CHAPTER 3

RESEARCH METHODOLOGY

3.1 Chemicals and equipment

Chemicals, materials and instruments used in this study are listed below:

3.1.1 Potassium persulfate (Merk, Germany)

3.1.2 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) (Sigma, Chemical Co, USA)

3.1.3 Potassium di-hydrogen phosphate (KH_2PO_4) (BDH Prolabo®, Belgium)

3.1.4 Di-potassium monohydrogen phosphae (K_2HPO_4) (BDH Prolabo®, Belgium)

3.1.5 Sodium di-hydrogen phosphate (NaH_2PO_4) (Ajax Finechem Pty. Ltd (New Zealand)

3.1.6 Hydrogen peroxide (30%)(Merck, Germany)

3.1.7 Horseradish peroxidase (HRP) (Sigma, Chemical Co, USA)

3.1.8 Hypoxanthine (Sigma, Chemical Co, USA)

3.1.9 Xanthine oxidase (Sigma, Chemical Co, USA)

3.1.10 Nitro blue tetrazolium (NBT) (Sigma, Chemical Co, USA)

3.1.11 2-Deoxy-D-ribose (Sigma, Chemical Co, USA)

3.1.12 Iron (III) perchlorate ($FeCl_3$) (Sigma-Aldrich, Chemical Co, USA)

3.1.13 L-ascorbic acid (Sigma, Chemical Co, USA)

3.1.14 Thiobarbituric acid (TBA) (Sigma, Chemical Co, USA)

3.1.15 Trichloroacetic acid (TCA) (Sigma, Chemical Co, USA)

3.1.16 Sodium nitroprusside (SNP) (Sigma-Aldrich, Chemical Co, USA)

3.1.17 Sulfanilamide (Sigma-Aldrich, Chemical Co, USA)

3.1.18 N-1-napthylethylenediamine dihydrochloride (NED) (Sigma-Aldrich, Chemical Co, USA)

3.1.19 Sodium carbonate (Na_2CO_3) (Merck, Germany)

3.1.20 Gallic acid (Sigma-Aldrich, Chemical Co, USA)

3.1.21 Folin-Ciocalteu (Sigma-Aldrich, Chemical Co, USA)

3.1.22 Ortho-Phosphoric acid (85%) (Merck, Germany)

3.1.23 Methanol (HPLC) (Lab-Scan, Thailand)

3.1.24 Acetonitrite (HPLC) (RCI-LAB-SCAN, Thailand)

3.1.25 Ethyl acetate (Merck, Germany)

3.1.26 Glacial Acetic acid (Merch, Germany)

3.1.27 Catechin (Sigma, Chemical Co, USA)

3.1.28 Epicatechin gallate (ECG) (Sigma, Chemical Co, USA)

3.1.29 Epicatechin (EC) (Sigma, Chemical Co, USA)

3.1.30 Epigallocatechin gallate (EGCG) (Sigma, Chemical Co, USA)

3.1.31 Epigallocatechin (EGC) (Sigma, Chemical Co, USA)

3.1.32 Myricetin (Sigma, Chemical Co, USA)

3.1.33 Quercetin (Sigma, Chemical Co, USA)

3.1.34 Daidzin (Sigma, Chemical Co, USA)

3.1.35 Kaempferol (Sigma, Chemical Co, USA)

3.1.36 Genistin (Sigma, Chemical Co, USA)

3.1.37 Distilled water

3.1.38 Centrifuge (Xiang Yi Centrifuge Instrument Co, Ltd)

3.1.39 UV-Visible Spectrophotometer (GENESYS 10S VIS, England)

3.1.40 High Performance Liquid Chromatography (HPLC) UV-Vis detector by 1100 (Hewlett-Packard, Germany)

3.1.41 Reversed Phase C18 HPLC Column (25 cm x 4 mm i.d. and 5 µm particle diameter) (Phenomenex, USA)

3.1.42 3- CAT Research ELISA™ (Cat. No. BA E-5600, Labor Diagnostika Nord GmbH & Co. KG, Germany)

3.1.43 Balance (Ohaus EO2140, Switzerland)

3.1.44 Micro centrifuge (Hycon, USA)

3.1.45 Automatic pipette (Gilson, France)

3.2 *Vernonia cinerea* Less. preparation

Naturally mature VC plants after grown at least one year were collected on summer period (March-April, 2014) from a local clean area, without toxic insecticide spraying, at Amphoe Hang Dong, Chiang Mai province, Thailand. VC characteristics were identified using voucher specimens deposited at the CMUB herbarium, Department of Biology, Faculty of Science, Chiang Mai University. The flower, stem and leaf of VC were separated and washed four times with clean water (Nestle Pure Life, Nestle (Thai) Ltd, Thailand) before cutting into small pieces of approximately one inch long and dry heating in an oven at 60 ºC. All of the dry materials were kept in a sterile bottle (20% of moisture) containing a small bag of anti-moisture silica-gel pills. VC extract was prepared from this experiment by following a previous study (**Leelarungrayub *et al.*, 2010**) with a water-extracted protocol, as used generally in an anti-smoking clinic. One-hundred and thirty milliliters of VC condensed juice was prepared by mixing the dry materials (stem, leaf, or flower part) with clean water (20:390, *w;v*) and boiling in a traditional pot until the water evaporated. Extracts were prepared from each 130 milliliters of VC condensed juice by the freeze drying technique (LYOMASTER^{TM}, Germany).

3.3 Antioxidant activities and active compound *in vitro* assays

3.3.1 Antioxidant activities *in vitro*

3.3.1.1 Total antioxidant capacity (TAC)

Total antioxidant capacity of each VC extracts from the stem, flower, and leaf was assayed by the ABTS cation radical decolorization method (**Re *et al.*, 1999**). Stock ABTS cation radical was produced by activating 2, 2-Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) (14 mmol/L) with potassium persulfate (14 mmol/L), and leaving in the dark overnight. The working ABTS cation radical was diluted from stock ABTS radicals with deionized water until starting absorbance at 0.7 ± 0.02 before adding extract solution. 10 µL of extract solution; from the stem, flower, or leaf (10 mg/mL) was added to 990 µL of working ABTS cation radical solution in a plastic cuvette (size 1.5 mL), and gently alternated inversely 9 times before adding to

the spectrophotometer at 734 nm by spectrophotometry. Decreased absorbance was recorded continuously every 1 min for 3 min and finally calculated to ΔA/min, automatically by spectrophotometry. Total antioxidant capacity (TAC) of the VC extract was calculated by comparing with the ΔA/min of standard Trolox.

3.3.1.2 Nitric oxide scavenging assay

Nitric oxide was evaluated by a Griess reagent by following the Promega instruction (**Sumanont *et al.*, 2004**). Firstly, 0.2 mL of extract solution (at different concentrations of between 20-100 mg/mL) was added to 1.8 mL of sodium nitroprusside (SNP) and kept at room temperate (25 °C) for 180 min. After that, 1 mL of solution was mixed with 1 mL of 0.1% of N-1-napthylethylenediamine dihydrochloride (NED) in water and left in the dark for 5 min. Then, mixed with 500 µL of 1% sulfanilamide (Sulfa) in 5% phosphoric acid and kept in the dark for 5 min. After that, a slightly pink color was produced. Nitric oxide was calculated by comparing with the absorbance of standard sodium nitrite ($NaNO_3$) by spectrophotometry at 540 nm. The result presented in an inhibitory concentration at 50% (IC50).

3.3.1.3 Superoxide radical scavenging assay

Superoxide radical scavenging activity was evaluated following the Nakamura's protocol (**Nakamura *et al.*, 1998**). Firstly, 100 µL of extract solution (at different concentrations of between 20-100 mg/mL) was added to 760 µL of hypoxanthine (1.1 mmol/L), 100 µL of nitroblue tetrazolium (NBT) (300 µmol/L) and 40 µL of xanthine oxidase (1.67 U/mL). After that, the absorbance change was recorded continuously every 15 seconds as well as an absorbance reading by spectrophotometry at 560 nm. Finally, the result was represented in an inhibitory concentration at 50% (IC50).

3.3.1.4 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was evaluated following the Schleriser's protocol (**Schleriser *et al.*, 2002**). Firstly, 200 µL of KH_2PO_4-KOH (100 mmol/L) was added to 200 µL of deoxyribose (15 mmol/L), 100 µL of ethylene diaminetetraacetic acid **(**EDTA**)** (1 mmol/L), 100 µL of H_2O_2 (10 mmol/L), 100 µL of extract solution (at different concentrations of between 20-100 mg/mL) and 100 µL of

ascorbic acid (1 mmol/L). After being kept at room temperate (37 ºC) for 60 min, 1 mL of thiobarbituric acid (TBA) (1%) with trichloroacetic acid (TAC**)** (2.8%) was added before boiling at 80 ºC for 20 min. A slightly pink color was produced with an absorbance reading by spectrophotometer at 532 nm. Finally, the results represented in an inhibitory concentration at 50% (IC50).

3.3.2 Active compounds

3.3.2.1 Total phenolic

The total content of phenol in each VC extracts was determined by the previous study (**Singleton and Rossi, 1965**). Fifity µL of extract solution (20 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteau reagent and kept in the dark for 10 min before 500 µL of 20% sodium carbonate was added. After that, the tube was incubated for 2 hours, and the absorbance of clear supernatant from short high speed centrifugation for 3 min was read by spectrophotometry at 765 nm. The total phenolic content was calculated by comparison to standard gallic acid.

3.3.2.2 Total tannin assay

The total tannin content in different VC extracts was determined by modification of the previous protocol (**Polshettiwar *et al.*, 2007**). The extract (100 µL) was mixed with 0.5 mL of Folin-Denis reagent and Na_2CO_3 (0.5% *w;v*) solution and up to 5 mL of distilled water. After mixing for up to 30 min, absorbance had measured by spectrophotometry at 755 nm. The total tannin was expressed as equivalent to tannic acid (g TE) at one gram of extract.

3.3.2.3 Catechins

All catechin compounds such as EGC, C, EGCG, EC and ECG were analyzed using high-performance liquid chromatography (HPLC) by following the previous protocol (**Bronner and Beecher, 1998**). All compounds were separated on a RP-C18 column with a mobile phase of water/acetonitrite/methanol/ ethyl acetate/ glacial acetic acid (89:6:1:3:1, *v:v:v:v:v*) at a flow rate of 0.7 mL/min. Catechin compounds were detected by measuring UV absorbance at 280 nm, and those in each VC extracts had calculated by comparing the absorbance of each standard; i.e. catechin

(C), epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epigallocatechin (EGC), respectively **(Figures 5.2-5.6)**.

3.3.2.4 Isoflavone

Isoflavone as daidzin and genistin were analyzed using high-performance liquid chromatography (HPLC) by following the protocol of Khoo (**Khoo and Ismail, 2008**). All compounds of isoflavone in each extracts at 5 g were extracted in 10 mL of acidified methanol at 1% HCl and refluxed at 70 ºC for 2 hours before evaluated with HPLC. Daidzin and genistin compounds were separated on a RP-C18 column with a mixed solution of acetonitrite, methanol and 0.2 M ammonium acetate buffer (pH 4.6) (10:50:40, *v:v:v*) as a mobile phase at a flow rate of 1.0 mL/min. Isoflavone compounds were detected under UV absorbance at 260 nm. Each isoflavone compounds were calculated by comparing with the absorbance of each standard daidzin or genistin **(Figures 5.8-5.9)**.

3.3.2.5 Flavonoid

Flavonoid as Quercetin, Kaempferol and Myricetin were analyzed using high-performance liquid chromatography (HPLC) by following the protocol of Tokusoglu (**Tokusoglu *et al.*, 2003**). Each extract at 5 grams had be acidified with 10 mL of methanol (1% HCl) and refluxed at 70 °C for 2 hours before measuring with HPLC. Each compound was separated on a RP-C18 column with a mixed solution of a acetonitrile and phosphate buffer (0.025M) (25:75/ V: V) as mobile phase at a flow rate of 1.2 mL/min. Each flavonoid compound was detected under UV absorbance at 266 nm, and calculated by comparing the absorbance of each standard quercetin, kaempferol and myricetin **(Figures 6.1-6.3)**.

3.3.2.6 Nitrite and Nitrate

Nitrite and nitrate were analyzed using high-performance liquid chromatography (HPLC) by following the protocol (**Lulla *et al.*, 1984**). Compounds were separated on an RP-C18 column with a mixed solution of (0.2%) sodium acetate and (2.5%) glacial acetic acid at a flow rate of 1.0 mL/min and identified at 313 nm. The standard references were sodium nitrite and potassium nitrate **(Figures 6.5-6.6)**.

3.3.2.7 Nicotine

Nicotine was evaluated using high-performance liquid chromatography (HPLC) by following the previous protocol (**Mangathyaru *et al.*, 2006**). Nicotine was identified on an RP-C18 column with a methanol as a mobile phase at a flow rate of 1.0 mL/min under UV absorbance at 260 nm. The nicotine concentration in each extracts was calculated by comparing to the standard nicotine **(Figure 6.8)**.

3.3.2.8 Caffeine

Caffeine was evaluated with high-performance liquid chromatography (HPLC) by following the Harris's protocol (**Harris, 1999**). Caffeine in each extracts was separated on an RP-C18 column with a mixed solution of 0.5% phosphoric acid and 40% methanol at a flow rate of 1.0 mL/min under UV absorbance at 280 nm. The concentration of caffeine in each extracts was calculated by comparing to the standard caffeine **(Figure 7.0)**.

3.4 Catecholamine and Oxidative stress assays

3.4.1 Animal preparation

This animal study protocol was approved by the Ethics Committee at the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand [CODE: TS54001]. Male and female wistar rats (aged 5-8 weeks, weighing 250-300 g) were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. They were housed in individual cages, and maintained in an ambient temperature of 24±1 ºC. All of the rats were selected by a simple sampling method and identity numbers were put on their tails. The rats had fasted for 16 hours before the experiment.

They were then divided randomly into six groups with 5 male and 5 female rats for (1) control with normal saline (0.9%), (2) only nicotine, (3) bupropion (150 mg/kg body weight), (4) leaf extract (10 g/kg body weight), (5) flower extract (10 g/kg body weight) and (6) stem extract (10 g/kg body weight). (**Table 3.1**).

Table 3.1 Grouping and the number of Wistar rats in the experiment.

Group	Test	Wistar rat		Total
		Male	Female	
1	DW + 0.9% normal saline (0.15 mL) (control group)	5	5	10
2	DW + nicotine (0.6 mg/kg/BW)	5	5	10
3	Bupropion (150 mg/kg/BW) + nicotine (0.6 mg/kg/BW)	5	5	10
4	Leaf extract (10 g/kg BW) + nicotine (0.6 mg/kg/BW)	5	5	10
5	Flower extract (10 g/kg BW) + nicotine (0.6 mg/kg/BW)	5	5	10
6	Stem extract (10 g/kg BW) + nicotine (0.6 mg/kg/BW)	5	5	10

Note: BW = body weight

Rats in group 1 were fed with distilled water and injected subcutaneously one hour later with 0.15 mL of normal saline (0.9%) daily for 20 days.

Rats in group 2 were fed with distilled water and injected sub-cutaneously one hour later with nicotine at 0.6 mg/kg body weight daily for 20 days.

Rats in group 3 were fed with bupropion (150 mg/kg body weight) and injected subcutaneously one hour later with nicotine at 0.6 mg/kg body weight daily for 20 days.

Rats in group 4 were fed with leaf extract at 10 g/kg body weight, and injected subcutaneously one hour later with nicotine at 0.6 mg/kg body weight daily for 20 days.

Rats in group 5 were fed with flower extract at 10 g/kg body weight and injected subcutaneously one hour later with nicotine at 0.6 mg/kg body weight daily for 20 days.

Rats in group 6 were fed with stem extract at 10 g/kg body weight and injected subcutaneously one hour later with nicotine at 0.6 mg/kg body weight daily for 20 days.

After 20 days, all of the rats (n=10) were water-deprived overnight before the blood was taken from the right ventricle to evaluate the catecholamine neurotransmitters (dopamine, noradrenaline and adrenaline) in plasma by a 3-CAT research ELISA ™ Kit. Moreover, 6 sampling fresh plasmas (3 males and 3 females) from 10 rats were

evaluated for total antioxidant capacity (TAC) (**Re *et al.,* 1999**), and malondialdehyde (MDA) (**Rumley *et al.,* 2004**).

3.4.2 Catecholamine assay

The method to determine all catecholamine neurotransmitters (dopamine, noradrenaline and adrenaline) in plasma was performed by following the guideline in the 3- CAT Research ELISA™ protocol. 500-750 µL of plasma, 30 µL of standard and 30 µL of control were filled in each well with distilled water to a final volume of 750 µL and added 50 µL of assay buffer with 50 µL of extraction buffer into all wells. Covered the plate with adhesive foil and shaked 60 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Removed the foil and emptied the plate. Then 1 mL of wash buffer into all wells and shaked 5 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Washed one more time and after added 150 µL of acylation buffer into all wells with 25 µL of acylation reagent into all wells. Shaked 20 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Emptied the plate and blot dried by tapping the inverted plate on absorbent material and 1 mL of wash buffer into all wells with shaking for 5 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Blot dried by tapping the inverted plate on absorbent material again and washed one more time. 200 µL of hydrochloric acid into all wells and covered plate with adhesive foil with shake 10 min at RT (20-25 °C) on an o shaker (approx. 600 rpm). Then, added 50 µL of enzyme solution to all wells in which contained of 190 µL of the standards, controls and plasma. Covered plate with adhesive foil and shaked 1 min at RT (20-25°C) on a shaker to mix. Incubated for 2 hours at 37°C. The following volumes of the supernatants were needed for the subsequent ELISA: adrenaline 75 µL, noradrenaline 75 µL and dopamine 75 µL.

Standard adrenaline, noradrenaline and dopamine preparation

Pipetted 75 µL of standards, controls and plasma from the enzyme plate into the respective pre-coated mikrotiter strips. 50 µL of the respective antiserum was loaded into all wells. Covered the plate with adhesive foil and incubated for 1 min at RT (20-25 °C) on a shaker. Incubated for 15-20 hours (overnight) at 2-8 °C. Removed the foil and discarded or aspirated the contents of the wells and washed each well 4 times thoroughly with 300 µL of wash buffer. Blot dried by tapping the inverted plate on

absorbent material. Added 100 µL of enzyme conjugate into all wells. Covered the plate with adhesive foil and incubated 30 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Removed the foil and discarded or aspirated the contents of the wells and washed each well 4 times thoroughly with 300 µL wash buffer. Blot dried by tapping the inverted plate on absorbent material. Then added 100 µL of substrate into all wells with incubate 20-30 min at RT (20-25 °C) on a shaker (approx. 600 rpm). Finally loading 100 µL of stop solution into all wells and read the absorbance within 10 minutes by using a microplate reader set to 450 nm.

3.4.3 Malondialdehyde (MDA) by TBARs

A modified version of the original protocol of Chirico (1994) used high-performance liquid chromatography (HPLC) (**Rumley *et al.*, 2004**), from which 200 µL of plasma was mixed with 750 µL of Ortho-phosphoric acid (2.5%, *v:v*) and vortexed. Then, 500 µL of 2- Thiobarbituric acid (TBA) (0.2 mol/L) in Tris (hydroxymethyl)-aminomethan (0.14 mol/L) was added. After incubation in a water bath (90°C) for 30 min, all samples were cooled and centrifuged at 10,000 rpm for 3 min. Twenty µL of samples was injected into the rheodyne valve with a 20 µL fixed loop, and the peak of MDA-TBA adduct was identified at 532 nm by a C-18 reverse-phase column under pure isocratic methanol (HPLC grade), with a flow rate of 1.0 mL/min. The MDA (µmol/L) was calculated by comparing with standard tetramethoxypropane (TMP).

3.4.4 Total antioxidant capacity (TAC)

The protocol for evaluated the TAC in plasma was following as same as the protocol of TAC evaluation in extract (**3.3.1.1**) (**Re *et al.*, 1999**).

3.5 Chromosome aberration assay

3.5.1 Animals preparation

After, all male and female wistar rats (aged 5-8 weeks, weighing 250-300 g) from the National Laboratory Animal Center, Mahidol University, were housed in individual cages, and maintained in an ambient temperature of 24±1 ºC for 24 hr. They were then divided randomly into six groups with each of 5 male and 5 female rats for a single dose administration with: (1) distill water as a control group, (2) stem extract at 2

g/Kg BW, (3) leaf extract at 2 g/Kg BW (4) flower extract at 2 g/Kg BW and (5) Cyclophosphamine at 50 mg/Kg BW (**Table 3.2**), before testing the chromosome abbreviation.

Table 3.2 Grouping and the number of Wistar rats in the experiment.

Group	Test	Wistar rat		Total
		Male	Female	
1	Distilled water (control group)	5	5	10
2	Stem extract at 2 g/Kg BW	5	5	10
3	Leaf extract at 2 g/Kg BW	5	5	10
4	Flower extract at 2 g/Kg BW	5	5	10
5	Cyclophosphamine at 50 mg/Kg BW	5	5	10

Note: BW = body weight

3.5.2 Mammalian bone marrow chromosome aberration test

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations in bone marrow cells of animals, usually rodents that induced by extracts, distilled water or cyclophosphamide (**Preston *et al.*, 1987; Tice *et al.*, 1994**). Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy indicates that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberration is of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes are involved in cancer in humans and experimental systems.

After 24 hours of single dose administration **(Table 3.2)**, colchicine was injected to arrest the chromosome. Then, the rats were sacrificed humanely by inhaling carbon dioxide, and their bone marrow cells were removed from the femur thigh bone in order to evaluate the chromosomal aberration (gap, break, exchange and multiple

aberrations) (**Ito *et al.*, 2001**). After femurs were removed, the femoral marrow cells were flushed out with 3 mL of HBSS medium for chromosome analysis **(Preston *et al.*, 1987; WHO, 1985).** The bone marrow cells were washed in hypotonic solution (0.075 mol/L of KCl) and fixed (glacial acetic acid: methanol, 1: 3 *v:v*). All of the slides were coded and stained with 10% Giemsa (**Preston *et al.*, 1987**), and normal cells in mitosis stage were counted and compared with the total of 1,000 cells on each slide, in order to present the percentage of mitotic index (%MI). Types of chromosome aberration such as gap, break, exchange and multiple aberrations were also scored and recorded **(Ito *et al.*, 2001).**

3.6 Statistical analysis

Data were expressed as mean and standard error of mean (Mean ± SEM). All parameters were compared statistically by independent measurement in analysis of variance (ANOVA) and the Bonferroni test in the statistical software package, SPSS for windows version 11. The level of significance was established at $p < 0.05$.

3.7 Data collection location

The study was conducted in Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, and the Biomechanics Laboratory on the 4th floor of the Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University.