

ORIGINAL CONTRIBUTION

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Antidiabetic, anti-inflammatory and cytotoxic potential of *Theobroma cacao* Linn. husk aqueous extracts

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Abstract

Background: *Theobroma cacao* Linn. husks are considered agricultural wastes, but studies show that they exhibit natural compounds that may be used in alternative medicine. Hence, this study was conducted to determine the antidiabetic, anti-inflammatory, and cytotoxic potential of *T. cacao* husk aqueous extracts (TCE).

Results: A significantly higher glucose dialysis retardation index (GDRI) was shown by 10% TCE than the rest of the concentrations (1%, 3%, 5%, 7%) ($P < 0.05$), and its activity is comparable with 5% Metformin (positive control) after 30 min, 60 min, 120 min and 180 min of incubation. The 7% TCE also showed significantly higher GDRI than the 5%, 3%, and 1% concentrations ($P < 0.05$), and its activity is comparable with 5% Metformin after 60 min and 180 min of incubation. Moreover, 10% TCE exhibited a significantly higher glucose uptake percentage than the rest of the samples ($P < 0.05$) and has activity comparable with 5% Metformin. The anti-inflammatory assay showed a significantly higher inhibition rate of 10% TCE than the rest of the concentrations ($P < 0.05$), and its activity is comparable with 5% Diclofenac (positive control). The cytotoxicity assay showed that the percentage mortality of brine shrimps after 24 h of exposure to the different TCE samples is less than 50%.

Conclusion: This study concludes that *T. cacao* husk aqueous extract has potential antidiabetic and anti-inflammatory properties without being toxic to cells.

Keywords: Diabetes, Inflammation, Cytotoxicity, *Theobroma cacao* Linn. Husks, Alternative medicine

Background

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, protein, and fat metabolism. It is one of the most common forms of chronic diseases, globally affecting almost all ethnic groups. Around 285 million people worldwide have diabetes, most of them living in developing countries. The World Health Organization (WHO) estimates that the number of people with diabetes mellitus could increase to 438 million by 2030 worldwide [1]. There are over 3.7 million cases of diabetes recorded in the Philippines in 2017, with a prevalence of 6.2% [2].

Inflammation is a tissue reaction that delivers mediators of host defenses, such as circulating cells and proteins, to sites of infection and tissue damage. However, uncontrolled inflammation may worsen tissue injuries and lead to other chronic diseases [3]. Treatment of most inflammatory illnesses with steroidal anti-inflammatory drugs and nonsteroidal anti-inflammatory drugs may cause adverse effects in the body, such as gastric ulcers and renal failure if used for prolonged periods. Thus, there is an increased research interest in discovering alternative anti-inflammatory agents with little or no side effects [4].

Theobroma cacao Linn. (*T. cacao*) is a tropical tree species of the family Sterculiaceae. The recovery of the beans from the fruit, which is the primary economic part of *T. cacao*, generates large amounts of waste in the

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form of cocoa husks (CH) [5]. Nevertheless, some studies have presented alternatives to create valuable products out of CH, representing up to 75% of the weight of the fruit [6]. CH is a source of natural compounds that may be used as alternative antioxidant medicine [7] and anti-bacterial treatment [8]. There are currently no published researches about the alternative potential of CH as an antidiabetic and anti-inflammatory agent. Still, it is reported to be rich in fiber and bioactive polyphenols that may be used in generating pharmaceuticals [9]. *T. cacao* is the main ingredient of chocolate and other derived products such as cocoa liquor, butter, cake, and powder. In contrast, the produced husks left during the extraction of beans are often discarded. Cocoa husks have also been explored as animal feed, a precursor in the activated carbon production, fertilizer, and thermoplastic polyurethane composites [10]. Cocoa husks vary in color, usually from maroon to green, and thickness when ripe depending on their clone. These husks are also being used as a source of potash, colorant, gum, and anti-hypercholesterolemia supplement [11]. Cocoa husks may also be a valuable source of dietary fiber-rich food materials that may significantly reduce the risk of the development of various free radical-induced diseases [12].

The Philippines is one of the potential producers of *T. cacao* in the world. Mainly, Davao Region in the south of the Philippines is one of the country's biggest producers of this crop. The Philippine Statistics Authority – Bureau of Agricultural Statistics reported that the region produced 77% of the country's *T. cacao* supply from 2008 to 2012 [13]. This industry, however, may also bring a severe problem to the Philippines due to the continuous generation of CH. Reports show an estimate of seven tons of CH produced for each ton of dry seed [8]. These husks are generally left behind in the farms and may provide nutrients for various pathogenic microorganisms [14]. Hence, this study was conducted to determine the antidiabetic, anti-inflammatory, and cytotoxic potential of aqueous extracts from *T. cacao* husks, which are considered agricultural wastes. The antidiabetic potential of TEC was determined using in vitro methods namely glucose diffusion assay and glucose uptake by yeast cells assay. Egg albumin denaturation assay is the in vitro method used to determine the anti-inflammatory potential of the extract. Brine shrimp lethality assay was the cytotoxicity method used.

Methods

Collection and drying of *T. cacao* husk powder

The *T. cacao* plant was authenticated at the Ateneo de Davao University (AdDU) Biological Collections, Davao City, Philippines. The voucher specimen (AdDU2019.01) was kept in the AdDU Herbarium for

future reference. *T. cacao* husks (1 kg) were collected from Calinan, Davao City, Philippines. They were washed with distilled water three times to remove contaminants and blot-dried with paper towels. The husks were peeled using a stainless peeler, placed in plastic trays, and air-dried at room temperature for four weeks. The peelings were transferred in different, clean trays every day for four weeks to avoid contamination. At the end of the fourth week, the peelings were oven-dried (BIO-BASE BOV-T105F, Metro Manila, Philippines) for 24 h at 37°C. The dried peelings were milled using an electric blender (AVINAS AV-126, Bulacan, Philippines) and sifted using 0.75 mm sieves (MonotaRO, Japan). The powder (20 mg) was placed in a wide-mouthed amber glass bottle and kept at room temperature until further use.

Preparation of *T. cacao* husk aqueous extract

T. cacao powder (1 g) was suspended to 100 ml of deionized water and heated in a 60°C water bath (Thermo Fisher Scientific, Waltham, USA) for 1 h. The water bath temperature was checked every 5 min to ascertain that it is within the temperature range indicated. After heating, the suspension was filtered using a cheesecloth to a beaker. The filtrate was centrifuged (Fisher Scientific, Waltham, USA) at 3,000 rpm for 5 min, and the supernatant (20 ml) was placed in an amber glass bottle.

Preparation of *T. cacao* husk extract concentrations and positive controls

TCE was prepared by diluting different amounts of aqueous *T. cacao* extract to varying amounts of deionized water to make 10%, 7%, 5%, 3%, and 1% concentrations. Metformin and Diclofenac were the positive controls used for the antidiabetic assays and anti-inflammatory assay, respectively. A 5 mg Metformin tablet was dissolved in 100 ml deionized water to make a 5% concentration. The same procedure was used in preparing a 5% concentration of Diclofenac. TCE concentrations for the brine shrimp lethality assay were made by diluting different amounts of aqueous cacao extract to varying quantities of artificial seawater to make 10%, 7%, 5%, 3%, and 1% concentrations. The artificial seawater was prepared by dissolving 3.8 g of non-iodized rock salt in 100 ml deionized water. All solutions were stored in amber glass bottles and kept in a refrigerator (4°C) until further use.

Glucose diffusion assay

The protocol used in the study was based on Ahmed et al. [15]. This assay was conducted to determine if the TCE samples can inhibit the movement of glucose across a dialysis membrane, indicating a potential to reduce sugar levels in the bloodstream. Reaction mixtures

consisting of 1 ml of 25 mmol/L glucose solution, 1 ml of 0.15 mol NaCl solution, and 1 ml of the different TCE samples and 5% Metformin were prepared in separate dialysis bags (Thermo Fisher Scientific, Waltham, USA). The dialysis bags were tied and immersed in separate beakers containing 10 ml of deionized water and 40 ml 0.15 mol NaCl solution. The reaction mixtures were incubated at room temperature. An aliquot (2 ml) of dialysate from every mixture was transferred using a micro pipettor in different test tubes after 30, 60, 120, and 180 min of incubation. Benedict's reagent (Chem Vest Commercial Trading, Davao, Philippines) (2 ml), which served as an indicator of glucose content, was added to every test tube, shaken carefully for 1 min, and heated in a water bath at 70°C for 5 min. An aliquot (500 µl) of the mixture from each test tube was then transferred in a microwell, and the absorbance was read at 540 nm using Epoch Microplate Spectrophotometer (Biotek, Winooski, USA). The experiment was conducted in triplicates and three independent trials. Glucose dialysis retardation index (GDRI) was calculated using the formula:

$$GDRI (\%) = 1 - \frac{\text{Glucose content (Samples } \frac{\text{mg}}{\text{mL}})}{\text{Glucose content (Without Sample } \frac{\text{mg}}{\text{mL}})} \times 100$$

where: samples = glucose content of the samples and controls.

Glucose uptake by yeast cells assay

The method used in the study was based on Tarnam et al. [16]. An antidiabetic substance can increase glucose uptake in different cells and tissues, reducing its amount in the bloodstream. This assay was conducted to test if the TCE samples are potentially antidiabetic by determining if they can increase glucose uptake using yeast cells as model specimens. Commercial baker's yeast was dissolved in distilled water and centrifuged (Fisher Scientific, Waltham, USA) at 3000 rpm for 5 min. The supernatant (1 ml) was added to 9 ml of distilled water to make a 10% (v/v) yeast suspension in a beaker. In different test tubes, 1 ml of the TCE samples and 5% Metformin were added separately using a micro pipettor to 1 ml of varying glucose solutions (5 mmol/L, 10 mmol/L, and 25 mmol/L) and incubated for 10 min at 37°C. To each of the mixtures, 100 µl of yeast suspension was added to start the reaction. The mixtures were vortexed for 1 minute and incubated at 37°C for 60 min. The mixtures were added with 2 ml of Benedict's reagent and heated at 60°C in a water bath for 5 min. An aliquot (300 µl) from every mixture was transferred in a

microwell, and absorbance was read at 540 nm using Epoch Microplate Spectrophotometer (Biotek, Winooski, USA). The experiment was conducted in triplicates and three independent trials. The percentage glucose uptake by yeast cells was calculated using the formula:

$$\text{Percent Glucose Uptake by Yeast Cells} = \frac{\text{Abs Sample} - \text{Abs Blank}}{\text{Abs Untreated Control} - \text{Abs Blank}} \times 100$$

where: Abs Sample = absorbance of the reaction mixtures; Abs Untreated Control = absorbance of the mixture without the samples or positive control; Abs Blank = absorbance of deionized water

Egg albumin denaturation assay

The protocol used in the study was based on Ullah et al. [17]. This assay determined if the TCE samples can inhibit protein denaturation, an event that activates inflammatory responses in the body. The assay was conducted by mixing 200 µl of egg albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (PBS) (pH 6.4), and 2 ml of the samples or control. The mixtures were incubated at 37°C for 15 min and heated in a water bath at 70 °C for 5 min. The mixtures were cooled for 5 min, and 500 µl of every mixture was transferred in a microwell. The absorbance of every sample was read at 660 nm using Epoch Microplate Spectrophotometer (Biotek, Winooski, USA). The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Percent Inhibition of Protein Denaturation} = 1 - \frac{\text{Abs Sample} - \text{Abs Blank}}{\text{Abs Untreated Control} - \text{Abs Blank}} \times 100$$

where: Abs Sample = absorbance of the reaction mixtures; Abs Untreated Control = absorbance of the mixture without the samples or positive control; Abs Blank = absorbance of deionized water.

Brine shrimp lethality assay

The protocol used in the study was based on Meyer et al. [18] and Guevarra [19]. This assay was conducted to determine the cytotoxicity of the TCE samples. Artemia salina eggs (5 mg) were hatched in a shallow rectangular dish (approximately 1 inch deep) filled with artificial seawater. The rectangular dish is clamped with a plastic divider punched with 2 mm holes to make two unequal compartments. The eggs were sprinkled into the larger chamber and illuminated for 48 h. The hatched

brownish nauplii that moved to the illuminated smaller compartment were collected with a Pasteur pipette and used for the assay. An aliquot (3 ml) from every TCE sample was placed in a microwell, and ten brine shrimps were transferred in every well using pipettes. The number of alive and dead brine shrimps was counted after 24 h of exposure to the TCE samples. Brine shrimps were considered dead if they did not exhibit any movement during several seconds of observation. The experiment was conducted in five replicates and three independent trials. Percentage mortality was calculated using the formula: % Mortality of Brine Shrimps=(No.of Dead Brine Shrimp/s)/(Total No.of Brine Shrimps) ×100.

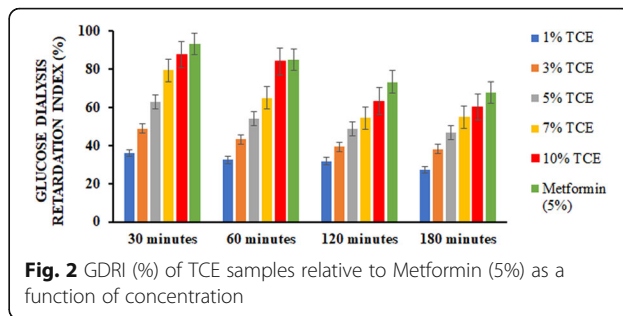
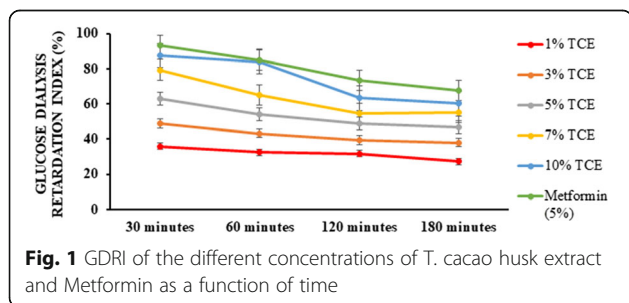
Statistical analysis

All values from the antidiabetic and anti-inflammatory bioassays were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test. The values of $P \leq 0.05$ are considered statistically significant. This test determined if the results of the antidiabetic and anti-inflammatory assays of the extracts have significant differences compared to the results of the positive controls.

Results

Glucose diffusion assay

Glucose diffusion assay was conducted to determine the inhibitory effect of the TCE concentrations relative to 5% Metformin on the movement of glucose across a dialysis membrane. Figure 1 reveals that the GDRI results of the TCE samples decrease with time. It is observed that at 120 min of incubation, the GDRI of the samples seems to have already reached saturation. Figure 2 shows a dose-dependent inhibitory action at 30, 60, 120, and 180 min of incubation. GDRI (%) decreased with decreasing TCE concentration. Furthermore, 10% TCE showed a significantly higher GDRI than the rest of the concentrations ($P < 0.05$), and its potential is comparable with 5% Metformin. The 7% TCE also showed significantly higher GDRI than 5%, 3%, and 1% TCE ($P < 0.05$), and its potential is comparable with 5% Metformin after 60 min and 180 min of incubation.



Glucose uptake by yeast cells assay

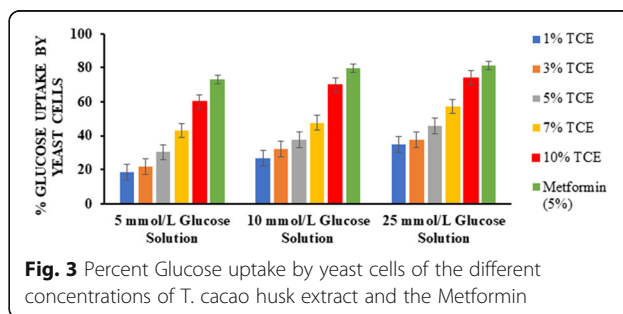
Glucose uptake by yeast cells assay was conducted to determine if the TCE concentrations can induce movement of 5 mmol/L, 10 mmol/L, and 25 mmol/L glucose solutions into yeast cells. Figure 3 shows the percentage glucose uptake by yeast cells of the different TCE samples relative to 5% Metformin. Data from the assay show that the rate of glucose uptake increases with increasing sample concentration using different glucose solutions. It is also shown that 10% TCE has a significantly higher glucose uptake percentage than the rest of the samples in all glucose solutions ($P < 0.05$) and has activity comparable with 5% Metformin.

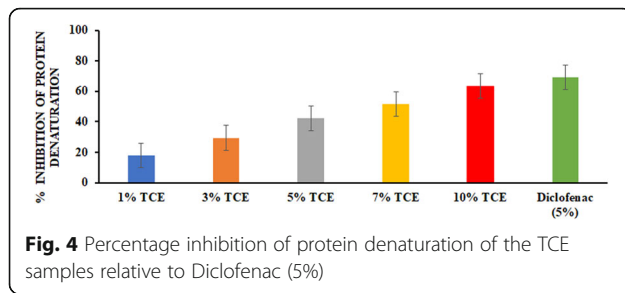
Egg albumin denaturation assay

Egg albumin denaturation assay was conducted to determine the inhibitory potential of TCE extracts on protein denaturation. Figure 4 shows the TCE samples’ inhibitory rate relative to 5% Diclofenac (positive control). Data from the assay reveals that the samples inhibit protein denaturation in a dose-dependent manner. Results also show that 10% TCE has a significantly higher inhibition rate than the rest of the samples ($P < 0.05$) and has activity comparable with 5% Diclofenac.

Brine shrimp lethality assay

Brine shrimp lethality assay was conducted to determine if the different TCE samples are toxic to cells. Figure 5 shows the percentage mortality of brine shrimps after 24 h of exposure to the different TCE samples. Data show that the percentage mortality of brine shrimps is dose-dependent, with the 1% TCE exhibiting a 0%



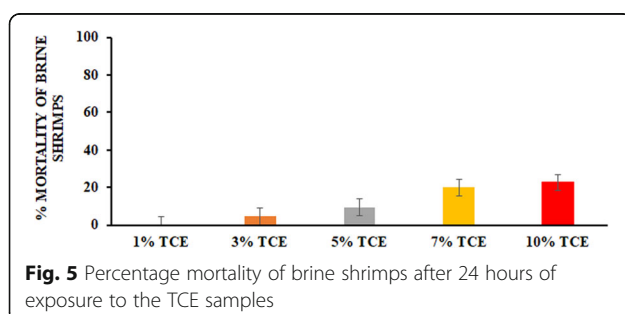


result. It is also revealed that all the TCE samples are non-toxic since the mortality rate of brine shrimps did not reach 50%.

Discussion

The GDRI of the different TCE concentrations decreased with time from 30 to 180 min based on the glucose diffusion assay. This trend may be attributed to the fiber contents of *T. cacao* husks. Yapo et al. [12] showed that *T. cacao* husk contained 59% dietary fiber, including 11% soluble dietary fiber and 48% insoluble dietary fiber. Mishra and Jha [20] demonstrated that the glucose diffusion rate of fibers from *Tamarindus indica* and *Cassia fistula* decreased with time. At the beginning of dialysis, diffusion of glucose was affected by adsorption and viscosity of fibers which slowed the diffusion rate of glucose even if the concentration in the dialysis bag was high. As the adsorption saturated, the diffusion of glucose was affected only by the viscosity of the fibers. Moreover, the diffusion rate was not significantly decreased even when the glucose concentration in the dialysis bag was reduced.

The potential ability of TCE samples to retard glucose diffusion across the membrane may also be attributed to the fiber content of the husk and the viscosity of the extract. This data agrees with the study of Ahmed et al. [15], which showed that *Psyllium* fibers have significant inhibitory effects on the movement of glucose into an external solution across a dialysis membrane compared to a control. The retardation in glucose diffusion may be due to the physical obstacle created by the fiber particles and the entrapment of glucose within such fibers preventing postprandial glucose rise. *T. cacao* husk fibers



may inhibit glucose diffusion in the small intestine through adsorption or inclusion of glucose within the structure of the fiber particles. The formation of a viscous matrix in an aqueous solution may delay gastric emptying and slow glucose access to the small intestine's absorptive epithelium, thereby reducing postprandial glucose peaks. Rehman et al. [21] also noted that dietary fibers might have inhibitors against alpha-amylase, an enzyme that digests starch, inhibiting starch digestion and reducing postprandial hyperglycemia. Mishra and Jha [20] also reported that fibers have the effects of hampering the diffusion of glucose and postponing the absorption and digestion of carbohydrates, thus resulting in lowered blood glucose levels. In this study, a similar mechanism for reducing glucose diffusion may be due to the viscosity and fiber components of the TCE samples.

Results of the study also show that the samples can induce glucose uptake by yeast cells. This potential may be attributed to the polyphenol contents of TCE, as reported by Adi-dako et al. [5]. This observation is consistent with the study of Nagano et al. [22], which demonstrated that black tea polyphenols promoted glucose transporter 4 (GLUT4) translocation and glucose uptake through P13K- and AMPK-dependent pathways in L6 cells. The translocation of GLUT4 in the plasma membrane facilitates the influx of glucose into the cells, reducing glucose levels in the bloodstream. Lankatillake et al. [23] reported that in muscle tissue, insulin promotes the translocation of GLUT4 transporters to the cell surface, thereby enhancing insulin-stimulated glucose uptake by the cells. Adipocytes also express the insulin-sensitive GLUT4 transporter. Therefore, insulin increases fatty tissue glucose uptake by enhancing the expression of GLUT4, which in turn increases glycolysis. TCE may exhibit similar mechanisms with insulin in promoting glucose uptake into cells, thereby lowering blood glucose levels.

Data of the study also reveals that TCE can inhibit protein denaturation. The capacity of the samples to inhibit protein denaturation may also be attributed to its polyphenol contents, including flavonoids [5]. Bouhlali et al. [24] reported that the interaction of proteins with polyphenolic compounds improved their thermal stability. This finding is supported by the study of Ali et al. [25] that showed improvement of thermal stability in the proteins soybean glycinin, bovine serum albumin (BSA), and bovine p-lactoglobulin triggered by phenolic compounds. This result may suggest that phenolic interactions strongly affect the secondary structure of proteins. Ojha et al. [26] also reported that the interaction of BSA with ferulic acid improved its thermal stability. Bouhlali et al. [24] found strong correlations between the inhibition of protein denaturation effect and the flavonoid compounds caffeic acid, ferulic acid, luteolin,

rutin, and quercetin. Similar phytochemicals may explain the potential of TCE to inhibit protein denaturation in this study. Murakami et al. [27] also demonstrated that polyphenols could increase the expression of some heat shock proteins (HSP) that maintain the stability of proteins and prevent it from being denatured even in the presence of a stressor like heat. HSP expression, which are highly conserved families of proteins, allows misfolded and unfolded proteins to achieve functional conformation. Their expression and activity status are considered critical determinants of homeostasis, health, and longevity. Continuous HSP expression prevents protein denaturation, thereby avoiding events that may lead to inflammatory responses in the body.

Data also shows that the TCE samples used in the study are non-toxic since the mortality of brine shrimps did not reach 50% in all concentrations after 24 hours. These results are consistent with the data of Campos Filho et al. [28], reporting that the potential damage of *T. cacao* in leukocyte DNA is relatively low, even in high concentrations. In the study of Santos et al. [29], *T. cacao* husk extracts up to 10 µg/ml concentrations did not cause significant DNA breakage on mammalian cells. The DNA lesions observed in the study may be strand breaks relevant to DNA modifications such as gene mutations. However, small lesions may be repaired without resulting in genetic changes. These data may imply that the TCE samples used in the study may not damage the structure of DNA and cell membranes, possibly indicating low toxicity. Determining the bioactive properties of TCE might provide alternative treatments for diabetes and inflammation while reducing the agricultural wastes generated from the continuous production of such husks. The determination of the antidiabetic and anti-inflammatory potential of TCE may also further establish *T. cacao* as a priority crop in the Philippines to help address sustainable growth in the country.

Conclusion

This study shows that TCE has antidiabetic and anti-inflammatory potentials without being toxic to cells. To date, no study was conducted on the possibility of such extract as an alternative medicine for diabetes and inflammation. This study used in vitro antidiabetic and anti-inflammatory assays, and the conduct of in vivo methods to support the study results is warranted. Conducting further cytotoxic investigations is also recommended.

Abbreviations

TCE: *Theobroma cacao* Linn. husk aqueous extracts; GDRI: Glucose Dialysis Retardation Index

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Authors' contributions

The author(s) read and approved the final manuscript.

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Availability of data and materials

The data and materials in the study are available from the authors on reasonable request.

Declarations

Ethics approval and consent to participate

This is an in vitro study that does not involve animals and human subjects. Hence, ethics approval is not applicable.

Consent for publication

All authors agreed to the publication of the research.

Competing interests

Not applicable.

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