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Cytotoxic activity of the phenolic extract of virgin coconut oil on human hepatocarcinoma cells (HepG2)

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Abstract

Phenolic compounds are secondary metabolites widely distributed in plants. This study aims to extract the phenolics in Virgin Coconut Oil (VCO) and study its cytotoxic effect on human Hepatocellular Carcinoma (HepG2). Using Fast Blue BB, the crude phenolic extract of VCO yielded 25.54 ppm gallic acid equivalence per 0.5 mg/ml sample. The antioxidant capacity of the phenolic extract gave an IC_{50} > 0.5 mg/ml against DPPH radical. Likewise, the phenolic extract was cytotoxic at IC_{50} of 1,958.5µg/ml and 1,563.59 µg/ml for 48 and 72 h against HepG2 cells. Compared to that of an anti-cancer drug 5-fluorouracil, which registered an IC_{50} of 5.41 µg/ml and 2.17 µg/ml for 48 and 72 h and gallic acid standard which gave an IC_{50} of 137.2 µg/ml and 106.85 µg/ml for 48 and 72 h, the phenolic extract of VCO is an antioxidant and cytotoxic agent on HepG2 cells.

Introduction

Cancer is a chronic morbid disease that can develop in any individual due to certain factors such as heredity, poor diet, unhealthy lifestyle and continuous exposure to various carcinogenic elements. According to GLOBOCAN (2008), liver cancer is the 5th most common cancer in men and 7th in women, 85% of which mostly occur in the developing countries. It is involved in several other liver diseases such as hepatitis and cirrhosis and is regarded as the third leading cause of death in the Philippines. Being a serious and life threatening illness, efforts have been made in order to discover and develop safer and affordable ways of treatment and prevention for the said disease.

Virgin coconut oil (VCO) is fast becoming as a functional food oil of the Philippines (Marina *et al.*, 2009). Hence, the study was conducted to support, in part, this claim. VCO contains sufficient amount of phenolic compounds that may be useful in eliciting several biological functions including cellular protection against oxidative stress (Dia *et al*., 2004; Marina *et al*., 2009; Seneviratne and Dissanayake 2008). According to Marina *et al.* (2008), VCO contains caffeic acid, p-coumaric acid, ferulic acid, protocatechuic acid, vanillic acid and syringic acid.

As reported also in the study of Marina *et al.* (2009), fermented VCO has a strong scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH). Likewise, it can elevate the level of antioxidant enzymes and reduce lipid peroxide content both *in vivo* in rats and *in vitro*. Nevin and Rajamohan (2004) corroborated these findings and reported that polyphenols in VCO can indeed inhibit lipid peroxidation better *in vitro* compared to copra oil and ground nut oil.

As an antioxidant, VCO can protect the cells against the ravaging effects of free radicals. Being unstable they are reactive and can cause severe damage to normal cells leading to several diseases such as cancer. Scant data are available on the anticancer potential of VCO. With these in mind, the study aimed to unravel the cytotoxic activity of VCO against hepatocarcinoma cell lines.

Materials and Methods

Chemicals

Analytical grade methanol and hexane were purchased from BELMAN Laboratories, Philippines while 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Fast Blue BB reagent were obtained from Invitrogen. All tissue culture media including Fetal Bovine Serum (FBS) and other supplements, Trypan Blue Dye, and dimethysulphoxide (DMSO) were provided by Globetek Pro Science Foundation, Inc., Philippines.

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Extraction of phenolic compounds from VCO

The VCO sample was provided by the Philippine Coconut Authority, Quezon City. It was produced through wet process from fresh grated mature coconuts by PASCIOLCO AGRI-VENTURES, Quezon Province.

Extraction procedure was followed based on the method described by Nevin and Rajamohan (2004) with some modifications. Ten grams of VCO was mixed with 50 mL hexane and successively extracted three times using 20 mL portions of 60% methanol using a separatory funnel. Then, the lower layer which contains the polar fractions of the mixture was collected[7]. The sample collected was concentrated in a rotary evaporator (Eyela, Philippines) and was then lyophilized.

The actual percentage yield was computed using this equation:

$$\%\ Yield = \frac{Mass\ of\ extract}{Mass\ of\ VCO\ used}\ x\ 100\%$$

Determination of total phenolic content by fast blue BB method

The method discussed by Medina (2011) was adopted for this experiment. The Fast Blue BB assay was based on the method developed by Medina (2011) with some modifications. This assay was preferred over Folin-Ciocalteu Method to measure the total phenolic content in VCO because aside from being novel it is simple, more sensitive and rapid.

The phenolic extract was dissolved in 60% methanol. A 0.1µL aliquot of 0.1% Fast Blue BB reagent was added to the sample in each well of a 96-well plate and was mixed for 1 min. Then 0.1mL of 5% NaOH was added. The reaction was allowed to complete for 90 min at room temperature. A well containing only 60% methanol was used as the blank. The optical density was measured at 420 nm and the total phenolic was estimated from the gallic acid standard curve.

Evaluation of antioxidant activity of VCO using DPPH assay

DPPH assay was performed as described in the procedure of Sharma and Bhat (2009) with some modifications. The phenolic extract of VCO samples was dissolved in 60 % methanol to obtain the following concentrations: 10, 20, 50, 75, 100, 150, 200, 250, 500 µg/ml. Using a 96-well plate, the sample solutions (50 µl per well) were mixed with 100 µl of 50 µM DPPH dissolved in methanol, and then incubated for 30 min. The absorbance was measured at 515 nm with Corona Microplate Reader SH-1000 (Hitachi, Japan). Ascorbic acid was used as the positive control. The percent antioxidant activity was plotted against concentration (µg/ml). The assay was performed in triplicate.

Cell culture and preparation

Culture of hepatocarcinoma cells (HepG2)

Human hepatocellular carcinoma (HepG2) was provided by Globetek Pro Science Foundation, Inc., Philippines. The study was performed in the Tissue Culture Laboratory of the University of Santo Tomas Research Center for the Natural and Applied Sciences. HepG2 was cultured in a growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) in an incubator at 370C and 5% CO_2 until it became 80% confluent.

Cell count and treatment

The number of viable cells that will be used for the analysis was determined using Trypan Blue exclusion test as discussed by Strober (2001). This assay is based on the principle that only live cells possess intact cell membranes that exclude the dye. As viewed under the microscope, viable cells appear colorless whereas dead cells are colored blue.

The cells were trypsinized, transferred to a conical tube and centrifuged at 1000 rpm for 5 min. The supernatant was removed and 1 mL of serum free culture medium was added to the cell pellet. A 10 µL of the cell suspension was placed in an eppendorf tube, mixed to avoid lysis, after which 10 µL of 0.4% of trypan blue was added and mixed gently. Using a micropipet, 10 µL of the cell suspension was drawn out and placed on the edge of the chamber of the hemocytometer. A hand tally counter was used to count the number of cells in eight sets of the 16 squares area. The cell density was computed by using the formula:

$$Cell\ Density = (dilution\ factor\ x\ cell\ count)10000$$

The cell density was adjusted to 2 x 10^4 cells/mL and the cells were seeded in a 96-well microplate. The plates containing the cells were incubated for 24 hours at 37°C to stabilize the cells and allow cell attachment. After incubation, the culture media of the cells was replaced with different concentration of VCO extract and incubated at 48 h and 72 h. The serum-free culture medium and the Gallic acid-treated served as the negative and positive control media, respectively. Considered one of the effective chemotherapeutic drugs for liver cancer, 5-

Fluorouracil was used in the experiment as a standard chemotherapeutic drug (American Cancer Society, Inc., 2012).

Evaluation of the cytotoxicity of VCO against HepG2 cell by MTT assay

After the incubation time, 48 and 72 hrs, the culture media was pipetted out from the 96-well plate. The plates were washed with PBS. One hundred microliters of PBS was then added to each well and 10 µL of 5 mg/mL MTT was then mixed. The plates were incubated for 4 hrs at 37°C. The cells were viewed for its intracellular precipitate using an inverted microscope. When purple precipitates were clearly visible under the microscope, 75 uL of PBS was removed and 100 µL of dimethyl sulfoxide was added to. The plate was left in the dark at 25°C for 10 min and was measured colorimetrically at 540 nm (Invitrogen 2002).

Statistical analysis

Two-way ANOVA was used for the statistical treatment of data using SPSS Statistics 17.0. All values less than 0.05 were considered significant.

Results and Discussions

Extraction of the phenolic compounds from VCO

The solubility of phenolic compounds depends upon the polarity of the solvent used, as well as interaction of phenolics with other food constituent and formation of insoluble complexes. In relation to this, the most commonly used reagents for phenolic extraction are methanol, ethanol, acetone, water, ethyl acetate, propanol, dimethlyl formamide and other combinations (Marian Naczk, 2004). For the study, methanol was used for the extraction of phenolic compounds in VCO since it was reported as the best extraction solvent (Chirinos *et al*., 2007). The actual percentage yield of phenolics obtained was 0.22%.

Total phenolic content of VCO

Measurement of total phenolics using Fast Blue BB diazonium salt is based on the coupling of phenolic compounds with the salt to form azo-complex, aromatic diazonium ions normally couple with active substrate such as phenols (Smith and March, 2007). The diazonium group of Fast Blue BB salt contain, -+N=N+- in which the nitrogen is retained in coupling with the reactive group (-OH, hydroxyl group) of the phenolic group. The coupling of the Fast Blue BB occurs para- to the phenolic activating group followed by substitution in the ortho-position; these occur in a slightly alkaline solution where they are converted

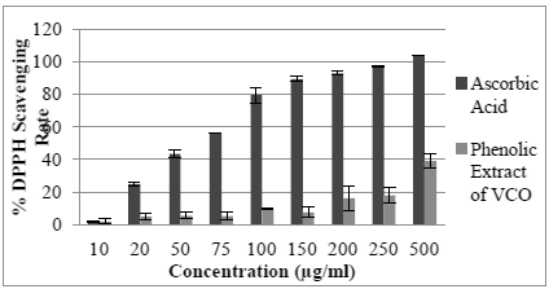

Figure 1. Concentration-dependent scavenging activity of ascorbic acid and the phenolic extract of VCO

to the more active phenoxide ions (Medina, 2011). The total phenolic content was estimated at 36.54 ppm Gallic Acid Equivalence per 0.5 mg/mL VCO sample.

DPPH assay

The antioxidant capacity of phenolic compounds depend on its structure (Smith and March, 2007), therefore it is important to know the concentration of the phenolic extract in VCO. Figure 1 shows a concentration-dependent relationship between concentration and antioxidant activity of the extract. The phenolic extract of VCO was able to scavenge 39.1% DPPH radicals at >500 µg/ml. Ascorbic acid, being a potent water-soluble chain-breaking antioxidant; a better scavenger compared to other standards i.e., propyl gallate and butylated hydroxytoluene (Sharma and Bhat, 2009), was used in the study to compare the antioxidant power of the phenolic extract from VCO. In related study by Santiago and Valerio (2013), they discussed that the phenolic extract from VCO can also inhibit nitric oxide (NO•) and production of lipid peroxides. These findings may support the observed antioxidant activity of the VCO extract used in the study.

Cytotoxic effect of phenolic extract on HepG2 cells

Firstly, the cytotoxicity of VCO extract was determined by MTT assay. The decrease in cell proliferation shows that the extract is toxic to cancer cells and may potentially modulate or possibly halt its growth. Viable and metabolically active cells can cleave the MTT dye to produce formazan crystals, and quantified colorimetrically (Stockert *et al*., 2012).

The standard chemotherapeutic drug 5-Fluorouracil gave an IC_{50} of 5.41 µg/ml at 48 h and 2.17 µg/ml at 72 h incubation period against HepG2 cells (Figure 2). This is expected since 5-Fluorouracil is known to interfere with the metabolism of nucleoside which can be incorporated into the RNA and DNA. Furthermore, 5-Fluorouracil inhibits deoxythymidine monophosphate (dTMP) production when converted instead to fluorodeoxyuridine monophosphate is likely

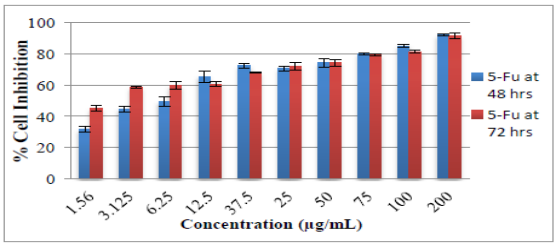

Figure 2. Time- and Concentration-dependent inhibition of HepG2 cells treated with 5- Fluorouracil

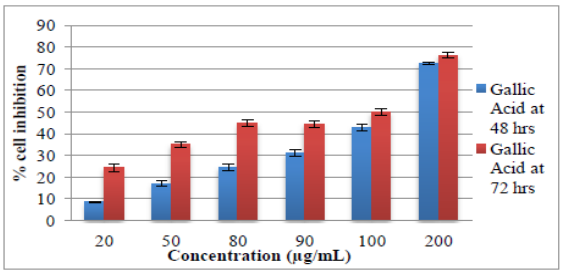

Figure 3. Time- and Concentration-dependent inhibition of HepG2 Cells Treated with Gallic Acid

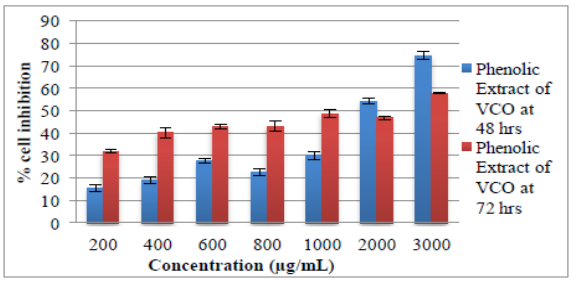

Figure 4. Time- and Concentration-dependent inhibition of HepG2 Treated with Phenolic Extract of VCO

to form a stable complex with thymidylatesynthase. Since dTMP is essential for DNA replication and repair, inhibition of its production may cause cell death (Zhang *et al*., 2008).

On one hand, gallic acid, exhibited an IC_{50} of 137.2 µg/ml and 106.85 µg/ml after 48 and 72 h exposure, respectively (Figure 3). As reported in the study conducted by Seneviratne and colleagues (2009), gallic acid was found to be a major component of VCO. Studies showed that gallic acid has cytotoxic activity via activating the reactive oxygen species (ROS)-mediated apoptotic pathway (Chen *et al*., 2009; Ho *et al*., 2010). On the contrary, the phenolic extract of VCO demonstrated an IC_{50} of 1,958.5 µg/ml and 1,563.59 µg/ml at 48 and 72 h, respectively compared to 5-Fluorouracil and gallic acid (Figure 4). This cytotoxic activity of the phenolic extract of VCO may be attributed to its stimulatory activity towards hydroxyl radicals (•OH) (Santiago and Valerio, 2013). The reactivity of •OH inside the body may cause DNA damage towards cancer cells that promotes cell death (Manian *et al.*, 2008).

Figure 4 shows the cytotoxicity activity of the phenolic extract of VCO on HepG2. As shown, there is an increase in percent cell death of HepG2 cells lines with increase in concentration of the phenolic extract and time of incubation. The phenolic extract was cytotoxic to HepG2 cancer cells implying that VCO may be used to prevent liver cancer. Furthermore, since the phenolic extract was able to scavenge 39.1% DPPH radicals at concentrations >0.5 mg/ml, VCO may be co-administered as an antioxidant together with other chemopreventive drugs such as 5- Fluorouracil to maximize its health benefits.

DNA damage due to ROS is widely accepted as one of the major processes involved in cancer initiation. Unrepaired DNA causes several mutations which ultimately lead to cancer (Waris and Ahsan, 2006). Prevention of cancer at an early stage decreases the possibility of complications. Antioxidants can protect normal cells and minimize further tissue damage brought about by toxic free radicals (National Cancer Institute). Vitamin C is a known water soluble, chain-breaking antioxidant, which was used as the positive control in thr study. On the other side, at a higher concentration, Vitamin C may acts as pro-oxidant that can activate the production of hydrogen peroxide (SOTT.net Signs of Time). This plausible mechanism is vital in killing tumor cells thereby preventing further proliferation. Though the VCO extract has a lower antioxidant activity compared to that of Vitamin C, it was able to scavenge 39.1% DPPH free radicals. This suggests that VCO has antioxidant capacity to scavenge antioxidants.

Statistical analysis

At $\alpha = 0.05$, the cytotoxic activity of the crude PE of VCO against HepG2 was comparable to that of the standard gallic acid at 137.2 µg/ml and 106.85 µg/ml after 48 and 72 h exposure. However, there was a significant difference between HepG2 treated with 5-FU and the crude PE of VCO (p value < 0.001) suggesting that the crude PE of VCO was less potent than 5-FU against HepG2 cell proliferation. In addition to that, the length of incubation time with the crude PE of VCO has a significant effect on the HepG2 cell growth, with 72 hours as the most optimum ($p = 0.001$).

Conclusion

The phenolic extract of Virgin Coconut Oil is a scavenger of free radical that may prevent human hepatocarcinoma cells proliferation. It may be concluded that the phenolic content of VCO is an antioxidant and a cytotoxic agent against HepG2 cells.

Acknowledgment

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