Total Phenolic Content, Antioxidant and Cytotoxic Activity of Cocoa (*Theobroma cacao* L.) Polyphenols Extracts on Cancer Cell Lines

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ABSTRACT

Introduction: Cancer chemopreventive agents from natural sources have been actively investigated over the years to seek prevention against cancer. In this study, cocoa polyphenols extract (CPE) was examined to explore its antioxidant and cytotoxicity activities. **Methods:** CPE was analysed for total phenolic content (TPC) and antioxidant activity (DPPH radical scavenging activity and FRAP ferric-reducing antioxidant power assays). *In vitro* cytotoxicity effect of CPE against HepG2, HT-29, HeLa, MCF-7, MDA-MB-231 and WRL-68 cell lines after 48 h exposure was measured by MTT assay. **Results:** The study showed that CPE had higher total phenolic content (13560.0±420.1 mg GAE/100g dry weight of sample) than vitamin E ($p<0.05$). CPE exhibited strong antioxidant activity comparable with ascorbic acid in both DPPH (IC_{50} = 14.73±1.47 μg/ml) and FRAP (2130.33±2.33 μM of FE/1 mg of dry weight of sample). The cytotoxicity study showed that CPE exhibited the highest cytotoxicity effect against MCF-7 with lowest IC_{50} value (3.00±0.29 mg/ml) compared to other cancer cell lines after 48 h treatment (p<0.05). **Conclusion:** Our results indicate that CPE demonstrated high total phenolic content, free radical scavenging activity, ferric reducing ability and cytotoxicity activity towards HepG2, HT-29, HeLa, A549, MDA-MB-231 and MCF-7 cancer cell lines. Further isolation of bioactive constituents from CPE should be done to characterise its potential chemopreventive activity as well as to elucidate the mechanism of cancer cell death induced by CPE.

Keywords: Cocoa polyphenols extract, DPPH, FRAP, MTT assay,TPC

INTRODUCTION

An antioxidant can be described as any substance at a low concentration which has the capability to decrease and delay the development of rancidity or other unpleasant odours or taste due to oxidation (Halliwell *et al.*, 1995) and able to defend the individual body against free radicals which may possibly cause pathological conditions such as Parkinson's disease, anaemia, ischemia, asthma, and the ageing process (Oke & Hamburger, 2002). Cancer is a major public health problem in Malaysia. About

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18,219 new cases were diagnosed in 2007 and registered at the National Cancer Registry (NCR). According to the 2007 NCR statistics, the cancer incidence rate for males and females were 8,123 (44.6%) and 10,096 (55.4%) respectively (Zainal Ariffin & Nor Saleha, 2011).

Cocoa tree or scientifically known as *Theobroma cacao* L., belongs to the family of *Sterculiaceae* which originated from the area of central, southern and southeastern Mexico (Rusconi & Conti, 2010). Catechin, epi-catechin, flavanol glycosides and antho-cyanins procyanidins are among the polyphenols identified in cocoa beans and cocoa products (Rimbach *et al.*, 2009). Several studies report that cocoa phenolics contain bioactive compounds that possess antioxidant, anticarcinogenic, and anti-radical properties (Ren *et al.*, 2003; Sanbongi *et al.*, 1998; Wollgast & Anklam, 2000). Another study has shown that the level of 8-hydroxy-2'-deoxyguanosine, a biological marker of oxidative DNA damage in rat testes decreased after consumption of cocoa polyphenols, suggesting a potential function of cocoa in cancer (Orozco, Wang & Keen, 2003).

Surgery, hormontherapy, chemotherapy and other complementary therapies are the conventional strategies for cancer treatment. However, these treatments cannot absolutely prevent intermittence and metastasis of the tumour. Polyphenol compounds from fruits and vegetables have gained much attention over the years because of the antioxidant and free radicals associated with compounds that indirectly reflect potential effects on human health. Although several studies have reported on the use of cocoa polyphenols against cancer cell lines such as prostate cancer cells (22Rv1 and DU145) and colon cancer cells (Caco-2) (Jourdain *et al.*, 2006; Carnésecchi *et al.*, 2002), studies pertaining to CPE against other cancer cell lines are still scarce. Moreover, a new, safe and effective chemopreventive agent discovered from nutritional foods that could

potentially delay and inhibit the development of cancer warrants further investigation. Therefore, this study aims to evaluate total phenolic content, free radical scavenging activity, ferric reducing antioxidant power of CPE and also determine the cytotoxicity activity of CPE against normal and cancer cell lines.

METHODS

Materials

Gallic acid, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, trolox, 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), ascorbic acid, thiazolyl blue tetrazolium bromide, phosphate buffer saline (PBS) tablet and trypan blue were purchased from Sigma. Butylated hydroxyl toluene (BHT) was purchased from Supelco Analytical. Ferrous sulphateheptahydrate ($FeSO_4.7H_2O$) and glacial acetic acid were purchased from Merck. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin andtrypsin-EDTA were purchased from PAA Laboratories GmbH. Sodium carbonate (Na_2CO_3) and methanol (CH_4O) were purchased from Systerm. Ferric (III) chloride-6-hydrate($FeCl_3.6H_2O$)and absolute ethanol (C_2H_6O)were purchased from HmbG chemicals. Dimethyl sulfoxide (DMSO) was purchased from Fischer Scientific. Hydrochloric acid (HCl) was purchased from R&M chemicals. Vitamin E (Sime Darby) was kindly given by Dr Huzwah Khazaai' while CPE was kindly given by Cocoa Board Malaysia.

Total phenolic content

The amount of total phenolic content (TPC) was evaluated according to the method described by Velioglu *et al.* (1998) with a slight modification. The sample was prepared at a concentration of 1 mg/ml. About 100 μl of sample and 0.75 ml of Folin-Ciocalteu reagent (previously diluted 10-fold

with distilled water) were added into a test tube and mixed. The mixture was left at room temperature (25ºC) for 5 min. Then, 0.75 ml of 6% (w/v) sodium carbonate was added to the mixture and mixed gently. After 90 min incubation at room temperature (25ºC), the absorbance was read at 725 nm using a spectrophotometer (T60 UV–VIS Visible spectrophotometer, USA). The standard calibration (0.01-0.05 mg/ml) curve was plotted using gallic acid. Vitamin E, ascorbic acid, BHT and trolox were used as reference. TPC was expressed as gallic acid equivalents (GAE) in milligram per 100 g dry weight of sample.

DPPH radical scavenging activity

DPPH assay was conducted according to the method described by Blois (1958) with modification using 96 well plates. Firstly, 50 μl of 15.26 - 1000 μg/ml of sample was added to each well.Then, 195 μl of 0.1mM DPPH solution was added to each well and the plate was incubated for 1 h. After 1 h, the absorbance was read at 540 nm using microplate ELISA reader (BioTek® EL808). Only 50 μl of methanol was used as a blank. Vitamin E, ascorbic acid, BHT and trolox were used as reference. The assay was performed in triplicate. The percentage of free radical scavenging activity was calculated using the following formula:

Percentage of inhibition free radical (%)
= Absblank –Abssample x 100%
       Absblank

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was determined according to the method described by Benzie & Strain (1996) with some modifications. FRAP reagent was prepared by mixing acetate buffer (300 mM, pH=3.6), 2,4,6-Tripyridyl-s-Triazine (in 40 mMHCl) and ferric chloridehexahydrate (20 mM) in a ratio of 10:1:1 respectively. Subsequently, the reagent was kept at 37°C prior to use. About 20 μl of final concentration of CPE(1 mg/ml) was added into 96-well plates, followed by adding 180μl of FRAP reagent to each well. Then, the plate was immediately shaken in a microplate ELISA reader (BioTek® EL808) for 30 sec and then incubated for 10 min at 37ºC prior to reading on a microplate ELISA reader at 595 nm. Ferrous sulphate($FeSO_4$) at concentrations ranging from 0 to 250μM in distilled water was used as a standard to generate the calibration curve by linear regression. Vitamin E, ascorbic acid, BHT and trolox were used as reference. TPC was expressed as ferric equivalents (FE) in μM per 1 mg dry weight of sample.

Cell culture

Liver cancer (HepG2), colon cancer (HT-29), cervical cancer (HeLa), hormone dependent breast cancer (MCF-7), non-hormone-dependent breast cancer (MDA-MB-231) and normal human liver (WRL-68)cell lines were purchased from American Type Culture Collection (ATCC). Cell lines were maintained in suitable media (HepG2, HeLa, MCF-7 and MDA-MB-231 in RPMI 1640; HT-29 and WRL-68 in DMEM) supplemented with 10% FBS and 1% antibiotics (100 IU/ml penicillin and 100μg/ml streptomycin). Cells were grown in 25 cm^2 and 75 cm^2 tissue culture flasks in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

Treatment with CPE

Working solutions of CPE were prepared in ten-fold serial dilution with the appropriate cell culture medium. Stock solution of CPE (10 mg/ml) in culture medium was freshly prepared and filtered using 0.2 μm filters. Working concentrations of CPE(0.001, 0.01, 0.1, 1 and 10 mg/ml) were prepared. An aliquot of 200 μl of each working concentration was added into each designated well. Control wells were added with 200 μl of culture medium while the blank wells (without cells) were only added with 200 μl of respective working

concentrations. Plates were incubated at 37°C and 5% CO_2 for 48 h. The experiment was performed in triplicate.

MTT assay

After 48 h, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for 3 h. Formazan crystals formed by mitochondrial reduction of MTT were solubilised in DMSO (100 μl/well) and the absorbance was read at 570 nm after 10 min incubation using the microplate ELISA reader (BioTek® EL808). Percentage of cell viability was calculated according to the following formula:

% cell viability:

$$\frac{Abs\,treatment - Abs\,blank\,treatment}{Abs\,control - Abs\,blank\,control} \times 100\%$$

Inhibition of cell viability was expressed as IC_{50}.

Statistical analysis

Each experiment was performed in triplicate. Data was presented as mean ± S.E.M. and analysed using one way analysis of variance (ANOVA) and multiple comparison by Duncan's test in which $p < 0.05$ was deemed as statistically significant.

RESULTS

Total phenolic content

TPC of antioxidant standard and CPE were determined as shown in Table 1. In this study, the linear regression of gallic acid was represented by the equation y= 7.453x + 0.013, r^2 = 0.991. Analysis of TPC indicated that ascorbic acid exhibited the highest TPC (13790.92±513.13 mg GAE/100g dry weight of sample) followed by CPE (13558.99± 420.10 mg GAE/100g dry weight of sample), trolox (13365.99±363.78 mg GAE/100g dry weight of sample), BHT (12430.72±437.24 mg GAE/100g dry weight of sample) while vitamin E demonstrated the lowest TPC (8328.38±187.98 mg GAE/100g dry weight of sample). The present study demonstrated that there is significant difference ($p < 0.05$) in TPC of CPE in comparison with vitamin E. Nevertheless, there is no significant difference ($p > 0.05$) in TPC of CPE with trolox, ascorbic acid and BHT.

DPPH scavenging activity

Based on Figure 1, the scavenging activity increased with increasing sample concentration. Trolox exhibited the highest

Table 1. TPC, IC_{50} and ferric reducing antioxidant power value of CPE. Vitamin E, ascorbic acid, BHT and trolox were used as positive control.

Sample	TPC (mg GAE/100g dry sample)	DPPH IC_{50} (μg/ml)	FRAP FE (μg/ml)
Vitamin E	8328.38±187.98[a]	44.73±4.73[f]	2085.67±21.40[i]
Ascorbic acid	13790.92±513.13[c]	10.00±1.44[d]	2283.70±9.07[g]
BHT	12430.72±437.24[b]	25.57±1.95[e]	1464.30±67.3[h]
Trolox	13365.99±363.78[bc]	7.78±0.74[d]	2258.30±38.5[g]
CPE	13558.99±420.10[bc]	14.73±1.47[d]	2130.33±2.33[g]

TPC was expressed as mg GAE/100 g of dry weight of sample.
Values are represented as mean ± S.E.M (n = 3); those with different superscripts are significantly different at $p < 0.05$, analysed by multiple comparison (Duncan test), ANOVA to compare the values between samples.
CPE= Cocoa polyphenols extract; TPC = Total phenolic content; BHT = Butylated hydroxyl toluene; GAE = Gallic acid equivalent.
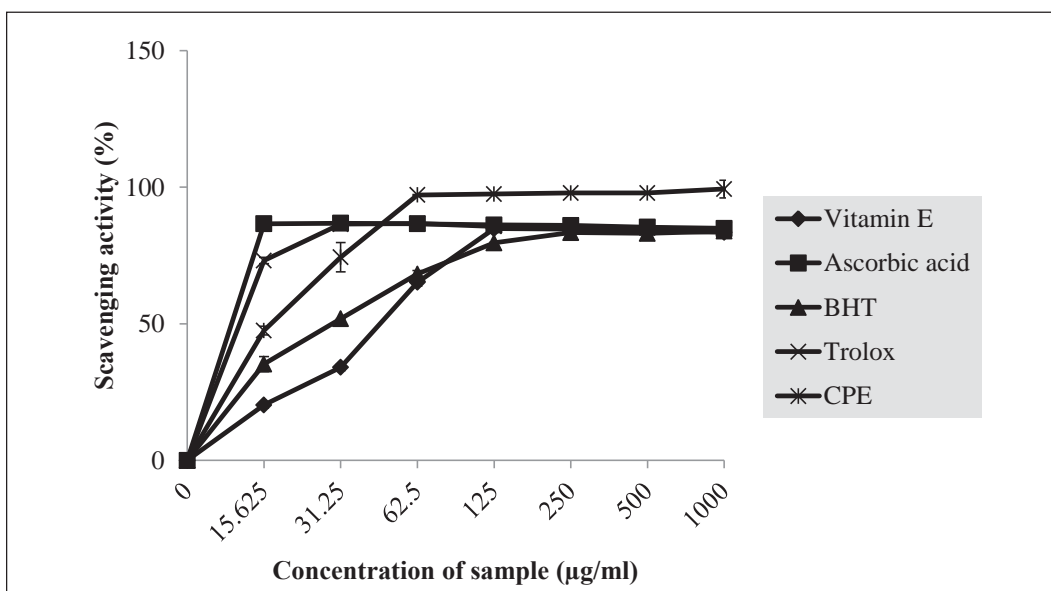
Figure 1. Percentage scavenging activity of CPE from concentration of 0 to 1000 µg/ml in comparison with vitamin E, ascorbic acid, BHT and trolox.

scavenging activity with 50% inhibition at concentration of 7.78±0.74 μg/ml followed by ascorbic acid (10.00±1.44 μg/ml) > CPE (14.73±1.47 μg/ml) > BHT (25.57±1.95 μg/ml) > vitamin E (44.73±4.73 μg/ml). In this study, the lower IC_{50} value indicated the intense ability of the sample to act as a DPPH scavenger while the higher IC_{50} value indicated lower scavenging activity with more scavengers being required to achieve 50% scavenging action. Based on the IC_{50} value in Table 1, CPE demonstrated no significant difference(p>0.05) with trolox and ascorbic acid. However, CPE showed significant difference (p<0.05) in comparison with BHT and vitamin E for DPPH assay.

Ferric reducing antioxidant power (FRAP) assay

The linear regression of $FeSO_4$is represented by the equation y= 0.001x + 0.169, r^2= 0.931. The study found the reducing ability of standard and CPE to be in the range of 1464.30–2283.70μM of FE/1 mg of dry weight of sample as shown in Table 1.

Antioxidant potentials in vitamin E, ascorbic acid, BHT, trolox and CPE were estimated from their ability to reduce 2,4,6-tripyridyl-*s*-triazine (TPTZ)-Fe (III) complex to TPTZ-Fe (II). Ferric reducing ability exhibited by the sample in increasing order: ascorbic acid (2283.70±9.07 μM of FE/1 mg of dry weight of sample) >trolox (2258.33±38.50 μM of FE/1 mg of dry weight of sample μM) >CPE (2130.33±2.33 μM of FE/1 mg of dry weight of sample) >vitamin E (2085.67±21.40μM of FE/1 mg of dry weight of sample) > BHT (1464.30±67.30 μM of FE/1 mg of dry weight of sample). The FRAP values for the CPE showed a significant difference (p<0.05) compared to vitamin E and BHT. However, therewas no significant difference (p>0.05) in FRAP value of CPE in comparison to FRAP value of trolox and ascorbic acid.

Cytotoxicity assay

The study showed CPE induced cytotoxicity against HepG2, HT-29, HeLa, MCF-7 and MDA-MB-231 (Figure 2). After 48 h incubation, CPE induced cytotoxic effect in
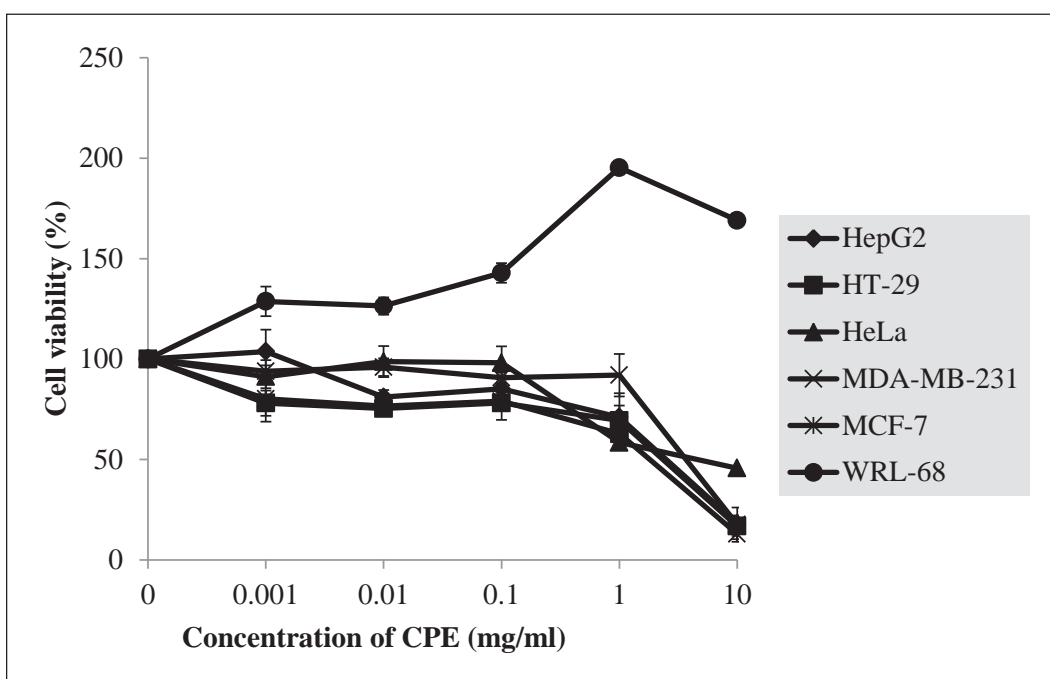
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Figure 2.Percentage of cell viability of HepG2, HT- 29, HeLa, MDA-MB-231, MCF-7 and WRL-68 cell lines against concentration of CPE from 0.001-10 mg/ml after 48 h treatment. The percent viable cell was calculated in comparison to untreated cells taken as 100%. Values are expressed as mean±S.E.M.

Table 2. IC_{50} value (mg/ml) of CPE in HepG2, HT-29, HeLa, MDA-MB-231, MCF-7 and WRL-68 cell lines.

Cell lines	IC_{50} value (mg/ml)
HepG2	4.50±0.29[a]
HT-29	5.00±0.00[a]
HeLa	5.50±0.29[a]
MDA-MB-231	5.33±0.67[a]
MCF-7	3.00±0.29[b]
WRL-68	-

Values are represented as mean ± S.E.M (n = 3); those with different superscripts are significantly different at $p<0.05$, analysed by multiple comparison (Duncan test), ANOVA to compare the values between samples. There is no IC_{50} value for WRL-68.

HepG2, HT-29, HeLa, MDA-MB-231 and MCF-7 with IC_{50} values of 4.50±0.29 mg/ml, 5.00±0.00mg/ml, 5.50±0.29 mg/ml, 5.33±0.67 mg/ml and 3.00±0.29 mg/ml respectively (Table 2). As observed from MTT assay, a high concentration of CPE was required to induce 50% cytotoxicity to HepG2, HT-29, HeLa and MDA-MB-231 for the cytotoxicity assay. CPE showed potent cytotoxicity effect against MCF-7 with the lowest IC_{50} value (3.00±0.29 mg/ml) compared to other cell lines. Our study

showed a significantly different *(p<0.05)* IC_{50} value of MCF-7 with IC_{50} value of HepG2, HT-29, HeLa and MDA-MB-231for cytotoxicity assay. Based on the results, no IC_{50} value of CPE against WRL-68 was obtained. The graph showed that CPE promotes proliferation activity of WRL-68 with increasing concentration of CPE. However, CPE induced WRL-68 cells death when the concentration of CPE was 10 mg/ml.

DISCUSSION

Polyphenols are antioxidant bioactive compounds that exhibit protective activity against critical diseases such as coronary heart diseases, cancer and neuro-degenerative diseases through their antioxidant and free radical scavenging capacities (Bravo & Saura-Calixto1998; Wan *et al.*, 2001). Research done by Martin *et al.*(2008) show that TPC of CPE was 2 g GAE/100g on a dry matter basis. However, our study showed that TPC of CPE was 13560.0 ±420.1 mg GAE/100g extract. A previous study used a mixture of methanol, acetone and water to extract polyphenol compounds from cocoa while in our study, CPE was extracted using a mixture of ethanol and water.That study documented that TPC of cocoa beans from Malaysia (71.42-82.68 mg GAE/g was higher compared to TPC of cocoa beans from Venezuala (64.3 mg GAE/g), Peru (50.0 mg GAE/g) and Dominican Republic (40.0 mg GAE/g). Nevertheless, the TPC of cocoa beans from Malaysia was lower compared to TPC of cocoa beans from Ecuador (84.2 mg GAE/g) (Tomas-Barberan *et al.*, 2007). According to Huda-Faujan *et al.* (2007), various phenolic compounds contribute to different outcomes when a TPC assayis conducted. Therefore, different levels of TPC in a compound may be due to the different geographical origins of plant, different extracting solvents, and procedures used to express the TPC by different investi-

gators.This study showed that CPE contained large amounts of phenolic compounds with the potential to act as a natural source of phenolic antioxidants, with a noticeably higher phenolic content of catechin, epicatechin and procyanidins.

DPPH is a stable, free radical, and conversion to 1, 1-dipheny I-2-picryl-hydrazine occurs when it reacts with an antioxidant. Khalid *et al.* (2011) reported that plants with free radical scavenger activity exhibit proton-donating ability and serve as free radical inhibitors. Thus, we suggest that the effect of free radical scavenging activity of CPE on DPPH radicals is due to the hydrogen donation ability of polyphenol compounds from cocoa beans. Our study showed that CPE exhibited dose dependent DPPH radical scavenging activity. Thus, it offers the potential for use as a radical inhibitor or scavenger, acting possibly as a primary antioxidant that reacts with free radicals, hence limiting free radical damage in the human body.

The EC_{50} value of aqueous extracts of cocoa beans has been reported to be 2.4±0.1 mg/ml (Azizah *et al.*, 2007). However, in our study,the IC_{50} value of CPE was 14.73µg/ml, lower in comparison to the aqueous extract of cocoa beans. Thus, we assume that extracting solvent used significantly affected antioxidant activity of the cocoa. Based on the antioxidative activity performed by the DPPH assay, it is possible that CPE has the potential to be developed as a natural antioxidant power and functional food.

FRAP assay is a new method of assessing the 'antioxidant power'. Reduction of ferric to ferrous ions at lower pH causes the formation of a ferrous-tripyridyltriazine complex which has an intense blue colour solution (Benzie & Strain 1996). The reducing capability of the compound possibly serves as an important indicator of its potential antioxidant activity (Balasubramanian & Ragunathan 2012). In our study, the FRAP assay was utilised because it is rapid, simple, reproducible and

linearly related to the molar concentration of the antioxidants present (Benzie, Wai & Strain, 1999). In fact, it has been reported that the reducing power of a substance is probably due to its hydrogen donating ability (Ferreira *et al.*, 2007). In the present study, we suggest that CPE might contain higher amounts of reductones comparable to other synthetic antioxidants. Therefore, CPE can function as an electron donor to react with free radicals and convert them into more stable products which finally impede the free radical chain reactions.

There has been a rapid increase in the discovery of chemopreventive agents from natural sources worldwide. The present study showed that CPE is only sensitive to cancer cell lines and is non-sensitive to normal cell lines. Based on this study, we suggest that CPE induces cancer cell death while simultaneously promoting normal cell line growth. However, at higher concentrations of CPE (10 mg/ml), CPE induced a cytotoxic effect on normal cell lines. We hypothesise that CPE may exert toxic pro-oxidant effects on the WRL-68 cell line. This hypothesis is supported by a study done by Lapidot, Walter & Kanner (2002) who found that elevation doses of dietary antioxidant also function as a pro-oxidant in cell culture and cause cellular damage. Furthermore, CPE exhibited most sensitive inhibitory activity against estrogen receptor-positive human breast cancer cell (MCF-7) compared to estrogen receptor-negative (MDA-MB-231). This finding is supported by the study of Jourdain *et al.* (2006) who showed that the inhibitory effect of CPE was more pronounced in androgen-sensitive prostate carcinoma (22 Rv1) cell line than in androgen-non responsive prostate carcinoma (DU145)cell line. Difference in hormonal sensitivity may explain the differences in the cytotoxicity action of CPE against these two tumor cells.

A study by Carnésecchi *et al.* (2002) showed that flavanol and procyanidin enriched fraction in cocoa inhibit the growth of Caco-2 cells by causing a blockade at the G2/M phase of the cell cycle with a significant decrease in ornithine decarboxylase and S-adenosylmethionine decarboxylase activities.This study showed that flavanols and procyanidins in cocoa exhibit antitumor properties.

Another study by Ramljak, Romancyzk & Metheny-Barlow (2005) revealed that pentamericprocyanidin derived from cocoa was more cytotoxic against MDA-MB-231 compared with MCF-7. This finding was contrary to our finding that found CPE to be highly toxic against MCF-7 compared to MDA-MB-231. Our study examined the crude extract which consisted of all compounds in the extract such as catechin, epicatechin and procyanidin whereas the study of Ramljak *et al.* (2005) only used a single compound (pentamericprocyanidin). We hypothesise that different compounds, other than procyanidin in CPE, may exert different effects. However, the mechanism of action of CPE against cancer cell lines cannot be confirmed yet.Apart from procyanidin, catechin and epicatechin are also strong inhibitors of cancer cell proliferation (Kampa*et al.*, 2000; Damianaki *et al.*, 2000 ; Notas *et al.*, 2006) interacting with different cellular mechanisms, including cell cycle modulation, cell growth arrest that ultimately leads to apoptosis (Kampa *et al.*, 2002).

CONCLUSION

Generally, we can conclude that CPE has the potential to function as an antioxidant which correlates to the specificity of the extract in targeting different cancer cell lines. Moreover, it might be developed as a chemopreventive agent as it impedes the initiation stage of cancer. However, it is not fully known how CPE induces cancer cell death. Based on antioxidant assay and initial screening work reported here, further studies could be performed by isolating bioactive compounds from CPE of

Malaysian cocoa beans, mode of cell death as well as elucidate the signaling pathway implicated in the cytotoxicity mechanism against cancer cells at molecular level.

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