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Original Article

The most commonly used spices in Thai traditional medicine: *in vitro* evaluation of anti-hyperglycemic, antioxidant, polyphenol content, and nitric oxide production inhibitory activities

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Abstract

Background and purpose: Diabetes mellitus is a persistent hyperglycemic condition. Thai cuisine and medicine incorporate spices: nutmeg, mace, clove buds, cardamom, cinnamon, and coriander. The *in vitro* impacts of these spices on anti-diabetic, antioxidant, anti-inflammatory, and total phenolic and flavonoid content were assessed.

Experimental approach: Alpha-amylase and alpha-glucosidase inhibition assays were conducted. Antioxidant potential was measured through DPPH and ABTS assays. Anti-inflammatory activity was determined by inhibiting nitric oxide generation in RAW 264.7 cells. Total phenolic content was quantified using the Folin Ciocalteu method, while total flavonoid content was estimated *via* the aluminum chloride colorimetric method.

Findings/Results: Ethanolic and aqueous extracts of a blend of spices (Siam cardamom, nutmeg, mace, and clove buds), denoted as 4-GlurE and 4-GlurA, displayed concentration-dependent inhibition of alpha-glucosidase, with IC_{50} values of 0.373 and 0.435 mg/mL, respectively. 4-GlurE and 4-GlurA exhibited antioxidant activity, by $ABTS^{\bullet+}$ radical and DPPH scavenging capabilities. 4-GlurE demonstrated anti-inflammatory potential by reducing nitric oxide generation (IC_{50}: 43.95 ± 2.47 µg/mL). 4-GlurE and 4-GlurA possessed total phenolic content (TPC) of 122.47 ± 1.12 and 148.72 ± 0.14 mg GAE/g, respectively. 4-GlurE exhibited a higher total flavonoid content (TFC) compared to the aqueous extract (340.33 ± 4.77 and 94.17 ± 3.36 mg QE/g). Cinnamon and clove aqueous extracts were more potent than acarbose in alpha-glucosidase inhibition with the highest antioxidant activity. Polyphenol levels (TPC and TFC) exhibited strong correlations with antioxidant capacity.

Conclusions and implications: Findings are consistent with the traditional use of 4-Glur, with cinnamon, for diabetes prevention and treatment.

Keywords: Alpha-amylase; Alpha-glucosidase; Antioxidant; Diabetic; Spices.

INTRODUCTION

Noncommunicable diseases (NCDs), also referred to as chronic diseases, have emerged as a global health crisis, responsible for a staggering 71% of all deaths worldwide (1). The leading NCDs causing this epidemic are cardiovascular disease, cancer, respiratory conditions, and diabetes (2).

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Diabetes mellitus (DM), a metabolic disorder characterized by dysfunctional pancreatic beta-cells, is a significant contributor to the burden of NCDs. Type II diabetes accounts for over 90% of all diabetes cases globally and is marked by chronic hyperglycemia, which is linked to a range of debilitating complications including cardiovascular disease, diabetic retinopathy, kidney failure, neuropathy, and ocular damage (3-5).

In the realm of diabetes management, alpha-amylase and alpha-glucosidase inhibitors like acarbose, voglibose, and miglitol have been pivotal in curbing glucose absorption and regulating blood sugar levels in diabetic patients (6,7). However, these medications are often accompanied by distressing gastrointestinal side effects (8,9). Thus, there is a growing interest in exploring novel anti-diabetic treatments, particularly those derived from natural sources, to mitigate adverse effects and enhance patients' quality of life. Spices, with their rich history of therapeutic use spanning centuries (10), represent one such natural resource garnering attention for their potential in diabetes management.

This study examined the six spices most frequently utilized in Thai traditional medicine (TTM): clove (*Syzygium aromaticum*), Siam cardamom (*Amomum testaceum*), cinnamon (*Cinnamomum burmannii*), coriander (*Coriandrum sativum*), nutmeg, and mace (*Myristica fragrans*). Among these, a specific combination of four spices, namely Siam cardamom, clove, nutmeg, and mace, known as "4-Glur," is commonly employed in equal proportions within TTM (11). These spices have enjoyed historical usage in TTM for their carminative properties, effectiveness in combating flatulence, and enhancement of blood circulation. In contemporary medical research, these spices have also demonstrated antioxidative and glucose-lowering potential.

The primary objective of this study was to investigate the potential hypoglycemic, anti-inflammatory, and antioxidant effects of these individual spices and different extracts of the combination of four spices including Siam cardamom, clove, nutmeg, and mace (known as "4-Glur").

This investigation entailed inhibiting alpha-amylase and alpha-glucosidase, assessing nitric oxide (NO) production, and conducting radical scavenging assays. Additionally, the total phenolic and flavonoid content (TPC and TFC) of these spices was quantified, contributing to the development of products aimed at preventing or treating diabetes and associated chronic diseases. Moreover, this study explored the correlations between antioxidant activities and (2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$)), polyphenol content (TPC and TFC), inhibition of NO production, and inhibition of alpha-glucosidase.

MATERIALS AND METHODS

Plant materials

Plant materials, including nutmeg and mace of *Myristica fragrans* Houtt, *Syzygium aromaticum* (L.) Merr. et Perry, *Wurfbainia testacea* (Ridl.) Škorničk. & A.D.Poulsen (synonym *Amomum testaceum* Ridl (fruit)), *Coriandrum sativum* L (seed), and *C. burmannii* (Nees and T. Nees) Blume (bark), were purchased from a herbal shop in Bangkok. The voucher specimens were identified and deposited at the Southern Center of the Thai Medicinal Plant at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand (Table 1).

Preparation of the extracts

The plants were washed and dried in an oven at no more than 50 ℃. The dried materials were ground into a coarse powder to produce crude extracts. The 4-Glur remedy was prepared by combining clove, Siam cardamom, mace, and nutmeg powders. Six plants and the 4-Glur remedy were macerated with 95% ethanol (liquid (ethanol) to solid (plant material) ratio = 3:1) used in the extraction process for three days to produce the ethanolic extract used in the extraction process. Specifically, it means that for every 3 parts of ethanol, there is 1 part of the solid plant material. So, if you were to use this ratio with 100 units (it could be grams, milligrams, or any other unit of measurement) of plant material, you would mix it with 300 units of 95% ethanol. This ratio is often used in plant extraction processes to ensure efficient extraction of bioactive compounds from the plant material into the ethanol solvent.

Table 1. Six spices commonly used in Thai traditional medicine from 48 oral remedies published in the Thai National List of Essential Medicines (11) and the voucher specimens.

Scientific name	Common name	Code	Voucher Specimen	Part used	Number of remedies
***Wurfbainia testacea* (Ridl.) Škornič. & A.D.Poulsen**	Siam cardamom	WT	SKP206012001	Fruit	17
***Cinnamomum burmannii* (Nees & T. Nees) Blume**	Cinnamon	CB	SKP09603022101	Bark	12
***Coriandrum sativum* L.**	Coriander	CS	SKP012/199031901	Seed	8
***Myristica fragrans* Houtt.**	Nutmeg	MFn	SKP121130601	Seed	22
***Myristica fragrans* Houtt.**	Mace	MFm	SKP121130601	Aril of seed	19
***Syzygium aromaticum* (L.) Merr. & L.M. Perry)**	Clove	SA	SKP123190101	Flower	23

The extract was filtered and double-macerated, and the filtrates were merged and evaporated until dry. For the aqueous extract, each plant material and the 4-Glur remedy were separately boiled for 15 min in water and then filtered, and the process was repeated twice. The combined filtrates were transferred to a double boiler and simmered until two-thirds of their volume was reduced. A third of the remaining filtrate was lyophilized in a freeze dryer to produce the powder extract. All fourteen extracted substances were placed in glass vials and refrigerated at -20 °C until use.

Chemicals and reagents

Food-grade 95% ethanol was purchased from CMJ Anchor (Thailand). Distilled water was obtained from Milford® (USA). Analytical grade reagents, including absolute ethanol, isopropanol, and dimethyl sulfoxide (DMSO), were purchased from Labscan®. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypan blue stain 0.4%, and trypsin-EDTA were obtained from Gibco® (USA). Lipopolysaccharide from *Escherichia coli* 055:B5 (LPS), phosphoric acid 85%, N-(1-naphthy) ethylene-diamine dihydro-chloride, sulfanilamide, and thiazolyl blue tetrazolium bromide (MTT), gallic acid, sodium nitrite, aluminum chloride, alpha-glucosidase (*Saccharomyces cerevisiae*), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), bovine serum albumin (BSA), p-nitrophenyl α-D-glucopyranoside, alpha-amylase from porcine pancreas type VI-B, soluble starch ACS reagent, quercetin, and acarbose were purchased from Sigma-Aldrich® (USA). Sodium hydroxide was obtained from Univar® (Australia). Sodium bicarbonate was purchased from BHD® (England). Phosphate-buffered saline (PBS) was obtained from Amresco® (USA). Hydrochloric acid (HCl) was obtained from Merck® (Germany). Butylated hydroxytoluene (BHT) was purchased from Fluka® (Germany). $ABTS^{\bullet+}$, Folin-Ciocalteu's reagent, and DPPH were obtained from Fluka® (USA). Sodium potassium tartrate, disodium hydrogen phosphate heptahydrate, 3,5-dinitrosalicylic acid, sodium carbonate (Na_2CO_3), sodium chloride, and phosphoric acid were purchased from Merck® (Germany).

In vitro assessment of NO-producing inhibitory effect

A mouse macrophage leukemia-like RAW 264.7 cell line (ATCC TIB-71) was cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. The NO inhibitory effect was evaluated using a modified method from Makchuchit and co-workers (12). RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated in a CO_2 incubator at 37 °C with 5% CO_2 and 95% humidity for 24 h. Afterward, the medium was replaced with fresh medium containing LPS (10 ng/mL) and various concentrations of the samples. The plate was incubated for more than 24 h. NO production was determined by measuring nitrite accumulation in the supernatant using Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine

dihydrochloride in distilled water). Cell viability after treatment with the extracts was assessed using the MTT colorimetric method to ensure that the cells were healthy and that the concentrations used to study NO production were non-cytotoxic (cell viability > 70%). Briefly, after 24 h of incubation with samples and LPS, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 2 h. The medium was removed, and 100 µL of isopropanol containing 0.04 M HCl was added to dissolve the formazan crystals. The absorbance of the plate was measured using a microplate reader at 570 nm. The percentage inhibition was calculated using the following equation (1), and the IC_{50} value was determined using GraphPad Prism software (CA, USA).

$$\text{The percentage of inhibition} = \left[\frac{\text{Mean of OD control} - \text{mean of OD sample}}{\text{Mean of OD control}}\right] \times 100 \quad (1)$$

Where the mean of OD control equals the mean of OD control (+LPS) - mean of OD control (-LPS); and the mean of OD sample equals the mean of OD sample (+LPS) - mean of OD sample (-LPS).

Preparation of sample solutions

To prepare the sample solutions, the extract was dissolved in either sterile DMSO or sterile distilled water. For non-aqueous extracts, the concentration was set at 50 mg/mL, while for aqueous extracts, the concentration was 10 mg/mL, and the solution was filtered through a 0.22 µm syringe filter. Next, the stock sample solution was diluted with DMEM medium to produce working sample solutions with concentrations of 2, 20, 100, and 200 µg/mL. To ensure the final DMSO concentration was below 0.2% v/v in all working solutions, it was carefully monitored.

Antioxidant activities

DPPH radical scavenging assay

The DPPH radical scavenging assay was conducted based on the method of Yamasaki and colleagues (13), with BHT as the positive control. The stock solutions of the aqueous and ethanol extracts were prepared at a concentration of 1 mg/mL by dissolving them in deionized water and absolute ethanol, respectively. These stock solutions were then diluted to varying concentrations of 2-200 µg/mL. To perform the assay, 100 µL of each sample solution was mixed with an equal volume of 6×10^{-5} M DPPH solution in ethanol in a 96-well plate. The plate was then incubated at room temperature for 30 min while protected from light. The scavenging effect of the extracts was measured using a microplate reader at 520 nm, and the Prism software was used to calculate the half-maximal effective concentration (EC_{50}) following equation (1).

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$ABTS^{\bullet+}$ radical scavenging assay

The method of Re and colleagues was used to determine the total antioxidant capacity of the extracts (14). Trolox was employed as a positive control. A solution of $ABTS^{\bullet+}$ radical was prepared by reacting 7 mM $ABTS^{\bullet+}$ stock solution in distilled water with 2.45 mM potassium persulfate and allowed to stand in a dark place at room temperature for 12-16 h. The $ABTS^{\bullet+}$ stock solution was then diluted with deionized water to obtain an absorbance of 0.68-0.72 at 734 nm. The assay was performed in 96-well microplates, in which 20 µL of the extracts (at concentrations of 1-100 µg/mL) were combined with 180 µL of $ABTS^{\bullet+}$ solution. After 6 min of reaction, the absorbance was measured at 734 nm using a microplate reader. The percentage of inhibition values of samples was calculated using equation (1), and the EC_{50} was computed using the Prism program.

In vitro antidiabetic activity

Inhibitory effect on alpha-glucosidase activity

The inhibition of the alpha-glucosidase enzyme was evaluated using the Wongnawa method with slight modifications (15). The ethanolic extracts were dissolved in DMSO, while the aqueous extracts were dissolved in distilled water at 10 mg/mL concentration in pH 6.8 phosphate buffer diluted working solutions. In 96-well microplates, 20 µL of the sample (50-1000 µg/mL in phosphate buffer, pH 6.8), 80 µL of 100 mM phosphate buffer, and 50 µL of alpha-glucosidase enzyme (0.2 U/mL in phosphate buffer) were mixed and incubated for 15 min at 37 °C. Subsequently, 50 µL of a substrate (5 mM p-nitrophenyl α-D-

glucopyranoside) was added to the mixture and incubated at 37 °C for another 15 min. The reaction was stopped by adding 100 µL of 1 M Na_2CO_3, and the released p-nitrophenol was measured at 405 nm using a microplate reader. The percentage of inhibition was calculated using equation (1), and the IC_{50} was determined using the Prism program with a sample size of n = 3.

Inhibitory effect on alpha-amylase activity

The method used to evaluate the inhibitory effect on alpha-amylase activity was based on the procedure described by Lordan and colleagues (16). Initially, 100 µL of the extract was mixed with an equal amount of alpha-amylase from porcine pancreases (0.5 mg/mL) in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride). Then, 100 µL of a starch solution (1% w/v in the buffer) was added, and the reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped, and the reducing sugar product was measured by adding 200 µL DNS reagent (96 mM 3,5-dinitro salicylic acid, 3.5 M sodium potassium tartrate in 2 M NaOH) to the reaction mixture. The tubes were heated at 90-100 °C for 5 min and then cooled on ice for 5 min. Next, 50 µL of the reaction mixture was transferred to 96-well microplates and diluted with 200 µL of ultrapure water. The microplates were read at an absorption wavelength of 405 nm using a microplate reader. The percentage of inhibition was calculated using equation (1), and the IC_{50} value was determined using the Prism program (n = 3).

Polyphenol content

TPC

The TPC of the crude extract was determined using the Folin-Ciocalteu's reagent and expressed as gallic acid equivalents (GAE) (17). The sample solution was prepared at 0.5 mg/mL and 1 mg/mL. The reaction was carried out in a 96-well microplate by mixing the sample solution (20 µL), Folin-Ciocalteu's reagent (100 µL), and sodium carbonate solution (80 µL). The plate was incubated at room temperature for 30 min, protected from light, and then measured using a microplate reader at a wavelength of 520 nm. To generate a calibration curve, gallic acid was prepared at concentrations ranging from 2.5 to 100 µg/mL.

TFC

The TFC was determined using the aluminum chloride colorimetric method (18). This method involves the reaction of phenolic hydroxyl groups in flavonoid molecules with $AlCl_3$, producing a yellowish-absorbing complex. In a microcentrifuge tube, 500 µL of quercetin standard solution (20-480 µg/mL) or samples, 75 µL of 5.0% $NaNO_2$, and 150 µL of 10% of $AlCl_3$ were mixed and allowed to react at room temperature for 5 min. Subsequently, 500 µL of 1 M NaOH and 275 µL of deionized water were added, mixed, and allowed to sit at room temperature for 30 min. The absorbance of the reaction was measured at a wavelength of 415 nm using a spectrophotometer, and the TFC of the sample was determined from the standard curve of quercetin. The flavonoid content was expressed as milligram quercetin equivalents per gram of dried extract ($mgQE.g^{-1}$ dried extract).

Analysis of TTM remedies and their utilization of various spices

The data presented in Table 2 was derived from an analysis of TTM remedies and the use of various spices in these remedies. To retrieve this data, we followed a methodology that involves several steps to collect this data. First, we defined the research objective which in this case is the usage of spices in TTM remedies. We collected data by identifying TTM remedies from the Thai National List of Essential Medicines for each remedy in the sample, we collected data on the spices used in the remedy. We assessed the treatment purposes or indications associated with each spice in the remedy and the percentage yield of ethanolic and aqueous extracts for each spice. The data were analyzed, organized, and categorized. The frequency of each spice's usage in the remedies and the percentage yield of extracts for both ethanolic and aqueous extraction methods were calculated. We grouped the treatment purposes into categories (A-G) for analysis. The data are presented in a tabular format to make it visually understandable and accessible for readers. We further analyzed the data to draw conclusions about which spices are commonly used for specific treatment purposes in TTM remedies.

Table 2. Number distribution of spices commonly used in Thai Traditional Medicines remedies (from 48 oral remedies) published in the Thai National List of Essential Medicines (11) and percentage of yield of ethanolic and aqueous extracts.

Spices	Number of remedies	Treatment purposes of the remedies							% of yield	
		A	B	C	D	E	F	G	Ethanolic	Aqueous
WT*	17	3	1	9	2	1	1	0	0.91	6.90
CB*	12	5	1	3	1	0	2	0	10.20	11.59
CS*	9	2	0	6	0	0	1	0	7.10	10.62
MFn*	22	4	1	10	4	1	1	1	15.0	22.01
MFm*	19	4	1	8	4	1	1	0	12.11	10.84
SA*	23	4	1	10	2	1	4	1	11.54	11.19
4-Glur	15	3	1	7	2	1	1	0	18.19	13.74
From total	48	5	1	16	6	6	8	6		

*, Defined in Table 1; A, increase blood circulation; B, blood tonic; C, gastrointestinal symptoms relief, D, muscle pain relief; E, antipyretic; F, cough and cold relief; G, obstetrics syndromes relief.

Statistical analysis

The experiments were conducted in triplicate, and the results were reported as mean ± SEM. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's multiple comparison tests to compare the samples with the positive control, and the correlation analysis calculated the correlation coefficient (r) by using the GraphPad Prism (GraphPad®, USA). *P*-values < 0.05 were considered statistically significant. Heatmap Illustrator (HemI) version 1.0 software was used to analyze the heat map and Hierarchical cluster analysis (19,20).

RESULTS

Distribution of spices commonly used in TTM remedies.

Table 2 shows that Siam cardamom, cinnamon, coriander, clove, nutmeg, and mace are the most commonly used spices in TTM as listed in the National List of Essential Medicines (NLEM) of Thailand. These spices are used for treating gastrointestinal disorders, regulating blood flow, and reducing fever. The 4-Glur spices are also widely used in TTM remedies for oral administration, with 15 out of 48 remedies (31.25%) in the NLEM containing them. Table 2 indicates that the nutmeg aqueous extract (MFnW) had the highest yield, with a percentage of 22.01%. As for the 4-Glur spices, the ethanolic extract had a higher yield (18.19%) than the aqueous extract (13.74%).

In vitro anti-inflammatory: NO inhibitory effect.

All 14 extracts underwent testing for their anti-inflammatory properties against LPS-induced NO production in RAW 264.7 cells, with prednisolone used as a positive control (Table 3). While the ethanolic extract of 4-Glur (4-GlurE) showed high inhibition of nitric oxide, it was not as active as the positive control (prednisolone)

The aqueous extracts of 4-GlurA and others did not inhibit NO production with IC_{50} values greater than 100 µg/mL. Ethanolic extracts of *C. burmannii* (CBE) and nutmeg of *M. fragrans* (MFnE) were found to inhibit NO production with IC_{50} values of 79.98 ± 2.02 and 89.73 ± 4.56 µg/mL, respectively. Other ethanolic extracts did not demonstrate any inhibitory effects on NO production, with IC_{50} values exceeding 100 µg/mL. All extracts were screened for cytotoxicity using the MTT assay at a concentration of 50 and 100 µg/mL to eliminate the possibility that the observed inhibition of NO production was due to cell death (the cytotoxic value had to be below 30%). From the results recorded in Table 3, all extracts were found to be non-toxic to the tested cells except for the MFmE and *S. aromaticum* ethanolic extract (SAE). Importantly, the findings of this study supported the notion that combining four herbs (4-Glur) produced superior NO inhibition and no cytotoxicity as compared to a single herb alone.

Table 3. The percentage of spice extracts on lipopolysaccharide-induced nitric oxide production from RAW264.7 cells and cytotoxicity of plant extracts. Data are expressed as mean ± SEM, n = 3.

Plants	Code	%Inhibition of nitric oxide production and (% of cytotoxicity)						IC_{50} (µg/mL)
		0.5 µg/mL	1 µg/mL	5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	
***Wurfbainia testacea* (Ridl.) Škorničk. & A.D.Poulsen**	WTE	-	-	-	-	19.56 ± 3.25 (0.09 ± 6.74)	49.72 ± 0.40 (13.33 ± 6.63)	> 100*
	WTA	-	-	-	-	12.15 ± 0.21 (-20.72 ± 8.44)	24.36 ± 2.04 (-24.17 ± 5.12)	> 100*
***Cinnamomum burmannii* (Nees and T. Nees) Blume.**	CBE	-	-7.27 ± 1.00 (-6.68 ± 0.61)	-3.97 ± 0.75 (-3.74 ± 1.67)	6.49 ± 0.40 (25.96 ± 1.79)	25.96 ± 1.79 (-8.25 ± 3.02)	64.11 ± 0.85 (-8.92 ± 1.19)	79.98 ± 2.02*
	CBA	-	-	-	-	6.72 ± 0.90 (-6.83 ± 0.98)	5.15 ± 2.36 (-16.25 ± 6.61)	> 100*
***Coriandrum sativum* L.**	CSE	-	-	-	-	1.77 ± 3.55 (-23.38 ± 4.26)	13.58 ± 1.59 (-4.75 ± 1.89)	> 100*
	CSA	-	-	-	-	0.57 ± 0.21 (-15.70 ± 7.91)	-0.92 ± 1.57 (-17.99 ± 8.51)	> 100*
***Myristica fragrans* Houtt. (Nutmeg)**	MFnE	-	-5.48 ± 2.79 (5.46 ± 5.31)	-5.23 ± 4.08 (3.38 ± 1.54)	2.85 ± 3.04 (9.85 ± 2.35)	26.73 ± 3.02 (3.83 ± 1.39)	54.98 ± 0.89 (14.48 ± 4.12)	89.73 ± 4.56*
	MFnA	-	-	-	-	4.50 ± 0.19 (-14.16 ± 3.86)	0.62 ± 0.81 (-14.53 ± 0.38)	> 100*
***Myristica fragrans* Houtt. (Mace)**	MFmE	-	-	-	-	7.66 ± 2.02 (29.49 ± 5.47)	30.19 ± 1.14 (34.09 ± 6.35)	> 100*
	MFmA	-	-	-	-	9.47 ± 3.32 (-3.00 ± 2.27)	9.83 ± 3.06 (-6.01 ± 4.15)	> 100*
***Syzygium aromaticum* (L.) Merr. & L.M. Perry)**	SAE	-	-	-	-	17.97 ± 6.21 (30.25 ± 0.77)	35.89 ± 6.72 (37.38 ± 3.20)	> 100*
	SAA	-	-	-	-	-0.64 ± 1.91 (-5.22 ± 2.31)	-0.03 ± 0.66 (-2.83 ± 1.83)	> 100*
95% EtOH of 4-Glur	4-GlurE	1.09 ± 0.83 (-15.48 ± 6.76)	-	-0.72 ± 1.96 (-2.14 ± 6.65)	18.13 ± 2.35 (-7.24 ± 1.43)	63.52 ± 5.77 (2.60 ± 5.15)	86.15 ± 4.36 (12.39 ± 3.82)	43.95 ± 2.47*
aqueous extract of 4-Glur	4-GlurA	-	-	-	-	-0.50 ± 2.51 (-7.13 ± 6.12)	-4.11 ± 2.15 (-12.22 ± 6.47)	> 100*
Prednisolone (positive control)		0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	
		-10.59 ± 0.51 (1.38 ± 1.39)	14.45 ± 6.48 (9.38 ± 0.70)	53.72 ± 2.08 (21.14 ± 1.38)	82.26 ± 8.34 (16.50 ± 4.73)	91.80 ± 5.13 (23.44 ± 0.82)	-	0.56 ± 0.20

* $P < 0.05$ indicates significant differences in comparison with the positive control (prednisolone).

In vitro antioxidant activity.

The antioxidant activity of ethanolic and aqueous extracts of 4-Glur was assessed by DPPH radical scavenging assay and the results were presented as EC_{50} values in Table 4 and Fig. 1A. The EC_{50} values of both extracts were similar and not significantly different from the positive control (BHT). However, *C. burmannii* (cinnamon) and *S. aromaticum* (clove) aqueous extracts (CBA and SAA), and SAE showed strong antioxidant effects. The 4-Glur remedy was effective in scavenging DPPH free radicals in both ethanol and aqueous extracts. Although there was no significant difference in the ability of extracts to resist oxidation, SAA and CBA showed antioxidant activity (by DPPH assay) 2.32 and 2.01 times higher than the positive control (BHT).

Based on the results presented in Table 4 and Fig. 1B, it can be observed that both SAE and SAA extracts exhibited high $ABTS^{\bullet+}$ radical scavenging. Additionally, CBA and 4-GlurE also showed antioxidant activity, although it was not significant compared to the positive control (Trolox). Notably, 4-GlurE exhibited higher antioxidant activity than 4-GlurA, albeit not significantly. Moreover, the results of the 4-Glur remedy were consistent with those of the DPPH assay, as both the ethanolic and aqueous extracts of 4-Glur demonstrated excellent antioxidant properties, with no significant difference compared to the positive controls.

Table 4. Correlation coefficients of antioxidation activities (DPPH and ABTS assay), polyphenol content (TPC and TFC), alpha-glucosidase inhibition and nitric oxide production inhibition.

Activities	DPPH	ABTS	TPC	TFC	Alpha-glucosidase
DPPH	1.00				
ABTS	0.8499*	1.00			
TPC	-0.7506*	-0.7796*	1.00		
TFC	-0.6799*	-0.7176*	0.8177*	1.00	
Alpha-glucosidase	0.4491	0.6311*	-0.3867	-0.3659	1.00
Nitric oxide inhibition	0.2462	0.2320	0.0483	-0.0720	0.1240

*$P < 0.05$ Indicates significant correlation; DPPH, (2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TPC, total phenolic content; TFC, total flavonoid content.

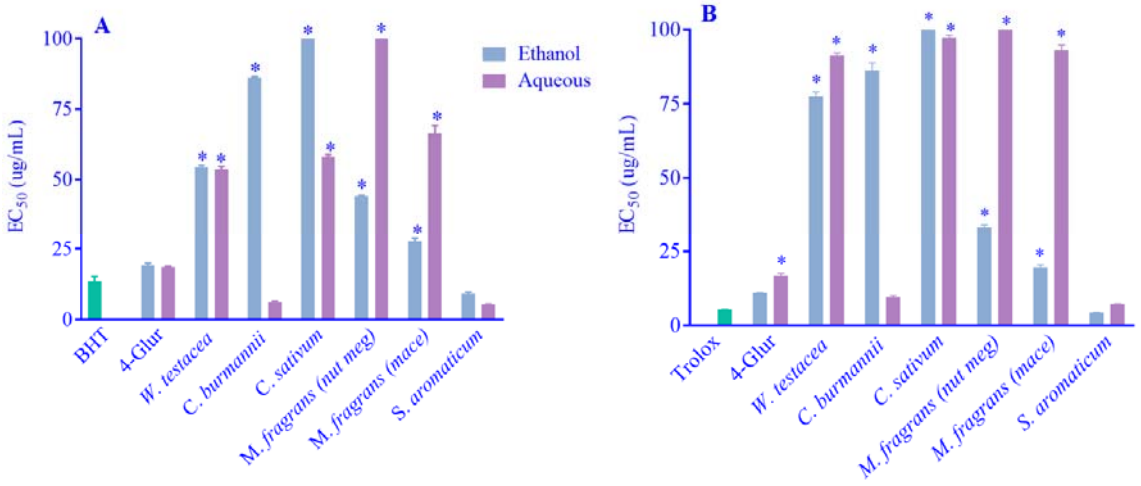

Fig. 1. The EC_{50} (µg/mL) for various extracts from spices (A) by DPPH radical scavenging compared to standard positive control BHT and (B) by $ABTS^{\bullet+}$ radical scavenging compared to standard positive control Trolox. Data are presented as mean ± SEM, n = 3. *$P < 0.05$ indicates significant differences compared to the positive control groups. DPPH, (2,2-diphenyl-1-picrylhydrazyl; $ABTS^{\bullet+}$, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BHT, butylated hydroxytoluene; C, *Cinnamomum; M, Myristica; S, Syzygium.*

In vitro alpha-glucosidase and alpha-amylase inhibitory activity

Figure 2A presents the results of the alpha-glucosidase inhibitory assay study, which reports the IC_{50} values. The CBA and SAA extracts demonstrated the best alpha-glucosidase inhibition, surpassing the positive control (acarbose). Additionally, 4-GlurA inhibited alpha-glucosidase, which was insignificant compared to acarbose.

Figure 2B shows that the CBA extract exhibited an inhibitory effect on the alpha-amylase enzyme, which was less potent than acarbose. However, the CBA extract was the only extract that demonstrated this activity. The other extracts showed low alpha-amylase inhibitory activity with IC_{50} values greater than 1.0 mg/mL.

Before determining the IC_{50}, the percentage of alpha-glucosidase inhibition of the extracts at a concentration of 0.5 mg/mL was also evaluated (Fig. 3). The results revealed that CBA, SAA, and MFmE were significantly more effective than acarbose. However, the percentage inhibition of 0.5 mg/mL for 4-GlurA, MFnE, CSE, and 4-GlurE did not differ significantly from acarbose. The high antioxidant activity and the presence of phenolic and flavonoid content were attributed to these findings. CBA, SAE, SAA, 4-GlurA, and 4-GlurE were found to have high antioxidant activity and contained very high phenolic content.

Total phenolic and flavonoid content

Figure 4A demonstrates that CBA had the highest TPC. This was followed by SAE, SAA, 4-GlurA, and 4-GlurE. The CBA extract exhibited a significantly higher TPC compared to the ethanol extract, while the TPC of other spices showed slight differences between their aqueous and ethanol extracts. Furthermore, 4-GlurA had a higher TPC than 4-GlurE. The ethanolic and aqueous extracts of the *W.testacea* are not significant.

Figure 4B represents the TFC of the different extracts. CBA had the highest flavonoid content, followed by SAE, MFmE, 4-GlurE, SAA, and MFnE. The most ethanolic extracts showed significantly higher flavonoid content than the aqueous extracts. Except for *C.burmannii* and *C.sativum*, aqueous extracts showed significantly higher flavonoid content than the ethanolic extracts. Additionally, 4-GlurE contained a higher flavonoid content than 4-GlurA, about 3.6 times.

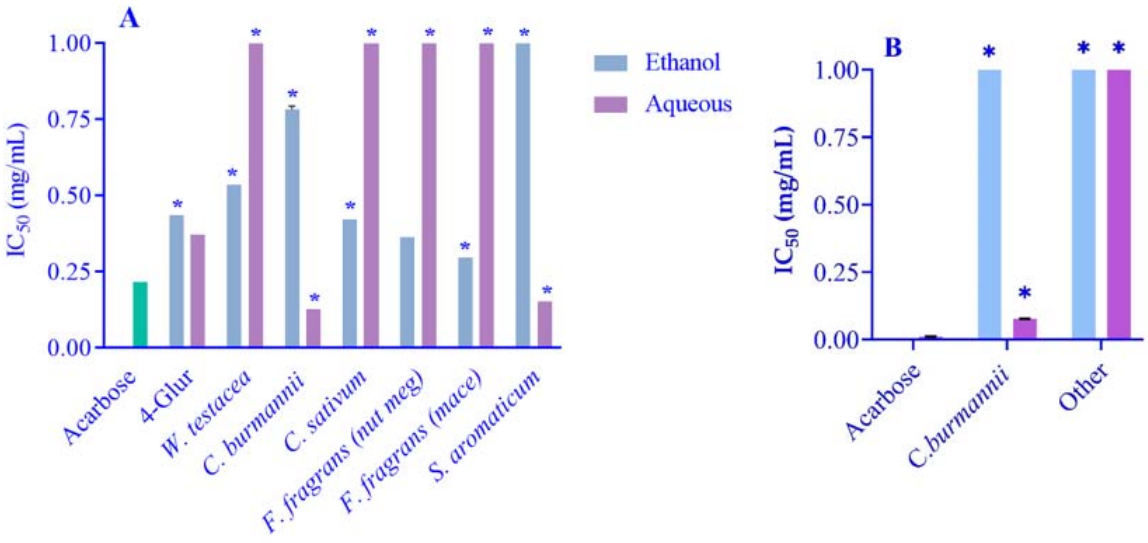

Fig. 2. The IC_{50} (µg/mL) on various extracts from spices (A) by alpha-glucosidase inhibition and (B) by alpha-amylase compares with standard positive control (acarbose). Data are expressed as mean ± SEM, n = 3. *P < 0.05 indicates significant differences compared to the positive control group. C, *Cinnamomum; M, Myristica; S, Syzygium.*

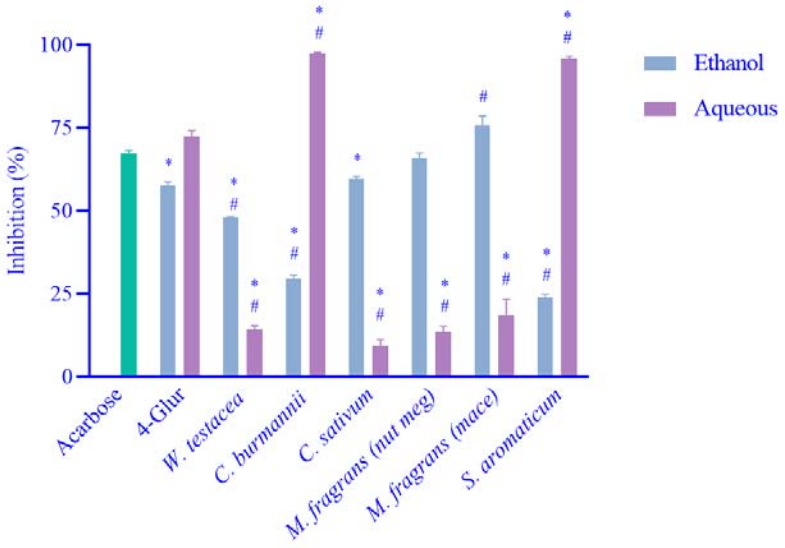

Fig. 3. The percentage of alpha-glucosidase inhibition at the concentration of 0.5 mg/mL of each spice extract compared with the positive control (acarbose). Data are expressed as mean ± SEM, n = 3. *$P < 0.05$ indicates significant differences compared to the positive control group; $^{\#}P < 0.05$) versus 4-GlurA. C, *Cinnamomum; M, Myristica; S, Syzygium*.

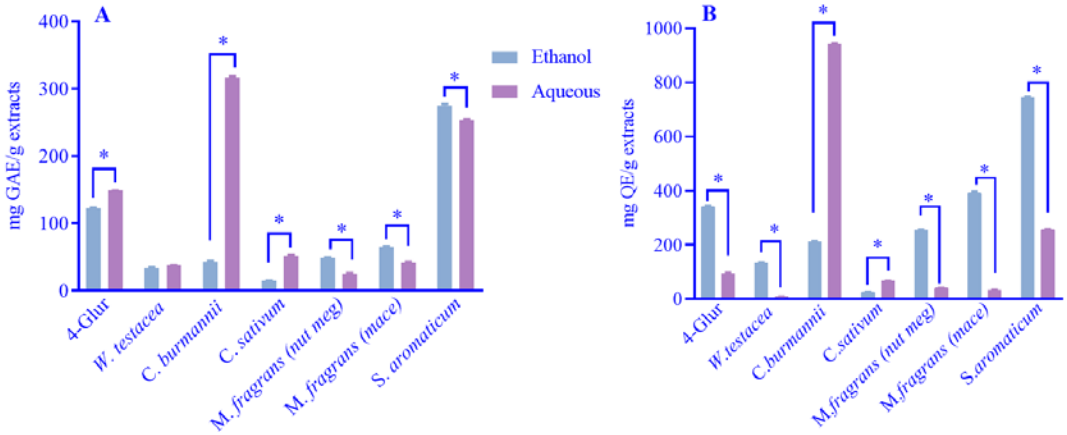

Fig. 4. (A) Total phenolic content and (B) total flavonoid content of each spice extract. Data are expressed as mean ± SEM, n = 3. C, *Cinnamomum; M, Myristica; S, Syzygium*; mg GAE/g extract, milligram gallic acid equivalents per gram of dried extract; mg QE/g extract, milligram quercetin equivalents per gram of dried extract. *P < 0.05 indicates significant differences compared between ethanolic and aqueous extracts.

Polyphenolic correlation analysis

The correlation analysis among the polyphenol (TPC and TFC), alpha-glucosidase inhibition, and antioxidant activities (by DPPH and ABTS assay; Fig. 5A-H and Table 4) and hierarchical clustering (Fig. 6A). From Table 4 and Fig. 5, the correlation coefficient between antioxidant activities EC_{50} by two assays (DPPH and ABTS scavenging assay) revealed a strong positive correlation of 0.8499. Antioxidant activities EC_{50} by two assays (DPPH and ABTS scavenging assay) were significantly correlated with TPC at r = -0.7506 and r = -0.7796, respectively. When TPC was high, the EC_{50} of DPPH and ABTS was also low (high antioxidant). Similar to TFC correlated with DPPH and ABTS at r = -0.6799 and r = -0.7176, respectively. In addition, TPC and TFC have shown a strong correlation of r = 0.8177. The correlation between TPC and TFC and alpha-glucosidase inhibition was weak (r < 0.4). The correlation between antioxidants (DPPH and ABTS) and alpha-glucosidase inhibition was moderate (r = 0.4 - 0.7). Antioxidant and NO production inhibition showed a weak correlation (r = 0.1-0.39), whereas NO production inhibition did not correlate with TPC, TFC, or alpha-glucosidase inhibition (r < 0.1).

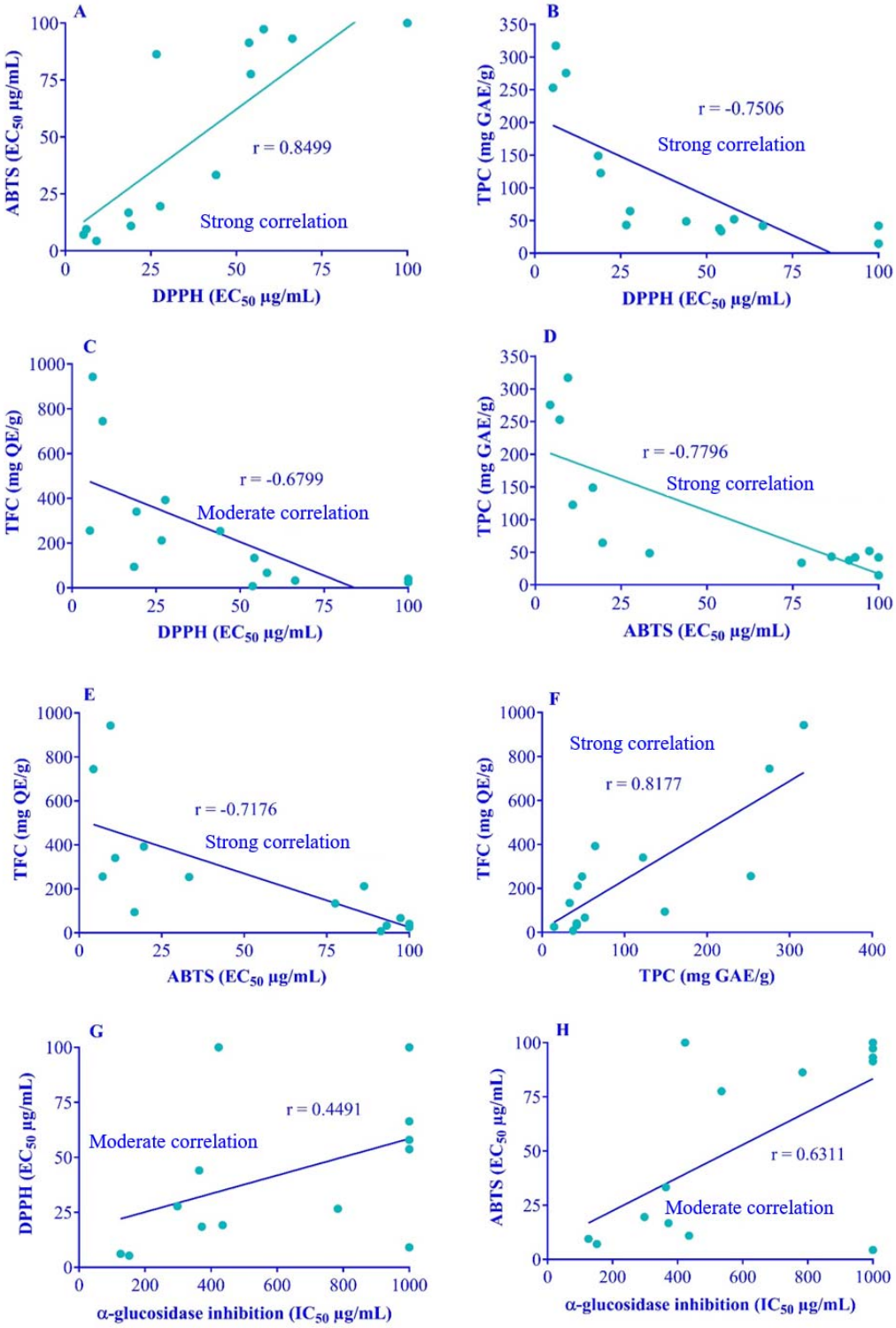

Fig. 5. Correlation scatter plot demonstrating the relationship amongst antioxidation activities, polyphenol content, and alpha-glucosidase inhibition; (A) DPPH and ABTS, (B) DPPH and TPC, (C) DPPH and TFC, (D) ABTS and TPC, (E) ABTS, and TFC, (F) TPC and TFC, (G) alpha-glucosidase inhibition and ABTS, and (H) alpha-glucosidase inhibition and DPPH. DPPH, (2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TPC, total phenolic content; TFC, total flavonoid content.

DISCUSSION

The present study confirmed the alpha-glucosidase inhibitory activity of coriander, cinnamon, nutmeg, and clove, consistent with previous reports (21). Sham Shihabudeen *et al.* found that cinnamon extract had a more potent alpha-glucosidase inhibitory effect than acarbose *in vitro*. Cinnamaldehyde, which makes up 65-80% of cinnamon bark extract, was more effective than metformin in lowering haemoglobin A1c, plasma blood glucose, and lipid profile *in vivo* (22-24). Additionally, clinical trials have shown that cinnamon extract can lower both fasting blood sugar and haemoglobin A1c levels (25).

In the present investigation, SAA extract demonstrated highly potent inhibitory activity against alpha-glucosidase, consistent with previous research (26). Oleanolic acid (OA) and maslinic acid isolated from clove also effectively inhibited alpha-glucosidase, comparable to acarbose. Treatment with maslinic acid or OA for five weeks reduced the expression of alpha-glucosidase, alpha-amylase, and glucose transporter in the small intestines of streptozotocin-induced diabetic rats, resulting in lower glucose absorption into the bloodstream (27). OA also exhibited a significant protective effect against oxidative stress induced by tert-butyl hydrogen peroxide in liver cells, according to preliminary research (28). Furthermore, in an *in vivo* study, OA administration to diabetes-induced rats restored the depletion of glycogen and glycogenic enzymes in hepatic tissues and muscles caused by diabetes to nearly normal levels (29).

The study measured the alpha-glucosidase inhibitory activity of different spice extracts and reported the results as IC_{50} values. Moreover, all spice extracts had higher alpha-glucosidase inhibitory activity compared to alpha-amylase inhibitory activity. This finding is consistent with Fernando's investigation of coriander, cinnamon, nutmeg, and clove extracts (30), which also demonstrated less effective alpha-amylase inhibition than acarbose.

Our findings were consistent with those of Hayward, who reported a high TPC for cinnamon extracts (31). Adefegha and colleagues conducted an *in vitro* study on a clove polyphenol-rich extract and found that it was a potent antioxidant and inhibited alpha-glucosidase more effectively than alpha-amylase (32).

To assess the correlation among the polyphenol (TPC and TFC), alpha-glucosidase inhibition, and antioxidant activities (by DPPH and ABTS assay), we performed correlation analysis (Fig. 5A-H and Table 4) and hierarchical clustering (Fig. 6A).

From Table 4 and Fig. 5, the correlation between antioxidant (DPPH and ABTS) and alpha-glucosidase inhibition was moderate, whereas some previous studies reported a strong correlation between antioxidant, TPC, TFC, and alpha-glucosidase inhibition (33). Besides, antioxidant and NO production inhibition showed a weak correlation, whereas NO production inhibition did not correlate with TPC, TFC, or alpha-glucosidase inhibition as previously reported (34).

HemI software categorized fourteen spice extracts based on their TPC, TFC, antioxidation, and alpha-glucosidase inhibition activities. The Euclidean metric was chosen to measure the similarity between patterns. The heat map was created to illustrate the intensity of activities, with the horizontal dimension displaying the relationships between similar extracts. According to Fig. 6A, Hierarchical cluster analysis categorized 14 spice extracts into three groups. The extracts exhibiting potent antioxidants, extremely high TPC and TFC, and potential alpha-glucosidase inhibition were grouped into group 1. Group 2 consisted of extracts with strong antioxidants, high TPC or TFC, and moderate alpha-glucosidase inhibition properties. The extracts with low or moderate antioxidants, TPC and TFC activity, and alpha-glucosidase inhibition were grouped in group 3. The Venn diagram (Fig. 6B) demonstrated that both SAA and CBA had all four properties (potential antioxidant, high alpha-glucosidase inhibition, high TPC, and high TFC), corresponding to group 1 of the categories determined by Hierarchical cluster analysis.

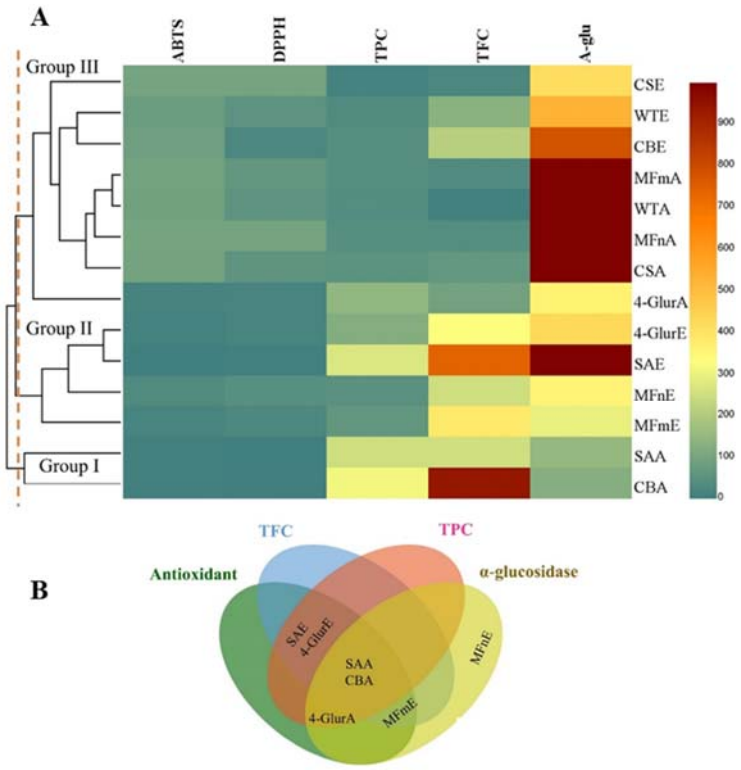

Fig. 6. Polyphenol content (TPC and TFC), antioxidant activities, and alpha-glucosidase inhibition were used to create (A) a heat map and perform a hierarchical cluster analysis of spice extracts (19,20). (B) The Venn diagram from the top 5 extracts of each activity demonstrates that only two extracts, SAA and CBA, possess all four characteristics (antioxidant (DPPH and ABTS), alpha-glucosidase enzyme inhibition, high TPC, and high TFC). DPPH, (2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TPC, total phenolic content; TFC, total flavonoid content; CSE, Coriandrum sativum L. ethanolic extract; CSA, Coriandrum sativum L. aqueous extract; CBE, Cinnamomum *burmannii e*thanolic extract; CBA, Cinnamomum *burmannii* aqueous extract; WTE, *Wurfbainia testacea e*thanolic extract; WTA, *Wurfbainia testacea* aqueous extract; MFnE, *Myristica fragrans* Houtt. (nutmeg) ethanolic extract; MFnA, *Myristica fragrans* Houtt. (nutmeg)aqueous extract; MFmE, *Myristica fragrans* Houtt. (mace) ethanolic extract; MFmA, *Myristica fragrans* Houtt. (mace) aqueous extract; SAE, *Syzygium aromaticum* ethanolic extract; SAA, *Syzygium aromaticum* aqueous extract.

The antioxidant activity of both the ethanolic and aqueous extracts of 4-Glur was excellent, as indicated by their EC_{50} values in the DPPH assay presented in Fig. 1A. According to the ABTS assay, the ethanolic extract showed higher antioxidant activity than the aqueous extract (Fig. 1B). The 4-GlurE and 4-GlurA extracts contained high concentrations of phenolic compounds, with the aqueous extract having a higher TPC than the ethanolic extract (Fig. 4A and B).

This study from the correlation coefficient, heat map, and Hierarchical cluster analysis revealed that antioxidant activity, as measured by the DPPH and ABTS assays, has been shown to correlate with TPC and TFC levels in previous studies (35-38). Whereas, some studies have found no correlation (39). Nonetheless, dietary phenolic and flavonoid compounds are potentially safer to use than synthetic antioxidants.

The extracts from CBA demonstrated a high flavonoid content, particularly quercetin, which has been shown to possess inhibitory activity against alpha-glucosidase (40). *In vitro* studies have also demonstrated quercetin's ability to inhibit alpha-alpha but not porcine pancreatic alpha-amylase (41). Additionally, *in vivo* studies have revealed that quercetin reduces blood glucose, malondialdehyde, and NO levels while increasing antioxidant enzyme activity and pancreatic insulin content. Furthermore, quercetin has been found to preserve the integrity and structure of pancreatic cells (42).

In the case of the 4-Glur remedy, combining the four spices has resulted in synergistic effects in terms of their antioxidant, anti-inflammatory, and alpha-glucosidase-inhibitory activities. This study is relevant to the use of 4-Glur in TTM since it is a component of the blood circulation-improving drug known as Ya-Hom, which is frequently utilized for the elderly (43,44). The 4-Glur is a less harmful and less expensive alternative to acarbose and has been found to reduce gastrointestinal side effects such as bloating, stomach pain, and discomfort. Furthermore, each spice in 4-Glur has a hot and spicy taste, which can help reduce flatulence and carminatives (43,44). Cinnamon has been utilized for ages in various cultures and has been found to have numerous health benefits, such as reducing blood sugar, having anti-inflammatory properties, and possessing anti-microbial activity (45). Combining cinnamon with other spices (4-Glur) to create a 5-combination spice is feasible, and this study has shown that cinnamon can inhibit both alpha-glucosidase and alpha-amylase.

Studying traditional medicines from diverse cultures, such as Ayurveda in India, Traditional Chinese Medicine in China, and Iranian Traditional Medicine in Persia, holds great ethnopharmacological and therapeutic significance, especially when exploring their potential effects on diabetes (46).

CONCLUSION

In conclusion, our study has shed light on the remarkable potential of the 4-Glur remedy, specifically through its ethanolic (4-GlurE) and aqueous (4-GlurA) extracts, as powerful agents in combating chronic diseases associated with oxidative stress. We have demonstrated their potency in inhibiting alpha-glucosidase activity and their high antioxidant activity, with *S. aromaticum* exhibiting the highest levels of phenolic and flavonoid compounds among the spices studied.

One intriguing discovery that emerged from our research is the unique anti-inflammatory property exhibited by 4-GlurE, as it effectively inhibited NO secretion. Furthermore, the application of the TTM method, combining the four spices, 4-Glur, *S. aromaticum*, and possibly cinnamon (5-Glur), revealed a synergistic effect. This synergy significantly enhanced their antioxidant, anti-inflammatory, and alpha-glucosidase inhibition properties, surpassing the expectations of the sum of individual results. It is noteworthy that the CBA extract also displayed remarkable results in antioxidant tests and alpha-glucosidase inhibition, which further accentuates the overall efficacy of the 4-Glur remedy.

In light of these findings, our research underscores the promise of the 4-Glur remedy, with the potential inclusion of cinnamon (5-Glur), in both preventing and treating chronic diseases associated with oxidative stress. However, we acknowledge that further research, including *in vivo* and clinical studies, and *via* bioinformatics methods such as docking or molecular dynamics simulation in future works is imperative to comprehensively evaluate their effectiveness across a wider spectrum of chronic conditions. These findings collectively position the 4-Glur remedy as a promising candidate for alternative medicine in the management of chronic diseases, with particular emphasis on the novel discovery of 4-GlurE's unique anti-inflammatory properties and the demonstration of synergy within this herbal combination.

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Conflicts of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

A. Itharat conceived and supervised the project. C. Choockong performed the experiments, analyzed, and wrote the manuscript in consultation with A. Itharat, W. Pipatrattanaseree and N.M. Davies. N. Intharit performed antioxidant activity. S. Sukkhum performed polyphenol content. T. Ninlaor and K. Piwngam verified the

analytical methods. N.M. Davies re-analyzed data and provided additional analytical and grammatical changes to the manuscript. All authors read and approved the finalized article.

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