Pharmacogn J. 2020; 12(6)Suppl: 1670-92
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Antihypertensive Assay-Guided Fractionation of *Syzygium polyanthum* Leaves and Phenolics Profile Analysis Using LC-QTOF/MS

Azlini Ismail[1,*], Erlena Nor Asmira Abdul Rahim[2], Muhammad Nor Omar[2], Wan Amir Nizam Wan Ahmad[3]

ABSTRACT

Introduction: *Syzygium polyanthum* leaves extract that contains gallic acid as the major phenolic compound has shown significant antihypertensive effect, however the amount of gallic acid was inversely-related with magnitude of this effect. This study aimed to conduct bioassay-guided fractionation of *S. polyanthum* leaves with gallic acid as a reference compound, and to screen for other possible compounds responsible for the antihypertensive effect. **Methods:** *S. polyanthum* leaves were extracted using n-hexane, ethyl acetate, methanol, and water. The most active crude extract was fractionated using column chromatography and analyzed for total phenolic content (TPC) (n=3). Crude extracts and the derived fractions were intravenously administered into pentobarbital-anaesthetized Spontaneously Hypertensive rats (n=5) for recording of blood pressure parameters. Liquid Chromatography-Quadruple Time-Off-Flight/Mass Spectrometry was used for determination of chemical composition. One-way and two-way ANOVA were used for statistical analysis using GraphPad® PRISM Version 6. **Results:** Fractionation of aqueous *S. polyanthum* leaves extract (ASP) afforded nine fractions, later combined into three fractions (F1ASP, F2ASP, and F3ASP) based on the thin-layer chromatography profiles. ASP has the highest TPC while F2ASP has the lowest TPC. All fractions exhibited significant antihypertensive property, but F2ASP was the most active fraction. Few phenolics with related antihypertensive effects such as 1-galloyl glucose (a gallic acid-derivative majorly found in F2ASP and F3ASP), and other compounds such as polydatin, sesamol, brazilin, eugenol, ellagic acid, kukoamine A, and cyclocurcumin were found across all active fractions. **Conclusion:** These phenolics may partly contribute to the antihypertensive effect of *S. polyanthum* leaves, thus further isolation study is recommended.

Key words: Antihypertensive, Bioassay-guided, LCMS, *Syzygium polyanthum*, Total phenol content (TPC).

Azlini Ismail[1,*], Erlena Nor Asmira Abdul Rahim[2], Muhammad Nor Omar[2], Wan Amir Nizam Wan Ahmad[3]

[1]Department of Fundamental Dental and Medical Sciences, Kuliyyah of Dentistry, International Islamic University Malaysia, Indera Mahkota, 25200 Kuantan, Pahang, MALAYSIA.
[2]Department of Biotechnology, Kuliyyah of Science, International Islamic University Malaysia, Indera Mahkota, 25200 Kuantan, Pahang, MALAYSIA.
[3]School of Health Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, MALAYSIA.

Correspondence

Azlini Ismail

Department of Fundamental Dental and Medical Sciences, Kuliyyah of Dentistry, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, MALAYSIA.

Phone no: +014-5010081

Email: dr_azlini@iium.edu.my

History

- Submission Date: 27-07-2020;
- Review completed: 26-08-2020;
- Accepted Date: 02-09-2020.

DOI : 10.5530/pj.2020.12.227

Article Available online

http://www.phcogj.com/v12/i6s

INTRODUCTION

Hypertension is a major public health problem. According to the World Health Organization[1], an uncontrolled rise in blood pressure may predispose a patient to a heart attack which will eventually lead to heart and kidney failures, stroke, and cognitive impairment. It was estimated that the worldwide prevalence of hypertension exceeded 1.3 billion, representing 31 % of all adults.[2] Throughout the years, the condition of raised blood pressure among the hypertensive patients was uncontrolled.[3] While there are available antihypertensive drugs in the market, the global condition remains stagnant since the treatment is expensive, thus an average or a poor society did not afford to receive the best treatment regime. In addition, the concomitant drugs' side effects such as dizziness, abnormal heart rate, sore throat, sexual dysfunction, thrombocytopenia, and hyperglycemia[4] are undesirable, and this untoward reaction actually occurs more easily when drugs are used in combination.[5] The expensive cost and the side effects of the currently-available antihypertensive drugs have enforced the research for new alternative antihypertensive drugs which should be at least equally effective, but yet inexpensive.

Some natural compounds from medicinal plants were found to exhibit significant antihypertensive effect[6], however, there is also a huge number of potential medicinal plants with antihypertensive properties that remains to be explored. *Syzygium polyanthum* (Wight) Walp, also known as 'salam' or 'serai kayu' is one of the medicinal herbs that is traditionally consumed as an alternative treatment for reducing blood pressure among Malay folks. *S. polyanthum* has been known as an antihypertensive medicinal plant and this is strongly supported by previous findings. Sukrasno *et al* [7] reported the hypotensive effect of orally-administered aqueous extract of *S. polyanthum* leaves in normotensive Wistar rats. *S. polyanthum* leaves extracts have shown a significant reduction in blood pressure of anaesthetized Spontaneously Hypertensive Rats (SHR) and normal Wistar Kyoto (WKY) when intravenously administered.[8] When fed orally, *S. polyanthum* leaves extract significantly reduced the systolic blood pressure in SHR.[9,10] Histological studies showed significant improvement in Bowman's capsule and glomerulus morphology of

Cite this article: Ismail A, Rahim ENAA, Omar MN, Wan Ahmad WAN. Antihypertensive Assay-Guided Fractionation of *Syzygium polyanthum* Leaves and Phenolics Profile Analysis Using LC-QTOF/MS. Pharmacogn J. 2020;12(6)Suppl:1670-92.

treated SHR's kidney, comparable to normal kidney structure and slight improvement of pedicels and thoracic aorta.[10, 11]

Ramli *et al*[11] suggested that the reduction in systolic blood pressure (SBP) in hypertensive rats might be due to the major composition of phenolics in the extract. Ismail *et al*[9] showed the presence of gallic acid, a major phenolic compound present in *S. polyanthum* leaves extract. Gallic acid was previously reported to normalize blood pressure of diabetic rats[12] and attenuate hypertension in NG-nitro-L-arginine methyl ester-induced hypertensive rats.[13] However, our previous study showed there was no correlation between the amount of gallic acid with the magnitude of antihypertensive effect for the tested *S. polyanthum* leaves extracts, suggestive of the presence of synergism between compounds that contributes to the net antihypertensive effect.[9] Therefore, this study aimed to perform bioassay-guided fractionation of *S. polyanthum* leaves to screen for other potential bioactive compounds responsible for its antihypertensive effect.

MATERIALS AND METHODS

Plant authentication

The leaves, flowers, buds, and stem parts of *S. polyanthum* were sent for authentication at UKMB Herbarium, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The plant was verified as *Syzygium polyanthum* Wight Walp. on 2nd May 2017. The voucher herbarium specimen (PIIUM 0282) was deposited in Herbarium, Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang, Malaysia.

Animal

Seventy-five of 3-month old male Spontaneously Hypertensive Rats (SHR), weighing around 250 to 280 grams were placed in standard rat cages and acclimatized for 7 days in standard environmental conditions (25 °C with 60-70 % humidity) on a 12-hour light-dark cycle. Tap water and rat pellet were given *ad libitum* and the animal bedding was changed once in a week. All experimental protocols regarding the animal study were approved by the Animal Ethics Committee, Universiti Sains Malaysia (USM/Animal Ethics Approval/2016/ (102) (757).

Sample extraction

Two kg of *S. polyanthum* leaves were collected from Taman Pertanian Jubli Perak Kuantan, Pahang, Malaysia. The leaves were left dried in a drying cabinet for a week at 50 °C. After a week, the dried leaves were ground into powder using a laboratory blender prior to extraction. The powdered sample was extracted using ultra-sound assisted extraction (UAE) method as described by Rahim *et al* .[14] This method enhances solvent penetration through plant cells with the aid of sound waves,[11, 15, 16] and usually provides sufficient yield for phenolic compounds.[17] Hot distilled water (80 °C) and also solvents with varying polarities including n-hexane, ethyl acetate, and methanol were used to prepare different crude extracts of *S. polyanthum* leaves.

For each sequential solvent extraction method, 250 g of powdered *S. polyanthum* leaves were initially soaked in 700 ml n-hexane. The soaked sample was then placed in a bath-sonicator (WiseClean, Switzerland) at 24 °C by frequency range from 40 to 80 λ for 30 minutes and then filtered with Whatman filter paper No.1. The filtrate was then concentrated using a rotary evaporator (Buchi R-200, Switzerland). This concentrated filtrate was designated as hexane extract of *S. polyanthum* (HSP). The remaining powder residue from hexane extraction was then soaked with 700 ml of ethyl acetate, and then it was also sonicated using the bath-sonicator (WiseClean, Switzerland) at 24 °C by frequency range from 40 to 80 λ for 30 minutes. The soaked sample was then filtrated with Whatman filter paper No. 1, and was subsequently left dried in the fume hood (Rico, Malaysia). This concentrated filtrate was designated as ethyl acetate extract of *S. polyanthum* (ESP). Next, the remaining residue from the ethyl acetate extraction was then soaked in methanol for three cycles of 700 ml (first cycle), 400 ml (second cycle), and 400 ml (third cycle), respectively. In between each cycle, the soaked sample was sonicated using the bath-sonicator (WiseClean, Switzerland) at 24 °C by frequency range from 40 to 80 λ for 30 minutes and then filtrated with Whatman filter paper No. 1. The filtrates from the three cycles were then combined and then concentrated using a rotary evaporator. This concentrated filtrate was designated as the methanol extract of *S. polyanthum* (MSP).

Meanwhile, for water extraction, another 250 g of powdered sample was used and soaked in a pre-heated distilled water at 80 °C using a hot plate for three cycles of 700 ml (first cycle), 400 ml (second cycle), and 400 ml (third cycle) of distilled water for 30 minutes. In between each cycle, the soaked sample was also sonicated using the bath-sonicator (WiseClean, Switzerland) at 24 °C by the frequency range from 40 to 80 λ for 30 minutes and then filtrated with Whatman filter paper No. 1. The three filtrates from each cycle were combined and then stored in a -80 °C freezer before being lyophilized using a freeze dryer (CHRIST Model Beta 1-8 LO, Germany) for 12 days. This lyophilized sample was designated as the aqueous extract of *S. polyanthum* (ASP). All samples were stored at -20 °C in a freezer (Wiseclean, Switzerland) before further use.

Bioassay-guided fractionation

Since ASP was the crude extract with the most prominent antihypertensive effect, it was then subjected to fractionation. Before fractionation, the thin layer chromatography (TLC) is performed to study the characteristics of the extract and to optimize the solvent system to achieve a good separation during fractionation.[18] TLC plates (8 x 8") were firstly cut into a measurement of 10 cm x 2 cm and were allowed to dry overnight at 37 °C in an incubator oven (Memmert, Germany). The crude extract was firstly developed with 100 % n-hexane, ethyl acetate, dichloromethane, methanol, and acetonitrile. The crude extract was then run with the solvent system of ethyl acetate: methanol: acetonitrile (8:1:1) with an additional one drop of formic acid. The additional one drop of formic acid was used to enhance the separation of the four spots. Gallic acid was used as a reference compound (standard) based on finding from our previous study that gallic acid was found as a major phenolic compound in the aqueous and methanolic extracts of *S. polyanthum* leaves.[9] The spots and the standard were visualized under UV lamp (Leybold Didactic GmbH, Germany) of short and long wave and by ferric chloride spraying detection reagent.

For fractionation, a 30 cm-height of silica column using a 25 mm glass column was prepared by mixing 35 g of silica gel 60 (0.063-0.200 mesh) with 100 % ethyl acetate. The column was allowed to stand overnight for complete packing. ASP slurry was prepared in a combined solvent mixture of methanol and water (50:50) to enhance the solubility of methoxylated and hydroxylated compounds.[19] Then, the ASP slurry was run in the column chromatography using a gradient elution technique with a binary solvent system of ethyl acetate and methanol, allowing polarity changes during the fractionation. Gradient elution usually offers better speed, separation, and retention reproducibility compared to isocratic elution for wide range polarities of organic compounds.[20] The gradient solvent system of ethyl acetate (100 %), ethyl acetate: methanol (7:3), ethyl acetate: methanol (5: 5), ethyl acetate: methanol (3:7), and methanol (100 %) were consequentially employed and finally, the column was washed with 100 % methanol. Nine fractions were collected in a 15 ml centrifuge tube and characterized by TLC profiling with a solvent system of ethyl acetate: methanol (9.5:0.5) with a drop of formic acid. The spots were visualized using a UV lamp, 50 % sulphuric acid spraying reagent, vanillin-sulphuric acid reagent and ferric chloride spraying detection reagent. These spraying reagents were

prepared according to the methods stated in Pirrung [21] and Mohrig *et al* .[22] Similar fractions (similar TLC profile) were pooled and combined to give the final three fractions designated as F1ASP, F2ASP, and F3ASP. These fractions were then dried in an incubator oven (Memmert, Germany) and stored at -20 °C in a refrigerator (SuperFreezer 340W 1D, Korea) for further analysis.

Determination of antihypertensive effect of crude extracts and fractions

This *in vivo* antihypertensive study was conducted based on several previous studies.[8, 23, 24] A BIOPAC Data Acquisition System, attached to an arterial pressure transducer with an amplifier recorder (MP30, BIOPAC Data Acquisition System) was employed for measurement of blood pressure parameters and the data were displayed using BIOPAC Student Lab Pro® v3.6.7.

Each rat was weighed using a laboratory weighing balance and anaesthetized with 60 mg/kg sodium pentobarbital *via* intraperitoneal injection. The reflex of the rat was checked by pinching the tail and the toe. The rat was placed in a rat's container until no reflex reaction occurred. Later, the rat was brought to the surgery table before performing a tracheotomy. An additional amount of 10 mg/kg sodium pentobarbital was given throughout the experiment to maintain the anaesthetic condition whenever necessary. The body temperature of rats was maintained at 37 ± 1 °C using an overhead lamp. The skin on the anterior side of the neck was carefully cut-off using a surgical scissor. A small incision was made (1.5-2 cm) on the skin layers of the anterior side of the neck. A slit incision was made on the rat platysma muscles. By using two forceps with teeth, the skin was separated *via* blunt dissection technique while taking extra precautions not to disturb the larynx, hyoid bone, and thyroid cartilage. The trachea was then identified and forceps were used to slightly pull up the trachea and then a thread was eventually passed underneath it. The front part of the trachea was then half-incised for the insertion of modified intravenous drip tubing. The tube was thick with a length around 3 to 4 cm. The thread under the trachea was then used to fix the inserted tube to the trachea. Tracheotomy was performed to aid the respiration process since the employed sodium pentobarbital usually increases the bronchial secretion. Continuous monitoring of the rats' respiration was performed throughout the experiment.

After tracheotomy, cannulation of the carotid artery was performed. The dark red, elastic, rounded, and thick vessel of the carotid artery was identified along the vagus nerve which was white-in-color on either side of the trachea. Separation of the vagus nerve, connective tissue, and *longus capitis* (a longitudinal bundle of muscle located adjacent to the trachea) was carried out. The cephalic end of the carotid artery was tied with a thread and another end near to the heart was temporarily clamped with a bulldog clamp. These were done to prevent misreading of actual blood pressure due to the division of pressure between the brain and carotid cannula.[23]

The carotid artery was half-incised, and then a cannula, pre-filled with heparinised saline (5 IU/ml) that was connected to a pre-calibrated pressure transducer was inserted into the carotid artery. The heparinised saline was a solution mixture of heparin and 0.9 % normal saline. Another end of the cannula was connected to a three-way stopcock, attached to a saline-filled tuberculin syringe. After the cannulation, the bulldog clamp was released slowly. Free transmission of pressure in the cannula must be ensured for continuous monitoring of accurate mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) that can be seen at the data acquisition system (Biopac System, USA).

A small incision (1-2 cm) was made on the epidermis of the right tight where the left jugular vein was located. A matrix of connective tissue was cleaned carefully *via* blunt dissection using two forceps with teeth. The jugular vein was differentiated from the nerve fibre and a catheter was cannulated before drug administration. During cannulation, threads were first passed under the vein. Once the vein has been isolated, the upper part of the rounded-vein was then half-incised to allow the insertion of a cannula, filled with heparinised saline (5 IU/ml). The thread was then tightened at the upper part (the part closer to brain) after the cannula has been inserted. Another thread was used to tie the vein along the inserted catheter. The cannulation line was flushed with heparinised saline (0.2 ml) to prevent thrombosis.[23]

To determine the crude extract with the most prominent antihypertensive effect, the four crude extracts (ASP, HSP, ESP, and MSP) were dissolved in 0.9 % normal saline to achieve dosages of 1, 10, 40, and 70 mg/kg based on a previous related paper by Ismail *et al* .[8] In the subsequent study to determine the most active fraction, the fractions were dissolved with the same vehicle as in the previous experiment with the crude extract and prepared to achieve the dosages of 10, 20, 30, 40, 50, and 60 mg/kg. All prepared extracts and fractions were vortexed using a vortex machine (PV1 Grant-bio, England) immediately before use. Normal saline (0.9%) was used as negative control while captopril at 5 mg/kg was used as a positive control drug according to Abdulazeez *et al* .[24] Captopril is an angiotensin-converting enzyme inhibitor that is used as one of the first-line antihypertensive drugs for the treatment of hypertension and congestive heart failure. Captopril was prepared by dissolving the drug into 0.9 % normal saline. A fixed volume of 0.2 ml for the extracts and fractions at increasing dosages were sequentially administered into each rat (n=5). The baseline for all blood pressure parameters such as MAP, SBP, and DBP of rats were ensured to return to the baseline value before administration of each subsequent dosage.

Total phenolic content analysis

The total phenolic content of the ASP crude extract and the three derived fractions (F1ASP, F2ASP, and F3ASP) were determined using Folin-Ciocalteu assay with ACS reagent grade gallic acid as a standard. Two-hundred µl of sample for ASP, F1ASP, F2ASP, F3ASP, and gallic acid (as a standard) were pipetted into individual test tubes. Eight-hundred µl of distilled water and 500 µl of Folin's Reagent were added together into the test tubes containing-samples and standard. Each sample was prepared in triplicates. The standard was prepared from 30 to 200 µg/ml of gallic acid dissolved in AR methanol. All samples (ASP, F1ASP, F2ASP, and F3ASP) were prepared in 1 mg/ml of AR methanol. All of them were allowed to stand in the dark for 5 minutes. After that, 1.5 ml of 20 % w/v sodium carbonate (Na_2CO_3) was added and all the mixtures were incubated at room temperature in a dark condition for 2 hours. Two ml of prepared mixtures of samples (ASP, F1ASP, F2ASP, and F3ASP) and standard (gallic acid, 30 to 200 µg/ml) were then transferred into a plastic cuvette for measurement. The absorbance was measured at the wavelength of 760 nm against a blank (distilled water) using a UV-VIS spectrophotometer (Perkin Elmer, Malaysia). Blainski *et al* [25] reported that the maximum absorption can be produced at this specific wavelength. Moreover, the long-wavelength absorption of the chromophores minimizes the interference of the sample matrix that is often coloured.[26] The measured absorbance for standard (gallic acid, 30 to 200 µg/ml) and each respective sample in triplicates were averaged and a standard curve graph was plotted.

LC-QTOF/MS analysis for identification of phenolic compounds in the most active crude extract and active fractions

Identification of the compounds in the ASP, F1ASP, F2ASP, and F3ASP were conducted using a modified method described by Terpinc *et al* .[27] LC-MS instrument used was a Waters, VION Ion Mobility QTOF MS. HPLC system was a binary pump with solvent gradient of water (A) and

acetonitrile (B): 99 % A, 1 % B from 0 to 0.5 min; 65 % A, 35 % B from 0.5 to 16 min; 0 % A, 100 % B from 16 to 18 min; and 99 % A, 1% B from 18 to 20 min). Negative ion electrospray ionization (ESI) was used without solvent splitting. The sample was filtered by a filter membrane with a 25 mm diameter and 0.45 µm pore size. Ten µl (1 mg/ml in methanol, HPLC grade Merck, Germany) of the sample was injected into the instrument. Reversed-phase HPLC (RP HPLC) separation was carried out using ACQUITY UPLC HSS T3 (2.1 x 100 mm x 1.8 µm) column protected by guard column. The mass spectrometer was operated in negative ion mode with parameters: capillary voltage of 1.5kV; start time of 0.00 min and end time of 20.00 min; source temperature of 120 °C; desolvation gas flow of 350 L/h; column temperature of 40 °C; and flow rate of 0.60 ml/min. All of the phytochemical compounds in the LC-MS were based on an accuracy of less than 5 ppm mass error.

Statistical analysis

The recorded MAP, SBP and DBP changes were expressed as mean percent changes ± standard error of mean (S.E.M). All statistical tests were analyzed using GraphPad® Prism Version 6 software. A two-way ANOVA test was performed to determine the significant differences between multiple doses of extracts and fractions. Unpaired T-test was done only to ensure there was no significant difference (P>0.05) if the plateau effect occurred on high dosages. A post-hoc Sidak test was performed for multiple pairwise comparisons between the doses. The ED_{50} values for MAP, SBP, and DBP reductions by ASP and fractions were computed by the software based on the constructed dose-response curves. TPC was analyzed by one way ANOVA, followed by post-hoc Sidak multiple comparison test between the doses. All tests were two-tailed and a P value less than 0.05 was considered significant (P<0.05).

RESULTS AND DISCUSSION

Yield of extraction

In total, 1.45 kg of dried *S. polyanthum* leaves used in this study. The mean average yield for HSP, ESP, MSP and ASP were 1.72 ± 0.83 %, 3.62 ± 1.97 %, 6.39 ± 1.25 % and 5.00 ± 2.59 %, respectively. It was observed that methanol gave the highest yield among the four extracts while hexane gave the lowest yield. In agreement with this finding, Jumaat *et al*[28] reported that their extraction with n-hexane, a solvent with a polarity index (P') of 0.1 gave low extraction yield as compared to methanol. Extraction with n-hexane is crucial to break down the cell wall which is coated with the non-polar phospholipids.[29] Ethyl acetate, a solvent with a polarity index (P') of 4.4, dissolves any hydrophilic, lipophilic compounds and hydrophobic chain lipids such as waxes and fats[29] while methanol is a solvent with a polarity index (P') of 5.1 that partially dissolves some other non-water soluble compounds[30] and extracts polar compounds like sugars, amino acids, glycosides and phenolic compounds with low and medium polarity.[31] Water, on the other hand, is a universal solvent that is widely being used in extracting phytochemicals from traditional medicine[30]. It mostly dissolves proteins, carbohydrates[32], and glycosides.[31] The extraction with water, a solvent with a polarity index (P') of 10.2 usually did not dissolve any hydrophobic hydrocarbon compound. This is perhaps the reason that the yield of water extract was lower as compared to methanol. Thus, optimal temperature (80 °C) and ultrasound wave from sonication in the ultrasound-assisted extraction technique plays an important role to enhance water as a solvent to permeate the plant cell wall.[15, 33] Do *et al*[32] and Dhawan and Gupta[34] also showed a lower percentage yield of water extract compared to methanol. This was probably due to the non-solubility of neutral lipids (non-polar hydrophobic) in water, while methanol dissolves a higher amount of polyphenols compared to water due to its inherent efficiency to degrade cell wall comprising of non-polar components. Tiwari *et al*[30] suggested the presence of active polyphenol oxidase enzyme in water extract which may be responsible for degradation of some polyphenols in water extract, whereas the enzyme is non-active in methanol extract. This may justify the higher yield in methanol extract as compared to water extract.

Bioassay-guided fractionation

Fractionation was done on ASP, the most prominent crude extract found in the first phase of the antihypertensive study. When ASP crude extract and the reference compound (gallic acid) was run with TLC using a solvent system of ethyl acetate: methanol: acetonitrile (8:1:1) with one drop of formic acid, four different spots were visualized with good separation when viewed under the UV lamp and sprayed with $FeCl_3$ reagent (Figure 1). These spots were identified with R_f values of 0.21, 0.24, 0.66, and 0.70. Only a spot with an R_f value of 0.21 appeared to be slightly-tailing. The reference compound, gallic acid resulted

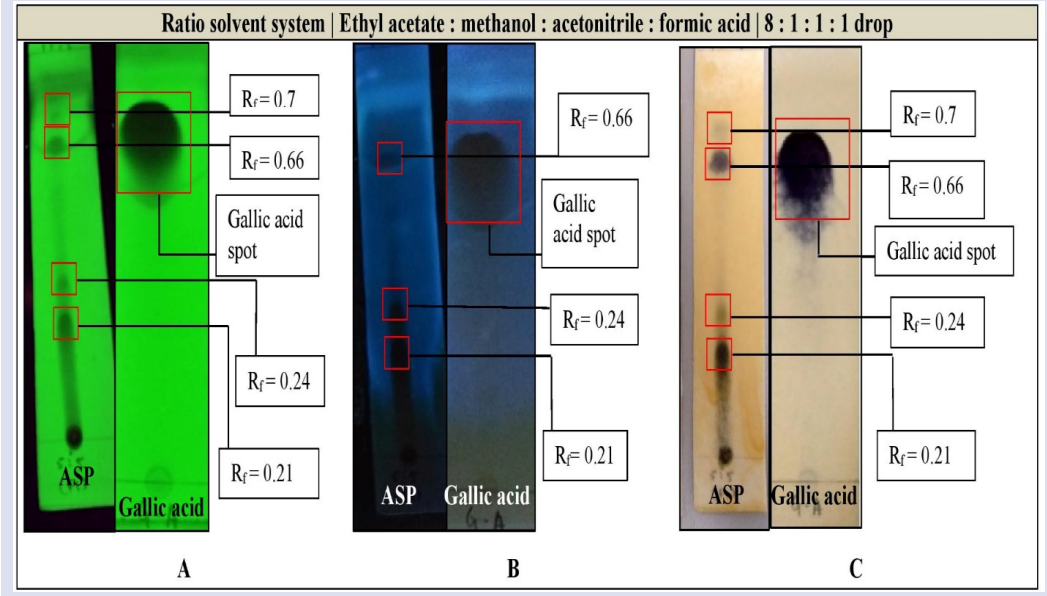


Figure 1: TLC profiles of ASP crude extract and reference (standard) gallic acid with the mobile system of ethyl acetate: methanol: acetonitrile (8:1:1) with one drop of formic acid with detection using A) UV short wave (254 nm), B) UV long wave (365 nm) and C) $FeCl_3$ reagent.

in a very huge spot at R_f = 0.68. In comparison to that, from the four spots developed for the crude ASP extract, two spots with R_f = 0.66 and R_f = 0.7 were very close to gallic acid spots, and they were probably pyrogallic acid, a derivative of gallic acid which was later identified in LC-MS chromatogram of ASP crude extract and F1ASP. Pyrogallic acid is a compound that can be derived from gallic acid via decarboxylation reaction.[35] Based on these TLC profiles, a combination of ethyl acetate and methanol were selected as the binary solvent system for fractionation of ASP using column chromatography.

When the sample was loaded with the starting solvent system (25 ml of 100 % ethyl acetate), three different colours of bands of brown, green, and yellow started to appear. The first (25 ml of 100 % ethyl acetate) and the second ratio solvent system (50 ml of 70 % ethyl acetate and 30 % methanol mixture) allowed the bands to separate. However, the brown and green bands were strongly attracted to the silica column even though the third ratio solvent system (50 ml of 50 % ethyl acetate and 50 % methanol mixture) has been added. The strong attraction was most probably due to the strong polarity of compounds. For the third ratio, the yellow band started to elute and fractions started to be collected in a fixed volume-dependent manner.[36] Altogether, a total of nine pale-yellowish fractions were collected; F1 and F2 were eluents collected when the ratio of solvent system used was ethyl acetate: methanol (5:5); F3, F4, and F5 were eluents collected when the ratio of solvent system used was ethyl acetate: methanol (3:7); F6, F7, and F8 were eluents collected when the ratio of the solvent system was 100 % methanol; while F9 was an eluent collected by washing the column again with 100 % methanol. The yield of each fraction was shown in Table 1.

F9 has the highest yield (2.57 g) while F1 has the lowest yield (0.34 g) after being dried in an incubator. When F1 until F9 were spotted on TLC with a solvent system of ethyl acetate: methanol (9.5:0.5) with a drop of formic acid, numerous spots developed with different R_f values when visualized using UV 254 nm (short wave), UV 365 nm (long wave), $FeCl_3$ reagent, 50 % sulphuric acid, and vanillin-sulphuric acid as shown in Figure 2. The summary of the spots developed on TLC profiles of F1 to F9 under different visualization agents was indicated in Table 2.

Fractions with the same spots developed (similar R_f value) during TLC analysis were pooled as one fraction. F1, F2, F3, and F4 were pooled as F1ASP since they shared a similar spot of S5 and have few more spots such as S1, S4, S6, and S7 while at the same time, there were some spots which were only visualized by vanillin reagent. The subsequent fractions of F5, F6, F7, and F8 have two similar spots of S2 and S5 and thus, they were pooled as F2ASP. The final fraction, F9 was designated as F3ASP as only one single spot of S5 with an R_f of 0.50 ± 0.04 cm was present. Spot S5 which was also identified in the TLC of ASP was suggested as 1-galloyl-glucose, a compound which was found in LC-MS chromatogram of ASP crude extract and all fractions. 1-galloyl-glucose can be produced by esterification of UDP-glucose and gallic acid, the first step of hydrolysable tannin biosynthesis in biosynthetic shikimic acid pathway.[37] However, identification using preparative TLC and high-performance liquid chromatography analysis is required for further confirmation. The reference compound (gallic acid) appeared as a spot with an R_f value of 0.68 ± 0.03 cm. There was no such spot with the same R_f value as gallic acid observed in the TLC of any fractions. The closest ones were S6 with an R_f value of 0.64 ± 0.03 cm and S7 with

Table 1: Yield of fractions derived from ASP crude extract.

Solvents (Gradient elution)	Ethyl acetate (100 %)	Ethyl acetate: methanol (7: 3)	Ethyl acetate: methanol (5: 5)	Ethyl acetate: methanol (3: 7)			Methanol (100%)			Methanol (Wash) (100 %)
Volume of binary solvent (ml)	25	50	50	50			50			100
Fractions (Weight in grams)	-	-	**F1** (0.34)	**F2** (0.84)	**F3** (0.48)	**F4** (0.45)	**F5** (0.76)	**F6** (0.64)	**F7** (0.88)	**F8** (0.52)	**F9** (2.57)
New fractions after pooling	-	-	**Fraction 1 (F1ASP)** (2.11g)					**Fraction 2 (F2ASP)** (2.80g)			**Fraction 3 (F3ASP)** (2.57g)
Percentage yield (%)	-	-	7.03 %					9.33 %			8.57 %

Table 2: Summary of the spots developed on TLC profiles of F1 to F9 and reference (standard) gallic acid under different visualization agents.

Visualization methods/ reagents	Description	S1 (R_f = 0.20 ± 0.01 cm)	S2 (R_f = 0.10 ± 0.02 cm)	S3 (R_f = 0.30 ± 0.01 cm)	S4 (R_f = 0.31 ± 0.02 cm)	S5 (R_f = 0.50 ± 0.04 cm)	S6 (R_f = 0.64 ± 0.03 cm)	S7 (R_f = 0.76 ± 0.03 cm)	Gallic acid (R_f = 0.68 ± 0.03 cm)
UV 254	Visualization of the quenched organic compounds including aromatic and conjugated double bonds.	√	√	√	√	√	√	√	√
UV 365	Visualization of most polycyclic compounds.	√	√		√	√			√
$FeCl_3$ reagent	General detection on phenolic compounds.	√	√		√	√	√		√
50 % sulphuric acid	General detection on steroids.	√			√		√		
Vanillin-sulphuric acid	General detection on higher alcohol, aldehydes, and ketones.	√			√		√		

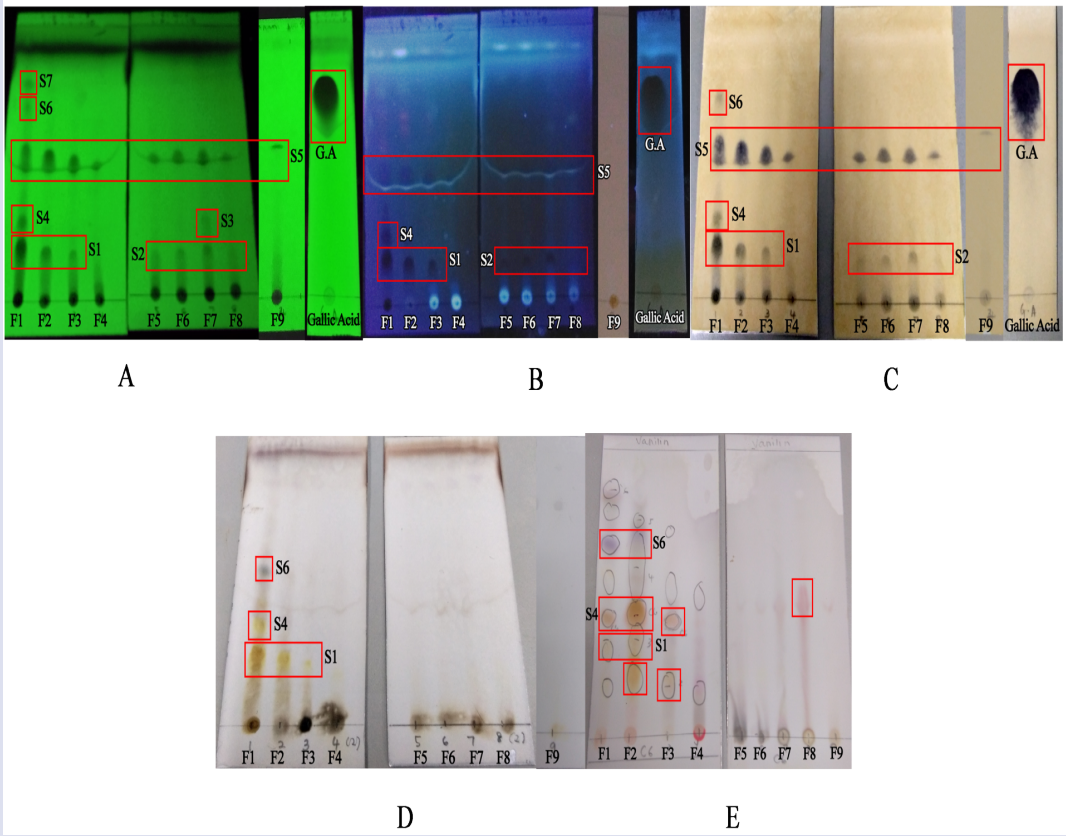


Figure 2: TLC profiles for fractions F1 to F4 (F1ASP), F5 to F8 (F2ASP), F9 (F3ASP), and reference (standard) gallic acid, visualized with A) short UV wave (254 nm); B) long UV wave (365 nm); C) $FeCl_3$ reagent; D) 50 % sulphuric acid, and E) vanillin-sulphuric acid.

an R_f value of 0.76 ± 0.03 cm. After pooling, F2ASP gave the highest yield of 2.80 g while F1ASP gave the lowest yield of 2.11 g (Table 1).

Antihypertensive effects of S. polyanthum leaves crude extracts and fractions

Three blood pressure parameters were measured in this study which includes mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP). MAP is the average arterial pressure throughout one cardiac cycle and it is usually influenced by cardiac output and systemic vascular resistance.[38] SBP is the pressure measured during systole or heart contraction, while DBP is a pressure during diastole or relaxation period. While MAP is a better indicator of perfusion to vital organs than systolic blood pressure (SBP), SBP is a bigger risk factor than DBP for cardiovascular disease in elderly patients.[39] Considering the individual importance of each parameter, this study includes all these three blood pressure parameters.

Figure 3 shows dose-response curves for MAP, SBP, and DBP of SHR when administered with HSP, ESP, MSP, and ASP crude extracts. The mean baselines for MAP, SBP, and DBP (n=20) before intravenous administration of these crude extracts were 185.77 ± 5.05 mmHg, 218.41 ± 5.86 mmHg, and 157.04 ± 5.27 mmHg, respectively. The negative control (vehicle that dissolved the extracts) did not give any significant changes to the baseline of all blood pressure parameters (n = 5).

ASP crude extract caused significant reductions in MAP at 40 mg/kg and 70 mg/kg by 35.07 ± 3.80 % (P<0.001) and 67.82 ± 8.44 % (P<0.001), respectively (Figure 3A). The maximum reduction in MAP was observed at dose 70 mg/kg ASP crude extract. Meanwhile, MSP crude extract also caused significant reduction in MAP at 40 mg/kg and 70 mg/kg by 15.66 ± 1.51 % (P<0.001) and 20.12 ±1.19 % (P<0.001), respectively. ESP crude extract only caused a significant reduction in MAP only at the highest dose of 70 mg/kg by 15.39 ± 3.58 %. On the other hand, there was no significant reduction of MAP for HSP crude extract.

There were also significant reductions in SBP by ASP crude extract at 10 mg/kg, 40 mg/kg and 70 mg/kg by 21.97 ± 3.79 % (P<0.05), 35.76 ± 4.74 % (P<0.001) and 73.75 ± 6.93 % (P<0.001), respectively (Figure 3B). On the other hand, MSP crude extract caused a significant reduction in SBP at 40 mg/kg and 70 mg/kg by 20.58 ± 1.50 % (P<0.001) and 16.19 ± 1.80 % (P<0.001), respectively. Similar to reductions in MAP, ESP crude extract only caused a significant reduction in SBP of SHR only at the highest dose of 70 mg/kg by 16.51 ± 3.82 % (P<0.001) while HSP gave no significant reduction in SBP at all.

Meanwhile for DBP, both ASP and MSP crude extracts gave significant reductions in DBP at 40 mg/kg and 70 mg/kg. ASP significantly reduced DBP by 37.31 ± 4.21 % (P<0.001) at 40 mg/kg and by 72.94 ± 7.76 % (P<0.001) at 70 mg/kg (Figure 3C). While for MSP, it significantly reduced DBP by 17.79 ± 2.24 % (P<0.001) at 40 mg/kg and by 21.84 ± 1.23 % (P<0.001) at 70 mg/kg. ESP crude extract could only give a significant reduction (P<0.01) at 70 mg/kg by 15.39 ± 3.58 %. However, there was no significant difference observed in DBP when administered with HSP crude extract at all dosages.

From the pattern of dose-response curves in Figure 3, HSP crude extract did not exhibit any significant antihypertensive effect and since it only extracted non-polar compounds, this has suggested that non-polar compounds did not significantly contribute to the antihypertensive

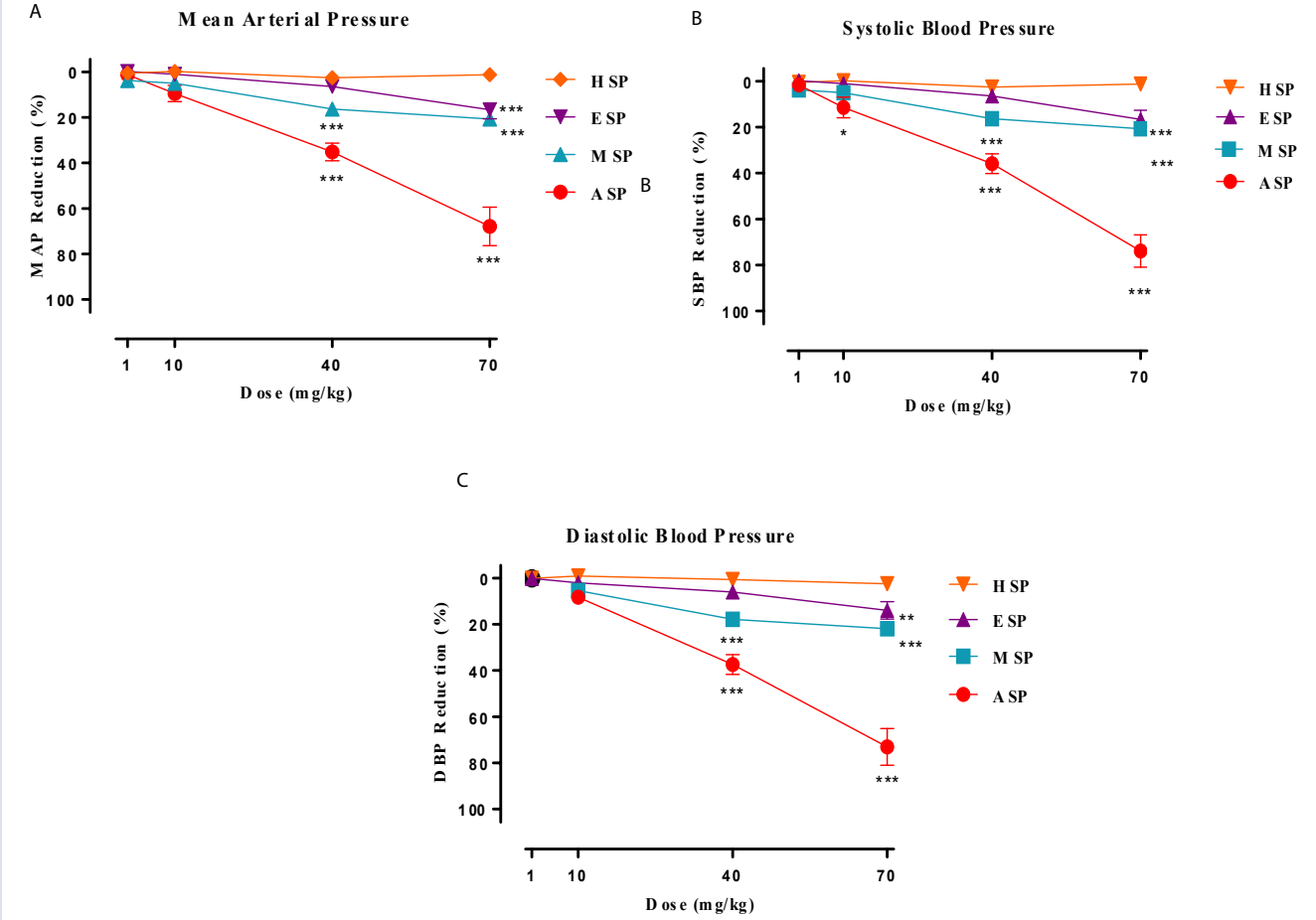


Figure 3: Dose-response curves for A) mean arterial pressure, B) systolic blood pressure, and C) diastolic blood pressure of Spontaneously Hypertensive Rats (n=5) when intravenously administered with n-hexane (HSP), ethyl acetate (ESP), methanol (MSP), and aqueous (ASP) crude extracts at 1, 10, 40 and 70 mg/kg. *P<0.05, **P<0.01 and ***P<0.001, all *vs.* negative control (0.90 % normal saline) by using two-way ANOVA with post-hoc Sidak multiple comparison test.

effect for *S. polyanthum* leaves. On the other hand, it was observed that ASP crude extract has the most prominent antihypertensive effect as it caused more reduction in all blood pressure parameters, especially at dosages of 40 mg/kg and 70 mg/kg when compared to other crude extracts. In summary, though ESP and MSP have significantly reduced the blood pressure of SHR, the antihypertensive effects by these two extracts were not as prominent as ASP. Besides the fact that ASP showed the most prominent antihypertensive effect, aqueous extraction is usually advantageous from the pharmacological point of view. Aqueous extract usually is the safest solvent with less toxicity for animal study[40], and at the same time it is cost-effective and is usually used to mimics the traditional preparation.[30] Considering all these findings, ASP was further fractionated in the subsequent study.

The subsequent antihypertensive study was conducted to identify the most active fraction. By using the same blood pressure parameters, the effect of fractions (F1ASP, F2ASP, F3ASP) was evaluated and compared with the original crude ASP extract. Normal saline (0.90 %) was used as the negative control while captopril (5 mg/kg) was used as the positive control. Figure 4 shows the magnitude of changes in MAP, SBP, and DBP when administered with the three fractions, in comparison with those exhibited by negative and positive controls as well as with the original ASP crude extract.

The mean baselines for MAP, SBP, and DBP (n=20) of SHR in this experiment were 171.19 ± 4.54 mmHg, 198.71 ± 4.09 mmHg, and 144.51 ± 3.94 mmHg, respectively. As shown in Figure 4A, there was no significant reduction observed on MAP of SHR with normal saline. Crude ASP extract at doses of 30, 40, 50, and 60 mg/kg significantly reduced MAP of SHR by 23.30 ± 1.08 % (P<0.01), 32.85 ± 3.75 % (P<0.001), 25.78 ± 7.09 % (P<0.01), and 29.24 ± 9.10 % (P<0.01), respectively. Meanwhile for the fractions, F1ASP at doses of 30, 40, 50, and 60 mg/kg significantly reduced MAP by 25.41 ± 3.57 % (P<0.01), 32.05 ± 6.66 % (P<0.001), 25.46 ± 4.55 % (P<0.05), and 27.39 ± 2.42 % (P<0.001), respectively. F2ASP at dosages of 20, 30, 40, 50, and 60 mg/kg significantly reduced MAP by 30.87 ± 6.70 % (P<0.001), 37.94 ± 5.84 % (P<0.001), 36.66 ± 5.41 % (P<0.001), 35.45 ± 0.93 % (P<0.001), and 27.65 ± 9.98 % (P<0.001), respectively. Nevertheless, for F3ASP, the MAP was significantly reduced only at two doses of 30 and 40 mg/kg by 25.64 ± 5.67 % (P<0.001) and 33.70 ± 5.45 % (P<0.001), respectively. In addition, 5 mg/kg of captopril (positive control) has significantly reduced MAP by 29.29 ± 3.18 % (P<0.001, Figure 4A). In comparison to positive control, the significant reduction in MAP by ASP (30, 40, 50 and 60 mg/kg), F1ASP (30, 40, 50, and 60 mg/kg), F2ASP (20, 30, 40, 50, and 60 mg/kg), and F3ASP (30 and 40 mg/kg) was not significantly different with the reduction by captopril at 5 mg/kg. This finding has indicated a comparable reduction in MAP between the positive control with ASP and the fractions at these dosages.

Meanwhile for SBP, ASP crude extract at dosages of 30, 40 and 50 mg/kg significantly reduced SBP by 27.16 ± 3.02 % (P<0.01), 32.80 ± 3.55 % (P<0.001), and 26.19 ± 13.52 % (P<0.01), respectively (Figure 4B).

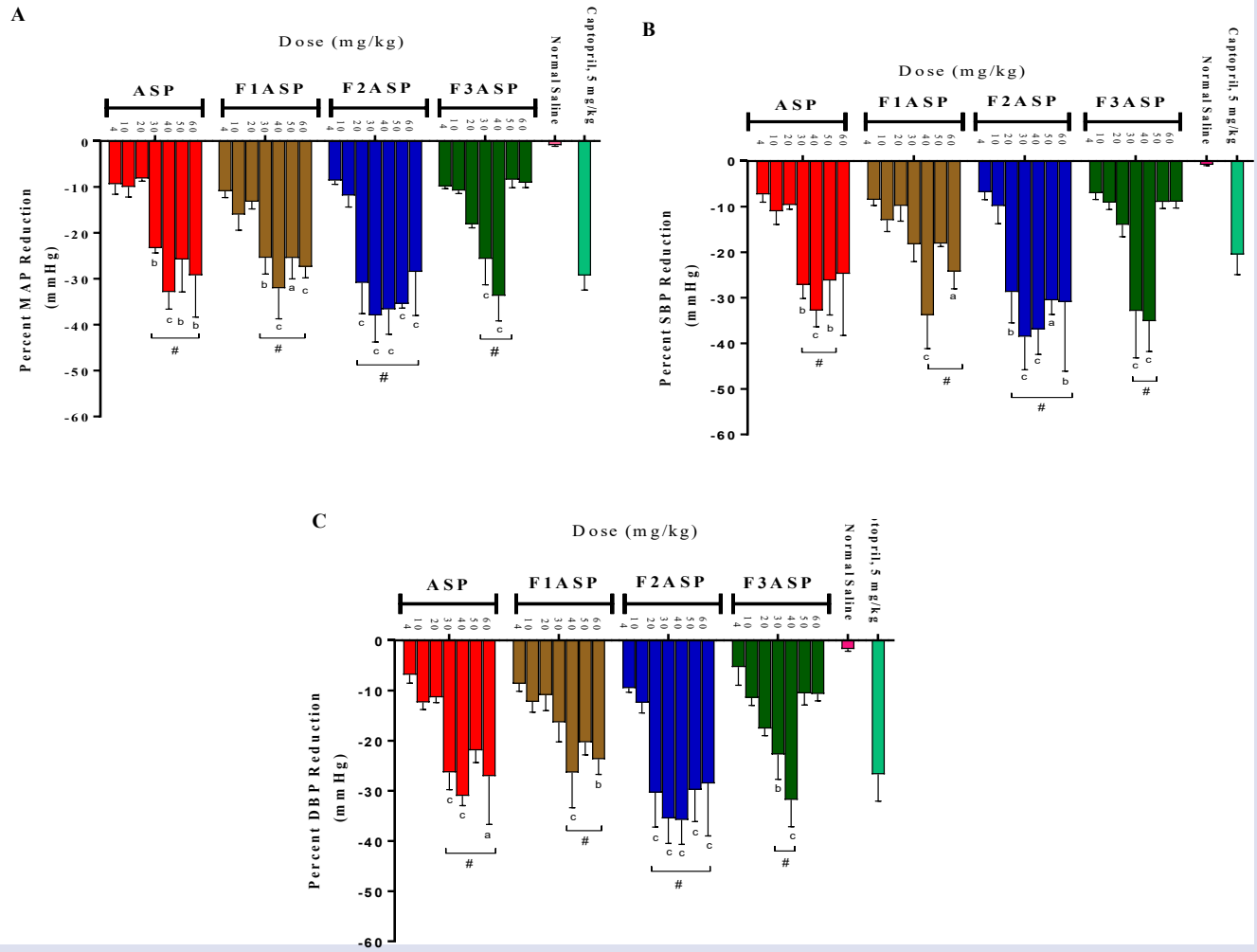


Figure 4: Effects of ASP, F1ASP, F2ASP, F3ASP, captopril (positive control), and normal saline (negative control) on A) MAP, B) SBP, and C) DBP of anaesthetized SHR (n=5). Mg/kg: Milligram per kilogram, MAP: Mean arterial pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, mmHg: Millimeters Mercury, a: P<0.05, b: P<0.01, c: P<0.001, all vs. normal saline and #P>0.05, all vs. Captopril, 5 mg/kg (this shows comparable effect with positive control drug). All data were analyzed using two-way ANOVA with post-hoc Sidak multiple comparison test.

For the fractions, F1ASP only significantly reduced SBP at two dosages of 40 and 60 mg/kg by 33.79 ± 7.35 % (P<0.001) and 24.25 ± 3.78 % (P<0.05), respectively. Meanwhile F2ASP at dosages of 20, 30, 40, 50, and 60 mg/kg significantly reduced SBP by 28.72 ± 6.78 % (P<0.01), 38.54 ± 7.26 % (P<0.001), 36.92 ± 5.53 % (P<0.001), 30.56 ± 3.08 % (P<0.05), and 30.93 ± 15.17 % (P<0.01), respectively. With the similar pattern observed for MAP changes, the SBP was significantly reduced by F3ASP only at two dosages of 30 and 40 mg/kg by 32.87 ± 10.32 % (P<0.001) and 35.11 ± 6.69 % (P<0.001), respectively. The significant reductions in SBP by ASP (30, 40, 50 and 60), F1ASP (30, 40, 50, and 60 mg/kg), F2ASP (20, 30, 40, 50, and 60 mg/kg), and F3ASP (30 and 40 mg/kg) for all dosages were not significantly different than the reduction by positive control (captopril) at 5 mg/kg (Figure 4B). These findings showed a comparable reduction in SBP of SHR by the positive control with ASP and the fractions at these dosages.

As illustrated in Figure 4C, ASP at doses of 30, 40, and 60 mg/kg significantly reduced DBP by 26.30 ± 3.45 % (P<0.001), 35.08 ± 4.37 % (P<0.001), and 27.04 ± 9.65 % (P<0.05), respectively. For the fractions, only two dosages of F1ASP at 40 and 60 mg/kg significantly reduced DBP by 26.36 ± 6.99 % (P<0.001) and 23.67 ± 3.06 % (P<0.01), respectively. F2ASP at dosages of 20, 30, 40, 50, and 60 mg/kg significantly reduced DBP by 30.37 ± 6.83 % (P<0.001), 35.48 ± 5.01 % (P<0.001), 35.81 ± 4.86 % (P<0.001), 29.78 ± 6.343 % (P<0.01), and 28.52 ± 10.48 % (P<0.001), respectively. In spite of that, for F3ASP, DBP was also significantly reduced only at two doses of 30 and 40 mg/kg by 22.73 ± 4.98 % (P<0.01), and 31.80 ± 5.33 % (P<0.001), respectively. Five mg/kg of captopril (positive control) significantly reduced DBP by 28.52 ± 10.48 % (P<0.001) (Figure 4C). These significant reductions in DBP by ASP (30, 40, 50, and 60 mg/kg), F1ASP (40, 50, and 60 mg/kg), F2ASP (20, 30, 40, 50, and 60 mg/kg), and F3ASP (30 and 40 mg/kg) were not significantly different than the reduction by captopril (5 mg/kg). This finding has also indicated a comparable reduction in DBP by the positive control with ASP and the fractions at these dosages.

Dose-response curves for the effect of each fraction on MAP, SBP, and DBP were then constructed and then compared with ASP crude extract (Figure 5). Both ASP and F1ASP started to cause significant reductions in MAP, SBP, and DBP at 30 mg/kg, then it caused a maximum reduction in MAP, SBP, and DBP at 40 mg/kg, and then the curve has started to become plateau afterward. In contrast to ASP and F1ASP, F2ASP started to produce significant MAP, SBP and DBP reduction at a low dose of 20 mg/kg, and then the effect has become plateau from 30 mg/kg until 60 mg/kg. In fact, the maximum reduction in MAP by F2ASP at 30 mg/kg was actually higher (P<0.05) than the other fractions and also ASP crude extract (Figure 5A). F3ASP showed the same trend as ASP and

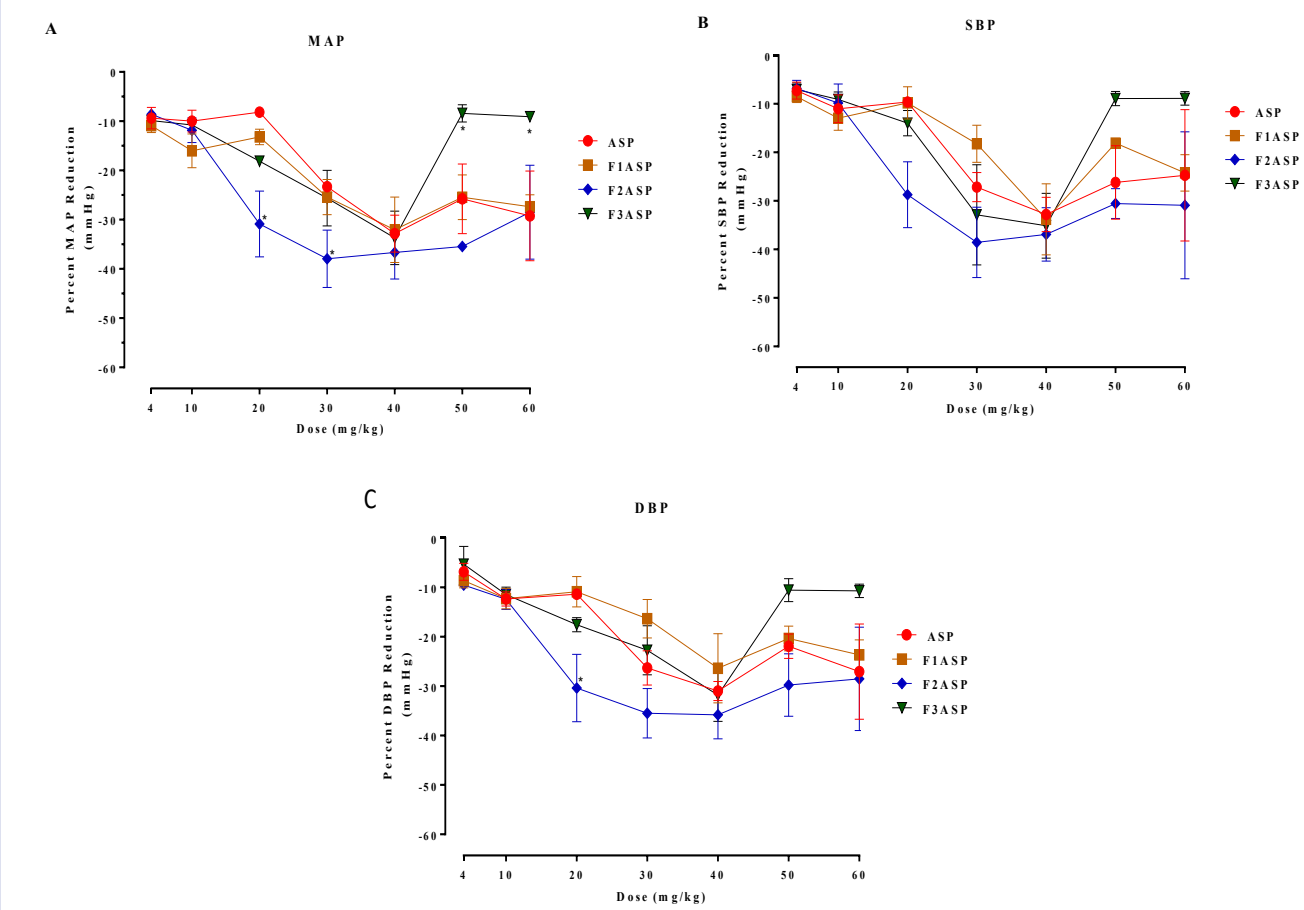


Figure 5: Dose-response curves for the effects of intravenously-administered ASP, F1ASP, F2ASP, and F3ASP on A) MAP, B) SBP, and C) DBP of anaesthetized SHR (n=5). ASP: Aqueous extract of *S. polyanthum* leaves, mg/kg: Milligram per kilogram, MAP: Mean arterial pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, mmHg: Millimeters Mercury, *P<0.05, all vs ASP by using two-way ANOVA post hoc Sidak multiple comparison test.

F1ASP at low dosages, and then the effects became reduced at 50 mg/kg and maintained at 60 mg/kg. It is postulated that for F3ASP, there are different receptors involved at low and high dosages. When the reduction in blood pressure has reached maximum through activating the first receptor, then at the higher dosages, the fraction might activate another receptor system that causes attenuation of the antihypertensive effect. The involvement of receptors can be further investigated through in-depth pharmacodynamics studies.

To analyze the potency of the ASP crude extract and fractions, their ED_{50} was then determined using GraphPad® Prism Version 6.00 software based on the constructed dose-response curves. ED_{50} is an effective dose that produces 50 % of the maximal effect. The ED_{50} values for MAP, SBP and DBP reduction by F2ASP were 14.16 mg/kg, 19.17 mg/kg and 13.80 mg/kg, respectively. These ED_{50} values were actually lower than the ED_{50} values for ASP crude extract (29.48 mg/kg, 29.18 mg/kg and 23.53 mg/kg, respectively) and F1ASP (29.08 mg/kg, 30.00 mg/kg and 30.15 mg/kg, respectively). On the other hand, F3ASP's pattern of reduction in blood pressure differed from the other fractions. After the antihypertensive effect by F3ASP reached maximum, then the effect was significantly reduced at subsequent dosages (50 mg/kg and 60 mg/kg). Thus, the ED_{50} value of F3ASP could not be determined in this study. Altogether, F2ASP was more potent as compared to ASP and F1ASP and thus was considered as the most active fraction. The high potency of F2ASP might be due to the high concentration of the bioactive compound in this fraction compared to its crude extract itself. In agreement, Idris *et al*[41] also found a higher antihypertensive effect of the fraction compared to crude extract and suggested the probability of an increased concentration of active compounds during the partitioning process.

Total phenolic content

This study examined the total phenolic content of the crude ASP extract and the three derived fractions (F1ASP, F2ASP, and F3ASP) by using Folin-Ciocalteu assay. Folin-Ciocalteu assay was generally a modified method from analysis of protein and is widely used for determination of the total phenolic content of various plant extracts.[25, 26, 42, 43] This assay was chosen to be used in this study as it is commercially available and has a standard procedure.[26] This assay utilizes Folin-Ciocalteau reagent that determine phenols and easily-oxidized substances by forming a blue color complex form, reducing the yellow color of heteroply phosphomolibdate-tungstate anions.[25] The concentration of phenols can be determined by the blue color formed. However, this reaction's mechanism is not solely used for specific determination of only phenolics, instead, it can be used for determining any reducing compounds that can react with the phosphotungstic reagent.[42]

Figure 6 shows the standard curve of gallic acid with an R_2 value of 0.992. The TPC for ASP, F1ASP, F2ASP, and F3ASP is shown in Table 3. ASP crude extract had the highest total phenolic content (232.81 ± 0.67 mg GAE/g), followed by F1ASP (76.15 ± 3.75 mg GAE/g), F3ASP (36.45 ± 1.35 mg GAE/g) and lastly F2ASP (30.52 ± 5.83 mg GAE/g). TPCs for all fractions were significantly different (P< 0.001) than ASP, in which all of them have lower TPC than ASP. The TPC

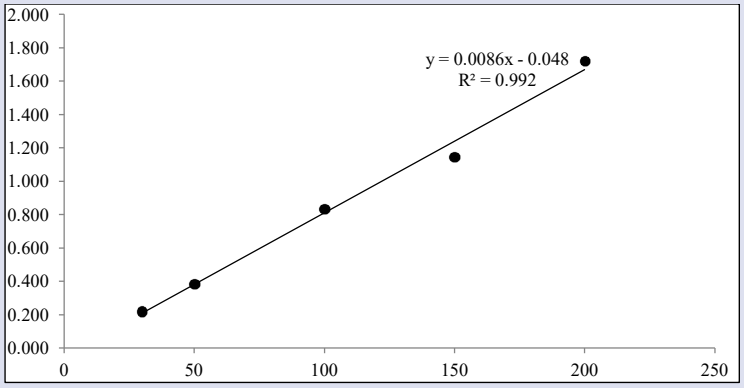


Figure 6: Standard curve of gallic acid which was used to determine the total phenolic content of ASP crude extract and fractions, expressed as mg/g gallic acid equivalent (GAE).

Table 3: Total phenolic content of ASP crude extract and fractions.

No.	Sample	Total phenolic content (GAE mg/gram)			Mean± SD
		Trial 1	Trial 2	Trial 3	
1	F1ASP	78.60	71.83	78.012	76.15 ± 3.75***
2	F2ASP	25.01	36.63	29.907	30.52 ± 5.83***
3	F3ASP	38.01	35.67	35.674	36.45 ± 1.35***
4	ASP	232.04	232.083	233.29	232.81 ± 0.67

Note. Symbol*** showing P<0.001, all *vs* ASP using one way ANOVA with post-hoc Sidak multiple comparison test.

of both F2ASP and F3ASP were not significantly different. Thus, the order of TPC of *S. polyanthum* leaves from the highest to the lowest order was ASP>F1ASP>F3ASP=F2ASP. Since the reaction of Folin's reagent also based on a redox reaction, the TPC assay would detect all substances that were oxidized[44], and this may include several potential reductants such as the reducing sugars glucose and fructose. This might cause significant effect on the accuracy of the TPC assay.[43] Note that from LC-MS analyses, the ASP crude extract and all fractions contained a lot of glucosides (glucose bounded to another functional group) in which the glucose part may affect the reactions. Besides, this assay involves an oxidation reaction where the blue chromophore is formed by a phosphotungstic-phosphomolybdenum complex in which the maximum absorption depends on the alkaline solution and the concentration of phenolic compounds oxidized.[25] Thus, the TPC assay would only detect phenolics that can function as reductants in a redox-linked colorimetric method. In addition, less availability of hydroxyl group or non-oxidized phenolics could also contribute to the low concentration of phenolics and eventually affected the total phenolic content analyzed.

In comparison with previous studies on the TPC of *S. polyanthum* leaves, it was found that the TPC of water extract of *S. polyanthum* leaves collected in Singapore was 11.21 mg GAE[44], a value which was lower than our present finding. Safriani *et al*[45] also reported a lower TPC of water extract (≈40.0 mg GAE/g) compared to the current findings. However, Har and Ismail[46] found that the methanolic extract of *S. polyanthum* leaves contained 1,125 mg GAE/g, which indicated for higher TPC than our ASP crude extract (232.81 ± 0.67 mg GAE/g). The higher phenolic content of methanol extract compared to the water extract used in our current study was probably due to the higher efficiency of methanol in extracting polyphenol. Methanol actually causes cell wall degradation causing more polyphenols to be released from the cells.[30]

Phenolic compounds identified using LC-QTOF/MS

The different magnitude of antihypertensive effects by the extracts and the fractions may be affected by their varying phytochemical composition. Thus, LC-QTOF/MS analyses were then run for the most prominent crude extract, ASP, as well as for the three derived fractions (F1ASP, F2ASP, and F3ASP). Since this LC-QTOF/MS analysis was conducted in negative mode, only compounds with negative ions at high pH were detected. Figure 7 showed the LC-MS chromatograms of the blank (methanol) and ASP crude extract while Table 4 listed all the eluted compounds. In total, there were 216 peaks eluted out using the binary gradient elution with some redundant compounds detected as different peaks and at different retention times. Thus, in total, only 93 single compounds were actually detected in this analysis. In terms of composition, ASP crude extract was composed of gallotannins, phenolic acids, glucosides, flavonoids, and simple phenols. The highest intensity compound (highest percent response) was 2,4,7-trihydroxy-9, 10-dihydrophenanthrene which was eluted at 11.90 min with an intensity of 14.36 %. Another two highest intensity compounds were osmanthuside H (4.33 %) and sinapaldehyde (3.56 %). Meanwhile compound with the least intensity was 3, 4-dihydroxyphenothyl-3-O-β-D-glucopyranoside by 0.06 %.

Figure 8 shows LC-MS chromatograms for the blank methanol and F1ASP while Table 5 listed all the eluted compounds. In total, there were 76 peaks eluted out using the binary gradient elution with some redundant compounds. To be exact, only 46 single compounds that were detected in this analysis. The highest intensity compound (highest percent response) was feroxin A at 5.76 min with an intensity of 8.44 %. Another two highest intensity compounds were 2,4,7-trihydrohydroxy-9,10-dihydrophenanthrene (7.79 %) and 1-galloyl-glucose (6.90 %). Meanwhile, the compound with the least intensity was cyclocurcumin with an intensity of 0.38 %.

Figure 9 shows the LC-MS chromatogram for the blank methanol and F2ASP while Table 6 listed all the eluted compounds. There were 13 peaks present in the chromatogram with few compounds that occurred in redundancy. Thus, there were only six compounds to be exact in F2ASP. These phytochemical compounds were either gallotannins, simple phenols, or isoflavanoids. The highest intensity of compound (highest percent response) in F2ASP was 1-galloyl-glucose; it was eluted at 1.33 min with an intensity of 20.24 %. Another two highest

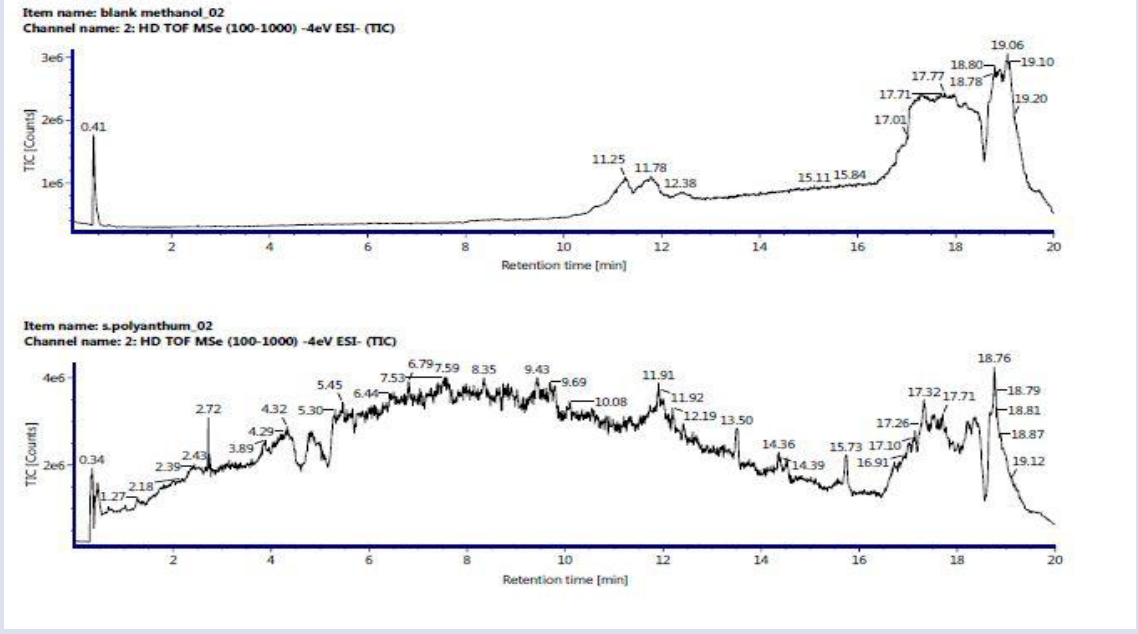


Figure 7: Chromatogram of blank methanol and crude ASP extract.

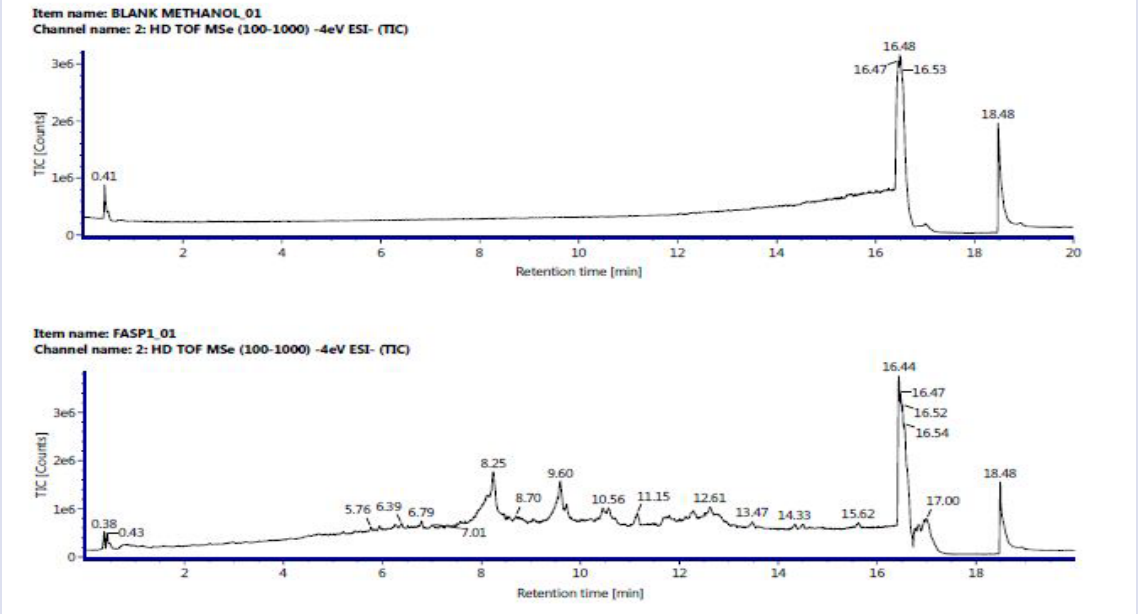


Figure 8: Chromatogram of blank methanol and F1ASP.

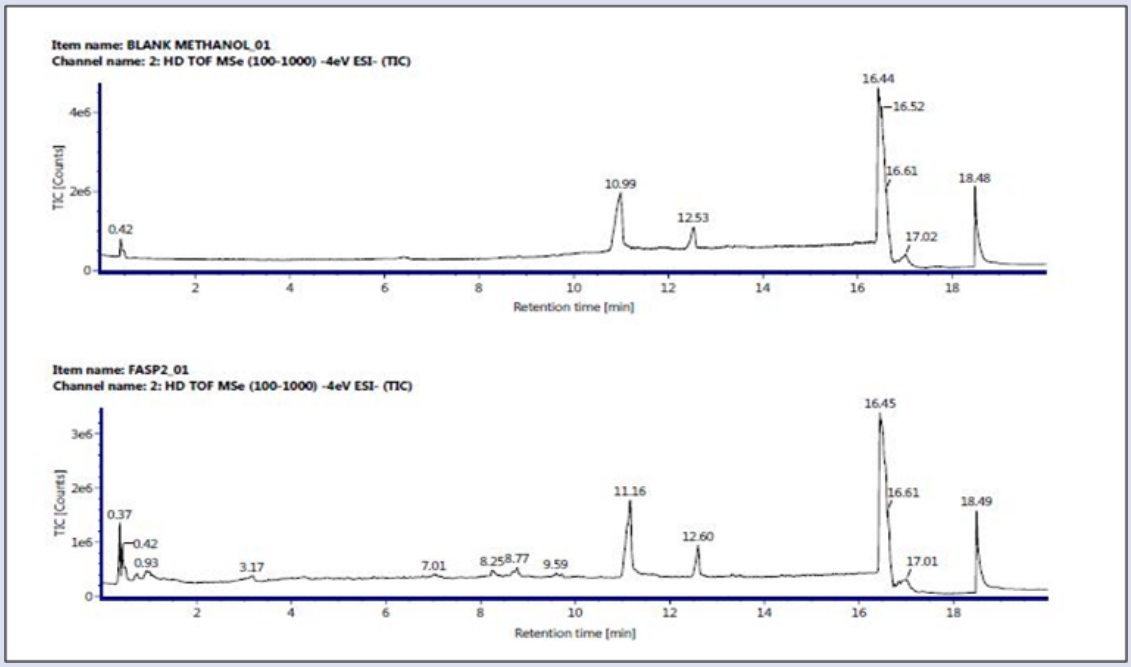


Figure 9: Chromatogram of blank methanol and F2ASP.

Table 4: Phytochemical compounds in ASP crude extract using LC-MS.

No.	Compound	Molecular Formula	RT (min)	Chemical Classes	Response %	[M⁺]
1	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.43	Gallotannin	0.15	331.07
2	Cassialactone	$C_{16}H_{16}O_{6}$	0.44	Simple phenol	0.12	349.09
3	Pyrogallic acid	$C_{6}H_{6}O_{3}$	0.53	Phenolic acid derivative	0.11	125.02
4	Pyrogallic acid	$C_{6}H_{6}O_{3}$	0.79	Phenolic acid derivative	0.07	125.02
5	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.91	Gallotannin	0.35	331.07
6	2,3-(S)-Hexahydroxydiphenol-D-glucose	$C_{20}H_{18}O_{14}$	1.05	Glucoside	0.35	481.06
7	Pyrogallic acid	$C_{6}H_{6}O_{3}$	1.19	Phenolic acid derivative	0.26	125.02
8	Cassialactone	$C_{16}H_{16}O_{6}$	1.26	Simple phenol	0.10	349.09
9	Pyrogallic acid	$C_{6}H_{6}O_{3}$	1.42	Phenolic acid derivative	0.07	125.02
10	Methyl-β-orsellinate	$C_{9}H_{10}O_{4}$	1.53	Ester phenol	0.11	181.05
11	3,4-Dihydroxy phenothyl-3-O-β-D-glucopyranoside	$C_{12}H_{16}O_{8}$	2.01	Glucoside	0.06	333.08
12	2,4,5Trihydeoxy benzaldehyde	$C_{7}H_{6}O_{4}$	2.07	Simple phenol	0.11	153.02
13	Cassialactone	$C_{16}H_{16}O_{6}$	2.19	Simple phenol	0.08	349.09
14	1-Galloyl-glucose	$C13H16O_{10}$	2.24	Gallotannin	0.18	331.07
15	1-Galloyl-glucose	$C_{13}H16O_{10}$	2.31	Gallotannin	0.67	331.07
16	Caesalpins J	$C_{17}H_{16}O_{6}$	2.96	Simple phenol	1.21	361.09
17	Osmanthuside H	$C_{19}H_{28}O_{11}$	3.07	Glycoside phenol	0.92	431.16
18	Feroxin A	$C_{17}H_{24}O_{8}$	3.26	3-O Glucoside	0.19	401.14
19	3'-O-Methylbrazilin	$C_{17}H_{16}O_{5}$	3.36	Simple phenol	0.20	345.10
20	Feralolide	$C_{18}H_{16}O_{7}$	3.42	Dihydro-isocoumarin	0.10	343.08
21	Darendoside A	$C_{19}H_{28}O_{11}$	3.88	Phenethyl alcohol glycosides	2.99	431.16
22	Caesalpins J	$C_{17}H_{16}O_{6}$	3.93	Simple phenol	1.18	361.09
23	Tachioside	$C_{13}H_{18}O_{8}$	3.98	Phenolic glycoside	0.09	301.09
24	Tachioside	$C_{13}H_{18}O_{8}$	3.98	Phenolic glycoside	0.11	301.09
25	Dendrocandin B	$C_{27}H_{30}O_{8}$	4.05	Bibenzyl phenols	0.09	481.19
26	Osmanthuside H	$C_{19}H_{28}O_{11}$	4.33	Glycoside Phenols	4.33	431.16
27	Odoriflavene	$C_{17}H_{16}O_{5}$	4.36	Isoflavene	0.22	345.10
28	Sesamol	$C_{7}H_{6}O_{3}$	4.40	Hydroquinone derivative	0.24	183.03
29	2,4,4′,6′-Tetrahydroxy-benzophenone	$C_{13}H_{10}O_{5}$	4.40	Benzophenone	0.22	245.05

30	Haematoxylin	$C_{16}H_{14}O_6$	4.41	Phenocyanin	0.14	347.08
31	Feroxin A	$C_{17}H_{24}O_8$	4.42	3-O Glucoside	0.34	401.14
32	Caesalpins J	$C_{17}H_{16}O_6$	4.49	Simple phenol	1.21	361.09
33	Cistanoside H	$C_{22}H32O_{13}$	4.55	Glucoside	0.09	503.18
34	Polydatin	$C_{20}H_{22}O_8$	4.58	Glucoside	0.29	435.13
35	Caesalpins J	$C_{17}H_{16}O_6$	4.79	Simple phenol	0.10	361.09
36	10-O-Methyl protosappanin B	$C_{17}H_{18}O_6$	4.85	Dibenzoxocin derivative	0.08	363.11
37	Moracin F	$C_{16}H_{14}O_5$	4.86	2-arylbenzofuran flavanoid	0.15	285.08
38	2,3,5,4'-Tetrahydroxystilbene-2,3-O-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	4.93	Glucoside	1.31	567.17
39	Methyl-β-orsellinate	$C_9H_{10}O_4$	4.94	Ester phenol	0.20	227.06
40	Protosappanin C	$C_{16}H_{14}O_6$	5.02	Dibenzoxocin derivative	0.07	347.08
41	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	5.27	Phenanthrene phenol	0.50	273.08
42	Kakuol	$C_{10}H_{10}O_4$	5.33	Prophiophenone derivative	0.09	239.06
43	Kakuol	$C_{10}H_{10}O_4$	5.71	Prophiophenone derivative	0.17	239.06
44	Aspidinol	$C_{12}H_{16}O_4$	5.79	Simple phenols	0.18	269.10
45	Feroxin A	$C_{17}H_{24}O_8$	5.80	3-O Glucoside	0.19	401.14
46	Feroxin A	$C_{17}H_{24}O_8$	5.80	3-O Glucoside	0.20	401.14
47	Feroxin A	$C_{17}H_{24}O_8$	5.80	3-O Glucoside	2.25	401.14
48	Maclurin	$C_{13}H_{10}O_6$	5.92	Benzanoid phenolic	0.41	261.04
49	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	6.01	Simple phenol	0.13	267.09
50	Moracin M-3′-O-β-Dglucopyranoside	$C_{20}H_{20}O_9$	6.07	Glycoside	0.12	449.11
51	Protosappanin A	$C_{15}H_{12}O_5$	6.09	Dibenzoxocin derivative	0.07	317.07
52	Methyl-5-Ocaffeoylquinate	$C_{17}H_{20}O_9$	6.15	Phenolic acid derivative	0.07	413.11
53	3,4-Dihydroxy phenethanol	$C_8H_{10}O_3$	6.26	Simple phenol	0.15	153.06
54	Cimidahurine	$C_{14}H_{20}O_8$	6.27	Phenylpropanoid glycoside	0.56	315.11
55	Cimidahurine	$C_{14}H_{20}O_8$	6.27	Phenylpropanoid glycoside	0.08	315.11
56	Aspidinol	$C_{12}H_{16}O_4$	6.29	Simple phenols	0.18	223.10
57	Protosappanin C	$C_{16}H_{14}O_6$	6.30	Dibenzoxocin derivative	0.12	347.08
58	10-OMethyl-protosappanin B	$C_{17}H1_8O_6$	6.41	Dibenzoxocin derivative	0.11	363.11
59	Cassialactone	$C_{16}H_{16}O_6$	6.43	Simple phenol	0.08	349.09
60	2,7-Dihydroxy-4-methoxyphenanthrene-2-O-glucoside	$C_{21}H_{22}O_8$	6.43	Phenanthrene phenol	0.14	447.13
61	1-O-Methyl-3,5 Odicaffeoylquinic acid methyl ester	$C_{27}H_{28}O_{12}$	6.45	Phenolic acid	0.08	589.16
62	Kakuol	$C_{10}H_{10}O_4$	6.47	Prophiophenone derivative	0.40	239.06
63	Aspidinol	$C_{12}H_{16}O_4$	6.49	Simple phenols	0.11	223.10
64	Dihydroeugenol	$C_{10}H_{14}O_2$	6.53	Simple phenols	0.13	211.10
65	Ciwujiatone	$C_{22}H_{26}O_9$	6.59	Lignan	0.06	433.15
66	Phenol	C_6H_6O	6.91	Simple phenols	0.08	139.04
67	2,7-Dihydroxy-4-methoxyphenanthrene-2-O-glucoside	$C_{21}H_{22}O_8$	6.96	Phenanthrene phenol	0.19	447.13
68	Nobilin D	$C_{16}H_{18}O_6$	6.97	Prenol lipid	0.07	305.10
69	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	6.99	Phenanthrene phenol	0.09	477.14
70	Cimidahurine	$C_{14}H_{20}O_8$	7.05	Phenylpropanoid glycoside	0.17	315.11
71	Kakuol	$C_{10}H_{10}O_4$	7.13	Prophiophenone derivative	0.09	239.06
72	Kakuol	$C_{10}H_{10}O_4$	7.13	Prophiophenone derivative	0.16	239.06
73	Phenol	C_6H_6O	7.14	Simple phenols	0.40	139.04
74	Phenol	C_6H_6O	7.14	Simple phenols	0.19	139.04
75	Kakuol	$C_{10}H_{10}O_4$	7.14	Prophiophenone derivative	1.17	239.06
76	Tachioside	$C_{13}H_{18}O_8$	7.15	Phenolic glycoside	0.74	301.09
77	Phenol	C_6H_6O	7.15	Simple phenols	0.16	139.04

78	Moracin F	$C_{16}H_{14}O_5$	7.16	2-arylbenzofuran flavanoid	0.69	285.08
79	Gingerone	$C_{11}H_{14}O_3$	7.26	Simple phenols	0.41	239.09
80	Dihydroeugenol	$C_{10}H_{14}O_2$	7.28	Simple phenols	0.53	211.10
81	2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucopyranoside	$C_{20}H_{22}O_9$	7.39	Glucoside	0.07	405.12
82	Cassialactone	$C_{16}H_{16}O_6$	7.41	Simple phenol	0.24	349.09
83	Nobilone	$C_{14}H_{10}O_4$	7.42	Simple phenols	0.18	287.06
84	Gingerone	$C_{11}H_{14}O_3$	7.42	Simple phenols	0.67	239.09
85	Nobilone	$C_{14}H_{10}O_4$	7.45	Simple phenols	0.96	287.06
86	Homoarbutin	$C_{13}H_{18}O_7$	7.49	Glucoside	0.09	331.10
87	Apocynin B	$C_{24}H_{20}O_{10}$	7.50	Simple phenol	0.13	513.11
88	Dihydroeugenol	$C_{10}H_{14}O_2$	7.55	Simple phenol	2.42	211.10
89	3,4-Dimethoxyphenol	$C_8H_{10}O_3$	7.56	Simple phenol	0.33	153.06
90	Kakuol	$C_{10}H_{10}O_4$	7.56	Prophiophenone derivative	0.35	239.06
91	Cimidahurine	$C_{14}H_{20}O_8$	7.56	Phenylpropanoid glycoside	1.39	315.11
92	Echinacoside	$C_{35}H_{46}O_{20}$	7.56	Glucoside	1.60	831.26
93	Sinapaldehyde	$C_{11}H_{12}O_4$	7.57	Lignin	3.56	253.07
94	3,4-Dimethoxyphenol	$C_8H_{10}O_3$	7.57	Simple phenol	0.12	153.06
95	Eugenol	$C_{10}H_{12}O_2$	7.57	Simple phenol	0.07	209.08
96	Brazilin	$C_{16}H_{14}O_5$	7.60	Pigment phenol	1.19	285.08
97	Tachioside	$C_{13}H_{18}O_8$	7.64	Phenolic glycoside	0.07	301.09
98	2-Hydroxy-4-methoxybenzaldehyde	$C_8H_8O_3$	7.70	Simple phenol	0.34	151.04
99	Ellagic acid	$C_{14}H_6O_8$	7.72	Phenolic acid	0.08	300.99
100	Aspidinol	$C_{12}H_{16}O_4$	7.81	Simple phenols	0.09	269.10
101	Moracin F	$C_{16}H_{14}O_5$	7.83	Flavanoid	0.22	285.08
102	Tachioside	$C_{13}H_{18}O_8$	7.84	Phenolic glycoside	0.19	301.09
103	Phenol	C_6H_6O	7.84	Simple phenols	0.16	139.04
104	Renifolin	$C_{18}H_{24}O_7$	8.02	Glucoside	0.25	397.15
105	Nobilone	$C_{14}H_{10}O_4$	8.12	Simple phenols	0.22	287.06
106	Protosappanin C	$C_{16}H_{14}O_6$	8.16	Dibenzoxocin derivative	0.09	301.07
107	3,4-Dihydroxybenzamide	$C_7H_7NO_3$	8.18	Amide phenol	0.09	152.04
108	3,4-Dihydroxybenzamide	$C_7H_7NO_3$	8.18	Amide phenol	0.09	152.04
109	(±)-Isoduartin	$C_{18}H_{20}O_6$	8.24	Isoflavan	0.26	377.12
110	Renifolin	$C_{18}H_{24}O_7$	8.31	Glucoside	0.10	397.15
111	Aspidinol	$C_{12}H_{16}O_4$	8.42	Simple phenols	0.21	269.10
112	Eugenol	$C_{10}H_{12}O_2$	8.47	Simple phenols	0.12	209.08
113	Gingerone	$C_{11}H_{14}O_3$	8.53	Simple phenols	3.48	239.09
114	Obovatol	$C_{18}H_{18}O_3$	8.55	Biphenolic	0.07	327.12
115	2′-Hydroxy-7,3′,4′-trimethoxy-isoflavan	$C_{18}H_{20}O_5$	8.55	Isoflavan	0.16	315.12
116	Arbutin	$C_{12}H_{16}O_7$	8.60	Glucoside	0.11	317.09
117	Moracin F	$C_{16}H_{14}O_5$	8.62	2-arylbenzofuran flavanoid	0.19	285.08
118	Smilaxin	$C_{17}H_{16}O_6$	8.63	Streoid glycoside	0.09	361.09
119	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	8.64	Glucoside	0.52	477.14
120	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	8.64	Phenanthrene glucoside	0.16	477.14
121	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	8.72	Phenanthrene phenol	2.96	273.08
122	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	9.02	Simple phenol	0.24	267.09
123	Torachrysone-8-O-β-Dglucopyranoside	$C_{20}H_{24}O_9$	9.04	Glucoside	0.12	407.14
124	Brazilein	$C_{16}H_{12}O_5$	9.08	Simple phenols	0.10	
125	Moracin F	$C_{16}H_{14}O_5$	9.09	2-arylbenzofuran flavanoid	0.16	285.08
126	Aspidinol	$C_{12}H_{16}O_4$	9.15	Simple phenols	0.68	269.10

127	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	9.24	Simple phenols	0.10	267.09
128	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	9.26	Simple phenols	0.07	267.09
129	Polydatin	$C_{20}H_{22}O_8$	9.42	Glycoside	0.12	435.13
130	Echinacoside	$C_{35}H_{46}O_{20}$	9.42	Phenyl propanoid glucoside	1.57	831.26
131	Sinapaldehyde	$C_{11}H_{12}O_4$	9.42	Lignin intermediate	1.80	253.07
132	3,4-Dimethoxyphenol	$C_8H_{10}O_3$	9.43	Simple phenol	0.06	153.06
133	Cimidahurine	$C_{14}H_{20}O_8$	9.43	Phenylpropanoid glycoside	0.36	315.11
134	±)-Isoduartin	$C_{18}H_{20}O_6$	9.43	Isoflavan	1.38	377.12
135	Nobilone	$C_{14}H_{10}O_4$	9.51	Simple phenols	0.11	287.06
136	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	9.53	Simple phenol	0.12	267.09
137	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	9.54	Simple phenol	0.60	267.09
138	Kuzubutenolide A	$C_{23}H_{24}O_{10}$	9.64	Glucoside	0.10	459.13
139	Moracin C	$C_{19}H_{18}O_4$	9.65	Glucoside	0.26	355.12
140	Albaspidin AA	$C_{21}H_{24}O_8$	9.76	Phloroglucinol derivative	0.24	449.15
141	Renifolin	$C_{18}H_{24}O_7$	9.81	Glucoside	0.16	397.15
142	Aspidinol	$C_{12}H_{16}O_4$	9.86	Simple phenols	1.23	269.10
143	Gingerone	$C_{11}H_{14}O_3$	9.87	Simple phenols	0.70	239.09
144	Nobilin B	$C_{17}H_{20}O_6$	10.07	Prenol lipid	0.11	319.12
145	Torachrysone-8-O-β-Dglucopyranoside	$C_{20}H_{24}O_9$	10.10	Glucoside	0.12	407.14
146	Nobilin C	$C_{18}H_{22}O_6$	10.10	Prenol lipid	0.16	379.14
147	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	10.14	Gallotannin	0.09	331.07
148	Asebotin	$C_{22}H_{26}O_{10}$	10.15	Dihydrochalcone glucoside	0.24	449.15
149	Renifolin	$C_{18}H_{24}O_7$	10.20	Glucoside	0.21	397.15
150	3,4-O-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	10.33	Phenolic acid	0.09	561.13
151	2-((3R,4R)-7-Hydroxy-4-(4-hydroxy-5-((R)-7-hydroxychroman-3-yl)-2-methoxyphenyl) chroman3-yl)-5-methoxycyclohexa 2,5-di-ene-1,4-dione	$C_{32}H_{28}O_9$	10.36	Simple phenol	0.09	555.17
152	Asebotin	$C_{22}H_{26}O_{10}$	10.49	Dihydrochalcone glucoside	0.09	449.15
153	Sinapaldehyde	$C_{11}H_{12}O_4$	10.59	Lignin intermediate	0.43	253.07
154	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	10.59	Phenanthrene phenol	2.99	273.08
155	p-Tolualdehyde	$C_7H_6O_2$	10.59	Benzenoid	0.07	167.04
156	Ciwujiatone	$C_{22}H_{26}O_9$	10.66	Lignan	0.13	433.15
157	Cearoin	$C_{14}H_{12}O_4$	10.66	Simple phenol	0.29	289.07
158	Albaspidin AA	$C_{21}H_{24}O_8$	10.67	Phloroglucinol derivative	0.11	403.14
159	Torachrysone-8-O-β-Dglucopyranoside	$C_{20}H_{24}O_9$	10.75	Glucoside	0.54	407.14
160	Ciwujiatone	$C_{22}H_{26}O_9$	10.81	Lignan	0.10	433.15
161	Cassialactone	$C_{16}H_{16}O_6$	10.84	Simple phenol	0.08	349.09
162	Renifolin	$C_{18}H_{24}O_7$	10.90	Glucoside	0.34	397.15
163	4-(4'-Hydroxy-3',5'-dimethoxyphenyl)-3-buten-2-one	$C_{12}H_{14}O_4$	10.91	Simple phenol	0.12	267.09
164	Sinapaldehyde	$C_{11}H_{12}O_4$	10.97	Lignin intermediate	0.64	253.07
165	4,4′-Dihydroxy-3,5-dimethoxybibenzyl	$C_{16}H_{18}O_4$	11.03		0.28	319.12
166	Protosappanin A	$C_{15}H_{12}O_5$	11.07	Dibenzoxocin derivative	0.34	271.06
167	Aspidinol	$C_{12}H_{16}O_4$	11.13	Simple phenols	0.33	223.10
168	Thannilignan	$C_{19}H_{22}O_5$	11.15	Lignan	0.34	329.14
169	Sinapaldehyde	$C_{11}H_{12}O_4$	11.17	Lignin intermediate	0.64	253.07
170	Cistanoside H	$C_{22}H_{32}O_{13}$	11.26	Glucoside	0.20	503.18
171	Renifolin	$C_{18}H_{24}O_7$	11.30	Glucoside	0.15	397.15

172	Protosappanin A	$C_{15}H_{12}O_5$	11.43	Dibenzoxocin derivative	1.18	271.06
173	2,3,5,4'-Tetrahydroxystilbene-2-O-(6"-O-α-glucopyranosyl)-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	11.53	Glucoside	0.17	567.17
174	Dendrocandin C	$C_{16}H_{18}O_5$	11.55	Bibenzyl phenols	0.38	289.11
175	3,4-Dihydroxybenzamide	$C_7H_7NO_3$	11.57	8380	0.27	152.04
176	Thannilignan	$C_{19}H_{22}O_5$	11.78	Lignan	0.18	375.15
177	Cistanoside H	$C_{22}H_{32}O_{13}$	11.80	Glucoside	0.08	503.18
178	p-Tolualdehyde	$C_7H_6O_2$	11.88	Benzenoid	0.57	167.04
179	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	11.90	Phenanthrene phenol	14.36	273.08
180	Tachioside	$C_{13}H_{18}O_8$	11.90	Phenolic glycoside	0.21	301.09
181	Eugenol	$C_{10}H_{12}O_2$	11.96	Simple phenol	0.17	209.08
182	3,4-Dimethoxyphenol	$C_8H_{10}O_3$	11.97	Simple phenol	0.45	153.06
183	Sinapaldehyde	$C_{11}H_{12}O_4$	11.97	Lignin intermediate	0.72	253.07
184	2,3,5,4'-Tetrahydroxystilbene-2-O-(6"-O-α-Dglucopyranosyl)-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	12.04	Glucoside	0.12	567.17
185	Phenol	C_6H_6O	12.09	Simple phenols	1.15	139.04
186	10-OMethyl protosappanin B	$C_{17}H_{18}O_6$	12.16	Dibenzoxocin derivative	0.08	363.11
187	Phenol	C_6H_6O	12.36	Simple phenols	0.09	139.04
188	1,4-Dihydroxy-2-methoxybenzene	$C_7H_8O_3$	12.37	Benzenoid	1.10	139.04
189	tran-Ferulaldehyde	$C_{10}H_{10}O_3$	12.37	Aldehyde	0.10	223.06
190	(3R)-3′,8-Dihydroxyvestitol	$C_{16}H_{16}O_6$	12.51	Isoflavane	0.15	349.09
191	Caesalpins J	$C_{17}H_{16}O_6$	12.59	Simple phenol	0.08	361.09
192	Polydatin	$C_{20}H_{22}O_8$	12.67	Glucoside	0.08	435.13
193	2,3,5,4'-Tetrahydroxystilbene-2,3-O-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	12.80	Glucoside	0.16	567.17
194	Aspidinol	$C_{12}H_{16}O_4$	12.88	Simple phenol	0.12	223.10
195	Aspidinol	$C_{12}H_{16}O_4$	12.88	Simple phenol	0.35	223.10
196	Protosappanin A	$C_{15}H_{12}O_5$	13.10	Dibenzoxocin derivative	0.35	271.06
197	Darendoside A	$C_{19}H_{28}O_{11}$	13.15	Phenethyl alcohol glycosides	0.41	431.16
198	Moracin M-3′-O-β-Dglucopyranoside	$C_{20}H_{20}O_9$	13.38	Glucoside	0.12	403.10
199	Dendrocandin C	$C_{16}H_{18}O_5$	13.43	Bibenzyl phenols	0.09	289.11
200	7,2′,3′-Trihydroxy-4′-methoxy-isoflavan	$C_{16}H_{16}O_5$	14.14	Isoflavane	0.28	287.09
201	2,3,5,4'-Tetrahydroxystilbene-2-O-(6"-O-α-Dglucopyranosyl)-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	14.27	Glucoside	0.10	567.17
202	Dendrocandin C	$C_{16}H_{18}O_5$	14.59	Bibenzyl phenols	0.10	335.11
203	Aspidinol	$C_{12}H_{16}O_4$	15.01	Simple phenol	1.05	223.10
204	Moracin M-3′-O-β-Dglucopyranoside	$C_{20}H_{20}O_9$	16.84	Glucoside	0.08	403.10
205	Dendrocandin E	$C_{15}H_{16}O_5$	16.87	Bibenzyl phenols	0.13	321.10
206	Xanthohumol	$C_{21}H_{22}O_5$	16.91	Chalcone	0.08	399.15
207	2,7-Dihydroxy-4-methoxyphenanthrene-2-O-glucoside	$C_{21}H_{22}O_8$	16.91	Phenanthrene phenol	0.15	447.13
208	Kuzubutenolide A	$C_{23}H_{24}O_{10}$	17.26	Glucoside	0.07	505.13
209	Cyclocurcumin	$C_{21}H_{20}O_6$	17.26	Diarylheptanoid	0.10	413.12
210	Dendrocandin E	$C_{15}H_{16}O_5$	17.26	Bibenzyl phenols	0.15	321.10
211	Nobilin A	$C_{17}H_{20}O_5$	17.26	Prenol lipid	0.09	349.13
212	Syringylethanone	$C_{10}H_{12}O_4$	17.26	Lignin	0.10	241.07
213	Dendrocandin C	$C_{16}H_{18}O_5$	17.36	Bibenzyl phenols	0.07	335.11
214	Polydatin	$C_{20}H_{22}O_8$	17.56	Glycoside	0.08	435.13
215	Moracin O	$C_{19}H_{18}O_5$	17.89	Glucoside	0.17	371.11
216	Gigantol	$C_{15}H_{16}O_4$	17.95	Bibenzyl phenols	0.07	305.10

Note. RT: Retention Time, [M^+]: Molecular ion mass (m/z)

Table 5: Phytochemical compounds in F1ASP using LC-MS.

No.	Compound	Molecular Formula	RT (min)	Chemical Classes	Response %	[M+]
1	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.44	Gallotannin	2.25	331.07
2	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.74	Gallotannin	0.38	331.07
3	Pyrogallic acid	$C_6H_6O_3$	0.80	Phenolic acid	0.49	
4	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.01	Gallotannin	0.99	331.07
5	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.27	Gallotannin	2.46	331.07
6	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.58	Gallotannin	6.90	331.07
7	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	2.22	Gallotannin	0.49	331.07
8	2,4,5-Trihydeoxybenzaldehyde	$C_7H_6O_4$	2.34	Benzaldehyde	0.43	153.02
9	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	3.03	Gallotannin	0.70	483.08
10	Polydatin	$C_{20}H_{22}O_8$	3.53	Glucoside	0.73	435.13
11	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	3.77	Gallotannin	1.64	483.08
12	Polydatin	$C_{20}H_{22}O_8$	3.84	Glucoside	2.57	435.13
13	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	4.22	Gallotannin	0.63	483.08
14	2,3,5,4'-Tetrahydroxystilbene-2, 3-O-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	4.32	Glucoside	0.53	568.17
15	Sesamol	$C_7H_6O_3$	4.34	Hydroquinone derivative	2.75	183.03
16	2,3,5,4'-Tetrahydroxystilbene-2, 3-O-β-Dglucopyranoside	$C_{26}H_{32}O_{14}$	4.44	Glucoside	0.38	567.17
17	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	4.65	Gallotannin	0.86	483.08
18	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	4.88	Gallotannin	2.63	483.08
19	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	4.95	Gallotannin	3.15	483.08
20	2,6-Di-O-galloyl-β-Dglucose	$C_{20}H_{20}O_{14}$	5.16	Gallotannin	0.50	483.08
21	6′-O-Galloylhomoarbutin	$C_{20}H_{22}O_{11}$	5.19	Galloglucoside	0.52	483.08
22	Darendoside A	$C_{19}H_{28}O_{11}$	5.48	Phenethyl alcohol glucosides	0.39	431.16
23	Aspidinol	$C_{12}H_{16}O_4$	5.76	Simple phenols	0.78	269.10
24	Feroxin A	$C_{17}H_{24}O_8$	5.76	3-O Glucoside	8.44	401.14
25	Feroxin A	$C_{17}H_{24}O_8$	5.76	3-O Glucoside	0.44	401.14
26	Moracin M-3′-O-β-Dglucopyranoside	$C_{20}H_{20}O_9$	5.82	Glucoside	0.77	449.11
27	Meliadanoside B	$C_{15}H_{20}O_8$	5.87	Glucoside	1.86	373.11
28	1,3,6-Trigalloyl-β-Dglucose	$C_{27}H_{24}O_{18}$	6.38	Gallotannin	2.99	635.09
29	Feroxidin	$C_{11}H_{14}O_3$	6.99	Simple phenol	1.98	329.14
30	Thannilignan	$C_{19}H_{22}O_5$	8.54	Lignan	0.47	
31	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	8.54	Phenantherene glucoside	0.62	477.14
32	Thannilignan	$C_{19}H_{22}O_5$	8.68	Lignan	0.38	329.14
33	Kuzubutenolide A	$C_{23}H_{24}O_{10}$	8.82	Glucoside	1.88	459.13
34	Smilaxin	$C_{17}H_{16}O_6$	9.09	Steroid glycoside	0.50	315.09
35	Polydatin	$C_{20}H_{22}O_8$	9.09	Glycoside	0.52	435.13
36	Renifolin	$C_{18}H_{24}O_7$	9.27	Glucoside	0.44	397.15
37	Protosappanin A	$C_{15}H_{12}O_5$	9.45	Dibenzoxocin derivative	0.37	271.06
38	1-O-Methyl-3,5-Odicaffeoylquinic acid methyl ester	$C_{27}H_{28}O_{12}$	9.67	Phenolic acid	3.35	543.15
39	Polydatin	$C_{20}H_{22}O_9$	9.76	Glycoside	0.79	435.13
40	Feroxidin	$C_{11}H_{14}O_3$	9.78	Simple phenol	2.06	
41	2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucopyranoside	$C_{20}H_{22}O_8$	9.94	Glucoside	0.37	405.12
42	1-O-Methyl-3,5-Odicaffeoylquinic acid methyl ester	$C_{27}H_{28}O_{12}$	9.96	Phenolic acid	0.47	543.15
43	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	10.20	Phenanthrene phenol	0.87	273.08
44	Polydatin	$C_{20}H_{22}O_8$	10.20	Glycoside	0.47	435.13

45	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	10.23	Phenanthrene phenol	2.38	273.08
46	Asebotin	$C_{22}H_{26}O_{10}$	10.25	Dihydrochalcone glucoside	0.38	449.15
47	Renifolin	$C_{18}H_{24}O_7$	10.79	Glucoside	0.42	397.15
48	Asebotin	$C_{22}H_{26}O_{10}$	10.92	Dihydrochalcone glucoside	0.57	449.15
49	Asebotin	$C_{22}H_{26}O_{10}$	11.33	Dihydrochalcone glucoside	0.56	449.15
50	Dendrocandin C	$C_{16}H_{18}O_5$	11.47	Bibenzyl phenols	0.59	289.11
51	(R)-Prechrysophanol	$C_{15}H_{14}O_4$	11.66	Preanthraquinone	1.40	303.09
52	Erigoster A	$C_{27}H_{26}O_{13}$	11.74	Caffeoyl conjugate derivative	0.38	557.03
53	7,2′,3′-Trihydroxy-4′-methoxy-isoflavan	$C_{16}H_{16}O_5$	12.22	Isoflavane	1.73	287.09
54	Phenol	C_6H_6O	12.28	Simple phenol	0.66	139.04
55	4-Hydroxyacetophenone	$C_8H_8O_2$	12.75	Simple phenol	0.38	181.05
56	Aspidinol	$C_{12}H_{16}O_4$	12.77	Simple phenols	0.79	223.10
57	Aspidinol	$C_{12}H_{16}O_4$	12.77	Simple phenols	1.66	223.10
58	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	12.77	Glucoside	0.44	431.13
59	Protosappanin A	$C_{15}H_{12}O_5$	12.94	Dibenzoxocin derivative	0.85	271.06
60	3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	$C_{22}H_{24}O_9$	12.98	Phenanthrene glucoside	1.19	431.13
61	Albaspidin AA	$C_{21}H_{24}O_8$	13.32	Phloroglucinol derivative	0.62	449.15
62	p-Tolualdehyde	$C_7H_6O_2$	13.44	Benzenoid	1.39	167.04
63	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	$C_{14}H_{12}O_3$	13.45	Phenanthrene phenol	7.79	273.08
64	2,7-Dihydroxy-4-methoxyphenanthrene-2-O-glucoside	$C_{21}H_{22}O_8$	13.86	Phenanthrene phenol	0.73	401.12
65	Methyl-β-orsellinate	$C_9H_{10}O_4$	14.91	Ester phenol	0.59	181.05
66	(3R)-3′,8-Dihydroxyvestitol	$C_{16}H_{16}O_6$	14.91	Isoflavane	0.74	303.09
67	Flavanthrinin	$C_{15}H_{12}O_3$	15.06	Phenanthrenoid	1.48	285.08
68	Haematoxylin	$C_{16}H_{14}O_6$	15.29	Phenocyanin	0.59	347.08
69	Cyclocurcumin	$C_{21}H_{20}O_6$	15.81	Diarylheptanoid	0.38	413.12
70	Moracin O	$C_{19}H_{18}O_5$	15.82	Glucoside	1.00	371.11
71	Methyl-β-orsellinate	$C_9H_{10}O_4$	16.45	Ester phenol	0.68	181.05
72	7,2′,3′-Trihydroxy-4′-methoxy-isoflavan	$C_{16}H_{16}O_5$	16.45	Isoflavane	2.64	287.09
73	7,2′,3′-Trihydroxy-4′-methoxy-isoflavan	$C_{16}H_{16}O_5$	16.45	Isoflavane	1.70	287.09
74	Renifolin	$C_{18}H_{24}O_7$	16.56	Glucoside	0.96	351.15
75	Thannilignan	$C_{19}H_{22}O_5$	16.56	Lignan	0.51	375.14
76	Polydatin	$C_{20}H_{22}O_8$	16.63	Glucoside	0.53	435.13

Note. RT: Retention Time, [M⁺]: Molecular ion mass (m/z)

Table 6: Phytochemical compounds in F2ASP using LC-MS.

No.	Compound	Molecular Formula	RT (min)	Chemical Classes	Responses %	[M⁺]
1	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.43	Gallotannin	8.34	331.07
2	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.80	Gallotannin	4.44	331.07
3	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.06	Gallotannin	6.27	331.07
4	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.33	Gallotannin	20.24	331.07
5	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.57	Gallotannin	17.05	331.07
6	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	2.21	Gallotannin	2.41	331.07
7	Sesamol	$C_7H_6O_3$	4.37	Hydroquinone derivative	7.73	183.03
8	Feroxin A	$C_{17}H_{24}O_8$	5.56	3-O Glucoside	5.60	401.14
9	Feroxin A	$C_{17}H_{24}O_8$	5.75	3-O Glucoside	11.84	401.14
10	Sinapaldehyde	$C_{11}H_{12}O_4$	7.03	Lignin intermediate	4.90	253.07
11	Sinapaldehyde	$C_{11}H_{12}O_4$	9.00	Lignin intermediate	3.49	253.07
12	7,2′,3′-Trihydroxy-4′-methoxy-isoflavan	$C_{16}H_{16}O_5$	12.48	Isoflavane	5.37	287.09
13	Kukoamine A	$C_{28}H_{42}N_4O_6$	16.97	Benzenoid	2.32	529.30

Note. RT: Retention Time, [M⁺]: Molecular ion mass (m/z)

intensity compounds were feroxin A (11.84 %) and sesamol (7.73 %). Apart from that, the compound with the lowest intensity in F2ASP was kukoamine A (2.32 %), eluted at 16.97 min.

F3ASP eluted the minimum number of compounds as compared to ASP, F1ASP, and F3ASP. Only 5 peaks eluted out including some that existed in redundant as can be seen in Figure 10 while Table 7 listed all the eluted compounds. To be exact, only 3 compounds were identified in F3ASP with negative mode ionization of LC-MS. Only the phenolic groups of gallotannin and simple phenols were identified in this fraction. Again, as in F2ASP, 1-galloyl-glucose was observed with the highest intensity of 34.45 % at 4.14 min. Another highest intensity compound was polydatin (30.51 %). The compound with the lowest intensity was feroxin A, by 16.39 %.

Table 8 summarizes the phytochemical compounds related to antihypertensive activity which were present in the ASP crude extract and the three derived fractions (F1ASP, F2ASP, and F3ASP). The possible phenolic compounds that have potential in contributing to the antihypertensive effect by *S. polyanthum* are 1-galloyl glucose, polydatin, sesamol, brazilin, eugenol, ellagic acid, kukoamine A and cyclocurcumin. 1-galloyl glucose or glucogallin is a compound that is present across all fractions as well as in the crude ASP extract. In fact, it becomes the major compound in F2ASP and F3ASP, whereby the concentration of this compound intensified by 30 times as compared to its original crude extract. Previously, this compound was shown to inhibit the angiotensin-converting enzyme I (ACE-I) activity by the formation of chelate complexes within the active site of ACE-I.[47] Inhibition of this enzyme indicates huge potential in reducing blood pressure and this is actually the mechanism of action of captopril, the positive control drug used in this study. Polydatin is a major compound found in F3ASP, while it is also present in smaller amounts in ASP and F1ASP. Polydatin, a glucoside of resveratrol can upregulate the level of nitric oxide (NO) and it also decreases the levels of endothelin (ET) and angiotensin II and thus depresses blood pressure in pressure-overload rats.[48] Sesamol which was found in ASP crude extract, F1ASP, and F2ASP was found to exhibit an antihypertensive effect in uninephrectomized deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats at a specific dosage of 50 mg/kg.[49] Other than sesamol, brazilin which was found only in ASP crude extract was previously reported to induce vasorelaxation in rat aortic rings through both endothelium-dependent and independent pathway[50] by activating calcium-dependent nitric oxide synthesis.[51] Vasorelaxation is one of the main mechanisms of actions that may result in an antihypertensive effect.

Not only these, eugenol which was also found in ASP crude extract was previously reported to relax mesenteric arteries, and thus reducing systemic blood pressure by activating endothelial cell TRPV4 channels.[52] It was also reported to have significant inhibition on ACE activity by 28 % in the serum of untreated diabetic rats.[53] Ellagic acid, another phenolic acid compound found in ASP crude extract was

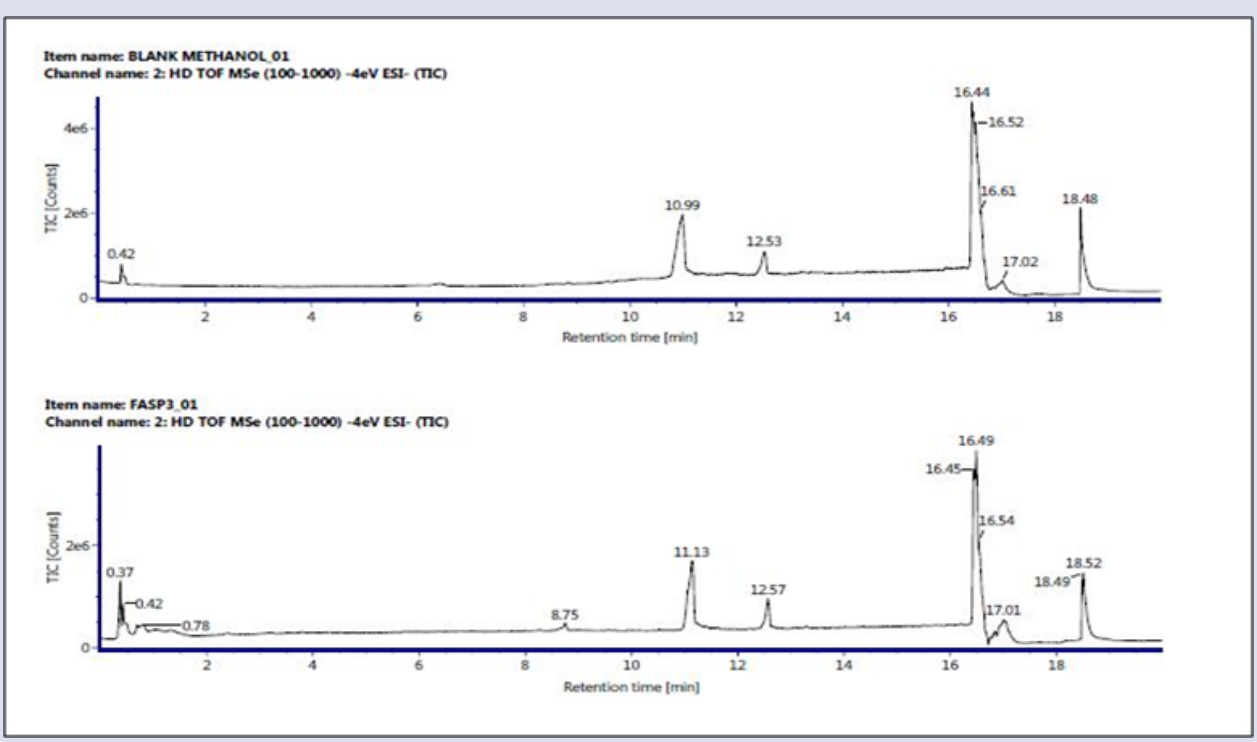


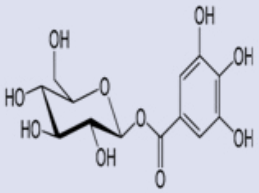
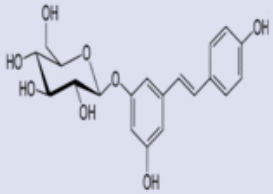
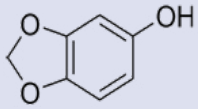
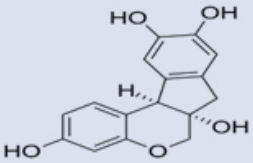
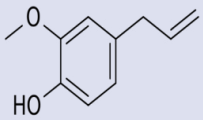
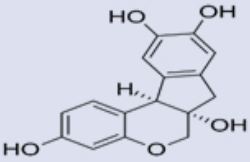
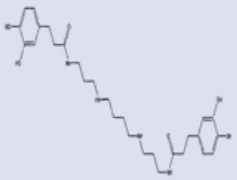
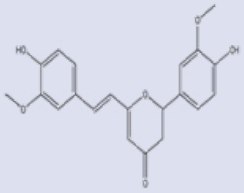
Figure 10: Chromatogram of blank methanol and F3ASP.

Table 7: Phytochemical compounds in F3ASP using LC-MS.

No.	Compound	Molecular Formula	RT (min)	Chemical Classes	Response %	[M⁺]
1	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.43	Gallotannin	9.37	331.07
2	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	0.80	Gallotannin	9.28	331.07
3	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	1.43	Gallotannin	34.45	331.07
4	Polydatin	$C_{20}H_{22}O_8$	4.14	Glucoside	30.51	435.13
5	Feroxin A	$C_{17}H_{24}O_8$	5.74	3-O Glucoside	16.39	401.14

Note. RT: Retention Time, [M⁺]: Molecular ion mass (m/z)

Table 8. Bioactive phenolic compounds in the crude aqueous extract of *S. polyanthum* leaves and its derived fractions with previous reported activities related to antihypertensive effect.

Compound	Chemical Structure	Highest intensity (%)			
		ASP	F1ASP	F2ASP	F3ASP
1-galloyl-glucose		0.67 %	6.90 %	20.24 % (major compound)	34.35 % (major compound)
Polydatin		0.29 %	2.57 %	-	30.51 % (major compound)
Sesamol		0.24 %	2.75 %	7.73 %	-
Brazilin		1.19 %	-	-	-
Eugenol		0.17 %	-	-	-
Ellagic acid		0.08 %	-	-	-
Kukoamine A		-	-	2.32 %	-
Cyclo-curcumin		0.10 %	0.38 %	-	-

able to attenuate β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p47phox expression which is responsible for increased vascular oxygen radical, and this can eventually prevent any oxidative stress and reinstate nitric oxide bioavailability.[54] Nitric oxide is an important endothelium-derived relaxing factor that might cause vasorelaxation, reducing the total peripheral resistance, and this might have contributed to the antihypertensive effect. Furthermore, kukoamine A which was found only in F2ASP was shown to induce hypotension in rats at a dose of 5 mg/kg when administered intravenously.[55] Other than that, cyclocurcumin that was found in ASP crude extract and F1ASP in the current study, were previously shown to significantly inhibit the contraction of the vascular muscle of isolated rat aorta ring.[56]

CONCLUSION

This study found 1-galloyl glucose as the major compound with several other phenolic compounds such as polydatin, sesamol, brazilin, eugenol, ellagic acid, kukoamine A, and cyclocurcumin in the active antihypertensive crude extract and fractions of *S. polyanthum* leaves. These phenolic compounds have proven biological activities related to the antihypertensive effect, thus, they may be in part, responsible for the antihypertensive effect by *S. polyanthum* leaves and thus further isolation is recommended.

ACKNOWLEDGEMENT

This research is funded by the Ministry of Higher Education, Malaysia with grant number FRGS/1/2018/SKK10/UIAM/02/1. The authors would like to acknowledge the staff at Natural Product Laboratory, Kulliyyah of Science, International Islamic University Malaysia, and the staff from Biomedicine Unit, School of Health Sciences and Animal Research and Service Centre, Universiti Sains Malaysia for providing technical support in carrying out this experiment.

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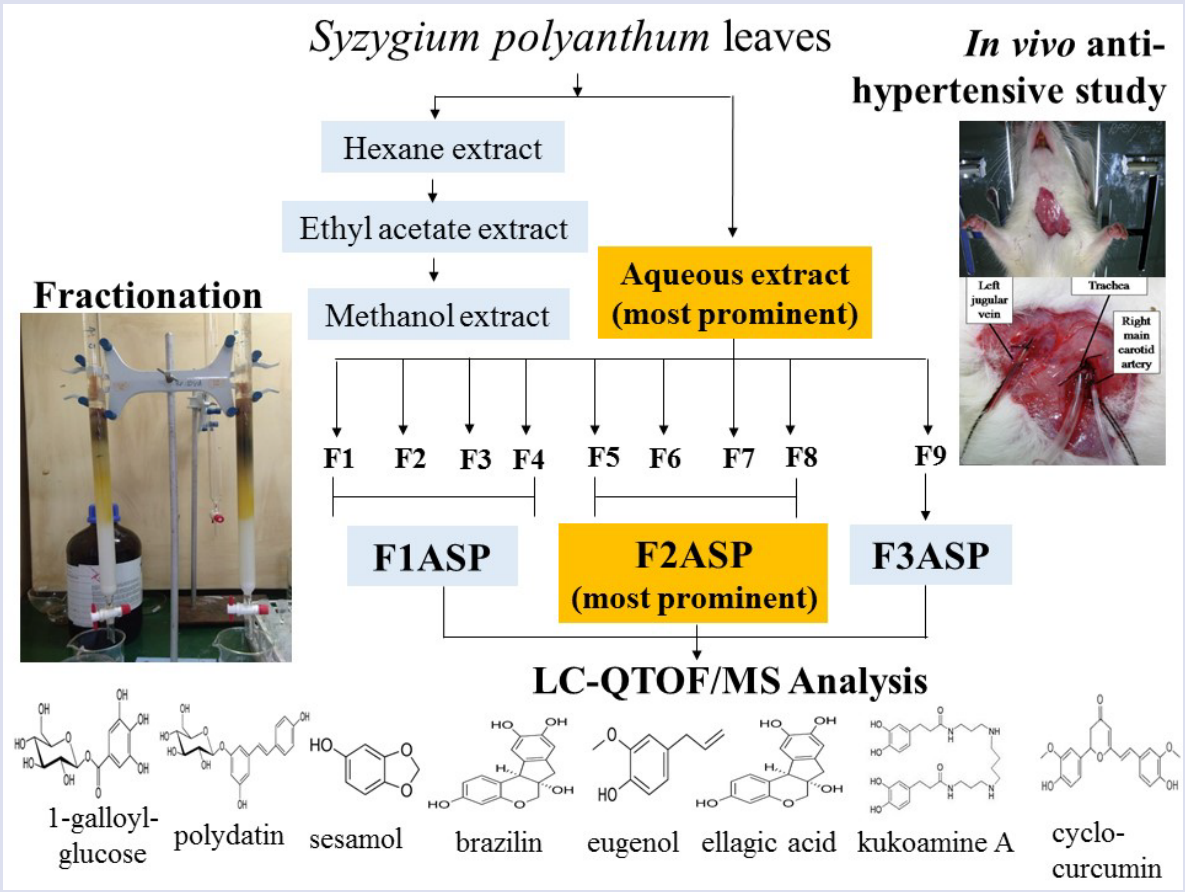
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Azlini Ismail: Azlini Ismail is an Assistant Professor (senior lecturer) at Department of Fundamental Dental and Medical Sciences, Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM). Her research interests include cardiovascular pharmacology, pharmacognosy, natural product, and traditional medicine.

Erlena Nor Asmira Abdul Rahim: Erlena Nor Asmira is an MSc Biotechnology (IIUM) and Bsc Chemistry (UiTM Pahang) holder currently working as Sales Executive cum. Biotechnologist at Asia Botanicals Sdn Bhd. Her current work is related with food supplement, natural products and traditional medicine.

Muhammad Nor Omar: Muhammad Nor Omar is a professor at Department of Biotechnology, Kulliyyah (Faculty) of Science, International Islamic University Malaysia (IIUM). He focus his research in the area of natural products besides developing products from palm oil oleochemicals.

Wan Amir Nizam Wan Ahmad: Wan Amir Nizam Wan Ahmad is currently a Senior Lecturer at School of Health Sciences, Universiti Sains Malaysia. His research interest include natural product pharmacology, cardiovascular disease and animal model of disease.

Cite this article: Ismail A, Rahim ENAA, Omar MN, Wan Ahmad WAN. Antihypertensive Assay-Guided Fractionation of *Syzygium polyanthum* Leaves and Phenolics Profile Analysis Using LC-QTOF/MS. Pharmacogn J. 2020;12(6)Suppl:1670-92.