CHAPTER 2

MATERIALS AND METHODS

Materials

1.Turmeric rhizome

2. Zedoary rhizome

3. Black polyethylene bags (Chalermsak Trading, Thailand), 8 inch width, 16 inch length and 0.1 mm thickness

4. Paper bags (Chalermsak Trading, Thailand), 8 inch width and 16 inch length, 0.15 mm thickness (80 gram)

5. Chemicals

5.1 Chemicals for the determination of total curcuminoids

5.1.1 Methanol (A.R.grade, Lab-Scan, Thailand)

5.1.2 Standard curcumin (A.R.grade, Fluka, Switzerland)

5.1.3 Tetrahydrofuran (A.R.grade, Lab-Scan, Thailand)

5.2 Chemicals for the determination of volatile oil content

5.2.1 Xylene (A.R. grade, J.T. Baker, U.S.A.)

5.3 Chemicals for the determination of moisture content

5.3.1 Toluene (A.R.grade, Lab-Scan, Thailand)

5.4 Solvents used for the extractions of powdered turmeric and zedoary rhizomes

5.4.1 95% Ethanol (commercial grade, L.B Science, Thailand) was pre-distilled before used.

5.5 Chemicals and media for determination of antibacterial activity

5.5.1 Dimethyl sulfoxide (A.R.grade, Merck, Germany)

5.5.2 0.5 McFarland standard (Appendix B)

5.5.3 0.85% NaCl (Appendix B)

5.5.4 0.1 M Phosphate buffer pH 4.5 (Appendix B)

5.5.5 Standard tetracycline (A.R.grade, Fluka, Switzerland)

5.5.6 Tetracycline 30 μg/disc (Oxoid, England)

5.5.7 Media (Appendix B)

5.5.7.1 Muller hinton agar (Merck, Germany)

5.5.7.2 Tryptic soy agar (Difco, France)

5.6 Chemicals for determination of antioxidant activity

5.6.1 Absolute ethanol (A.R. grade, Merck, Germany)

5.6.2 Butylated hydroxytoluene (A.R. grade, Fluka, Switzerland)

5.6.3 1,1-Diphenyl -2 - picrylhydrazyl (A.R.grade, Fluka, Switzerland)

6. Instruments

Instruments	Model	Company
Autoclave	HL-340	Hualey, Taiwan
Vortex	G-560E	Scientific, USA
Incubator	DIN 40050-IP 20	Memmert, Germany
Hot air oven	DIN 12880-KI	Memmert, Germany
pH meter	PHM 82	Radiometer, Denmark
Rotary vacuum evaporator	A-3S	Eyela, Japan
UV-vis spectrophotometer	Genesis5	Miltonroy, USA
UV-vis spectrophotometer	Spectro UV- vis RS	Labomed, USA
Dean stark apparatus	-	L.B. Science, Thailand
Azeotropic apparatus	-	L.B. Science, Thailand
Water bath	WB -14	Memmert, Germany

Methods

1. Quantitative analysis of curcuminoids and volatile oil in turmeric and zedoary rhizomes at different growth stages.

1.1 Quantitative analysis total curcuminoids in turmeric and zedoary rhizomes.

1.1.1 Preparation of plant materials.

Fresh rhizomes of turmeric and zedoary were collected from Krasasin district, Songkhla province at three stages of growth (6, 9 and 12 months). The rhizomes were washed with water, cut into slices about 0.5 cm thickness and dried in a hot air oven at 50 °C for 4 days. The dried rhizomes were pulverized and passed through a sieve No. 20.

1.1.2 Quantitative determination of total curcuminoids.

The analytical procedure was a UV-visible spectrophotometric method described in the Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998).

1.1.2.1 Preparation and quantitative analysis of standard curcumin solutions.

The preparation of standard curcumin solutions was performed by accurately weighing ten mg of standard curcumin (97% purity) and transferring to a 25 ml volumetric flask. Methanol was then added to dissolve the standard substance, and the volume adjusted with methanol and the sample mixed well to provide the stock solution with concentration of 0.39 mg/ml). From this stock solution, 20, 40, 50, 60 and 80 µl were accurately pipetted and transferred into five different 10 ml volumetric flasks, diluted to volume with methanol and shaken well to obtain 0.78, 1.56, 1.95, 2.34 and 3.12 x 10^{-3} mg/ml of standard solutions, respectively. The absorption intensities of the standard solutions were

measured at 420 nm and methanol was used as a blank. The calibration curve was plotted between absorbance and concentration of the standard solutions (see Appendix A, Figure A-1 and Figure A-2).

1.1.2.2 Preparation of samples and determination of total curcuminoids in samples.

Samples were prepared by accurately weighing 300 mg of powdered turmeric or zedoary rhizomes. This was transferred to 10 ml volumetric flasks, added and adjusted to volume with tetrahydrofuran. The mixture was set aside at room temperature for 24 hrs with frequent shaking. Each sample was assayed in triplicate (n=3). One ml of the clear supernatant solution was accurately pipetted and transferred into a 25 ml volumetric flask, diluted to volume with methanol and mixed well. One ml of this solution was pipetted and transferred into a 50 ml volumetric flask, diluted to volume with methanol and mixed well. The UV-spectrophotometric method was used for extinction of the test samples. The concentration of curcumin in the test samples was interpreted from the calibration curve (Appendix A, Figures A-1 and A-2) and the percentage of total curcuminoids was calculated as total curcuminoids content in the samples in the unit of % w/w.

1.2 Quantitative determination of volatile oil in turmeric and zedoary rhizomes.

1.2.1 Preparation of plant materials.

Preparations of plant materials were carried out using a similar procedure as previously described in section 1.1.1.

1.2.2 Determination of volatile oil content.

Volatile oil content was determined by a method described in the Thai Herbal Pharmacopoeia (method 7.3H) (Ministry of Public Health, 1998). Ten grams of the powdered turmeric or zedoary were accurately weighed and placed in a 500 ml round-bottomed flask, and one hundred ml of water was added into the flask with a few boiling chips. The flask was connected to a Dean stark apparatus (Figure 2-1). The Dean stark apparatus was filled with water to the standard line and 2.0 ml of xylene was added. The reflux condenser (Figure 2-2) was then attached to the Dean stark apparatus. The flask was heated at 130 – 150 °C and the distillate was collected at a rate of 2-3 ml per minute for 5 hrs. After cooling (about 1 hr), the total volume of the distillate was measured and subtraction of the volume of added xylene gave the volume of volatile oil obtained from plant material. The volatile oil content was calculated and expressed as a volume of the oil per one hundred grams of sample (% v/w). This experiment was carried out in triplicate.

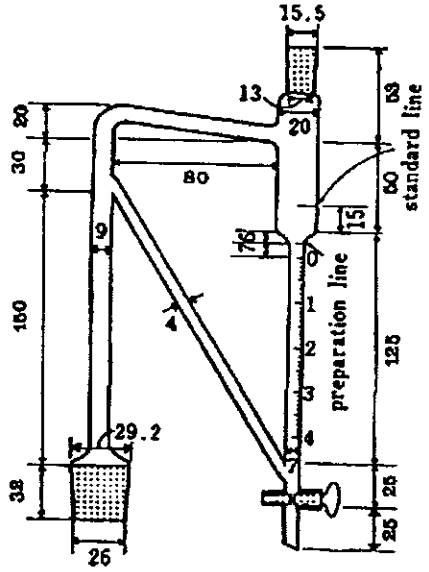

Figure 2-1 Dean stark of volatile oil steam distillation apparatus.

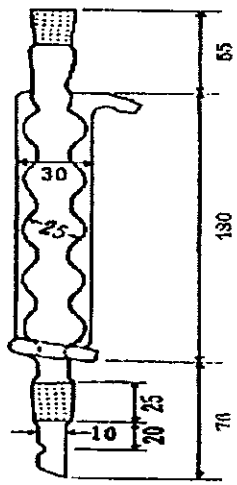

Figure 2-2 Reflux condenser of volatile oil steam distillation apparatus.

2. Quantitative analysis of total curcuminoids, volatile oil and moisture contents in turmeric and zedoary rhizomes during storage at room temperature and determination of theirs volatile oil compositions.

2.1 Determination of transmittance of light through black polyethylene and paper bags.

Pieces of black polyethylene and paper bags were attached in turn to spectrophotometer tubes. The transmittance spectra of the black polyethylene and paper bags was monitored spectrophotometrically by measuring transmittance from 200 to 800 nm.

2.2 Preparation and storage conditions of plant materials.

Fresh turmeric and zedoary rhizomes (12 months old) were collected from Phang-Nga and Songkhla province, respectively. The rhizome of zedoary was separated as bulb and finger. The rhizomes of turmeric and zedoary were washed with water, cut into slices about 0.5 cm thickness and dried in a hot air oven at 50 °C for 4 days. About half of the dried rhizomes were pulverized and passed through sieve No. 20. The dried sliced and powdered rhizomes were separately packed, either in the closed black polyethylene or paper bags and stored at room temperature (28-31 °C) over a 12-15 month period.

2.3 Quantitative analysis of total curcuminoids in turmeric and zedoary rhizomes.

The UV-visible spectrophotometric analytical procedure was as described in the Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998).

2.3.1 Preparation and determination of standard curcumin solutions.

Preparation and determination of the standard curcumin solutions were carried out as previously described in section 1.1.2.1.

2.3.2 Preparation of samples and quantitative determination of total curcuminoids in samples.

The dried sliced and powdered turmeric and zedoary rhizomes were stored in the closed black polyethylene and paper bags at room temperature over a 12-15 month period. Turmeric samples were assayed at zero time (i.e. before storage) and at 3, 6, 12 and 15 month periods. Zedoary samples were assayed at zero time, and at 3, 6, 9 and 12 month periods. The dried slices were ground to a fine powder and passed through a sieve No. 20., prior to weighing and determining total curcuminoids content. The assay procedure was as previously described in section 1.1.2.2.

2.4 Quantitative analysis of volatile oil content in turmeric and zedoary rhizomes.

The dried sliced and powdered rhizomes of turmeric and zedoary were prepared as previously described in section 2.3.2. The procedure for determining volatile oil content was as described in section 1.2.2.

2.5 Determination of volatile oil composition in turmeric and zedoary rhizomes.

The volatile oils obtained from powdered turmeric and zedoary rhizomes stored in paper bags before (zero time) and after 6 and 12 months storage were collected in glass vials and stored in a refrigerator at 4 °C until analysis. These oil samples were analyzed for theirs chemical compositions by gas chromatography - mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph coupled to a HP 5972 mass selective detector. Determination of volatile oil composition in these rhizomes at zero time and after 6 months storage, the systems were connected with a 15 m x 0.25 mm (i.d.) capillary column, 0.25 μ m film thickness and determination of volatile oil composition, after 12 months storage, the system was connected with a 30 m x 0.25 mm (i.d.) capillary column, 0.25 μm film thickness. Helium was used as a carrier gas. The column temperature was programmed from 100 to 320 °C at 7 °C/min. Temperature for GC injector was set at 250 °C.

2.6 Quantitative analysis of moisture content in turmeric and zedoary rhizomes.

The dried sliced and powdered turmeric and zedoary rhizomes were prepared as previously described in section 2.3.2 and their moisture content was determined by the azeotropic distillation method as described in the Thai Herbal Pharmacopoeia (method 4.12) (Ministry of Public Health, 1998). Two hundred ml of toluene and 2 ml of water were introduced into a dry 500 ml flask with a few boiling chips. The flask was then attached to the azeotropic apparatus as shown in Figure 2-3. For determination of water, the flask was heated at 130 – 150 °C for 2 hrs and allowed to cool afterward for 1-2 hrs. The water volume was measured. Twenty-five grams of the powdered rhizomes of turmeric or zedoary were placed in a 500 ml flask and heated gently for 15 minutes. The distillate was collected at the rate of 2 drops per second until most of water was distilled over and the rate of distillation was then increased to about 4 drops per second. The inside of the condenser was rinse with toluene and the flask was continuously heated for 5 minutes. After cooling (about 1 hr), the water volume was measured, and moisture content then calculated and expressed as milliliter of water per one hundred grams of sample (% v/w).

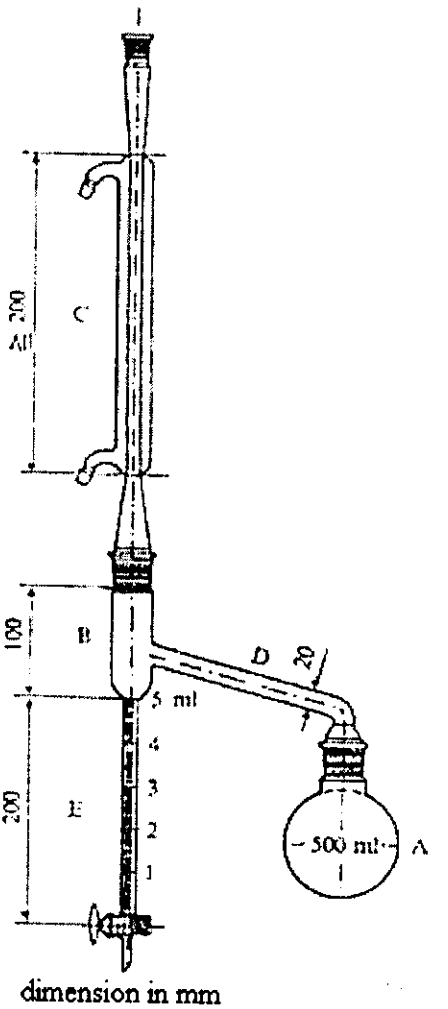

Figure 2-3 Azeotropic apparatus for determination of moisture content.

3. Quantitative analysis of total curcuminoids of turmeric and zedoary during storage at accelerated temperatures.

A study of the stability of curcuminoids under accelerated condition

was performed using the International Conference on Harmonization (ICH) guidelines and the method described in the text Drug Stability (Carstensen and Rhodes, 2000; ICH, 2000).

3.1 Determination of transmittance of light through black polyethylene and paper bags.

Determination of transmittance of light through black polyethylene and paper bags was performed as previously described in section 2.1.

3.2 Preparation and storage conditions of plant materials at accelerated temperatures.

The dried sliced and powdered rhizomes of turmeric and zedoary were prepared as previously described in section 2.2. The rhizomes were separately stored in closed black polyethylene and paper bags and kept in a desiccator at 45°, 55° and 70 °C in a hot air oven, respectively. The desiccator contained a jar of saturated NaCl solution to provide an atmosphere of 75 ± 5% relatively humidity (RH) (adopted from the handbook Pharmaceutical Excipients, Wade and Weller, 1994).

3.3 Quantitative determination of total curcuminoids.

The samples at each storage periods were taken from the desiccator on days 0, 7, 14, 28, 45, 60 and 90, and allowed to cool to room temperature. The dried slices were ground to a fine powder and passed through a sieve No. 20 prior

to assay for total curcuminoids. The powdered rhizomes were then weighed and assayed for total curcuminoids as previously described in section 1.1.2.

4. Antioxidant activity assay of turmeric and zedoary rhizomes at different storage periods.

4.1 Preparation of turmeric and zedoary crude extracts.

Two hundred grams of powdered turmeric, zedoary (bulb) and zedoary (finger) rhizomes were accurately weighed and packed in black polyethylene bags before (zero time) and after 6 and 12 months storage were placed in three different 1000 ml flasks. Five hundred ml of 95% ethanol was then added. The content thoroughly mixed and allowed to stand overnight at room temperature. The samples were then filtered, and the extracts evaporated to dryness under reduced pressure using a temperature of 40 °C to give the dried ethanolic extracts (about 24 – 100 g). These dried extracts were then reconstituted in absolute ethanol (see section 4.2) for determination of antioxidant activity.

4.2 Measurement of radical scavenging activity.

Antioxidant activity of turmeric and zedoary ethanolic extracts was assayed according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. The assay procedure was modified from that described by Yamasaki *et al.* (1994). DPPH is considered as an unstable radical because of the paramagnetism conferred by its odd electron. Its solution (in absolute ethanol) has a deep violet

color and shows a strong absorption band at 520 nm. The DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule, in which the absorption vanishes and the resulting decolorization depends on the number of electrons taken up (Blois, 1958). A DPPH solution having concentration of $6x10^{-5}$ M was used in this study. Five mg of each crude extract was accurately weighed and transferred into a 5 ml volumetric flask, diluted to volume with absolute ethanol and shaken well to provide the stock solution (concentration 1 mg/ml). From this stock solution, 125 µl was accurately pipetted and transferred into a 5 ml volumetric flask, diluted to volume with absolute ethanol and shaken well to obtain 25 µg/ml of solution. Each sample was further diluted for 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (500 µl) was mixed with an equal volume of $6x10^{-5}$ M DPPH solution (in absolute ethanol) and allowed to stand at room temperature for 20 min. The absorbance (A) was then measured at 520 nm. Butylated hydroxytoluene (BHT), a well known synthetic antioxidant, was used in the same system as a positive standard. The radical scavenging activity of the extracts corresponded to the intensity of quenching DPPH. Radical scavenging activity was calculated from the following equation:-

$$\% \text{ Radical scavenging activity} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of DPPH solution without sample solution and A_{sample} is the absorbance of DPPH solution with sample solution.

5. Antibacterial activity assay of turmeric and zedoary rhizomes at different storage periods.

5.1 Preparation of turmeric and zedoary crude extracts.

Preparation of turmeric and zedoary crude extracts was performed as previously described in section 4.1.

5.2 Bacterial cultures.

Gram-positive bacteria, namely *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* and gram-negative bacteria, *Escherichia coli* ATCC 25922 used in this study were kindly provided by Miss Sopa Khamme, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical sciences, PSU. The cultures were maintained on Muller hinton agar (MHA) slant and sub-cultured monthly.

5.3 Preparation of inoculum.

Staphylococcus aureus ATCC 25923, *Bacillus subtilis* and *Escherichia coli* ATCC 25922 were transferred from MHA slants and inoculated onto TSA plates. The cultures were used as an inoculum after incubation at 37 °C for 18-24 hrs.

5.4 Testing of antibacterial activity.

The antibacterial activity of turmeric and zedoary extracts was evaluated using a disc-diffusion and agar dilution method. The assay procedures were modified from that described by Lorian (1996).

5.4.1. Disc-diffusion method.

Following the initial incubation on TSA plates, organisms were suspended in steriled 0.85% NaCl and their concentration equilibrated to a 0.5 McFarland standard (1×10^8 cfu/ml). Using a steriled cotton swab, each culture was spread onto a MHA plate (10-cm diameter) prior to the addition of the impregnated discs. The 6-mm sterile blank paper discs were then saturated with 10 µl of each extract per disc, corresponding to 1.3, 2.5, 5.0 and 10.0 mg of dried extracts (in DMSO). The impregnated discs were applied to the surface of the agar plates which had previously been inoculated with the above organisms. Tetracycline (30 µg/disc) and DMSO saturated discs were used as positive control and solvent control, respectively. The plates were incubated at 37 °C for 24 hrs, followed by the measurement of clear zone diameter of inhibition and expressed in mm. The experiment was carried out in triplicate.

5.4.2 Agar-dilution method.

The dried ethanolic extracts of turmeric and zedoary rhizomes were dissolved in DMSO to obtain the highest concentration of 1000 mg/ml. Each extract was further diluted for 10 concentrations (two-fold dilutions). Sixty µl of

each dilution was pipetted into tubes containing 6 ml melted MHA and mixed thoroughly. The mixtures were then poured into sterilized plates (6-cm diameter) and allowed to set and dry at room temperature. DMSO and tetracycline solutions were used as a positive and negative control, respectively. The preparation of tetracycline solutions was performed by accurately weighing 3.2 mg of standard tetracycline and transferring to a 10 ml volumetric flask. 0.1 N HCl was then added to dissolve the standard substance, and the volume adjusted with 0.1 N HCl and the sample mixed to provide the stock solution (concentration 320 µg/ml). One ml of this stock solution was mixed with 1 ml of 0.1 M phosphate buffer pH 4.5 (concentration 160 µg/ml). This tetracycline solution was further diluted for 8 concentrations (two-fold dilutions). Six hundred µl of each tetracycline solution were pipetted into the tubes containing 5.4 ml of melted MHA and mixed thoroughly. The mixtures were then poured into sterilized plate and allowed to set and dry at room temperature. Following the initial incubation on TSA plates, organisms were suspended in and their concentration equilibrated to a 0.5 McFarland standard (1×10^8 cfu/ml). Each suspension was then diluted with steriled 0.85% NaCl to 1×10^7 cfu/ml. Two µl of each culture was added to each MHA plate seeded with each tested sample. The plates were incubated at 37 °C for 24 hrs. After the specified time period, the resulting zone surrounding each plate was observed and interpreted as minimal inhibitory concentration (MIC) of the tested sample. Tests were done repeatedly three times to ensure reliability of the results.

6. Statistical analysis.

Data for curcuminoids, volatile oil and moisture contents from turmeric and zedoary rhizomes stored under different storage conditions at room temperature were subjected to analysis of variance (ANOVA) with Bonferroni adjustment and the mean comparisons were performed using completely randomized factorial design. Data for curcuminoids content from turmeric and zedoary rhizomes at different growth stages and data for IC_{50} values of ethanolic extracts of turmeric and zedoary rhizomes at different storage periods were subjected to analysis of variance and the Duncan's multiple range test was used to determine significant difference between means.