

Original Article

BIOLOGICAL ACTIVITIES AND CHARACTERIZATION OF THE POD EXTRACTS FROM SOMPOI
(*ACACIA CONCINNA* LINN) GROWN IN NORTHERN THAILAND

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ABSTRACT

Objective: Sompoi or Shikakai (*Acacia concinna* Linn.) pod extracts were produced and investigated for antioxidant and antityrosinase activities together with the determination of their total phenolic and flavonoid content. In addition, phytochemical screening, cytotoxic effect and HPLC-fingerprint of the extract were also performed.

Methods: The pods of *A. concinna* were extracted using three different solvents including 95%ethanol, 50%ethanol and deionized water and named as ethanolic extract (ET), freeze-dried hydroethanolic extract (HEF), Spray-dried hydroethanolic extract (HES), freeze-dried aqueous extract (HF), and spray-dried aqueous extract (HS). Antioxidant activity of the extracts were determined by three different assays including 1-Diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)-radical scavenging assay and linoleic acid peroxidation assay. Total phenolic content in each extract was evaluated using folin-ciocalteu spectrophotometric assay and the total flavonoid content was also determined using aluminium chloride colorimetric assay. Cytotoxic effect on human peripheral blood mononuclear cells (PBMCs) was investigated using MTT assay. HPLC-fingerprint of the selected extract was finally analysed.

Results: HES showed the greatest radical scavenging activity on DPPH and ABTS assays with IC_{50} of 0.8280 ± 0.0131 mg/ml and 0.1418 ± 0.0012 mg/ml, respectively. Moreover, it showed the highest inhibition on lipid peroxidation with IC_{50} of 1.8751 ± 0.0307 mg/ml. Among five extracts, HES contained the highest amount of gallic acid and rutin expressed as gallic acid equivalent value (GAE) of 100.51 ± 0.06 mg/g and rutin equivalent value (RE) of 14.67 ± 0.01 mg/g. Therefore, its highest antioxidant activity was due to its highest phenolic and flavonoid content. The %viability of HES-treated PBMCs was comparable to ascorbic acid at each individual concentration. The HPLC-fingerprint of HES, detected at the wavelength of 280 nm, showed one major peak at 9.88 min that can be used as a marker of the HES.

Conclusions: *A. concinna* pod extracts showed good *in vitro* antioxidant and antityrosinase activities with moderate total phenolic and flavonoid content. HES showed the greatest activity with safety profile comparable to ascorbic acid. It might be a promising approach for application in the cosmetic or cosmeceutical products.

Keywords: Shikakai, Sompoi, Antioxidant activity, Antityrosinase activity, Cytotoxic effect, HPLC-fingerprint, Chemical screening, MTT assay.

INTRODUCTION

Aging problem has been of great interested since the ancient time. It is the phenomenon of cellular degradation overtakes that leads to many degenerative diseases such as cardiovascular disease, neurodegenerative disease and even skin aging [1]. Skin aging is a complex process influenced by several factors including hormonal changes, environmental exposure (smoking, pollution, UV irradiation) and genetics. Skin aging can be divided into two types including intrinsic and extrinsic. Intrinsic skin aging is a skin aging type which is influenced by metabolic processes and hormones [2] whereas; extrinsic skin aging is related to the exposure of UV [2, 3]. Photo-aging is the most common cause of facial skin aging. Clinical signs of photo-aging include loss of elasticity, deep wrinkle, roughness, dryness and irregular pigmentation [3]. Moreover, UV-exposure leads to generation of reactive oxygen species (ROS). The imbalance of ROS and antioxidant processes within the cells cause oxidative stress that can destroy cell components including lipid, protein and DNA which leads to skin aging [1, 4]. UV-irradiation also increases matrix metalloproteinase enzymes (MMPs) in the skin cells which is mainly collagenase that can decrease the amount of collagen in the dermis [2, 5]. Tyrosinase, a rate-limiting enzyme that catalyse melanin synthesis in melanocytes, is also increased by UV-exposure. Topical treatment of skin aging including antioxidant substances have been agreed to scavenge ROS and stimulate self-repair [4]. Application of antityrosinase substances may inhibit melanogenesis in the skin cells leading to the repair of irregular skin pigmentation [6].

Nowadays, herbal extracts in cosmetic industry are growing of great interested because of their efficacy and safety. Since the ancient time, herbal extracts have been used in anti-aging cosmetic

formulations [5]. Various phytochemical constituents in the herbal extracts include carotenoids, flavonoids and phenolics that generated antioxidant and antityrosinase activities [4].

Acacia concinna Linn. (Sompoi or Shikakai) belonging to the Leguminosae family [7], is a medicinal plant widely grown in Southern Asia including India, Myanmar and Thailand [8]. In the North of Thailand, the pods of Sompoi were used as one ingredient of Thai traditional holy water to sacrifice senior people. The ethanolic extract from the pod of *A. concinna* has been used in anti-dandruff shampoo due to its antidermatophyte and antibacterial activities. Saponins in the pods acts as detergent [7, 8]. Moreover, an ointment of the extract has been used for skin disease [7]. However, the antioxidant and antityrosinase activities of the pod extracts of *A. concinna* have been rarely investigated. The purposes of this study were to investigate antioxidant and antityrosinase activities of the pod extracts of *A. concinna* grown in Northern Thailand. Phytochemical constituents, cytotoxic effect and HPLC-fingerprint of the selected extract were also determined.

MATERIALS AND METHODS

Plant material

A. concinna pods were collected at maturity form between May-September, 2014 in Chiang Mai Province, Thailand. The seeds were removed, and then the pods without seeds were dried in hot-air oven at 45 °C for 24 h before further use.

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), linoleic acid, 2,2'-

Azobis(2-amidinopropane) dihydrochloride (AAPH), tyrosinase from mushroom and L-tyrosine were purchased from Fluka (Buchs, Switzerland). Folin-ciocalteu's reagent was purchased from Merck (Darmstadt, Germany). L-ascorbic acid, trolox, quercetin, gallic acid, beta-arbutin, Histopaque-1077, Thiazolyl Blue Tetrazolium Blue (MTT) powder and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Solvents for extraction were analytical grade. For HPLC-analysis, all solvents were HPLC grade.

Extraction of *A. concinna* pods

A. concinna pods were ground and extracted using three different solvents including 95% ethanol, 50% ethanol in water that were extracted by maceration technique for 48 h, and distilled water that was boiled for 1 h. Each extractant was filtered by filtration paper No.1 and the marc was re-extracted by the same process twice. The collected filtrate were dried in different processes. The ethanolic extract (ET) was concentrated using the rotary evaporator. The hydroethanolic extract was evaporated and then dried by freeze drier (HEF) or spray drier (HES). The aqueous extract was dried by freeze drier (HF) or spray drier (HS).

Chemical screening of *A. concinna* pod extracts

The pod extracts of *A. concinna* were screened for their chemical constituents including alkaloids, flavonoids, phytosterols, saponins, tannins, and phenolics. The chemical reagents and methods used were dragendorff's reagent, Shinoda's test, Lieberman-Burchard test, frothing test, 1% gelatin solution, and 1% FeCl₃ solution for detection of alkaloids, flavonoids, phytosterols, saponins, tannins and phenolic compound, respectively [9, 10].

Determination of antioxidant activity

1) DPPH-radical scavenging assay

Scavenging activity of each extract on DPPH radicals was determined using the method of Thaipong *et al.* (2010) with some modifications [11]. The extracts were dissolved in DMSO in the concentration range of 1.25-40.00 mg/ml. The aliquot of sample (20 µl) was mixed with 120 mM DPPH in methanol (180 µl) and kept in the dark for 30 min. Then the absorbance of the solution was measured at 520 nm using Beckman coulter®, DTX 880 multimode detector, Austria. Quercetin and trolox served as standards. The percentage inhibition was calculated using the following equation: %inhibition = $[(Ac-As)/Ac] \times 100\%$, where Ac was the absorbance of control and As was the absorbance of sample. Those showing good activities were tested in various concentrations and calculated for the concentration that reduced the DPPH absorbance by 50% (IC₅₀).

2) ABTS-radical scavenging assay

ABTS stock solution was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution and left the mixture until the reaction complete at room temperature for 16 h [12]. ABTS stock solution was then diluted to an absorbance of 0.70±0.02 at 734 nm. The extracts were dissolved in 70% ethanol in five serial concentrations. The aliquot of sample (10 µl) was added to 1 ml of diluted ABTS solution. The experiments were done in triplicate. The mixture was measured the absorbance at 734 nm after 6 min. The percentage inhibition and IC₅₀ were then calculated using the following equation; %inhibition = $[(Ac-As)/Ac] \times 100\%$, where Ac was the absorbance of control and as was the absorbance of sample.

3) Linoleic acid peroxidation assay

The inhibition of linoleic acid peroxidation of each extract was determined using the method of Olszewska with some modifications [13]. Each extract and standard was dissolved in 70% methanol in five serial concentrations. Phosphate buffer (PBS) pH 7.0 (1.40 ml), 1.3% linoleic acid in methanol (1.40 ml) and deionized water (0.70 ml) were mixed with the aliquot of sample (0.30 ml) in test tubes. AAPH solution in the concentration of 46.35 mM (0.20 ml) was added to start lipid peroxidation process and the tubes were incubated in the dark at 50 °C for 4 h or until the absorbance of the control was 0.550±0.020 at 500 nm. The degree of lipid peroxidation was determined by ferric-thiocyanate method. The reaction mixture (0.10 ml) was mixed with 20 mM FeCl₂ solution in 3.5% HCl (0.10

ml), 10% NH₄SCN solution (0.10 ml) and 75% methanol (9.70 ml) for 3 min. The absorbance was measured at 500 nm using UV-VIS spectrophotometer (Shimadzu, UV-2450). The experiments were done in triplicate. Percentage of the inhibition was determined using the following equation; %inhibition = $[(Ac-As)/Ac] \times 100\%$, where Ac was the absorbance of control and As was the absorbance of sample. The IC₅₀ was calculated from the regression curve of the concentration.

Determination of antityrosinase activity

The extracts were determined for the inhibitory effect on tyrosinase enzyme using modified Manosroi *et al.* protocol [14]. Each extract and standard was diluted in 20% tween20 in 0.1 M PBS pH 6.8 in five serial concentrations. The aliquots of sample (70 µl) were mixed with PBS (70 µl), and 1.66 mM tyrosinase solution in PBS (70 µl) in 96-well plate. The mixtures were left at room temperature for 10 min. Then, L-tyrosine substrate (70 µl) in PBS was added and incubated at room temperature for another 20 min. The absorbance was measured using multimode detector at 450 nm. The percentage of mushroom tyrosinase inhibition was calculated by following equation; %inhibition = $[(Ac-As)/Ac] \times 100\%$, where Ac was the absorbance of control and As was the absorbance of sample. The % inhibition was plotted versus the concentration to determined IC₅₀. In this experiment, beta-arbutin was used as a standard.

Determination of total phenolic content

Total phenolic content of each extract was determined using folin-ciocalteu's reagent following the protocol of Alhakmani [15]. Gallic acid was used as a standard. Each extract and standard was dissolved in the mixture of ethanol and distilled water (2:3). The aliquot of the extracts (0.50 ml) and gallic acid solution were mixed with diluted folin-ciocalteu solution (diluted 1:10 in deionized water). Then, 4 ml of sodium carbonate solution was added. The mixture was left at room temperature for 30 min. The absorbance of the mixture was determined at 765 nm using UV-VIS spectrophotometer (Shimadzu, UV-2450). The absorbance of the extracts were compared with gallic acid calibration curve and presented as Gallic acid equivalent (GAE) values in terms of mg gallic acid/g of the extract. These were done in triplicate.

Determination of total flavonoid content

Total flavonoid content was determined using the method of Samatha *et al.* with some modifications [16]. An aliquot (1 ml) of the extract, distilled water (10 ml) and 5%NaNO₂ (0.3 ml) were mixed and incubated for 5 min. Then, 10%AlCl₃ (0.3 ml) was added into the mixture. After 1 min of incubation, 1 M NaOH (2 ml) was added into the mixture. The absorbance of the solution was determined using UV-VIS spectrophotometer (Shimadzu, UV-2450) and compared with rutin calibration curve. Then, rutin equivalent (RE) values of the extracts were calculated and presented as mg rutin/g of the extract.

Determination of cytotoxic effect

HES was selected to investigate cytotoxic effect on human PBMCs using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay due to its highest antioxidant and antityrosinase activities. Ficoll-Hypaque gradient centrifugation was selected to isolate PBMCs from heparinized venous blood of healthy volunteers. PBMCs were plated in 96-well plate in cell concentration of 1×10⁵cells/well and cultured in complete RPMI 1640 media with or without HES or ascorbic acid in 37 °C, 5% CO₂ and 90% humidity incubator for 48 h. Following treatment, the medium was removed and then, 15 µl of MTT dye solution at 0.33 g/l was added and incubated for 4 h. The supernatant was removed and 100 µl of DMSO was added to dissolve the crystal formazan. The absorbance was read at wavelength 540 nm using multimode detector. %Cell viability was calculated by the following equation: %cell viability = $(Ae/Av) \times 100\%$, where Ae was the absorbance of the sample and Av was the absorbance of vehicle control.

High performance liquid chromatography (HPLC) analysis

The extract that showed the highest antioxidant and antityrosinase activities was selected to analyse for HPLC-fingerprints which

performed with HP HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) equipped with CHEM STATION software, a degasser G1322A, a binary gradient pump G1311A, a column oven G1316A and UV-Visible detection system G1314A. Sample (2 mg/ml) was filtrated through a 0.45 µm filter before injected into the HPLC column. The injection volume was 10 µl and the flow rate was 0.9 ml/min. the temperature of the system was 27 °C. The column was inertsil ODS-3 (5 µm, 4.6×250 mm ID).

The maximum absorbance of the extract by UV-spectrophotometry was 280 nm. The solvent A was methanol. The solvent B was 0.01% phosphoric acid. The gradient elution was used for separate the extract. The program of elution was as follows: 0-20 min, 90-10% (A), 10-90% (B); 20-21 min, 10% (A), 90% (B).

Statistical analysis

Statistical analysis was performed by one-way ANOVA using SPSS statistic 17.0 program (IBM SPSS Statistics Inc.) All assays were done in triplicate experiment. The results were demonstrated as mean±standard deviation (S. D.) from three independent analysis. P-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Percentage yields of the extracts

The percentage yield of the extracts and their characteristics were shown in table 1. ET gave the highest percentage yields of 42.36±1.70%. The hydroethanolic extract and aqueous extract were dried in two different processes including freeze drier and spray drier. The freeze-dried extracts (HF and HEF) significantly showed higher yield than spray-dried extracts (HS and HES).

Table 1: Yields of *A. concinna* pod extracts obtained from various solvents

Extracts	Yield %	Descriptions
ET	42.36±1.70	Sticky and dark brown
HF	41.67±2.10	Dry dark brown powder
HS	18.24±2.70	Dry dark brown powder
HEF	34.35%±1.90	Dry dark brown powder
HES	19.22±1.30	Dry dark brown powder

Chemical screening of the extracts

Alkaloids, flavonoids, saponins, and phenolics were found in all extracts while phytosterols was found only in ET. In addition, tannin was found only in aqueous extracts (HF, HS) because of its hydrophilicity (data not shown).

Different compounds are soluble in different kinds of solvents and the polarity of the solvent is an important factor to extract different phytochemical constituents. Ethanol can extract both hydrophilic and hydrophobic phytochemical constituents, while only aqueous extracts contain tannins which are hydrophilic constituents.

Table 2: IC₅₀ values of *A. concinna* pod extracts determined by DPPH, ABTS, linoleic acid peroxidation and antityrosinase activity assays

The extracts	IC ₅₀ (mg/ml)			
	DPPH assay	Linoleic acid peroxidation assay	ABTS assay	Antityrosinase activity assay
ET	2.0487±0.1675	5.5011±0.4569	0.5660±0.0033	1.7055±0.0733
HF	1.6976±0.0764	2.4683±0.2624	0.4072±0.0046	1.6807±0.0729
HS	1.2509±0.1120	2.6729±0.3889	0.4176±0.0051	1.5333±0.0193
HEF	1.1340±0.0603	2.0670±0.0575	0.3858±0.0034	1.6429±0.0447
HES	0.8760±0.0131	1.8751±0.0307	0.1418±0.0012	1.1013±0.0130
Standards				
Trolox	0.0057±0.0004	0.0217±0.0005	0.0016±0.0001	-
Quercetin	0.0043±0.0004	0.0902±0.0068	0.0007±0.0001	-
Ascorbic acid	0.0299±0.0004	0.0944±0.0033	0.0004±0.0001	-
Gallic acid	0.0016±0.0001	0.1287±0.0024	0.0006±0.0001	-
Beta-arbutin	-	-	-	0.2816±0.0251

Determination of antioxidant activity

1) DPPH-radical scavenging assay

DPPH assay has been accepted as an antioxidant screening test for natural substances [17]. The IC₅₀ values of the extracts were demonstrated in table 2. HES showed the highest scavenging DPPH-radical activity. The aqueous extracts showed better scavenging activity than the ethanolic extract. Therefore, the active compounds of the extracts may be quite polar or more hydrophilic.

2) ABTS-radical scavenging assay

The results are shown in table 2. HES presented the strongest scavenging activity in ABTS assay while ET showed the weakest activity. The result from ABTS-radical scavenging assay was in a good agreement with DPPH-radical scavenging assay.

IC₅₀ values from ABTS-radical scavenging assay showed better antioxidant activity than that from DPPH-radical scavenging assay because of the higher amount of the hydrophilic active compounds in the extracts than the hydrophobic compounds. Therefore, using ABTS-radical scavenging assay is proper for this extracts. However, both DPPH and ABTS assay are generally performed to confirm the scavenging ability of the extracts.

3) Linoleic acid peroxidation assay

Lipid peroxidation that occurs in cell membrane was initiated from ROS that oxidized the unsaturated bond of lipid to lipid peroxides and aldehyde that responsible in long term damage of the skin cells [1, 17]. In this study, the protective effect on lipid peroxidation of *A. concinna*. pod extracts was determined using linoleic acid peroxidation assay. Linoleic acid acts as the unsaturated fatty acid that was oxidized by AAPH. The IC₅₀ values of the extracts were 1.8751±0.0307 to 5.5011±0.4569 mg/ml as shown in table 2. HES exhibited the highest activity.

Determination of antityrosinase activity

Tyrosinase is the rate-limiting enzyme in melanogenesis in the skin cells that cause melasma and dark spots [4, 6]. The extracts were determined for inhibitory effect on mushroom tyrosinase enzyme. Their IC₅₀ values were demonstrated in table 2 and compared with a standard, beta-arbutin. From the results, HES showed the greatest inhibition on hydroxylation of L-tyrosine, among the extracts. However, beta-arbutin presented better result than HES.

Determination of total phenolic content

Phenolic compounds that are widely found in herbs, have been accepted for their strong antioxidant activities [4, 18]. The *A. concinna* pod extracts were determined the total phenolic content. GAE value was calculated by gallic acid calibration curve following linear equation; y = 0.00746x+0.00309, correlation coefficient of R²= 0.999 and demonstrated in fig. 1. Since HES showed the highest GAE value at 100.506±0.060 mg/g of the extract, it also exhibited the highest activities in every antioxidant experiment.

Determination of total flavonoid content

The total flavonoid content of the extracts expressed as RE were calculated by rutin calibration curve following linear equation; $y = 0.00106x + 0.02092$, correlation coefficient of $R^2 = 0.986$. RE values in a comparison with GAE values of each extracts are shown in fig. 1. HES presented the highest RE value of 33.562 ± 0.030 mg/g of the extract. Therefore, the flavonoids in the extracts may also act as antioxidant. Furthermore, GAE and RE values of the extracts were compared. The results demonstrated that the extracts may consist of phenolic compounds as principal constituents that play an important role in antioxidant and antityrosinase activities rather than their flavonoid content.

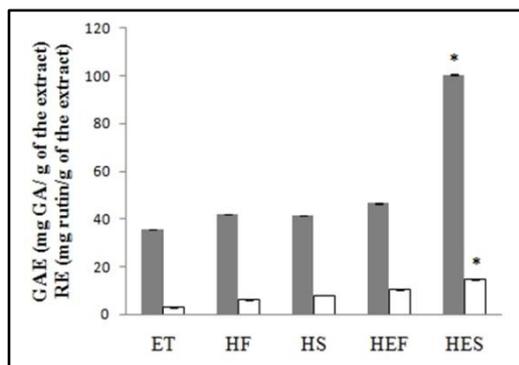
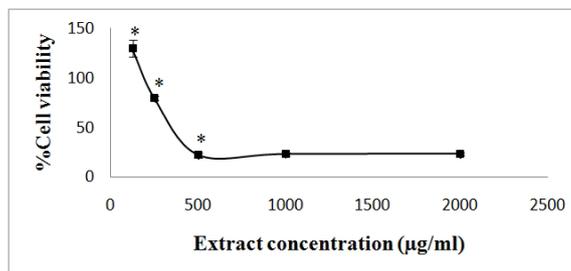


Fig. 1: GAE values (■) and RE values (□) of *A. concinna* pod extracts using various solvents and extraction methods (*P<0.05)

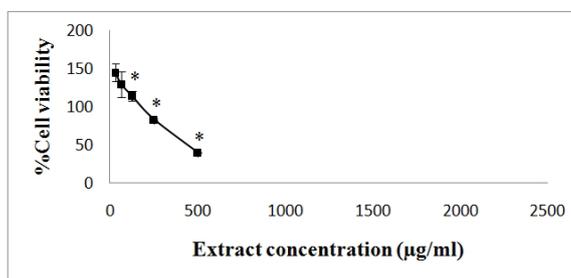
Cytotoxic effect of HES

To determine the cytotoxic effect of HES on PBMCs, %cell viability was evaluated using MTT assay. PBMCs were treated with different concentrations of the extract ranging from 125 to 2000 µg/ml for 48 h, whereas ascorbic acid that used as a control, its concentrations were ranging from 31.25 to 500 µg/ml. The concentration that reduces the %cell viability by 20% (IC₂₀ value) of HES and ascorbic acid on PBMCs were 280.85 ± 16.53 µg/ml and 295.94 ± 17.58 µg/ml, respectively. In general, %cell viability higher than 80%, indicated that the test sample was accepted for human and safe to use [19]. In addition, the %cell viability of cells treated with HES and ascorbic acid were decreased in a dose-dependent manner as shown in fig. 2. The pH values of HES and ascorbic acid were also determined and the %cell viability were compared as shown in table 3. The results demonstrated that pH of the sample and ascorbic acid may affect on cell viability. At the concentration of 125 µg/ml, both HES and ascorbic acid showed more than 100% cell viability. But at high concentration (>500 µg/ml), the %cell viability

were decreased due to their acidity. These results presented that the safety profile of HES was comparable to ascorbic acid. Interestingly, we found that at low concentration (125 µg/ml), both ascorbic acid and HES could promote cell growth.



(A)



(B)

Fig. 2: %Cell viability of human PBMCs treated with HES (A) and ascorbic acid (B) in various concentrations (n=3, *P<0.05)

Table 3: Cell viability of human PBMCs and pH of HES and ascorbic acid at the concentration of 125 µg/ml

Extract and standard	%cell viability	pH
HES	129.50 ± 8.40	4.32
Ascorbic acid	114.01 ± 6.50	4.02

High performance liquid chromatography (HPLC) analysis

HES was selected to analyse for HPLC fingerprint due to its highest antioxidant and antityrosinase activities. As shown in fig. 3, the HPLC chromatogram demonstrated one major peak at the retention time of 9.88 min which was neither gallic acid (retention time at 8.21 min) nor rutin (retention time at 16.22 min). This peak could be recognized as a marker of HES. However, it was interesting for further identification.

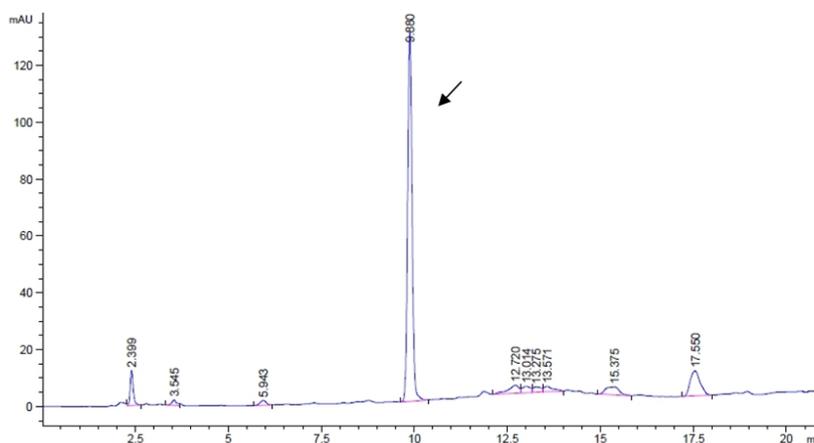


Fig. 3: HPLC-chromatogram of HES using UV-detector at wavelength of 280 nm

CONCLUSION

In conclusions, the pod extracts of *A. concinna* consisting phenolic compounds exhibited good *in vitro* activities in both free radical scavenging and mushroom tyrosinase inhibition. The HES extract revealed the highest activities with highest phenolic content. Its cytotoxic effect on PBMCs was comparable to ascorbic acid. Therefore, it might be a promising approach for application in the cosmetic or cosmeceutical products.

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CONFLICT OF INTERESTS

Declared None

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