Southeast-Asian J. of Sciences, Vol. 9, No. 1 (2022) pp. 142-152

EXTRACTION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM STACHYS AFFINIS.

Dung Thi Nguyen[1], Nguyen Thanh Thao Vo[1], Phuong Nhi Thi Tran[1], Tam Thi Doan[1], Loan Thi Ha [1], Phan Phuoc Hien [2]

[1] *Biotechnology Center of Ho Chi Minh City, Ho Chi Minh City, Viet Nam*

[2] *Institute of Applied Science and Technology, Van Lang University, HCM city Vietnam*
e-mail: hien.pp@vlu.edu.vn

Abstract

The Stachys affinis is a rare medicinal plant that is rich in protein, carbs, phenolics, flavonoids, and vitamins. It is mostly composed of stachyose, which has been shown to be is beneficial to health. The purpose of the present study is to investigate phytochemical screening to identify some components and active ingredients in extracts from S. affinis and to detect the principal class of chemicals contained. The findings of phytochemical screening demonstrated the abundance of bioactive components in S. affinis extracts. The methanol extract had a high amount of polyphenol, and polysaccharide content, especially stachyose content. There was 97.69% total polyphenol content, 58% extract of polysaccharide content with 14.73% stachyose content. So it can be recommended that S. affinis This medicinal plant might be used as raw material for functional food and pharmaceutical.

1. Introduction

Stachys affinis also called *Stachys sieboldii* is widely used in various traditional

Key words: bioactive components, methanol extract, polysaccharide, stachyose, *Stachys affinis.*

medicines and consumed as edible plants, vegetables also food additives in China and Japan. Many studies in the world have shown that Stachys affinis were rich in protein, carbohydrates, phenolics, flavonoids and vitamins, especially containing large amounts of stachyose (Lukasz et al., 2011). The natural sourced bioactive compounds had been noticed due to their therapeutic potentials such as anti-microbial, antioxidant, anti- inflammatory, and anti-cancer, anti-diabetes activity, hypotensive activity (Hayashi et al., 1994, Skaltsa et al., 2000, Maleki et al., 2001).

Stachyose has the potential to partly exert anti-diabetes activity by regulating energy metabolism and gut microbiota (Liang et al., 2020). The results of studying the mechanism of stachyose on type 2 diabetes mellitus treatment in mouse models show that stachyose has the effect of regulating the level of insulin, low-density lipoprotein cholesterol, and triglycerides. The polysaccharides of S. affinis had high capture activity against superoxide anions, hydroxyl and ATBS radicals (Feng et al., 2015). In vitro and in vivo experiments have shown that polysaccharides have hypoglycemic effects and pancreatic- cell dysfunction, its antidiabetic effect is equivalent to those of synthetic diabetic agents (Wu et al., 2016).

In Vietnam, The Biotechnology Center of Ho Chi Minh City is a pioneer organization in the cultivation, research and exploitation of biological activities from S. affinis. Extraction is the main process by which bioactive compounds may be abtained from biomass materials. Water and methanol were suboptimal extraction solvents for organic. This study aimed to identify some active ingredients in aqueous from S. affinis. The results of the study are a premise for the study of the application of this active ingredient in biological activities to support the functions of this precious medicinal herb.

2. Materials and method

Plant material

S. affinis were cultivated under greenhouse conditions in the Biotechnology Center of Ho Chi Minh City (Ho Chi Minh City, Vietnam) and the tubers were collected after five months old.

Preparation of methanol extract of Stachys affinis

The tubers of Stachys affinis were thoroughly washed and dried at 50oC until constant weight. Dried tubers were then ground into a fine powder and kept in a desiccator at a temperature of 40^0C until extracted. The extracts of tubers were extracted by immersion extraction technique (solid-liquid extraction) (Han et al., 2007). Briefly, 10 g of powder sample was homogenized in 40 mL of solvent (water and methanol). The homogenate was allowed to stand for 24 hrs

at room temperature with intermittent shaking and then filtered through No. 1 Whatman filter paper. The extract from each filtrate was evaporated using a vacuum rotary evaporator at 40^0C, and then vacuum freeze-dried to form a dry paste according to Vietnam Pharmacopoeia. The percentage of extract yield was calculated as % yield = [Weight of sample extract/Initial weight of sample] x 100. The extracts obtained were stored at 4^0C until analysis.

Analysis of the phytochemical contents

For preliminary analysis, the extracts obtained were subjected to qualitative tests for the identification of various plant constituents. Tests for reducing sugars, phenolic compounds, flavonoids, saponins, LAC, PAC, Organic Acids, tannin, anthocyanin, and alkaloids standard procedures to identify the constituents as described by standard methods of Cauley (the University of Medicine and Pharmacy Bucharest). The principle of this method is to separate different groups of organic compounds based on the different solubility of these compounds in solvents of different polarity, and then identify the groups of compounds in each extract by a characteristic reaction. It was determined by solubility tests, color reactions by reagent characteristics and precipitation.

Total phenolic content assay

The total phenolic contents of extracts of Stachys affinis was were determined using the Folin-Ciocalteu reagent as described by Ainsworth et al., 2007. Briefly, samples were inserted into different test tubes and mixed thoroughly with 5 mL Folin-Ciocalteu reagent (previously pre-dilute 10 times with distilled water). After 3 mins, 4 mL of 7.5% sodium carbonate (Na_2CO_3) was added and allowed to react for an hour at room temperature in the dark. The absorbance was measured at 765 nm using microplate reader spectrophotometers (Molecular devices, VERSAmax tunable, California, USA). Samples were measured in three replicates. The standard curve of gallic acid solution (500; 250; 125; 62.5; 31.25 và 15.625 μg/mL) was prepared using a similar procedure. The results were expressed as mg GAE/g extract sample.

Determination of total alkaloid content

The total alkaloid content was determined according to the UV-Spectrophotometer method (Shamsa et al., 2008). This method is based on the reaction between alkaloid and bromocresol green. The extract was dissolved in MeOH, then added HCl_2N and filtered. 1 mL of this solution was transferred to a separatory funnel and the pH of the phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1 mL of this solution was transferred to a separating funnel and then 5 mL of bromocresol solution along with 5 mL of phosphate buffer was added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fractions were collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm.

Determination of Saponin - Determine Total Saponin Content

Qualitative Saponin by TLC method

Determine to identify saponin compounds by thin layer chromatography (TLC) with dynamic solvent butanol: ethanol: ammoniac (6:3:3) and observe the sample at 365 nm wavelength.

Quantitative Saponin by Vanillin – Sulfuric acid assay

The vanillin-sulphuric acid assay for determining the total saponin content (TSC) of Stachys affinis extracts (Anh et al., 2018) by incubating 0.25 mL of the extracts, standards, or reagent blank with 0.25 mL of 8% (w/v) vanillin in ethanol and 2.50 mL of 72% (v/v) sulfuric acid in water for 15 min at 60^0C in a shaking water bath, with the standards and the reagent blank made up with the solvent used for Stachys affinis extracts. After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extracts is measured at 560 nm using a Cary 50 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The TSC content was calculated based on the oleanolic acid standard curve.

Total Polysaccharides Content Measurement

The total polysaccharides content was determined according to the Phenol-Sulfuric acid method (Nielsen et al., 2010). The Stachys affinis extract was dissolved in 100mL of distilled water to at 20 μg/mL concentration. From this 1mL was used for the polysaccharide content analysis. 0.5 mL of 5% phenol was added to the 1mL of sample solution or blank, followed by 2.5mL of concentrated H_2SO_4. The absorbance was measured after 10 minutes at 490 nm against blank. The experiment was carried out in triplicate. A stock solution of 100μg/mL of glucose was prepared in distilled water, using for standards.

Determination of stachyose content

The stachyose content of the extracts was determined by the ultra-performance liquid chromatography- tandem mass spectrometry (UPLC - MS) method as described by Xue et al. (2018) with modifications. The chromatographic analysis was carried out by using the UPLC system: Waters Acquity QDA - Quaternary Solvent Manager R – Sample manager FTN-R - QDa Detector with conditions: flow rate, 0.6 mL/min; column temperature, 30^0C; Sample injection, 5 μL; and detection mass spectrometer probe ESI (-); m/z (stachyose) 665, m/z (hydrocortisone) 407; Cone voltage, 20 V. UPLC column: C18 (250 mm x 4.6 mm, 5 μm); Mobile phase: Solvent A: methanol; solvent B: formic acid 1%. Data were collected and analyzed stachyose content (%).

$$X\% = (S_{Sta\ test}/S_{HCtest})/(S_{Stast}/S_{HCst})^* (m^*_{st}H/m_{test})^*(Df_{test}/Df_{st})^*100$$

$S_{Sta\ test}$: Peak area of Stachyose in the chromatogram of the test solution;

$S_{HC\ test}$: Hydrocortisone peak area in the chromatogram of the test solution;

$S_{Sta\ st}$: Stachyose peak area in the chromatogram of the standard solution;

$S_{HC\ st}$: Hydrocortisone peak area in the chromatogram of the standard solution (g) ;

mst: weight of standard Stachyose balance (calculated on anhydrous preparation) (g)

H: standard content (calculated on anhydrous preparation);

m_{test}: weight of test sample;

Df_s: Test dilution (100);

Df_{St}: Standard Dilution (100).

Statistical Analysis

The results are expressed as mean values of three replications ± standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA).

3. Results and discussion

Extraction yield The selection of the solvent is crucial for extraction. In this study, Stachys affinis extracts were obtained by using water and methanol solution. As shown in Figure 1, the extraction efficiency for different solvents were was different. The extraction efficiency of aqueous extract has higher than that of methanol extract. This could be explained by the cultivar of extraction conditions applied in the analysis and based on the law of similarity and intervisibility, solvents with a polarity value near to the polarity of the solute are likely to perform better.

The preliminary phytochemical analysis

The results of phytochemical screening Stachys affinis are rich in bioactive compounds. All extracts are rich in polyphenols, saponins and reducing sugars. The methanol extract had an abundance of alkaloids, saponin, and polyphenols while the aqueous extract had a strong presence of polysaccharides. Flavonoids, Organic Acids, Tannin, and Anthocyanin were absent in both aqueous and methanol extracts (Tab.1)

Total phenolic content

The total phenolic content of the extracts was tested using the diluted Folin-Ciocalteu reagent. Table 1 showed the total phenolic content of extracts. High levels of phenolic compounds (total polyphenol) were recorded. Results clearly showed that the content of total phenolic in terms of gallic acid equivalent (standard curve equation: Y = 0.047X 0.0449, R^2 = 0.9975) and indicated that phenolic contents of methanol extract had higher than the amount of total

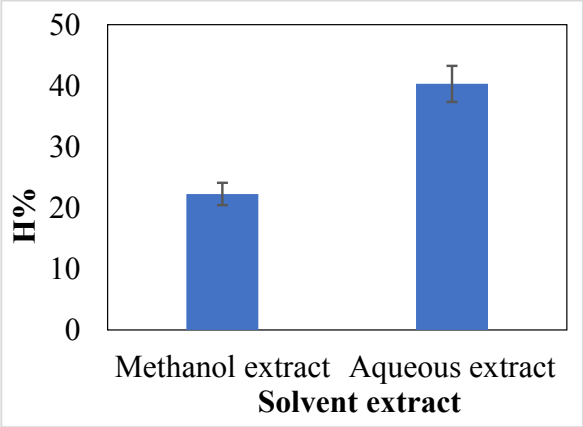

Fig 1. Extraction yield of Stachys affinis extract

Chemical groups	**Method to determine/reagent**	**The results (+)**	**Methanol extract**	**Aqueous extract**
Polyphenol	$FeCl_3$ 5%	dark green appears	+	+
Alkaloid	reagent Wagner	Precipitation appears	+	-
Saponin	Vanillin+H_2SO_4 72%	Red colour appears	+	+
Flavonoid	Alkaline reaction: NaOH 10% (1:1) →H_2O (1:1)	Precipitation appears → soluble precipitate	-	-
Organic Acids	Na_2CO_3	Bubbles	-	-
reducing sugars	reagent Fehling, Water heater insulation	Red colour appears	+	+
Tannin	$FeCl_3$ 1%	moss green/dark green appears	-	-
Anthocyanin	+HCl 10 %+KOH 10%	(Red/H^+), (Blue/OH^-)	-	-

Tab 1. Phytochemical screening of the methanol and aqueous extract of Stachys affinis.

phenolic content of aqueous extract (with 97.69 GAE mg/g extract). Several researchers have reported that polyphenols are highly active compounds against cancer, antioxidant, and antiviral agent (Zhang et al., 2022). Extraction yield and polyphenol content not only depended on the extraction method but also on the solvent used for extraction.

Tab 2. Total phenolic content of the extracts of *Stachys affinis*

Sample	Methanol extract GAE (mg/g extract)	Aqueous extract GAE (mg/g extract)
	97.69 ± 8.37^{a}	4.17 ± 0.24^{b}
Gallic acid standard $Y = 0.047x + 0.0449$ ($R^2 = 0.9975$)		

Tab 2. Total phenolic content of the extracts of Stachys affinis

Determination of total alkaloid and polysaccharide content Total alkaloids and polysaccharides were measured in two types of extraction solvents. The table demonstrated that the concentration was higher in the methanol extract than in the water sample (Tab. 3). For the alkaloid content, the methanol extract had a concentration of 0.334 mg/g, which was 1.5 times higher than in the water sample, only 0.192 mg/g. While for the polysaccharides, two samples showed almost comparable extraction efficiency in the range from of 56 to 58 mg/g. Extraction with methanol solvent had more efficient than with water solvent.

Tab 3. Total alkaloid and polysaccharide content of the extracts of *Stachys affinis*

Sample	Alkaloid total (mg/g extract)	Polysaccharide total (% extract)
Methanol extract	0.334 ± 0,043	58.43 ± 1.9
Aqueous extract	0.192 ± 0,011	56.04 ± 3.33

Tab 3. Total alkaloid and polysaccharide content of the extracts of Stachys affinis

Determination of Total Saponin Content

Qualitative Saponin by TLC method

The TLC result illustrated the methanol extracted corresponds to the OA standard (Fig. 2). Compare the Rf values of the sample and the equivalent standard (Rf sample = Rf standard = 0.77). It can be concluded that the extract of S. affinis has the presence of oleanolic acid or saponin.

Quantitative Saponin by Vanillin – Sulfuric acid assay

According to Rahimi et al. (2009), saponins have the ability tocan protect

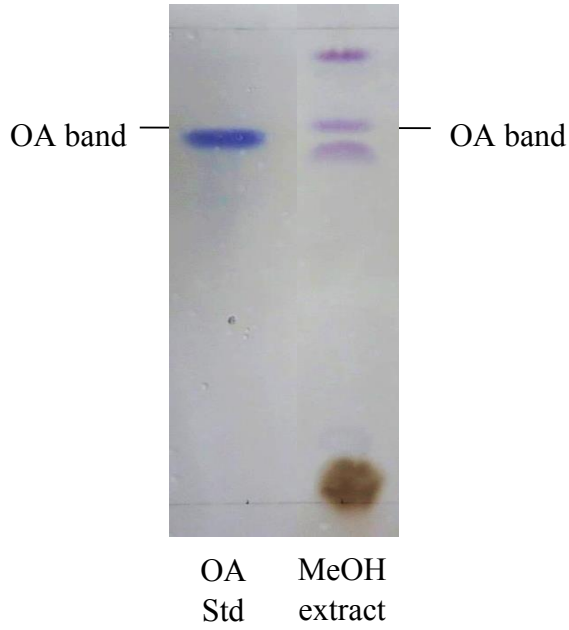

Fig 2. Saponin qualitative results of methanol extract and standard oleanolic acid (OA).

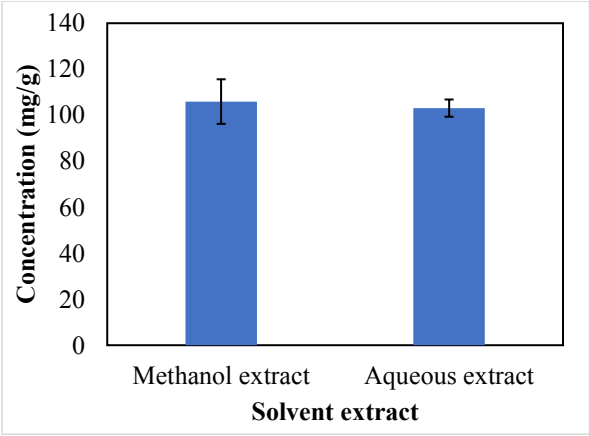

Fig 3. The saponin content of the extracts of *Stachys affinis*

liver function, anti- inflammatory, and antiviral agents. In addition, saponins can also treat cancer and diabetes. The analysis showed that the saponin content in the sample was relatively high. OA standards equivalent in methanol and aqueous extracts are 105.94, and 103.09 mg/g extract, respectively. The results showed that the saponin content of S. affinis extracted with methanol solvent had higher recovery efficiency than in water solvent conditions.

Determination of stachyose content

Stachyose is an oligosaccharide that can be consumed directly and benefits the digestive system, consisting of galactose, glucose and fructose. In S. affinis tubers have up to 80-90% stachyose, and tuberous vacuoles are rich in stachyose (Luo et al., 2014). In two different extraction methods, the stachyose content of S. affinis extracted with methanol solvent had higher recovery efficiency than in water solvent conditions but no statistically significant difference. There were 14.73% and 11.35%, respectively, as presented in table 4.

Tab 4. Stachyose content of the extracts of *Stachys affinis*

Sample	Stachyose (%)
Methanol extract	14.73
Aqueous extract	11.35

Tab 4. Stachyose content of the extracts of Stachys affinis

In general, extraction is the crucial process to obtain the highest biological activity from biomass materials, which maximizes the number of target compounds. The extraction yield and biological activity of the resulting extract are affected by the extraction technique and the extraction solvent. Methanol, even though an organic liquid, is a polar solvent, It is easily evaporated and has high solvation properties. Thus, it can readily dissolve polar organic substances which are widespread in nature, and beneficial to man and other living organisms. soIt that, it is commonly used as a solvent for the extraction of natural materials.

4. Conclusions

According to our results, the extracts of Stachys affinis were rich in bioactive compounds, such as polyphenols, alkaloids, steroids, saponin, stachyose and reducing sugar. The methanol extract had a higher total phenolic content and stachyose contents than the aqueous extract. These phytochemicals observed in this study can further screen genotypes with high bioactive compounds and purification of phytochemical compounds to further potentiate their pharmacological uses. Therefore, extracts of Stachys affinis can be explored as a promising

source of bioactivity compounds in pharmaceutical fields.The presence of these phytocompounds observed in this study can be further to screen genotypes with high bioactive compounds and purification of phytochemical compounds, which are valuable to produce potentiates its pharmacological uses further making it a good candidate for the food and pharmaceutical fields.

Acknowledgements

The authors gratefully acknowledge the support from the Biotechnology Center of Ho Chi Minh cityCity, Viet Nam for their generous support of this research project.

References

[1] Lukasz J.L., Svanberg I., Köhler P., *Marsh woundwort, Stachys palustris L. (Lamiaceae): an overlooked food plant,* Genetic Res. Crop Evol., **58**(5)(2011), 783-793.

[2] Hayashi K., Nagamatsu T., Ito M., Hattori T., Suzuki Y., *Acteoside, a Component of Stachys Sieboldii MIQ, May Be a Promising Antinephritic Agent (2): Effect of Acteoside on Leukocyte Accumulation in the Glomeruli of Nephritic Rats,* Japanese J. Pharm., **66**(1)(1994), 47-52.

[3] Skaltsa H., Bermejo P., Lazari D., Silvan A.M., Skaltsounis A.L., Sanz A., Abad M.J., *Inhibition of prostaglandin E2 and leukotriene C4 in mouse peritoneal macrophages and thromboxane B2 production in human platelets by flavonoids from Stachys chrysantha and Stachys candida,* Biol. Pharmaceutical Bulletin, **23**(1)(2000), 47-53.

[4] Maleki N., Garjani A., Nazemiyeh H., Nilfouroushan N., Sadat A.E., Allameh Z., Hasannia N., *Potent anti-inflammatory activities of hydroalcoholic extract from aerial parts of Stachys inflata on rats,* J. Ethnopharm., **75**(2-3)(2001), 213-218.

[5] Liang L., Liu G., Yu G., Zhang F., Linhardt R.J., Li Q., *Urinary metabolomics analysis reveals the anti-diabetic effect of stachyose in high-fat diet/streptozotocin-induced type 2 diabetic rats,* Carbohydrate Polym., 229(2020), (115534).

[6] Feng K., Chen W., Sun L., Liu J., Zhao Y., Li L., Wang Y., Zhang W., *Optimization extraction, preliminary characterization and antioxidant activity in vitro of polysaccharide from Stachys sieboldii Miq,* Tubers. Carbohydrate Polym., 125(2015), 45-52.

[7] Wu J., Shi S., Wang H., Wang S., *Mechanisms underlying the effect of polysaccharide in the treatment of type 2 diabetes: A review,* Carbohydrate Polym., **25**(144)(2016), 474-494.

[8] Han N.V., Nghi D.H., 2007. *Kỹ thuật chiết xuất dược liệu,* NXB Y học, 25-41.

[9] Ainsworth E.A., Gillespie K.M., *Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent,* Nature Protocols, **2**(4)(2007), 875-877.

[10] Shamsa F., Monsef H., Ghamooshi R., Verdian-rizi M., *Spectrophotometric determination of total alkaloids in some Iranian medicinal plants,* Thai. J. Pharm. Sci., **32**, (2008), 17-20.

[11] Anh V.L., Sophie E.P., Minh H.N., Minh, Paul D.R., *Improving the Vanillin-Sulphuric Acid Method for Quantifying Total Saponins,* Technologies, **6** (3)(2018), 84-95.

[12] Nielsen S.S., *Phenol-Sulfuric Acid Method for Total Carbohydrates.,* Food Analysis Laboratory Manual, Springer, 2010, 47-53.

[13] Xue S., Wang L., Chen S., Cheng Y., *Simultaneous analysis of saccharides between fresh and processed Radix rehmanniae by HPLC and UHPLC-LTQ-Orbitrap-MS with multivariate statistical analysis,* Mol., **23**(3)(2018), 541.

[14] Zhang Z., Li X., Sang S., McClements D.J., Chen L., Long J., Jiao A., Jin Z., Qiu C., *Polyphenols as Plant-Based Nutraceuticals: Health Effects, Encapsulation, Nano-Delivery, and Application,* Foods, **11**(2022), 2189.

[15] Rahimi R., Ghiasi S., Azimi H., Fakhari S., Abdollahi M., *A review of the herbal phosphodiesterase inhibitors; future perspective of new drugs,* Cytokine, **49**(2)(2009), 123-129.

[16] Luo G.H., *History of culture and spread of Chinese artichoke,* Agri. History of China (in Chinese), (6)(2014), 21-24.