CHAPTER II

LITERATURE REVIEWS

Cancer is basically a disease of cells characterized by a shift in the control mechanisms that govern cell proliferation and differentiation. Cells that have undergone neoplastic transformation usually express cell surface antigens that may be of normal fetal type, may display other signs of apparent immaturity, and may exhibit qualitative or quantitative chromosomal abnormalities, including various translocations and the appearance of amplified gene sequences. Such cells proliferate excessively and form local tumors that can compress or invade adjacent normal structures. Quantitative abnormalities in various metabolic components also occur in tumor cells. The invasive and metastasis process as well as a series of metabolic abnormalities resulting from the cancer cause illness and eventual death of the patient unless the neoplasm can be eradicated with treatment *(4)*.

2.1 Causes of cancer

The incidence, geographic distribution and behavior of specific types of cancer are related to multiple factors, including sex, age, genetic predisposition and exposure to environmental carcinogens. Of these factors, environmental exposure is probably the most important. Chemical carcinogens (particularly those in tobacco smoke) as well as azo dyes, aflatoxins, asbestos and benzene have been clearly implicated in cancer induction in humans and animals *(4)*.

Oncogenes and tumor suppressor genes

Certain viruses have also been implicated as causative agents in animal cancers and are responsible for some human cancers as well. Oncogenic RNA viruses all appear to contain a reverse transcriptase enzyme that permits translation of the RNA message of the tumor virus into the DNA code of the infected cell. Thus, the information governing transformation can become a stable part of the genome of the host cell. Some cellular genes are homologous to the transforming genes of the retroviruses, a family of RNA viruses. These genes, known as oncogenes, have been shown to code for specific

growth factors and their receptors and may be amplified (increased number of gene copies) or modified by a single nucleotide in malignant cells *(4)*.

Another class of genes, tumor suppressor genes, may be deleted or damaged, with resulting neoplastic change. A single gene in this class, the p53 genes, has been shown to have mutated from a tumor suppressor gene to an oncogene in a high percentage of cases of several human tumors, including liver, breast, colon, lung, cervix, bladder, prostate and skin. The normal wild type form of this appears to play an important role in suppressing neoplastic transformation; mutation in this gene place the cell at high risk *(4)*.

At present, there are three main approaches for treating established cancer, surgical excision, irradiation and chemotherapy, and the role of each of these depends on the type of tumor and stage of its developments. Chemotherapy is the main method of treatment for only a few cancers but it is increasingly used as an adjunct to surgery or irradiation for many types of tumor cancers *(17)*.

2.2 Chemotherapy

Chemotherapeutics can be used to cure cancer, prevent its spread, relieve cancer symptoms or prolong survival. For patient with a systemic cancer, such as leukemia, chemotherapy can be curative. In other patients, these drugs can be used as adjunct therapy based on the premise that micrometastasis, although undetectable, exist. In other patients with an advanced neoplastic disorders, chemotherapy can be palliative to reduce tumor size and relieve pain and symptoms *(18)*.

Chemotherapeutic drugs can be divided into *(4,17-18)*:

1.Polyfunctional Alkylating Agents

Alkylating agents contain a bisamine, ethyleneimine, or nitrosourea moiety. They exert cytotoxic effects via transfering of their alkyl groups to various cellular constituents. Alkylations of DNA within the nucleus probably represent the major interactions that lead to cell death. The major site of alkylation within DNA is N7 position of guanine. These interactions can occur on a single strand or on both strands of DNA through cross-linking, as most major alkylating agents are bifunctional, with two reaction groups. Cross-linking of DNA appears to be

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

the major cytotoxic action of alkylating agents, and replicating cells are most susceptible to these drugs. Thus, although alkylaing agents are not cell cycle-specific, cells are most susceptible to alkylation in late G_1 and S phase of the cell cycle and express block in G_2. The major toxicity of alkylating agents is on the bone marrow and results in dose related suppression of myelopoiesis. Most alkylating agent used widely in clinic are nitrosoureas, procarbazine, dacarbazine and cisplatine.

2. Antimetabolites.

Antimetabolites structurally resemble DNA base pairs, these drugs can become involved in the synthesis of nucleic acids and proteins. Antimetabolites differ sufficiently from the DNA base pairs to interfere with this synthesis. Because the antimetablites are cell cycle-specific and primarily affect cells that actively synthesize DNA, these drugs are referred to as S phase-specific. Antimetabolites, which are classified by the metabolite affected, include folic acid antagonist (e.g. methotrexate), purine antagonists (e.g. mercaptopurne, thioguanine, fludarabine phosphate and cladribine) and pyrimidine antagonists (e.g. fluorouracil, capecitabne, cytarabine and gemcitabine). Toxic effects are observed in the bone marrow, skin and gastrointestinal mucosa.

3. Anticancer Antibiotics

All of the anticancer antibiotics are products of various strains of the soil microbe *Streptomycin*. They exert their cytotoxic action through four major mechanisms. These are 1) inhibition of topoisomerase II; 2) DNA intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission; 3) binding to cellular membrane to alter fluidity and ion transport; and 4) generation of semiquinone free radicals and oxygen free radicals through an enzyme-mediated reductive process. Anticancer antibiotics include the antracyclines, mitoxantrone, dactinomycin, mitomycin and bleomycin. The major toxicities are bone marrow suppression and cardiotoxicity.

4. Hormonal and antihormonal agents

Steroid hormones and antisteroid drugs are actively involved in the stimulation and control of proliferation and function of certain tissues. Sex

hormones or their antagonists are most effective in tumors arising from cells that are normally hormone dependent, namely breast and prostate. Drugs in this class affect malignant cells either directly by having cytotoxic action or indirectly by suppressing the production of other hormones via feedback mechanism.

5. Plant Alkaloids

5.1 Vinca alkaloids

Vinca alkaloids are derived from *Vinca rosea*, the periwinkle plant. Their mechanism of action involves depolymerizaton of microtubules, which are the part of the cytoskeleton and the mitotic spindle. The drug binds specifically to the tubulin in dimeric form. The drug-tubulin complex adds to the forming end of the microtubules to terminate assembly, and depolymerization of the microtubules then occurs. This results in mitotic arrest at metaphase. Several agents are use in clinic, such as vinblastine, vincristine and vinorelbine. The major toxicities are bone marrow suppression and alopecia.

5.2 Epipodophyllotoxin

Two compounds, etoposide and teniposide are semisynthetic derivatives of podophyllotoxin, which is extracted from the mayapple root *(Podophyllum peltatum)* . They block cell division in the late S-G2 phase of the cell cycle. Their primary mode of action involves inhibition of topoisomerase II, which results in DNA damage through stand breakage induced by the formation of a ternary complex of drug, DNA and enzyme.

5.3 Camptothecins

The camptothecins are natural products that are derived from The Camptotheca tree. They inhibit the activity of topoisomerase I, the key enzyme responsible for cutting and religating single DNA stands. Inhibition of the enzyme results in DNA damage. These drugs are use in clinically, such as topotecan and irinotecan. Myelosuppression and diarrhea are the two most common adverse events.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

5.4 Taxanes.

Paclitaxel and docetaxel are alkaloid esters derived from the Western yew *(Taxus brevifolia)* and European yew *(Taxus baccata)*. The drugs function as a mitotic spindle poison through high-affinity binding to microtubules with enhancement of tubulin polymerization. This promotion of microtubule assembly by paclitaxel occurs in the absence of microtubule-associated proteins and guanosine triphosphate and results in inhibition of mitosis and cell division. Their major toxicities are bone marrow suppression and peripheral sensory neuropathy.

6. Miscellaneous Anticancer drugs

6.1 Imatinib

Imatinib is an inhibitor of the tyrosine kinase domain of the Bcl-Abl oncoprotein and prevents the phosphorelation of the kinase substrate by ATP. It is used for the treatment of chronic myelogenous leukemia. The toxicities are nausea and vomiting.

6.2 Hydroxyurea

Hydroxyurea is an analog of urea whose mechanism of action involves the inhibiton of DNA synthesis in the S phase by inhibiting the enzyme ribonucleotide reductase, resulting in depletion of deoxynucleotide triphosphate pools. It is mainly used in chronic myelogenous leukemia. Myelosuppression is the dose-limiting toxicity.

6.3. Mitotane

This drug is a dichloro analog of the insecticide. It is used in the treatment of an adrenocortical carcinoma. The drug produces tumor regression and reduces the excessive adrenal steroid secretion. The toxicities are nausea and vomiting.

2.3 Problems of cancer therapeutic *(4,17-18)*

Although many drugs are effective for the treatment of cancer in many stages, but anticancer agents present many problems. The major problems are in the following:

2.3.1 Common toxicities; such as nausea, vomiting, alopecia, mucosal ulcerations, falling hair, diarrhea, fever, myelosuppression, immunosuppression.

2.3.2 Specific toxicities; such as

- hepatotoxicity from mercaptopurine asparagenase
- neurotoxicty from carboplatin, docetaxel
- nephrotoxicities from cisplatinetc, fluorouracil
- cardiotoxicity from daunorubicin,doxorubicin

2.3.3 Drug resistance; the drug resistance can be either primary resistance, absence of response on the first exposure or acquired resistance which develops in a number of drug-sensitive tumor types.

The problems of currently used anticancer drugs lead to development of new drugs. Many new anticancer drugs are derived both from traditionally methods by synthesizing analogs of currently used drugs or screening from natural resources, and from new technologies using DNA recombination and cloning techniques for producing monoclonal antibodies and cytokines. In Thailand, it has plenty of natural resources for medical used. There are many medicinal plants which are used in Thai traditional medicine. Some of them are claimed for treatment of cancer, including *Murdannia loriformis* (Hassk.) *(6), Brucea javanica* Linn.Merr *(7), Rhinacanthus nasutus* Linn.Kurz *(8)* and *Stephania venosa BL.* Spreng *(9-11).*

2.4 *Stephania venosa* (Bl.) Spreng

Stephania venosa (Bl.) Spreng belongs to the family of Menispermaceae. It has many local names including boraphet phung chang (southwestern); plao lueat khruea (northern); cho koe tho (karen / northern); kratom lueat (northeastern); kling klang dong (southwestern); boraphet yang daeng.

Figure 1. *Stephania venosa* (Bl.) Spreng tuber

This plant is also distributed in Vietnam, Malaysia, Sumatra, Java, Sabah, Celebes and Philippines. It is slender climber, containing red sap, entirely glabrous; leafy stems herbaceous, annual, arising from a large exposed rhizome, up to 40 cm diameter; perennial stems less than 1 m long and up to 4 cm diameter. Leaves broadly triangular-ovate, margin often slightly lobed (6-11 by 7-12 cm), base truncate or slightly cordate, apex obtuse and mucronulate, lower surface minutely papillose, glaucous, with reticulation usually drying reddish - brown; submembranous; petioles 5-15 cm. Male inflorescence is an axillary umbelliform cyme, 4-16 cm long. Male flowers are on pedicels (1-2 mm) which have 6 greenish sepals (2-2.5 mm long), usually unequal, outer 3 oblaneolate, inner 3 obovate, unguiculate. Petal 3, orange, obdetoid-obovate, 1.25 mm long. Synandrium is 1-1.75 mm long. Female inflorescences are much more condensed than the male, sometimes subcapitate. Female flowers very shortly pedicellate, asymmetrical. *Sepal* 1, with/without elliptic, 0.75 mm long. *Petals* 2, ± suborbicular, 0.75 mm long. *Carpels* subellipsoidal, 1.5 mm long. *Drupes* Obovate in outline, 7 mm long. Endocarp perforates, dorsally bearing 4 rows of 12-16 subcapitate projections *(8-11,19)*.

The tuber of this plant contains a wide variety of isoquinoline alkaloids, including, apoglaziovine, ayutianine, N-carboxamidostepharine, crebanine, 4-α-hydroxycrebanine, 7-oxocrebanine, dehydrocreanine, kamaline, kikemanine, nuciferoline, mecambroline, tetrahydroplamatine,β-n-oxide-stephadiolamine,4-α-hydroxy N-carboxamidostepharine, stepharinosine, o-methy stepharinosine, stesakine, sukhodianine, acetylsukhodianine, β-n-oxide-sukhodianine, thailandine, thairugosamine, β-n oxide ushinsunine, β-n oxide , 4-α- hydroxy ushinsunine, uthongine and etc *(20-21).*

The tubers of *S. venosa* are used as nerve tonic, aphrodisiac, appetizer, antiasthmatic, antimicrobial, hypoglycemic and anticancer agents in Thai folk medicine *(8,10,22-23).* Eight isoquinoline alkaloids; stephanine, crebanine, sukhodianine, dehydrocrebanine, dehydrostephanine, tetrahydropalmatine, jatrorhizine and stepharine, were identified from *S.venosa* which demonstrated antimalarial activity *(24).* The methanol extract from the root *S.venosa* at concentration of 0.1 mg/ml inhibited more than 90 % acetylcholinesterase (AchE) activity *(25).* The water and ethanol extracts from the tuber of *S.venosa* showed cytotoxic activity against brine shrimp with the IC_{50} at 184.9 and 90.8 μg/ml, respectively. The water extract was two times less active than the ethanol extract. The ethanol extract was exhibit cytotoxic activity against human cancer cell line MCF-7 (breast adenocarcinoma) with the IC_{50} at 11.6 μg/ml. Palmatine and crebanine purified from the ethanol extract, were found to possess pronounced cytotoxic activity against MCF-7 with IC_{50} at 5 – 6 μg/ml and against brine shrimp with LC_{50} value of 74.4 and 29.6 μg/ml, respectively *(16).*

The pharmacological activities of the water extract of *S.venosa* tuber on human PBMCs were also investigated. The extract had cytotoxic effect with the IC_{50} 300 μg/ml and antiproliferative effect with the IC_{50} 40 μg/ml on human PBMCs (15). It also possed apoptotic activity on these cells. The tuber of *S.venosa* has been used in Thai traditional medicine as anticancer agents in the forms of boiled solution, liquor and pills. It is interesting to evaluate the potency of anticancer effects in each formulation.

2.5 Methods for anticancer evaluation.

Cytotoxicity, antiproliferative and apoptosis activities are criteria used for screening any antitumor/anticancer agents. Screening methods for evaluating these activities are in the following.

2.5.1 Cell viability assessment methods

1. Trypan blue exclusion

Cell staining by trypan blue dye has been used as the standard method for determining cell viability. Viable cells can exclude trypan blue, while death cells are stained by this dye. Trypan blue exclusion is a rapid method for assessing cell viability and cell death in response to environmental insult *(26-27)*.

2. Propidium iodide staining

Cell death can be assessed using propidium iodide, a DNA intercalating agent. Viable cells can exclude propidium iodide, while the nuclei of dead cells are stained with this reagent *(28)*.

3. Carboxyfluorescein diacetate (CFDA) staining

CFDA is a fluorescent dye which selectively stains living cells. It diffuses into a cell where it is de-acetylated and then concentrated. Living cells are much more intensely stained with this dye than death cells *(29)*.

4. Neutral red staining

Neutral red stains viable cells. This dye is absorbed by viable cells and is concentrated in the lysosomes. Quantitation of neutral red staining is used in monitoring cytotoxicity and proliferation assays *(30-31)*. Result obtained when measuring viability by neutral red uptake correlate well with viability measurements by trypan blue exclusion *(31)*. As with determination of trypan blue exclusion, determination of percentage neutral red staining requires only a light microscope.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

5. Lactate dehydrogenase (LDH) assay

Released LDH is a biologically stable enzymatic marker that linearly correlates with cell death. Cell viability was determined by assaying the medium from each well for LDH activity. The assay is based on the reduction of NAD by the LDH-catalyzed conversion of lactate to pyruvate. Then reduced NAD (NADH) is utilized in the conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically *(32)*.

6. AlarmarBlue reduction

It is a method for measuring cell proliferation and cytotoxicity by using alamarBlue. As a tetrazolium salts alamarBlue monitors the reducting environment of the proliferation cell. AlamarBlue is soluble, stable and nontoxic in culture medium, therefore the continuous monitoring of cells in culture is permitted. Specifically, alamarBlue does not alter the viability of cells in cultures for various times as monitored by trypan blue exclusion *(33-37)*.

AlamarBlue contains a oxidation-reduction (redox) indicator. Cellular proliferation induces chemical reduction of the media which resulted in a change in redox color from blue to red. The intensity of red color reflected the extent of cellular proliferation. The reaction mixture was measured the optical density (OD) at 570 nm (reduced form) and 600 nm (oxidized form). Specific OD, obtained by subtracting the absorbance at 600 nm from that of 570 nm, was used in the calculation for cytotoxicity and proliferation *(33-37)*.

2.5.2 Cell Proliferation Assessment Methods.

1. ^{3}H-thymidine incorporation

^{3}H-thymidine incorporation into cell is a method for quantifying cell proliferation, reflected by DNA synthesis. DNA synthesis is measured

by incorporation of the radioactive exogenous precursor ^{3}H-thymidine into proliferating cells *(37)*.

2. Bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU), a thymidine analog, replaces ^{3}H-thymidine. BrdU is incorporated into newly synthesized DNA strands of actively proliferating cells. Following partial denaturation of double stranded DNA, BrdU is immunochemically allowing the assessment of the population of cells, which are actively synthesizing DNA *(38-40)*.

3. Tetrazolium salt reduction

Tetrazolium salt reduction, as an indicator of cell growth, has been used in models for screening cytocidal chemical agents *(41-42)* or cell growth promoting factors *(43)*. The internal environment of proliferating cells is in more reduced state than that of non-proliferating cells. This reduced state can be measured using tetrazolium salts. The most frequently used of these salts are MTT (3-(4,5-dimethylethiazol-2-yl) 2, 5 diphenylttrazolium bromide), XTT [sodium 3'-(1-phenylamino)-carbonyl]-3,4 tetrazolium-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate], and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyp)-2-(4-sulfophenyl)-2H-tetrazolium].

The yellow tetrazolium salts MTT is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan is nonsoluble in water and can be solubilized with DMSO or HCl/isopropanol *(44-47)*. XTT is based on the cleavage of the yellow tetrazolium salt, XTT, to form an orange formazan dye by mitochondrial dehydrogenase in metabolic active cells. It is soluble in culture medium. The use of XTT requires the presence of PMS (phenozine methosulfate) for efficient reduction *(48-49)*. MTS is an alternative tetrazolium salt which also has utility in cell proliferation assays. It is bioreduced by cells into a soluble formazan product. The conversion of MTS into aqueous soluble formazan is accomplished by

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

mitochondrial dehydrogenase enzymes found in metabolically active cells *(50-51)*.

2.5.3 Apoptosis assessment methods

1. Hoechst 33342 staining

Hoechst 33342 staining is useful for monitoring cell death by apoptosis because the dye selectively stains nuclei of apoptotic cells. The cells under investigation are stained with this compound and inspected microscopically. The pattern of staining is indicative of the state of the cell. Specifically, the chromatin of normal cells shows only faint staining while cells at the early stage of apoptosis display increased condensation and brightness, and cells in later stages of apoptosis exhibit chromatin condensation and nuclear fragmentation *(32)*.

2. TdT – mediated dUTP nick end labeling (TUNEL) assay

The TUNEL assay allows detection and quantification of apoptotic cell death. End-stage apoptotic is commonly characterized by nuclear condensation and fragmentation by endonucleases. In this technique, the 3' ends of the DNA fragments generated in apoptotic cells are labeled with biotin-coupled uridine by using the enzyme terminal dideoxynucleotidyl transferase (TdT). The biotin label is then detected with enzyme tagged streptavidin, which binds to biotin. When the colorless substrate of the enzyme is added to a tissue section or cell culture, it is reacted upon to produce a colored precipitate only in cells that have undergone apoptosis *(52)*.

3. Internucleosomal DNA fragmentation analysis

The fragmentation of nuclear DNA into mono- or oligomers of 180-200 base pairs is a biochemical hallmark of apoptosis in many cells. It is the result of an endogenous endonuclease activity capable of inducing double-stand breaks at internucleosomal sites. Apoptotic cell death can be evaluated qualitatively as a “ladder” of DNA by electrophoresis *(53)*.

4. Annexin V- Fluorescence isothiocyanate-conjugated (FITC)

In normal cells plasma membranes exhibit significant phospholipids asymmetry, with phosphatidylcholine and spingomyelin predominantly on the external leaflet, and most of the membrane's phosphatidylethanolamine and phosphatidylserine on the inner leaflet. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine (PS) from the cytoplasmic interface to the extracellular surface *(54)*. This loss of membrane asymmetry can be detected by utilizing the binding property of annexin V to PS. According to this property, annexin V labeled with FITC is widely used for apoptotic cell detection. Cells progressing though apoptosis are monitored according to their annexin V-FITC and propidium iodide (PI) staining pattern using fluorescent microscopy or flow cytometry *(55)*. Viable cells will be negative for annexin V FITC and PI. Staining cells that are induced to undergo apoptosis will be positive for Annexin V FITC but negative for PI. Both cells in later stages of apoptosis and necrosis will be positively stained for both annexin V FITC and PI *(56-58)*.

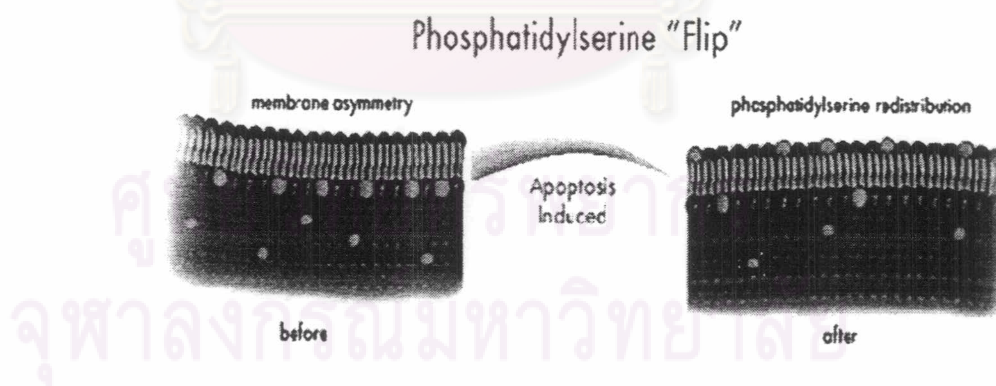
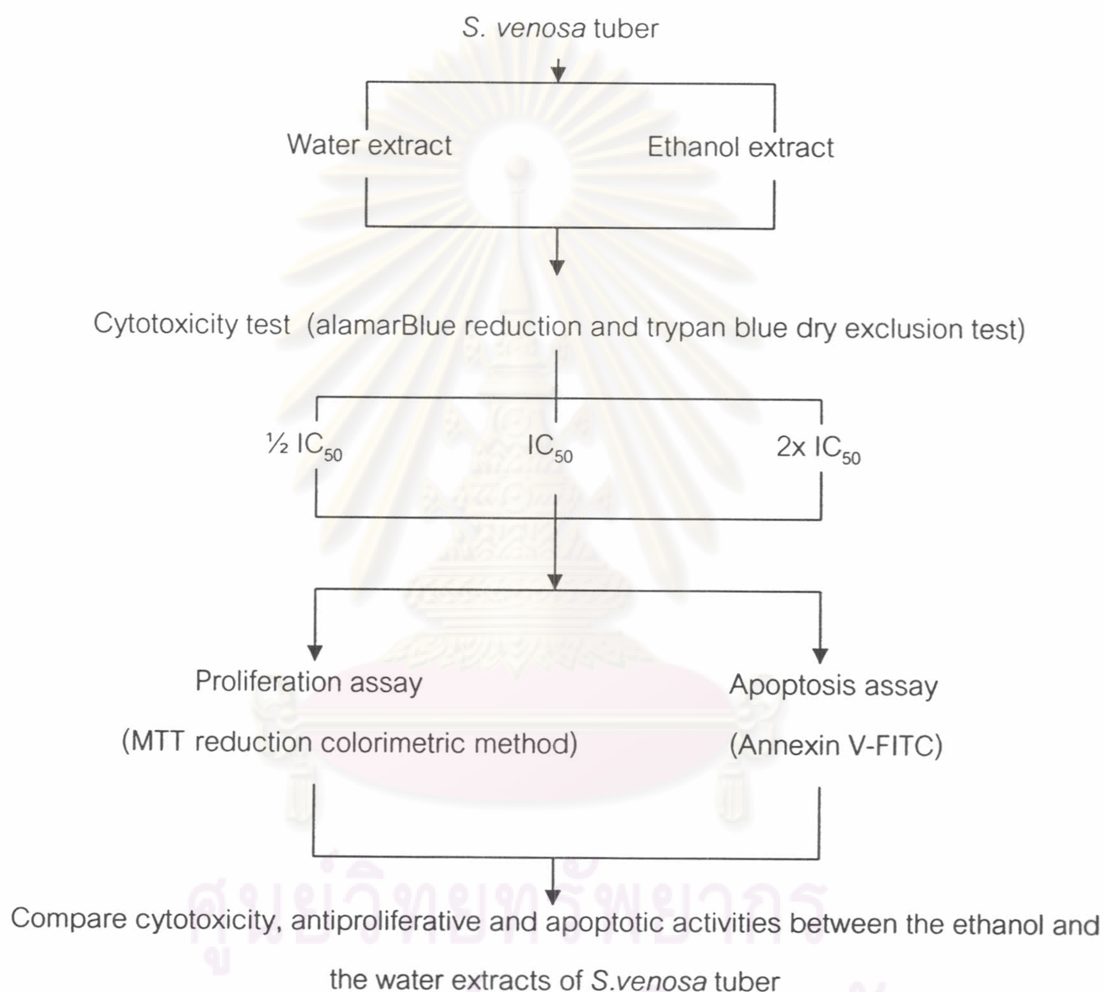


Figure 2. Loss of membrane asymmetry (phosphatidylserine redistribution) is a very early event in apoptosis *(54)*.

In this study, trypan blue dry exclution and alamarBlue reduction were used for determining cytotoxic activities of *S.venosa* extracts on human PBMCs. MTT assay was used for evaluating *S.venosa* antiproliferative effect. Annexin V-FITC assay was used for detecting apoptotic activity of *S.venasa* on human PBMC.

A conceptual framework



ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย