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Phytochemical Contents and Multifunctional Bioactivities of Cocoa Pod Husk Extracts

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ABSTRACT

Cocoa pod husk (CPH), a main residue of cocoa processing, is commonly discarded or underutilized despite its potential to be a sustainable source of health-promoting phytochemicals. Investigating its biological activities could support both environmental sustainability and the development of value-added functional products. This study aimed, for the first time, to comprehensively evaluate the antiglycation, tyrosinase inhibitory, and acetylcholinesterase inhibitory activities of CPH extracts, together with their total phenolic and flavonoid contents and antioxidant capacities. Total phenolic and flavonoid contents were determined using standard colorimetric assays. The activity of antioxidant was assessed by DPPH and ABTS radical scavenging methods. Anti-tyrosinase and anti-acetylcholinesterase activities were analyzed using *in vitro* enzyme activity assays, while antiglycation activity was examined using bovine serum albumin (BSA)-glucose and BSA-methylglyoxal systems. Among the tested extracts, the ethanolic extract demonstrated greatest total phenolic (84.18 mg GAE/g extract) and flavonoid contents (73.78 mg QE/g extract). It also showed the strongest antioxidant capacities, yielding IC_{50} values of 15.75 µg/mL (DPPH) and 19.86 µg/mL (ABTS). In addition, this extract demonstrated the most potent inhibitory effects against tyrosinase (IC_{50} = 155.58 µg/mL) and acetylcholinesterase (IC_{50} = 85.23 µg/mL), as well as the highest antiglycation activity of BSA-MGO and BSA-glucose systems. These results suggest that CPH represents a valuable source of multifunctional biologically active compounds, supporting its potential use in functional foods and nutraceuticals while contributing to the sustainable valorization of cocoa industry by-products.

Keywords: Cocoa pod husk, Tyrosinase inhibitory activity, Acetylcholinesterase inhibitory activity, Antiglycation activity

Introduction

Theobroma cacao L. had long been employed for both food and medicinal purposes, and its industrial demand continues to increase. In particular, cocoa beans serve as an essential source material for products for example chocolate, cocoa powder and cocoa butter. The worldwide consumption of cocoa is steadily rising each year, raising concerns regarding the sustainability of the cocoa industry. Cocoa processing generates substantial quantities of cocoa pod husk (CPH) as a by-product, which constitutes approximately 52–76% of the whole cocoa fruit. These components are often considered waste and are commonly utilized as organic fertilizers on farms, facilitating nutrient recycling back into the soil after decomposition. However, the accumulation of such waste can result in environmental pollution and the proliferation of pests and diseases if improperly managed. [1-3] In recent years, the isolation of value-added compounds from agro-industrial residue has attracted growing scientific interest.

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Non-edible agricultural residues, traditionally viewed as waste, often contain abundant phytonutrients and bioactive compounds that can be converted for application in value-added products, including food additives, nutraceuticals, phytotherapeutics, and cosmetic products.[1,4] Concurrently, increasing consumer recognition of the association between diet and health has driven demand for functional foods enriched with biologically active compounds, particularly those with antioxidant properties. Among these, phenolics, flavonoids, and anthocyanidins are important secondary metabolites exhibiting strong antioxidant potential. These compounds play critical roles in physiological regulation and in mitigating the danger of chronic diseases, including cancer, diabetes, heart-related disorders and neurodegenerative conditions. Consequently, recent research has focused on finding natural antioxidants in agricultural residues to drive the development of functional food formulations and health-beneficial products.[5-9] In previous reports, the determination of the bioactive compound content and the antioxidant activity in cocoa residues was studied most commonly.[10,11]It was found that the cocoa pod husk (CPH), which is a cocoa bean residue, contains macronutrients such as proteins, fibers, and carbohydrates, as well as minerals.[12-16]Moreover, numerous studies have reported that the main compounds responsible for the multifunctional properties of cocoa by-products are flavanol compounds such as epicatechin and catechin. In addition, methylxanthines, including theobromine and caffeine, are also present in cocoa and have been connected with various potential health-related effects including antibacterial, antiviral, anticarcinogenic, and anti-inflammatory activities.[17-19]The intake of biologically active substances has been demonstrated to confer antioxidant and anti-mutagenic properties. By preventing the oxidative degradation of biomolecules such as DNA, lipids, carbohydrates and proteins, these compounds mitigate the damaging effects of free radicals. Consequently, it

participates in reducing the tendency to develop chronic diseases, including cancer, diabetes, and cardiovascular syndromes.[20,21] These bioactive constituents make CPH a promising natural resource for further utilization. Several studies have investigated the utilization of cocoa-based phytochemicals in skincare applications, showing that compounds such as catechin can protect against oxidative damage by modulating antioxidant enzyme activity.[16,17,22] In addition, CPH has been explored as a feed component for poultry, cattle, and swine and, more recently, as an ingredient in tea beverages due to its high fiber and protein content. Cocoa husk tea has been related to several physiological benefits, including lipid reduction, relaxation, and improved sleep quality.[1]

Despite increasing evidence that cocoa pod husk (CPH) is rich in many active compounds with strong antioxidant potential, most previous studies have focused primarily on its phytochemical composition and free-radical scavenging activity. However, oxidative stress is only one of several biochemical pathways involved in chronic diseases. To fully assess the health-related value of CPH, it is therefore essential to assess its effects on key disease-related biological targets, particularly those associated with neurodegeneration, skin disorders, and metabolic complications. Acetylcholinesterase (AChE) inhibition is an important therapeutic approach for Alzheimer's disease, as it enhances cholinergic neurotransmission by preventing acetylcholine degradation, and many plant polyphenols are known to act as natural AChE inhibitors.[23-26] Tyrosinase inhibition is relevant to the control of melanin synthesis and is therefore important for managing hyperpigmentation and for cosmetic applications, where plant-derived phenolics are favored for their safety and dual antioxidant–depigmenting effects.[27-29] Antiglycation activity is also critical, as the generation of advanced glycation end products (AGEs) contributes to diabetic complications and tissue aging; polyphenols can inhibit these processes by trapping reactive carbonyl intermediates and reducing oxidative stress. [30,31] Despite its promising potential, the broader biological activities of CPH particularly its acetylcholinesterase (AChE) inhibitory, tyrosinase inhibitory, and antiglycation properties have not yet been systematically studied. Therefore, in this work, we aimed for the first time to evaluate the AChE inhibitory, tyrosinase inhibitory, and antiglycation properties of CPH extracts in association with their total phenolic and flavonoid levels including antioxidant capacities. This comprehensive approach enables a more complete understanding of CPH bioactivity and supports its sustainable development as a high-value ingredient for food, nutraceutical, and cosmeceutical applications, while also contributing to environmental sustainability and added income for cocoa farmers.

Materials and Methods

Chemicals and reagents

L-tyrosine, mushroom tyrosinase, kojic acid, Folin–Ciocalteu's reagent, quercetin, gallic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE, type VI-S, from electric eel), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), galantamine, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), bovine serum albumin (BSA), methylglyoxal (MGO), and acarbose were acquired from Sigma-Aldrich (St. Louis, MO, USA). All organic solvents were bought from Merck Co. (Darmstadt, Germany).

Preparation of dried plant materials

Cocoa pod husks (CPHs) were obtained in November 2023 from the Community Enterprise for Cocoa in Na Ngua, Mueang Phetchabun, Phetchabun Province, Thailand. The plant material was taxonomically authenticated, and a voucher specimen (No. NU-H:2906/2023) was deposited in the NU Botanical Herbarium, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand. The plant sample was manually cut into small pieces and freeze-dried for three days to preserve the integrity of its phytochemical constituents. The dried sample was finely pulverized using a high-speed mixer. The resulting powder was preserved in airtight storage containers at 4 °C until further analysis.

Preparation of Cocoa Pod Husk (CPH) crude extracts

Crude extract preparation was carried out according to the previously published method [26] with minor adjustments. Briefly,5.00 g of the dried sample was sequentially extracted using different solvents including hexane, ethyl acetate, and ethanol. The initial extraction was performed with hexane (100 mL), during which the sample was thoroughly shaken and then centrifuged to separate the phases. The hexane phase was collected, and the residual plant material was re-extracted twice to improve recovery. The combined hexane fractions were reduced to dryness under vacuum using a rotary evaporator (R-114 RE Buchi, Thailand) and subsequently dried under vacuum at ambient temperature to obtain the hexane crude extract. This same procedure was repeated with ethyl acetate (100 mL) and ethanol (100 mL) on the residual plant material to obtain the corresponding ethyl acetate and ethanol crude extracts. All dried extracts were precisely weighed and stored at 4 °C.

Determination of Total Phenolic Content (TPC)

Total phenolic content was evaluated employing the Folin–Ciocalteu colorimetric assay, a well-established method recognized for its simplicity, sensitivity, and reproducibility, through gallic acid employed as the reference standard.[26,32] Briefly, 0.5 mL of crude extract (1 mg/mL) was made up to 1 mL with methanol prior to the addition of 1.5 mL of Folin–Ciocalteu reagent. The mixture was subsequently neutralized by adding 2 mL of 7.5% (w/v) sodium carbonate. The mixtures were maintained at ambient temperature for 30 minutes to obtain color generation. Absorbance was observed at 765 nm via a microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand). All analyses were obtained in three replicates. Total phenolic content was estimated from a calibration curve of gallic acid and reported as milligrams of gallic acid equivalents (mg GAE) per gram of crude extract.

Evaluation of Total Flavonoid Content (TFC)

Total flavonoid content was evaluated by an aluminum chloride–based colorimetric assay, as described in a previously reported protocol[32] using quercetin to generate the standard calibration curve. For the assay, 0.3 mL of the extract was combined with 3.4 mL of 30% methanol, 0.15 mL of 0.5 M sodium nitrite ($NaNO_2$), and 0.15 mL of 0.3 M aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$). After 5 minutes, 1 mL of 1 M sodium hydroxide (NaOH) was applied to the reaction solution. The solution was then mixed and maintained at room temperature for 60 minutes. Absorbance was measured at 420 nm against a blank using microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand). The flavonoid content was estimated from the standard calibration curve and presented in relation to milligrams of quercetin equivalents (mg QE) per gram of crude extract.

Evaluation of Antioxidant Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity method

The antioxidant activity of the samples was tested on the basis of their free radical scavenging capacity against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as reported by Alías,[33] with slight adjustments. Briefly, 100 µL of crude extract solutions at various concentrations were transferred into wells of microplate. DPPH solution (0.33 mM, 100 µL) was subsequently applied to each well. The mixtures were thoroughly mixed and maintained for 30 minutes under dark conditions at ambient temperature. The absorbance was then recorded at 517 nm using a microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand). Ethanol acted as the blank, while Trolox was used for the reference standard. All assays were conducted with three replicates to confirm reliability. The percent scavenging of DPPH radicals was determined employing the absorbance values of the control and tested samples according to Equation 1. The IC_{50} was quantified employing nonlinear dose–response curve fitting, plotting % inhibition against extract concentration.

$$[(A_c - A_s)/A_c] \times 100 \quad (1)$$

where A_c denotes the absorbance of the control sample (all reagents excluding the sample), and A_s represents the absorbance of the reaction mixture containing the sample.

-2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity method

The antioxidant capacity of the crude extracts was also tested using the ABTS free radical cation decolorization assay as previously described [33,34] with slight adjustments. The $ABTS^{\bullet+}$ stock solution was achieved through combining equal volumes (1:1, v/v) of 10 mM ABTS and 3.5 mM potassium persulfate, followed by kept under dark conditions at ambient temperature for 16 hours to ensure fully developed radical formation. The $ABTS^{\bullet+}$ solution was first diluted in ethanol to obtain an initial absorbance (A_0) of 0.70 ± 0.02 at 734 nm. In the assay, each sample solution or Trolox standard (100 µL) was combined with 100 µL of the $ABTS^{\bullet+}$ reagent. After vortex mixing, the reaction solution was allowed to stand in the dark for 30 minutes at ambient temperature, and absorbance was then recorded at 734 nm through a microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand), with ethanol as the blank. The radical scavenging percentage was obtained employing the equation as in the (DPPH) free radical assay. The IC_{50} value was subsequently determined.

Investigation of Acetylcholinesterase (AChE) inhibitory activity

The acetylcholinesterase inhibition was evaluated employing a modified Ellman's spectrophotometric assay, employing acetylthiocholine iodide (ATCI) as the substrate.[35,36] In this reaction, AChE catalyzes the hydrolysis of ATCI to yield thiocholine and acetic acid. The amount of the resulting thiocholine is determined by a reaction with DTNB, normally known as Ellman's reagent, to yield 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, where the latter product a yellow coloration measurable at a wavelength of 405 nm. The AChE inhibitory activity of each crude extract was evaluated utilizing 96-well microplates read on a microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand). The sample solution at several concentrations was added into a reaction mixture consisting of 25 µL of 0.5 mM ATCI, 125 µL of 3 mM DTNB and 50 µL of Tris(hydroxymethyl)aminomethane buffer (pH 8). The enzymatic reaction was started from addition of 25 µL AChE solution (0.28 U/mL). The absorbance was recorded at 405 nm at 10-second intervals for a total duration of 2 minutes. Each experiment was performed in triplicate. Prior to each measurement, the enzyme concentration was screened to ensure it fell within the range of 90–120% activity by substituting the sample solution with buffer and measuring absorbance at 405 nm. AChE inhibitory activity (%) was calculated using Equation (2).

$$[(V_{control} - V_{sample})/V_{control}] \times 100 \quad (2)$$

where $V_{control}$ is the mean velocity of the control reaction)comprising all reactants without the sample(and V_{sample} is the mean velocity of the reaction containing crude extract. The IC_{50} was subsequently determined.

Evaluation of Tyrosinase Inhibitory Activity

The tyrosinase inhibiton of the plant extracts was estimated employing a spectrophotometric assay according to the enzymatic oxidation of L-tyrosine as previously described [27] with slight modifications. In brief, 50 µL of L-tyrosine solution (1 mg/mL) was combined with 50 µL of each sample extract at varying concentrations in a 96-well microplate. The reaction was performed by adding 50 µL of tyrosinase enzyme solution (200 U/mL) to well. The mixtures were incubated at 37 °C for 60 minutes. The formation of dopachrome, the oxidation product of L-tyrosine, was quantified through absorbance measurement at 475 nm by a microplate reader (Synergy H1 Hybrid Reader, Agilent, Thailand). A reaction solution lacking the enzyme was used for the blank, while a mixture containing the solvent alone (without plant extract) served as the negative control. Kojic acid was employed as the positive control. Tyrosinase inhibition (%) was calculated using Equation (3).

$$[(V_{control475} - V_{sample475})/V_{control475}] \times 100 \quad (3)$$

where $A_{control\ 475}$ and $A_{sample\ 475}$ denote the absorbance for the control and the test sample, respectively, at 475 nm. The half-maximal inhibitory concentration (IC_{50}), representing the extract concentration expected to inhibit 50% of tyrosinase activity, was then quantified using GraphPad Prism version 5.

Determination of antiglycation activity

Bovine Serum Albumin (BSA)-glucose Model

The activity inhibiting protein glycation was evaluated following the method of Rakariyatham [37] with slight modifications. In brief, bovine serum albumin (BSA, 10 mg/mL) was incubated with 1.10 M glucose with or without of test samples at various concentrations. All samples were prepared in 50 mM phosphate buffer (pH 7.4) comprising 0.02% sodium azide (NaN_3) to prevent microbial contamination. The reaction solutions were maintained at 37 °C for 14 days. The generation of advanced glycation end-products (AGEs) was investigated by recording fluorescence signal by a microplate fluorescence reader, with excitation and emission wavelengths set at 360 nm and 420 nm, respectively. Aminoguanidine (AG), a well-characterized synthetic antiglycation agent, was served as a positive control at 1 mg/mL. The AGE formation inhibition percentage was quantified according to the following Equation (4).

$$[(F_{control} - F_{control\ blank}) - (F_{extract} - F_{extract\ blank})] / [(F_{control} - F_{control\ blank}) \times 100 \quad (4)$$

where $(F_{control} - F_{control\ blank})$ represents the net fluorescence signal of BSA incubated with glucose (excluding sample), and $(F_{extract} - F_{extract\ blank})$ represents the net fluorescence signal of BSA and sugars incubated with the sample. The blank samples, which exclude either sugars or sample, account for background fluorescence in each condition. The IC_{50} value, indicating the concentration of extract needed to suppress 50% of AGE formation, was determined by using GraphPad Prism version 5.

Bovine Serum Albumin (BSA)-methylglyoxal (MGO) Model

The BSA-MGO assay was evaluated following Rakariyatham's method[37] with slight modifications. Briefly, bovine serum albumin (BSA, 20 mg/mL) and methylglyoxal (MGO, 60 mM) were prepared in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.02% sodium azide (NaN_3) to prevent microbial expansion. Samples at various concentrations were applied to a reaction solution comprising 1 mL of the BSA-MGO solution and the reaction was subsequently incubated in the dark for 14 days at 37 °C, with aminoguanidine (1 mg/mL) employed as the positive control for protein glycation inhibition. Following incubation, the generation of advanced glycation end-products (AGEs) was quantified by monitoring fluorescence signals at excitation/emission wavelengths of 370/420 nm using a microplate fluorescence reader. All tested were conducted in three replicates. The AGE formation inhibition percentage was estimated employing the same equation as the BSA-glucose model, and the IC_{50} for each sample was determined.

Statistical analysis

Statistical analyses of all biological activities from crude extracts obtained using different solvents were investigated by one-way ANOVA at a 95% confidence interval, applying the general linear model in SPSS version 14.0 for Windows. A *p*-value below 0.05 was regarded as statistically significant. IC_{50} values were quantified by GraphPad Prism version 5 software, and all data are illustrated as mean ± standard deviation.

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Results and Discussion

Investigation of total extraction yield (%) and Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of Cocoa Pod Husk (CPH) crude extracts

As indicated in Table 1, total phenolic content ranged from 22.66 to 84.18 mg GAE/g crude extract, whereas total flavonoid content ranged from 18.75 to 73.78 mg QE/g crude extract. Statistical variation ($p < 0.05$) was observed among the extracts for both parameters. The ethanolic extract comprised the greatest concentrations of phenolics and flavonoids, followed by the ethyl acetate and hexane extracts. This trend can be ascribed to the higher polarity of phenolic and flavonoid constituents; whose hydroxyl groups enhance solubility in ethanol. In addition, phenolic and flavonoid compounds bound to sugar molecules (glycosylated forms) tend to dissolve more readily in polar solvents, as hydrogen bonding between the oxygen atoms of ethanol and the hydrogen atoms of glycosylated phenolics/flavonoids facilitates solubility.[38,39] These results are in agreement with previous studies reporting that alcohols are effective organic solvents for the isolation of phenolic and flavonoid compounds.

Investigation of antioxidant activity of Cocoa Pod Husk (CPH) crude extracts

Figures 1 and 2 demonstrate a high positive correlation between the DPPH and ABTS radical quenching assays, confirming the consistency of both methods. All crude extracts exhibited a dose-dependent enhancement of radical scavenging capacity, with antioxidant activity rising as extract concentration increased. Among the extracts, the ethanolic extract exhibited the greatest activity, with inhibition values of 92.09% (DPPH) and 92.71% ($ABTS^{\bullet+}$) at 200 µg/mL, along with the weakest IC_{50} values (15.75 µg/mL for DPPH and 19.86 µg/mL for $ABTS^{\bullet+}$), as shown in Table 2.

Table 1: Extraction yield (%) and Total phenolic and flavonoid contents found in cocoa pod husk (CPH) crude extracts

Crude samples	Extraction yield (%)	Total phenolic content GAE in mg/g crude extract	Total flavonoid content QE in mg/g crude extract
Hexane extract	3.02	22.26 ± 0.30[a]	18.75± 0.70 [a]
Ethyl acetate extract	4.45	65.06 ± 0.60 [a]	46.02± 0.50 [a]
Ethanolic extract	7.89	84.18 ± 0.70 [a]	73.78± 0.40 [a]

Data are expressed as mean ± standard deviation (SD).[a]Values within the same column bearing different superscript letters are significantly different ($p < 0.05$).

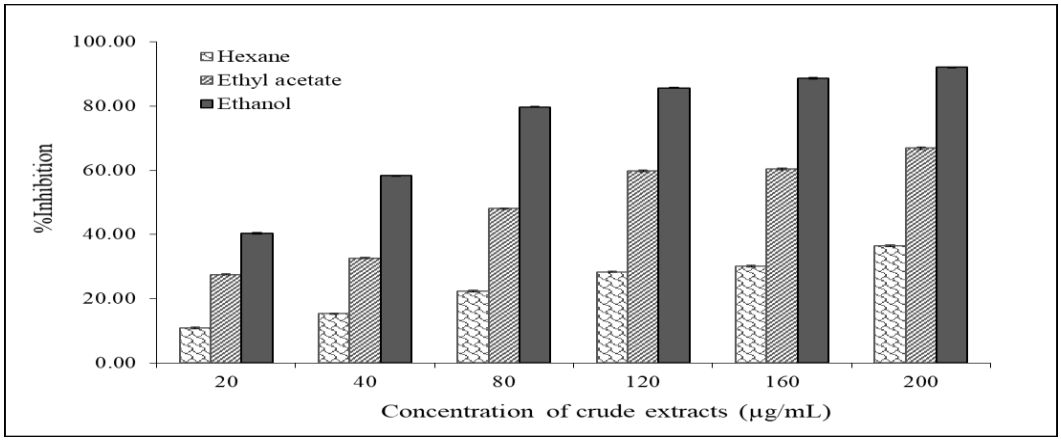

Figure 1: Antioxidant activity of the crude extracts at various concentrations tested by the DPPH radical scavenging assay; data represent mean ± SD, $n = 3$.

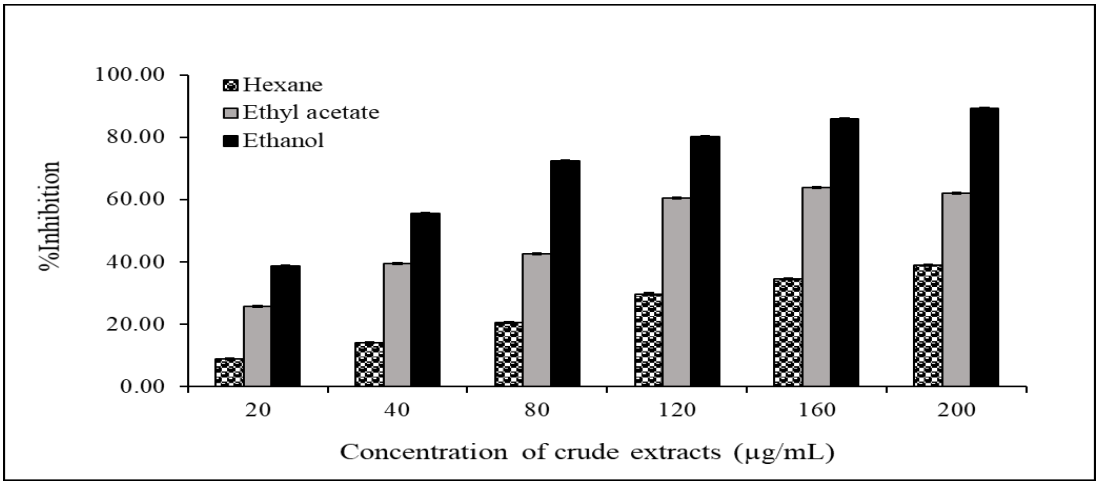

Figure 2: Antioxidant activity of the crude extracts at various concentrations assessed by the ABTS radical scavenging method. Data are indicated as mean ± SD (n = 3).

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Table 2: *In vitro* antioxidant activity by DPPH and ABTS radical scavenging methods (%inhibition and IC_{50} value) of cocoa pod husk crude extracts and Trolox

Samples	% Inhibition		IC_{50} µg/mL (Mean ± SD)	
	DPPH assay	ABTS assay	DPPH assay	$ABTS^{\bullet+}$ assay
Hexane extract (200 µg/mL)	36.48 ± 0.13	38.96 ± 0.15	242.45 ± 0.05	221.56 ± 0.09
Ethyl acetate extract (200 µg/mL)	66.89 ± 0.08	61.94 ± 0.18	88.76 ± 0.08	91.64 ± 0.08
Ethanolic extract (200 µg/mL)	92.09 ± 0.15	89.24 ± 0.10	15.75 ± 0.11	19.86 ± 0.12
Trolox (50 µg/mL)	92.23 ± 0.12	90.45 ± 0.18	5.0 ± 0.12	6.35 ± 0.13

Data are expressed as mean ± standard deviation (SD). Values within the column are significantly different ($p < 0.05$).

Previous studies have identified cocoa pod husk as an abundant source of bioactive compounds including lignin, phenolics, tannins, and alkaloids that contribute to its antioxidant potential. [16,17,19] The strong scavenging effect of the ethanolic extract is likely due to the higher solubility of these compounds in ethanol. Similar findings have been reported in earlier studies, which showed that ethanolic extracts often contain the majority of active compounds from plant materials and exhibit the strongest antioxidant activity.[40,41] Trolox, used as the positive control, demonstrated the highest inhibition at 50 µg/mL and the lowest IC_{50} value as presented in Table 2. The antioxidant capacity of the ethanolic extract was slightly less than the Trolox standard. These results suggest that the ethanolic extract can function as a free radical inhibitor, highlighting its potential as a valuable natural antioxidant source. Moreover, the findings showed a positive correlation with total phenolic and flavonoid contents. Consistent with earlier reports, the antioxidant activity of cocoa pod husk extracts is primarily attributed to phenolic compounds, whose radical scavenging properties are strongly influenced by the quantity and location of hydroxyl (OH) groups on the aromatic ring. In general, a higher degree of hydroxyl substitution enhances free radical scavenging activity by increasing hydrogen atom donation. Therefore, higher phenolic and flavonoid compositions are directly related to stronger antioxidant capacity, as reflected by the inhibition percentages. This observation aligns with previous studies reporting strong positive relationships between antioxidant activity and phenolic and flavonoid levels, where increased concentrations of these compounds enhance antioxidant properties.[40,42]

Investigation of acetylcholinesterase (AChE) inhibitory activity of Cocoa Pod Husk (CPH) crude extracts

Acetylcholinesterase (AChE) plays a central role in terminating cholinergic signaling by hydrolyzing acetylcholine in the synaptic cleft. In Alzheimer's disease and related neurodegenerative disorders, reduced acetylcholine levels contribute to memory impairment and cognitive decline. Inhibition of AChE prolongs cholinergic transmission and is therefore considered a promising therapeutic strategy. Many plants and phytochemicals have demonstrated AChE inhibitory activity, supporting the search for natural sources of neuroprotective agents.[21-22] In this study, the AChE inhibitory activity of cocoa pod husk (CPH) extracts was tested at concentrations between 20 and 200 µg/mL (Figure 3).

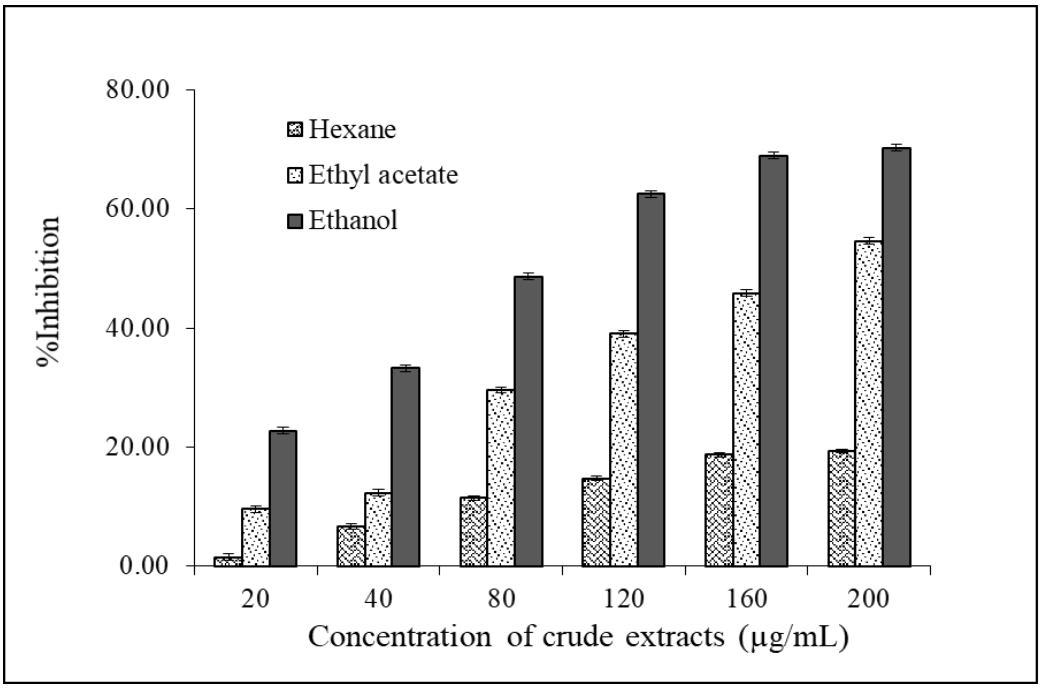

Figure 3: Acetylcholinesterase (AChE) inhibitory activity of the crude extracts at several concentrations; data are denoted as mean ± SD (n = 3).

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All extracts exhibited concentration-dependent inhibition, with the ethanolic extract showing the highest activity (70.23% inhibition at 200 µg/mL), indicating ethanol as the most effective solvent for extracting bioactive inhibitors. The ethyl acetate extract demonstrated moderate inhibition (54.64% at 200 µg/mL), while the hexane extract showed the lowest level of capacity. From the determination of the IC_{50} values (Table 3), it was found that the ethanolic extract produced the strongest AChE inhibition with an IC_{50} of 85.23 µg/mL, which is nearly two-fold more potent than the ethyl acetate extract (IC_{50} = 171.25 µg/mL) and substantially higher than the hexane extract (IC_{50} not reached).

Table 3: In vitro acetylcholinesterase (AChE) inhibitory activity (%inhibition and IC_{50} value) of cocoa pod husk crude extracts and galantamine.

Sample	% Inhibition	IC_{50} µg/mL (Mean ± SD)
Hexane extract (200 µg/mL)	19.34 ± 0.33	ND
Ethyl acetate extract (200 µg/mL)	54.64 ± 0.53	171.25 ± 0.45
Ethanolic extract (200 µg/mL)	70.23± 0.55	85.23 ± 0.41
Galantamine (positive control, 50 µg/mL)	96.47 ± 0.45	18.67 ± 0.39

Data are expressed as mean ± standard deviation (SD). Values within the column are significantly different ($p < 0.05$). ND = not detectable

This result indicates that the major AChE-inhibitory constituents in CPH are predominantly polar compounds, which are more efficiently extracted by ethanol. Such compounds are likely to include phenolic acids, flavonoids, and alkaloids, all of which have been reported to interact with the catalytic or peripheral anionic sites of AChE.[43,44] In particular, alkaloids containing heterocyclic nitrogen atoms can become positively charged and bind strongly to the anionic active site of AChE, thereby blocking substrate access, whereas phenolic compounds may inhibit the enzyme by producing π–π interactions and hydrogen bonds with essential residues.[45] The strong activity of the ethanolic extract therefore reflects its higher enrichment in these bioactive polar constituents.Galantamine, used as the positive control, showed almost complete AChE inhibition (96.47%) at 50 µg/mL through an IC_{50} of 8.67 µg/mL, confirming its superior potency. Despite being less active than galantamine, the CPH extracts exhibited appreciable AChE inhibitory effects, indicating that this agricultural by-product represents a promising and sustainable source of neuroactive phytochemicals with potential for development into functional foods or lead compounds for the treatment of neurodegenerative diseases.

Evaluation of Tyrosinase inhibitory activity of Cocoa Pod Husk (CPH) crude extracts

Tyrosinase, a copper-containing oxidase, promotes the transformation of L-tyrosine to L-DOPA and then to dopaquinone, which are key steps in melanin biosynthesis. Its inhibition can occur through copper chelation at the active site, competition with natural substrates, or conformational changes that reduce enzymatic activity. Because tyrosinase inhibition prevents excessive melanin accumulation, tyrosinase inhibitors are widely studied for cosmetic applications and therapeutic use in pigmentation disorders.[29,46] In this work, the tyrosinase inhibitory activity of cocoa pod husk (CPH) extracts were evaluated at concentrations varying from 20 to 300 µg/mL (Figure 4). The results showed a concentration-dependent increase in inhibition, with the ethanolic extract demonstrating the strongest effect (>80% at higher concentrations), the ethyl acetate extract showing moderate activity, and the hexane extract exhibiting only weak inhibition. These findings highlight the role of solvent polarity in extracting bioactive compounds, with ethanol effectively recovering phenolics and flavonoids known for their tyrosinase-inhibitory properties. Thus, ethanol appears to be the most suitable solvent for isolating inhibitory agents from CPH, supporting its potential use in cosmetic skin-lightening formulations and as an anti-browning agent in food preservation. Table 4 summarizes the *in vitro* tyrosinase inhibitory activity based on IC_{50} values. The results clearly demonstrate differences among the extracts, where a lower IC_{50} value reflects greater inhibition capacity. The ethanolic extract showed the strongest effect with the lowest IC_{50} (155.58 µg/mL), followed by the ethyl acetate extract with moderate activity (225.47 µg/mL).

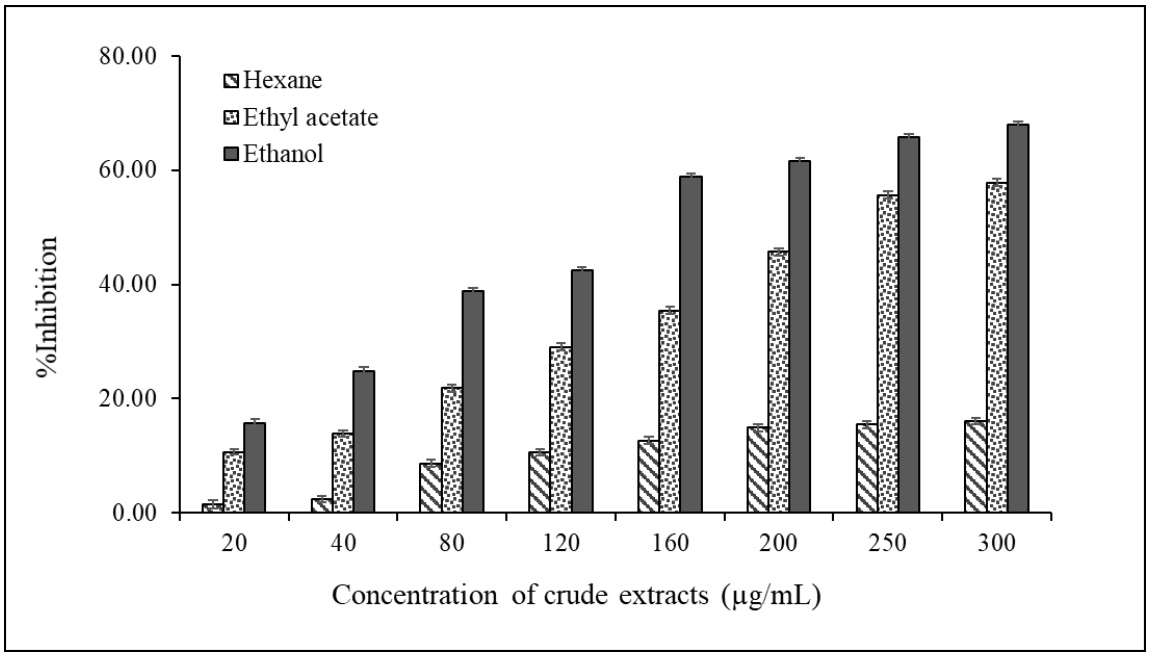

Figure 4: Tyrosinase inhibitory activity of the crude extracts; data reflected mean ±SD, = 3.

On the other hand, the hexane extract exhibited only weak inhibition and no measurable IC_{50}, indicating that non-polar solvents recover few bioactive compounds relevant to tyrosinase inhibition. Although less potent than kojic acid (IC_{50} = 10.32 µg/mL), the activity of the ethanolic extract is notable and can be ascribed to its high level of phenolic and flavonoid compounds. In particular, polymerized flavanols such as procyanidins can inhibit tyrosinase through copper chelation and substrate competition,[46,47]while flavonols may interact with the enzyme due to their structural similarity to the 3-hydroxy-4-keto moiety of kojic acid. Accordingly, the strong inhibitory effect of the ethanolic extract reflects its higher abundance of these bioactive polar compounds. In addition, the antioxidant properties of these compounds may further suppress melanogenesis by scavenging reactive oxygen species and reducing *o*-quinones back to *o*-diphenols, thereby enhancing overall tyrosinase inhibition.[47]

Table 4: Tyrosinase inhibitory activity (%inhibition and IC_{50} value) of cocoa pod husk crude extracts and kojic acid.

Sample	% Inhibition	IC_{50} µg/mL (Mean ± SD)
Hexane extract (300 µg/mL)	16.02 ± 0.58	ND
Ethyl acetate extract (300 µg/mL)	57.86 ± 0.58	225.47± 0.52
Ethanolic extract (300 µg/mL)	67.97± 0.61	155.58 ± 0.54
Kojic acid (positive control,50 µg/mL)	98.47 ± 0.44	10.32 ± 0.35

Data are expressed as mean ± standard deviation (SD). Values within the column are significantly different ($p < 0.05$). ND = not detectable

Evaluation of antiglycation activity of Cocoa Pod Husk (CPH) crude extracts

Advanced glycation end products (AGEs) are harmful compounds synthesized through the non-enzymatic Maillard reaction between reducing sugars and biomolecules such as proteins, lipids, or nucleic acids. Their collection contributes to oxidative stress, inflammation and tissue dysfunction, which play a key role in diabetes complications, cardiovascular disorders and neurodegenerative diseases. [31,48] Antiglycation activity is defined as the capacity of compounds to inhibit AGEs formation through mechanisms such as trapping reactive carbonyl intermediates (e.g., methylglyoxal), scavenging free radicals, chelating metal ions or disrupting AGE cross-linking. In this work, the antiglycation activity of the extracts was evaluated using two widely recognized in vitro models including BSA-glucose and BSA-methylglyoxal (MGO) which represent different stages of the glycation process.[30,48] The BSA-glucose system simulates the slow formation of amadori products during prolonged hyperglycemia, whereas the BSA-MGO model reflects a more rapid pathway of AGE generation under carbonyl stress. Together, these models provide a complete evaluation of antiglycation potential as test compounds may act either by delaying early-stage glucose-mediated reactions or by scavenging reactive dicarbonyl intermediates such as MGO.[49] The combined use of both models therefore offers valuable insight into the mechanisms by which natural products or synthetic agents moderate protein glycation and AGE accumulation. All crude extracts, along with aminoguanidine, were analyzed at 1000 µg/mL. The percentage inhibition of AGEs formation for each sample was determined and the results are presented in Table 5. As summarized in Table 5, all extracts exhibited inhibitory effects on AGEs formation at 1000 µg/mL, though with marked differences in potency.The ethanolic extract demonstrated the strongest inhibition in both models with 66.75% in the BSA-glucose and 62.38% in the BSA-MGO systems indicating superior antiglycation efficacy compared with the ethyl acetate and hexane extracts.

Table 5: *In vitro* antiglycation activity of cocoa pod husk crude extracts and aminoguanidine

Samples	% Inhibition at 1000 µg/mL	
	BSA-glucose	BSA-MGO
Hexane extract	22.32± 0.39	19.87± 0.27
Ethyl acetate extract	27.86± 0.32	26.83± 0.31
Ethanolic extract	66.75± 0.29	62.38± 0.28
Aminoguanidine (positive control)	89.53± 0.35	90.27± 0.34

Data are expressed as mean ± standard deviation (SD). Values within the column are significantly different ($p < 0.05$).

The positive control, aminoguanidine, produced the highest inhibition (>89%) in both assays, validating the reliability of the experimental models. The apparent activity of the ethanolic extract suggests that polar constituents, particularly phenolic and flavonoid compounds, play a key role in suppressing glycation reactions induced by both glucose and reactive carbonyl intermediates. In contrast, the weaker activity of the nonpolar hexane extract indicates a limited contribution from lipophilic compounds. These findings reveal that the antiglycation potential of the extracts correlates positively with solvent polarity, highlighting the importance of polar phytochemicals in AGE inhibition. Consistent with its strong antiglycation effect, the ethanolic extract also exhibited the greatest total phenolic and flavonoid levels coupled with strong antioxidant capacity. These results further demonstrate a positive association between phenolic/flavonoid content, antioxidant potential and antiglycation activity. Similar trends have been widely reported, with phenolic and flavonoid constituents identified as major contributors to antiglycation effects in various plant extracts.[30,31,49-51] Based on its potent activity, the ethanolic extract, along with aminoguanidine, was selected for further evaluation against both models at concentrations varying from 50 to 2000 µg/mL as presented in Figure 5. As shown in Figure 5, all samples exhibited a clear concentration-dependent increase in antiglycation activity indicating that higher concentrations enhanced the suppression of AGEs formation in both model systems. At 2000 µg/mL, the ethanolic extract demonstrated substantial inhibition of AGEs formation achieving 70.86% and 69.25% in the BSA-glucose and BSA-MGO models, respectively. These findings suggest that the ethanolic extract effectively suppresses both glucose- and methylglyoxal-mediated protein glycation, although its potency remains moderate compared with the positive control, aminoguanidine, which exhibited over 91% inhibition in both models. The IC_{50} values of aminoguanidine and the ethanolic extract were further determined and the results are summarized in Table 6.

As shown in Table 6, aminoguanidine, used as the positive control, exhibited strong antiglycation activity in both models (IC_{50} = 132.21 µg/mL for BSA-glucose and 152.67 µg/mL for BSA-MGO), confirming its high efficacy. Although less potent, the ethanolic extract showed consistent antiglycation activity in both systems (IC_{50} = 619.65 and 650.23 µg/mL), indicating its ability to inhibit both the early (glucose-mediated) and intermediate (MGO-mediated) stages of AGE formation. This activity can be ascribed to to its numerous phenolic and flavonoid constituents, which are known to trap reactive carbonyl species and suppress oxidative reactions involved in protein glycation. Previous research has reported that phenolic compounds namely catechin, epicatechin, and procyanidin B2 possess strong inhibitory effects on AGE formation.[49,50] Likewise, flavonoids including luteolin and rutin effectively inhibit methylglyoxal-mediated protein modification, while rutin, quercetin, and kaempferol act during the later stages of protein glycation in the BSA–glucose system.[50,52] These findings support the observed activity of the ethanolic extract. Moreover, the strong correlation between antioxidant and antiglycation activities suggests that hydroxylated phenolics, particularly those

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Table 6: *In vitro* antiglycation activity (%inhibition and IC_{50} value) of cocoa pod husk crude extracts and aminoguanidine

Samples	% Inhibition at 2000 µg/mL		IC_{50} µg/mL(Mean ± SD)	
	BSA–glucose	BSA–MGO	BSA–glucose	BSA–MGO
Ethanolic extract	70.86± 0.26	69.25± 0.29	619.65± 0.34	650.23± 0.33
Aminoguanidine	91.78 ± 0.29	91.64± 0.38	132.21± 0.37	152.67± 0.41

Data are expressed as mean ± standard deviation (SD). Values within the column are significantly different ($p < 0.05$).

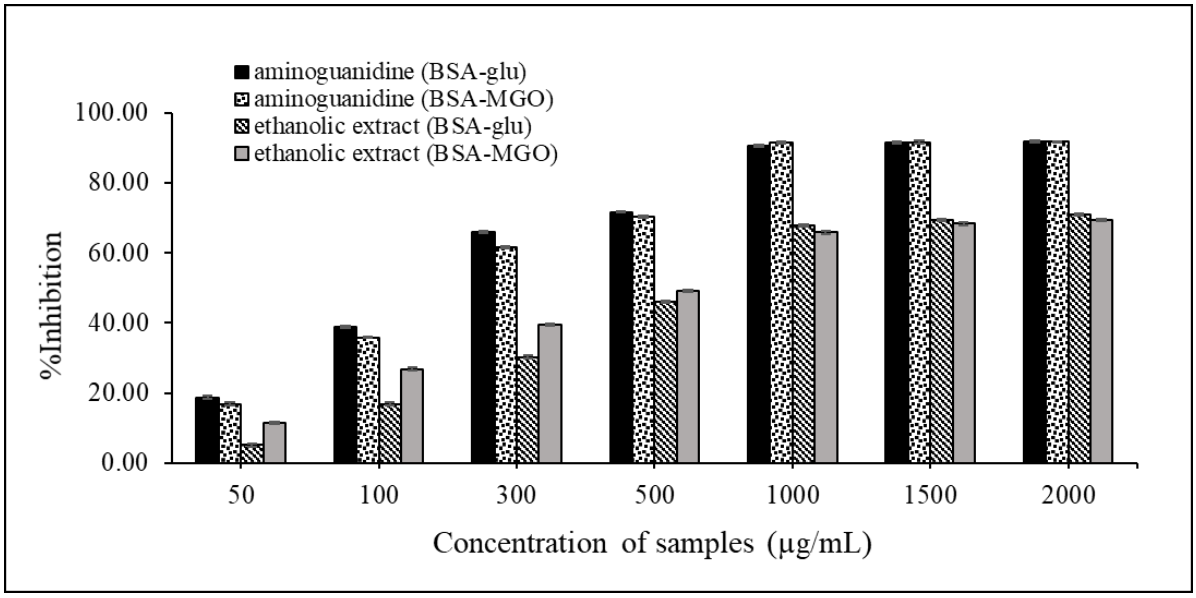

Figure 5: Antiglycation activity via BSA-glucose and BSA-methylglyoxal (MGO) models of the crude extracts at various concentrations. Data are showed as mean ± SD (n = 3).

with adjacent OH groups, play an essential role in reducing oxidative stress and limiting AGE formation.[53] Collectively, these results indicate that the ethanolic extract is a promising natural source and potential complementary agent for mitigating protein glycation, owing to its rich phenolic and flavonoid content.

Conclusion

This study systematically evaluated the total phenolic and flavonoid levels along with the biological activities including antioxidant, antiglycation, acetylcholinesterase (AChE) and tyrosinase inhibitory properties of cocoa pod husk extracts. The results demonstrated that the ethanolic extract was significantly richer in phenolic and flavonoid constituents than the ethyl acetate and hexane extracts, which was consistent with its superior performance across all bioassays. These findings establish cocoa pod husk as a previously underutilized source of phenolic-rich fractions with multifunctional in vitro bioactivities. However, further studies are required to isolate and identify the active constituents, assess their safety and toxicity, and validate their efficacy in suitable *in vivo* models. Such investigations will be essential to determine the potential of cocoa pod husk-derived compounds and for supporting the sustainable valorization of cocoa residues as functional ingredients with enhanced economic value.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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