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Myanmar traditional medicine formulations and their antioxidant, antiglycation and alpha-glucosidase inhibitory activities: Potentials for antidiabetes complications

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ABSTRACT

Myanmar Traditional Medicine (MTM) has been used since ancient times to treat life-threatening diseases like diabetes. In this study, various in vitro assays were used to prove that four MTM formulations were effective at treating diabetes. Antioxidant activities were determined using in vitro DPPH, nitric oxide (NO), and superoxide (SO) radical scavenging assays. The Folin-Ciocalteu method was used to quantify the total phenolic content, while the BSA-fluorescent antiglycation and α-glucosidase inhibitory assays were utilized to determine the antidiabetic activity of MTMs. Among the tested samples, MTM3 showed the best activities for almost all the biological assays tested in this experiment with the % inhibition of 82.89 ± 1.64 for NO and 65.02 ± 2.82 for SO radical scavenging activity, 92.12 ± 1.18 for α-glucosidase inhibitory activity and IC_{50} of 180.29 ± 1.6 µg/ml for the antiglycation activity. It also possessed the highest total phenolic content of 149.41 ± 3.7 mg GAE/g of extract/l among the tested samples. Therefore, the findings suggested that MTM could help diabetic patients improve their quality of life through antioxidant activity against several free radicals and their antiglycation and α-glucosidase inhibitory characteristics.

1. Introduction

Myanmar is a country rich in natural resources, with over 7.000 species of medicinal plants growing throughout the country and 70% of Myanmar's population relies on traditional medicine (Wai, 2015). Traditional medicine has been used to treat various diseases since ancient times and there is no doubt about its effectiveness, but it is still a traditional knowledge-based practice and needs to be promoted as evidence-based medicine through scientific validation. Although Western medicines and technology have become more influenced by the urban population, Myanmar Traditional Medicine (MTM) is still popular among rural people (Wai, 2015).

MTM covers all the fundamental traditional disciplines, as well as different treatises on traditional medicine and a variety of ways for prescribing a wide range of remarkably strong, efficacious, and low-toxicity traditional drugs (Mamatha, 2017; Ministry of Health, 2006). Ethnobotanical research, on the other hand, has become increasingly popular not only at the

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national level but also at the worldwide level, and it has revealed that a large gap exists between the scientific validation of ethnomedicine and the applications to which it is put (Rafe, 2017). MTM formulations have also received quite a little attention for evidence-based validation.

Diabetes mellitus is a prevalent chronic and metabolic disorder defined by elevated glucose levels resulting from either a lack of insulin or insufficient insulin production. The condition is characterized by long-term problems affecting the ocular, renal, cardiovascular, and neurological systems. This particular ailment is additionally linked to symptoms including excessive urination, exhaustion, loss of weight, impaired wound healing, impaired vision, and elevated amounts of glucose in the urine (Moradi et al., 2018). Diabetes is associated with an increase in oxidative stress, and there is substantial experimental and clinical evidence that reactive oxygen species (ROS) generation is elevated in diabetes (Matough et al., 2012). Non-enzymatic glycation is the primary factor responsible for spontaneous protein damage, resulting in a range of issues attributed to the development of non-reversible Advanced glycation end products (AGEs) and the induction of oxidative stress (Perera et al., 2013). Plant samples exhibiting both antioxidant and antiglycation capabilities possess enhanced potential for the treatment of a variety of biological illnesses, such as diabetes (Moe et al., 2018).

As globalization and east-west interactions intensify, the rising prevalence of type 2 diabetes in Asia has significant public health and socioeconomic consequences (Lee et al., 1998). This research attempted to validate the commercially available antidiabetic MTM on the aspects of antioxidant, antiglycation, and α-glucosidase inhibitory activities using in vitro assays and compared their effectiveness based on their formulations.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade chemicals such as 1,1-diphenyl- 2-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, sodium nitroprusside, sulphanilic acid, N-(1-naphthayl) ethylenediamine dihydrochloride, glacial acetic acid, Folin-Ciocalteu's reagent, sodium carbonate, ethylenediamine tetra-acetic acid (EDTA), nitro blue tetrazolium (NBT), riboflavin, acarbose, α-glucosidase, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), fructose, sodium azide, and bovine serum albumin (BSA) were purchased from Sigma Chemicals Co. (St. Louis, USA), or TCI Development Co. Ltd (Shanghai, China).

2.2. MTM formulations

Commercially available MTMs were purchased from Kyaukse district, Mandalay region in Myanmar, and brand names were given the codes MTM1, MTM2, MTM3, and MTM4. The formulations of purchased MTMs are shown in Table 1.

2.3. Antioxidant activity assays

2.3.1. In vitro DPPH free radical scavenging assay

The free radical-scavenging activity of the MTM formulations was determined with the DPPH free radical-scavenging assay as described by Lee et al. (1998). In a 96-well microplate, the reaction mixture containing 5 µl of the test sample (0.5 mg/ml in DMSO) and 95 µl of DPPH (300 mol/l in methanol) was deposited. The optical density (OD) was measured using a SPECTROstar Nano microplate reader (BMG LABTECH, Germany) at a wavelength of 515 nm after the microplate had been kept in the dark at 37 °C for 30 minutes. As the blank, DMSO was utilized, while ascorbic acid served as the standard. The inhibition rate (%) was calculated through comparison to the blank.

2.3.2. In vitro nitric oxide radical scavenging assay

Nitric oxide (NO) radical scavenging assay was performed as described by Hertog et al. (1993). 10 µl of the test sample (0.5 mg/ml) in DMSO, 20 µl of phosphate buffer saline (0.1 mol/l, pH 7.4), and 70 µl of sodium nitroprusside (10 mmol/l) were mixed, and incubated at 25 °C for 90–100 minutes to facilitate the formation of nitrite ions. After incubation, 50 µl of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added and permitted to stand for 5 minutes for diazotization to be completed. Then 50 µl of 0.1% w/v N-(1-naphthyl) ethylenediamine dihydrochloride was added, agitated, and left to stand for 20 minutes. Under the diffused light, a pink chromophore was created. Using a SPECTROstar Nano microplate reader, the reduction of the pink-colored chromophore was measured at 540 nm relative to the corresponding blank solution. Ascorbic acid served as the standard for comparison.

2.3.3. In vitro superoxide radical scavenging assay

The free radical scavenging activity of the MTM formulations was also determined with superoxide (SO) radical scavenging assay modified from the protocol described by Patel Rajesh and Patel Natvar (2011). The reaction mixture containing 10 µl of the test sample (0.5 mg/ml) in DMSO, 160 µl of potassium phosphate buffer (0.067 mol/l, pH 7.4), 15 µl of EDTA (4.5 mmol/l), 10 µl of NBT (1 mg/ml), and 5 µl of riboflavin (0.2 mg/ml) was incubated under fluorescence light for 5 minutes. Using a SPECTROstar Nano microplate reader, the absorbance of the solution was determined at 560 nm relative to the blank solution. Gallic acid was used as the reference standard.

2.4. Antidiabetic activity assays

2.4.1. In vitro α-glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity assay was performed as described by Abu-Zaiton (2010) to detect the antihyperglycemic potentials of the extracts. In a 96-well microplate, 10 µl of the test sample (0.4 mg/ml in DMSO), 20 µl of -glucosidase (0.5 unit/ml), and 120 µl of 0.1 M phosphate buffer (pH 6.9) were added together and incubated at 37 °C for 15 minutes. Then, 20 µl of 5 mM *p*-nitrophenyl-D-glucopyranoside in 0.1 M phosphate buffer (pH 6.9) was added to initiate the enzymatic reaction, followed by 15 minutes of incubation at 37 °C. The reaction was then neutralized by adding 80 µl of 0.2 M sodium carbonate solution, and absorbance was measured at a wavelength of 405 nm using a SPECTROstar Nano microplate reader. The reaction system without plant extracts was used as a control, while the reaction system without α-glucosidase was used as a blank to correct for background absorbance. Acarbose was utilized as the reference standard.

All of the aforementioned analyses were conducted at least in triplicate for each sample and positive control, and the inhibition rate was calculated using the following formula given below:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.4.2. In vitro antiglycation activity assay

In vitro, antiglycation activity of MTM formulations was determined by measuring the ability of the MTM to inhibit the formation of advanced glycation end products (AGE) as described by Yarizade et al. (2017). In a 96-well black fluorescence plate, the reaction mixture (200 µl), containing 10 µl BSA (10 mg/ml), 70 µl of sodium phosphate buffer (0.1 M, pH 7.4), 100 µl of fructose (500 mM), sodium azide (0.1 mM) and 20 µl test sample (various concentrations in DMSO), was incubated at 37 °C for 7 days in the dark. After incubation, AGE formation was measured at the fluorescence intensity of excitation (370 nm) and emission (440 nm) by using an Agilent Cary Eclipse Fluorescence spectrophotometer (G9800, US). Rutin was used as the standard. The reaction mixture without fructose was used as the negative control and that without extracts as the positive control. Inhibition rate (%) was calculated by using the following formula given below:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of control}}\right) \times 100$$

Table 1. Formulation of MTMs

Name	Ingredients	Formulation (mg/g of MTM)
MTM1	*Swertia angustifolia*	17.19
	Tinospora cordifolia	245.70
	Trigonella foenum-graecum	245.70
	Quercus intecforia	245.70
	Capsicum frutescens	245.70
MTM2	*Trigonella foenum-graecum*	139.46
	Curcuma longa	101.24
	Centella asiatica	354.34
	Andrographis paniculata	253.09
	Piper nigrum	101.24
	Tinospora cordifolia	50.62
MTM3	*Ferula asafoetida*	142.86
	Curcuma longa	142.86
	Cinnamomum zeylanicum	142.86
	Aloe barbadensis	142.86
	Croton roxburghianus	142.86
	Tinospora cordifolia	142.86
	Andrographis paniculata	142.86
MTM4	*Gnetum gnemon*	230.78
	Odoriferous medicinal salt	115.39
	Rock salt	115.39
	Salamoniac	115.39
	Glycyrrhiza glabra	115.39
	Five types of stones (salt + saltpetre + natron + charcoal ash + limestone)	48.08
	Five types of seeds from *Foeniculum vulgare*, *Hyoscyamus niger*, *Apium gravedens*, *Carum carui*, *Anethum graveolens*	48.08
	Curcuma longa	38.46
	Rauvolfia serpentina	38.46
	Terminalia citrina	19.23
	Ray sting	19.23
	Myristica fragrans	19.23
	Caesalpinia crista	19.23
	Piper longum	19.23
	Trigonella foenum-graecum	19.23
	Caryophyllus aromaticus	9.61
	Zingiber officinale	9.61

2.5. Total phenolic content measurement

The total content of phenolic compounds present in each MTM formulation was measured with the method described by Waterhouse (2002) with slight modifications. Briefly, 2 µl of the test samples (1 mg/ml in 70% methanol) or gallic acid at varying concentrations (0, 0.5, 1, 1.5, 2.0, 2.5, and 5 g/ml in 70% methanol) were dispensed into each well of the 96-well microplate. 148 µl of distilled water and 20 µl of the 1 N Folin-Ciocalteu's reagent were added. The reaction mixtures were thoroughly combined and incubated at room temperature for 5 minutes. The reaction was then neutralized by adding 30 µl of sodium carbonate at a concentration of 0.2 g/ml. After incubating the samples at 40 °C for 30 minutes, the optical density (OD) was measured using a SPECTROstar Nano microplate reader at a wavelength of 765 nm. Three replicate assays were performed on each sample. Total phenolic content (TPC) was computed as gallic acid equivalent (GAE) using the gallic acid standard curve equation ($y = 0.0007x$, $R^2 = 0.9985$).

2.6. Statistical analysis

All data were expressed as mean ± standard error of the mean of at least triplicate measurements. One-way analysis of variance (ANOVA) and Dunnett's or Tukey's multiple comparison tests were performed to compare the difference between the MTM activity and standard control or between each other. In each analysis, $p \leq 0.05$ was considered statistically significant. For the gallic acid standard curve, the optical density (OD) of the gallic acid was plotted against the concentrations of 0, 50, 100, 150, 250, and 500 mg/l. The linear regression points were used to determine the GAEs of the phenolic concentration in tested samples. Statistical analyses were performed using Microsoft Office 2010 and GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The IC_{50} was defined as the concentration of MTM required to inhibit 50 % of radicals or enzyme activity under assay conditions.

3. Results and discussion

Four commercial anti-diabetic MTMs were tested for their biological activities by using in vitro assays and their activities were compared

based on their formulations. In vitro DPPH radical scavenging assay, nitric oxide (NO) radical scavenging assay, and superoxide (SO) radical scavenging assay were used to determine the antioxidant activities. Folin-Ciocalteu's method was used to determine the total phenolic content and BSA-fluorescent antiglycation assay and α-glucosidase inhibitory activity were used to evaluate the antidiabetic activity of selected MTM.

Among the four tested samples for six biological activities evaluations, MTM3 showed the best activities for almost all the biological assays tested in this experiment with the % inhibition of 82.89 ± 1.64 for NO and 65.02 ± 2.82 for SO radical scavenging activity, 92.12 ± 1.18 for α-glucosidase inhibitory activity and IC_{50} of 180.29 ± 1.6 µg/ml for the antiglycation activity (Figure 1, 2, 3, 4 and Table 2). It also possessed the highest total phenolic content of 149.41 ± 3.7 mg GAE/g of extract/l among the tested samples (Figure 5).

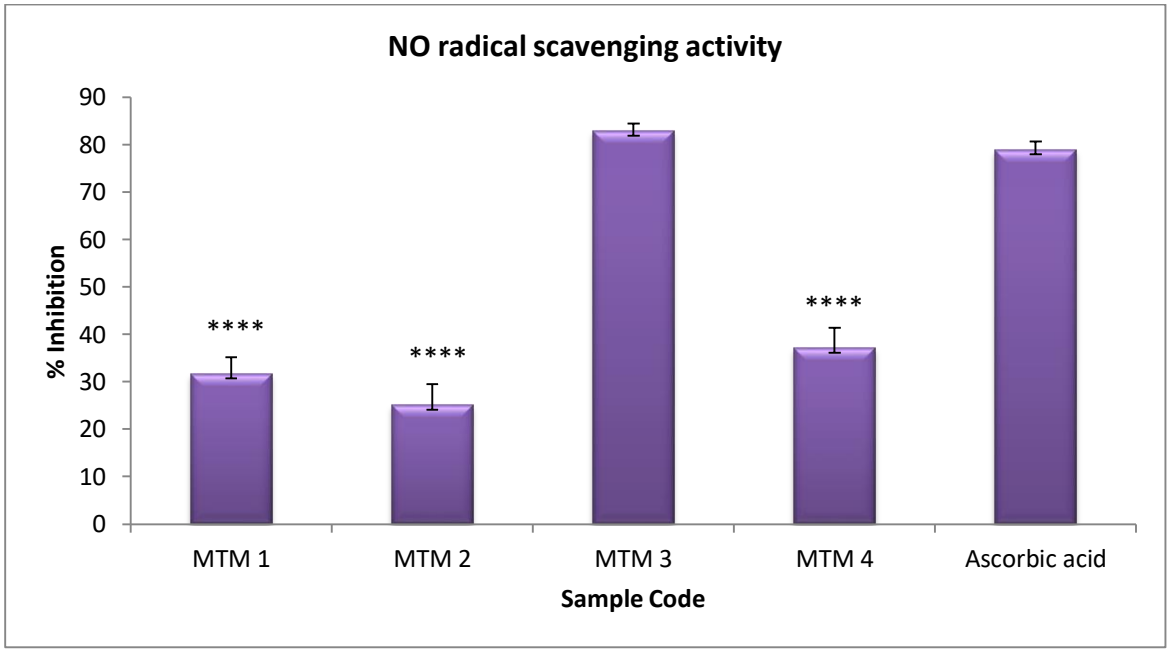

Figure 1. Comparison of antioxidant activities of Myanmar traditional medicine formulations through NO radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. NO: Nitric oxide

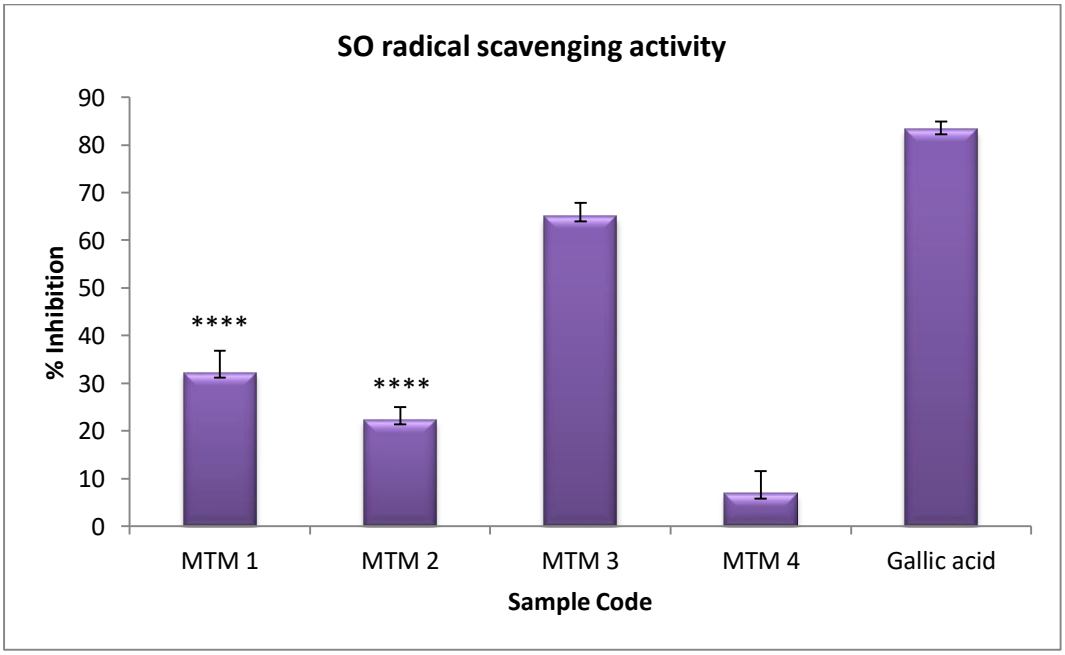

Figure 2. Comparison of antioxidant activities of Myanmar traditional medicine formulations through SO radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. SO: Superoxide

Figure 3. Comparison of antidiabetic activities of Myanmar traditional medicine formulations through α-glucosidase inhibitory activity assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.

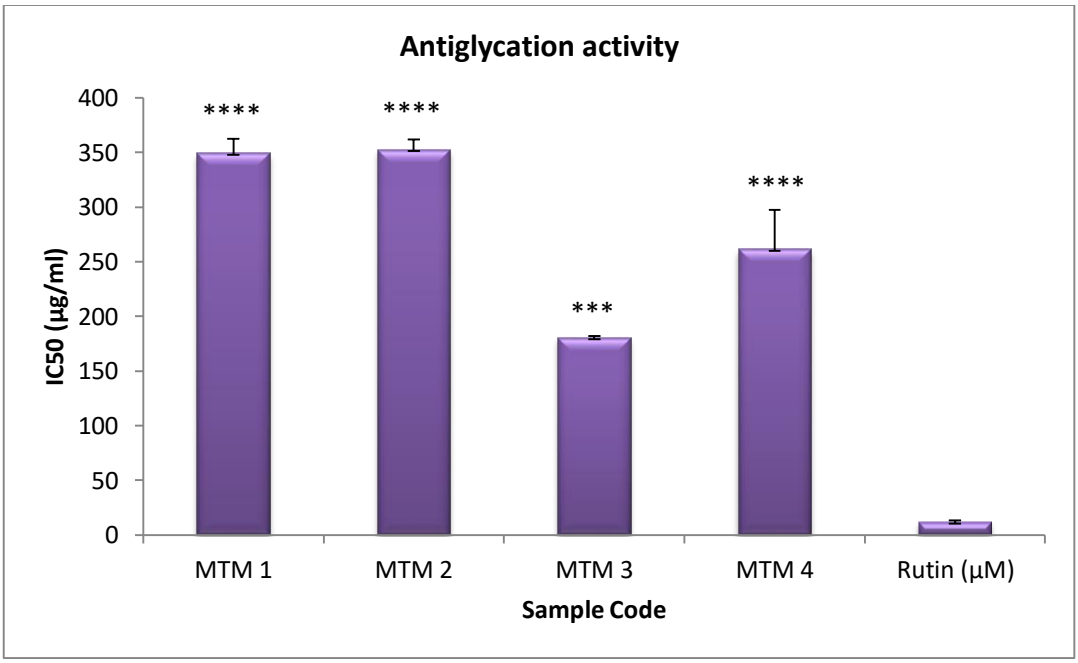

Figure 4. Comparison of antidiabetic activities of Myanmar traditional medicine formulations through antiglycation assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.

For the DPPH radical scavenging assay, MTM1 showed the best activity with a % inhibition of 53.66 ± 4.19 (Figure 6). It was also active in α-glucosidase inhibitory assay with a % inhibition of 62.39 ± 4.54 while positive control, acarbose, showed a % inhibition of 60.54 ± 5.57. MTM2 was not active for all the currently tested bioassays.

MTM4 was moderately active in antiglycation assay with IC_{50} of 260.88 ± 36.36 µg/ml and active in α-glucosidase inhibitory assay with % inhibition of 61.39 ± 0.96.

MTM3 was composed of seven traditional medicinal plants including *Ferula asafoetida*, *Curcuma longa*, *Cinnamomum zeylanicum*, *Aloe barbadensis*, *Croton roxburghianus*, *Tinospora cordifolia*, and *Andrographis paniculata*. Several research teams have studied *F. asafoetida*'s antidiabetic potential. *F. asafoetida* extract was examined for potential antidiabetic action against alloxan-induced diabetes in rats and associated hormones. *F. asafoetida* extract lowered blood glucose levels and increased serum insulin (Abu-Zaiton, 2010). Another study team determined the in vitro antidiabetic potential of *F. assafoetida* via DPP-IV and α-glucosidase inhibitory activities and concluded that *F. assafoetida* could be a

source for active ingredients as α-glucosidase and DPP-IV inhibitors to treat type 2 diabetes (Yarizade et al., 2017). It was also shown that ethanolic *F. assafoetida* oleo-gum-resin extract can regulate hyperglycemia and diabetes complications in streptozotocin (STZ)-induced diabetic rats (Latifi et al., 2019). The rest six medicinal plants used in this formulation were also known for their antidiabetic activities and several studies have been carried out to support their antidiabetic activity through in vitro and in vivo assays (Den Hartogh et al., 2019; Krishnakumar et al., 2014; Sharafeldin & Rizvi, 2015). *A. barbadensis* was also investigated for its antidiabetic activity through clinical trials (Yongchaiyudha et al., 1996). Therefore, formulation employing potent medicinal plants made this MTM3 effective to serve as an antidiabetic drug.

Table 2. In vitro biological activities and total phenolic content of Myanmar traditional medicine formulations

Name	DPPH (%)	NO (%)	SO (%)	Antiglycon (IC_{50})	α-Glucosidase inhibitory (%)	Total phenolic content (mg GAE/g extract)
MTM1	53.66 ± 4.19**	31.72 ± 3.48****	32.18 ± 4.70****	348.98 ± 13.65****	62.39 ± 4.54	31.78 ± 2.25[a]
MTM2	44.05 ± 0.87***	25.16 ± 4.37****	22.37 ± 2.61****	352.17 ± 9.98****	0.49 ± 0.06****	27.16 ± 1.69[a]
MTM3	48.57 ± 8.05**	82.90 ± 1.65	65.02 ± 2.82*	180.30 ± 1.63***	92.12 ± 1.18***	149.41 ± 3.64[b]
MTM4	36.37 ± 7.02***	37.09 ± 4.27****	6.82 ± 4.74****	260.88 ± 36.36****	61.39 ± 0.96	32.53 ± 1.59[a]
Ascorbic acid	84.78 ± 0.47	78.96 ± 1.71	-	-	-	-
Gallic acid	-	-	83.24 ± 1.73	-	-	-
Rutin (μM)	-	-	-	11.64 ± 1.86	-	-
Acarbose	-	-	-	-	60.54 ± 5.57	-

Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.
DPPH: 2,2-diphenyl-1- picrylhydrazyl. For total phenolic content assay, different letters (a to b) indicate the difference ($p < .05$) in each group.

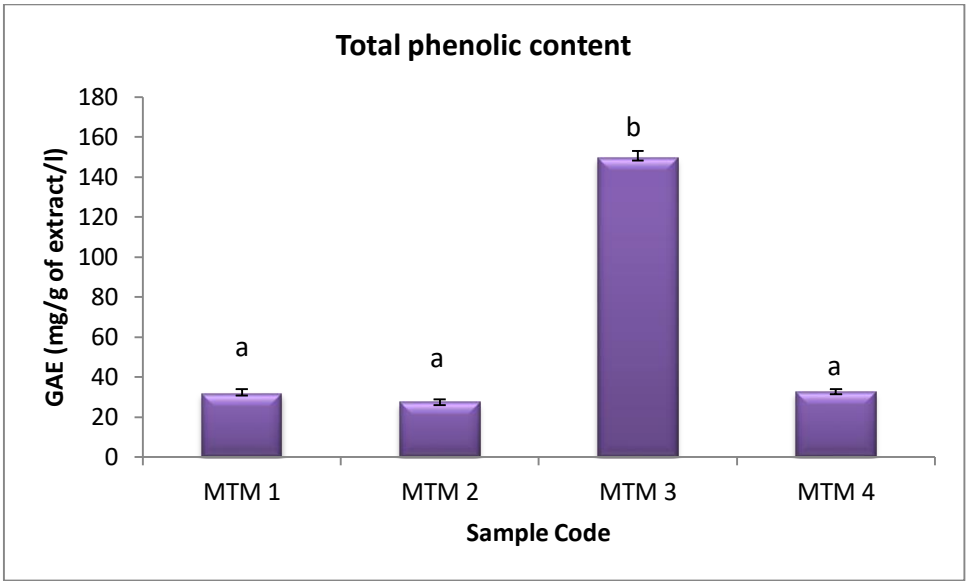

Figure 5. Comparison of TPC of Myanmar traditional medicine formulations determined by the Folin-Ciocalteu's assay and calculated as GAE in mg/g extract based on dry weight
Results were the mean of triplicates ± standard error of the mean. Different letters (a–b) indicated significant differences ($p < 0.05$) in each group.
GAE: gallic acid equivalent, TPC: total phenolic content

Another antidiabetic drug; MTM1 was comprised of five traditional medicinal plants including *Swertia angustifolia*, *T. cordifolia*, *Trigonella foecgraetun*, *Quercus intecforia*, and *Capsicum frutescens*. MTM2 was composed of six medicinal plants such as *T. foecgraetum*, *C. longa*, *Centella asiatica*, *A. paniculata*, *Piper nigrum*, and *T. cordifolia*. MTM4 is made up of 17 ingredients of which 12 are ingredients derived from medicinal plants and the rest are minerals, salts, and animal products which are normally used in MTM preparations.

It was observed that *T. cordifolia* was used in 3 formulations of MTM1, MTM2, and MTM3. *T. cordifolia* is a plant that is frequently used in Ayurvedic medicine for the control of diabetes. Studies conducted in the past have demonstrated that *T. cordifolia*, which is abundant in nutraceuticals, is an effective anti-diabetic plant material. It was shown that *T. cordifolia* aqueous extract dramatically lowered blood glucose and increased plasma insulin levels in alloxan-induced mildly diabetic rats, thereby demonstrating an insulinotropic activity (Joladarashi et al., 2014; Noor & Ashcroft, 1998; Noor et al., 1989).

T. foenum-graecum was included in 3 MTM formulations of MTM1, MTM2, and MTM4. The previous research revealed that a seed powder solution made from *T. foenum-graecum* has a substantial role to play in reducing dyslipidemia in individuals who have just been diagnosed with type II diabetes (Geberemeskel et al., 2019). *C. longa* was present in the formulation of MTM2 and MTM3. A comprehensive spectrum of physiological and pharmacological properties, including antioxidant, anti-inflammatory, anticancer, neuroprotection, and anti-diabetic actions was found in the bioactive constituents isolated from *C. longa* (Den Hartogh et al., 2019; Fazel Nabavi et al., 2015). MTM2 and MTM3 also used *A. paniculata* in the formulations. Previous research reported that *A. paniculata* or its most active constituent andrographolide demonstrated hypoglycemic and hypolipidemic effects in rats fed a high-fat, high-fructose diet (Nugroho et al., 2012).

There are several in vitro antidiabetic activity assays that could be used to study the antidiabetic activity of medicinal plants such as α-amylase inhibitory assay, dipeptidyl peptidase IV (DPP-IV) inhibition assay, PPARγ and GLUT-4 assay, PTP1B assay, glucose uptake assay, glucose adsorption assay, reporter gene assay, insulin secretion assay, calcium measurement assay, ATP measurement assay, and cAMP assay (Vhora et al., 2020). Therefore, it is imperative to carefully choose a suitable in vitro assay to assess the antidiabetic activity of the medicinal plants. A comprehensive examination of the multiple parameters involved in the pathway for glucose and hormone metabolism is essential. In the current research, two commonly used in vitro antidiabetic assays: BSA-fluorescent antiglycation assay and α-glucosidase inhibitory assay were used to determine the antidiabetic activity of MTM. MTM1, MTM3, and MTM4 which showed their antidiabetic activities through these two bioassays. However, MTM2 was not active for all the currently tested bioassays. Therefore, it is recommended that the aforementioned in vitro antidiabetic assays be employed to evaluate the comprehensive effects of the tested MTM to acquire a thorough understanding of their actions against diabetes mellitus.

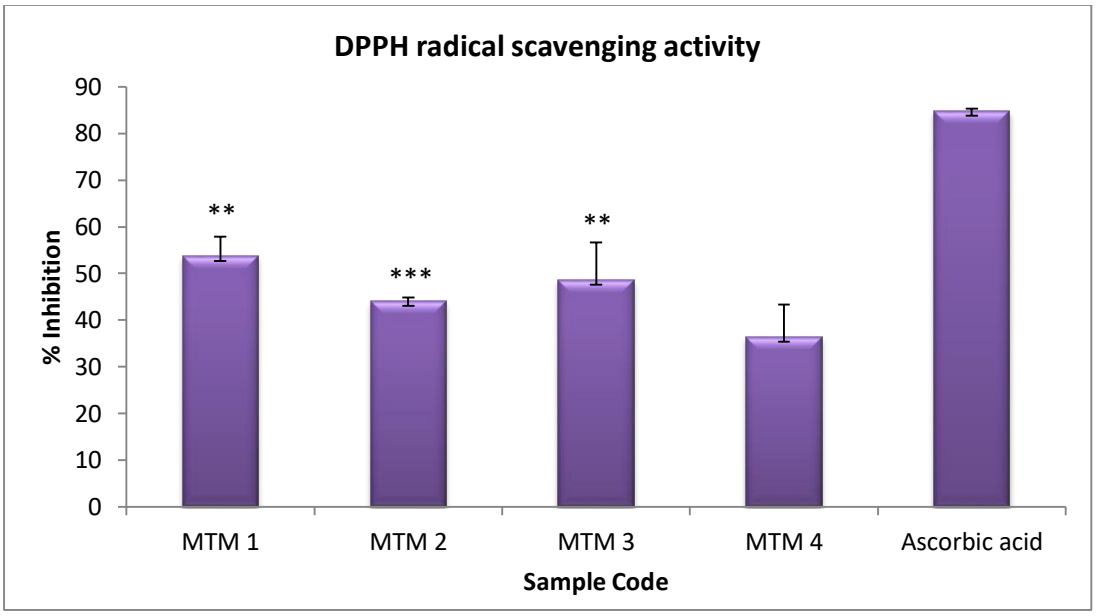

Figure 6. Comparison of antioxidant activities of Myanmar traditional medicine formulations through DPPH radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. DPPH: 2,2-diphenyl-1- picrylhydrazyl

4. Conclusions

In conclusion, MTM were extensively utilized to treat type 2 diabetes mellitus and were developed using traditional knowledge. Although their actions were very different from one another, recent evidence-based validation research demonstrated that these MTMs possessed antioxidant, antiglycation, and α-glucosidase inhibitory characteristics in a range of in vitro experiments. These formulations showed antioxidant activity against several free radicals in addition to their antiglycation and α-glucosidase inhibitory characteristics. Therefore, the antidiabetic activities of MTM could be confirmed by evidence-based validations of current research experiments as stated in their description as an antidiabetic drug, and diabetic patients could improve their quality of life by using these MTM formulations that were comprised of a potent combination of MTM plants.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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CRediT authorship contribution statement

The Su Moe: Conceptualization, Investigation, Data curation, Writing - original draft, Supervision
Htet Htet Win: Laboratory investigation, Practical, Investigation, Result analysis
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Supplementary File

None.

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