CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals As shown in appendix 1.

2.2 Preparation of shallot (Allium ascalonicum Linn.)

Shallot bulbs(Allium ascalonicum Linn.) were purchased from a local market in Chiang Mai, Thailand. The fresh samples of shallot bulbs were chopped into a small pieces, then freeze-dried by lyophilizer, ground to powder, and kept in a refrigerator for further study.

2.3 Preparation of sample extract.

One hundred grams of shallot powder were stirred with 600 ml absolute methanol at room temperature for 8 hours. In the following day the extract was again stirred, filtered through filter paper, and the remained on filter paper was again stirred with 300 ml absolute methanol for 4 hours. After filtration, the combined filtrates were evaporated to dryness in a vacuum rotatory evaporator at 50 °C. The dry residue was weighed and mixed with a known volume of distilled water, then centrifuged at 10,000 rpm for 20 inutes. The supernatant (water-soluble fraction) was saved for further study. The scheme of extraction method is shown in Fig. 2.

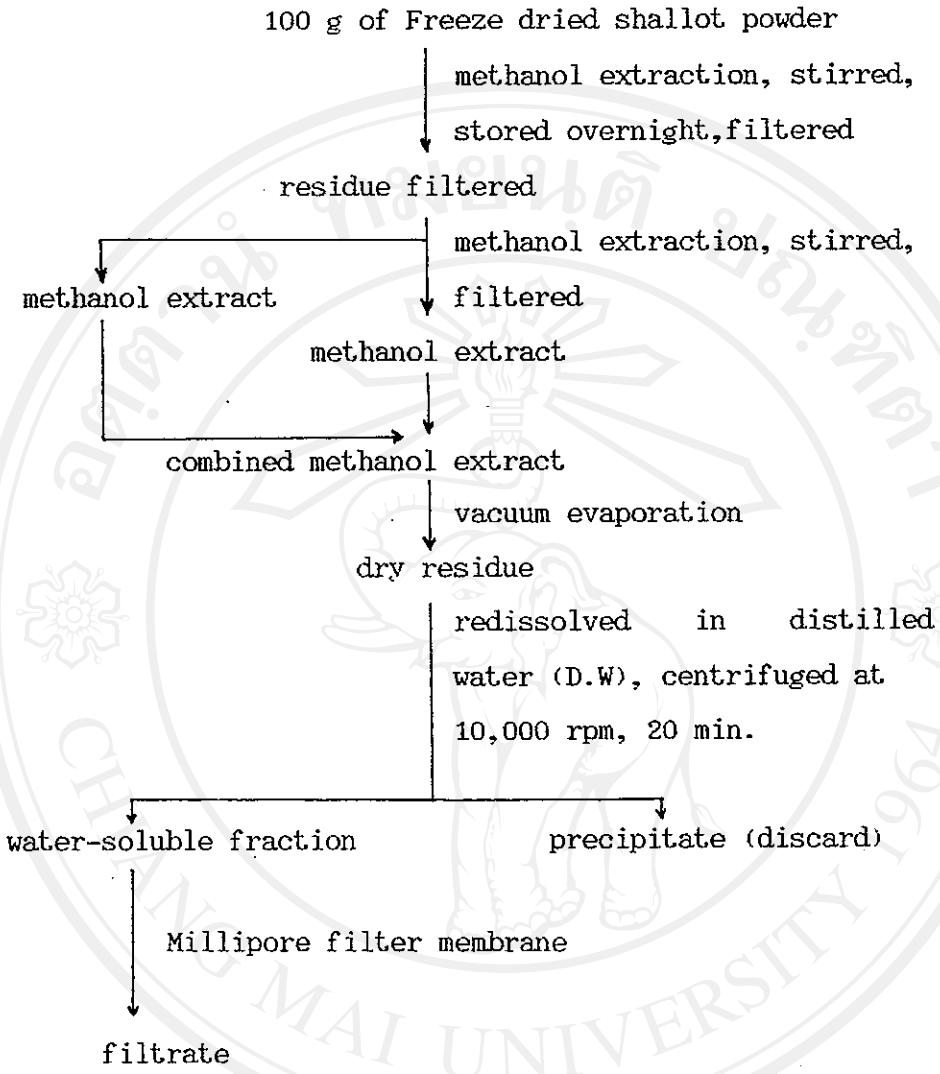

Fig.2 Scheme of sample extraction for mutagenicity assay.

2.4 Mutagenicity assay

The salmonella mutation, with preincubation technique described by Matsushima and his colleages (Mutsushima, T., et al, 1980), was used throughout this study. The assay were performed in either the presence or absence of S9 mix. The S9 mix was prepared by mixing 50 ul of the S9 fraction, obtained from the livers of rats pretreated with sodium phenobarbital, and 5,6-benzoflavone (β - napthoflavone) (Matsushima, T., et al, 1976), 2 μmole NADPH, 2 μmole NADH and 2.5 μmole G-6-P in a total volume of 0.5 ml.(See appendix 4)

2.4.1 Bacterial strains

The Salmonella typhimurium strains TA98 and TA100 were used. These tester strains were kindly given by Prof. Dr. Taijiro Matsushima, Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Japan. They were kept at -80° C until used. The genotypes of these tester strains were regularly checked to confirm the properties of the strains. Tester strains were cultured in Oxoid No.2 for 14 hours before tests.

2.4.2 Mutagenicity of shallot

The water-soluble fraction of methanol extract of shallot was sterized by passing through Millipore-filter membrane. The solution was then tested for its mutagenicity. The assay was performed as following ;

Procedure : Test materials (0.1 ml) were mixed with 0.5 ml of S9 mix or 0.5 ml of 100 mM sodium phosphate buffer, pH 7.4 and 0.1 ml of bacterial culture. The mixture was preincubated for 30 min at 30 °C, then 2 ml of molten top agar was added and the mixture was poured onto a minimal glucose agar plate. Revertant colonies were counted after incubation for 48 hours at 37 °C. The growths of tester strains in the background lawn of all plates were checked under the stereomicroscope and the toxicity was scored. Positive and negative control plates were included in this assay. The experiments were repeated twice and performed in triplicates for each concentration.

2.5 Modulation of shallot mutagenesis

Metabolic detoxification of chemicals may convert toxic metabolites to inactive, excretable products. Numerous xenobiotics have been shown to conjugate with glutathione or glucuronide in phase II conjugation reaction, resulting in subsequent changes in chemical toxicity (Hodgson, E. and Guthrie, F.E. 1980).

Thus this investigation was to determine the role of phase II : conjugation reaction in modulating the toxicity of shallot extract. Studies of the modulation of mutagenicity of shallot by chemicals, such as glutathione (GSH), glucuronide (UDPGA), cysteine and dithiothreitol(DTT), were performed. All tests were done in two separate experiments with three plates at each dose. Experimental design is shown in Fig. 3.

2.5.1 Effect of glutathione (GSH)

Procedure : Various concentrations of GSH (0.2 to 0.6 mmole/plate) were mixed with shallot extract in either the presence or absence of S9 mix (0.5 ml) and preincubated with 0.1 ml of bacterial culture at 30 °C for 30 minutes. After preincubation, 2 ml of molten top agar was added to the mixture and poured onto minimal glucose agar plate. After the plates were incubated at 37 °C for 48 hours, mutant colonies and toxicity were scored.

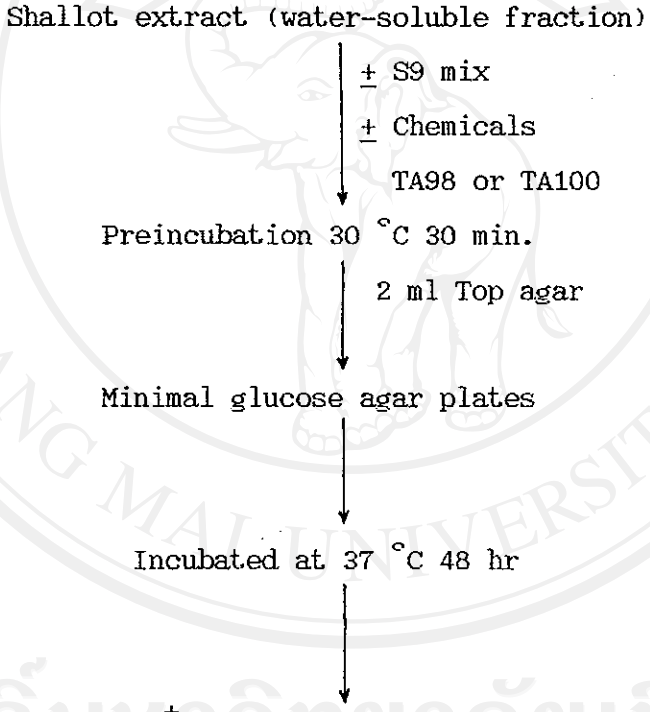

Fig.3 Scheme for study of mutagenicity or modulation of mutagenicity

2.5.2 Effect of uridine-5'-diphosphoglucuronic acid (UDPGA)

Procedure : Shallot extract was preincubated with UDPGA at various concentrations (1.0,2.0 and 4.0 mM), with and without S9 mix, and then mixed with bacterial suspension. The mixture was preincubated at 30 °C for 30 minutes, then 2 ml of top agar was added and poured onto the minimal glucose agar plates. The plates were scored for histidine revertants and toxicity after 48 hours incubation at 37 °C.

2.5.3 Effect of dithiothreitol (DTT)

Procedure : Dithiothreitol at concentrations of 1.25×10^{-2} to 5.0×10^{-2} mmole was studied for its ability to modify shallot extract mutagenesis using the same procedure as in 2.5.1

2.5.4 Effect of cysteine

Procedure: The same procedure as 2.5.1 was studied with cysteine at a concentration of 4.0×10^{-2} mmole instead of glutathione.

2.6 Effect of retinoic acid or ascorbic acid

Procedure : Retinoic acid at concentrations of 2×10^{-10} M to 2×10^{-6} M or ascorbic acid at 2×10^{-9} M to 2×10^{-5} M were studied for their ability to modify the mutagenicity of shallot.

2.7 Effect of incubating GSH with shallot extract prior to mixing with S9 mix.

Shallot extract was preincubated with GSH (0.4 mmole) at 37 °C for 30 minutes. The mixture was divided into two portions. The first one was tested its mutagenicity by standard pour plate (Ames test), another was tested by preincubation techniques. Flow chart of this method is shown in Fig. 4.

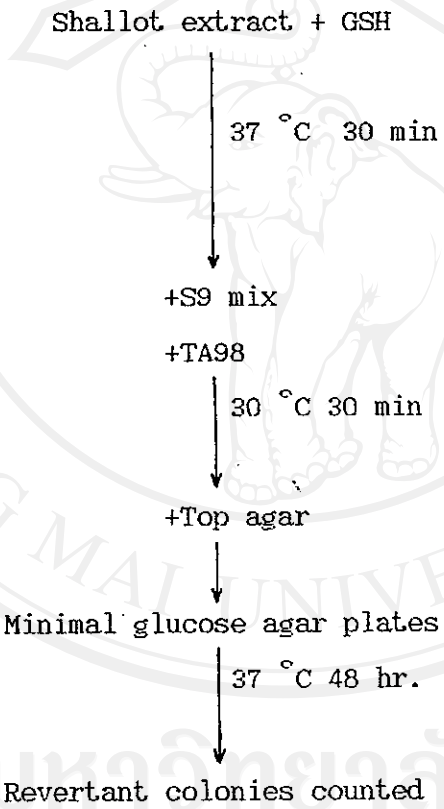

Fig.4 Flow chart of method used for studying effect of GSH on shallot extract prior to mutagenicity test.

2.8 Effect of nitrite treatment

Procedure : One ml of shallot extract was mixed with 1 ml of 0.1 M sodium nitrite. The pH of the mixture was adjusted to 3.0 with 6 M HCl. The mixture was incubated in the dark for 1 hour at 37 °C. Nitrosation reaction was stopped by an addition of 1 ml of 0.1 M ammonium sulfamate. The mixture was sterilized by passing through a 0.45 µm Millipore filter membrane. Aliquots (50 µl to 200 µl) of the filtrate were tested for mutagenicity by Salmonella mutation assay with the preincubation technique, using TA98 and TA100 as tester strains.

2.9 Modulation of quercetin mutagenesis by some chemicals

Procedure : Standard quercetin at concentrations of 5 to 20 µg was mixed with different chemicals, i.e., glutathione ($4x10^{-2}$ mmole), or DTT ($2.5x10^{-2}$ mmole), or cysteine ($4x10^{-2}$ mmole). The mutagenicity of the mixture was studied by Salmonella mutation assay, preincubation technique, using TA98 as tester strain.

2.10 Isolation and partial purification of mutagenic substances in shallot extract

The active mutagenic substances were isolated and partially purified from methanol extract of shallot by column chromatography as following ;

2.10.1 Chromatographic fractionation by Bio-gel P-10 column

Previously, it was demonstrated that the active mutagenic substance in shallot extract might be a compound with a low molecular weight of not more than 500 (Vinitketkumnuan, U. unpublished data). Therefore, after evaporation of the methanol extract to dryness, the extract was dissolved in distilled water and applied to a 2x45 cm. Bio-gel P-10 column. The substances were eluted with distilled water at the flow rate of 1 ml per minute. Fractions of 3 ml were collected. After the 70^{th} fraction, the eluent was changed to 50% methanol successively, with the same condition. The eluate was monitored by absorption at 370 nm.

Each fraction was dried under vacuum, and residues were dissolved in water and assayed for mutagenicity toward TA98 with metabolic activation.

2.10.2 Chromatographic fractionation by SEP-PAK cartridges

SEP-PAK is a commercially available uBonda Pak C_{18} cartridge column (Waters) which can be used for the rapid of determination of a suitable eluent for chromatography. The cartridge was pre-wet firstly with methanol and then water before applying the shallot extract. One ml of shallot extract was applied to the pre-wetted cartridge and eluted using the highest polarity solvent followed by weaker polarity solvents, i.e., distilled water, 15% methanol, 30% methanol, 50% methanol, 100% methanol, acetone and hexane, respectively.

Each fraction was dried and determination of mutagenic activity to TA98 with and without metabolic activation.

2.10.3 Partial purification by Sephadex LH-20 column chromatography.

The 50% MeOH eluent and 100% MeOH eluent from SEP-PAK separation contained mutagenic activity. Those fractions were further purified by rechromatography on 2x45 cm. Sephadex LH-20 column and were eluted with 100 % MeOH at a flow rate of 3 minutes per fraction. Fractions of 4 ml were collected and monitered at 280 nm and 370 nm. The mutagenic peaks were pooled from 5 chromatographic runs and rechromatographed on a second Sephadex LH-20 column (2x45 cm.) and again eluted with methanol. The partially purified mutagenic peaks were pooled again, and after drying the material was finally purified by Sephadex LH-20, 1x18 cm., and eluted with methanol at a flow rate of 0.8 ml per minute.

2.10.4 Characterization of the partially purified mutagenic substance(s)

2.10.4.1 R_f value on Silica gel 60 G thin-layer chromatography

The partial purified peak was subjected to Silica gel 60G thin layer chromatography with two different solvent systems as following ;

First system ; Chloroform:ethanol:butanone:acetyl acetone (16:10:5:1)

Second system ; Chloroform:methanol:water(65:45:12)

The R_f values of the partially purified peaks were determined and compared to authentic standards, quercetin and kaempferol.

2.10.4.2 Co-chromatographed with authentic standard on Sephadex LH-20 column.

The retention time of the partial purified mutagenic peak was compared to the authentic standard on Sephadex LH-20 column, 1x18 cm., eluted with 100% MeOH.

2.10.4.3 UV Spectra pattern

The ultraviolet absorption spectra of the partial purified peak was measured on a Beckman UV-spectro-photometer equipped with a recorder. The substance was dissolved in methanol, and the spectrum of the methanol solution was measured at the normal scan speed (100 nm per minute) using 2 ml of the solution.

The concentration of the solution was adjusted so that the optical density of the major absorption peak gave an optical density reading in the region of 0.2 to 1.0.