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COMBINED METHANOLIC EXTRACTS OF SELECTED MEDICINAL PLANTS IN SOUTHERN PHILIPPINES AS A POTENTIAL THERAPEUTIC TOOL FOR DIABETES AND OBESITY

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Type 2 Diabetes (T2D) is a significant public health problem with an increasing number of cases over the years. With this, the search for cheaper and natural alternatives also continues. Oxidative stress, obesity, and advanced glycation end products (AGEs) formation contribute to T2D pathogenesis.

The aim. *This study employs a range of functional assays to assess the antidiabetic, antioxidant, anti-obesity, and antiglycation activities of air-dried leaf methanolic extracts from the combined extract (CoM) of Clitoria ternatea, Ficus septica, Heliotropium indicum, and Celosia ignea. Additionally, quantitative screening was conducted to determine the presence of key bioactive secondary metabolites, particularly flavonoids and phenolic compounds.*

Materials and methods. *Glucose adsorption and glucose diffusion were utilized to measure the antihyperglycemic effects; BSA protein-methylglyoxal and BSA-glucose reactions were used as models for the glycation studies; the pancreatic lipase enzyme inhibition was employed to assess the sample extracts' potential lipid-lowering effects; and quantitative phytochemical screening for total phenolic compounds and total flavonoid contents was conducted for initial characterization of phytoconstituents presents.*

Results. *This study reported the glucose adsorption capacities of CoM at various concentrations (25, 50, 100 ppm) indicative of its potential antihyperglycemic effects. An in vitro glucose diffusion assay, on the other hand, showed a negative result (1.82±0.06 at 100-ppm) relative to the control. The CoM also exhibited antioxidant capacities via iron-reducing assay and H_2O_2 scavenging activity (57.86±8.28 % at 25-ppm). PPL inhibition was evaluated to indicate potential antiobesity and this study reported that CoM (75-ppm) inhibited 52.13±7.16 % enzyme activity. Antiglycation tests revealed that CoM extracts are potential inhibitors of AGEs formation as it (100-ppm) inhibited 72.23±2.71 % of the glycation (BSA-glucose model) and 55.46±13.43 % (BSA-MGO model). Phytochemical screening results support the presented properties with TPC and TFC of 11.29±2.10 GAE mg/g sample and 5.83±0.03 QE mg/ g sample, respectively.*

Conclusion. *Overall, the combined methanolic plant extracts, CoM, may be utilized as a treatment strategy for oxidative stress-driven metabolic disorders such as diabetes and obesity. While this provides promising results, further investigation must still be done on the bioactive compounds of the sample*

Keywords: *Type 2 Diabetes, oxidative stress, obesity, AGEs, metabolic disease*

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1. Introduction

Type 2 Diabetes Mellitus (T2DM) is a growing health concern where the body suffers from high blood glucose levels, known as hyperglycemia, resulting from insulin resistance and impaired insulin secretion [1]. The cases of diabetes mellitus (DM) continue to rise at an alarming rate, from an estimated 451 million adults in 2017 worldwide to 693 million by 2045, which is about a 35 % increase in DM cases [2]. Oxidative stress is one of the major factors affecting the pathogenesis of both types of diabetes mellitus, generating excessive amounts of free radicals that damage cellular organelles and enzymes, increase lipid peroxidation, and aid the development of insulin resistance. These oxidative stress effects may contribute to the development of diabetes complications [3]. DM is also linked to obesity as insulin resistance and pancreatic β-cell apoptosis can be traced back to long-term sedentary lifestyles and consumption of large amounts of fast food, from which obesity is frequently observed [4].

In a global report on diabetes by the International Diabetes Federation in 2021 [5], an estimated 537 million adults are living with diabetes, which shows a substantial increase compared to the record of 151 million diabetes cases in 2000. In the Philippines, DM is predicted to double more than double, affecting 7.8 million individuals by 2030. This poses a serious threat to the individual's health and also to the healthcare system since it has no cure but can only be managed and controlled. Treatments include injections and oral antidiabetic medications such as metformin, acarbose, and sulfonylureas, however, these therapies can lead to serious health complications [6]. With

this dilemma, a search for a safer and more effective medication grows restless. This led researchers to study plants in the pursuit of discovering such a cure.

Most plants have been utilized in the field of medicine due to their medicinal properties. Many medicinal plants possess pharmacological activities which is a potential source for novel treatment strategies [7]. And in this study, we evaluated the antidiabetic, antioxidant, anti-obesity, and antiglycation activities of the combined each methanol and hexane leaf extracts of the following plants: *Clitoria ternatea* "blue ternate", *Ficus septica* "lagnob", *Heliotropium indicum* "elepante", *Costus igneus* "insulin plant". This would establish a treatment strategy that can potentially ameliorate the obesity-diabetes nexus and thereby provide more effective and affordable treatment or prevention of this health challenge. The plants selected for this study were chosen due to their strong folkloric value and wide distribution throughout the region. Their traditional use in local medicine highlights their cultural significance and potential therapeutic properties, while their widespread availability ensures relevance and accessibility for further research and practical applications.

2. Planning (methodology) of research

The methodology includes the following in vitro assays to assess the biological activity of CoM:

1. Evaluate the potential of CoM as an antidiabetic agent and its antioxidant capacity by conducting a glucose adsorption determination assay and an in vitro glucose diffusion test and by assessing its iron chelating reducing ability and H_2O_2 radical scavenging activity, respectively.

2. Assess the anti-obesity capacity of CoM through a pancreatic lipase assay and antiglycation potential via a congo red assay and its ability to inhibit the formation of AGEs using the BSA-Glucose and BSA-MGO model systems.

3. Materials and methods

3. 1. Sample preparation

Collection and Identification.

The fresh leaves of *C. ternatea* and *F. septica*, and aerial parts of *H. indicum* were collected from Barangay San Manuel of Lala, Lanao del Norte, while the leaves of *C. igneus* were collected from Phase 1 Tipanoy, Iligan City, Lanao del Norte. The collected plants were identified and authenticated by Prof. Jaime Guihawan of the Biological Sciences, MSU-IIT.

Extraction and solvent partitioning.

The air-dried (room temperature) plant samples (≈100-grams each) were pulverized, soaked in 400-mL ethanol for 3-days and concentrated under vacuo using rotary evaporator (40 °C). The crude ethanolic extracts were then partitioned with methanol:hexane (1:1) to yield the crude methanol and hexane extracts. To obtain the methanol-soluble combo extract, a uniform amount (≈1.0-gram) of each of the individual crude methanol extracts (semi-dried) was mixed to obtain and evaporated to dryness under the fumehood at 37 °C. The resulting combined extract (CoM) was then appropriately stored in the fridge for further analysis.

3. 2. Evaluation of the antidiabetic activity of the plant combo

Glucose adsorption capacity determination.

A volume of 1mL of plant combo extracts (25, 50, and 100 ppm) was added to 4mL of glucose solution in two concentrations (2500 and 5000 ppm). The mixtures were incubated in a water bath at 37 °C for 6h. After incubation, 1 mL sodium carbonate and 1 mL picric acid were added to each test tube. The solution was then heated for 20 minutes at a nearly boiling point (~70–80 °C), cooled at room temperature, and the glucose content was read at 567 nm. A calibration curve of the standard glucose solutions was made. The concentration of bound glucose was calculated using the following formula [8]:

$$\text{Glucose bound} = \frac{G_1 - G_2}{G_2}, \quad (1)$$

where G_1 is the concentration of glucose in the initial solution; G_2 is the concentration of glucose after 6 h.

In vitro glucose diffusion of plant combo sample.

A 25 mL of glucose solution (10 mM) and the 2.5 mL samples of plant combo extracts (100-ppm) were dialyzed in dialysis bags against 200 mL of distilled water at 37 °C in a shaker water bath. The glucose content in the dialysate was determined after 3 hours using a glucose oxidase peroxidase diagnostic kit. A control test was carried out without a sample. Glucose dialysis retardation index (GDRI) was calculated using the following formula [9]:

$$\text{GDRI} = \frac{D_S}{D_O}, \quad (2)$$

where D_s is the concentration of the dialysate containing the sample (CoM), and D_0 is the concentration of the dialysate with no treatment of the sample.

3. 3. Antioxidant activity evaluation of the plant combo

Determination of reducing ability of iron chelators.

The reducing ability of the iron chelators was determined using the method of Oyaizu [10] with a slight modification. An aliquot of the plant combo extract (25, 50, 75, 100 ppm) was added to test tubes containing 1.25 mL of phosphate buffer (200 mM, pH 6.6) and 1.25 mL of 1 % potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were incubated in a water bath at 50 °C for 20 min and cooled rapidly on ice, followed by the addition of 1.25 mL of 10 % trichloroacetic acid (TCA) to stop the reaction. The supernatant was then obtained from the previous solution and added with distilled water and $FeCl_3$ (0.1 %) allowed to stand for 10 min at room temperature after which the absorbance was taken at 700 nm. An absorbance at 700 nm, which is due to the formation of Perl's Prussian blue potassium ferrocyanide-ferric complex, is a measure of the reducing ability of the chelator. Increasing absorbance at 700 nm indicates an increase in reductive ability.

H_2O_2 radical scavenging activity.

Determination of the H_2O_2 scavenging activity of the iron chelators was performed according to the meth-

od of Ruch et al. [11]. In a phosphate buffer (pH 7.4), a solution of hydrogen peroxide (40 mM) was prepared. The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. 1 mL of the hydrogen peroxide solution will be added with the iron chelators (500 µL each at 25, 50, 75, and 100 ppm). A reading against a black solution of phosphate buffer not containing hydrogen peroxide was done after 10 minutes to determine the absorbance of hydrogen peroxide at 230 nm. The percentage of hydrogen peroxide scavenged by the iron chelators and standard compounds (Vitamin C) were calculated using the following formula:

$$\text{Percent Scavenging } (\%) = \frac{A_C - A_S}{A_C} \times 100\ \%, \quad (3)$$

where A_c is the absorbance of the control, and A_s is the absorbance of the sample.

3. 4. Evaluation of the anti-obesity activity of the plant combo

Porcine pancreatic lipase inhibition.

The plant combo sample's porcine pancreatic lipase (PPL, type II) inhibitory activity was measured using p-nitrophenyl butyrate (p-NPB) as a substrate. The method used for measuring pancreatic lipase activity was modified from that previously described by [12, 13]. PPL stock solutions (1 mg/mL) were prepared in a 0.1 mM potassium phosphate buffer (pH 6.0) and the solutions were stored at −20 °C. To determine the lipase inhibitory activity, the extracts (25, 50, 75, 100 ppm) or Orlistat (same concentrations) as a positive control were pre-incubated with PPL for 1 h in a potassium phosphate buffer (0.1 mM, pH 7.2, 0.1 % Tween 80) at 30 °C before assaying the PPL activity. The reaction began by adding 0.1 µL NPB as a substrate, all in a final volume of 100 µL. After incubation at 30 °C for 5 min, the amount of p-nitrophenol released in the reaction was measured at 405 nm. The activity of the negative control was examined with and without an inhibitor. The inhibitory activity (I) was calculated according to the following formula:

$$\text{Inhibitory activity } (I\ \%) = \frac{A_0 - A_1}{A_0}, \quad (4)$$

where A_0 is the activity without an inhibitor, and A_1 is the activity with an inhibitor.

3. 5. Antiglycation activity evaluation

Congo red binding assay.

To preliminarily assess if CoM could potentially prevent protein tertiary structure modifications, a dye-based binding assay was utilized. This assay was performed based on a published method by [14]. Briefly, three groups: non-glycated (BSA only), (BSA (10 mg/mL), and D-glucose (90 mg/mL) in the absence or presence of CoM were prepared. All solutions were dissolved in 0.2-M phosphate buffer saline (pH 7.4) containing 3.0 mM sodium azide. The final volume of the incubation was 1.8-mL. Glycated groups were incubated at 60 °C for 1 day to accelerate the reaction. Non-glycated group was not incubated. After incubation, half of the samples were added to 0.5-mL Congo red solution (75 µM) in phosphate buffer saline-ethanol 10 % (v/v). The other half was then used for background correction. Absorbance readings were recorded at 530 nm by a UV-Vis spectrophotometer. Similar assays were conducted this time using 60 mM MGO instead of glucose.

BSA-glucose model system.

In a phosphate buffer (pH 7.4), BSA (10 mg/mL) and glucose (90 mg/mL) were dissolved separately. In a test tube, 0.5 mL of CoM (25, 50, 75, and 100 ppm) were mixed with 0.5 mL BSA and 0.5 glucose solution. 0.5 mL of aminoguanidine (AG) solution (1 mol/L) mixed with 0.5 mL of BSA and 0.5 mL of glucose were used to prepare a positive control. A volume of 0.3 NaN_3 (0.01 %) was added to the test solution to prevent microbe development. A negative control was prepared in the same manner using 0.5 mL PBS. The tubes were capped and incubated at 37 °C for 7 days in a dark, temperature-controlled incubator. The fluorescence intensity was read after incubation [15].

BSA-MGO model system.

BSA (2 mg/mL) and MGO (40 mg/mL) were dissolved separately in phosphate buffer (pH 7.4). The same procedure with BSA-Glucose Model was performed, but the incubation time was extended to 12 days.

3. 6. Phytochemical Analysis

Total phenolic content (TPC).

The total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method [16, 17]. Gallic acid (20, 40, 60, 80, 100, 120 ppm) solutions were used as the standards. A 500 µL of sample (100 ppm) or each standard solution was added with 250 µL Folin-Ciocalteu reagent and 750 µL of 20 % Na_2CO_3. Then the solution mixture was diluted up to 5 mL and incubated for 20 minutes at 40 °C water bath. After incubation, the absorbance was measured using a UV-Vis spectrophotometer at 733 nm. The total phenolic content of the CoM extract was calculated using the Gallic acid calibration curve. The results were expressed as Gallic acid equivalent (GAE) mg/g extract.

Total flavonoid content (TFC).

The total flavonoid content was determined using the Aluminum Chloride colourimetric method [18] with some modifications. Quercetin (20, 40, 60, 80, 100, 120ppm) solutions were used as standards. Briefly, a volume of 500 µL sample (100 ppm) or each standard solution was added with 1.5 mL methanol, 0.1 mL 10 % $AlCl_3$, and 0.1 mL of 1M CH_3COONa. The solutions were diluted up to 5 mL and incubated in a dark room at ambient temperature for 1 hour. The absorbance was recorded at 420 nm. The total flavonoid content of the CoM extract was estimated using the quercetin calibration curve. The results were expressed as milligram quercetin equivalent per gram sample (mg QE/ g sample).

3. 7. Statistical analysis

Results were expressed as mean±standard error of the mean from n=3 or more independent trials. Data evaluation was carried out using GraphPad Prism 9.5.1 (for

macOS). For analyses that involved only two independent conditions, a two-tailed unpaired t-test was used. One-way analysis of variance (ANOVA) was performed on analyses involving more than two conditions, and when results showed a significant difference ($p<0.05$), Post-hoc (Dunnett and Tukey's test) was performed.

4. Results

4. 1. Quantitative phytochemical screening

To determine the possible phytoconstituents present in CoM, a quantitative analysis on both total phenolic and flavonoid compounds was employed. As depicted in Table 1, the CoM pharmacological properties demonstrated above might be attributed to this class of secondary metabolites, which could potentially act as antioxidants, thereby sequestering reactive species that contribute deleterious effects to the optimum functions of some important biomolecules such as protein and DNA.

Table 1

Estimated Total Phenolic Compounds, TPC (GAE mg/g sample) and Total Flavonoids Compounds, TFC (QE mg/g sample) of CoM

Extract	TPC (GAE mg/g sample)	TFC (QE mg/g sample)
CoM	11.29±2.10	5.83±0.03

Note: data are presented as mean±SEM (n=3).

4. 2. Antidiabetic capacity of CoM

Glucose adsorption capacity.

The glucose adsorption capacities of CoM were determined using glucose solutions of two concentrations (2500- and 5000-ppm). The concentration of glucose that is adsorbed may be correlated with the hypoglycemic action of the extract. As expected, more glucose bound was detected when a higher concentration of glucose was used implicating the adsorption capacity of CoM. The combo was effective at the concentration range (25-, 50-, 100-ppm) employed with almost similar ($p>0.05$) capacities as depicted in Fig. 1. These exhibited adsorption capacities of CoM may be related to the dietary fiber content of the sample, as it has been determined that both soluble and insoluble fibers are shown to adsorb glucose [19].

All concentrations of CoM showed no significant difference in 2500 and 5000 ppm glucose. Hence, the percent glucose bound of all the concentrations, from 25 to 100 ppm, are consistent. This indicates that at low concentrations, the glucose adsorption capacity of CoM is already similar to that of the higher concentration. The extract's ability to adsorb glucose in the intestinal lumen may have the ability to reduce the postprandial rise in blood glucose levels [20].

In vitro glucose diffusion of the plant combo.

The inhibitory activity of CoM on glucose diffusion was determined by performing an in vitro glucose diffusion system where the retardation of glucose to the dialysate was observed. The movement of glucose diffusion across the dialysis membrane was monitored after 3 hours of incubation, which mimics the physiological time of digestion. The retardation of glucose diffusion can be attributed to the inhibition of α-amylase, an enzyme involved in the digestion of glucose, which results in the inhibition of the release of glucose from the starch. This inhibition of α-amylase by plant extracts might be due to several factors, including the presence of inhibitors in fibres, the concentration of fibres, starch, and enzyme interaction by the existing fibres in the plant sample, which results in a decrease in the accessibility of starch to the enzyme, and also the adsorption of enzyme on fibres. Reported from other studies that a decrease in α-amylase activity also led to a decrease in carbohydrate digestion and low levels of postprandial glucose levels [21, 22]. The glucose diffusion retardation (Fig. 2) of 100 ppm CoM was determined to be a ratio of 1.82±0.06 relative to the control, 1.00±0.00. Student's t-test was performed between the control and 100 ppm COM, and it has been determined that the values are statistically different (**$p<0.01$). This indicates that after 3 hours of incubation, the glucose concentration of the dialysate containing the combo sample is higher than that of the dialysate containing the control. Hence, CoM exhibited no glucose diffusion retardation after 3 hours of incubation. To properly monitor the diffusion rate of glucose to the dialysate, time interval dialysate absorbance monitoring is recommended.

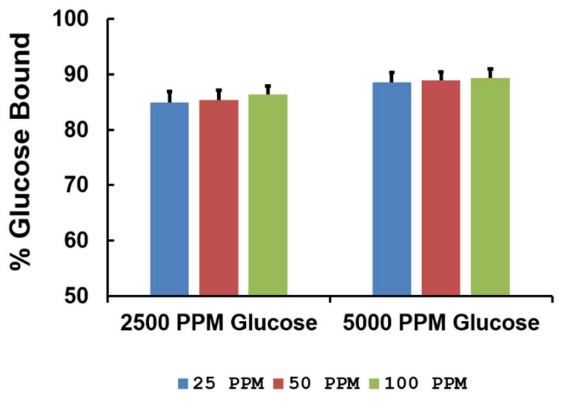

Fig. 1. The percent glucose bound of CoM in glucose at various concentrations (25, 50, and 100 ppm) (ns; Tukey's test)

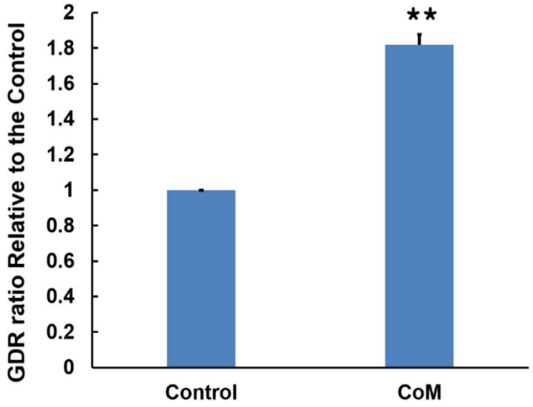

Fig. 2. In vitro glucose diffusion retardation of CoM relative to the control (100 ppm). ($p<0.01$; t-test, **$p<0.01$, wrt control)

4. 3. Antioxidant Activity of CoM

Metal chelating activity.

Molecular species such as proteins, lipids, and nucleic acids, are damaged by oxidative stress, which develops as a result of an imbalance between free radical generation and antioxidant defenses [23]. Antioxidants can be defined as reducing agents capable of inhibiting the oxidation of another molecule and thus neutralizing a free radical by donating an electron. The Fe^{3+}/ferricyanide complex is reduced to the ferrous form in the presence of reductants, such as antioxidant compounds. As a result, the formation of Perl's Prussian blue at 700 nm can be used to monitor Fe^{2+} [24]. All concentrations of CoM exhibited a dose-dependent manner of reducing ability although not as good compared to the positive control, ascorbic acid (Fig. 3). CoM at 100 ppm showed the highest reducing ability of 0.100±0.001, followed by 75, 50, and 25 ppm with reducing abilities of 0.085±0.006, 0.081±0.005, and 0.069±0.002, respectively. Ascorbic acid at 100 ppm exhibited a reducing power of 0.191±0.025. The results showed a linear relationship between the concentration and reducing ability; as the concentration increases, the reducing ability also increases. One-way ANOVA was performed and a significant difference of $p<0.0001$ was observed. Post-hoc: Tukey's test was done with respect to the Ascorbic acid (100-ppm). All concentrations of the sample except for 100 ppm have a significant difference of ****$p<0.0001$, while 100 ppm CoM has a significant difference of ***$p<0.001$. While it is true that CoM possesses Fe^{3+}-reducing ability, it is still far inferior compared to the reducing activity of Ascorbic acid, especially at 100 ppm.

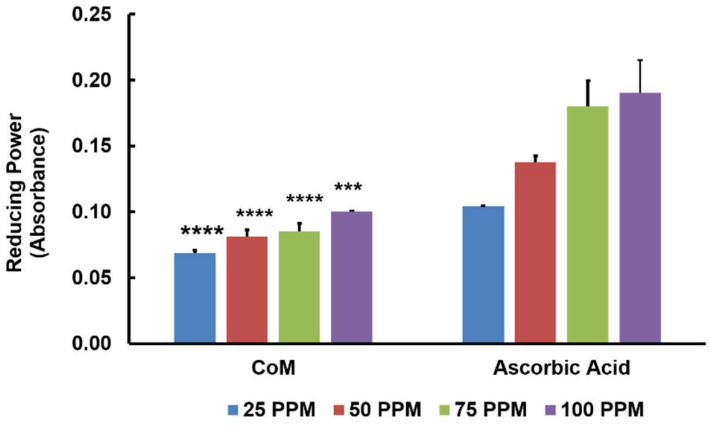

Fig. 3. The reducing power of CoM and Ascorbic acid (+) at various concentrations (25–100 ppm) (in terms of absorbance) ($p<0.0001$; one-way ANOVA, ***$p<0.001$, ****$p<0.0001$, wrt Ascorbic Acid (100 ppm); Tukey's test)

Radical scavenging activity.

Hydrogen peroxide (H_2O_2) is frequently used as an experimental source of oxygen-derived free radicals. It breaks down quickly into oxygen (O_2) and water (H_2O), and it may also produce hydroxyl radicals (OH). H_2O_2 itself is not that reactive, but when it produces OH radicals, lipid peroxidation, and DNA damage may occur [25]. Molecules that could scavenge H_2O_2 may provide a therapeutic role by preventing the formation of another harmful species such as hydroxyl radical. The concentrations of the hydrogen peroxide in the sample and ascorbic acid were read at 230 nm, and their percent H_2O_2 scavenging activities were calculated and presented in Table 2.

Table 2

The percent H_2O_2 radical scavenging activity of CoM and Ascorbic acid (+) at different concentrations (25–100 ppm) ($p<0.0001$; one-way ANOVA, *$p<0.05$, **$p<0.01$, wrt Ascorbic Acid (100 ppm); Tukey's test)

Concentration, PPM	% Scavenging activity	
	CoM	Asc. acid (+)
25	57.86±8.29*	53.46±6.84
50	29.64±6.73**	77.47±6.54
75	40.45±4.48**	78.90±5.76
100	40.73±7.27**	92.18±1.90

Note: Data are presented as mean±SEM (n=3)

All concentrations of CoM exhibited H_2O_2 scavenging activity with 25 ppm being the best scavenger with a percent H_2O_2 scavenging activity of 57.86±8.28 %, and this is followed by 100 and 75 ppm with almost similar activity of 40.73±7.28 % and 40.45±4.48 %, respectively. On the other hand, 50 ppm CoM exhibited the lowest activity with 29.64±6.73 %.

4. 4. Anti-obesity activity of the plant combo

Pancreatic lipase is a crucial digestive enzyme involved in both the metabolism and absorption of triglycerides to monoglycerides and free fatty acids. If lipase enzyme is inhibited, the total cholesterol concentration is reduced in the body. This provides potential methods for treating obesity [26].

Orlistat, a medication sold over the counter, is the industry standard for the treatment of obesity. While it's known as a weight loss treatment, it causes some gastrointestinal tract (GIT) side effects which include diarrhea, abdomen cramps, liquid stools, flatulence, and oily stools [27]. With that being said, a search for a better treatment for obesity with lesser side effects from plants, which are naturally available and could potentially be cheaper, is important. CoM, when compared to the positive control, shows comparable inhibitory activity on PPL (Fig. 4). All concentrations exhibited a relatively high PPL inhibition (%) with 75 ppm being the best with a percent inhibition of 52.13±7.16 %, the inhibitory activities of CoM at other concentrations follow close with values 49.06±26.74, 51.04±21.75, and 48.14±13.27 for 25, 50, and 100 ppm, respectively. It can be noted from the results, as well as from the figure above, that at the lowest concentration, 25 ppm, PPL inhibition is higher compared to the highest concentration, 100 ppm. While these data are close to each other, it is still safe to say that there is already a significant inhibitory activity even at low concentrations of CoM. ANOVA was performed and it was determined that CoM and Orlistat have no significant difference. Hence, the % PPL inhibitory activity of CoM is consistent with Orlistat.

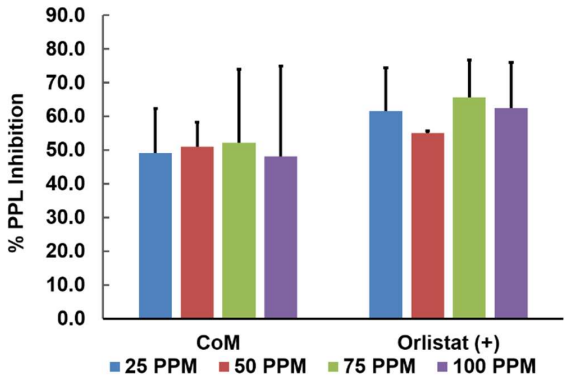

Fig. 4. The percent PPL inhibitory activity of CoM and Orlistat at various concentrations (25–100 ppm) (ns; Tukey's test)

4. 5. Antiglycation potential of CoM

Congo red binding assay.

The Congo red binding assay can be used to determine the extent of protein secondary structure modification. Congo red binds to a protein's β-sheet structure and exhibits a specific absorption at 530 nm. The hydrophobic regions between antiparallel β-strands provide a site for the Congo red dye to bind to. Congo red staining is performed to determine whether CoM possesses a protective ability on the secondary structure of BSA [28].

The protective activity (Fig. 5) of the CoM was determined by comparison of detected absorbances of samples with that of the glycated blank. The absorbances relative to control (ARC) were calculated and used as the basis of the protective activity. An ARC lower than the glycated ratio, 1, indicates that there is a protective activity that prevents the CR from being glycated. 100 ppm CoM has an ARC of 0.92±0.09, while 25 ppm CoM has an ARC of 0.99±0.04. The positive control, AG, on the other hand, has an ARC of 0.87±0.07. Dunnet's test was performed, and no significant difference was observed. Hence, there is a consistent inhibitory activity between the sample and positive control.

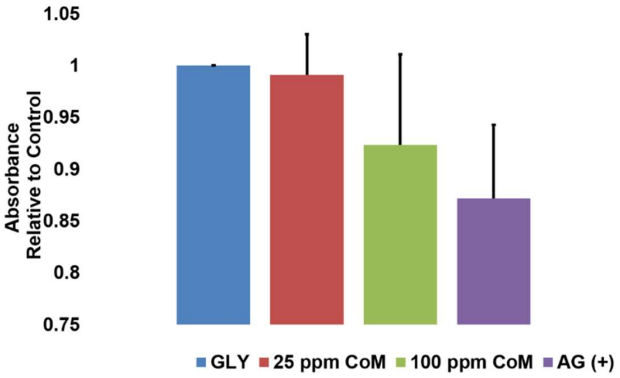

Fig. 5. The absorbance relative to the control of CR, CR-CoM complex (25 ppm and 100 ppm), and CR-AG complex at λ=530 nm (ns; Dunnet's test, wrt AG)

On BSA-glucose model system.

The formation of advanced glycation end products (AGEs) is inhibited by antiglycating agents by preventing glucose from forming adducts to biomolecules such as proteins, lipids, and/or DNA. The pathophysiology of aging, diabetes, and other chronic diseases are thought to be influenced by increased rates of AGE formation in tissue proteins during hyperglycemia, oxidative stress, or inflammation [29].

In this experiment, bovine serum albumin, or BSA, was used as a model protein. The BSA-glucose model was used as the final stage of AGE formation. The solution was incubated for 7 days because it is the physiological time for the glucose to form adducts to the BSA.

Presented in Fig. 6 is the percent inhibition of CoM at increasing concentrations of 25, 50, 75, and 100 ppm. Positive results were found in the investigation of the sample's antiglycation properties: 25 ppm with 21.67±9.45 %; 50 ppm with 43.29±6.68 %; 75 ppm with 55.19±7.47 %; 100 ppm with 72.23±2.71 %. It can be seen that as the concentration increases, the inhibitory activity on AGE formation of CoM also increases.

The positive control, AG, showed the highest inhibitory activity of 76.29±12.25 %. Data showed that 25 ppm CoM has a significant difference with AG (+). The remaining concentrations showed no significant difference wrt AG (+). This indicates that all concentrations of CoM, except 25 ppm, have anti-AGEs formation ability comparable to AG. This positive effect might be attributed to the inhibition of dicarbonyl intermediates' conversion to AGEs.

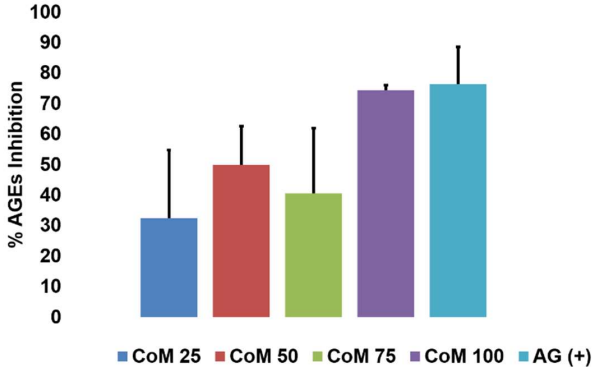

Fig. 6. The percent inhibition of CoM at various concentrations (25–100 ppm) and AG (+) in the BSA-Glucose model system ($p<0.01$; One-way ANOVA, $^{**}p<0.01$, wrt AG (+); Dunnet's test)

In Fig. 7, the percent inhibitory activity of CoM is presented. All concentrations of CoM exhibited an inhibitory activity with 100 ppm having the highest percent inhibition with a value of 55.46±13.43 %. 75, 25, and 50 ppm followed with percent inhibition of 37.65±4.41 %, 32.70±10.50 %, and 31.84±12.34 %, respectively. The positive control, AG, exhibited a percent inhibitory activity of 83.18±3.29 %. Statistical analysis (one-way ANOVA) was performed and a significant difference ($p<0.05$) was observed. 100 ppm CoM exhibited comparable activity to the positive control, AG, where it has no significant difference. The other concentrations (25, 50, and 75 ppm) have shown a significant difference with AG ($^{*}p<0.05$), indicating a difference in the inhibitory activities. Hence, the highest concentration of CoM (100 ppm) has a significant inhibitory activity on AGEs formation in the BSA-MGO system, which is the

middle stage of the formation of oxidative cleavage product, and this proposes good antiglycation properties.

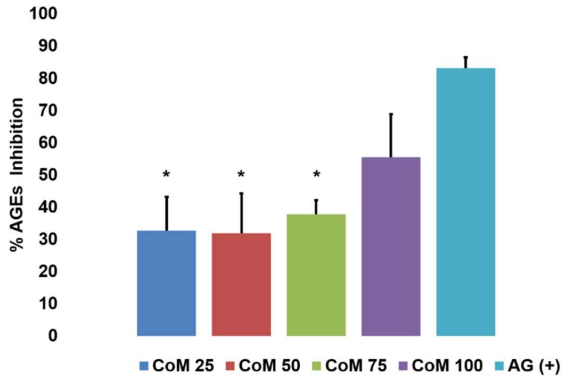

Fig. 7. The percent inhibition of CoM at various concentrations (25–100 ppm) and AG in the BSA-MGO model system ($p<0.05$; One-way ANOVA, *$p<0.05$, wrt AG (+); Dunnet's test)

5. Discussion

Plants remain a valuable source of bioactive compounds with considerable therapeutic potential. With ongoing scientific advancements, the integration of traditional knowledge and modern research methodologies holds great promise for the discovery and development of novel plant-based medicines. To the best of our knowledge, this study is the first to report the combined effects of the extracts used. Nonetheless, a limited number of previous studies have highlighted certain pharmacological properties of these individual extracts. For instance, extracts from *Clitoria ternatea* flowers have demonstrated antibacterial, antioxidant, anti-inflammatory, cytotoxic, and antidiabetic properties [30]. The plant contains various bioactive constituents, including alkaloids, glucosides, flavonoids, saponins, and tannins [31]. However, most studies to date have focused exclusively on the flowers, with little to no investigation into the potential therapeutic properties of the leaves. This study, therefore, aims to explore the medicinal potential of *Clitoria ternatea* leaves. *H. indicum* has been reported to exhibit a wide range of biological activities, including antimicrobial, antioxidant, antiviral, anti-inflammatory, antitumor, gastroprotective, and antihyperlipidemic effects [32]. However, studies investigating its antidiabetic potential remain limited. *F. septica* has a long-standing history of use in various traditional medicinal systems. It is known to contain several therapeutic compounds and exhibits a range of pharmacological activities, including analgesic, antifungal, diuretic, and laxative effects [33]. Extracts of *C. igneus* have demonstrated antidiabetic activity both in vitro and in chemically induced diabetic rat models using agents such as alloxan, dexamethasone, and streptozotocin [34].

In this study, the antidiabetic, antioxidant, anti-obesity, and antiglycation properties of the combination (CoM) of *C. ternatea, F. septica, H. indicum,* and *C. igneus methanol* (polar) extracts were assessed using different functional assays. The antidiabetic activity of CoM was determined using two assays, namely glucose adsorption capacity and in vitro glucose diffusion retardation. In glucose adsorption capacity determination, results showed that CoM extracts may have phytoconstituents that could be potentially effective antihyperglycemic agents. For in vitro glucose diffusion retardation assay, the diffusion of glucose to the dialysate is monitored to simulate the effect of fiber on the rate at which glucose is adsorbed in the gastrointestinal tract. After 3 hours of incubation, results showed that CoM was not capable of adsorbing glucose. The reason for this could be the lack of dialysate monitoring at time intervals, which could have given a more accurate glucose diffusion rate.

In antioxidant tests, all samples in both the determination of iron-reducing capabilities and H_2O_2 scavenging activity showed relatively good activity compared to the positive Ascorbic acid. In iron reducing assay, 100 ppm of the sample showed the highest activity among all concentrations of CoM with a power of 0.100±0.0001 (***$p<0.001$, wrt Asc. acid (100-ppm)), while in H_2O_2 scavenging activity test, 25 ppm showed the highest scavenging percentage of 57.86±8.28 % (*$p<0.05$, wrt Asc. Acid (100-ppm)). This observation provides a good prospect for the antioxidant potential of CoM.

Porcine pancreatic lipase inhibition assay was performed to assess the anti-obesity potential of CoM. Orlistat was used as a positive control due to its established weight loss capabilities. All concentrations showed relatively high percent PPL inhibition. 75 ppm CoM exhibited the highest percent inhibition, 52.13±7.16 %. Data showed that all concentrations have no significant difference compared to the positive, Orlistat, which indicates that the inhibitory activity of the sample is similar to the inhibitory activity of the positive.

The antiglycation potential of CoM was assessed using three assays, namely Congo red assay, BSA-glucose, and BSA-MGO models. Glycation is defined as the formation of adducts of sugar to the biomolecules which negatively affect the intracellular and extracellular structures and functions of cells. This forms AGEs, which accumulate and further cause adverse effects on the cells.97 Congo red assay showed that both high and low concentrations (25- and 100-ppm) of CoM possess inhibitory activity. Statistical analysis showed that CoM and AG have no significant difference, indicating that they have similar activities. Both BSA-glucose and BSA-MGO showed that 100 ppm CoM has the highest percent inhibition with a percentage of 72.23±2.71 % and 55.46±13.43 % for BSA-glucose and BSA-MGO, respectively. The percent inhibition of the positive control, AG, in BSA-glucose and BSA-MGO systems are 76.29±12.25 and 83.18±3.29, respectively. Dunnet's test with control showed that there is a significant difference of $p<0.01$ for BSA-glucose and $p<0.05$ for BSA-MGO. These assays show that there is a greater inhibition in BSA-glucose, which is the final stage of AGE formation, than in the BSA-MGO model which represents the middle stage of formation of oxidative cleavage product. This effect might be caused by blocking free radicals, which would reduce oxidative stress and the reduction of the production of reactive carbonyl and dicarbonyl groups can help inhibit the glycation func-

tions. The phytoconstituents in the CoM may serve as valuable source of therapeutic agents, playing a crucial role in promoting health and combating various diseases. These naturally occurring bioactive compounds detected including phenolics and flavonoids contribute to the promising pharmacological properties of these plants that are deemed beneficial in the management of type 2 diabetes, obesity, and other oxidative-driven diseases. Phenolic compounds and flavonoids are known for their strong antioxidant properties, which help neutralize free radicals and prevent oxidative stress-related diseases, they possess anti-inflammatory and immune-modulating effects, making them beneficial for conditions like cardiovascular diseases, neurodegenerative disorders, antimicrobial and anticancer properties [35–37].

Overall, the combination of different plant extracts can serve as both preventive or therapeutic agents for oxidative stress-driven metabolic disorders. With the data obtained, plant components in this combo through further evaluation could be utilized for something beneficial to people suffering from T2D and its complications as the search for natural medications for the mentioned disease continues; this can be developed into a cheap alternative to T2D medicines with further research. In conclusion, this study can serve as an additional resource for combinatory studies of plant extracts and their beneficial properties all the while looking into the effects of each component extract and the synergistic effects might have in the combo solution.

Practical relevance. Given the potential benefits of the combined extracts, our findings suggest that they may serve as an effective and accessible alternative remedy for managing blood sugar levels. However, to ensure their safety and efficacy, comprehensive toxicity studies must be conducted.

Research limitations. This study acknowledges certain limitations and strongly recommends further investigations using more physiologically relevant cellular or animal models of type 2 diabetes and obesity to enhance the translational value of the findings. Additionally, it would be worthwhile to explore the potential interactions between individual phytoconstituents within the extract, particularly how the presence of one compound may influence the biological activity of another. Understanding these synergistic or antagonistic effects could provide deeper insights into their mechanisms of action and optimize their therapeutic potential. Importantly, despite their potential, the development of "combo" as a source of plant-based medicine should take into consideration the standardization, toxicity and safety concerns.

Prospects for further research. The data from this study provides few avenues for further investigation to translate and maximize the potential of the "Combo" crude extracts into viable therapeutic products which may include but not limited to the identification and isolation of specific bioactive compounds responsible for pharmacological effects via integration of bioassays with metabolomics, understanding how the crude extracts exert their effects at the molecular and cellular level by using more advanced omics approach, and importantly to further document and validate more underexplored medicinal plants.

6. Conclusion

Type 2 diabetes remains a global health epidemic, affecting millions worldwide. The disease is closely linked to oxidative stress, which contributes to protein glycation and obesity. This study investigates the antidiabetic, antioxidant, anti-obesity, and antiglycation properties of CoM. The results demonstrate that CoM shows promise in several key areas: it enhances glucose adsorption and diffusion, binds and reduces iron, scavenges hydrogen peroxide, inhibits pancreatic lipase activity, and prevents the formation of advanced glycation end-products. These findings suggest that CoM may effectively inhibit factors that contribute to the development and progression of T2DM. While further study and characterization are necessary, the plants analyzed in this study may contain promising molecular candidates for pharmaceutical research and development. Moving forward, developing a standardized CoM formulation is highly desirable, as it could provide synergistic effects, thereby enhancing its therapeutic potential in both integrative and traditional medicine. Additionally, rigorous evaluation of the CoM formulation using relevant cellular and animal models for various pharmacological conditions is strongly recommended to validate its efficacy and safety.

Conflict of Interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence

The authors confirm that they use artificial intelligence technologies for proofreading and refining the structure. All data and analysis were verified and conducted by the authors.

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