

# IDENTIFICATION OF BIOACTIVE COMPOUNDS IN WATER EXTRACT OF SARANG SEMUT (*MYRMECODIA PENDANS*) BULB AND ITS POTENTIAL FOR THE INHIBITION OF HELA AND MDA-MB-231 CANCER CELLS

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**Abstract** - Sarang semut (*Myrmecodia pendans*) or ant-nest plant is a potential medicinal plant. The water extract of this plant is well-known to cure many kinds of disease including cancer. Phytochemicals present in this plant are suspected to be responsible for its health benefits. Water extract obtained at 100°C and 30 min extraction shows the highest radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the highest cytotoxicity towards cervix (HeLa) and breast (MDA-MB-231) cancer cells. The water extract is fractionated using n-hexane, ethyl acetate and n-butanol. The n-butanol fraction is found to contain the highest amount of total phenolic content, DPPH scavenging and cancer cell cytotoxic activities. Identification of bioactive compounds in the n-butanol fraction is performed using high performance liquid chromatography coupled with mass spectrometry and ultraviolet detector. Forty-eight antioxidant compounds are identified in the water extract, and 37 compounds among them are reported for the first time.

**Key words** - Anticancer; antioxidant; Sarang semut; phenolic compounds; water extract

## 1. Introduction

Serious chronic diseases and high cost of modern synthetic medicines have led to the increase of interest in traditional medicine. Antioxidants extracted from plants play a vital role in health protection [1]. The National Cancer Institute has reported more than 30,000 plants that exhibit anticancer activity. Aromatic plants and herbs have long been used as medicine and many of these can be found in ancient records [2]. Antioxidants can protect biomolecules against free radical attacks that may result in cardiovascular diseases, cancers, neurodegenerative diseases, inflammation and allergies [1-3, 7].

Sarang semut is a newly reported and not well-studied medicinal plant. It is a native plant of Papua, Indonesia and is also found in Australasian regions, Malay Peninsula, Cambodia, Sumatra, Java, Philippines, Vietnam and Cape York to Solomon Islands. The water extract of sarang semut, obtained by boiling its hypocotyls in water, has been reported as an effective alternative medicine for treating diarrhea, uric acid disorder, ulcer, leukemia, tuberculosis, stroke, tumor, cancer, kidney and prostate problems [8-11]. However, researches and publications regarding the active constituents of sarang semut are very limited.

Qualitative test on sarang semut extracts revealed the presence of polyphenols, saponins, alkaloids, tannins, flavonoids, glycosides, tocopherols, and a variety of essential minerals, but only a few specific compounds were identified [8-9]. Bioactive compounds identified in the ethanol and supercritical CO<sub>2</sub> extracts of sarang semut were mostly phenolic compounds (kaempferol, luteoline, rutin, quercetin, apigenin, gallic acid, catechin, ferulic

acid, caffeic acid, p-coumaric acid, rosmarinic acid and procyanidin B<sub>1</sub>) [10-12]. Due to the limited reports on the isolation and identification of constituents in sarang semut extract, the objective of this study is to separate and identify phytochemical constituents of the water extract using HPLC-MS. Forty-eight antioxidant compounds are tentatively identified and 37 among them are reported for the first time in this plant. Additionally, radical scavenging activity using DPPH, and cytotoxicity towards cervix (HeLa) and breast (MDA-MB-231) cancer cells of the water extract are examined.

## 2. Material and methods

This study is divided into three parts: extraction, activity assay and identification of bioactive compounds. All experiments were performed in triplicate.

### 2.1. Materials

Dried sarang semut was obtained from Wamena, Papua, Indonesia. HPLC grade methanol, ethyl acetate, and n-butanol were purchased from Alfa Aesar (UK). n-Hexane (95% purity) was supplied by Tedia (Fairfield, OH). Analytical grade acetic acid, glacial acetic acid was purchased from Scharlau (Spain). Dimethyl sulfoxide (DMSO), DPPH, Folin Ciocalteu reagent, Eagle's Minimum Essential Medium, sodium bicarbonate, L-glutamine, antibiotic antimycotic solution (100×), sodium pyruvate, non-essential amino acid solution (100×), fetal bovine serum, phosphate buffer saline (PBS), trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (97.5%) were obtained from Sigma Aldrich (St. Louis, MO). Standards for verification and identification of compounds were purchased from Sigma Aldrich and Alfa Aesar (Heysham, Lancashire). The human cervix cancer cell line (HeLa) and breast cancer cell (MDA-MB-231) were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan).

### 2.2. Sample preparation

Samples were rinsed with water to remove impurities and dried in a freeze dryer. The dried samples were grounded into powder and sieved passing 120 mesh. The framework of this study is presented in Figure 1.



Figure 1. The framework of this study

### 2.3. Extraction and fractionation of bioactive compound

Sarang semut powder was extracted with water at 100°C with a solid to solvent ratio of 1:10(g/mL) at a constant stirring of 500 rpm for a predetermined time. The solid residues were re-extracted twice. The pooled extracts were then freeze dried. The dried crude extract was kept at -5°C prior to DPPH and cytotoxicity assays.

Water extract obtained was then fractioned and partitioned consecutively using n-hexane, ethyl acetate and n-butanol to obtain the n-hexane fraction, the ethyl acetate fraction, the n-butanol fraction, respectively while the remaining extract was referred to as the water fraction. The volume ratio of water extract and solvent was 1:1. The following assays were carried out for each fraction: antioxidant activity using DPPH, total phenolic content, UV visible spectrophotometric measurement, and cytotoxic test using HeLa cell and MDA MB 231.

### 2.4. Total phenolic content (TPC)

Quantification of TPC in extracts were carried out using the Folin-Ciocalteu assay [13]. Folin-Ciocalteu reagent (100  $\mu$ L, 0.2 mol/L) was added to 20  $\mu$ L sample for 4 min, and subsequently 80  $\mu$ L sodium carbonate solution (75 g/L) was added to the mixture. The sample was incubated for 2 h at room temperature and then the absorbance was measured at 760 nm using a microplate reader. The standard curve was constructed by the absorbance of gallic acid at different concentrations. TPC value was expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of sample [13].

### 2.5. Radical scavenging activity

Radical scavenging activity was performed using the DPPH method. Plant extract (2.5  $\mu$ L, from 0 to 5 mg/mL) was added to 250  $\mu$ L of 0.004% DPPH radical solution. The mixture was incubated at room temperature under dark condition for 30 min. The absorbance was measured at 517 nm. DPPH scavenging activity was calculated using the equation:

$$IC\ (%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where  $A_0$  is absorbance of the control reaction, and  $A_1$  is absorbance in the presence of plant extract.  $IC_{50}$  was graphically calculated by intersecting the concentration of extract that have 50% inhibition in a curve of extract concentration vs. the corresponding scavenging effect [14].

### 2.6. Cytotoxicity test

The medium used for cell culture was Eagle's Minimum Essential Medium containing 1.5 g/L sodium bicarbonate, supplemented with 1% antibioticantimycotic formulation, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 10% fetal bovine serum. Cancer cells were seeded into a 24 well-plate with a cell density of 15,000 cells/well and incubated for 24 h prior to addition of extracts at 37°C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity. After cell attachment was completed, the cells were washed with phosphate buffered saline (PBS). Mediums with various concentrations of water extract were then added and the cells were incubated for another 24 h. Medium without the addition of water extract was served as the control.

MTT assay of cancer cells was conducted using method described by Fahmi and Chang [15] with some modifications. After one day incubation, the medium was aspirated and the cells were washed with PBS, then 300 $\mu$ L of MTT solution in PBS (5 mg/ml) was added to each well and re-incubated at 37°C for 4 h. The MTT solution was removed and the formed formazan crystals were re-dissolved in 600  $\mu$ L DMSO and the absorbance was measured at 570 nm using a microplate reader. The percentage of cell inhibition was calculated using Equation (2):

$$\% \text{ cell inhibition} = 1 - \frac{\text{Absorbance of water extract}}{\text{Absorbance of control}} \times 100 \quad (2)$$

### 2.7. High performance liquid chromatography and mass spectrometry (HPLC-MS)

Aliquot of BF(20  $\mu$ L) was separated using a HPLC system which was coupled to a UV detector. Separation was performed using a Phenomenex Gemini 5  $\mu$ m NX-C18 110 Å (240 mm x 4.6 mm) with UV detection operated at 280 nm. Compounds were eluted using solvent A (methanol) and mixture solvent B (water: acetic acid, 99:1) with solvent gradient as follows: 0 min (5% A), 35 min (15% A), 45 min (15% A), 110 min (50% A), 115 min (50% A), 120 min (100% A), 122 min (100% A) and 125 min (5% A). The solvent flow rate was 1 mL/min.

The HPLC column was then coupled to a mass spectrometer. Electrospray ionization was operated in negative ion mode. The voltage of capillary, sampling cone, and extraction cone was set at 2 kV, 35 V and 3 V, respectively. The source and desolvation temperatures were 100 and 300°C, respectively. The gas flow for desolvation was 500 L/h. Full scan mass spectra were acquired from m/z 50 to 1200. Mass spectrometry data were analyzed using MassLynx V4.1.

The compounds in this work were identified by the MS/MS2 data, results of interpretation of the observed MS/MS2 spectra were compared with those reported in literatures. The following databases were also used as reference: Mass Bank (<http://www.massbank.jp>), mzCloud database (<https://www.mzcloud.org>). All identified chemical formula were validated using MassLynx V4.1 by typing the possible chemical formula to simulate the theoretical precursor ion MS and comparing them to existing databases and experiment result. After mass spectra interpretation, standards were injected to HPLC for confirming and quantifying the identified compounds.

## 3. Results and Discussion

Water extracts obtained at various extraction times were analyzed for DPPH free radical scavenging activity and cytotoxicity assay, and their  $IC_{50}$  are summarized in Table 1. Repeated and prolonged extraction (over 30 min for each extraction) with water at 100°C did not result in improved yields. An extraction time of 0.5 h yielded extracts with the highest radical scavenging activity. Cytotoxicity assays using HeLa cell and MDA MB 231 cell were also performed by incubating cells in the presence of crude water extract (500 to 2000 mg/mL) for 24 h. Cytotoxicity assay showed that water extract was capable

of inhibiting the growth of cervix and breast cancer cells linearly in a dose-dependent manner. The activity decreased slightly with increasing extraction time. This maybe the result of degradation caused by longer exposure of phenolic compounds to heat, light and oxygen [16]. Extract obtained at 0.5 h resulted in the highest activity; it was therefore used in the succeeding experiments.

**Table 1.** Crude extract yield (H, %) and  $IC_{50}$  of water extract (100°C, 500 rpm, sarang semut: Water = 1:10, w/v)

Extraction time (h)	H (%)	$IC_{50}$ (mg/mL)		
		DPPH	HeLa inhibition	MDA-MB-231 inhibition
0.5	13.85 ± 0.31	2.99 ± 0.14	700.02 ± 1.41	1116.09 ± 1.33
1	14.17 ± 0.55	3.20 ± 0.11	724.58 ± 1.29	1156.36 ± 2.17
2	14.22 ± 0.43	4.04 ± 0.09	732.11 ± 1.14	1189.23 ± 1.96
3	14.94 ± 0.56	4.39 ± 0.22	741.03 ± 2.01	1225.67 ± 1.31
Gallic acid (standard)	n.d.*	0.11 ± 0.01	n.d.	n.d.

\* not determined

### 3.1. Solvent partition of crude water extract

The crude water extracts (CWE) were partitioned using solvents of different polarities and the fractions obtained are n-hexane fraction (HF), ethyl acetate fraction (EAF), n-butanol fraction (BF) and water fraction (WF). The yield, DPPH scavenging activity and total phenolic content (TPC) of each fraction are presented in Table 2. Phenolic contents of various fractions vary from 120.47 to 794.84 mg GAE/g crude fraction, with BF having the highest TPC content and consequently better DPPH radical scavenging activity. These results are consistent with literature data, suggesting that TPC is positively correlated to radical scavenging activity [17]. BF was found to be most effective in inducing growth inhibition of cervix and breast cancer cells in a dosage-dependent manner, with an  $IC_{50}$  of 400 ppm for HeLa cell and 820 ppm for MDA-MB-231 cell.

**Table 2.** Fractionation yield (%), TPC, and radical scavenging activity of various solvent fractions

Sample	Based on initial weight (%)	Based on crude extract (%)	TPC (mg GAE/g fraction)	DPPH $IC_{50}$ (mg/mL)
HF	0.855	6.17	120.47 ± 0.66	13.32 ± 0.77
EAF	4.97	35.87	133.95 ± 0.41	7.74 ± 0.18
BF	0.465	3.36	794.84 ± 0.98	1.99 ± 0.10
WF	6.99	50.47	155.47 ± 1.12	2.73 ± 0.13
Loss	0.57	4.12	n.d.*	n.d.*
Gallic acid (standard)	n.d.*	n.d.*	n.d.*	0.11 ± 0.01

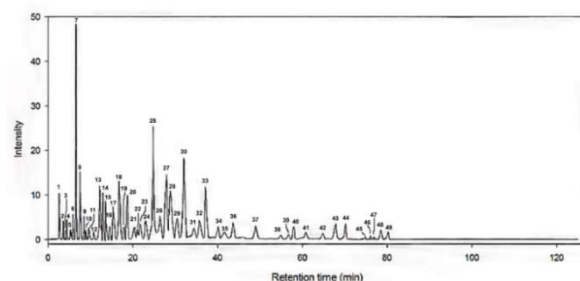
\* not determined

### 3.2. HPLC-MS analysis of BF

Further identification of active compounds extracted from sarang semut was conducted by using HPLC-MS. A total of 49 peaks (Figure 1) were detected, out of which 48 compounds were identified by comparing each peak with the mass spectrum in databases and in literatures. Thirty-seven among the 48 compounds identified were

reported for the first time. Although quantification of all compounds was not possible, thirty compounds were confirmed and quantified by comparing with standards. The 48 compounds identified can be grouped into organic acids, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and other compounds. A detailed discussion is provided in the following sections.

Analysis showed BF extract contained 4 organic acids, 14 hydroxybenzoic acids, 6 hydroxycinnamic acids, 20 flavonoids (8 flavanols, 2 anthocyanins, 6 flavonols, 1 chalcone, 2 flavones and 1 flavanolol), an unknown compound and 4 other compounds (stilbene, pyridene, anthocyanin, and phenyl ethyl alcohol). Each class of compound contributes different benefits for the health. Some organic acids possess biological activities including anti-inflammatory, radical scavenging, inhibition of platelet aggregation, cardiovascular protection and conversion of cholesterol to bile acids. Organic acids may be involved in the synthesis of neurotransmitters, hormones, and collagen [18, 19]. Hydroxybenzoic acids were reported to possess various pharmacological activities such as antioxidant, antimutagenic, antiviral, antibacterial, antifungal, antiprotozoal, nematocidal, insecticidal, anti-hypoglycemic, neuro-protective, anti-rheumatic, analgesic and cardio-protective. They also aid in the inhibition of platelet aggregation, exhibit cytotoxicity towards several cancer cell lines including prostate, cervix (HeLa), breast (MDA-MB-231) and leukemia (MOLT-3) [20-22]. Hydroxycinnamic acids have been reported to inhibit LDL oxidation, to chelate transition metals, to reduce anxiety and to prevent diabetes, atherosclerosis and cardiovascular diseases. These compounds also exhibited antioxidant, antibacterial, antiviral, antifungal and anticancer activities [23-27]. Flavonoids, the most abundant group of phenolic compounds, include flavonols, flavanols, isoflavones, flavanones, flavones, dihydroflavonols, anthocyanidins, and chalcones. Flavonoids are able to scavenge radicals, reducing lipid peroxidation, and preventing coronary heart disorder, diabetes and neurodegenerative diseases such as Parkinson and Alzheimer. They also possess antiviral, anti-inflammatory, hepatoprotective, anticancer, analgesic, antibacterial, and anti-mutagenic activities [19-20, 28].



**Figure 2.** HPLC chromatogram detected at 280 nm

## 4. Conclusions

Decoction allows the extraction of various active compounds from sarang semut. BF of the water extract results in the highest cancer cell inhibition as well as the highest TPC and DPPH activities. A total of 49 peaks have

been detected using HPLC-UV with the detector set at 280 nm. Forty-eight compounds (4 organic acids, 14 hydroxybenzoic acids, 6 hydroxycinnamic acids, 20 flavonoids, 5 others) were identified using HPLC-MS, in which 37 compounds were reported for the first time. Thirty compounds were confirmed and quantified by comparing with those of the authentic standards. These compounds are probably responsible for the observed radical scavenging activity and inhibitory effects on cervical and breast cancer cells.

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