Phenolic-dependent anti-lipid peroxidative, antimodulatory and antioxidant activity of virgin coconut oil *in vitro*

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Abstract

The phenolic profile of virgin coconut oil (VCO) was studied using HPLC and was found to contain 4.04×10^{-2} ppm gallic acid. Further test using Folin-Ciocalteu gave 3.658 mg/mL gallic acid equivalent (GAE). The same phenolic extract revealed a concentration-dependent activity against DPPH radical (IC_{50} = 14.9 mg/mL). Thiobarbituric acid reactive substances (TBARS) assay for the assessment of lipid peroxidation exhibited an IC_{50} of 4.32×10^{-1} mg/mL GAE. Also, it demonstrated an IC_{50} at 2.31×10^{-2} mg/mL GAE against nitric oxide (NO•). Stimulation of both hydroxyl (•OH) (SC_{50} = 1.29 mg/mL GAE) and H_2O_2 formation was observed. Cell viability test using alamarBlue® confirmed that 80%v/v VCO increased cell death of human hepatocarcinoma (Hep-G2) by 18.2% prior to treatment of H_2O_2. The data suggest that VCO may help inhibit general free radicals, lipid peroxidation and vasodilation-related diseases. Stimulation of H_2O_2 and •OH radicals are necessary in fighting pathogens and malignant cells.

Introduction

The market of virgin coconut oil (VCO) as functional food oil (Marina *et al.*, 2009) is growing fast not only in the Philippines, but also in other countries. Its butyric acid content has been associated as anticancer and lauric acid has been attributed as an antiviral agent. There was also a report showing its antibacterial activity against Staphylococcus aureus (Verallo-Rowel *et al.*, 2008). Its major phenolic consists of caffeic acid, p-coumaric acid, ferulic acid and catechin (Krishna *et al.*, 2010). Previous works showed that VCO scavenged 1, 1'-diphenyl-2-picrylhydrazyl (DPPH) radical and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonate)(ABTS) which are only general antioxidant tests (Hatano *et al.*, 1988; Sirisha *et al*., 2010) and are inadequate in proving its specific antioxidant activity. With these, it can be ascertained that VCO can provide health advantages that cannot be found in other oils.

In line with its free radical activity, many specific reactive oxygen species (ROS) are known to demonstrate different effects in the biological system – both beneficial and deleterious. Some of these are: hydroxyl radical (•OH), •OH forming hydrogen peroxide (H_2O_2), lipid peroxides and nitric oxide (NO•). Hydrogen peroxide, although not a free radical is a dangerous compound because it can easily cross the cell membrane and enter the nucleus leading to deformation of protein molecules. At worse, H_2O_2 may be converted to •OH in the presence of iron or any metal-ion promoter (Moreno *et al*., 2010). Hydroxyl radical on the other hand, is known as the most dangerous free radical that can attack a large variety of biomolecules and known for inducing DNA mutation.

Lipid peroxidation is the deterioration of lipids which contains carbon-carbon double bonds via oxidation. In this process, many toxic substances are formed as by-products (Devagasayam *et al*., 2003). These by-products exhibit its effect at a location other than its generation site. And because membrane lipids are specifically susceptible to lipid peroxidation, it is highly detrimental to the functioning of the cell. The end product of lipid peroxidation identified as malondialdehyde (MDA) can react with the DNA and cause mutations.

Nitric oxide is a highly reactive free-radical nascent molecule (Liu *et al*., 2009) which reacts with other free radicals like oxygen and heavy metals. Some of its function includes roles in the inflammation process (Marletta,1989), neurotransmission (Gillman, 1992), and vasodilation (Herman and Moncada, 2005; Moncada and Higgs, 2006). It is generated by the conversion of L-arginine of arginine ketoglutarate to L-citrulline mediated by nitric oxide synthase (NOS) (Knowles *et al*., 1989). Its vasodilatory function is attributable to NO binding to the ferrous

heme of soluble guanylate cyclase (sGC) which converts guanosine triphosphate (GTP) to its cyclic form (cGMP). An increase in the concentration of intracellular cGMP will affect the activity of protein kinase G (PKG) which results to vasodilation.

Though ROS are known to have negative effects in the biological system, our body's phagocytic cells and granulocytes uses a mixture of these oxidants – NO•, H_2O_2, •OH, and HOCl – in attacking pathogenic substances (Pollack and Leeuwenburgh, 1999). The phenolic content of VCO will be used in the antioxidant assays as inspired by the study of Nevin and Rajamohan (2004) that dwelled on DPPH assay and the review of Krishna and colleagues (2010) that showed the used of both DPPH and ABTS assay. One major point of this method using the phenolic extract is because of the oil constitution of VCO. This makes it very hard to react with assay systems that use water.

This study aims to critically assess the identity of the active compounds of VCO by HPLC and its behavior in various antioxidant tests – to observe changes in the amount of harmful free radicals in the biological system using DPPH, •OH and H_2O_2 assay, to detect its activity on lipid peroxides using TBARS assay, and to show its activity regarding nitric oxide using Griess method. These tests will provide adequate data for other application than its conventional uses.

Materials and Methods

Materials

All the solvents and reagents used in the study were given by the University of Santo Tomas – Research Center for the Natural and Applied Sciences (UST-RCNAS (Manila, Philippines). These chemicals were ordered from Belman Incorporada Laboratories (Quezon City, Philippines) and are all analytical grades. Standards used were acquired from Sigma-Aldrich (Singapore). Peripheral Blood Mononuclear Cells (PBMC) were extracted from the blood of healthy volunteer using Ficoll-density gradient centrifugation. The VCO used was accredited by the Philippine Coconut Authority (PASCIOLCO Agri-Ventures, Quezon, Philippines).

Extraction of phenolic from VCO

For the step of the extraction procedure of the phenolic compounds (Nevin and Rajamohan, 2004) 60% methanol in water was used to solvent extract the phenolic in VCO. This was done by adding 10 grams of VCO to 50 mL hexane. This mixture was mixed with 20 mL MeOH in a separatory funnel.

The funnel was swirled and the lower layer was collected. This was repeated for 5 times to ensure adequate extraction. After collecting the lower layer, it was subjected to reduced pressure evaporation at 40°C. Evaporation was halted after only 10-20 mL solvent remained. The solvent left in the evaporation process was subjected to freeze drying until none of the solvent is left. The resulting solid was weighed and diluted with 15 mL methanol and served as the stock solution.

Determination of constituents by HPLC

HPLC separation was done using Agilent 1200 Series with a reverse phase C18 column. A linear gradient elution was applied and elution was done using solvent A (1% acetic acid) and solvent B (methanol) as mobile phase. Throughout the analysis, the solvent gradient was programmed at 40%, 70%, 90% B to A within 30 minutes with a flow rate of 1.0 mL/min. The phenolic acid standards: gallic acid and ellagic acid, and flavonoid standards: quercetin and rutin was analyzed using the same conditions but different detector wavelengths (254 nm and 360 nm, respectively).

Folin-Ciocalteu's test for total phenolic content

The procedure was done by adding 50 µL of gallic acid standard/sample to 430 µL dH_2O then 20 µL of Folin-Ciocalteu's reagent was added. After mixing, 50 µL of saturated Na_2CO_3 was added then mixed. Lastly, 450 µL of dH_2O was added then incubated for 60 minutes. The reaction mixture was read at 765 nm. Gallic acid was used for the standard curve standard (Ye *et al.*, 2008).

Antioxidant assays

DPPH assay using VCO

The procedure used was similar to that previously described by Green (2004). DPPH assay was carried out by adding 150 µL of sample to 3 mL of 6×10^{-5} M DPPH solution in methanol then incubating it at room temperature in a dark location for 30 minutes. The reaction mixture was read at 517 nm. Gallic acid was used as positive standard.

Deoxyribose degradation assay for •OH radical scavenging

The procedure for deoxyribose degradation assay used was similar to that previously described in the study of Kunchandy and Rao (1990). It was carried out by mixing 200 µL of each of the following in order: 3 mM deoxyribose, 0.1 mM $FeCl_3$, 0.016% EDTA, 0.1 mM ascorbic acid and 0.019 M H_2O_2.

This mixture, totaling to 1 mL will be incubated at room temperature for 30 minutes. After incubation, 200 µL of the sample was added followed by addition of 500 µL of both 8% TCA and 0.67% TBA. The whole reaction mixture will be subjected to a 100°C water bath for 30 minutes and will be read at 430 nm. Gallic acid was used as positive standard for phenolic.

TBARS assay

The procedure used was similar to that previously described by Oyinbo *et al.* (2006), with slight modification. 600 µL of 0.67% TBA and 700 µL of 8% TCA were added to 150 µL chicken liver homogenate. After this, 150 µL of the sample was added. The reaction mixture was introduced to a 100°C water bath for 15 minutes. Gallic acid was used as positive standard. The pink chromogen was measured at 532 nm.

Nitric oxide assay

The procedure used was similar to that of previously described (Hazra *et al.*, 2008). In a tube, 10 mM sodium nitroprusside (SNP), PBS (pH 7.4), and various concentration of VCO phenolic extract was added, totaling to 3 mL. This mixture was incubated for 150 min at 25°C. After incubation, 1 mL of 0.33% sulfanilamide (in 20% acetic acid) was added to 0.5 mL of the reaction mixture. This was allowed to stand for 5 min. After 5 min, 1 mL of 0.1% w/v napthylethylenediamine dihydrochloride (NED) was added and again incubated for 30 minutes at the same temperature. Gallic acid was used as positive standard. The pink chromophore produced through the diazotization of nitrite ions with sulfanilamide and its consequent coupling with NED was measured at 540 nm.

H_2O_2 scavenging assay

Hydrogen peroxide scavenging activity

The procedure used was similar to that previously described (Sroka and Cisowski, 2003), with slightly modification. Hydrogen peroxide was prepared at 40 mM in phosphate buffer saline (pH 7.4). Various concentration of the phenolic extract was added to 2 mL H_2O_2 solution followed by 10 minutes incubation at room temperature. Absorbance of hydrogen peroxide was read at 230 nm against a blank solution.

H_2O_2 scavenging of VCO in cell culture (PBMC) using VCO

The procedure used was similar to that of previously described by Shi *et al.* (2009) with slight modification. PBMC was used as prescribed by Fabiani, R. colleagues (2009). It was extracted from a healthy volunteer using Ficoll-density gradient centrifugation. An 80 µM H_2O_2 was prepared in PBS (Jonas *et al.*, 1989). Cells with a 5×10^4 cells/well density were seeded to a 96-well plate and were incubated for 24 hours. After incubation, 100 µL of different concentrations of VCO 0-100% v/v with 10% increments prepared in RPMI was applied to each well. The plate was again incubated for 24 hours. After 24 hours, 10 µL of 80 µM H_2O_2 was added to each well and was incubated for 1 hour. After 1 hour, 10 µL of alamarBlue® was added to each wells and was incubated for another 4 hours. After 4 hours, the plates were read at 550 nm with a reference filter of 650 nm.

Results and Discussion

The extraction of phenols in VCO yielded 3.658 mg/mL gallic acid equivalent worth of Total Phenolic Content (TPC) based from the Folin-Ciocalteu's Test. The phenolic extract was subjected to different in vitro assays: TBARS, NO•, •OH, H_2O_2 (both with and without cells) scavenging, to critically assess its antioxidant behavior, anti-lipid peroxidation capacity and vasoconstrictive activity. Also, the cytotoxicity of VCO was observed in PBMC.

HPLC analysis of the phenolic extract of VCO showed the presence of gallic acid (4.04×10^{-2} ppm). Gallic acid has been confirmed to inhibit the growth of HeLa cervical cancer cells and human umbilical vein endothelial cells via apoptosis and/or necrosis (You *et al.*, 2010). Aside from this, peaks present for VCO chromatogram prescribed six (6) components that are still unidentified but may possibly be – epigallocatechin, syringic acid and p-coumaric acid – as reported by Krishna and colleagues (2010). The same way as for flavonoids, there were no peaks in the VCO chromatogram that match any of the flavonoid standards used.

This simple HPLC procedure provides reproducible peaks which suggest good separation and high resolution of constituents within a short analysis time. This method, using 1% acetic acid and methanol, can be used for the separation of other phenolics reportedly present in VCO.

DPPH Assay was done using VCO itself and was possible because the system is using methanol as a solvent which could possibly solubilize the phenolic that is present in VCO. Its mechanism lies when a hydrogen atom-donating antioxidant reacts with DPPH (Balasundram *et al.*, 2005). This assay is based on the colorimetric change that DPPH undergoes in the presence of an antioxidant. It can be quantified
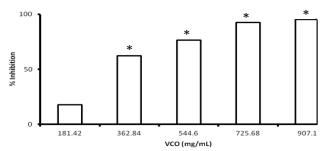
Figure 1. Concentration dependent DPPH scavenging of VCO phenolic extract. At this form, VCO showed weak scavenging of DPPH. Concentrations used were 181.42 mg/mL to 907.1 mg/mL. Analysis was done in triplicates. Differences were significant at $p < 0.05$.
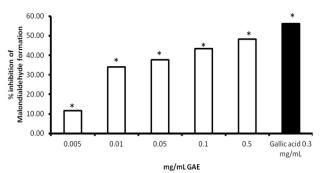

Figure 2. Concentration dependent inhibition of lipid peroxidation by VCO phenolic extract. Analysis was done in triplicates. Differences were significant at $p < 0.05$.

by measuring its absorbance, where in a decrease correlates with DPPH scavenging activity of the antioxidant. The computed half maximal inhibitory concentration (IC_{50}) is at 313.46 mg/mL. This data generally classifies VCO as weak antioxidant.

Thiobarbituric reactive substances (TBARS) assay was used to monitor the inhibition of lipid peroxidation of the phenolic extract of VCO. In this assay, malondialdehyde (MDA) forms an 1:2 adduct with thiobarbituric acid which then forms a pink chromogen measurable at 532 nm. VCO in its oil form was not used in this assay and the following assays because of its non-reactivity to the solvent system and its oil consistency affects the absorbance reading of the assay rendering it non-conclusive. The IC_{50} of the phenolic extract was 4.32×10^{-1} mg/mL gallic acid equivalent (GAE). This shows that the phenolic extract can greatly inhibit lipid peroxidation that can halt damage in the cellular membrane that can further leads to mutagenesis and carcinogenesis. The end product of peroxidation, MDA, may form chromosomal aberrations (Bird *et al.*, 1982) and also react to deoxyadenosine and deoxyguanosine in DNA forming DNA adducts, primarily pyrimido[1,2-a] purin-10(3H)-one (M1G) (Dedon *et al.*, 1998).

TBARS assay was performed with the methanolic extract of VCO. As shown in Figure 2, 0.5 mg/mL GAE was almost comparable to 0.3 mg/mL gallic

acid. Analysis was done in triplicates. Differences were significant at $p < 0.05$.

Nitric oxide scavenging assay was used to determine how much nitric oxide produced by Griess reaction can be scavenged by the Phenolic extract of VCO. In the Griess reaction, NO• is generated from the breakdown of SNP which when reacts to O_2, produces nitrite ions. Nitrite ions (NO^{2-}) form a pink solution measurable at 540 nm when reacted to sulfanilamide and NED. Data illustrated in Figure 3 shows that VCO exhibited an LC_{50} at 2.30×10^{-2} GAE against NO•.

Nitric oxide is a representative of reactive nitrogen species. It reacts with superoxide radical, forming peroxynitrite which is toxic to the cells. Intracellular peroxynitrite leads to cellular depletion of –SH groups, oxidation and nitration of lipids, and even mutation of DNA by breakage formation and deamination of DNA bases.

As from the mentioned role of nitric oxide in vasodilation, this inhibition clarifies a possible vasoconstrictive property of VCO which can be consequently tested *in vivo*. This vasoconstrictive effect, if proven, may be applied on management of diseases concerning vasodilation like erythermalgia and hypotension.

Hydroxyl radical scavenging assay was utilized to see how much •OH radical is inhibited by the VCO phenolic extract. This assay is based on the ability of an antioxidant to scavenge the highly-reactive •OH. This free radical can be generated via Fenton reaction where H_2O_2 reacted with the EDTA-bound Fe^{+2}. Unexpectedly, data from figure 4 depicted that there was a stimulatory effect to the •OH formation in the assay. The half maximal stimulatory concentration (SC_{50}) of the phenolic extract against •OH formation was 1.29 mg/mL GAE. Hydrogen peroxide scavenging assay was done to clarify the activity of the extract on •OH radicals. Anti-thyroid (Ross *et al.*, 1998) and bactericidal (Kohanski *et al.*, 2007) drugs are known to induce hydroxyl radical formation.

This stimulatory activity of the phenolic extract of VCO may be compared to ascorbic acid (Jansson *et al.*, 2003). Though it is a well known water-soluble antioxidant, it can induce the production •OH in high concentrations. This pro-oxidant activity may induce oxidative stress but may also help defend against pathogens. Its inhibition of other free radicals also suggests that it can act as an antioxidant.

Because in the presence of iron and EDTA, the decomposition of H_2O_2 to •OH is greatly favored, the results from •OH radical scavenging assay was clarified using H_2O_2 scavenging assay both *in vivo* and *in vitro*. The median stimulatory concentration
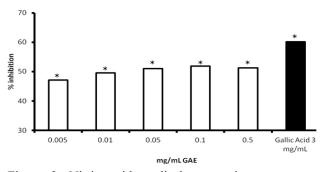
Figure 3. Nitric oxide radical scavenging assay was performed with the methanolic extract of VCO. Data showed that 2.31×10^{-2} GAE of the extract inhibited 50% of the NO• formed. Analysis was done in triplicates. Differences were significant ($p < 0.05$).
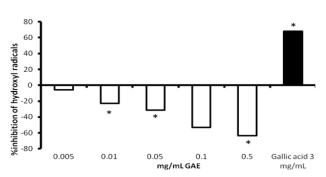

Figure 4. Hydroxyl radical scavenging assay exhibited a stimulatory effect on the generation of •OH radicals. Maximum stimulation at 63.29% while $SC_{50} = 1.29$ mg/mL GAE. Analysis was done in triplicates. Differences were significant ($p < 0.05$).
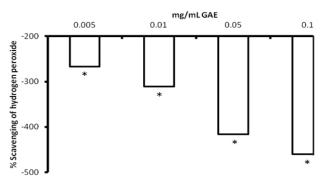

Figure 5. H_2O_2 scavenging assay exhibited maximum stimulation of H_2O_2 by 460% at 0.376 mg/mL GAE. SC_{50} was not computed because values were very high. Analysis was done in triplicates. Differences were significant ($p < 0.05$).

was concluded to be <0.005 mg/mL GAE. Using only 0.019 mg/mL GAE extract boosted the formation of H_2O_2 by 266.67% (Figure 5). Also, H_2O_2 induced toxicity was tested in PBMC cell culture. PBMC pre-treated with increasing concentrations of VCO was exposed with 80 μM H_2O_2 for 1 hour. The result showed that as the concentration of VCO increases, there is a significant increase in the percentage of cell death. This phenomenon may be attributed to the stimulatory activity of VCO on H_2O_2. Increasing VCO

concentration further increases the concentration of H_2O_2 which in turn causes apoptosis.

Although apoptosis inducing, VCO may be used as an antibacterial agent because of its H_2O_2 stimulation, it has been reported that H_2O_2, together with cationic proteins, lipopolysaccharides, myeloperoxidase and lactoferrin (Pollack and Leeuwenburgh, 1999) plays an important role in the body's resistance against bacterial infection (Clifford *et al.*, 1982). Hydroxyl radical, as its decomposition product, is also used by the body in fighting pathogens that are harmful once it causes infection (Pollack and Leeuwenburgh, 1999).

AlamarBlue® was used to measure cell viability with application of increasing concentrations of VCO (0%-80% at 20% increments) in hepatocarcinoma cells (HepG2). The results clearly show that 80% v/v VCO exhibited its maximum percentage of cell death at 20.88%. This collaborates with the stimulation of H_2O_2 and •OH. It also possible that since gallic acid is found to be present, though in minute amount, would inhibit malignant cells.

Conclusion

Virgin coconut oil is a scavenger of DPPH radicals, lipid peroxides and nitric oxides but not •OH radical and H_2O_2 in cell-free system and in cell culture. Its scavenging activity may be attributed to its phenolic, gallic acid as proven to be one, and flavonoid content observed in HPLC. Cytotoxicity test showed that 80% v/v VCO exhibited the maximum percentage of cell death at 20.88%. Other in vitro systems using serum, heart tissue or tissue culture of normal cell lines need to be assessed for the potential antioxidant capacity of VCO and validate its alleged health claims. Also, other assays may be used to assess specific damage to the cells like – Comet assay, TUNEL assay, and flow cytometric analysis. For the identification of the active compounds present in VCO, it is suggested to use other standards for both phenolic acids and flavonoids. Also, to further evaluate VCO's activity as an antioxidant, nitrative and oxidative assays using a different method like luminometry is suggested.

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