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Research Article

The Antioxidant Activity of Cocoa Polyphenolic Extract-Treated 3T3 Fibroblast Cells

Ranneh Y[1], *Ali F[1], Fadel, A[2]

[1]*Department of Nutrition and Dietetics, Faculty of Medicine and Health sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*
[2] *Manchester Research Centre, Hollings Faculty, Manchester Metropolitan University, United Kingdom*

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ABSTRACTS
The existence of reactive oxygen species among cells at high ratio is associated with different types of disorders. A link between exogenous antioxidant supplementation and reducing oxidative stress remains unclear. Since a grave concern has been raised regarding synthetic antioxidant usage, justifying an alternative treatment is required. Cocoa, a naturally occurring plant containing various functional compounds, was used to determine the cytotoxicity and antioxidant efficacy in 3T3 fibroblast cells. In the present study, ABTS and ORAC assays were deployed as a comprehensive analysis for evaluating the antioxidant activity of cocoa polyphenolic extract (CPE) *in vitro*. Pretreatment of cells with (250, 500, 1000 mg/ml) of CPE completely prevented any toxicity on 3T3 cells and enhanced antioxidant activity. Based on ABTS and ORAC assays, CPE had significantly ($P < 0.05$) potential antioxidant activities compared with Trolox equivalent. A high correlation between total antioxidant capacity and phenolic contents indicated that phenolic compounds from cocoa were a major contributor of antioxidant activity ($0.967 \leq r \leq 1.00$) .These results show that treatment of 3T3 cells in culture with CPE confers a significant protection against oxidation to the cells.

Keywords: reactive oxygen species; cocoa polyphenolic extract; 3T3 cells, ABTS; ORAC; antioxidants.

INTRODUCTION

Reactive oxygen species (ROS) and free radicals namely, superoxide radical ($O^{\cdot 2-}$), hydrogen peroxide (H_2O_2), Nitric oxide radical ($NO^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$) are molecules that have high reactions resulted from aerobic metabolism**.** Additionally, ROS exert potentially a decisive role in human body, specifically in physiological and pathological process [1]. Unpaired valence electrons and unstable bounds are the main characteristic of all ROS types. Because of their high activity, ROS is able to act, at high concentration, with the biological materials such as protein, lipid, and nucleic acid causing either negative functional alterations or destructive actions [2]. In addition, many chronic diseases such as cancer, atherosclerosis, and arthritis are attributable with high levels of ROS [3,4].

Since reducing the risk of chronic diseases such cancer and atherosclerosis has taken the attention of doctors, nutritionist, consumers and food industry, the chemoprevention of these diseases is considered as an effective plan[5]. Thus, antioxidant and redox system are promising approach for functional food that, nowadays, increase the intake of antioxidants to reduce chronic diseases-related to oxidative stress. At the same time, it is well-documented that polyphenols-extracted from fruits have a high scavenging ability [6].

Cocoa (*Theobroma cocoa* L.*)* is the food of the Gods as translated from Greeks and the native land of cocoa is Americas that produce cocoa seed which is the main part in chocolate and cocoa manufacturing [7]. Cocoa was applied for therapeutic purposes to cure several disorders such as fever, indigestion, angina, heart, liver and lung diseases [7,8]. Polyphenols that are widely founded in plants food are the primer antioxdative part of cocoa branching to various subclasses compounds such as flavanols and procyanidins. Reported in different studies, cocoa polyphenols have a potential healthy ability for several chronic diseases including cardiovascular illness, neurodegenerative disorders, and prostate cancer [10,11].

Most of the previous researches in functional foods have been classically used the direct chemical reactions to assess the total antioxidant capacity without using cell culturing analysis. This method is not absolutely rigorous to evaluate the antioxidant actions of natural compounds *in vitro.* Therefore, the aim of this study was to determine the total polyphenolic content in cocoa extract and quantification their antioxidant activity by ABTS and ORAC methods in 3T3 cell lines as a model system for TAC. In addition, the correlation between the antioxidant activity and polyphenolic content was also determined.

MATERIALS AND METHODS

Chemicals:The Malaysian cocoa powder was purchased from (KL-Kepong Cocoa Products Sdn. Bhd., Port Klang, and Selangor, Malaysia). The mouse embryo fibroblasts cells (3T3) were purchased from American Type Culture Collection (ATCC). DMEM with L-glutamine, fetal serum albumin (FBS), penicillin, streptomycin, 3-(4,5-

*Author for correspondence: Email: sshrmany@yahoo.co.uk

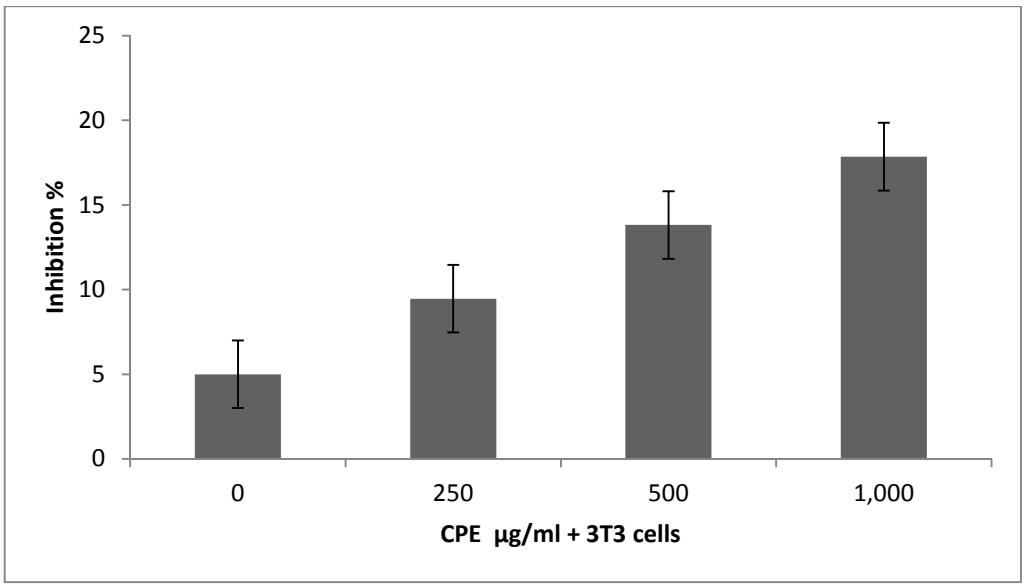


Figure 1: 3T3 fibroblast Cells were treated with three concentrations of CPE for 24 h. Cell viability was determined by MTT assay as described in Section 2. Results are expressed as mean ± SEM (n = 3).

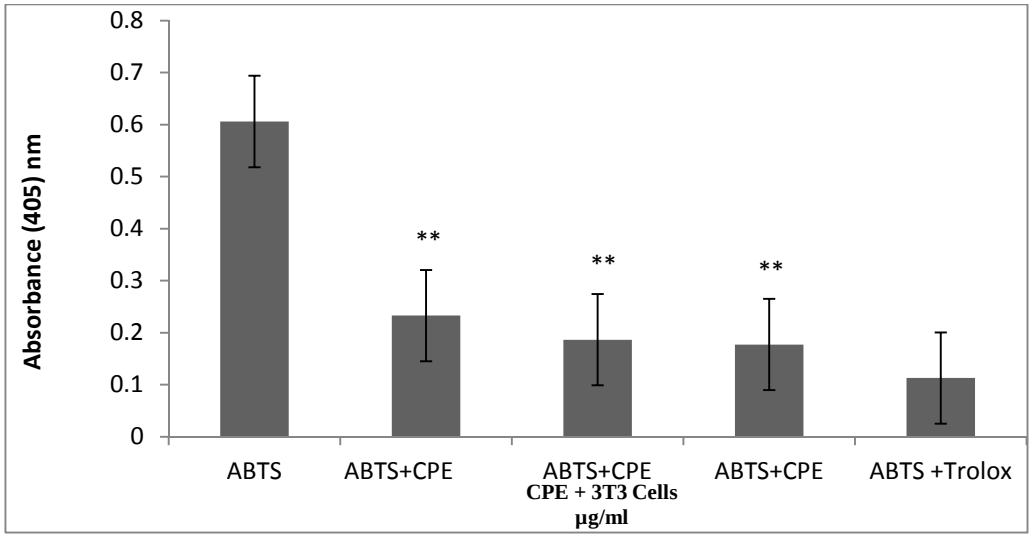


*Figure 2: Total Antioxidant capacity of 3T3 cells treated with CPE (250, 500 and 1000 µg/ml) compared with Trolex using ABTS· . The positive control contained 3T3 cells with Trolox while the negative control contained 3T3 cells with ABTS alone. Results are expressed as means ±SD (n=3). Differences between means were significantly different p < 0.05.. Tukey's test: **P< 0.01 versus ABTS. TE: Trolox equivalent per gram of extract*

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), gallic acid (GA), catechin and epicatechin were purchased from Sigma-Aldrich, Chemicals, (Co., USA). ORAC and ABTS kit was purchased from Zen-Bio, Inc., USA.

Preparation of cocoa phenolic extract: Cocoa phenolic extract was made whereby to previous method [12]. Malaysian cocoa powder was kindly gifted by KL-Kepong Cocoa Products Sdn. Bhd. (Port Klang, Selangor, Malaysia). Concisely, the extract was prepared through treating the defatted powder with 80% (v/v) ethanol for 2 hours. To remove the ethanol, a rotary evaporater (Buchi Rotavor R-200, Flawil, Switzerland) was used for 45 min at 55 °C. The following step was to keep the resulting extract at – 80 °C and to lyophilize it by using a freeze-dryer (The Virtis Company Inc., Gardiner, NY, USA) at −45 ◦C and 120 bar.

The Phenols and flavonoid contents of Cocoa extract: The total amounts of phenols and flavonoid contents were measured following to a method described by [13]. Total phenol content was detected by a method of Folin - Ciocalteu's phenol reagent using gallic acid as a standard and expressed as mg gallic acid equivalent (GAE)/100 mL extract. Total flavonoid was measured according to a method with 10% $AlCl_3.3H_2O$ solution using (+)-catechin as a standard and explained as mg catechin equivalent (CE)/100 ml extract.

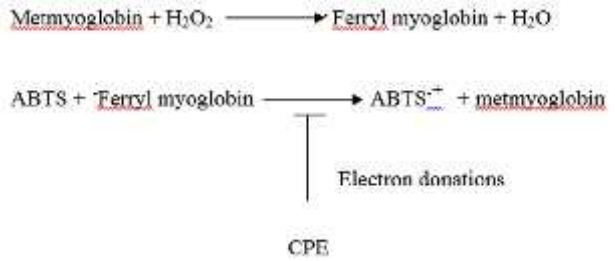
Cell Culture: Mouse embryonic fibroblast cells (3T3) were grown in DMEM media supported with 10 % v/v FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Then, the cells were incubated in

37º C at the incubator with 5% CO_2 with >95% humidity. Reaching to 80 – 90 % of confluence, fibroblasts (3T3) cells were scratched out and trypsinized, followed by centrifugation at 120 xg at 4 °C for 10 min. Different concentration of cocoa phenolic extract (CPE) 250, 500, and 1000 µg/ml was used to treat the cells.

Cell viability by MTT assay: The cytotoxicity of CPE on seeded fibroblasts (3T3) cells was colorimetrically evaluated by measuring the formation of formazin salts because of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagents. The cells were cultured for 18 h. Then, CPE in different concentrations 250, 500, and 1000 µg/ml were added. After 24 hours incubation, 20 µl of 5 mg/ml MTT reagents were added to each well. Then, the wells were incubated for 4 h at 37 °C and the formazen crystals were dissolved by adding 200 µl of dimethyl sulfoxide (DMSO) at 37 °C for 30 min. The optical density of the wells was read at 570 nm on a microplate reader (Molecular Devices Inc.,Sunnyvale, CA, USA). The rate of cell death was specified relative to control group.

Cell-based antioxidant assays-

ABTS assay: Before performing ABTS assay, the 3T3 fibroblast cells (1 X 10^5 cells/ml) were kept (5% CO_2, 37ºC) in 6-well plate containing different concentrations of CPE (250, 500, and 1000 µg/ml) for 24h. Then, myoglobin working solution was diluted with dilution buffer and kept on ice. The fibroblasts (3T3) cells were treated with Trolox (positive control) and assay buffer as a negative control. Ten micro-litters of cells suspensions were added to 96-well microtiter plate followed by adding 20 µl of Myoglobin working solution. Reaction was started by adding 100 µl of ABTS solution for 5 min (25°C). To stop the reaction, 50 µl of stop solution was added and the absorbance was read at 405 nm as described in the manufacturer instructions.



ORAC assay: Before conducting ORAC assay, the 3T3 fibroblast cells (1 X 10^5 cells/ml) were kept (5% CO_2, 37ºC) in 6-well plate containing different concentrations of CPE (250, 500, and 1000 µg/ml) for 24h. Antioxidant capacity was also analyzed in the cells using Oxygen Radical Absorbance Capacity (ORAC) assay using procedures outlined by the reagent provider (Zen-Bio, Inc., USA). AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) working solution was prepared by adding 2 ml assay buffer and kept on ice. Then, 25 µl of the AAPH working solution was added to each well containing cell suspensions and the kinetic fluorescence reading was read at 485 nm.

STATISTICAL ANALYSIS

Values were presented as means of three replicate determinations ± standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA) to test whether there is significant differences in antioxidant activity of natural and synthetic antioxidants, and the significance of the difference between means was determined by Tukey's test ($P < 0.05$) using SPSS for windows version 18.0. Pearson Correlation Coefficient was used to determine the correlation between the parameters studied in CPE.

RESULTS

Phenols and flavonoid content: The total phenols and flavonoid content in CPE are present in Table 1. The concentration of phenolic acid was higher than the flavonoid 114 mg/g > 94.95 mg/g respectively.

MTT assay: To test and prove the un-toxic effect of CPE, we examined the viability of the cells by using MTT assay. Shown in Figure 1, treating fibroblast cells with CPE in different concentration (250, 500, 1000 µg/ml) did not weaken the viable ability of cells with regard to untreated cells. The highest inhibition rate was approximately 20%. However, CPE at 1000 µg/ml concentration cannot affect the viability of 3T3 cells.

Cell-based antioxidant activity-

ABTS: The linearity of the calibration curve with Trolox was maintained with the Trolox concentration range from 4.7 µ M to 300 µM (R^2 =0.987). The oxidation of ABTS by ferryl myoglobin radical with H_2O_2 proceeded linearly as a function of time (Figure. 2). The fibroblast cells (3T3) treated with three various concentrations of CPE have shown significantly ($p < 0.05$) inhibitory effect against the oxidation of ABTS compared with the positive (Trolox+ the cells) and negative (ABTS + the cells) control (Figure. 3). The equations below demonstrate the mechanism of actions of CPE as a potent inhibitor towards ABTS free radicals in the reaction of the assay.

ORAC: The antioxidant ability of 3T3 cells-incubated with CPE was analyzed using the standard ORAC procedures, and the obtained results were normalized to the Trolox equivalents per gram on a freeze-dried basis. The results were illustrated in figure 4. Surprisingly enough, the 3T3 cells-treated with CPE were shown a significantly ($p < 0.05$) high florescence value by inhibiting the flourescin from oxidation compared with the positive and negative control.

DISCUSSION

Natural products have played a significant role not only in the clinical nutrition against several diseases but also in drug discovery and development, contributing in finding alternative therapies. Cocoa was administrated for several curative purposes to improve the status of several disorders such as fever, indigestion, angina, heart, liver and lung diseases [7,8]. It was believed that the preventive action of cocoa is attributed to the presence of different functional groups such as methoxy, phenoxy, and carbon-carbon double bound in its structure [14]. On the other hand, a variety of assays has been employed to assess the antioxidant activity of cocoa and its by-product. The *in*

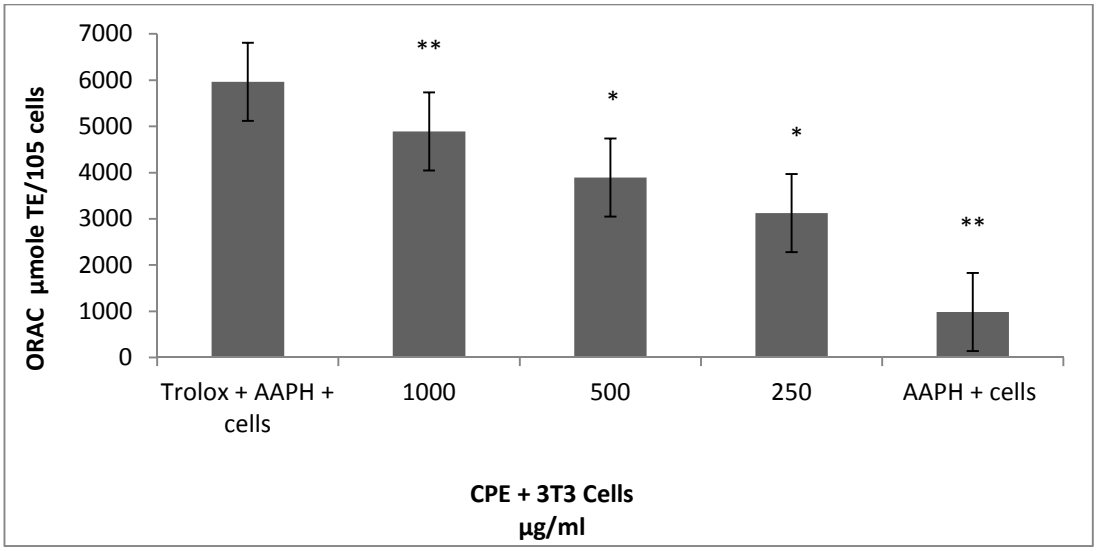


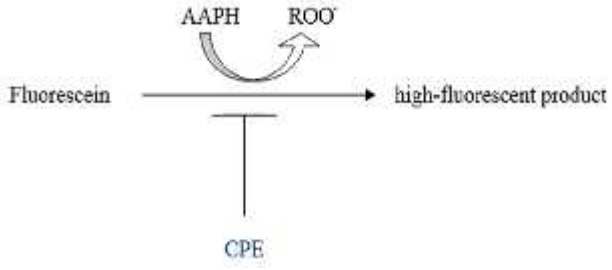
*Figure 3: Total Antioxidant ability of 3T3 cells treated with CPE (250, 500 and 1000 µg/ml) compared with Trolex using AAPH. The positive control contained 3T3 cells with Trolox while the negative control contained 3T3 cells with AAPH alone. Results are expressed as means ±SD (n=3). Differences between means were significantly different p < 0.05. Tukey's test: *P<0.05 ,**P< 0.01 versus AAPH. TE: Trolox equivalent per gram of extract.*

vitro assays through chemical reactions method, mostly, have been depended on measuring the radical scavenging activity [15, 13, 16] whereas rats and human subject have been used as *in vivo* model. Differently, the current study was performed on using cell-based assays to assess the antioxidant activity of CPE.

By using ethanol, we extracted the cocoa powder as described in section 2 followed by measuring the phenolic acids and flavonoid content. It is worth mentioning that Polyphenol contents of cocoa products depend on cocoa bean variety (genotype), postharvest processes (fermentation and drying), and roasting conditions [17]. This means that the geographical origin and cocoa processing have positively effect on the quantity and antioxidant activity of cocoa or cocoa products (i.e. Cocoa liquor, cocoa powder, chocolate and cake) and thereby, different health benefits on human health. For using polyphenols as a chemotheraputic and/or chemoprevntive agent, they should have not toxicity on cells. Currently, cocoa extract rich in phenolic compounds was not shown a toxic effect on 3T3 fibroblast cells incubated with different concentrations of this extract (Figure 1). Martín et al [18] have concluded that incubating ethanolic cocoa extract along with pancreatic β-cells has not damaged the viability of the cells. On the other hand, *Burkea africana* and *Syzygium cordatum*, two African plants rich in polyphenols, have increased the death population of 3T3-L1 cells [19].

TAC assays using 96-well plates have already been mentioned [20]. In our study, we used 96-well microplate to assess TAC of CPE-treated 3T3 cells with different concentrations (250, 500, and 1000 µg/ml) simultaneously. The ABTS·+ decolorization method assay measures ABTS·+ radical cation formation induced by metmyoglobin and hydrogen peroxide. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble vitamin E analog, serves as a positive control inhibiting the formation of the radical cation in a dose dependent manner [21]. In a previous research, it has been mentioned that ABTS assay using microplate would help in cutting the cost to 25%, increasing the analyzed sample and improving the analytical performance compared with other previously reported methods [22]. CPE extract-treated 3T3 cells have been perfectly demonstrated to inhibit the oxidation of ABTS by donation electron compared with Trolox (Fig. 9). The contribution of ABTS assay-microplate in evaluating TAC of our results was in agreement with [23]. In the same context, Chu et al [24] have found that *Spirulina platensis*, a commercial alga, has antioxidant protection on 3T3 cells using ABTS assay.

ORAC assay was created initially by Cao et al [25] and later developed by Ou and co-wrokers [26]. In the last version of ORAC assay, the fluorescent probe was fluorescein. Then, after identifying the oxidized form of fluorescein products, the determination of reaction mechanism was achieved to Hydrogen atom transfer mechanism [26]. The loss of fluorescein of fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH is an indication for the oxidative damage. When the antioxidant is present, ROO· strips a hydrogen atom from the antioxidant to create hydrogen peroxide (ROOH) which in turn inhibit the damage of fluorescein-stimulated by peroxyl radical (ROO·). In current study, 3T3 cells incubated with CPE in different concentrations (250, 500, 1000 µg/ml) inhibited the oxidation of flourescein compared with positive and negative control. Adamson et al [27] have concluded that cocoa and its-by products have shown a significant antioxidant capacity using ORAC assay. Measuring the antioxidant activity of different types of vegetables has shown a positive effect against the oxidation of perxoyl radicals [28]. The following equation is demonstrating the potentially possible mechanism by which CPE-treated 3T3 cells can prevent the oxidation of fluoresceine by hydrogen donation.



CONCLUSION

Taken together, using ABTS along with ORAC assay to measure the antioxidant activity of natural products with medicinally beneficial effect provides a comprehensive analysis for evaluating the antioxidant activity. In our research, 3T3 fibroblast cells supplemented with CPE reduced the oxidative damage of pro-oxidants, suggesting that CPE possess prominent medical properties and can be exploited as natural supplement to treat free radicals associated diseases. However, further studies to compare the antioxidant activity of CPE in cell-based assays and *in vivo* is still extensively required.

Abbreviations

Cocoa PolyPhenolic extract; CPE, ROS; Reactive oxygen specious, ROO·; peroxyl radical, MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, ABTS, 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid; ABTS•+, ABTS cation radical; TAC, total antioxidant capacity; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ORAC; Oxygen Radical Absorbance Capacity**.**

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Conflict of interest: The authors have no competing interests to disclose.

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