
**ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ
И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ**

**CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS
AND BIOLOGICALLY ACTIVE SUBSTANCES**

ISSN 2410-6593 (Print), ISSN 2686-7575 (Online)

<https://doi.org/10.32362/2410-6593-2021-16-3-232-240>



УДК 615.322

RESEARCH ARTICLE

Combination of *Phyllanthus amarus* Schum. & Thonn. and *Gymnema sylvestre* R. Br. for treatment of diabetes and its long-term complications

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Abstract

Objectives. The amount of patients with diabetes is increasing, and such patients experience several long-term complications. Therefore, finding a method to treat the disease and its complications is an urgent issue worldwide. In Vietnam, *Phyllanthus amarus* Schum. & Thonn. (PA) and *Gymnema sylvestre* R. Br. (GS) are common herbs used in traditional therapy including diabetes treatment. This study aimed to combine PA and GS to extend their bioactivities in antidiabetes, antioxidant, and anti-inflammatory treatments.

Methods. Here, PA and GS powders were mixed at different ratios for extraction. Ethanolic extract was used to detect bioactive compounds, bioactivities, and appropriate ratios of the mixtures.

Results. The optimal ratio for the PA and GS combination was 2 : 1 (g/g). The ethanolic extraction of the 2 : 1 sample at 50°C over two hours with a solid/liquid ratio of 1 : 10 achieved a high yield of 14.37%. This sample exhibited good α -glucosidase inhibition activity with a half-maximal inhibitory concentration (IC₅₀) of 9.74 μ g/mL, antioxidant activity with an IC₅₀ of 29.87 μ g/mL, and anti-inflammatory activity with an IC₁₅ of 400 μ g/mL.

Conclusions. The study confirmed that combining PA and GS can have high α -glucosidase inhibition as well as antioxidant and anti-inflammatory effects.

Keywords: antidiabetes, antioxidant, anti-inflammatory, *Phyllanthus amarus* Schum. & Thonn, *Gymnema sylvestre* R. Br., integration

For citation: Le T.M., Nguyen Ch.D.P., Ha A.C. Combination of *Phyllanthus amarus* Schum. & Thonn. and *Gymnema sylvestre* R. Br. for treatment of diabetes and its long-term complications. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2021;16(3):232–240. <https://doi.org/10.32362/2410-6593-2021-16-3-232-240>

НАУЧНАЯ СТАТЬЯ

Комбинация *Phyllanthus amarus* Schum. & Thonn. и *Gymnema sylvestre* R. Br. для лечения диабета и его долгосрочных осложнений

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Аннотация

Цели. Количество пациентов с диабетом растет, у них часто возникают долгосрочные осложнения, поэтому поиск методов лечения этого заболевания и коррекции его осложнений является важным для всего мирового медицинского сообщества. *Phyllanthus amarus* Schum. & Thonn. (PA) и *Gymnema sylvestre* R. Br. (GS) – распространенные во Вьетнаме лекарственные растения, используемые в традиционной медицине, включая лечение диабета. Цель данного исследования – скомбинировать PA и GS, чтобы расширить их биологическую активность и усилить антидиабетический, антиоксидантный и противовоспалительный эффект.

Методы. Порошки листьев PA и GS смешивали в различных соотношениях и экстрагировали 95% этанолом. Полученные этанольные экстракты использовались для определения биологически активных соединений, биологической активности и оптимального соотношения компонентов смеси.

Результаты. Оптимальное соотношение PA и GS, определенное в исследовании, равно 2 : 1 (г/г). Экстракция 95% этанолом данного образца (2 : 1) при 50 °C в течение двух часов при соотношении сырье/экстрагент 1 : 10 позволила получить высокий выход экстрактивных веществ, равный 14.37%. Этот образец продемонстрировал хорошую активность ингибирования α -глюкозидазы с половинной максимальной ингибирующей концентрацией (IC₅₀) 9.74 мкг/мл, антиоксидантную активность с IC₅₀ 29.87 мкг/мл и противовоспалительную активность с IC₁₅ 400 мкг/мл.

Выводы. Исследование подтвердило, что сочетание PA и GS может значительно ингибировать α -глюкозидазу, а также обладает антиоксидантным и противовоспалительным эффектами.

Ключевые слова: антидиабетическое, антиоксидантное, противовоспалительное, *Phyllanthus amarus* Schum. & Thonn, *Gymnema sylvestre* R. Br., интеграция

Для цитирования: Le T.M., Nguyen Ch.D.P., Ha A.C. Combination of *Phyllanthus amarus* Schum. & Thonn. and *Gymnema sylvestre* R. Br. for treatment of diabetes and its long-term complications. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2021;16(3):232–240. <https://doi.org/10.32362/2410-6593-2021-16-3-232-240>

1. INTRODUCTION

Diabetes mellitus is an endocrine disorder disease characterized by a hereditary or acquired deficiency in insulin excretion as well as reduced responsiveness of organs to the produced insulin [1]. According to International Diabetes Federation reports, there were about 463 million adults with diabetes in 2019, and the number is predicted to rise to 630 million by 2045 [2, 3]. Patients with diabetes mellitus are likely to have long-term complications, e.g., impaired wound healing, retinopathy, atherosclerosis, cataract, neuropathy, and nephropathy [4]. Inflammation and oxidation are the most common complications of diabetes [5]. Therefore, a medicine or drug for treating diabetes and its complications is being researched worldwide [1]. In these efforts, the use of natural compounds with antidiabetic properties has attracted much attention. However, the major drawback of herb-based medicine is that the bioactivities of the plants depend on the extraction conditions. A solution for this issue can be found in traditional medicine. For example, in traditional Vietnamese medicine, several human diseases can be treated using a combination of various herbs. Certain plants play the main role in treating the disease, while the others are used for treating the complications. However, the development of pharmaceutical preparations has led to lower and less efficient quantities of herbs being used in medication for human diseases. The traditional method shows promise in creating more valuable disease treatments, especially diabetes.

Phyllanthus amarus Schum. & Thonn (PA) is a small tropical herb in the *Phyllanthus* genus of the *Euphorbiaceae* family and can be found in Nigeria, India, China, Vietnam, and Thailand [6]. Its leaves are commonly used and highly valued in traditional medicine because of their beneficial properties [7]; several researchers have extracted and isolated bioactive compounds, such as polyphenols, flavonoids, tannins, triterpenes, sterols, and alkaloids, from PA leaves [8]. Because of these valuable ingredients, the leaves have been confirmed to have several potential bioactive applications, such as antihepatitis [9], antioxidant [10], anticancer [11], anti-inflammatory [12], antimalarial [13], antimicrobial [14], and antidiabetes [15].

Gymnema sylvestre R. Br. (GS), a plant in the *Asclepiadaceae* family, is commonly found across Asia, Africa, and Australia [16]. Its leaves are used in numerous traditional therapies for patients with diabetes and various other diseases [17]. The plant reportedly has potent anti-obesity activities, e.g., it may help lower weight gain and fat accumulation [18]. Isolated bioactive compounds of GS indicate the gymnemic acid group is the main active compound responsible for the plant's

antidiabetes activity. In addition, GS leaves have been reported to have diverse antiviral [19], antibiotic [20], and anticancer [21] bioactivities among others [16]. Similar to those of PA, most bioactivities of GS do not concurrently exist under the same extraction conditions. The plant has also been used in the production of tea brews, tea bags, and confections as well as in the management of sugar homeostasis and maintenance of obesity and blood cholesterol levels in a various foods [22].

Owing to their diverse bioactivities, PA and GS show potential for use in treating diabetes and its complications. Therefore, in this study, they are mixed at different ratios to improve their antidiabetes, antioxidant, and anti-inflammatory activities. The antidiabetes activity is measured via α -glucosidase inhibition, whereas the antioxidant and anti-inflammatory effects are determined via 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and albumin denaturation, respectively. Following traditional medicine, the herbs play the main role in the mixtures' antidiabetes activity, as verified via ethanolic extraction for further research.

2. MATERIALS AND METHODS

2.1. Materials

The PA and GS leaves were harvested from Binh Chanh District, Ho Chi Minh City, Vietnam, in October 2020. The identification was done at the Department of Ecology and Evolutionary Biology of the Faculty of Biology and Biotechnology, Ho Chi Minh City University of Science, Vietnam National University. After being harvested, the samples were rinsed with water, dried at room temperature, ground into a powder with a particle size of 3–5 mm, and stored in sealed bags.

The following pure-grade chemicals were purchased from commercial suppliers: ethanol (EtOH), methanol (MeOH), distilled water, sodium nitrite, sodium carbonate (Na_2CO_3), sodium hydroxide, aluminum chloride, and dimethyl sulfoxide (DMSO). The Folin–Ciocalteu reagent, quercetin, gallic acid (GA), *para*-nitrophenyl α -D-glucopyranoside (*p*-NPG), α -glucosidase, acarbose, bovine albumin, and DPPH were provided by *Sigma-Aldrich*, Singapore.

2.2. Preparation of extracts

A total of 50.00 g of the PA and GS powder mixture was extracted at different ratios with 500 mL EtOH 95% over two hours at 50 °C and a solid/liquid ratio of 1 : 10 g/mL. Afterward, the extracts were filtered using filter paper (15–20 μm) under vacuum conditions. The herbal residue was recycled for subsequent extraction under the same conditions. The two extractions were mixed and concentrated via rotary vacuum evaporation at 55 °C (Buchi R-215 Rotavapor). The moisture content of the extracts and powders were determined using Sartorius moisture analyzer MA37.

All experiments were performed in triplicate. The extraction yield was determined using Eq. 1:

$$\text{Extraction yield (\%)} = \frac{m_{\text{extract}}}{m_{\text{sample}}} \times 100\%, \quad (1)$$

where m_{extract} is the dry weight of the extract (g) and m_{sample} is the dry weight of the sample (g).

2.3. Phytochemical screening

Phytochemical screening of the PA and GS was conducted to detect the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, cardiac glycosides, and polyphenols [23–27]. The reagents, test method, and results are shown in table.

2.4. Determination of the total polyphenol excreted (TPE)

The phenolic level was determined by Folin–Ciocalteu assay, as described by McDonald *et al.* [28]. A total of 40 μL of each extract was dissolved in DMSO, and 200 μL of Folin–Ciocalteu reagent was mixed and homogenized in a sonication bath for five minutes at room temperature. Afterward, 600 μL of 20% Na_2CO_3 and 3160 μL of distilled water were added to the mixture. An extract without reagents was used as a sample. After incubating all samples at room temperature for 30 min, their absorbance was measured at 760 nm using a UV–Vis spectrophotometer (Thermometer, USA). The calibration curve for GA was created to calculate the phenolic content. The TPE was shown as the milligrams GA equivalent (GAE) per gram of the extract (dry weight). The equation of the calibration curve was $y = 1.2003x - 0.0034$, where $R^2 = 0.9979$.

2.5. *In vitro* α -glucosidase inhibitory assay

The antidiabetes activity of the extracts *in vitro* was measured via the α -glucosidase inhibition activity because the α -glucosidase enzyme plays an important catalytic role in converting polysaccharides to monosaccharides (glucose). Thus, inhibition of α -glucosidase lowers the glucose content.

The investigation regarding the α -glucosidase enzyme inhibitory activity of the extract was conducted following Liu's method [29]. The test was performed on 96 well plates. The extracts were dissolved in DMSO before the test, and 40 and 20 μL of the sample solution and α -glucosidase enzyme (1 U/mL), respectively, were added to the wells. Next, 100 μL of phosphate buffer (pH 6.8) was added to the mixture. The plate was incubated for five minutes at 37 °C. Then, 40 μL of 0.1 mM *p*-NPG was added to the reacting mixture, which was continuously incubated for 30 min at 37 °C. Subsequently, ~100 μL of 0.1 M Na_2CO_3 was added to terminate the reaction. The sample absorbance was measured at 405 nm using the UV–Vis spectrophotometer. Acarbose was employed as a positive

control. The percent inhibition of the α -glucosidase reaction was calculated as follows:

$$I\% = \left(\frac{A - B}{A} \right) \times 100\%, \quad (2)$$

where A is the absorbance at 405 nm of the blank (α -glucosidase and the substrate) and B is the absorbance at 405 nm of the extract (α -glucosidase, the substrate, and the sample).

The concentrations of the extracts resulting in the half-maximal inhibitory concentration (IC_{50}) of the enzyme activity were determined graphically.

2.6. *In vitro* antioxidant assay

The oxidation is one of the main complications of diabetes; thus, finding an agent with antioxidant and antidiabetes activities is a necessity. The antioxidant activity of the samples was investigated via DPPH free radical scavenging assay according to Stagos' method, with slight modifications [30]. A total of 120 μL of sample was added to 180 μL of DPPH dissolved in 80% MeOH. The mixture was incubated for 30 min at 30 °C in the dark. Then, the absorbance was measured at 517 nm using the UV–Vis spectrophotometer. Here, MeOH and ascorbic acid were used as the negative control and positive control, respectively. The percentage inhibition ($I\%$) was calculated using Eq. 3:

$$I\% = \left(\frac{A - B}{A} \right) \times 100\%, \quad (3)$$

where A is the absorbance at 517 nm of the DPPH radical of the negative control and B is the absorbance at 517 nm of the DPPH radical solution mixed with the sample.

The antioxidant activity was expressed by the IC_{50} value, representing the sample concentration required to inhibit 50% of the free radical scavenging activity.

2.7. *In vitro* anti-inflammatory assay

Inflammation is a complex process associated with the reaction of body tissues to infection, irritation, or other injuries. Therefore, it is involved in various diseases, including diabetes [31]. The *in vitro* anti-inflammatory activity of the extracts was evaluated via the extracts' protective activity against albumin denaturation, as described in previous studies with slight modifications [32]. The extracts were serially diluted in DMSO, which was used as the negative control.

The reaction mixture was prepared by adding 3 mL of bovine albumin dissolved in phosphate buffer with a pH of 6.4 into 2 mL of tested extract. The mixture was incubated for 5 min at 70 °C. After cooling to room temperature, the sample absorbance was measured at 660 nm using a UV–Vis spectrophotometer (Jasco, USA).

The percentage inhibition of the protein denaturation was calculated using Eq. 4:

$$I\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%, \quad (4)$$

where A_{control} is the absorbance of the negative control (DMSO) and A_{sample} is the absorbance of the extract.

2.8. Statistical analysis

All analyses were performed in triplicate, and the data were expressed as the mean value \pm standard deviation for each measurement.

3. RESULTS AND DISCUSSION

3.1. Phytochemical studies of PA and GS

Ethanol is a polar solvent commonly used in phytochemical extraction because EtOH has been suggested to extract the maximum amount of bioactive compounds from plants [33, 34]. According to the phytochemical screening results presented in table, all previously mentioned bioactive compounds were detected in both PA and GS. Alkaloids and terpenoids were present in large quantities in both herbs, which indicated that the plants have potent antidiabetes and antioxidant activities because of the specific activities of these biocompounds. In particular, the strong presence of tannins in the PA extract suggested the probability of such bioactivities. Tannins have been shown to have antibacterial, antiviral, antifungal, antidiabetic, and antioxidant activities and promote tissue recovery in cases of superficial burn injuries [35]. Furthermore, the existence of flavonoids in the PA extract suggested the ability to enhance the

current therapy options for type 2 diabetes mellitus [36]. The GS extract had a more vigorous reaction with the saponin and cardiac glycoside reagents than the PA extract. Saponins have been shown to exhibit hemolytic, antimicrobial, insecticidal, anthelmintic, analgesic, anti-inflammatory, sedative, and antitumor bioactivities [37]. The use of cardiac glycoside in clinical trials for the treatment of heart disease and atrial arrhythmia has been confirmed [38]. Because of the robust amount of key biocompounds possessing antidiabetes activity, the PA extract was predicted to exhibit better antidiabetes activity than the GS extract.

3.2. Effect of different PA and GS ratios on extraction yield and TPE

The TPE and extraction yields of the PA and GS mixtures of various ratios are presented in Fig. 1. The results showed that the differences in the extraction yield were insignificant (approximately 1%). The extraction yield was in the range of 13.77% (GS) to 15.22% (PA), increasing with the increase in the PA content in the powder. The GS content increased when the TPE of the extracts decreased, except in the 2 : 1 sample. In general, the TPE of all samples was in the range of 65–71 mgGAE/g and the difference was statistically significant. The TPE reached a maximum value of 71.06 mgGAE/g at the PA/GS ratio of 2 : 1, with the highest extraction yield from the PA. Phenolics are the primary bioactive materials in nature and have been reported to have multiple biological effects, including antidiabetes effects [39]. Thus, the high TPE values illustrated the extracts' potential antidiabetes activity.

Phytochemical screening of PA and GS extracted with EtOH solvents

Bioactive compounds	Test	Extracts	
		PA	GS
Alkaloid	Dragendorff [24]	++	++
	Bouchardat [24]	++	++
Flavonoid	Lead acetate 10% [24]	+	–
	Sulfuric acid 98% [26]	+	+
Tannin	Gelatin 1% [25]	++	+
Saponin	Foam [23]	+	++
	Liebermann–Burchard [27]	+	++
Terpenoid	Salkowski [26]	++	++
Cardiac glycoside	Keller–Kiliani [25]	–	+
Polyphenol	Ferric (III) chloride [25]	++	++

– Not detected; + Slightly positive reaction; and ++ Strong positive reaction

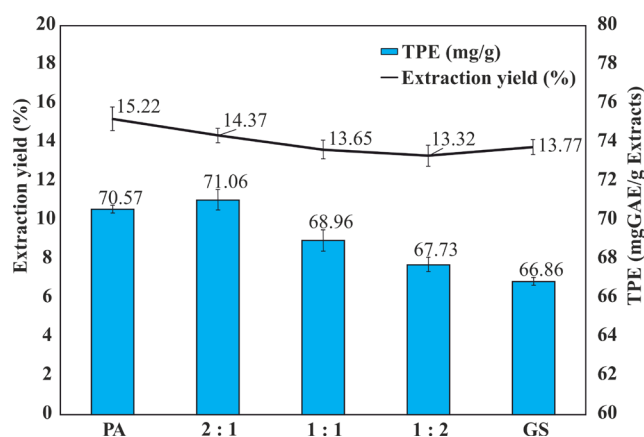


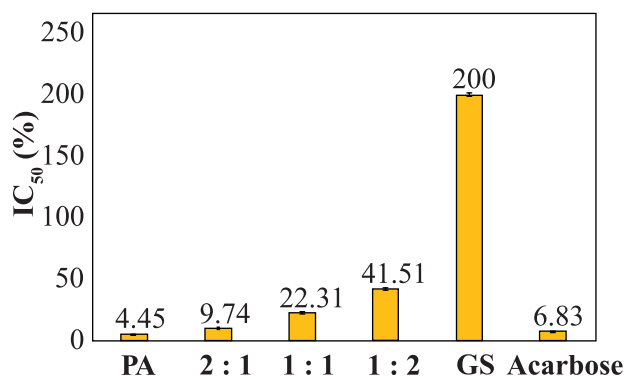
Fig. 1. Extraction yield and TPE in the PA and GS mixtures of various ratios. Here, 2 : 1 refers to the PA/GS ratio of 2 : 1, and from right to left, the PA content gradually decreases.

3.3. The effect of the different ratios of PA and GS on their bioactivities

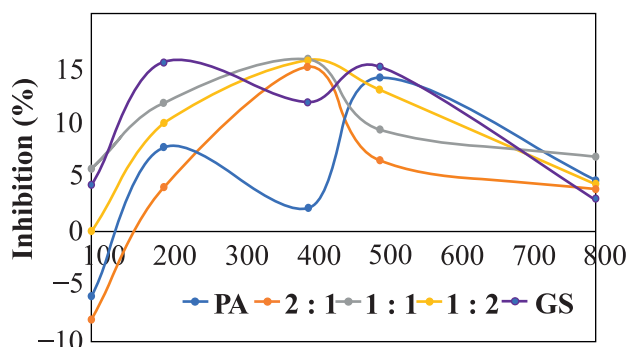
Figure 2 displays the bioactivities of the various extracts at different herbal powder ratios. The PA extracts showed strong inhibitory effects on α -glucosidase, with an IC_{50} value of 4.45 μ g/mL lower than that of the positive control acarbose (6.83 μ g/mL). The antioxidant and anti-

inflammatory activities of the PA samples were lower than those of the GS samples, but the PA's antioxidant activity was still higher than that of numerous herbs in the existing literature [40]. The GS extract exhibited the most antioxidant activity, with an IC_{50} value of 22.12 μ g/mL, as well as anti-inflammatory activity, with an IC_{15} value of 200 μ g/mL. Here, IC_{15} was used to evaluate the anti-inflammatory activity because all extracts' inflammatory inhibition was lower than 20%. William *et al.* reported that extracts with inflammatory inhibition above 20% following albumin denaturation can be considered anti-inflammatory agents [41].

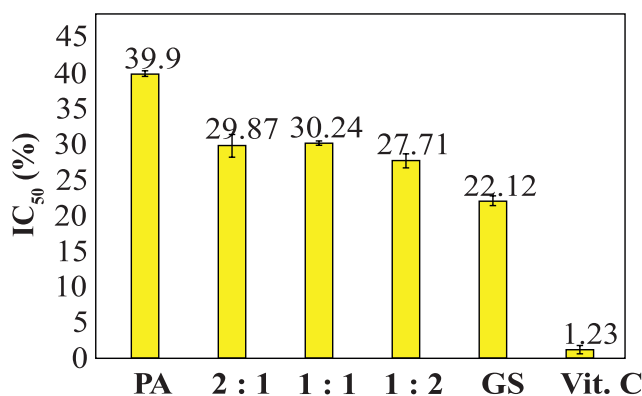
The efficiency of the plants' combination was determined using the bioactivities of the combined samples. The results indicated significant biological activity changes compared with the raw materials. The PA content was an important factor in the antidiabetes activity, and a decrease in PA led to a reduction of the activity in the extract. Mixing the PA with the GS powder significantly improved the α -glucosidase inhibition activity of the latter, which was demonstrated via the increased antidiabetes activity of samples 2 : 1, 1 : 1, and 1 : 2 compared with the GS powder alone. The PA/GS ratio of 2 : 1 showed antidiabetes activity (IC_{50} 9.47 μ g/mL) 21 times higher



a



b



c

Fig. 2. Bioactivities of the various extracts at different PA/GS ratios: (a) α -glucosidase inhibition activity, (b) anti-inflammatory activity, and (c) antioxidant activity.

than that of the GS extract alone. Moreover, the GS content was responsible for improving the antioxidant and anti-inflammatory activities of the mixture. The radical scavenging activity increased as the GS content increased in the samples at the ratios of 2 : 1, 1 : 1, and 1 : 2, with IC_{50} values of 29.87, 30.24, and 27.71 $\mu\text{g/mL}$, respectively. The combination of PA and GS enhanced the antioxidant activities approximately 1.7-fold compared with the PA extract alone. Increasing the GS concentration in the mixture had no discernible effect on the anti-inflammatory properties. Compared with the PA extract, the IC_{15} value of samples 2 : 1, 1 : 1, and 1 : 2 was almost 400 $\mu\text{g/mL}$, a 1.8-fold improvement. The enhancement of the bioactivities verified that the integration of PA and GS led to the discovery of a novel agent for treating diabetes and its complications.

The extraction yields and bioactivities of the five samples are shown in Fig. 3 to determine the optimal ratio for integration. The results illustrated that the PA extract exhibited the highest antidiabetes activity and extraction yield but the lowest antioxidant and anti-inflammatory activities. However, the GS extract

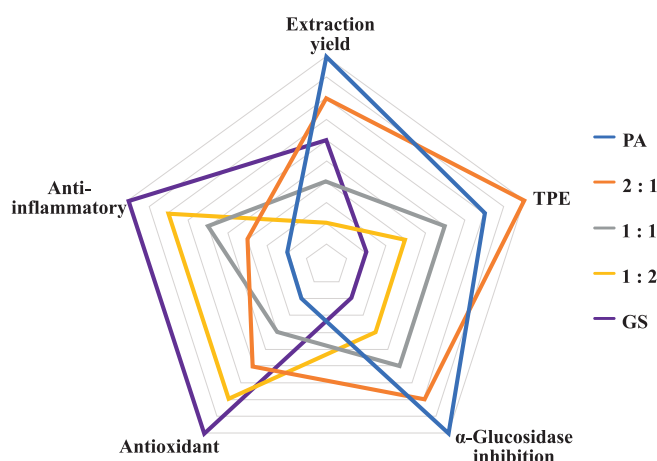


Fig. 3. Comparison of the samples' bioactivities, extraction yields, and TPE.

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exhibited high antioxidant and anti-inflammatory activities. The samples with a PA/GS ratio of 1 : 2 and 1 : 1 exhibited nearly equivalent activities, but their antidiabetes activity was lower than that of sample 2 : 1. Therefore, the powder with a PA/GS ratio of 2 : 1 was concluded to be suitable for developing a new agent with strong antidiabetes activity to treat diabetes and its complications.

4. CONCLUSIONS

The present study reported the extraction of PA and GS and the creation of mixtures of different ratios using EtOH. The combination of PA and GS achieved high bioactivities, such as antidiabetes, antioxidant, and anti-inflammatory. In the mixtures, PA played the main role in the antidiabetes activity, whereas GS yielded high antioxidant and anti-inflammatory activities. The mixture with the PA/GS ratio of 2 : 1 was the best sample because of its high TPE and antidiabetes and related bioactivities. The optimization of the bioactivities of this mixture will be reported in the next phase. In future work, the PA and GS combining extracts need to be studied *in vivo* for a comprehensive assessment for the plants' integration before practical application.

Acknowledgments

We acknowledge the support of time and facilities from Ho Chi Minh City University of Technology (HCMUT) and Vietnam National University Ho Chi Minh City (VNU-HCM) for this study.

Authors' contribution

Tan M. Le – conceptualization, formal analysis, conducting research, and writing the manuscript;
Chinh D.P. Nguyen – conducting research, writing the review, and editing the text;
Anh C. Ha – supervision, methodology, and conceptualization; writing the review and editing the text.

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Поступила: 16.04.2021; получена после доработки: 11.05.2021; принята к опубликованию: 21.05.2021.
The article was submitted: April 16, 2021; approved after reviewing: May 11, 2021; accepted for publication: May 21, 2021.

The text was submitted by the authors in English.

Edited for English language and spelling by Enago, an editing brand of Crimson Interactive Inc.