CHAPTER I

INTRODUCTION

Annonaceous plants is a large group of the old world plant family, comprises of 130 genera with more than 2,300 species worldwide (Alali *et al.*, 1999). Almost 320 secondary natural products, from 150 species belonging to 41 genera of the plant, were reviewed from 288 publications by Leboeuf *et al.* (1982). The phytochemistry of the plant has been studied for decades with several major reviews by the group of Professor Jerry L. McLaughlin in U.S.A. since 1990 (Rupprecht *et al.*, 1990; Fang *et al.*, 1993; Gu *et al.*, 1995; Zeng *et al.*, 1996 and Alali *et al.*, 1999). Alkaloid and non-alkaloid natural bioactive compounds have been identified from different parts of the plants. The compounds have been proven to be with several activities; antibacterial, antiparasitic, pesticidal, antiperkinsonian and antitumor activity. Acetogenins, a large group of the C35/C37 natural compounds was mainly found in the plants with the powerful antitumor and pesticidal activites (Alali *et al.*, 1999). The natural acetogenins from the plants has been expected to be a new chemotypes for commercialized antitumor and pesticidal agents. Recent activity testing from the crude extracts of some annonaceous plants reported by Kamuhabwa *et al.* (2000) shew the cytotoxic activities to the cancer cell lines. Among the above mentioned articles and reviews, none of which has been reported the effect of the lead compounds from the plants to the chromosome aberration or genotoxicity in animal cells.

The distribution of annonaceous plant in Thailand, with 41 genera and 195 species, has been reported by Chalearmkrin (2000). Several species has been used as antitumor and antiparasites for decades from generation to another by traditional medical practitioners (Wutthi-Dramawetch, 1977). In this study, 5 species in 3 genera available from the Chiang Mai basin were used; *Annona reticulata* L., *Annona squamosa* L., *Cananga odorata* (Lam.) Hook. F. & Thomson var. *odorata*, *Cananga odorata* (Lam.) Hook. F. & Thomson var. *fruticosa* (Craib) J. Sinclair and *Melodorum fruticosum* Lour. Cytotoxicity screening by using animal cell lines provides important preliminary data to help select plant extracts with potential mutagenesis approach. For adequate economic and laboratory time limitation reasons, only the ethanolic

extracts were used for all the plants. These extracts were subjected to a bioscreening assay using the two cultivated human cell lines; AMC-K46 (amniocytic cell line) and HeLa (cervical carcinoma cell line).

The AMC-K46 was established in 2003 from a male fetus cells retrived by the amniocentesis and became a continuous cell lines. The cells originally detached from different parts of the fetus including skin, the epithelial lining of gastro-intestinal tract, respiratory tract, sense organs and (probably) the mesenchymal cells, and suspend in amniotic fluid (Fauza, 2004). The primary cells are usually used in clinically prenatal diagnosis for the fetal chromosomal anomalies; Down's syndrome, Patau's syndrome, Edward's syndrome etc. The risk of the chromosomal anomalies increased with the advancement of maternal age (Mevatee, 1998). The cell line has been characterized on the 8^{th} to 12^{th} passages by Wisadkeaw (2004). Three types of cell morphology have been reported; epitheloidal, fibroblastoids and giant cells. Spontaneous transformation seems to be an important explanation to the aneuploidy of the cells (Walen, 2002), with 46-69 of chromosome number (Wisadkeaw, 2004). However, the characteristics related to the karyotype of the cells have been evaluated in this study following the guildline for cytogenetic nomenclature, ISCN (An International System for Human Chromosome Nomenclature 1995 (Le Beau, 2001)). Briefly, at least 15-30 metaphases are stained using G-banding technique with at least 2 metaphases chosen for chromosome analysis. The chromosomal structural rearrangement (e.g. translocations, deletions, or inversions) and chromosome number counting, the mosaicism (a state in which a single individual chromosome number has two or more different karyotype) are observed. The karyotype has been reported in the numerical changes (modal chromosome number, the total count of chromosome number of each cell in the predominant cell type in the population) and the structural changes.

The HeLa cell, the cancerous cell, which was established from the epithelial cervical carcinoma of a 31-year-old black woman in 1951 (Cavanagh, 1997). The cells is used as the important model for several field of studies (*in vitro* cytotoxicity, molecular approaches of cell biology etc.), including cytotoxic screening of plant crude extracts (Kamuhabwa, 2000). The research on HeLa cells has provided scientists with an enormous amount of basic knowledge about the physiology and

genetics of human and animal cells. In this study, some basic characteristics and the cytotoxic screening of the plant crude extracts has been approached for HeLa.

Screening for mutagenesis or genotoxicity has been proposed by several authors using *in vivo* animal models (Çelik *et al.*, 2005; D'Sauza *et al.*, 2005; Hu, Xu and Chen, 2005; Leopardi, *et al.*, 2005; Mouchet, *et al.*, 2005; Sadeghiani, *et al.*, 2005). *In vitro* testing is used to describe and evaluate the activity of toxic agent to the living organism. The laboratory animal models, invertebrate or vertebrate, have been used unnecessary in the past for topical or specific toxicity tests (Frame, *http://www.frame.org.uk/*). Human and animal cell culture technology is well developed at present to be used as the representative to the whole organism. In this preliminary study for mutagenesis screening, the *in vitro* approach of the plant crude extracts in human cell lines has been evaluated. The overlapping of the *in vivo* and the *in vitro* testing is upon the experimental design that can predict of what would be happened in the living animals. More advantages for the *in vitro* to the *in vivo* have been considered in this study. With the cultured animal (or human) cells as experimental models, the factors involved in the experiments could be controlled and together with the relevant economic points of view. The cytotoxic measurement can be considered within two criterias; the non-colorimetric and the colorimetric methodology (Chew *et al.*, 2000). The *non-colorimetric* methods are normally used for the prediction of the cosmetic allergy or bioactive compounds to specific cell models such as artificial human skin model, cornea cells, embryonic stem cells etc. The spectrum of measurements could be vary from the normal penetration or irritation to the inflammations and with the mechanism of damaging and the recovery of the cell models (Freshney, 2000). The stage of morphological damage including cell organelle and chromosome aberrations, including cell lysis studied by the light microscope, microphysiometer or electron microscopies are under the scope of this measurement. The *colorimetric* method is more reliable for the *in vitro* toxicity assay with the varieties of spectroscopic technologies. Cell viability assay, cell proliferative activity assay, measurement of cell deaths etc., are considered to be under this methodology. Many of the bioassay which used at the end of the exposure such as; neutral red uptake assay, coomassie blue (target protein label) assay, SRB

assay (total protein measurement) and MTT assay (mitochondria activity measurement) are among the popular cytotoxicity testing methods.

The MTT assay (or cell survival assay), is the end point of cellular cytotoxic treatment developed by Mossman in 1983 (Freshney, 1994). The principle of the MTT assay is the measurment of the cellular activity by the primary function of mitochondria ATP production. The important enzyme involved in the ATP production is the succinate dehydrogenase. The yellow MTT dye (or [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide]) is reduced by the enzyme to the insoluble purple formazan pigment in living cells. The concentration of the formazan pigments can be detected by the ELIZA spectrophotometric plate reader in a suitable solvent (DMSO, SDS etc.). The more concentrated solution of formazan detected from the exposed cultures, the higher activity of mitochondria expressed and also the more number of living cells.

The chromosome aberration is the large-scale numerical or structural alteration in eukaryotic chromosome. Chromosome deletion, breakage, translocation etc., can cause lethality or malfunctions to the cells (Adler *et al.*, 1985). To visualize and evaluate the chromosome aberrations the chromosomes are arrested in metaphase by a suitable spindle fiber inhibitors such as colchicine or colcemid. Then the cells are swelled by a hypotonic solution, fixed and spreaded on a glass slide. The chromosomes of the first mitotic cycle, after the active compound exposure, are the targeted chromosomes to be observed. The vulnerable phases of the cell cycle to the active agent are usually on the S and M phase (Mitchell, 2000). With the effect of the chemical agent on the S phase, where the DNA replication occurred, *the chromosome type of aberration* may be observed. On the other hand, with the effect to the M phase, *the chromatid type of aberration* would be peaked on.

OBJECTIVES

This study was initiated with the aims to;

(i) specify the optimum conditions for the AMC-K46 and the HeLa to be used in the cytotoxicity test on the plant crude extracts by MTT assay,

(ii) evaluate the *in vitro* cytotoxic activity of the crude extracts from some species of the annonaceous plants against the AMC-K46 and the HeLa.