *A. viridis*, and *S. androgynous* was carried out on HUVECs at different dilution concentrations (0.15, 0.31, 0.63, 1.25, 2.5 and 5 mg/ml) through evaluation of the IC_{50} (50% growth inhibition) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Procedure: HUVEC lines were grown in vascular cell basal medium. The cell was treated with each crispy vegetable sheets extract at different concentrations for 24 h in 96-well plate. 100 µl of MTT (10 mg/ml) was added to each well and then incubated at 37 ºC for 2 h. Then 100 µl DMSO was introduced to each well to make the formazan dissolve. This was followed by cell incubation at the same temperature for 10 min and absorbance was measured at 570 nm using micro plate spectrophotometer.

Statistical analysis: Data analysis of this study was taken in triplicate and expressed as mean with standard deviation (Mean ± SD). Statistical analysis between groups was analyzed using SPSS Version 24.

Values were considered significant ($p < 0.05$) at 95% level of confidence.

RESULTS

S. androgynous crispy vegetable sheet showed the highest TPC and TFC followed by 50:50% mixed crispy vegetables formula of *A. viridis* and *S. androgynous* while the crispy vegetable sheets of *A. viridis* recorded the lowest TPC and TFC. The phenolic content was 16.55±0.00; 36.55±0.01; 26.01±0.00 mg gallic acid equivalent/g of dry weight for *A. viridis*, *S. androgynous* and 50:50% mixed crispy vegetables respectively (Table 1). TFC of *A. viridis* was estimated to be 40.58±0.01 mg quercetin equivalent (QE)/g of dry sample weight; *S. androgynous* was 81.17±0.00 mg QE of dry sample weight and 50:50% mixed crispy vegetables formula was 59.38±0.00 mg/QE of dry sample weight as depicted in Table 1.

The antioxidant activity of the three crispy vegetables is presented in Table 2. The crispy vegetable of *S. androgynous* and 50:50% mixed formula of *S. androgynous* with *A. viridis* showed similar inhibition pattern of DPPH, ABTS radicals and FRAP as the values were not significantly ($p< 0.05$) different from each other. Crispy vegetables of *A. viridis* alone exhibited the lowest antioxidant activity against DPPH, ABTS and ferric radicals when compared to the 50:50% mixed crispy and *S. androgynous* crispy vegetable. The 50:50% mixed crispy vegetables formula expressed a better effect of NO production on HUVEC at the concentrations investigated as it promoted NO production in HUVECs more than double folds at 0.31 mg/ml when compared with the control group (Figure 2). In the oxidative stress-induced group with hydrogen peroxide, nitric oxide production was in double folds when compared with the control group. The highest NO production was observed in HUVECs treated with both 0.63 mg/ml of 50:50 crispy vegetables formula and H_2O_2; which was higher than the control in triple folds. Cytotoxicity activity of the three crispy vegetables on HUVEC is presented in Table 3. The three crispy vegetables showed a concentration-dependent cell viability effect on HUVEC i.e. the percentage of cell viability decreased when concentration of the crispy vegetables increased. The IC_{50} growth inhibition of 8.33±0.69 mg/ml 7.86±0.35 and 7.84±0.33 mg/ml was demonstrated by *A. viridis*, *S. androgynous* and 50:50% mixed formula respectively indicating that the mixed formula exhibited better cell viability effect since lower IC_{50} corresponds to better activity.

Table 1: Total Phenolic and flavonoid content of crispy vegetables of *A. viridis* and S. *androgynous*

Formula of crispy vegetables	Total Phenolic Content	Total Flavonoid Content
(mg GAE/g DW)	(mg QE/g DW	
A. viridis crispy	16.55±0.00[a]	40.58±0.01[a]
S. androgynous crispy	36.55±0.01[b]	81.17±0.00[b]
Mixed crispy of A. viridis and		
S. androgynous	26.01±0.00[c]	59.38±0.00[c]

DW= Dry weight; **GAE**= gallic acid equivalent; **QE** = quercetin

Values are mean of three replicates mean ± SD. Alphabet with different superscript are significantly different ($p<0.05$)

Table 2: Antioxidant activity of crispy vegetables of *A. viridis* and S. *androgynous*

Formula of crispy vegetables (%)	DPPH (%)	ABTS	FRAP
Amaranthus viridis	13.45±0.11[a]	26.35±0.17[a]	1.56±0.01[b]
Sauporus androgynous	15.43±0.07[b]	34.04±0.05[b]	1.73±0.01[a]
Mixed crispy of A. viridis and S. androgynous	14.23±0.43[b]	32.31±0.12[b]	1.81±0.03[a]

Values are mean of three replicates mean ± SD. Alphabet with different superscript down each antioxidant parameters are significantly different ($p<0.05$).

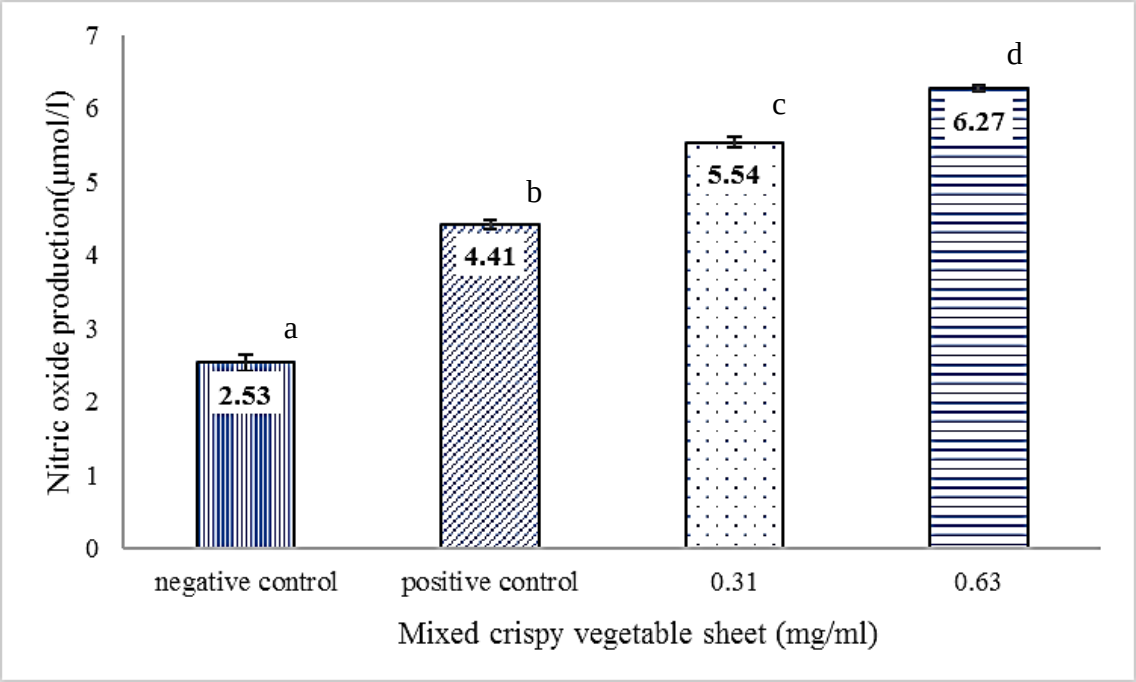


Figure 2: Rate of production of nitric oxide in HUVEC cells after treatment with mixed crispy vegetable sheet. Values are as mean values ± standard deviation (n =3)

Table 3: Cell viability (%) of crispy vegetable sheets of *A. viridis* and *S. androgynous*

Concentrations (mg/ml)	*A. viridis*	*S. androgynous*	50:50 mixed crispy vegetables (%) of cell viability
Negative control			100.00±0.05
Positive control			29.01±2.94
0.15	103.34±2.63	93.73±1.93	103.39±2.86
0.31	100.40±1.52	91.83±2.69	99.96±1.45
0.63	94.79±0.69	86.10±1.48	98.50±1.76
1.25	93.77±0.83	82.83±1.87	94.10±0.08

2.50	82.05±3.41	77.70±3.34	88.34±1.45
5.00	70.54±2.67	64.78±0.67	70.25±2.94
IC_{50}	8.33±0.69	7.86±0.35	7.84±0.33

DISCUSSION

The high TPC and TFC of the crispy vegetables of *A. viridis* and *S. androgynous* noticed in this study further substantiate the literature information on vegetables as good sources of flavonoids and phenols. We noticed that crispy vegetable sheet of *A. viridis* was lower in total phenol and flavonoid contents than in crispy vegetable of *S. androgynous* and in 50:50% combined formula. This could mean that *A. viridis* contained thermo labile volatile phenolic and flavonoids compounds which were probably lost during thermal processing of the vegetables into crispy sheets. Though these plants vegetables (*A. viridis and S. androgynous*) do not belong to the same species, however, the variation noticed in the quantity or yield of total phenol and total flavonoids may also be attributed to edaphic and climatic differences of the geographical location of the plants which affects the bioaccumulation of these polyphenols [24,25]. Some researchers have argued that edaphic and climatic factors affect the phenolic biosynthesis of plants and their antioxidant capacity [26]. Interestingly, the phenolic and flavonoid contents reported for the three crispy vegetable sheets in this study were higher than those for other twenty-one (21) commonly edible vegetables in Thailand reported by Tharasena and Lawan [27]. The *A. viridis* crispy TPC and TFC were also higher than previous report of Ahmed *et al*. [12] while the phenolic and flavonoids contents of *S. androgynous* vegetables sheet in this study were lower in quantity compared to the report of Chandra and Laveena [17].

In the current study, the antioxidant parameters investigated were DPPH, ABTS and FRAP. DPPH assay is a simple, fast; accepted and most widely used criteria for determining the antioxidant potential of an agent or plant [28,29]. The DPPH molecule is a stable free radical with an odd electron characterized by violet color which decolorizes upon contact with an antioxidant. The 50:50% mixed crispy vegetables formula demonstrated better antioxidant capacity against DPPH radical than the single *A. viridis* sample but in a similar manner with the single *S. androgynous* crispy vegetables. Nevertheless, the three vegetable samples under investigation were able to scavenge DPPH radical by donating hydrogen atom or an electron to it and converting it from a radical form (DPPH in violet colour) to DPPH-H (i.e.α; α-dihenylhydrazyl in a yellow color) a non-radical form (30). It has been reported that antioxidants either transfer electron or hydrogen atom to DPPH to neutralize its free radical character [31]. Ferric reducing power assay is used to measure the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form at low pH in the presence of an antioxidant [32]. The ABTS+ radical cation decolorization assay is another well-known technique for determining the antioxidant activity of an agent. In the assay of ABTS, ABTS+ chromophore which is blue/green in color is formed as a radical from the reaction of ABTS with potassium persulfate and its reduction is measured spectrophotometrically at 745 nm in the presence of an antioxidant

compound [30]. It is established that color reduction during the assay is an indication of ABTS radical inhibition [33] and such inhibition process may be facilitated by compounds like phenols [30]. Though the three crispy vegetables demonstrated antioxidant activity against ABTS but this activity was more evident in the mixed 50:50% crispy formula and *S. androgynous* crispy than in *A. viridis* crispy sample. Similar to DPPH, the 50:50% mixed crispy vegetables formula exhibited similar ferric reducing power with *S. androgynous* crispy vegetable but higher than the *A. viridis* vegetable sheet. FRAP assay for antioxidants is convenient, reproducible, and linearly concentration-dependent [34]. In the FRAP assay, the color changes from the yellow color test solution to either blue and/or green depending on the reduction capacity of the agent or compound under investigation [35]. This ferric reducing antioxidant power observed could be attributed to the presence of the flavonoids and phenols acting as reductants which reduced ferric ion (Fe^{3+}) complex to the ferrous (Fe^{2+}) by donating hydrogen atom to the ferric complex thereby breaking the radical chain of Fe^{3+}. We also noticed in our study that *A. viridis* and *S. androgynous* crispy demonstrated higher FRAP than earlier report of Tharasena and Lawan [27] on twenty-one commonly consumed edible vegetables in Thailand. Since the crispy vegetables sheet of *S. androgynous* among others recorded a higher TPC and TFC, it is not a coincidence to see that same crispy vegetable of *S. androgynous* demonstrated better antioxidant activity than the single crispy vegetable of *A. viridis* and mixed crispy vegetables formula of *A. viridis* with *S. androgynous* since the former contained higher amount of phenol and flavonoids than the latter. This pattern is in consonance with Barku *et al*. [36] who reported that the antioxidant activity of *Amaranthus spinosus* belonging to the same genus with *A. viridis* is directly proportional to its TPC content. Several studies have also documented the relationship between total flavonoids and phenol contents with the antioxidant activity of plants [19,37,38].

In spite of the low antioxidant activity of *A. viridis* crispy vegetable sheet, the 50:50% mixed crispy vegetables formula of *A. viridis* and *S. androgynous* demonstrated higher antioxidant activity similar to *S. androgynous* crispy sheets. This activity noticed could be due to the additive effects of the phenols and flavonoids in the mixture causing it to express this increased antioxidant property. It has been reported that the antioxidant activity of many edible fruits and vegetables increases with their polyphenol contents [39,40]. Furthermore, the antioxidant activity of polyphenols is influenced by where the hydroxyl groups are positioned and numbered in their aromatic structure because of their power to donate electron to free radicals and stabilize them [41,42]. The planar structure, number, and position of their hydroxyl groups, as well as the presence of the double bonds are important for metal chelation, free-radical scavenger capacities, inhibition of free radical producing enzymes and prevent oxidative stress disease [43–45]. Though in this study, the flavonoid and phenol constituents as well as the number of hydroxyl groups they contain were not determined, however, it is possible that the low antioxidant activity demonstrated by *A. viridis* crispy in inhibiting DPPH; ABTS and ferric ion radicals compared to the high antioxidant activity of *S. androgynous* crispy sheet and mixed crispy vegetables formula of *A. viridis and S. androgynous* in inhibiting these radicals is a reflection of the low number of hydroxyl groups likely to be present in the flavonoids constituents of *A. viridis* vegetable and vice-versa. According to Bouterfas *et al.* [46] polyphenols having a high

number of hydroxyl groups usually exhibit better antioxidant activity due to their power to donate several atoms to free radicals in order to stabilize them. This indicates the low antioxidant activity of *A. viridis* and the high antioxidant activity of *S. androgynous* crispy vegetable and mixed 50:50% formula. In addition, the antioxidant effect of flavonoids is a property of their low redox potential, which makes them thermo-dynamically able to reduce radical forms by transferring hydrogen from hydroxyl groups [47]. Phenolic compounds and flavonoids are believed to have therapeutic benefit against different diseases caused by oxidative stress [48,49] .

The concentration of hydrogen peroxide (H_2O_2) (180 µM) used to induced oxidative stress on HUVECs in this current study has been used in earlier studies of Drummond *et al*. [50] and Hafizah *et al*. [51]. The production of NO was higher in the hydrogen peroxide-treated group compared to the control group. This may be a response of self-protective mechanism of the HUVECs caused by H_2O_2 [52]. It is obvious that the 180 µM concentration of hydrogen peroxide employed in this study caused no lethality to HUVECs as the cells promoted NO production after the hydrogen peroxide challenge. However, H_2O_2 also induced oxidative damage of the synthesized NO, thus explaining why the production of nitric oxide in the hydrogen peroxide-treated group was not as high as seen in the mixed crispy group and in the combined mixed crispy with H_2O_2 groups (Figure 2). In our study, the responses to hydrogen peroxide are in consonance with previous study of [53]. The mixed crispy vegetables formula from this study was seen to increase NO production better than the control. This could also be an additive function of the phenols and flavonoids contents of *A. viridis* and *S. androgynous* in the mixed crispy vegetables formula which caused the activation and upregulation of eNOS in HUVEC, thereby increasing the NO output. Bioactive principle(s) in plant extracts, fruits and vegetables can act either in synergy or additive to promote therapeutic and biological activities of plants. Polyphenols like flavonoids have been reported to enhance the expression of eNOS and by inhibiting the action of NADPH oxidase thus increasing the production of NO [54]. Additionally, in pathological condition such as atherosclerotic lesion, oxidative stress generates superoxide radical, and superoxide can react with NO to produce peroxynitrite which reduces bioactivity of NO. Since the mixed crispy vegetable formula demonstrated antioxidant activity, it is noteworthy to say that another mechanism by which the formula increased NO production in HUVECs is by its antioxidant property. Antioxidants can enhance the synthesis of NO by protecting it against oxidative damage via ROS like H_2O_2 which the mixed crispy vegetable formula of *A. viridis* and *S. androgynous* exhibited in this study. Also, the three crispy vegetables caused over 80% cell viability *in vitro* in this study, thus the crispy vegetables may not pose any potential threat to the body nor increase the risk chances of CVD.

In general, our findings showed the crispy vegetables of *A. viridis* and *S. androgynous* are rich in TPC and TFC but exhibited weak antioxidant status against DPPH and ABTS+ radical compared to other 21 common edible vegetables in Thailand reported by Tharasena and Lawan [27] and earlier reports on fresh vegetables of *A. viridis* and *S. androgynous* [10,12,13,17,18,55]. It is possible that other chemical components that are present in the crispy vegetables are acting antagonistically with the polyphenols to induce this effect. It may also appear that the subjection of the vegetables to thermal heat during their processing into sheets played a significant role

in reducing the antioxidant power noticed. This finding is contrary to the submission of Tangkanakul *et al*. [56] on seven commonly consumed Thailand foods who reported that natural antioxidants retained their activity after thermal processing. Further studies are recommended to substantiate the effect of thermal processing on antioxidant capacity.

CONCLUSION

The study concludes that the antioxidant activity of the crispy vegetables reflects the total phenolic and total flavonoid content as their functional properties. Our findings also suggest that thermal processing of foods could have significant effect on the antioxidant activity of functional foods; however, crispy vegetables of *A. viridis* and *S. androgynous* may not pose potential threat to the body like the fresh vegetables.

List of abbreviations: DPPH- 1, 1-diphenyl-2-picryhydrazyl; ABTS- 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid; FRAP- Ferric reducing antioxidant power; HUVECs- Human umbilical vein endothelium cells; IC_{50}- 50% Inhibition concentration; eNOS- Endothelial nitric oxide synthase; ROS- Reactive oxygen species; RNS- Reactive nitrogen species; O^{2-} - Superoxide oxide; CVD- cardiovascular diseases; NO- Nitric oxide; H_2O_2- Hydrogen peroxide

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