"EXPLORING THE PAIN-RELIEVING POWER OF A POWDER" IN VITRO CYCLOOXYGENASE I (COX-1) INHIBITORY ACTIVITY OF UF-18 THEOBROMA CACAO POWDER

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Abstract

Cyclooxygenase 1 (COX-1) is an enzyme that forms prostanoids responsible for inflammatory response. There has been a link between inflammation and consuming cacao food products. Investigating cacao powder's inhibitory potential against COX-1 was to establish empirical data. Phytochemicals involved in the inhibitory potential of powder were determined using the methods of Harborne, Edeoga et al., and Onwukaeme et al. (1995) with modifications. We evaluated phytochemicals such as tannins, saponins, terpenoids, cardiac glycosides, alkaloids and phenolic compounds. Quantitative estimations show the amounts of 10.2±3 mg of tannins, 8.1±3 mg of saponins, 15.3±4 mg of terpenoids, 20.22±1 mg cardiac glycosides, 5.6±8 mg of alkaloids and 66.8±5 mg of phenols. In vitro COX-1 inhibition assay, we used 100 ppm of cacao powder dissolved in dimethyl sulfoxide (DMSO) in triplicates added to COX-1-hematin as an enzyme-cofactor solution with an incubation time of 15 minutes. After incubation, the assay solutions are added with 10-acetyl-3,7-dihydroxyphenoxazine to produce resorufin. The fluorescence of resorufin was monitored every 12 seconds at an excitation of 535 nm to determine the slope of inhibition. Using the average and the percentage inhibition (%) of cacao powder was calculated to be 69.39 ±7.73. It was considered to be a very active sample (Active Sample >50% Inhibition) concerning the standard drug Indomethacin (96.98±3.16 %). Multiple comparisons against the negative control were determined using Dunnet's T-Test, resulting in a significant mean difference of -0.11198 and -0.15632 for cacao powder and Indomethacin, respectively ($p \leq 0.05$). Hence, cacao powder is proven to be an effective anti-inflammatory alternative.

Keywords: Cyclooxygenase, Enzyme-Cofactor, Percentage Inhibition.

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1. Introduction

Around 40 million people are thought to suffer from arthritis. The two most prevalent types are rheumatoid arthritis, a chronic disease that causes inflammation of the joint lining, and osteoarthritis, a degenerative joint disease marked by a progressive loss of cartilage. The major enzyme responsible for inflammation in patients with rheumatoid arthritis is Cyclooxygenase 1 and 2. The enzyme cyclooxygenase (COX) is responsible for producing the prostanoids prostaglandins, prostacyclins, and thromboxanes, which cause inflammation (Turman et al., 2010). COX inhibition is how nonsteroidal anti-inflammatory medicines (NSAIDs) are frequently recommended to treat various forms of arthritis and function. Even though they are helpful, traditional NSAIDs like Motrin (ibuprofen), aspirin, and Aleve (naproxen) can lead to gastrointestinal issues like ulcers (Silvestrini, 2007). It's because they are non-selective, which blocks both kinds of cyclooxygenase. Traditional NSAIDs' inhibition of COX-2 helps to reduce inflammation, but it has the drawback of simultaneously inhibiting COX-1, which can result in side effects such as gastrointestinal bleeding. These medications are not advised for anyone who has had stomach ulcers, asthma, high blood pressure, renal illness, or liver disease because of this and similar consequences (Gragnolati, 2022). To address the side effects of COX inhibitors, traditional and alternative medicines are developed to act as COX inhibitors, thereby reducing inflammation with limited or no side effects. Aiming to "improve the quality and delivery of health care services to the Filipino people through the development of traditional and alternative health care and its integration into the national health care delivery system," Republic Act 8423 (R.A. 8423) or TAMA Act mandates the Philippine Institute of Traditional and Alternative Health Care (PITAHC). This act paves the way to discovering and developing alternative medicines to address common and chronic diseases. There needs to be more research on developing plant-based COX inhibitors, for it requires compounds that may selectively inhibit the said enzyme. On the other hand, there has been a link between the anti-inflammatory activity of cacao-food consumption, but empirical data needs to be established. About this empirical gap, the research aims to evaluate the cyclooxygenase-1 inhibitory potential of UF-18 cacao powder in vitro as a basis for future COX-2 inhibition and pre-clinical trials. Further, this research shall intensify the utilization of cacao powder as a nutraceutical and captivate the vision of the Philippine TAMA ACT: "People's health through traditional and alternative health care."

Objectives of the Study

Generally, the study aims to evaluate the cyclooxygenase I (COX-1) inhibitory activity of UF-18 Theobroma cacao powder. Specifically, it sought to determine the phytochemicals and their quantitative estimations and evaluate the percentage inhibition of cacao powder concerning a standard drug.

2. Materials and Methods

Research Design: Conducted this initial experimentation at the Terrestrial Natural Products Laboratory, UP Institute of Chemistry, UP Diliman, Quezon City. The researchers utilized an experimental research design, which follows a scientific approach to research. One or more independent variables are manipulated and applied to one or more dependent variables to measure their effect on the latter. Only one concentration of cacao powder was utilized to determine whether or not there would be percentage inhibition in an in vitro set-up.

Materials: The study utilizes 100 grams of UF-18 cacao powder obtained from CSU-Lasam Cacao Processing Center, and dimethyl sulfoxide, hematin solution, 2ml of Vanilin in ethanol, concentrated sulfuric acid, HCl solution, $CHCl_3$ solution, 15% $FeCl_3$ solution, Folin- Ciocalteu's reagent, 5.18 mL of 100 mM pH 8 Tris buffer, 10-acetyl-3,7-dihydroxyphenoxazine solution (Amplex Red solution), CLARIOstar® (BMG LABTECH) multifunctional microplate reader at an excitation wavelength of 535 nm, ELISA well plates and Indomethacin from Terrestrial Natural Products Laboratory, Institute of Chemistry, UP Diliman, Quezon City.

Methods: Phytochemical Screening and Quantitative Estimations of Phytochemicals Phytochemical screening procedures were based on Harborne (1984), Edeoga et al. (2005), and Onwukaeme et al. (2007) with slight modifications.

The following are tests done in triplicates to determine the presence and estimated amounts of phytochemicals: Test for Tannins- One (1) mL of distilled water was used to dissolve the 20.3 mg cacao powder. After mixing, give the residue time to settle. When 15% FeCl3 solution is added dropwise, blue-black or brownish-green precipitates will form; this is a sign that tannins are present. Put one millilitre of the test extract of the tannin solution into 10-millilitre volumetric flasks. Following the addition of potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml), sulfuric acid (4N, 2 ml) and iron (III) chloride (0.5% w/v, 2 ml) were added. The mixture was heated for 30 minutes at 70–20 0C in a water bath with periodic shaking before being diluted with distilled water to the proper concentration. At 780 nm, the absorbance was calculated against a blank for the reagent.

Test for Saponins- 102.1 mg of the powder was dissolved in 5 mL of distilled water. After mixing, the leftover residue was allowed to settle. The test tube's contents were then heated to a boil, cooled, and forcefully shaken. The development of foaming indicates the presence of saponins. Test extracts were dissolved in 80% methanol, 2ml of Vanilin in ethanol was added, mixed well, and heated on a water bath at 600°C for 10 minutes before the absorbance at 544 nm was measured in comparison to a reagent blank. As a standard material, diosgenin is utilized to compare the assay results to diosgenin equivalents.

Test for Terpenoids- The powder (39.4 mg) was mixed with two (2) mL CHCl3, and then concentrated H2SO4 was added. A reddish-brown interface and its quantitative equivalents indicate the presence of terpenoids.
Test for Flavonoids-added 3 drops of conc to 2 mL of ethanol after the powder (43.7 mg) had dissolved. HCl. The presence of flavonoids is shown by the pinkish-to-red-orange solution that forms when magnesium ribbon is added, and its quantitative equivalents are determined.

Test for Cardiac Glycosides- 2 mL of distilled water was used to dissolve the powder (41.3 mg). Drop by drop, added 1% FeCl3 to the supernatant and one (1) mL of concentrated H2SO4 without disturbing the solution. The supernatant was subsequently separated from the residue to enable better observation of the test result. The presence of a brown ring is indicated by its observation, and its quantitative estimations were determined using a spectrophotometer.

Test for Phenols- used a two (2) mL volume of distilled water to dissolve the powder (43.0 mg). When 1% FeCl3 solution is added dropwise, blue-black precipitate forms, indicating the presence of phenolic chemicals using the Folin-reagent, Ciocalteu's total phenolics content of various solvent extracts was measured (FCR). The procedure combined 0.4 ml of 1:10 v/v diluted FCR with various extract concentrations added f 4 ml of sodium carbonate solution following 5 minutes. After being filled to 10 ml with distilled water, the tubes remained at room temperature for 90 minutes. A spectrophotometer was used to test the sample's absorbance at 750 nm compared to the blank. It quantified the total phenolic content of the powder in terms of milligrams of catechol per gram of dry weight. The standard graph using a calibration curve was created using catechol solutions as the standard.

Test for Alkaloids- Three (3) mL of distilled water were used to dissolve the powder (60.1 mg). Alkaloids are present when a brown-black precipitate forms after Wagner's reagent is added dropwise and added 5 ml of pH 4.7 phosphate buffer, 5 ml of BCG solution, and 4 ml of chloroform to 1 ml of test extract. Then shook, the mixture The extracts were gathered in a 10-ml volumetric flask and diluted with chloroform to adjust the volume. At 470 nm, the absorbance of the chloroform complex was measured compared to a blank that was made in the same manner but without extract. Atropine is used as a reference substance to compare the assay results to Atropine equivalents.

Preparation of sample and blank solutions

Three solutions of 10 ppm or mg/L of cacao powder were prepared using dimethyl sulfoxide as solvent. A negative dimethyl sulfoxide control before the inhibition assay.

Preparation of COX-1 Inhibition Assay

5.18 mL of 100 mM pH 8 Tris buffer was introduced to a clean scintillation vial. Separately, 480 mL of 20 mM hematin and 96 mL of 250 U/mL sheep COX-1 enzyme were combined before being dispensed into the buffer-filled vial. Thus, the enzyme-cofactor

solution is created. Added 120 L of the enzyme-cofactor mixture to each test well after distributing 50 L of the same buffer. Additionally, Indomethacin is dissolved in 5% dimethyl sulfoxide as a standard reference solution.

COX-I Inhibition Assay

To the designated wells, 10 L of a 300 ppm sample, 160 mM of positive control, and DMSO, a negative control, were added. One trial and four replicates for each (n=1, t=4). An effective solvent healthy concentration of 5% DMSO is present in the sample, with Indomethacin as the positive and negative control. Where spent 15 minutes of incubation at 250C on the mixture. Following incubation, 10 L of 200 M Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) and 10 L of 2,000 M arachidonic acid were added to each well. N2 was used to mix and purge the reaction mixture quickly. We used The CLARIOstar® (BMG LABTECH) multipurpose microplate reader to observe the reaction for 2 minutes at 535 nm for excitation and 590 nm for emission. At 12-s intervals, the fluorescence intensity was measured. And we modified this procedure from the DOST-PCHRD publication Tuklas Lunas Protocols for Drug Discovery and Development (2020).

Statistical Analysis

Based on the average slope and the equation, the inhibitory activity of the sample and the positive control (Indomethacin) was calculated:

Figure 1. Percentage Inhibition of Sample or Positive Control Equation

$$\%\ Inhibitory\ activity = \frac{Slope_{uninhibited} - Slope_{inhibited}}{Slope_{uninhibited}} x\ 100$$

The slope is uninhibited is the slope of the line from the fluorescence intensity vs time plot of the negative control group. The slope is uninhibited is the slope of the line from the fluorescence intensity vs time plot of the samples or positive control. A sample is considered "active" if the COX-1 per cent inhibition is greater than or equal to 50% and the sample slope has a significant mean difference compared to negative control at $p < 0.05$. We determined the mean percentage inhibition and standard deviation. Further, multiple comparisons of samples against the negative control for COX-1 using Dunnett's t-test as a posthoc test were also determined using SPSS version 25.0. Also, we determined a graphical analysis of slopes of inhibition at a 12-s interval for concrete data analysis.

3. Results and Discussion

Phytochemicals such as tannins, saponins, terpenoids, cardiac glycosides, alkaloids and phenolic compounds were detected. Table 1 shows the phytochemicals present in the UF-18 cacao powder with its corresponding quantitative estimations. Quantitative estimations show the amounts of 10.2±3 mg of tannins, 8.1±3 mg of saponins, 15.3±4 mg of terpenoids, 5.6±8 mg of alkaloids and 66.8±5 mg of phenols. Phenols have the highest amounts of phytochemicals found in the powder. Similarly, in another study done by Lee et al. (2013), a comparison of black tea (124 mg of GAE and 34 mg of ECE, respectively), green tea (165 mg of GAE and 47 mg of ECE), and red wine, cocoa had substantially more significant levels of total phenolics (611 mg of gallic acid equivalents, GAE), and flavonoids (564 mg of epicatechin equivalents, ECE), per serving (340 mg of GAE and 163 mg of ECE). This is also parallel with the study of Kim et al. (2014) that cacao powder is rich in polyphenols, procyanidins as a form of catechin and epicatechin, and theobromine as a form of alkaloids and caffeine. According to reports, some molecular targets connected to the aetiology of chronic human diseases, including cardiovascular illnesses, cancer, neurodegenerative illnesses, obesity, diabetes, skin ageing and inflammation, are specifically modulated or interacted with by cocoa phytochemicals.

Table 1. Mean Estimates and Standard Deviation of Phytochemicals in UF-18 Cacao Powder

Phytochemicals	Mean Estimates & S.D. (mg)
Tannins	10.2±3
Saponins	8.1±3
Terpenoids	15.3±4
Alkaloids	5.6±8
Phenols	66.8±5
Cardiac Glycosides	20.22±1 mg

Figure 2 shows the kinetic curve of the presence of resorufin due to COX inhibition. The Y-axis of the curve emphasizes the uninhibited cyclooxygenase enzymes, while the X-axis represents the 12-s interval of resorufin detection. Blue lines represent the curve of blank solution or dimethyl sulfoxide, light brown lines represent cacao powder and dark brown lines represents the standard drug Indomethacin. According to the formula in Figure 1, the average slope of blank solution or negative control is obtained first before calculating percentage inhibition. Using SPSS version 25.0, the average slope of negative control or slope uninhibited is found to be 0.1614. From this value, the individual percentage inhibition of the four sample replicates and the standard drug is calculated to be 95.21 %, 94.10%, 96.86%, 101.29% and 63.59 %, respectively, 73.26 %, 64.59 %, 78.24 %, 61.46 % respectively. A similar trend of the kinetic curve was also observed by Alberto et al. (2019) with the utilization of dimethyl sulfoxide as negative control and Indomethacin as a standard reference drug. They found that Indomethacin almost inhibits 95 % of COX enzymes about the negative control. Hence, this kinetic curve proves that Indomethacin is a good reference drug for plant extracts in COX inhibition assays.

Figure 2. Kinetic Curve of Negative control (Blue), Cacao Powder (Light Brown) and Indomethacin (Dark Brown)

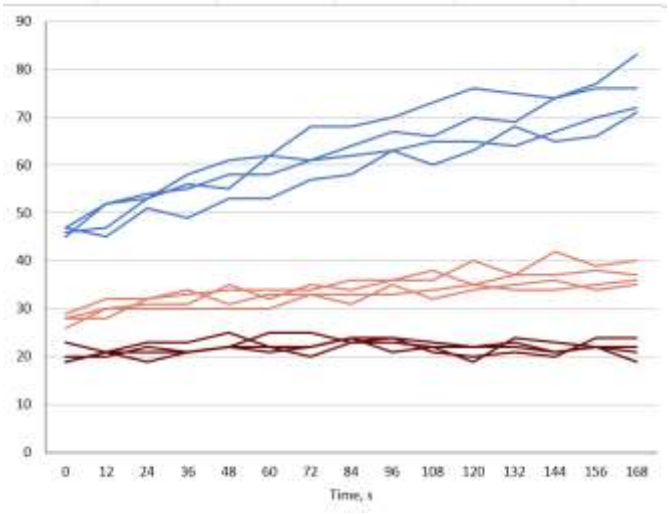

Table 2 summarises mean percentage inhibition (MPI), standard deviation and mean comparison against negative control using Dunnet's T-test cacao powder and Indomethacin. According to equation 1, a sample is considered "active" if the COX-1 per cent inhibition is greater than or equal to 50% and if the sample slope has a significant mean difference compared to negative control at $p < 0.05$. The table shows that cacao powder has an MPI of 69.39 ±7.73 compared to the reference drug Indomethacin (MPI=96.98±3.16). Hence, the UF-18 cacao powder is an active sample that can inhibit COX-1 in vitro at 10 ppm or mg/L concentration. These findings are also comparable with the study of Ranneh et al. (2016), where they observed that phenolic cocoa extracts inhibit pro-inflammatory mediator secretion

induced by lipopolysaccharide in RAW 264.7 cells. According to the findings, cocoa phenolic extracts dramatically reduce 5-Lipoxygenase activity (p 0.01). Cacao phenolic extracts also reduced the generation of Prostaglandin E2, reactive oxygen species, nitrogen oxide, and Tumor Necrosis Factor Alpha (TNF-a) in RAW 264.7 cells in a dose-dependent manner. Parsaeyen et al. (2021) also report similar findings on the beneficial effects of cocoa on lipid peroxidation and inflammatory markers in type 2 diabetic patients and investigation of probable interactions of cocoa-active ingredients with prostaglandin synthase-2 (PTGS-2/COX-2). This study found that cacao powder is effective against the said biomarkers. Many findings also suggest that the phenolic compounds of cacao powder are strongly associated with its anti-inflammatory activity. Lee et al. (2016) discovered that cocoa polyphenols inhibit phorbol ester-induced superoxide anion formation in cultured HL-60 Cells. Under stimulation with lipopolysaccharide, macrophages produce inflammatory molecules, including tumour necrosis factor (TNF)- and monocyte chemoattractant protein (MCP)-1. A cocoa flavonoid-enriched extract and the monomers epicatechin and isoquercitrin reduced this production (LPS; Ramiro et al., 2005a). Similarly, in stimulated whole blood cell cultures, epicatechin reduced interleukin (IL)-6 and IL-8 production (Al-Hanbali et al., 2009). Hence, the COX-1 inhibitory activity of UF-18 cacao powder is firmly attributed to its phenolic compounds detected on the phytochemical screening and quantitative estimations.

Table 2. Summary of Mean Percentage Inhibition (MPI), Standard Deviation and Mean Comparison Against Negative Control Using Dunnet's T-test

	MPI & SD (%)	Mean Difference with Negative Control	Standard Error	Sig.
Cacao Powder	69.39±7.73	-.11198**	.01238	0.000
Indomethacin	96.98±3.16	-.15632**	.01238	0.000

**The mean difference is significant at $p < 0.05$ as assessed by SPSS 25.0.

4. Conclusion and Recommendation

In the findings of this study, UF-18 cacao powder exhibited major phytochemicals such as tannins, saponins, terpenoids, alkaloids, cardiac glycosides and phenols. Moreover, the methods utilized for the quantitative estimations revealed the amounts of the said phytochemicals for a better understanding of the COX-1 inhibition potential of the said powder. These quantitative estimations shall pave the way to isolate and further study the phytochemicals which contributed to the anti-inflammatory potential of UF-18 cacao powder. The COX-1 inhibition assay directly measures the peroxidase (POX) activity which is reported to be directly proportional to the COX activity of the enzyme. The reduction of PGG2 due to POX is coupled with the oxidation of ADHP (10-acetyl-3,7-dihydroxyphenoxazine), or Amplex Red, which is subsequently converted to resorufin, a highly fluorescent compound. Resorufin fluorescence can be easily analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The in vitro experiment established clarity and empirical evidence on the link between consuming cacao food-based products and inflammation. It is further concluded that UF-18 cacao powder exhibited a significant COX-1 inhibition potential about a standard drug. Moreover, the phenolic compounds of the powder having the highest amounts are attributable to its anti-inflammatory activity, as described further by related literature. Hence, the researcher recommends conducting further studies on the COX-2 inhibition potential of the said powder and isolation of the phenolic compounds as a potential alternative medicine. Moreover, in vivo, studies may also be conducted specifically on the toxicological characteristic of the UF-18 cacao powder as a phase of its pre-clinical trial.

5. References

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