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Cacao's flair against colorectal despair: Elemental analysis, *in vitro* antioxidant and anticancer property of UF-18, *Theobroma cacao* powder against human colorectal cancer cells (HCT-116 Cells)

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Key words: Antioxidant property, Anticancer potential, Heavy metals, MTT assay

Abstract

The aim of the present study was to evaluate the safety, *in vitro* antioxidant and anti-cancer properties of UF-18 *Theobroma cacao* powder. The safety of cocoa powder was assessed by the elemental content through X-ray fluorescence spectroscopy. The antioxidant activity of the cocoa powder was evaluated using DPPH radical scavenging assay. The anticancer activity of cocoa powder was evaluated using the MTT assay against human colon cancer cells (HCT-116). Investigations showed that there was very low or trace amount of heavy metal Cadmium (0.48 %) in grams. On the other hand, potassium (K) has the largest content (40.83%). Cacao powder also contained 33.1096 % of Calcium (Ca), 10.46% of Iron (Fe), 6.16% of Phosphorous (P), 4.55% Silicon (Si). Other metallic elements present in minute quantities include 0.002 % Nickel (Ni), 0.2 % Manganese (Mn) and 0.2 % Titanium (Ti). The DPPH radical scavenging assay showed that cacao powder exhibits strong antioxidant activity, with an IC_{50} value of 17.60 µg/mL. The MTT assay showed that cacao powder has moderately strong anticancer activity against HCT-116 cells, with an IC_{50} value of 15.81 µg/mL. Cacao powder is a safe and effective anti-cancer agent as investigated *in vitro*. Potential health benefits merit further investigation, particularly *in vivo* and human clinical trials of the UF-18 cacao powder as anticancer alternative.

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Introduction

In the Philippines, there are 141, 021 total cancer cases with 86,337 total cancer-related deaths in 2018. Among the top 10 most common cancers are breast, cervix uteri, corpus uteri, leukemia, liver, lung, ovary, prostate, thyroid and colorectal cancer. Colorectal cancers (CRCs) are currently the third leading site of malignancy in the Philippines. The incidence of CRC cases has escalated from 5,787 in 2010 to 9,625 in 2015. Historical data estimate 3- and 5-year survival for colon cancer to be 38.1% and 33.9% and that of rectal cancer 31.3% and 20.0%, respectively (Sung *et al.* 2020). While colon cancer is rare in young adults before, it's the third most common cancer and second most common cause of cancer deaths worldwide. Nowadays, there has been many reports of colon cancer cases in the ages of early 20's and 30's and this is very alarming (World Health Organization, 2024).

Precision therapy, chemotherapy, surgery, and immunotherapy are the current treatments for colorectal cancer. Precision medicine in colorectal cancer faces challenges such as tumor heterogeneity, where different tumor regions may have unique genetic profiles including identifying genetic variants that can be used for targeted therapy maybe complicated by the evolving nature of the cancer genome (Smith *et al.*, 2020).

Chemotherapy is associated with various side effects, such as nausea, fatigue, and myelosuppression, which can compromise a patient's quality of life (Anand *et al.,* 2022). Surgery is a cornerstone in colorectal cancer, but can cause complications such as infection, bleeding and anastomotic leaks. Immunotherapy has shown promise in a variety of cancers, but colorectal cancer is less effective, in part due to low tumor mutation load and immunosuppressive tumor microenvironment (Carlsen *et al.,* 2022). Hence, there is a need to look for other alternative medicine and treatment.

In recent times, plant-based chemicals have become the standard in the treatment of cancer. Human health studies have been carried out for a long time on plant compounds, especially flavonoids (Fatima *et al.*, 2021). For instance, most of the antioxidants that are found in cacao include catechins and epicatechin that help combat or neutralize unsafe free radicals within the body (Sing *et al.*, 2011). These are the ones which research suggests contribute to the creation of cancer, initiating and promoting factors, so exploring them could prove to be beneficial in cancer prevention as well as management researches. Additionally, new proof indicates that cocoa phytochemicals may possess anti-cancer characteristics. Such effects consist of prohibiting growth of malignant cells, programmed cell death or apoptosis, having no inflammation and prevention of angiogenesis or the creation of new blood vessels in order to supply cancers (Pfeffer and Sing, 2018). The likelihood that cocoa might be an area for investigating anti-cancer alternatives is very promising. New clinical investigations are uncovering the many-faced anticancer effectiveness of cacao phytochemicals with results indicating that they can inhibit cancer cell propagation, induce programmed cell death (apoptosis), relieve infection and stop angiogenesis (Katz *et al.,* 2011). These findings show that cacao products possess great prospects for creative and supportive cancer treatment options.

Although, it is worth noting that the existing research does not offer a clear understanding of the mechanisms involved. Some existing research posits potential antioxidant and anti-cancer capabilities of the cacao phytochemicals. Nonetheless, there is a need for precise understanding of the correct molecular pathways. Mechanistic and *in vitro* assay studies can offer insights into the targets and pathways affected by cacao metabolites from which these compounds are derived, thus enhancing knowledge in regard to therapeutic potential among them especially for colorectal cancers (Di Mattia *et al.,* 2017). It is important to comprehend the aspects of bioavailability and metabolism of cacao-derived phytochemicals that might contribute to how good they could be for one's health. Exploring how these chemicals using cell-based setting must be established first prior to the conduct of *in vivo*, *in* *silico* and clinical studies. Such understanding can guide the use for enhancing antioxidant and anticancer effects particularly in cases such as colorectal cancer (Ortega *et al.,* 2017).

To address these research gaps, the study aims to evaluate the *in vitro* antioxidant and anticancer property of UF-18 *Theobroma cacao* powder. Specifically, it sought to assess the safety profile of the powder in terms of heavy metals, determine the percentage inhibition (IC_{50}) of the powder for antioxidant and anticancer property against human colorectal cancer cells (HCT-116 cells).

Materials and methods

Materials

The study utilizes 100 gram of UF-18 cacao powder obtained from CSU-Lasam Cacao Processing Center, 95 % ethanol, 10% fetal bovine serum (FBS), 1% antibiotic- antimycotic solution, 5% CO2, Dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution, HCT-116 cells, 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye 96-well microtiter plates, Doxorubicin, microplate reader, Erlenmeyer flask, 0.1 whatman filter paper, Buchner funnel, hot plate, UV-VIS spectrophotometer and extract refrigerator.

Research design

An experimental research design was utilized by the researchers for it follows scientific approach to research, where one or more independent variables were manipulated and applied to one or more dependent variables to measure their effect on the latter. The powder of UF-18 *Theobroma cacao,* the endemic variety in the municipality of Lasam, was utilized to determine whether or not there will be potential heavy metal in the elemental analysis, antioxidant activity in varying concentrations and anticancer properties in an *in vitro* set-up.

Elemental analysis for safety profile of powder

Elemental analysis of the cacao powder was determined using X-ray fluorescence spectroscopy adopted from the procedure of Pacubat (2022) at NASAT NanoTech Laboratories, Muntinlupa City. Fifty grams (50 g) of cocoa powder were subjected for the analysis done in triplicates. The measurement time for the voltage, which was operated at 50 kilovolts (kV), was 500 seconds. The spectroscopy collimator is 7 millimeters in diameter, and the applied current is 4 microamperes. The identification of elements and concentration by weight percentage (%) in grams were measured by spectroscopy utilizing the idea that excited electrons release x-rays that descend to the ground state level and are absorbed by the machine's detector.

MTT toxicity assay for anticancer property

The experiment was modified from the procedure by Mosmann (1983) at Mammalian Laboratory, Institute of Biology, UP Diliman, Quezon City, using MTT cytotoxicity test. 40,000 cells were plated on HCT-116 cells in a sterile 96-well microtiter plate then incubated overnight using McCoy's 5A Media containing10% fetal bovine serum (FBS) and 1% antibiotic- antimycotic. To promote cell adhesion, plates were incubated in a humidified incubator at 37°C and 5% CO_2 for an entire night. Sterilized DMSO was used to dissolve the cacao powder samples. Eight, two-fold dilutions of the material, ranging from 100 g/mL to 0.78125 g/mL, were utilized as treatments done in triplicates. It was controlled positively by Doxorubicin, negatively by DMSO, and it was done by treating cells first with diluted samples and controls after they cultured for 72 hours at 37°C and 5% CO_2. The MTT dye, 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, after the incubation period was added. It was followed by incubating the MTT-treated cells for three hours under 5% CO_2 at 37°C. DMSO was used to dissolve formazan crystals produced as a result of MTT dye reduction. Absorbances were taken at 570 nm wavelength using microplate reader. Percent Inhibition was computed through the following formula:

OD570nm (treated)/OD570nm (negative control) * 100 %

Statistical analysis

Mean percentage of the identified elements of the cacao powder was determined. GraphPad Prism was used to calculate the inhibition concentration at 50% (IC_{50}) by non-linear regression curve fitting at a p-value of 0.01. Active sample are those with IC_{50} values lower than 30 µg/mL in determining whether the treatment has anticancer property.

Results and discussion

Profile of cacao powder

There was very low or trace amount of heavy metal Cadmium, which is below the critical threshold of 30 µg/day consumption (Satarug *et al.,* 2010) found as shown in Table 1. On the other hand, potassium (K) has the largest content (40.83%), which can be deduced. The concentration of relatively high calcium (Ca) is 33.1096%. Cocoa nibs also contain a percentage concentration by weight of 10.46% of iron (Fe). Table 1 demonstrates the amount of phosphorus (P) in the weight of 6.16%. Fifty grams of cocoa contain 4.55% silicon (Si). Other elements present in minute quantities include nickel (Ni), manganese (Mn), copper (Cu) and titanium (Ti). The results in table 1 correspond with the Pacubat (2022) study on mineral and heavy metal analysis of cacao nibs. Therefore, the cocoa powder dangerous dose of heavy metals that would make it dangerous to humans when taken or used for laboratory activities.

Table 1. Elemental profile of UF-18 cacao powder

Element	Mean (Percent by weight in grams)	SD
Cadmium (Cd)	0.4892	±0.1230
Potassium (K)	40.83	±0.0614
Calcium (Ca)	33.1096	±0.0373
Iron (Fe)	10.46	±0.0004
Phosphorous (P)	6.16	±0.0918
Silicon (Si)	4.55	±0.0006
Nickel (Ni)	0.002	±0.0003
Manganese (Mn)	0.002	±0.0003
Titanium (Ti)	0.02	±0.0006

Antioxidant property of UF-18 cacao powder

The figure below shows that the DPPH radical scavenging trendline of the sample having concentrations which increases with increasing concentration, having a linear regression of y= 0.0541x + 41.97 and R^2= 0.346. This trendline shows that cacao powder has a positive, increasing curve and is suitable for antioxidant activity. The trendline in Fig. 1 shows that cacao powder has a positive, increasing curve and is suitable for antioxidant activity. With higher concentrations, there are more antioxidant molecules.

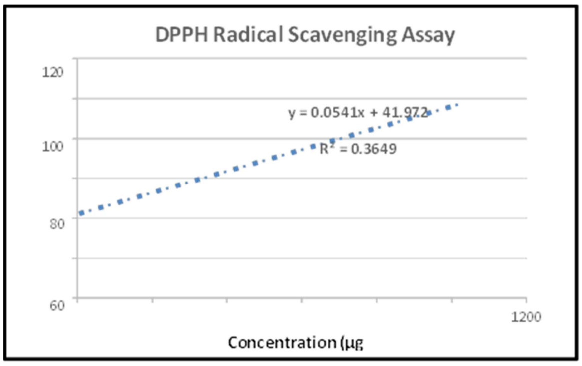

Fig. 1. Trendline of DPPH radical scavenging assay

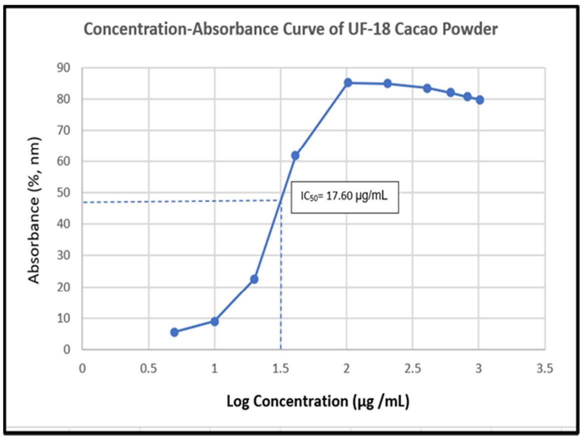

Fig. 2. IC_{50} of the antioxidant property of UF-18 cacao powder

Fig. 2 shows the concentration-absorbance curve of UF-18 cacao powder. The curve follows a sigmoidal trend, hence the IC_{50} can be computed. The antioxidant IC_{50} value of the sample is 17.60 µg/mL as can be gleaned on figure 2. This means that half of the DPPH radicals were scavenged at a concentration of 17.60 µg/mL. According to (Molyneux, 2004), a compound is classified as very strong when the IC_{50} value is <50 µg/mL, strong when the IC50 value is 50-100 µg/mL, moderate when the IC_{50} value is 101-150 µg/mL, and weak antioxidants when the IC50 value is >150 µg/mL. Accordingly, 17.60 µg/mL is a low IC_{50} value, indicating that the cacao powder sample is a strong antioxidant as investigated *in vitro*. Similarly, a study published by Zubayda *et al.* (2022) investigated a similar effect on cacao peel extract against DPPH and ABTS radicals. Hence, UF-18 cacao powder is a promising antioxidant alternative as investigated *in vitro*.

Anticancer property of UF-18 cacao powder

Table 2 shows the summary of mean IC_{50} of Doxorubicin and UF-18 cacao powder against human colorectal cancer cells (HCT-116). Cacao powder exhibited mean IC_{50} of 15.81±0.4 µg/mL as compared with the standard drug Doxorubicin, 1.096±0.76 µg/mL. Accordingly, a compound tested dose-dependently inhibits cancer cell proliferation at a sigmoidal trend with an IC_{50} of less than 30 µg/mL. Samples with IC_{50} values less than 30 µg/mL are considered active against cancer cells (Jokhadze *et al.* 2007). By convention, it is important to note that the ideal IC_{50} value for a particular cancer cell line or tumor type will vary depending on a number of factors, including the type of cancer, the stage of the disease, and the presence of any genetic mutations. Abu Bakar *et al.* (2019) discussed a general guide to *in vitro* IC_{50} values for cancer cells- Very strong: <5 µg/mL, Strong: 5–10 µg/mL, Moderate: 10–20 µg/mL, Weak: 20–100 µg/mL and Not active: >100 µg/mL. Hence, UF-18 cacao powder exhibited a moderately strong anticancer property against human colorectal cancer cell *in vitro* as can be gleaned in Table 2.

Table 2. Mean IC_{50} of standard drug doxorubicin and UF-18 cacao powder in human colorectal cancer-cells (HCT-116 Cells)

Sample	Mean IC_{50} (µg/mL)	Trendline	P-value
Doxorubicin	1.096±0.76	Sigmoidal (Active)	0.000
UF-18 cacao powder	15.81±0.4	Sigmoidal (Active)	0.000

Conclusion

Cacao powder is a safe, potent antioxidant, and a moderately strong anticancer agent as seen *in vitro*. It has the potential to be investigated as a dietary supplement as antioxidant drug and for the prevention and treatment of cancer. Key findings of this study show that cacao powder has trace amount of heavy metal, cacao powder has a high DPPH radical scavenging potential, indicating its strong antioxidant activity and cacao powder exhibits moderately, strong anticancer activity against human colorectal cancer cells *in vitro*. For future studies, the researchers may determine and isolate the phytochemical constituents of the cacao powder's active ingredient against colorectal cancer cell and may include an investigation of the anticancer effects of cacao powder *in vivo*, *in silico* and in human clinical trials. It is necessary to determine the optimal concentration of cacao powder to be used in cancer therapy.

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