# CHAPTER 2
# EXPERIMENT

## 2.1 Plant Material

The whole plants of *Sphaeranthus africanus,* were collected at Ayutthaya during October 1997. The voucher specimens ( 085404-87-154) have been deposited in the herbarium of the Royal Forest Department, Bangkok, Thailand

## 2.2 General Procedure

Melting points were determined with a Fisher-John melting point apparatus and are uncorrected. Chromatotron equipment on Harrison Research Model 7924T was operated for certain separation. Thin Layer Chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck's, Kieselgel 60 $PF_{254}$). Column chromatography was performed on silica gel (Merck's, Kieselgel 60 G).

The IR spectra were recorded on Nicole impact 410 FT-IR. Mass spectrum was obtained on Fisson MS800 mass spectrometer. The $^{1}H$ and $^{13}C$-NMR spectra including 2D-NMR were preformed in deuterated chloroform (unless specified otherwise) with tetramethylsilane (TMS) as an internal reference on Fourier Transformed Nuclear Magnetic Resonance Spectrometer of a Bruker, model AC-F200 and a Joel, model JNM-A500.

## 2.3 Chemicals

All solvents used in this research were purified prior to use by standard methodology except for those ,which were reagent grade. Merck's silica gel 60 G Art 7734 (70-230 mesh) and silica gel 7749 60 $PF_{254}$ containing gypsum were used as adsorbent for column chromatography and chromatotron technique. The spots were

visualized with $I_2$ and/or 10% $H_2SO_4$ in ethanol after detecting with UV lamp (254 or 365 nm)

## 2.4 Chemical Test

### 2.4.1 Liebermann Burchard's Test[18]

To a solution of the sample to be tested (2-3 mg) in dried chloroform (0.5 ml.) was added a few drops of acetic anhydride, followed by one drop of concentrated sulfuric acid. If an unknown was steroid, the color would gradually change from pink to deep green. In case of an unknown was triterpenoid, the color would change to reddish pink.

### 2.4.2 Cyanidin test[19]

This is a test for flavonoid compound. To an alcoholic solution of the sample (2-3 mg) was added a few pieces of magnesium and 1-3 drops of concentrated hydrochloric acid, observed the color change. If unknown was flavone, flavonol or flavonone, the color would be reddish, deep red or purple red, respectively.

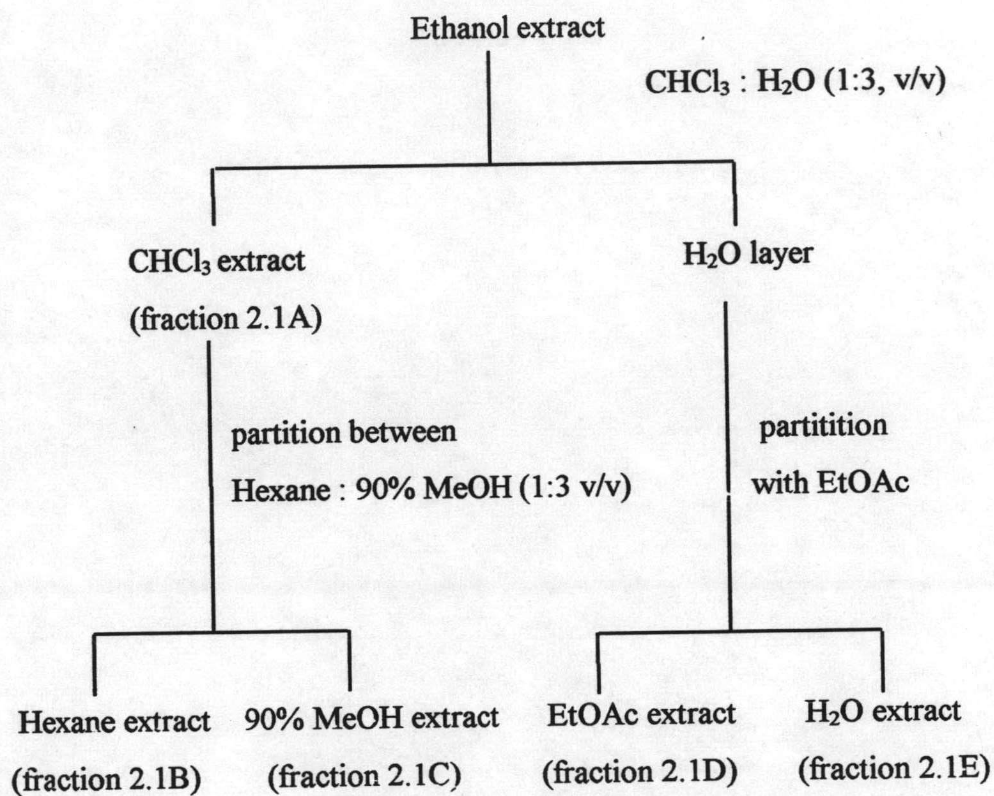
Other reagents for color tests used in this research, such as 2,4-DNP, 5% $FeCl_3$ and $Br_2$ in $CCl_4$ were carried out and observed by following the procedure as described in the textbook of Practical Organic Chemistry[20] and the systematic Identification of Organic Compound[21].

## 2.5 Extraction

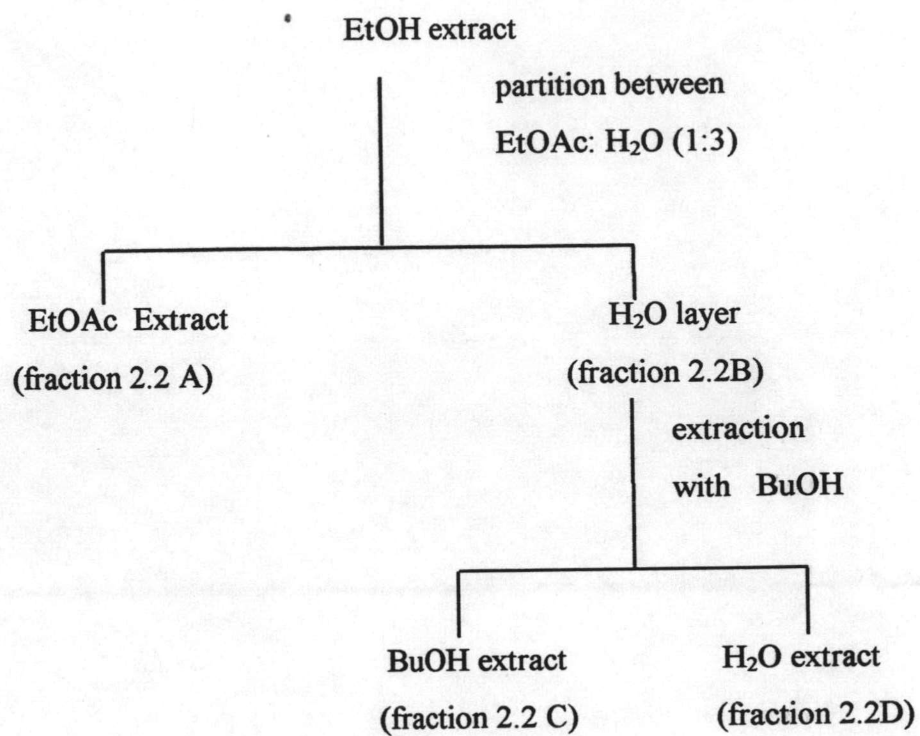
### 2.5.1 Extraction for Preliminary Screening Test

The whole plant of *Sphaeranthus africanus.* was sun-dried and minced to coarse pieces. The sample approximately 1 kg, was extracted by soaking in ethanol for three days at room temperature. The extraction was repeated for several times until the color of the last extract was clear. The solution was filtered and the solvent was evaporated, yielding an ethanol crude extract. The ethanolic extract (each 50 g ) was further extracted according to two extraction procedures (Scheme 2.1 and 2.2)

**Scheme 2.1** Extraction Procedure I



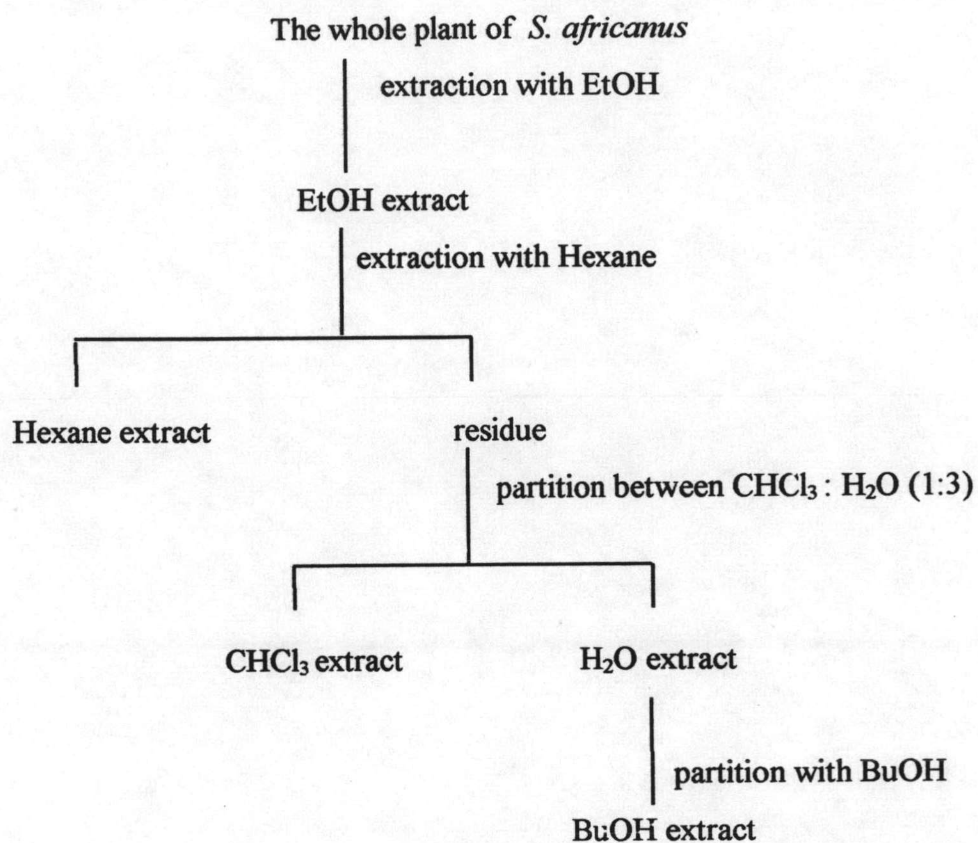
**Scheme 2.2** Extraction Procedure II



### 2.5.2 Extraction for Isolation

The sun-dried wholes plant of *S. africanus* was extracted with ethanol using the same above-mentioned procedure, giving the ethanolic crude extract. The ethanolic crude extract was further extracted with hexane. The residue was extracted by partition between chloroform and water in ratio 1:3 to gain a chloroform soluble fraction and a water soluble fraction. The water soluble fraction was extracted by partition with *n*-butanol to obtain a butanolic crude extract. The general scheme for the extraction is shown in Scheme 2.3

**Scheme 2.3** Extraction procedure III



## 2.6 Bioassay Experiment

Previously mentioned, one of the major goal is to search for bioactive compounds from *S. africanus*, which might possibly be used for agricultural and/or medicinal purposes. The following bioassay experiments were therefore performed.

### 2.6.1 Brine Shrimp Cytotoxicity Lethality Test[22,23]

A method utilizing brine shrimp *Artemia salina* is proposed as a simple bioassay for natural product research. The procedure determines $LC_{50}$ values in µg/ml of active compounds and extracts in brine shrimp medium. This method is rapid, inexpensive and convenient to guide phytochemical screening and fractionation. The general procedure follows the BSLT assay[24].

### 2.6.2 The Inhibitory Effect for Carcinoma Cell Lines

Several crude extracts from the whole plants of *S. africanus* were preliminary screened by using the MTT assay[25]. This method used seven Carcinoma Cell lines: Human Gastric Carcinoma (BGC-823), Human Hepatocellular Carcinoma (Bel-7402), Human Nasopharyngeal (KB), Human Leukemia Carcinoma (HL-60), Proliferation of Mouse (B) Lymphocyte, Proliferation of Mouse (T) Lymphocyte and Human Colon Carcinoma (HCT-8). These experiment were performed by researchers at Beijing Medical School, Beijing, China.

### 2.6.3 Cyclic AMP Phosphodiesterase Inhibition[26,27]

Cyclic AMP Phosphodiesterase (cAMP) has been used as a screening tool for the detection of biologically active substances and a variety of synthetic compounds. Therefore, the cAMP PDE can be used as a primary screening test for biological activity. Moreover, there are quite a number of enzymatic system that have been used as screening test for biological active substances, for example, rat liver cyclic AMP-dependent protein kinase protein (cAK), ariancalmodulin-dependent myosin light chain kinase (MLCK), rat brain $Ca^{2+}$-and embryo wheat embryo $Ca^{2+}$-dependent protein kinase (CDPK). The structure activity relations of natural occurring protein kinase

inhibitions may be useful for the development of synthetic chemotherapeutic agent that protein kinase inhibitors may be useful for the development of synthetic chemotherapic agents that are protein kinase inhibitor. cAMP-PDE assay solutions were as follows:

a) The enzyme solution contains phosphodiesterase (0.037 unit/ml), 5'nucleotidase (1.67 unit/ml), $MgCl_2$ (5 mM) and Tris-HCl(0.2 M)

b) The reaction mixture A contains malachite green (0.33 mM), polyvinyl alcohol (n = 1500)(3.87 g/l) and ammonium molybdate in 6NHCl (8.33 mM). The sample dissolved in 1.5% dimethyl sulfoxide (DMSO). The reaction was start by the addition of cAMP (10 mM, 100 μl) to the enzyme solution (400 ml) at 30 °C. After that, the sample solution (500 μl), the reagent mixture A (1.0 ml) and 25% sodium citrate(200 μl) were added to the above successively in every 5 min. The absorbance of the color complex was measured at 630 nm by UV-spectrophotometer against a mixed reagent blank. A calibration curve, obtained by this procedure using potassium dihydrogen phosphate solutions of known concentration, was used to determine the amount of phosphorus present in the assay. In the control experiment, DMSO was added instead of the solution of sample to minimize the effect the vehicle solvent. The effects of samples were compared with glycyrrhetic acid (reference compound for cAMP-PDE assay).